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**Medical treatment and monitoring for
disorders of cortisol and
adrenocorticotrophin excess and deficiency**

E. Daniel

**A thesis submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy**

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The University of Sheffield

The Medical School, Department of Oncology and Metabolism

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Table of Contents

DECLARATION	5
1. SUMMARY	6
2. INTRODUCTION	7
2.1. Coherence of work presented in this thesis	7
2.2. Contribution of the student	8
2.3. Publications from thesis	11
2.4. Aims and Objectives	13
2.4.1. Aims	13
2.4.2. Objectives	13
3. BACKGROUND	15
3.1. Cortisol production and regulation	15
3.1.1. Physiology	15
3.1.1.1. Cortisol	15
3.1.1.2. Glucocorticoid receptor	21
3.1.1.3. Cortisol production: the steroidogenesis pathway	22
3.1.1.4. Regulation of cortisol secretion: HPA axis and regulation of steroidogenesis	31
3.1.1.4.1. HPA axis	31
3.1.1.4.2. The regulation of steroidogenesis in the adrenal gland	34
3.1.1.5. Physiological cortisol secretion	35
3.1.1.6. Assessing physiological cortisol levels	38
3.1.2. Androgen effects on erythropoiesis	44
3.1.3. Disorders of cortisol secretion	47
3.1.3.1. Cushing's syndrome	47
3.1.3.2. Nelson's syndrome	53
3.1.3.3. Primary adrenal insufficiency	55
3.1.3.4. Congenital adrenal hyperplasia	59

3.2. Medical treatment and monitoring of disorders of cortisol and corticotrophin excess	72
3.2.1. Cushing’s syndrome	72
3.2.1.1. Medical Treatment	72
<i>Adrenal-targeted therapy for Cushing’s syndrome</i>	72
<i>Pituitary-targeted therapy for Cushing’s Disease</i>	76
<i>Glucocorticoid receptor targeted therapy for Cushing’s syndrome</i>	77
3.2.1.2. Monitoring of medical treatment	78
3.2.2. Nelson’s syndrome	79
3.2.2.1. Medical Treatment	79
3.2.2.2. Monitoring of medical treatment	82
3.3. Medical treatment and monitoring of disorders of cortisol deficiency	83
3.3.1. Primary adrenal insufficiency	83
3.3.1.1. Medical treatment	83
3.3.1.2. Monitoring of medical treatment	87
3.3.2. Congenital adrenal hyperplasia	90
3.3.2.1. Medical treatment	90
3.3.2.2. Monitoring of medical treatment	93
3.4. Hypothesis	95
4. METHODS	96
4.1. Overview	96
4.2. Data collection	96
4.3. Analytical biochemistry for cortisol quantification	98
4.3.1. Liquid chromatography tandem mass spectrometry	98
4.3.2. Immunoassays	101
4.4. Ethical Approval	104
4.5. Data management and statistics	105
5. RESULTS	107
5.1. Study 1: Medical therapy in Cushing’s syndrome	107
5.2. Study 2: Medical therapy in Nelson’s syndrome	129
5.3. Study 3: Administration of Hydrocortisone through nasogastric tubes	150

5.4. Study 4: Biomarkers for monitoring hormone replacement in CAH	173
5.5. Study 5: Pharmacokinetic analysis of Hydrocortisone granules	196
6. DISCUSSION	221
6.1. Why are physiological cortisol levels important?	221
6.2. Can medical therapies restore physiological cortisol secretion	226
<i>Cortisol deficiency</i>	226
<i>Cortisol excess</i>	227
<i>Compliance and adequate dose titration</i>	229
<i>Safety considerations</i>	230
6.3. Methodological challenges in studying conditions of cortisol excess and deficiency	231
6.3.1. Assessing treatment effectiveness	231
6.3.2. Analytical biochemistry for measuring steroids	233
6.3.3. Challenges in designing clinical trials for rare diseases	237
6.4. New biomarkers for monitoring treatment response	243
7. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH	245
ABBREVIATIONS	247
SUMMARY OF FIGURES	250
SUMMARY OF TABLES	250
ACKNOWLEDGEMENTS	251
APPENDIX	252
A.1. Metyrapone data collection form	252
A.2. Hydrocortisone measurement with LC-MS/MS protocol	256
REFERENCES	257

Declaration

I, the author, confirm that the Thesis is my own work.

I am aware of the University's Guidance on the Use of Unfair Means.

This work has not been previously been presented for an award at this, or any other, university.

1. Summary

Background: Disorders of cortisol secretion have high mortality and morbidity if inadequately treated. Medical treatment is an essential part of patient management and has improved prognosis and morbidity, however, there are unanswered questions about effectiveness, safety, accuracy and monitoring. The hypothesis in this thesis was that medical treatments can restore physiological cortisol and adrenocorticotrophin hormone (ACTH) levels in patients with disorders of cortisol excess and deficiency.

Methods: Five studies examined the treatment and monitoring of cortisol secretion disorders. Two studies examined medical treatment of cortisol and ACTH excess, Cushing's syndrome (CS) and Nelson's syndrome (NS), two studies examined new methods for replacing cortisol in children with adrenal insufficiency and one study examined potential for a novel biomarker of cortisol replacement in congenital adrenal hyperplasia (CAH).

Results: Study 1, demonstrated that medical therapy with the steroidogenesis enzyme inhibitor metyrapone was effective in restoring eucortisolaemia and reducing hypercortisolaemia in 50-80% of patients with CS. Study 2, demonstrated that medical therapy with pasireotide, a multi-receptor somatostatin analogue, reduced plasma ACTH levels in patients with Nelson's syndrome. Study 3, showed that it is possible to replace cortisol with hydrocortisone through nasogastric tubes, however, there are variable drug losses due to interaction with the administering equipment and the study provided practical solutions. Study 5, showed that a novel formulation of hydrocortisone granules administered sprinkled on soft food (applesauce or yoghurt) are bioequivalent to granules delivered directly to the back of the tongue. Study 4, showed that haemoglobin and haematocrit are positively correlated with androgen and steroid precursor levels in women with CAH and provide a novel biomarker.

Conclusions. Medical therapy for cortisol excess and deficiency can be improved. Metyrapone and pasireotide are effective in improving cortisol and ACTH levels in patients with CS and Nelson's syndrome, respectively. The replacement of cortisol in paediatric adrenal insufficiency can be done through nasogastric tubes if required and with food to improve accurate dosing in neonates, infants and children. Markers of erythropoiesis may be used as a biomarker to monitor disease control in women with CAH.

2. Introduction

2.1. Coherence of work presented in this thesis

The work presented in this thesis examines the effectiveness of medical treatment for disorders of cortisol excess and deficiency and explores the monitoring of response to medical treatment. The five studies that form the spine of the thesis assess the optimisation and effectiveness of medical therapy, how response to treatment is objectively defined and how old and new biomarkers can be used to define the response to medical therapy.

The first two studies assess the effectiveness of medical treatment for cortisol excess (Cushing's syndrome) and adrenocorticotrophin hormone excess (Nelson's syndrome), describe the variations in the monitoring of response in clinical practice (cortisol excess) and apply a monitoring approach based on clinical, imaging and biochemical data (adrenocorticotrophin excess). The next three studies explore the medical treatment of Congenital adrenal hyperplasia (CAH) and disorders of cortisol deficiency that require long-term treatment and monitoring. The fourth study tests the association of markers of erythropoiesis, which are easily accessible and standardised tests as biomarkers of monitoring response to medical treatment in women with CAH. The third and fifth studies explore issues related to the accuracy of cortisol dose replacement, which is the cornerstone of medical therapy in conditions of cortisol deficiency. The fifth study assesses the effect of co-administration with food in the pharmacokinetics of a new paediatric formulation of immediate release hydrocortisone and the third assesses different delivery methods and accuracy of nasogastric administration of hydrocortisone, which is a route of medicine administration necessary in some children and adults that require hydrocortisone replacement.

2.2. Contribution of the student

The candidate has performed the significant majority of work presented in this thesis and was the primary contributor in the five published scientific papers that form the body of work of the thesis. The papers were published between 2015 and 2019 during the period of PhD studies.

The candidate was the first author in four and the senior author in one of the five studies included in the thesis. The studies have been performed with the collaboration of internal and external co-authors and a detailed contribution of the student per study presented in the thesis is as follows:

1. *Effectiveness of Metyrapone in Treating Cushing's Syndrome: A Retrospective Multicentre Study in 195 Patients*

The candidate was the first author of the published paper and contributed to the design of the study and the data collection proforma, collected the local data from Sheffield Teaching Hospitals, led the co-ordination of data collection from the twelve participating centres, collated and analysed data from all the centres, produced the first draft of the manuscript and then edited, and revised the manuscript, including referee's comments, and produced the final version.

Statement and signature by Prof Newell-Price, senior author:

Dr Daniel's contributions are accurately described above.



2. *A prospective longitudinal study of Pasireotide in Nelson's syndrome*

The candidate collated the data from the four participating centres and co-ordinated biochemical and radiological analysis. The candidate analysed and interpreted the data, produced the first draft of the paper, edited and revised the manuscript, including referee's comments, and produced the final version.

Statement and signature by Prof Newell-Price, senior author:

Dr Daniel's contributions are accurately described above.



3. *Accuracy of hydrocortisone dose administration via nasogastric tube*

The student designed the experimental protocol, used preliminary data to update the design of future experiments, performed 90% of all experiments, analysed the samples, interpreted the data and performed the statistical analysis, wrote the first draft of the paper, edited and revised the manuscript and was the first author of the published paper.

Statement and signature by Prof Ross, senior author:

Dr Daniel's contributions are accurately described above.



4. *Androgens correlate with increased erythropoiesis in women with congenital adrenal hyperplasia:*

The student had a major role in the concept of the study and deciding which data would be collected by designing the relevant proforma, collected local data and performed the preliminary data analysis, edited and revised the manuscript and was the senior author of the published manuscript.

Statement and signature by Prof Ross, major contributing co-author:

Dr Daniel's contributions are accurately described above.



5. *Hydrocortisone Granules Designed for Children with Taste Masking and Age Appropriate Dosing are Bioequivalent When Sprinkled onto Food or Given Directly:*

The student interpreted the study report, produced the first manuscript including discussing the data in the context of the literature, edited and revised the manuscript and was the first author in the published paper.

Statement and signature by Prof Ross, major contributing co-author:

Dr Daniel's contributions are accurately described above.



2.3. Publications from the thesis

There have been five publications of original research papers in peer reviewed medical journals from this thesis ¹⁻⁵. Data have also been presented in part in national and international conferences during the time course of the PhD studies. The manuscripts submitted in this thesis are the authors' accepted version post peer-review with links to the publisher version to comply with the publishers' copyright policies and embargo restrictions as outlined on sherpa.ac.uk/Romeo (accessed 4 September 2019).

Three review papers were also undertaken during the PhD studies following extensive literature review and were published in peer-reviewed journals; these papers do not form part of the thesis but the experience has informed the writing of the background chapter ⁶⁻⁸.

The first scientific paper included in this thesis was selected by the Editor of the prestigious journal of the Endocrine Society, The Journal of Clinical Endocrinology and Metabolism, as 'The Best of JCEM in 2016' ¹. This was an award given to 10 articles in 2016 and this paper was the only one in its category, hypothalamus and pituitary, to receive the award and was selected as the most highly-rated peer reviewed article in its category. It was presented in an award session at the annual conference of the Endocrine Society in Orlando, Florida, USA in 2017 (ENDO2017).

The five peer-reviewed original research publications that form part of this thesis are:

1. Daniel E, Aylwin S, Mustafa O, Ball S, Munir A, Boelaert K, Chortis V, Cuthbertson DJ, Daousi C, Rajeev SP, Davis J, Cheer K, Drake W, Gunganah K, Grossman A, Gurnell M, Powelson AS, Karavitaki N, Huguet I, Kearney T, Mohit K, Meeran K, Hill N, Rees A, Lansdown AJ, Trainer PJ, Minder AE, Newell-Price J. Effectiveness of metyrapone in treating Cushing's Syndrome: a retrospective multicenter study in 195 patients. *J Clin Endocrinol Metab.* 2015 Nov; 100(11): 4146-54. doi: 10.1210/jc.2015-2616
<https://academic.oup.com/jcem/article/100/11/4146/2836118>

2. Daniel E, Debono M, Caunt S, Girio-Fragkoulakis C, Walters SJ, Akker SA, Grossman AB, Trainer PJ, Newell-Price J. A prospective longitudinal study of Pasireotide in Nelson's syndrome. *Pituitary* (2018) 21: 247. <https://doi.org/10.1007/s11102-017-0853-3>
<https://link.springer.com/article/10.1007%2Fs11102-017-0853-3>

3. Daniel E, Whitaker MJ, Keevil B, Wales J, Ross RJ. Accuracy of hydrocortisone dose administration via nasogastric tube. *Clin Endocrinol*. 2019; 90: 66– 73. <https://doi.org/10.1111/cen.13876>
<https://onlinelibrary.wiley.com/doi/full/10.1111/cen.13876?af=R>

4. Karunasena N, Han TS, Mallappa A, Elman M, Merke DP, Ross RJ and Daniel E. Androgens correlate with increased erythropoiesis in women with congenital adrenal hyperplasia. *Clin Endocrinol*. 2017; 86(1): 19-25. doi:10.1111/cen.13148
<https://onlinelibrary.wiley.com/doi/full/10.1111/cen.13148>

5. Daniel E, Digweed D, Quirke J, Voet B, Ross RJ, Davies M. Hydrocortisone Granules Designed for Children with Taste Masking and Age Appropriate Dosing are Bioequivalent When Sprinkled onto Food or Given Directly on the Tongue. *J Endocr Soc*. 2019;3(5):847-856.
<https://academic.oup.com/jes/article/3/5/847/5364743>

2.4. Aims and Objectives

2.4.1. Aims

The aims were to:

- i. Assess new and existing treatments for disorders of cortisol and adrenocorticotrophin hormone excess and deficiency
- ii. Discover and assess new monitoring tests
- iii. Improve long-term management of patients with disorders of cortisol excess and deficiency

2.4.2. Objectives

The objectives were to:

1. Assess an existing medical treatment for Cushing's syndrome:
 - i. Collect biochemical and clinical data on patients undergoing medical treatment with metyrapone for Cushing's syndrome from multiple centres in the UK.
 - ii. Analyse data and assess the effectiveness of metyrapone in treating hypercortisolaemia in patients with Cushing's syndrome.
 - iii. Analyse safety data from patients receiving metyrapone to establish the safety of this treatment.
2. Assess a new treatment for Nelson's syndrome
 - i. Collect and organize samples and imaging of patients with Nelson's syndrome treated with pasireotide in four centres in the UK and organize biochemical analysis from the reference lab.
 - ii. Assess the effectiveness of pasireotide in treating patients with Nelson's syndrome using statistical analysis.
 - iii. Analyse the safety data to assess the safety of using pasireotide in patients with Nelson's syndrome.
3. Assess the feasibility of oral and nasogastric administration of hydrocortisone for children and young children with Congenital adrenal hyperplasia and primary adrenal insufficiency

- i. Collect information on nasogastric administration of hydrocortisone in paediatric patients and neonates from literature and from interviews with staff of endocrine and neonatal hospital units.
 - ii. Develop a protocol for testing in vitro the administration of a new formulation of hydrocortisone and two commonly used formulations through nasogastric tubes.
 - iii. Perform and biochemical analysis of the samples to assess the delivery of the new formulation and the two current formulations through the nasogastric tube
 - iv. Perform statistical analysis of the results to assess how the delivery of the new formulation compares with the delivery of the two current formulations
4. Discover new biomarkers for monitoring disease control in adult patients with Congenital adrenal hyperplasia (CAH)
- i. Collect monitoring and clinical information from patients with CAH treated at Sheffield Teaching Hospitals.
 - ii. Perform a statistical analysis to check if markers of erythropoiesis are associated with worst disease control (using the androgen levels as a surrogate marker) in women with CAH.
 - iii. Confirm any significant findings by testing the correlations of markers of erythropoiesis and androgens in patients treated in a second cohort from another specialist centre (the National Institutes of Health, USA).
5. Test whether the pharmacokinetics of a new hydrocortisone replacement formulation for children with CAH could be altered by different administration methods
- i. Interpret the pharmacokinetic report of a clinical study to assess the differences between three different administration methods of hydrocortisone granules.
 - ii. Perform a literature review to understand
 - a. the problems of drug administration in young children and practical approaches adopted by carers and the flexibility offered by different methods of drug administration.
 - b. the possible implications of mixing medication with food in the medication's absorption and pharmacokinetic parameters.
 - c. national and international regulations on pharmacokinetic studies.
 - iii. Write a research paper based on the study report and the review of literature performed by the student.

3. Background

3.1. Cortisol production and regulation

3.1.1. Physiology

3.1.1.1. Cortisol

Cortisol is a glucocorticoid steroid hormone, which is produced by the adrenal cortex under tight regulation from centres in the hypothalamus in the brain and the pituitary gland. It has important actions on homeostasis, stress response and is essential for life. It is secreted to the blood stream and travels through the circulation to reach distant organs and tissues where it exerts varied actions in multiple cell types (Table 1)⁹. The term glucocorticoid, for this group of steroid hormones, reflects the important effects on glucose homeostasis including an increase in the release of glucose in the circulation.

Steroid hormones are organic compounds that share a core carbon structure consisting of seventeen carbon atoms arranged in four fused rings; three cyclohexane and one cyclopentane. Functional chemical groups [such as hydroxyl (OH), carbonyl (C=O), hydrocarbyl (C_aH_b)], attach on carbon points on the core structure and diversify the chemical and physical properties of steroids and their biological activity¹⁰. There are three categories of steroid hormones based on their biological activity; mineralocorticoids, glucocorticoids and sex steroids. Cortisol is the most potent endogenous glucocorticoid and is a 21-carbon steroid (C₂₁H₃₀O₅, 11β,17α,21-trihydroxypregn-4-ene-3,20-dione) with hydroxyl groups at carbons 11, 17, and 21 and oxo groups at positions 3 and 20 (Figure 1)¹¹. Cortisol is a lipophilic, low-molecular weight molecule that can diffuse through cell membranes. In the circulation it mainly travels bound with reversible bonds to carrier proteins. These proteins also act as a reservoir and prevent cortisol metabolism in the liver.

The structure and biological properties of steroid hormones produced by the adrenal glands became an area of intense research interest in the 1930s following the recognition that adrenal glands were essential for life and that extracts from adrenal glands could sustain life in adrenalectomised animals and improve symptoms of patients with adrenal insufficiency^{12, 13}. Research groups at the time working on

Table 1: Tissue-specific glucocorticoid effects ^{9, 14, 15}

Immune system	Immunosuppression and anti-inflammatory action Reduction of lymphocytes and eosinophils in the blood Induction of lymphocyte apoptosis Inhibition of synthesis of immunoglobulins and cytokines Inhibition of macrophagocytic activity and differentiation Inhibition of prostaglandin synthesis
Carbohydrate metabolism	Increased glucose release and diabetogenic effect Impaired glucose tolerance Activation of gluconeogenesis in the liver Increase in hepatic glycogen deposition Inhibition of glucose uptake in the muscle and fat Activation of lipolysis and increase in free fatty acids in the circulation Resistance to insulin through permissive effect on catecholamines and glucagon
Protein catabolism	Muscle wasting, progressive proximal myopathy Insulin resistance in myocytes Catabolic effects in connective tissue with reduction of collagen production Thinning of the skin and stretch marks due to loss of dermal collagen Retardation or cessation of linear growth in children
Adipose tissue	Redistribution of body fat with increase in visceral adiposity Activation of lipolysis Increase in total cholesterol, triglycerides and fall of HDL-Cholesterol Adipogenesis through induction of adipogenic genes
Electrolyte and water homeostasis, blood pressure control	Sodium retention Potassium loss Metabolic alkalosis Water retention and increase in total body water Increase in free water clearance Increase in blood pressure Increased sensitivity to catecholamines-induced vasoconstriction
CNS	Depression Euphoria Psychosis Effects on memory and cognitive function Induction of neuronal death (hippocampus)
Bone	Osteoporosis due to inhibition of osteoblast function Osteonecrosis (avascular necrosis) Reduction of calcium absorption from the intestine Increase renal calcium excretion Increase in parathyroid secretion
Eyes	Glaucoma, increase in intra-ocular pressure Cataract
Gastrointestinal	Peptic ulcer Pancreatitis with glucocorticoid excess
Endocrine	Suppress thyroid axis through inhibition of TSH secretion Inhibition of peripheral conversion of T4 to T3 Inhibition of gonadal axis: inhibition of GnRH pulsatility and LH/FSH release Inhibition of IGF1 in glucocorticoid excess

isolation of compounds from the adrenal cortex recognised these compounds to be steroids; Kendall, a chemist at Mayo Clinic, and Reichstein, a chemist at Basel University in Switzerland independently discovered cortisone (Kendall's Compound E) and investigated its biological activity ¹⁶. The anti-inflammatory effects of cortisol were confirmed when in 1949 Kendall and Hench, a physician at Mayo Clinic, used cortisone to treat patients with severe rheumatoid arthritis with clinical and biochemical improvement ¹⁷. Cortisone, a glucocorticoid with a structure similar to cortisol, is converted to biologically active cortisol after administration. For this breakthrough work, the three received the Nobel prize in Medicine in 1950 (Table 2) ¹⁶. Following the studies on the anti-inflammatory effects of cortisone, adrenocorticotrophin hormone (ACTH), a hormone produced by the pituitary gland and essential signal for cortisol synthesis, was administered to patients with chronic inflammatory diseases resulting in disease remission but also side effects, namely glycosuria, cushingoid features, and potassium depletion ¹⁵.

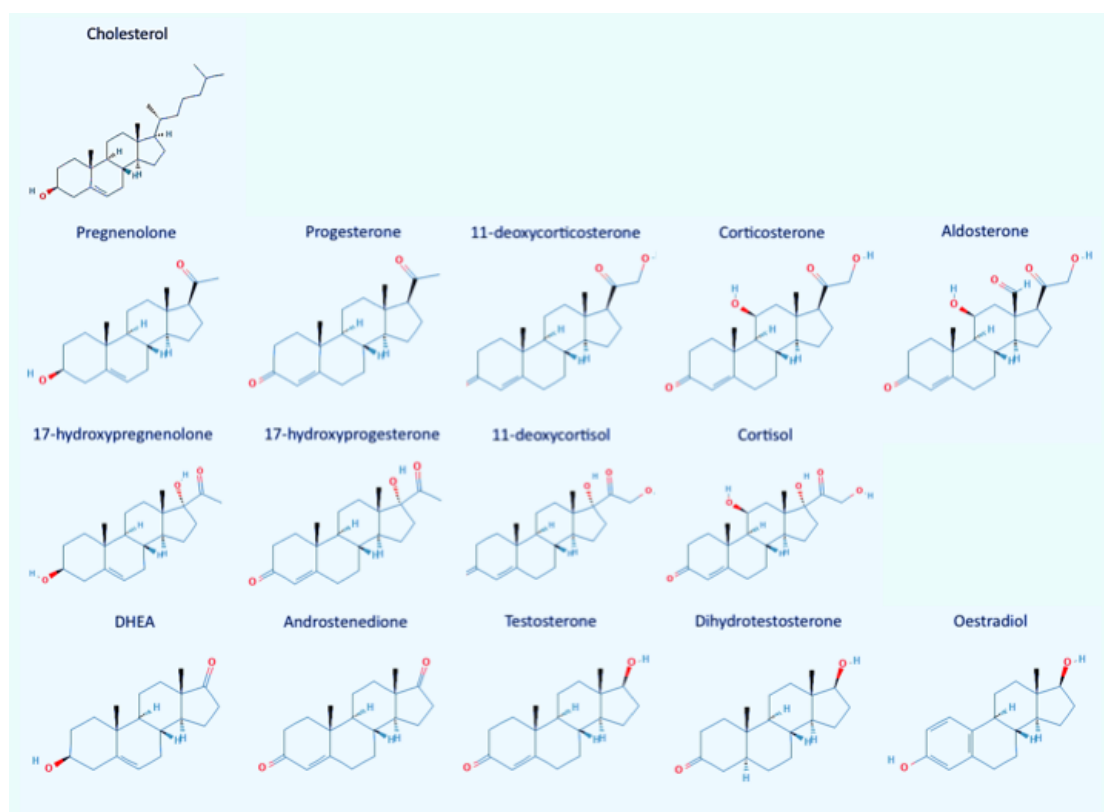


Figure 1: Molecular structures of endogenous steroids

Molecular structures downloaded from <https://pubchem.ncbi.nlm.nih.gov>, accessed 19 April 2019.

Table 2: Nomenclature of endogenous glucocorticoids and mineralocorticoids

In the 1930s research groups led by E.C. Kendall at Mayo Clinic, USA, T. Reichstein at Basel University in Switzerland, and O. Wintersteiner at Columbia University, USA were isolating steroid compounds from animal adrenal extracts. The compounds were named by alphabetical letters in order of discovery by each team. Current steroid nomenclature is defined either by trivial names or by the chemical structure as defined by the International Union of Pure and Applied Chemistry (IUPAC) ^{16, 18-21}.

Trivial names	Initially known as	Synonyms	IUPAC names	Chemical formula
Cortisone	Kendall's compound E, Reichstein's substance Fa Wintersteiner's compound F	17-hydroxy-11- dehydrocorticosterone	17 α ,21-dihydroxypregn-4-ene-3,11,20-trione	C ₂₁ H ₂₈ O ₅
Cortisol	Kendall's compound F, Reichstein's substance M, Wintersteiner's compound F	17-hydroxy-corticosterone, 11 β -hydrocortisone	11 β ,17 α ,21-trihydroxypregn-4-ene-3,20-dione	C ₂₁ H ₃₀ O ₅
11-Deoxycortisol	Reichstein's substance S		17 α ,21-dihydroxypregn-4-ene-3,20-dione	C ₂₁ H ₃₀ O ₄
11-Deoxycorticosterone (DOC)	Kendall's desoxy compound B Reichstein's substance Q	Deoxycorticosterone, 21-hydroxyprogesterone	21-hydroxypregn-4-ene-3,20-dione	C ₂₁ H ₃₀ O ₃
Corticosterone	Kendall's compound B Reichstein's substance H	17-deoxycortisol, 11 β ,21-dihydroxyprogesterone	11 β ,21-dihydroxypregn-4-ene-3,20-dione	C ₂₁ H ₃₀ O ₄
Aldosterone			11 β ,21-dihydroxy-3,20-dioxopregn-4-en-18-al	C ₂₁ H ₂₈ O ₅

Since then, glucocorticoids have been used extensively to treat inflammatory conditions and many synthetic glucocorticoids have been produced for clinical use. In the 1950s the pharmaceutical steroid production flourished; initially, production of significant amounts of synthetic steroids was based on modifications of bile acids but soon cost-effective production methods were discovered allowing the production of progesterone and glucocorticoids from plant steroids ²². An example was diosgenin which is derived from a wild Mexican yam (*Dioscorea* species) and its discovery helped make cortisone widely available ²³. Synthetic glucocorticoids differ in their potency of anti-inflammatory and mineralocorticoid action, duration of action, and mode of administration and these factors determine their clinical use. In recent years, the most commonly used synthetic steroids are prednisolone, prednisone that is converted to prednisolone by 11-hydroxylation in the liver, methylprednisolone, and dexamethasone ²⁴. Hydrocortisone is also prescribed widely; it is cortisol when administered as a medication and is on the World Health Organisation's list of essential medicines.

Following production by the adrenal gland, cortisol is excreted in the blood and travels through the circulation mainly bound on carrier proteins. Cortisol binds with high affinity to cortisol binding globulin (CBG), an alpha₂-globulin produced by the liver that circulates in the blood with levels around 700 nmol/L ²⁵. The majority of cortisol in the blood is bound to CBG (80%), with less bound on albumin (15%) and a small amount is unbound, free cortisol (5%) ^{24, 26}. Albumin is a water-soluble protein produced by the liver and as the most abundant protein in the circulation, it maintains the colloid osmotic pressure of the blood but also is an important carrier protein for hydrophobic molecules. When cortisol is quantified in blood or plasma samples both bound and unbound cortisol account for the total circulating concentration. However, only unbound, free cortisol is biologically active as it can passively travel through the cell membrane and enter cells in the target tissues where it exerts its biological actions. Free cortisol is present in the saliva and excreted in the urine.

Binding proteins can affect cortisol concentration in the blood. The levels of CBG in the blood may be affected by hormones, physiological changes, acquired and congenital conditions ²⁶. CBG levels are significantly increased by oestrogens, pregnancy, hypothyroidism, some forms of chronic active hepatitis and drugs such as oestrogen-containing oral contraceptives and mitotane ^{27, 28}. CBG is reduced in

hyperthyroidism, critical illness and acute inflammation, post-surgery, nephrotic syndrome, and liver disease such as cirrhosis^{27, 29}. Cortisol itself downregulates production of CBG by the liver³⁰. When the levels of CBG are increased, the total cortisol (bound and unbound) levels are increased too and the opposite occurs with reduced CBG levels. Cortisol binds to albumin with low affinity and provided the levels of albumin in the blood are stable, albumin buffers changes in the plasma distribution of cortisol for example when there is a change in the rate of cortisol secretion or CBG concentration^{26, 30}.

Free cortisol diffuses through the cell membranes and enters cells in multiple target tissues. Inside the cells, the levels of cortisol are affected by the action of two enzymes; 11 β -Hydroxysteroid dehydrogenase type 1 and type 2 (11 β HSD1 and 11 β HSD2). These are oxidoreductases that transfer electrons using the cofactors NADPH and NADP⁺ and modify the carbon-11 position of cortisol's chemical structure; 11 β HSD1 adds a hydroxy-group whereas 11 β HSD2 catalyses the opposite reaction removing 2 hydrogen atoms to form a keto-product. The reaction catalysed by 11 β HSD1 converts inactive cortisone to cortisol and this enzyme has a widespread localisation in tissues. 11 β HSD2 inactivates cortisol to cortisone and is localised in aldosterone-sensitive tissues such as the kidney and restricts cortisol activating the mineralocorticoid receptor in these tissues^{14, 31}.

The half-life of cortisol in the circulation is 60-90 min^{14, 24, 32}. It is metabolised primarily by the liver where a group of enzymes that include 11 β -HSD, 5 β -reductase, 5 α -reductase, and 3 α -HSD perform reduction, hydroxylation at C6 and C20, oxidation at C17 and conjugation with glucuronic acid or sulphate¹⁴. Conjugation increases the solubility of inactive cortisol metabolites in aqueous solutions that can therefore be excreted in the urine as tetrahydrocortisol metabolites in high concentrations³³. Less than 1% of cortisol is excreted by the kidneys as free cortisol³⁴. Cortisol clearance is increased by hyperthyroidism, IGF-1, cortisol and enzyme inducers such as rifampicin and phenytoin (through the induction of 6 β -hydroxylation). On the other hand, cortisol clearance is reduced by hypothyroidism, chronic renal disease (due to impaired conversion to cortisone), increasing age, pregnancy and stress.

3.1.1.2. Glucocorticoid receptor

In the cells, cortisol exerts its biological actions by binding to nuclear receptors. It is a ligand for two receptors, the glucocorticoid receptor (GR or NR3C1) and the mineralocorticoid receptor (MR or NR3C2), which also binds mineralocorticoid hormones to high affinity. The glucocorticoid receptor has low affinity for mineralocorticoids and high affinity for glucocorticoids and is the main receptor through which cortisol exerts its biological actions. GR and MR are members of the steroid hormone receptor subfamily (subfamily 3) together with the oestrogen, progesterone, and androgen receptors. Steroid receptors function as transcription factors regulating the transcription of hormone-responsive target genes. GR is encoded by the gene *NR3C1* located on chromosome 5. GR protein has the following structure: a N-terminal transactivation domain responsible for interaction with co-activators and other transcription factors, a DNA-binding domain containing zinc-binding motifs for DNA binding and receptor dimerisation, and a C-terminal domain responsible for ligand binding^{35, 36}. There are two main isoforms of the glucocorticoid receptor in human, GR α and GR β and these are produced by alternative splicing of the gene and differ in the C-terminal ligand-binding area³⁶. GR α is a 777 amino acid protein and is responsible for hormone-responsive biological activity. GR β is a 742 amino-acid protein lacking the important helices for the formation of the ligand-binding pocket, therefore it does not bind ligands and mainly acts as a negative inhibitor of GR α with some transcriptional activity regulating sensitivity to glucocorticoids^{14, 36, 37}. GR α and GR β have further multiple isoforms each due to different translation initiation sites that differ in their N-terminal domain and their transcriptional activity³⁵.

When free cortisol from the circulation diffuses through membranes and reaches cells in the target tissues it travels into the cytoplasm and binds to the GR α . Upon ligand binding the steroid receptors activate, the ligand-receptor complex translocates to the nucleus where it binds to specific DNA sequences in the promoter region of target genes called the glucocorticoid-response elements (GRE)^{35, 38}. The target genes for transcription are multiple and vary according to the tissue. The main mechanism of transcription regulation by the complex cortisol-GR α involves GR α homodimerisation, recruitment of co-activators or co-repressors and transcription induction or repression of target genes (transactivation or transrepression) through binding on GREs^{39, 40}. Transactivation involves conformational changes and stabilisation of the RNA polymerase II complex following receptor-DNA binding and the GR α -ligand complex

may be directly bound to GREs or tethered through interaction with other transcription factors ⁴¹. There are other mechanisms for transcription regulation by GR α that are non-genomic or do not require DNA binding of GR α to GREs; for example, indirect suppression of transcription through binding of the complex cortisol-GR α to other transcription factors such as the immunomodulator NF- κ B and subsequent prevention of NF- κ B binding to DNA and NF- κ B-dependent transcriptional activation ^{35, 39}.

The function of GR α is supported by various other proteins. For example, in the ligand-free state the GR α interacts with the heat shock proteins 90 and 70 (HSP90 and HSP70), which are chaperone proteins that stabilise and activate proteins through the induction of conformational changes. The interaction of GR α with HSP90 and HSP70 together with other co-chaperone proteins keeps the receptor in a high affinity state for ligand binding and therefore aids ligand-binding and subsequent activation ^{42, 43}. GR α also interacts with the testicular orphan nuclear receptor 4 (TR4), which is a nuclear receptor that acts as a regulator of transcription (activator or repressor) and is expressed in corticotroph cells ⁴⁴. Activated TR4 binds to the promoter region of the POMC gene and promotes POMC expression and this is induced by receptor phosphorylation through the MAPK/ ERK pathway ⁴⁵.

The recycling of the GR α receptor involves dissociation from the ligand after transcription, interaction with heat-shock proteins and return to the cytoplasm or degradation of ligand-bound GR α in the nucleus via the ubiquitin-proteasomal pathway ^{35, 46}. The transcriptional activity of GR is tightly regulated by multiple mechanisms; pre-receptor ligand availability through ligand activation or inactivation by 11 β -HSD type 1 and type 2 in the cells, interaction of ligand and receptor facilitated by chaperone and co-chaperone proteins, intracellular receptor circulation facilitated by chaperone proteins, and modification of receptor function by phosphorylation, ubiquitination, SUMOylation and acetylation ^{38, 46-48}.

3.1.1.3. Cortisol production: the steroidogenesis pathway

Cortisol is synthesised by the cells of the zona fasciculata of the cortex of the adrenal gland in a process called steroidogenesis. The adrenal cortex cells have steroidogenic

capacity; they express steroidogenic enzymes necessary for the biosynthesis of steroid hormones. The cells of the adrenal cortex are organised in three separate zones with distinct histological characteristics and different steroid synthesis specialisation; the zona glomerulosa cells produce mineralocorticoid steroid hormones, the zona fasciculata cells mainly produce glucocorticoid steroid hormones but also small amounts of sex steroids, and the zona reticularis cells mainly produce sex steroids with small amounts of glucocorticoids ⁴⁹.

Steroidogenesis is a complicated biochemical pathway through which steroid hormones are synthesized from a cholesterol molecule through multiple enzymatic steps. The end steroid products differ in biologic activity and receptor specificity and belong to three categories; mineralocorticoids, glucocorticoids, and sex steroids (Figure 2). Immediately after production the steroid hormones are released to the circulation and travel to target tissues where they act on nuclear steroid receptors to exert varied actions essential for homeostasis, stress response, metabolism, growth and reproduction. Steroidogenesis can occur at various tissues, most importantly the adrenals and gonads by cells that have the ability to express steroidogenic enzymes, most notably the P450_{scc} or cholesterol side-chain cleavage enzyme which catalyses the first enzymatic step of the pathway to convert cholesterol to pregnenolone. Conversion of testosterone to the more potent androgen dihydrotestosterone and aromatisation of androgens to oestrogens occurs in steroidogenic cells but also extra-glandular tissues such as the adipose tissue, brain and genital skin that express the enzymes that catalyse these modifications. Throughout life the production rate of steroids is relatively stable for mineralocorticoids and glucocorticoids but changes for sex steroids at different ages ⁵⁰⁻⁵².

The majority of steroidogenic enzymes belong to the cytochrome P450 family of mixed-function oxidases, which are membrane-associated enzymes essential in the biosynthesis of steroids and sterols and drug metabolism ⁵³. There are 57 cytochrome P450 enzymes in humans, 50 are microsomal and are located in the endoplasmic reticulum and 7 are located in the mitochondria; mitochondrial P450 are involved in biosynthesis and microsomal in biosynthesis and drug metabolism. They have an overlap of function and can each catalyse multiple reactions, therefore a single enzyme defect may not cause a complete block to the enzymatic reaction it principally catalyses ⁵⁴. The main microsomal P450 enzymes that catalyse reactions in the steroidogenesis

pathway are P450c17 and P450c21 (Figure 2, Table 3). P450aro is also microsomal and essential for steroidogenesis of sex steroids in the gonads but is not present in the adrenals and is not necessary for adrenal steroidogenesis. The mitochondrial P450 enzymes are P450scc, P450c11 β , and P450c11AS^{51, 55}. Steroid substrates must transfer between these locations in the cell for consecutive reactions to take place.

P450 enzymes contain one heme group and catalyse oxidation-reduction reactions for which they rely on transfer of electrons from NADPH through electron donor proteins; microsomal P450 enzymes receive electrons from P450-oxidoreductase (POR) and mitochondrial from adrenodoxin and adrenodoxin reductase that are located in the mitochondrial matrix^{56, 57}. POR is a flavoprotein bound to the endoplasmic reticulum; it receives two electrons from NADPH that then transfers to the heme iron atom of microsomal P450 enzymes such as P450c17 through electrostatic interactions and conformational change^{58, 59}. Cytochrome b5 is a heme-protein and facilitates the allosteric interaction and electron transfer between POR and microsomal CYP450 enzymes such as P450c17^{58, 60}.

Other enzymes involved in steroidogenesis include members of the family of Hydroxysteroid dehydrogenases (HSDs). HSDs are oxidoreductases that catalyse the oxidation of hydroxysteroids to ketosteroids (dehydrogenases) or the reduction of ketosteroids to hydroxysteroids (reductases) using NAD⁺/NADP⁺ (dehydrogenases) or NADPH/NADH (reductases) as cofactors^{61, 62}. HSDs are membrane-associated enzymes located in the endoplasmic reticulum, each enzyme can catalyse multiple reactions and their expression is tissue-specific. They are bidirectional in vitro but in vivo they mainly function in one direction determined by the availability of the cofactors. Structurally they are members of the short-chain dehydrogenases/reductases (SDRs) (3 β HSDs, 11 β HSDs, 17 β HSD types 1-4) or aldo-keto reductases (AKRs) (17 β HSD5) superfamilies⁶². Proteins involved in cholesterol biosynthesis, storage, and cell uptake also facilitate steroid biosynthesis and examples include the HMG CoA reductase which is the rate-limiting enzyme in cholesterol synthesis, the LDL receptor that takes up lipoprotein-stored cholesterol from the circulation, hormone-sensitive lipase (HSL) that releases cholesterol from lipid droplets and sterol protein 2 that transfers cholesterol to the mitochondria⁶³⁻⁶⁵.

Figure 2: The steroidogenesis pathway

The steroidogenesis pathway is a biochemical pathway consisting of multiple enzymatic steps that leads to the production of steroid hormones; mineralocorticoids, glucocorticoids, and sex steroids. Hormones produced by the adrenal cortex are in black. Alternative pathways of androgen production have recently been elucidated and contribute significantly to the androgen excess in CAH due to 21-hydroxylase deficiency (Backdoor pathway and 11-oxygenated C19 androgen synthesis).

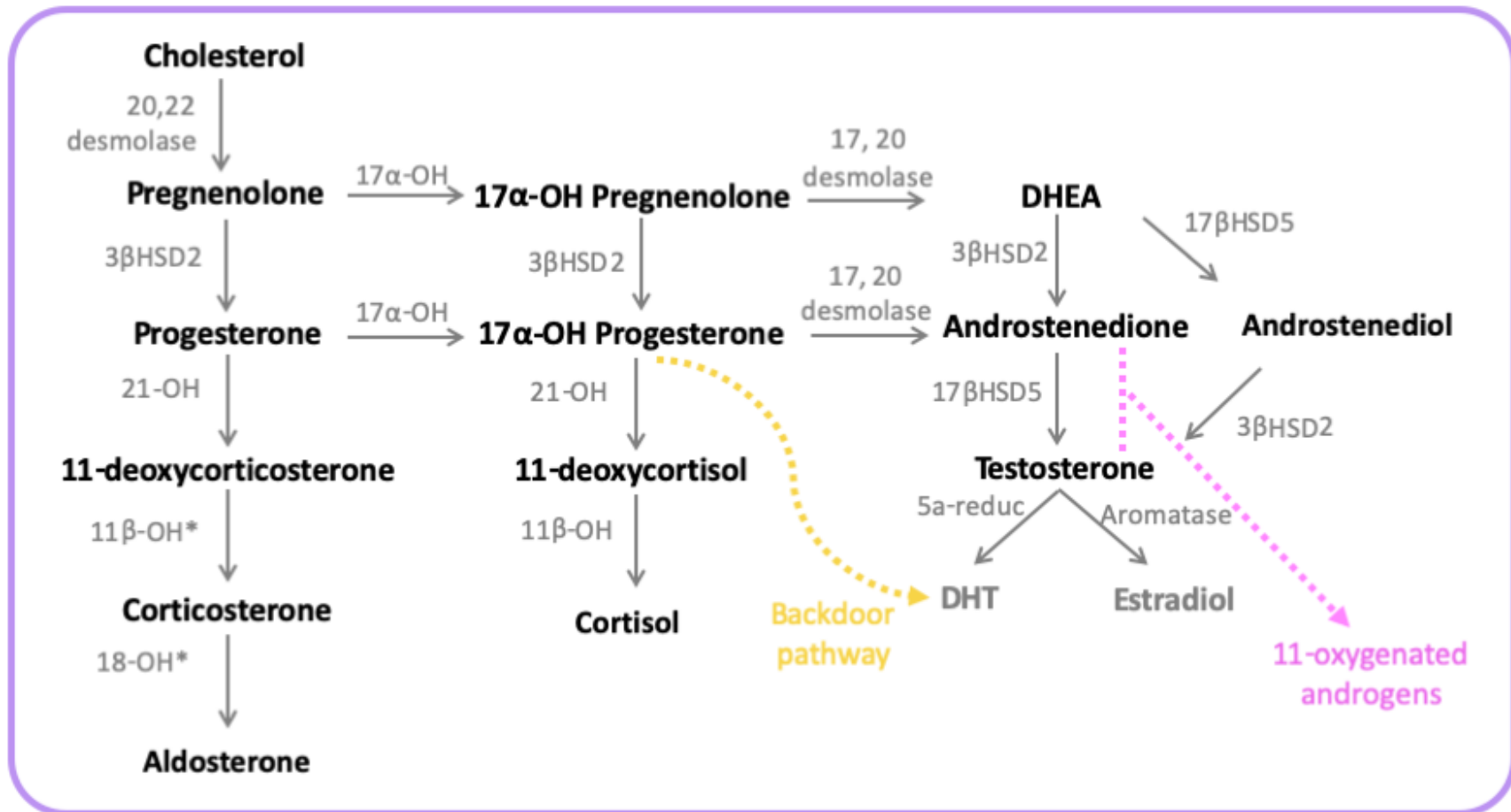


Table 3: Enzymes and genes involved in steroidogenesis in human ^{51, 53, 66}

Gene	Protein Name	Synonyms	Protein function
<i>CYP11A1</i>	CYP11A1	P450scc cholesterol side-chain cleavage enzyme 20, 22 desmolase	Expressed in all the steroidogenic cells including the adrenocortical, ovarian theca, ovarian corpus luteum, Leydig and placenta cells. Localized on the matrix side of the inner mitochondrial membrane. Catalyses a 3-step reaction: hydroxylation at C22, hydroxylation at C20, cleavage of side chain between C20 and C22.
<i>HSD3B2</i>	HSD3B2	3 β - hydroxysteroid dehydrogenase type 2	Type 2 is expressed in the adrenals and gonads and type 1 is expressed in the placenta, brain, liver, breast. Localised in the endoplasmic reticulum membrane. It is an oxireductase that catalyses the conversion of Δ^5 -3 β -hydroxysteroids to Δ^4 -3 β -ketosteroids.
<i>CYP17A1</i>	CYP17A1	P450c17 17 α -hydroxylase/ 17, 20 desmolase	Expressed in the adrenal cortex (only in zona fasciculata and zona reticularis) and gonadal steroidogenic cells (testicular Leydig, and ovarian theca). Not expressed in the placenta. It is localised in the endoplasmic reticulum and has two activities: 17 α -hydroxylation of C21 steroids and cleavage of the C17-C20 bond to form C19 androgen precursors. The 17,20 lyase activity requires the presence of b5cytochrome in the zona reticularis and the two activities are independently regulated.
<i>CYP21A2</i>	CYP21A2	P450c21 21- hydroxylase	Only expressed in the adrenal cortex, in all three zones. It is localised in the endoplasmic reticulum and catalyses the 21-hydroxylation of steroids.
<i>CYP11B1</i>	CYP11B1	P450c11 β 11 β - hydroxylase	Expressed only in the adrenal cortex (zona reticularis/ zona fasciculata). It is localised in the inner mitochondrial membrane and catalyses the 11 β -hydroxylation of C21 steroids.
<i>CYP11B2</i>	CYP11B2	P450c11AS P450c18 P450aldo Aldosterone synthase	Expressed only in the adrenal cortex (zona glomerulosa). It is localised in the inner mitochondrial membrane and catalyses three sequential reactions in C21 steroids: 11 β - hydroxylation, 18-hydroxylation, oxidation of C18-hydroxyl group to yield C18-aldehyde.
<i>CYP19A1</i>	CYP19A1	P450aro Aromatase	It is localised in the endoplasmic reticulum and expressed in Leydig, Sertoli, granulosa cells, adipose tissue, brain and placenta. It converts C19 androgens to C18 estrogens through three sequential reactions: two oxidations at C19 followed by an oxidative aromatisation with C1 β hydrogen abstraction C10-C19 cleavage.
<i>FDX1</i>	FDX1	Adrenodoxin Adrenal ferredoxin Ferredoxin-1	Expressed in the adrenal cortex, gonads, and kidneys. It is localised in the mitochondria matrix and is an electron transfer protein, transferring electrons between CYP11A1 and adrenodoxin reductase.

Gene	Protein Name	Synonyms	Protein function
<i>FDXR</i>	FDXR	Adrenodoxin oxidoreductase Adrenodoxin reductase	Widely expressed including adrenal cortex, gonads, and brain. It is localised in the inner mitochondria membrane and transfers electrons to the mitochondrial P450 enzymes.
<i>STAR</i>	STAR	Steroidogenic acute regulatory protein (StAR)	Transfers cholesterol from the outer mitochondrial to the inner mitochondrial membrane for steroidogenesis to start. Rapid expression controlled by ACTH signalling via cAMP ^{67, 68} .
<i>CYB5A</i>	CYB5A	Cytochrome b5 (microsomal)	Small, membrane-bound (endoplasmic reticulum), heme-containing protein with diverse function. Catalyses conversion of C21 to C19 steroids together with P450c17 in androgenic tissues (zona reticularis and testicular Leydig cells). Interacts with P450 enzymes allosterically but also through electron transfer through POR ^{58, 60, 69} .
<i>POR</i>	POR	P450 oxidoreductase NADPH-cytochrome P450 reductase	Localised in the endoplasmic reticulum, it transfers electrons to cytochrome P450 microsomal enzymes and other enzymes such as cytochrome B5 and is required for their catalytic activity ⁵¹ .
<i>AKR1C3</i>	AKR1C3	17 β HSD type 5 Aldo-keto reductase family 1 member C3	Localised in the cytoplasm. Types 3 and 5 convert androstenedione to testosterone and DHEA to androstenediol. Type 3 is present in the testis and type 5 is present in small amount in the zona reticularis. Type 1 converts oestrone to oestradiol and is present in the breast, ovary and adipose tissue.
<i>SRD5A2</i>	SRD5A2	5 α -reductase	Expressed in the endoplasmic reticulum in the skin, testes, and prostate. Converts testosterone to 5 α -dihydrotestosterone.

Figure 2 illustrates the enzymatic steps that make up the steroidogenesis pathway and shows Cholesterol as the essential and parent molecule from which all steroid hormones are synthesised. The transfer of cholesterol from the outer to the inner mitochondrial membrane is a prerequisite step for steroidogenesis to begin and is facilitated by the steroidogenic acute regulatory protein (StAR) as cholesterol is a hydrophobic molecule and there is an aqueous barrier between the outer and inner mitochondrial membranes that cholesterol must pass through ^{68,70,71}. It is in the inner mitochondrial membrane where the first modification to the cholesterol molecule occurs catalysed by P450scc or cholesterol side-chain cleavage enzyme; this is the first enzymatic step in steroidogenesis. The pool of cholesterol used for steroidogenesis in the adrenal cells comes from low- and high-density lipoprotein stores, free cholesterol newly-synthesised in the endoplasmic reticulum, and insoluble cholesterol transported on transfer proteins ⁵¹. StAR is essential for the transfer of cholesterol from the outer mitochondrial membrane to the P450scc and without its action steroidogenesis cannot begin however a small low-grade cholesterol influx to the mitochondria occurs independent from StAR allowing for a low-grade basal steroidogenesis ^{68,72}. StAR has a C-terminal that is responsible for cholesterol binding and lies in the cytoplasmic aspect of the outer mitochondrial membrane and a N-terminal that is responsible for mitochondrial import and transfers one cholesterol molecule at a time ^{73,74}. The C-terminal is essential for the function of StAR and steroidogenesis but the N-terminal affects functionality less as truncated forms of StAR at the N-terminal do not alter steroidogenesis in cultured cells ^{73,75}.

There is tissue-specific variation in the expression of steroidogenic enzymes and the biochemical output of the pathway. The essential enzymes are expressed in all steroidogenic cells, P450scc being the most important. The zona fasciculata cells of the adrenal gland express all enzymes that catalyse steps to cortisol production. The zona glomerulosa cells specialise in the production of mineralocorticoids; 17 α -hydroxylase is not expressed in these cells therefore glucocorticoids and sex steroids are not produced and aldosterone is the final product of steroidogenesis ⁵³. Zona fasciculata and zona reticularis cells do not express P450c11AS (Aldosterone synthetase) and do not produce aldosterone. In the gonads, testicular (Leydig) and ovarian (thecal) steroidogenic cells express P450c17 but not P450c21 and P450c11 β therefore cannot produce mineralocorticoids and glucocorticoids, and only produce the androgens testosterone and androstenedione ⁷⁶. Leydig, Sertoli and ovarian granulosa

cells express aromatase (P450aro) that catalyses the formation of oestrogens from androgens.

Once cholesterol is transferred to the inner mitochondrial membrane, P450scc removes the 6-carbon side chain from the cholesterol molecule to form pregnenolone, a 21-carbon steroid through three enzymatic reactions; a 20-hydroxylation, a 22-hydroxylation and cleavage of the C20-C22 carbon-carbon bond. Pregnenolone, 17 α -hydroxy-pregnenolone and dehydroepiandrosterone (DHEA) are Δ 5 steroids as they retain a double bond between carbon atoms 5 and 6 of the cholesterol B-ring and have no hormonal biological activity. The enzyme 3 β -hydroxysteroid dehydrogenase type 2 (3 β HSD2) converts the Δ 5 steroids to the corresponding Δ 4 steroids progesterone, 17 α -hydroxyprogesterone and androstenedione, which are biologically active. These reactions occur in the mitochondria (pregnenolone) or the cytoplasm (17 α -OH-pregnenolone and DHEA), therefore the localisation of 3 β HSD2 in the cell and the chaperone proteins that regulate its localisation may influence the amount of steroids produced during steroidogenesis in the cell ⁵¹. P450c17 (17-hydroxylase) is encoded by the *CYP17A1* gene and the enzyme produced has a combined 17-hydroxylase and 17,20-lyase activity and catalyses the production of 17 α -hydroxy-pregnenolone from pregnenolone and 17 α -hydroxy-progesterone from progesterone with equal efficiency ^{58, 77, 78}.

In the zona glomerulosa progesterone is converted to 11-deoxycorticosterone by P450c21 (21-hydroxylase) that also catalyses the production of 11-deoxycortisol from 17-hydroxyprogesterone in the cells of the zona fasciculata and zona reticularis. Then, 11-deoxycorticosterone and 11-deoxycortisol transfer to the inner mitochondrial membrane, where they undergo 11-hydroxylation to form corticosterone and cortisol, the final product of glucocorticoid synthesis. In the zona fasciculata the reaction is catalysed by the mitochondrial enzyme P450c11 β whereas in the zona glomerulosa it is catalysed by mitochondrial enzyme P450c11AS or aldosterone synthase which has 11-hydroxylase, 18-hydroxylase, and 18-methyloxidase activity and catalyses the final three steps to the production of aldosterone, the most potent mineralocorticoid ⁵¹.

In the androgenic tissues, such as the zona reticularis and testicular Leydig cells, the 21-carbon steroid precursors 17-hydroxypregnenolone and 17-hydroxyprogesterone

are converted to 19-carbon androgens DHEA and androstenedione respectively by the 17, 20-lyase activity of P450c17. 17,20-lyase catalyses the cleavage of the C17-C20 carbon bond to yield C19 sex-steroids. The reaction catalysed by 17,20-lyase leading to DHEA production is about 50 times more efficient than the reaction leading to androstenedione production; DHEA is produced abundantly in the adrenal as 17-hydroxy-pregnenolone is the preferred substrate for 17,20-lyase and significant androstenedione levels are produced mainly when 17-hydroxyprogesterone concentrations are very high such as in Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency^{58, 60}. The 17,20 lyase activity of P450c17 is specific to androgenic tissues and is promoted by an abundance of electron-transfer protein POR, the phosphorylation of P450c17 and the interaction of P450c17 and cytochrome b5^{60, 79-81}. DHEA and DHEAS concentrations reflect the activity of steroid production from the zona reticularis. DHEA levels increase as a response to ACTH signalling whereas DHEAS, which is protein-bound, shows stable concentrations but overall much higher than DHEA (100-1000 times); both increase rapidly before the onset of puberty however they have no known hormonal action or receptor and exert indirect endocrine effects following biochemical transformation to sex steroids and activation of the androgen and estrogen receptors⁵¹.

The final steps in the synthesis of sex steroids include the conversion of DHEA to androstenediol and androstenedione to testosterone which is catalysed by 17 β HSD; type 3 is present in the testis and type 5 is expressed in low levels in the zona reticularis allowing only a small amount of the potent androgen testosterone to be produced in the adrenal⁸². In extra-adrenal tissues such as the ovarian granulosa cells and adipocytes, P450aro (aromatase) catalyses the production of oestrogens using androgens as substrates; androstenedione is converted to oestrone and testosterone to oestradiol and in oestrogenic tissues (ovary, breast, adipocytes) 17 β HSD type 1 converts oestrone to oestradiol¹⁴.

In addition to the classic steroidogenesis pathway described above, there are alternative biochemical pathways that contribute to a significant amount of production of potent androgens using early steroid precursors as substrates⁸³. 11-hydroxylation of adrenal androgens catalysed by P450c11 β and driven by ACTH produces 11-oxygenated C19 androgens that can activate the androgen receptor⁸⁴⁻⁸⁶. 11-Oxygenated androgens, are potent androgens and are significantly elevated in

patients with CAH due to 21-hydroxylase deficiency and correlate with clinical markers of poor disease control ^{86, 87}. In particular 11-ketotestosterone is the main circulating androgen in women and children suffering from the genetic deficiency ⁸⁸. Furthermore, the 'backdoor pathway' uses 17-hydroxyprogesterone as substrate and produces the potent androgen dihydrotestosterone through androsterone catalysed by type 1 5 α -reductase, 3 α -HSD, and P450c17 and androstenediol catalysed by 17 β HSD3/5 without the intermediates DHEA, androstenedione, and testosterone ⁶². These pathways are very active when there is substrate abundance such as in genetic deficiency of 21-hydroxylase and could contribute to the development of some clinical features of the condition, such as testicular rest tumours and the masculinisation of female fetuses ^{87, 89, 90}.

3.1.1.4. Regulation of cortisol secretion: HPA axis and regulation of steroidogenesis

3.1.1.4.1. HPA axis

The production of cortisol is tightly regulated by centres in the hypothalamus and pituitary that together with the adrenal cortex form the hypothalamus-pituitary-adrenal axis. This is a stress-responsive dynamic system, the anatomic components of which lie in the hypothalamus, pituitary or hypophysis, and adrenal glands.

The hypothalamus is a small and important area of the brain, evolutionary conserved and essential for life, that co-ordinates neuroendocrine, autonomic and behavioural responses to environmental and intrinsic stimuli. The hypothalamic neurones are organised in nuclei that specialise in important survival functions such as regulating sleep, arousal, temperature, feeding, drinking, rivalry, and reproduction; the function of the hypothalamic nuclei is to receive stimuli from the cortex and other parts of the central nervous system, internal and external stressors and regulate responses through neuronal connections, and release of hypothalamic factors to the bloodstream or the cerebrospinal fluid ^{91, 92}. Nuclei important for the regulation of HPA axis are the

suprachiasmatic nucleus that controls circadian rhythms and regulates the pattern of production and secretion of hypothalamic factors such as the corticotropin-releasing hormone (CRH) and vasopressin (AVP), and the supraoptic and paraventricular nuclei which contain neurons that produce AVP and CRH ^{92, 93}.

The pituitary gland is about 1cm in diameter and is located in the sella turcica of the sphenoid bone and connects to the hypothalamus with a thin streak of tissue, which is the pituitary stalk or infundibulum. The anterior part of the gland is called the adenohypophysis and contains hormone-producing cells; the lactotroph, somatotroph, thyrotroph, gonadotroph, and corticotroph cells that produce and release ACTH. The posterior pituitary (neurohypophysis) contains the bodies of the hypothalamic neurons that produce and store the hormones AVP and oxytocin ¹⁴. The pituitary has an extremely rich blood supply made up of the inferior and superior hypophyseal artery but particularly important for the vascular supply and function of the anterior pituitary are the portal veins ¹⁴. These are capillaries that spread from the level of the hypothalamus to the pituitary and provide the means for vascular transport of hypothalamic factors such as CRH that are released by the hypothalamic neurons to the anterior pituitary where they regulate the function of the anterior pituitary and the release of pituitary hormones.

The adrenal gland is a small pyramidal structure that lies above each kidney surrounded in a capsule of connective tissue. The adult adrenal gland is made of two areas with distinct physiological function and structure: the cortex and the medulla. They derive from separate embryonic tissues; the adrenocortical cells from the mesoderm and the adrenal medulla from the neural crest ⁴⁹. The adrenal cortex is about 2 mm thick and contains specialised cells arranged in three concentric bands that express steroidogenic enzymes and synthesise steroid hormones and the adrenal medulla cells produce catecholamines ⁹⁴. Steroidogenesis starts early in fetal life; at 6-7 weeks the fetal adrenal cortex is formed, at 8-10 weeks it produces cortisol transiently, and at 12 weeks it starts producing high amounts of DHEA and DHEAS that fall rapidly after birth ⁴⁹. However, fetal adrenal steroidogenesis is not essential for fetal development and survival ⁵¹. By pre-puberty the adrenals mature in structure and the three concentric zones of steroid producing cells become distinct; a narrow outer zona glomerulosa containing small columnar cells with a few lipid droplets arranged in small nests, a wide middle zona fasciculata containing polygonal cells with prominent

lipid-rich vacuolated cytoplasm arranged in vertical columns, and an inner zona reticularis with dark-stained cells due to presence of lipofuscin granules ^{49, 94}.

Hypothalamic neurons of the paraventricular nucleus secrete CRH and AVP in response to the oscillations of the central pacemaker in the suprachiasmatic nucleus, stressors, and other central stimuli ⁹⁵. CRH, a 41-amino acid peptide hormone and AVP, a 9-amino acid oligopeptide hormone stimulate the pituitary corticotroph cells to secrete ACTH; CRH is the main signal and AVP potentiates its function ⁹⁵. CRH released by the hypothalamic neurones travels to the pituitary and exerts its biological actions through binding to CRH-R1, a G-protein-coupled receptor on the cell surface of the pituitary corticotroph cells ⁹⁶. When CRH binds to CRH-R1, it leads to a conformational change of the receptor and activation of a stimulatory G-protein alpha subunit (Gas) ⁹⁷. The downstream intracellular signalling is mediated through cAMP and protein kinase A signalling pathways and results in transcription of the pro-opiomelanocortin (*POMC*) gene and release of ACTH in the circulation ^{96, 97}.

POMC codes for pro-opiomelanocortin (*POMC*), a 266-amino-acid precursor polypeptide and upon cleavage gives ACTH, a 39-amino-acid polypeptide, and β -lipotropin. Further cleavage of ACTH gives α -melanocyte-stimulating hormone (α -MSH) among other *POMC* products ^{14, 98}. ACTH production and release in the circulation follows a circadian rhythm with ultradian pulsatility with nadir levels between midnight to 2am, peak on awakening and gradual fall throughout the day and this circadian rhythmicity is regulated by the master clock in the suprachiasmatic nucleus through vasopressin ⁹⁹. There are about 20 pulses of ACTH secretion in the 24-hour day and the amplitude of these pulses also follows a circadian rhythm with larger pulses in the morning ^{14, 100}. The generation of the ultradian pulsatile pattern of ACTH and cortisol release (see 3.1.1.5 below) is likely determined in the sub-hypothalamic level by the interaction between cortisol release in response to ACTH in the adrenals followed by a delayed negative feedback of cortisol-GR α signalling to the anterior pituitary ^{93, 101, 102}.

ACTH released by the anterior pituitary into the systemic circulation acts at the adrenal cortex by binding to the highly tissue-restricted melanocortin type 2 receptor (MC2R), a G-protein-coupled receptor, on the cell membrane of adrenocortical cells of zona

fasciculata and reticularis to stimulate the transcription of genes involved in the steroidogenesis pathway through mainly a cAMP-dependent protein kinase A signalling pathway¹⁰³. Cortisol, an end product of the steroidogenesis pathway is then released to the circulation within a few minutes of ACTH stimulation and travels to target tissues and cells where it exerts its biological effects through binding to the GR α ¹⁰¹. Central centres in the hippocampus, hypothalamus, and the corticotroph cells in the pituitary gland also express GR α and at these central centres the cortisol-GR α complex translocate to the nucleus and bind to regulatory areas on the DNA to inhibit *POMC*, *CRH* and *AVP* mRNA and therefore reduce ACTH secretion in a typical negative feedback pathway.

3.1.1.4.2. The regulation of steroidogenesis in the adrenal gland

Steroid hormones are not stored in the adrenocortical cells and are produced *de novo* and released when needed and ACTH is the signal for the secretion of glucocorticoids and adrenal androgens¹⁴. The regulation of glucocorticoid synthesis is through the HPA axis and specifically by two different actions of ACTH; an acute response and a chronic response. Through the cortisol-mediated central effects to the hypothalamus and pituitary (negative feedback pathway) the end product of the pathway participates in the dynamic control of the system by inhibiting the effect of ACTH.

The acute response regulates substrate supply and is through non-genomic mechanisms. ACTH causes rapid hormone-dependent mobilisation of cholesterol molecules from lipid droplets to the inner mitochondrial membrane over 15-60 minutes^{51, 74, 103, 104}. Cholesterol mobilisation to the inner mitochondrial membrane is essential for steroidogenesis reactions to start and ACTH drives this through an increase in StAR protein expression mediated by cAMP and PKA signalling and post-translational phosphorylation and phosphorylation of the hormone sensitive lipase (HSL)^{67, 68, 70, 105, 106}. The acute response to ACTH is rapid and very efficient in producing cortisol in response to stress in the adrenal cortex^{53, 68, 70, 107}. The pulsatile pattern of ACTH secretion is critical to the acute effects on inducing steroidogenesis^{93, 108}.

The chronic response to ACTH is through the trophic effect of ACTH to the adrenal tissue causing hypertrophy and hyperplasia over months, and by induction of transcription of steroidogenesis enzymes' genes and *MC2R* over hours and days^{51, 53, 109}. ACTH causes an increase in the expression of all cytochrome-450 steroidogenic enzymes and electron-donor proteins through cAMP signalling within a few hours after stimulation (4 hours in cultured bovine adrenocortical cells)^{53, 109}. Furthermore, ACTH promotes expression of LDL receptors and HMG-CoA reductase and is essential for the expression of enzymes (17 α -hydroxylase) and factors such as the steroidogenic factor 1 (SF1) that promote differentiation and induce growth of the steroidogenic cells^{14, 53, 110}.

Together with ACTH, other factors have a role in the regulation of adrenal steroidogenesis. In the zona glomerulosa cells of the adrenal cortex the regulation of mineralocorticoid synthesis is by angiotensin II and potassium that induce StAR and CYP11B2 activity through protein kinase C pathway¹¹¹. There is evidence of a glucocorticoid-induced intra-adrenal negative regulation of steroidogenesis^{93, 112} and during inflammatory stress immune factors may contribute to the regulation of steroidogenesis¹⁰⁰. Prolactin, a polypeptide hormone secreted by the anterior pituitary, has varied effects on gonadal steroidogenesis and has also been investigated as a regulator of adrenal steroidogenesis as prolactin receptors are present in all three zones of the human adrenal cortex and stimulation with prolactin increased release of cortisol, aldosterone and sex steroids in human adrenal primary cultures¹¹³⁻¹¹⁵.

3.1.1.5. Physiological cortisol secretion

Cortisol is produced when needed from the adrenal cortex and is released to the circulation and not stored. In normal circumstances, cortisol production follows a circadian rhythm pattern with peak levels (around 450 nmol/L) in the early morning about 30min after awakening that reduce during the day to above 100 nmol/L and reach nadir levels (around 50 nmol/L) at midnight^{99, 116, 117}. Superimposed on this, there is an ultradian variability due to pulsatility. There are multiple (15-20) pulses of cortisol secretion throughout the 24-hour day with variable amplitude that decreases during the day and determines the circadian variation of cortisol levels^{32, 93, 118}. Significant

peaks of cortisol secretion are seen after mealtimes, physical or other stress^{99, 116}. The release of ACTH follows a similar pattern with peaks preceding cortisol peaks by about 10 minutes⁹⁹.

Eucortisolaemia is a state of physiological cortisol levels in the blood. It is not a straightforward exercise to define eucortisolaemia and set reference ranges of normal cortisol levels because cortisol secretion and its concentration in the blood depends on many physiological and pathological factors; the time of the day and the presence of stressors, sleep patterns and shift work. There is also significant inter and intra-individual variation of cortisol secretion which is likely due to the time relation of cortisol sampling to the secretion pulses¹¹⁹. Eucortisolaemia has been used in the literature to refer to restoration of cortisol levels following treatment for cortisol excess and in this context the term has been used interchangeably with long-term cure. Many definitions have been proposed in this context; serum cortisol levels within the normal reference range for daytime serum cortisol (220-690 nmol/L or 8-25 mcg/dL and 140-690 nmol/L or 5-25 mcg/dL), normalisation of urinary free cortisol (UFC), or serum cortisol less than 48% of the upper limit of normal (≤ 12 mcg/dl or 331 nmol/L)¹²⁰⁻¹²⁵. Others have defined eucortisolaemia as a combination of clinical resolution of symptoms, restoration of diurnal rhythm of cortisol secretion, normalisation of UFC, suppressibility of cortisol in a dexamethasone suppression test (ONDST) and stimulability with a short synacthen test (SST)¹²⁶. In healthy individuals or patients suspected of adrenal insufficiency eucortisolaemia has been used to indicate a normal response to a stimulatory test.

Hypercortisolaemia is the presence of excessive levels of cortisol in the circulation and hypercortisolism is the effects of chronically elevated cortisol levels in the tissues. Chronic, inappropriate and excessive cortisol levels will eventually cause clinical signs and symptoms characteristic of Cushing's syndrome (see 3.1.2.1), but these can be non-specific in early onset or mild disease. It is particularly important to be able to recognise and define eucortisolaemia in patients with proven cortisol excess that are undergoing medical or other treatments aiming to restore cortisol levels to physiological and in suspected or borderline hypercortisolaemia. On the other end of the spectrum, cortisol deficiency is defined as the presence of excessively low cortisol levels in the blood and this definition is reliant on the time of the day the samples were measured.

On defining physiological cortisol levels there are certain factors that need to be considered. Firstly, there is diurnal variation in cortisol secretion with levels changing throughout the 24-hour day cycle therefore there is not one rigid reference range that can be applied. The physiological cortisol secretion pattern can be described by a number of parameters which are; the time of peak cortisol in the morning, the level of peak cortisol, the duration of the quiescent period when cortisol levels reduce to less than the mean level for the 24-hour period, and the nadir cortisol levels at midnight ¹²⁷. Secondly, there are pulses of cortisol secretion superimposed on the circadian variation, which are affected by physical and mental stressors like eating patterns and the time of the day. For these reasons only broad cortisol reference ranges can be given as the levels are affected by multiple factors. Even with these broad reference ranges, the values are far from rigid in defining normal levels of cortisol and any attempt to classify levels as normal or abnormal should place special consideration on the presence of physical stressors. With the exemption of early morning cortisol values that reflect early peak and late evening values that reflect nadir levels, random cortisol measurements are of very limited use in assessing for eucortisolaemia. Attempts to characterise 24-hour patterns of cortisol secretion offer more information but may be inconsistent as there is significant inter-individual variability of cortisol levels.

Serum or plasma steroid estimations reflect production rates as these compounds are not stored and are released in the circulation as they are produced. Accurate analytic methods have estimated the daily cortisol production rate to be around 5.3–6.1 mg/m²/24hours or 10 mg/24hours and 24-hour cortisol concentration was 180 nmol/L in pubertal males ^{14, 32, 118, 128, 129}. These estimates are lower than older studies that have defined the historical approach to cortisol replacement. Quantification of basal and pulsatile levels of cortisol is currently undertaken in research studies, are not in use in clinical practice and serve as a research tool to study cortisol secretion patterns; the secretion rate of basal cortisol has been found to be 0.1 µmol/L/24hours and 3.5 µmol/L/24hours during pulses ¹³⁰.

3.1.1.6. Assessing physiological cortisol levels

Biochemical assessment of physiological cortisol secretion requires multiple tests that are complementary and test the integrity and function of the HPA axis at multiple levels and includes: 1. Assessment of sufficiency of cortisol levels and diurnal variation with early morning and midnight cortisol levels and ACTH levels (physiological results assumed if agreement in all of the following: morning cortisol levels in the peak reference range as defined by the assay, midnight cortisol levels in the nadir range, ACTH levels in the normal range). 2. Assessment of responsiveness of the adrenal cortex to ACTH stimulation with dynamic endocrine testing, either a short-synacthen test (using synthetic ACTH₁₋₂₄, SST) or an insulin tolerance test (using a stimulation test that causes endogenous ACTH release from the pituitary, ITT) with assessment of baseline and response blood cortisol levels. 3. Assessment of daily production of cortisol with 24hour urine tests measuring free cortisol excreted in the urine or multiple time-point estimation of cortisol in the blood (cortisol day curves or 24hour cortisol profiles) 4. Assessment of response of the HPA axis to negative feedback exerted by glucocorticoids in the hypothalamus and pituitary (suppression tests using the synthetic glucocorticoid dexamethasone).

In the blood, cortisol is measured in the plasma or serum as total or less commonly as free cortisol. Total cortisol is measured as a surrogate of free cortisol, which is the biologically active fraction. Overall there is an equilibrium of free to total cortisol ratios however this is not always the case and total cortisol may not reflect accurately the biologically active cortisol amount in many situations¹³¹. Physiological, pathological conditions and drugs can alter CBG levels in the circulation and affect the total cortisol quantification; conditions that increase CBG levels result in increased total cortisol and conditions that decrease CBG levels cause a reduction therefore CBG levels or the presence of conditions that influence them should be taken into consideration when assessing for cortisol excess or deficiency^{30, 132}. Examples frequently encountered in clinical practice include assessment of acutely unwell and critical care patients who are likely to have a reduction in CBG levels or patients taking oral oestrogens or pregnant women who may have increased CBG levels resulting in falsely normal cortisol results^{28, 131}. Medications also affect CBG levels and the mode of administration matters as well; oral oestrogens increase CBG and total cortisol levels but transdermal oestrogens have minimal effects on CBG and total cortisol due to

higher levels in the hepatic circulation achieved by the oral route and hepatic induction of CBG production^{132, 133}. During stress CBG levels reduce allowing a higher increment of free cortisol levels; for example, free cortisol as a ratio to total cortisol doubles after a SST in normal individuals^{25, 26}. The equilibrium of free to bound cortisol is also affected when the affinity of cortisol for CBG reduces which occurs with rising temperature allowing more free cortisol in febrile conditions¹³⁴. Furthermore, high cortisol levels from endogenous or exogenous origin could saturate CBG binding capacity which is around 400-500 nmol/l when CBG levels are normal and when total cortisol increases above this concentration free cortisol levels increase rapidly and then are rapidly cleared through excretion in the urine^{26, 134-136}.

Cortisol assays are available routinely in secondary care settings and measure the total cortisol in the blood, which is 95% bound to CBG and albumin, and 5% free cortisol^{26, 130}. Free cortisol is present in the saliva and urine and cortisol levels in these samples reflect serum free cortisol and show good correlation with total plasma cortisol¹³⁶. Plasma free cortisol can be calculated based on total cortisol using a simple equation or can be measured directly using complicated laboratory techniques (ultrafiltration, equilibration dialysis or gel filtration), which are time-consuming and difficult to standardise and perform routinely and lack a validated reference range^{27, 137, 138}. Calculated free cortisol based on total cortisol suffers from varying affinity of cortisol to CBG and some reports indicate that it may underestimate free cortisol levels^{138, 139}. Specific sampling methods should be followed for blood and urine tests and without adherence to these methods the results may not be interpretable or accurate and the timing of sampling is usually needed to aid interpretation.

The majority of laboratories in the UK use immunoassays to measure cortisol in the blood, urine or saliva however the gold standard analytical technique is mass spectrometry¹³⁸. Gas chromatography–mass spectrometry (GC-MS) is a highly sensitive, specific and accurate method and is considered the gold standard for steroid hormone analysis; however, it is labour intensive and not considered suitable for a busy clinical laboratory whereas liquid chromatography tandem mass spectrometry (LC-MS/MS) is similarly sensitive and specific and applicable to use in clinical practice¹³⁸. Mass spectrometry techniques report lower cortisol values than many immunoassays^{140, 141}. Chemiluminescence immunoassays are frequently used in the commercial laboratories and most secondary care laboratories and are easily

automated suitable for high throughput clinical laboratories. Immunoassays have good results but are less specific due to competition of plasma proteins with assay antibodies and cross-reaction with other steroids for example synthetic steroids such as prednisolone and steroid precursors such as 11-deoxycortisol in the serum and steroid metabolites in the urine samples ¹³⁸.

Early morning serum/plasma cortisol is used to test for adrenal (cortisol) deficiency. Levels above 500 nmol/L exclude adrenal deficiency although the cut off between assays varies and new assays that suffer less from cross reactivity with cortisol precursors have lower cut off values and local guidelines are followed. Early morning cortisol levels <100 nmol/L strongly indicate adrenal deficiency especially if there are no concomitant steroid medications and night-day sleep cycle has not been disturbed. Levels in between may indicate adequate cortisol production or partial deficiency and must be tested further with a dynamic test; a short synacthen test (SST) or an insulin tolerance test (ITT) ¹⁴².

Midnight serum cortisol and late-night salivary cortisol tests assess the disturbance of diurnal production, which is characteristic of disorders of cortisol excess. Serum midnight cortisol is a sensitive test but requires admission for at least 48 hours prior to phlebotomy and is therefore inconvenient to perform; a sleeping level above 50 nmol/L (1.8 µg/dL) has 100% sensitivity for the detection of CS ¹⁴³. At a cut-off value of 200 nmol/L (7.5 µg/dL), there is 96% sensitivity and 100% specificity for differentiating between CS and pseudo-Cushing states ¹⁴⁴.

Free salivary cortisol is used for the assessment of diurnal cortisol secretion in conditions of cortisol excess with late night values (LNSC) showing high sensitivity and specificity ^{145, 146}. LNSC is a non-invasive test that reflects the levels of free cortisol in the blood and convenient for outpatient investigation as it can be performed by the patient in their own environment. It is very useful in differentiating between true cortisol excess and pseudo-Cushing states, and as it is not affected by CBG levels it is a good test to do in pregnancy or in patients taking oestrogens or having other conditions that affect CBG ^{27, 147-149}. This method involves the passive collection of saliva through a straw or swab device and the patients should refrain from eating, drinking, brushing their teeth or smoking for two hours prior to collection. Cross contamination with

steroids (inhaled or oral) and blood in patients with gingivitis is a problem³³. Salivary cortisol assays are widely available with appropriate reference ranges for late night values. The analytic technique needs to be sensitive to very low levels of cortisol and specific to avoid cross-reaction with cortisone and ideally should be LC-MS/MS due to better performance compared with immunoassays^{33, 137}. There is significant inter-patient variability therefore at least 2 samples are needed¹⁵⁰⁻¹⁵². LNSC levels below 2-3 nmol/L are proposed to exclude CS¹³⁷. Salivary cortisone is currently investigated as a better marker for serum free cortisol and one not contaminated by oral hydrocortisone^{153, 154}.

Cortisol day curves (CDCs) have been used in clinical practice to estimate adequacy of medical treatment for Cushing's syndrome and hydrocortisone replacement in adrenal insufficiency¹⁵⁵. There are several protocols which involve measuring multiple (4-6) cortisol levels during a fraction of the 24hour period, a commonly used method involves measuring cortisol levels at 5 time-points starting from 08:00h until 18:30^{156, 157}. The levels are then interpreted against hydrocortisone administration times or an average of all time-points is calculated. CDCs have been validated against daily production rates in eucortisolaemic and metyrapone-treated Cushing's patients with calculated mean cortisol between 150-300 nmol/L corresponding to the normal cortisol production rate¹⁵⁶. CDCs use total cortisol measurements and are therefore affected by CBG fluctuations. They require frequent blood sampling, are labour intensive and need to be undertaken in a hospital setting, and not used routinely by the majority of clinicians.

Urinary free cortisol (UFC) measures the free, unbound cortisol excreted in the urine over 24 hours and this amount is estimated around 60 nmol/day¹⁵⁸. UFC levels are not affected by the diurnal variation of cortisol production as they are 24-hour collections or presence of conditions that elevate CBG (pregnancy, oestrogens) and fluctuations of CBG levels²⁸. Urine samples cannot be used for quantifying pulsatile cortisol secretion or detection of cortisol deficiency and they are not recommended for patients with significant renal impairment. UFC is a standard test for detecting hypercortisolaemia and is an essential part of the biochemical work-up for suspected cortisol excess. There is significant inter-patient variability in UFC results and up to 10% of patients eventually diagnosed with cortisol excess have been shown to have a normal UFC level; therefore, in the assessment of patients for hypercortisolism it is

common practice to analyse multiple UFC collections^{33, 146, 159, 160}. In clinical practice UFC is usually measured by immunoassays however this method suffers from specificity problems due to the presence of many cross-reacting steroid metabolites and other compounds in the urine and levels of free cortisol can be 2-fold overestimated when measured by immunoassays compared with accurate methods such as high performance liquid chromatography or LC-MS/MS^{33, 140, 158, 161}. The addition of chromatography for purification prior to immunoassay improves specificity¹³⁷. There are practical problems affecting accuracy due to incomplete collections and patients may find 24-hour collections troublesome to perform.

The dynamic tests used to assess the integrity of the HPA axis are the ITT or the SST. ITT is the gold standard and has been validated against a physical stressor (elective surgery) however it has multiple contraindications (epilepsy, cardiovascular disease), needs close monitoring and is unpleasant for patients¹⁶². It tests the ACTH secreting reserve at the level of the pituitary and is based on hypoglycaemia-induced ACTH secretion from the pituitary and subsequent cortisol release from the adrenals. The SST is also a good test with peak cortisol results correlating closely to ITT, is simpler to perform and has been adopted as the first line test in everyday clinical practice¹⁶³. It is based on the failure of the adrenals to respond to an acute ACTH stimulus due to adrenal atrophy induced by chronic adrenocorticotrophin deficiency. SST tests the function of the adrenal cortex directly and the pituitary and hypothalamus indirectly and is not useful in the immediate period after acute pituitary or hypothalamic dysfunction causing adrenal deficiency¹⁶⁴. Both tests require trained staff and monitored conditions with ITT being more intensive. Normal values for SST are an increase in cortisol to above 500 nmol/L but cut-offs depend on the cortisol assay used to analyse the samples and the current cut off at Sheffield Teaching Hospitals being 430 nmol/L¹⁶⁵.

The dexamethasone suppression tests are commonly used to assess the loss of negative feedback regulation of the HPA axis and they are useful in assessing for conditions of cortisol excess. There are two tests, the 1 mg overnight test and the 2 mg 48-hour low-dose test. These are based on the principle that administration of exogenous glucocorticoids should suppress ACTH and endogenous cortisol production due to corticotroph cell response to negative glucocorticoid feedback⁹⁹. Both tests can be performed in an outpatient setting. The overnight dexamethasone test (1 mg of dexamethasone at 11 pm and measurement of cortisol early the next

morning) has high specificity and is an excellent screening test. The low-dose dexamethasone-suppression test (0.5 mg of dexamethasone every 6 hours and cortisol measurement at baseline and at 48 hours) has a slightly better performance.

The administration of synthetic glucocorticoids affects the assessment of endogenous cortisol levels not only because of suppression of endogenous cortisol production but also because of interference with analytical methods. Hydrocortisone is measured as cortisol in the blood. Prednisolone cross-reacts with most commonly used commercial cortisol assays therefore measurement of cortisol levels in patients treated with prednisolone is not accurate with standard immunoassays and more accurate analytical methods are needed to increase specificity such as MS ¹⁶⁶. Prednisolone binds to CBG and is affected by CBG fluctuations. Dexamethasone does not bind to albumin nor has any significant cross-reactivity therefore it is possible to measure endogenous cortisol levels accurately in patients treated with dexamethasone.

Cross-reaction of endogenous steroids can also affect cortisol measurement. Cross-reactivity of steroid precursors mainly affects serum and plasma cortisol quantification and cross-reactivity with steroid metabolites mainly affects urine cortisol quantification ¹³⁸. 11-deoxycortisol, a precursor of cortisol is structurally similar to cortisol and cross-reacts in immunoassays and can affect interpretation of the biochemical assessment of the HPA axis in patients with endogenous overproduction of this steroid. Quantification of cortisol levels in samples taken from patients treated with medications that increase the endogenous production of 11-deoxycortisol such as the steroidogenesis enzyme metyrapone may show falsely raised values due to cross reaction when immunoassays are used as the analytic method and this could cloud assessment of HPA axis and underestimate the risk of adrenal insufficiency or response to treatment in these patients. Tetrahydrocortisol metabolites in the urine also cross-react with commonly used immunoassays.

3.1.2. Androgen effects on erythropoiesis

Erythropoiesis is the generation of new erythrocytes to deliver oxygen to the tissues and is a complex process that takes place in the bone marrow and occurs in eight stages. In the early stages, pluripotent haemocytoblast stem cells differentiate into erythroid progenitor cell types and then erythroblasts. The terminal stages of erythropoiesis involve maturation of erythroblasts into anuclear reticulocytes that are released in the circulation and mature into erythrocytes after 24-48hours¹⁶⁷. Decrease in oxygen availability in the blood stimulates the production of erythropoietin (EPO) by the kidneys, primarily in adults, and the liver, primarily in fetuses and newborns. EPO is the key factor regulating erythropoiesis and promotes the differentiation of late erythroid progenitor cells to erythroblasts. Erythrocytes released in the circulation survive for about three months and are then destroyed by macrophages in the spleen and liver and new erythrocytes are produced under EPO control at a steady pace to maintain the necessary amount of erythrocytes in the circulation to deliver oxygen to the tissues¹⁶⁸.

The early stages of erythroid progenitor cell differentiation are regulated by multiple factors including glucocorticoids, insulin growth-factor 1, stem cell factor, interleukin-3 and interleukin-6¹⁶⁹. EPO binds to its receptor (EPO-R) at the cell surface of erythroid progenitors and this initiates downstream signalling including activation of JAK2/STAT5 and MAPK/PI3K pathways that regulate gene transcription to promote differentiation, proliferation and inhibition of apoptosis¹⁶⁸. Maturation of erythrocytes also depends on the availability of iron, B12, folic acid and copper and hormones such as androgens, thyroxin and growth hormone^{170, 171}. Regulators of iron uptake and metabolism also modulate erythroid maturation and examples of these proteins are; the main iron-transport protein transferrin, the central regulator of iron homeostasis hepcidin, and the only known cellular iron-exporter protein ferroprotein¹⁷². Hepcidin is a negative regulator of iron levels and controls systemic iron homeostasis by binding to and inactivating ferroprotein, which exports iron from absorptive enterocytes, macrophages and hepatocytes promoting iron recycling^{173, 174}. Elevated hepcidin reduces iron availability and predispose to 'iron-restricted' erythropoiesis and low hepcidin levels increase iron availability and predispose to iron overload.

Steroid hormones affect erythropoiesis. Patients with cortisol excess present with polycythaemia. Glucocorticoids and bone morphogenetic protein 4 together with hypoxia-induced factor-1 α (HIF-1 α) are important for stress erythropoiesis ¹⁷⁵. Sex steroids and androgens in particular have a significant effect in erythropoiesis and although the mechanism is not clear, it is linked to enhanced EPO action. Androgen therapy likely increases EPO levels, although this is disputed by some studies. Animal studies show increase in EPO levels with testosterone and anabolic androgen therapy and several clinical studies also report an increase in EPO levels in anaemic men and women with the maximum effect seen after few weeks of androgen treatment together with improvement of anaemia ¹⁷⁶⁻¹⁷⁸. The mechanism through which androgens increase EPO production is not known and in contrast to these findings, other clinical studies showed a dose-dependent increase in biomarkers of erythropoiesis, haemoglobin and haematocrit, without changes in EPO ^{179, 180}. Similarly, anti-androgen therapy has been found to reduce EPO production in patients treated with cyproterone but not in men with prostate cancer treated with androgen-deprivation therapy (GnRH agonist leuprolide acetate) and castration-levels of testosterone ¹⁸¹. Additionally, there is evidence of a direct action of androgens on erythropoiesis in the bone marrow; androgens promote erythroid differentiation to erythroblast synergistically with EPO ^{176, 182, 183}, and anti-EPO pre-treatment abolishes androgen-induced erythropoietic effects ^{184, 185}. Androgen treatment in women with breast cancer was associated with an increase in haemoglobin, erythrocytes and haematocrit associated with histological evidence of erythroid cell hyperplasia in the bone marrow ^{186, 187}. Androgens also enhance erythropoiesis by indirect effects on iron metabolism and availability for erythropoiesis such as promoting iron incorporation into erythrocytes and suppressing hepcidin levels and therefore increasing iron availability in a mechanism that is independent on 5 α -reductase activity and conversion to DHT ^{176, 184, 188-192}.

There are sex differences in erythropoiesis with adult men having higher haemoglobin and erythrocyte counts than women and this is likely due to differences in androgen levels ¹⁹³. On the contrary, no sex-specific differences exist in EPO production and levels are similar in men and women ¹⁹⁴. Sex-specific differences in haemoglobin, haematocrit and erythrocyte counts start at puberty when differences in sex steroids are established, are greatest in young adults and are not due to menstrual loss as differences persist throughout adulthood and post menopause, and in women with hysterectomies ¹⁹⁵⁻¹⁹⁸. Women have higher rates of anaemia throughout adulthood and

men with haemolytic anaemias have higher erythrocyte mass than women with the same disease ^{199, 200}.

Several observational studies show a positive correlation of total and free testosterone levels with haemoglobin and haematocrit levels and lower testosterone levels are associated with a risk of anaemia ^{201, 202}. Population studies showed positive correlation of testosterone and haemoglobin or haematocrit in men ²⁰³, and in 905 men and women 65 years-old and above, those with total and free testosterone levels in the lowest quartile at baseline were more likely to have anaemia than those in the highest quartile ²⁰⁴. Interventional studies consistently show that testosterone therapy in men increases haemoglobin and haematocrit ²⁰¹. Endogenous androgen excess such as in patients with CAH is associated with increased erythropoiesis and polycythaemia in patients with poor biochemical control ^{205, 206}.

A dose-dependent induction of erythropoiesis is a well-recognised response to testosterone and androgen therapy and older people and women may be more responsive ^{179, 207}. Polycythaemia is a common side-effect of testosterone replacement in hypogonadal men and usually improves after treatment dose reduction or treatment withdrawal ²⁰⁸. Increase in erythrocyte mass is seen in women with breast malignancy treated with androgens and testosterone and anabolic steroids with androgenic action have been used for treatment of anaemias for decades and improve erythropoiesis in some patients ¹⁹⁰. Prior to the introduction of recombinant human EPO, androgens were used for the treatment of anaemia due to chronic kidney disease and are still used for the treatment of mild forms of acquired aplastic anaemia ^{209, 210}.

In contrast, hypogonadism is associated with reduced erythropoiesis, anaemia and androgen replacement corrects this ^{178, 211, 212}. Bilateral orchidectomy results in a 1.2 g/dl median decrease in post-operative haemoglobin levels ²¹³. Hypogonadism in patients with end stage renal failure predisposes to anaemia refractory to erythropoiesis-stimulating agents indicating that some important effects of testosterone on erythropoiesis are independent to EPO-induced signalling, and could affect iron availability for erythropoiesis ²⁰². Moreover, profound hypogonadism due to androgen deprivation therapy in patients with prostate cancer and normal baseline testosterone levels was associated with a reduction in erythropoiesis and anaemia;

serum EPO levels were unchanged with treatment, however, androgen-derivation therapy induced changes in markers of iron availability and these findings could be explained by drug-induced 'slowing' of bone-marrow erythropoiesis or reduced iron availability or 'iron-restriction anaemia' due to increased hepcidin levels ¹⁸¹.

3.1.3. Disorders of cortisol secretion

3.1.3.1. Cushing's syndrome

Cushing's syndrome (CS) is a group of disorders caused by chronic exposure to excessive levels of glucocorticoids ²¹⁴. The source of glucocorticoid excess maybe endogenous due to overproduction of cortisol by the adrenal cortex or exogenous due to chronic administration of glucocorticoid-containing medicines at supraphysiologic doses, usually prescribed for the treatment of inflammatory conditions. Endogenous glucocorticoid excess is a rare condition with an incidence of 1 per 250,000 population ²¹⁵. In this thesis CS signifies CS due to endogenous glucocorticoid excess.

The biochemical features of CS are hypercortisolaemia, loss of the negative feedback that cortisol exerts on the hypothalamus and pituitary, and loss of the circadian rhythm of cortisol secretion ^{99, 160}. The typical clinical features were first described by Harvey Cushing in the first case report of a woman with CS in 1912 ²¹⁶⁻²¹⁸. Chronic hypercortisolism causes protein wasting, with clinical signs of skin thinning, proximal myopathy, skin striae, and easy bruising. Typically there is an altered centripetal fat distribution due to visceral fat accumulation, glucose intolerance, vascular disease and hypertension, hypogonadism, osteoporosis, life-threatening infections, mental and cognitive changes ^{216, 219-221}. The condition is more prevalent in women and may persist for years prior to presentation especially if hypercortisolaemia is mild in which case patients frequently present with cardiovascular and metabolic complications due to chronic exposure to cortisol excess. Some patients present with acute life-threatening infections, frequently atypical, and this acute presentation is more common when the

circulating levels of cortisol are very elevated; electrolyte abnormalities such as hypokalaemia is also more common with significant hypercortisolaemia due to saturation of the 11 β HSD2 in the kidney which allows excess cortisol to activate the mineralocorticoid receptor ²²². If left untreated, chronic hypercortisolaemia is associated with significant morbidity and mortality usually due to vascular disease or infections ²²³.

CS is caused either because of overproduction of ACTH (ACTH-dependent, 80% of all causes of CS) or because of autonomous overproduction of cortisol from the adrenal glands (ACTH-independent, 20%) (Table 4) ^{14, 215}. In ACTH-dependent CS the cause is usually a corticotroph adenoma of the pituitary gland over-secreting ACTH and driving cortisol production by the adrenal gland and this is called Cushing's Disease (CD) ^{216, 220}. A less frequent cause is ectopic ACTH release from tumours ²²⁴. ACTH-independent CS is usually caused by an adrenal tumour that autonomously over-secretes cortisol escaping from the regulation of ACTH and the HPA axis. The adrenal tumour is most frequently a benign adenoma and less frequently a malignant adrenal carcinoma, or rarely by adrenal hyperplasia conditions.

Corticotroph adenomas are rare intracranial tumours and account for 10-15% of all pituitary tumours ²²⁵. There are almost always benign tumors and very rarely are caused by malignant pituitary carcinomas or pituitary blastomas ²²⁶. The majority (80-90%) are microadenomas having a diameter of less than 10 mm however occasionally can be macroadenomas and very rarely locally aggressive tumours of significant volume exerting pressure or invading surrounding structures such as the optic chiasm and the cavernous sinuses and these aggressive tumours frequently have histological features of Crooke's cell adenomas ²²⁵. Corticotroph adenomas oversecrete ACTH that causes excess cortisol production through induction of steroidogenesis by the adrenal glands. Women are affected more frequently than men.

The pathophysiological mechanisms leading to CD are not entirely clear; however, recent advances in genetics and identification of specific somatic mutations in a large population of patients with corticotroph adenomas have significantly increased our understanding on what triggers corticotroph tumorigenesis. Corticotroph adenomas,

Table 4: Causes of Cushing's syndrome ^{14, 215, 227, 228}

Cause	Pathophysiology
ACTH-dependent (80%)	
Cushing's Disease (65%) <ul style="list-style-type: none">90% microadenomas10% macroadenomas	Corticotroph pituitary adenoma oversecreting ACTH. Genetics: rarely associated with familial endocrine syndromes (MEN1, MEN4, AIP), somatic gain-of-function <i>USP8</i> mutations in sporadic tumours.
Ectopic ACTH secretion (15%)	Paraneoplastic syndrome, secretion of ACTH from tumours of neuroendocrine cell origin. Frequently associated with small cell lung carcinoma, bronchial and thymic carcinoid, gastrointestinal neuroendocrine tumours, medullary thyroid carcinoma, pheochromocytoma.
Ectopic CRH secretion	Rare, paraneoplastic secretion of CRH by a tumour of neuroendocrine cell origin.
ACTH-independent (20%)	
Adrenal adenoma (12%)	Autonomous cortisol production from adrenocortical adenoma cells through overactivation of the cAMP and β -catenin pathways. Genetics: somatic mutations of <i>PRKACA</i> , <i>CTNNB1</i> , <i>GNAS1</i> , <i>PRKAR1A</i> genes.
Adrenocortical carcinoma (5%)	Autonomous cortisol production from adrenocortical carcinoma cells due to overactivation of the β -catenin pathway. Genetics: somatic mutations of <i>CTNNB1</i> gene.
Primary pigmented nodular adrenocortical disease (PPNAD)	Multiple adrenal nodules (micronodular adrenal hyperplasia) with limited pigment, due to overactivation of the cAMP pathway. May be isolated or with Carney complex. Genetics: germline <i>PRKAR1A</i> loss of function mutations, inactivating mutations in phosphodiesterase 11A (<i>PDE11A</i>) gene.
Bilateral macronodular adrenal hyperplasia (ACTH-independent macronodular adrenal hyperplasia)	Multiple adrenal nodules with diameter greater than 1 cm, usually sporadic but rarely familial with autosomal dominant inheritance. There is aberrant G-protein-coupled receptor expression and autocrine ACTH production. Genetics: inactivating mutations of armadillo repeat containing 5 (<i>ARMC5</i>) gene, rarely <i>GNAS</i> mutations and somatic <i>MC2R</i> mutations.
McCune-Albright syndrome	Post-zygotic activating mutations of <i>GNAS</i> gene encoding for the stimulatory alpha subunit (Gsa), which cause overactivation of cAMP signalling.

similar to other pituitary adenomas, are monoclonal tumors arising from a single cell that multiplies to cause tumor growth therefore somatic mutations in the tumour have been explored as the trigger to tumorigenesis ²²⁹⁻²³². Most corticotroph adenomas occur sporadically and only very rarely are part of a familial endocrine genetic syndrome, most commonly due to germline mutations of the tumor suppressor *MEN1* gene that causes multiple endocrine neoplasia type 1 syndrome (MEN1), the aryl-hydrocarbon receptor-interacting protein gene (*AIP*), and the *CDKN1B* gene (or p27/Kip1) that encodes for p27, a cell cycle inhibitor and causes multiple endocrine neoplasia type 4 (MEN4) ²³³⁻²³⁸. Sporadic corticotroph tumours have been found to harbour somatic mutations in genes encoding for GR and GR chaperone protein HSP90, cell cycle proteins and protein regulators, and the Ubiquitin specific peptidase 8 gene (*USP8*) and only rarely in genes that cause CD by germline mutations (*MEN1*, *AIP*, *CDKN1B*) ²³⁹. Of these somatic mutations those affecting the *USP8* gene are particularly frequent and found in about 50% of sporadic corticotroph adenomas, more likely in female patients or microadenomas ²⁴⁰⁻²⁴³. *USP8* gene encodes for a protein member of the ubiquitin-specific processing protease family that targets proteins for ubiquitination, that is targeting proteins for degradation by the endosome-lysosome system through ubiquitin tags ²⁴⁴. *USP8* cleaves the ubiquitin tags (deubiquitination) and is involved in epidermal growth factor receptor (EGFR) trafficking, inhibition of EGFR degradation at the lysosomes, receptor recycling to the cell surface and augmentation of EGFR-induced MAPK signalling leading to high *POMC* mRNA expression ^{240-242, 245-247}.

Multiple mechanisms are most likely involved in the pathogenesis of CD. Excess cortisol production in CD should cause negative feedback at the level of the pituitary and hypophysis and inhibit CRH and ACTH release however there is evidence that there is resistance to this mechanism; although some negative feedback is maintained in ACTH-secreting adenomas it seems the sensitivity is set at a much higher set point ^{219, 248, 249}. Recent studies show that the testicular nuclear receptor, TR4, is overexpressed in corticotroph tumour cells and promotes resistance to the negative glucocorticoid feedback. When ligand-activated GR α translocate to the nucleus of corticotroph cells, GR α interacts with TR4 and this interaction overrides the negative regulation of GR α on *POMC* transcription ²⁵⁰. Recent genetic studies indicate that EGFR signalling, a transmembrane receptor for the epidermal growth factor (EGF), and the ubiquitin system for protein tagging for its degradation have a key role in the tumorigenesis of corticotroph adenomas. EGFR signalling is a powerful proliferation

signal and promotes corticotroph cell proliferation and ACTH secretion by down-regulation of p27/Kip1 gene that encodes for a cyclin-kinase inhibitor protein that regulates cell cycle progression²⁵¹. Aggressive corticotroph tumors have high levels of EGFR/ EGF expression and inhibition of EGFR signalling by gefitinib, an EGFR kinase inhibitor, inhibits *POMC* expression and corticotroph cell proliferation in cell cultures, decreases tumour growth and cortisol levels and improves clinical features in an animal model of CD²⁵¹⁻²⁵⁶. A possible role of sex steroids in the pathogenesis could also be contemplated given the strong female predominance in adults whereas in children presenting with CD before puberty there is a male predominance²¹⁸.

Ectopic ACTH syndrome (EAS) is caused by paraneoplastic production of ACTH from tumours of neuroendocrine cell origin. The tumours that most commonly cause ectopic ACTH production are small cell lung cancer, neuroendocrine (carcinoid) tumours, pheochromocytoma, and medullary thyroid cancer^{215, 257, 258}. There is approximately equal prevalence of the disease in men and women; it is uncommon in children but more frequent in older adults^{222, 258}. The presentation of CS may be different in EAS and CD; in EAS there is often a history of cancer known to cause EAS or a new diagnosis of lung cancer, there is frequently more aggressive disease with higher cortisol levels, hypokalaemia and acute complications of hypercortisolism such as psychosis and severe infections^{221, 259, 260}.

ACTH-independent CS is due to disorders affecting the adrenal glands and most commonly due to tumours of the adrenal cortex. Benign adrenal adenoma is the cause in 60% of ACTH-independent CS followed by adrenocortical carcinoma in 40% of cases. In patients with adrenal carcinoma co-secretion of other adrenal steroids (androgens) is common and may be a prominent feature in the clinical presentation. Overactivation of cAMP (benign disease) and b-catenin (benign disease and ACC) signalling is a pathogenic feature of adrenal CS (Table 4). Benign adrenal adenoma presents with features of CS, usually due to chronic and mild hypercortisolism. Commonly at presentation patients may have hypertension and glucose intolerance or even evidence of vascular disease. A small number of benign adrenal adenomas develop as part of a familial genetic syndrome such as MEN1 or Gardner's syndrome where the genetic culprit is mutations of the *MEN1* gene or germline loss of function mutations of the *APC* gene²²⁸. The majority of benign adrenal adenomas causing CS are sporadic tumours and in 50% harbour somatic mutations of the *PRKACA* gene,

which encodes for a catalytic subunit of protein kinase A and leads to overactivation of cAMP signalling and increased steroidogenesis. Adrenocortical carcinoma is an aggressive disease that mostly affects women and has a biphasic age distribution peaking in the 3rd and 5th decades. It can present with distant metastasis at presentation, features of severe CS or androgen excess and prognosis is usually guarded. Adrenal carcinomas are monoclonal in origin and may be part of a familial neoplasia syndrome such as MEN1, Rubinstein-Taybi syndrome (germline *CREBBP* or *EP300* loss of function mutations), Li-Fraumeni syndrome (germline *TP53* loss of function mutation), familial adenomatous polyposis syndrome (loss of function of the *APC* gene), or Beckwith-Wiedemann syndrome^{228, 261}. Most primary adrenal cancers are sporadic and in 30% harbour somatic mutations of the *CTNNB1* gene encoding for beta-catenin, a protein involved in adrenocortical cell proliferation²⁶². Bilateral macronodular adrenal hyperplasia, previously termed ACTH-independent macronodular adrenal hyperplasia, presents with multiple adrenal nodules and hyperplasia with mild to moderate cortisol excess and is associated with germline and somatic mutations of the armadillo repeat containing 5 (*ARMC5*) gene that encodes for a tumour suppressor protein.

It is important to differentiate the cause of CS in patients presenting with cortisol excess, as the treatment is very different depending on the cause. The prognosis also varies between benign and malignant disease and between benign adrenal and pituitary disease¹⁴⁶. The differential diagnosis requires careful biochemical investigation that follows published guidelines and the first clues for the diagnosis come from the history and clinical examination¹⁴⁶. A careful biochemical investigation is required to confirm hypercortisolism by demonstrating: 1. Loss of negative feedback control. 2. Loss of the circadian rhythm of cortisol secretion. 3. Excessively high cortisol production. The biochemical tests employed for this investigation and the outcomes that confirm hypercortisolism are: 1. Failure to suppress cortisol to less than 50 nmol/L following a dexamethasone suppression test (either the overnight or the low dose two-day test). 2. High midnight cortisol levels in the serum or saliva indicating loss of the circadian pattern of cortisol secretion. 3. Excess cortisol secretion by the adrenal glands demonstrated by quantifying free cortisol excretion in a 24-hour urine collection^{7, 215, 263}. Quantification of plasma ACTH levels then differentiates the causes of hypercortisolism to ACTH-dependent where ACTH levels are high or normal, or ACTH-independent where ACTH levels are low or suppressed^{7, 146, 215}.

Following the confirmation of biochemical hypercortisolaemia and quantification of ACTH the anatomical level of possible disease is imaged with radiological studies of the adrenals (ACTH-independent CS) or the pituitary (ACTH-dependent disease). In cases of ACTH-dependent disease localisation studies and confirmation of pituitary source of excess ACTH may be needed and these are with CRH-stimulation test and inferior petrosal sinus sampling. When EAS is suspected, a whole-body cross-sectional imaging using computerised tomography is needed to look for tumours, but these may remain occult^{7, 215}.

3.1.3.2. Nelson's syndrome

Nelson's syndrome (NS) is caused by corticotroph tumour growth in patients with Cushing's disease that occurs following surgical resection of both adrenal glands. In these patients the bilateral adrenalectomy was performed to treat the hypercortisolism caused by the corticotroph adenoma as surgical removal of the adrenal glands removes the site of production of cortisol and immediately cures the hypercortisolaemia rendering the patient cortisol deficient and in need of cortisol replacement long-term. In a proportion of patients who undergo bilateral adrenalectomy in this context the corticotroph pituitary adenoma may progress further and increase in size causing symptoms due to volume effects on the surrounding neurological structures.

NS may be an incurable condition that can become life threatening and has limited treatment options. It was first described by Nelson in 1958 who described a case series of patients with pituitary adenomas who underwent adrenalectomies and developed deep pigmentation, high ACTH levels, and restricted visual fields due to an expanding pituitary tumour 1-8 years following the adrenalectomies^{264, 265}. The clinical presentation is with hyperpigmentation of the skin in the majority of patients, and symptoms due to mass effects from the expanding pituitary adenoma such as headache, visual field defects, and external ophthalmoplegia. NS develops at mean 15years post-bilateral adrenalectomy (but has developed up to 43 years later), and

mortality is high (12%) with late diagnosis. The corticotroph tumour can be small or large and locally invasive^{266, 267}.

The incidence of NS depends on the criteria set for diagnosis but in case series it is estimated that up to 30% of patients with functioning corticotroph adenomas (CD) treated with bilateral adrenalectomies develop NS eventually^{268, 269}. An increase in the volume of a corticotroph adenoma following bilateral adrenalectomy is common on imaging (MRI) and up to 50% of patients develop NS based on imaging criteria for tumour progression²⁷⁰⁻²⁷². Although it is known that bilateral adrenalectomy can cause NS in patients with CD it is an effective cure for hypercortisolism and is indicated in patients where rapid control of hypercortisolism is needed for acute and life-threatening complications, in women of reproductive age that plan to start a family soon and prefer to avoid medical therapy, and in patients where other treatments for CD have failed to control hypercortisolaemia.

The biochemical hallmark in NS is increasing levels of ACTH following adrenalectomy. There are no universally agreed criteria for the biochemical diagnosis of NS and the criteria used are debated. The following diagnostic criteria are commonly used; 1. an expanding pituitary tumour on imaging compared with pre-adrenalectomy, 2. an elevated plasma ACTH level above 200ng/l 2-hours after the morning dose of glucocorticoid, 3. hyperpigmentation^{268, 273}.

Progressive growth of corticotroph adenomas following bilateral adrenalectomy could be either because of the natural course of the adenoma or the loss of cortisol negative feedback to the pituitary and hypothalamus on ACTH and CRH release following treatment of hypercortisolaemia. Although the sensitivity to cortisol negative feedback is reduced in CD, there is some residual effect and this is withdrawn following adrenalectomy for the treatment of cortisol excess²⁷⁴. Furthermore patients with NS have likely had multiple treatments prior to bilateral adrenalectomy including radiotherapy to the corticotroph adenoma and development of somatic mutations in the corticotroph tumours following radiotherapy, for example in the tumour suppression *p53*, could change the natural course and contribute to the development of more aggressive tumours²⁷⁵. Data also show that expansion of a corticotroph tumour following bilateral adrenalectomy is more likely when there is a residual pituitary

tumour on MRI, an aggressive subtype based on clinical and histology evidence, lack of prophylactic neoadjuvant pituitary radiotherapy at the time of bilateral adrenalectomy, younger age at adrenalectomy, large tumour size at the time of CD presentation, and a rapid rise of ACTH levels in the first year following bilateral adrenalectomy (for example an increase higher than 100ng/l in 1 year could be indicative)^{271, 274, 276}. Hyperpigmentation is a common feature and is due to activation of the melanocortin 1 receptor (MC1R), a transmembrane GPCR receptor in the skin melanocytes that is involved in the regulation of skin and hair colour. High circulating levels of ACTH and other *POMC*-derived melanostimulating peptides bind and activate MC1R leading to release of dark melanin pigment^{277, 278}.

3.1.3.3. Primary adrenal insufficiency

Primary adrenal insufficiency (PAI) is a group of disorders characterised by failure of the adrenal cortex and reduced production of steroid hormones, most notably glucocorticoids and mineralocorticoids, despite normal or increased ACTH stimulation. It was first described by Thomas Addison in 1849 following autopsy studies in patients with anaemia and hyperpigmentation⁸². It is a rare condition with incidence 5-6 per million but life threatening if left untreated²⁷⁹⁻²⁸². It is caused by various disease processes most commonly autoimmune and infective adrenalitis due to tuberculosis or HIV infection (Table 5). Autoimmune adrenalitis is the most common cause in the developed world with an increasing incidence and can be isolated or part of a syndrome affecting other endocrine glands or systems^{279, 281, 283}. Autoimmune adrenalitis occurs in any age but is rare in young children.

In adrenal insufficiency there is reduced production of steroid hormones due to interruption of steroidogenesis or adrenocortical cell failure. Deficiency of mineralocorticoid and glucocorticoid hormones could lead to adrenal crisis with hypotension, intravascular volume depletion and electrolyte abnormalities in periods of stress and illness; this is a life-threatening condition characterised by hypovolaemic shock which could lead to ischaemia and death. Other symptoms include fatigue and lack of energy, anorexia and weight loss, nausea, vomiting and abdominal pain, myalgia, salt craving and postural symptoms²⁸⁴. Patients may have evidence of hyperpigmentation due to compensatory ACTH secretion and stimulation of the MC1R

in the skin melanocytes. There may also be raised creatinine and hypotension exacerbated by standing posture due to volume depletion, hyperkalaemia and hyponatraemia, anaemia, hypoglycaemia, and abnormalities in sexual development due to abnormal secretion of adrenal androgens²⁸⁵.

Table 5: Causes of Primary adrenal insufficiency ^{283, 284, 286, 287}

Autoimmune adrenalitis/ Addison's disease

- Isolated
- Part of Autoimmune polyglandular syndrome type 1 or type 2

Infective adrenalitis

- Bacterial (TB)
- Viral (HIV, CMV)
- Fungal (Candidiasis, histoplasmosis)

Haemorrhage and infarction (affecting both adrenals)

- Sepsis (such as meningococcal sepsis)
- Anticoagulation drugs
- Anticardiolipin syndrome

Genetic

- Congenital adrenal hyperplasia due to defects in steroidogenesis enzyme genes
- Congenital adrenal hypoplasia due to *NROB1* gene mutations (X-linked recessive), IMAGE syndrome (*CDKN1C* gene), deletion of multiple genes on chromosome Xp21,
- ACTH insensitivity syndrome due to *MC2R* mutations, Triple A syndrome
- Keams-Sayre syndrome due to mitochondrial DNA deletions
- X-linked Adrenoleukodystrophy due to *ABCD1* gene mutations
- Wolman's disease

Adrenal metastases

Bilateral, usually from primary in the lung, breast, colon, lymphoma, melanoma

Infiltration

- Haemochromatosis
- Primary amyloidosis
- Lymphoma
- Sarcoidosis

Surgery

Post bilateral adrenalectomy for Cushing's disease, bilateral Cushing's syndrome or bilateral pheochromocytomas

Abbreviations: GC: glucocorticoids, MC: mineralocorticoids, SS: sex steroids

In autoimmune adrenalitis, adrenocortical or 21-hydroxylase autoantibodies initially trigger a mononuclear infiltration of the adrenal cortex consisting of activated lymphocytes, plasma cells and macrophages associated with depletion of suppressor T-lymphocytes and activated T-lymphocytes in the peripheral blood ²⁸³. There is subsequent disturbance and loss of normal cell organisation and architecture with loss of the cortical zonation and adrenocortical cell necrosis. As the disease progresses there are nodules of functioning cells which are eventually destroyed and replaced by fibrous tissues and in the final stages the adrenals become atrophic and small ²⁸³.

The diagnosis of primary adrenal insufficiency is made with biochemical testing of the adequacy of cortisol levels. Low early morning cortisol levels indicate the diagnosis especially if lower than 140 nmol/L and need further evaluation with ACTH levels to exclude a central (pituitary/hypothalamic) pathology and a dynamic test for cortisol secretion. SST using synthetic ACTH₁₋₂₄ (250mcg or lower doses in children) administered intravenously or intramuscularly confirms the diagnosis; in primary adrenal insufficiency basal and stimulated cortisol levels are suboptimal and ACTH is usually above 100ng/L or two times the upper limit of normal for the assay used ^{284, 286, 288, 289}. The stimulated cortisol level cut off in the SST depends on the local assay but overall levels above 500 nmol/l (18mcg/dl) at 30 or 60 min exclude AI ^{284, 286, 290}. UFC levels are not useful for diagnosis of adrenal insufficiency due to low sensitivity ¹⁶⁴. The mineralocorticoid deficiency is demonstrated with raised renin levels; aldosterone is low or low-normal ²⁸⁹. Adrenal androgen deficiency may exist and is evident by low DHEA levels.

The age and features at presentation are important clues for diagnosing the cause of PAI. When patients present with features of congenital adrenal hyperplasia including hyperandrogenaemia or virilisation at birth or young age the evaluation seeks to identify the enzymatic block with biochemical analysis of precursor steroid levels and genetic tests. In autoimmune adrenalitis of recent onset adrenal cortex autoantibodies or autoantibodies against 21-hydroxylase are detectable in the blood and patients are screened for concurrent autoimmune conditions such as hypothyroidism, diabetes, and gonadal failure due to high incidence of other autoimmune conditions in patients with PAI ^{283, 291}. Boys and young men with no obvious cause are tested for the rare X-linked adrenoleukodystrophy by assessment of very long chain fatty acids ^{284, 292}. Imaging tests show atrophic adrenals in autoimmune disease but bulky in infective

causes with or without calcifications and with structural findings such as bilateral masses in metastatic disease ^{283, 293, 294}.

3.1.3.4. Congenital adrenal hyperplasia

Congenital adrenal hyperplasia (CAH) is a group of genetic disorders with autosomal recessive inheritance. The genetic defect affects one of the steroidogenesis enzymes causing significant loss of enzyme functionality. As a result, the enzymatic step(s) of the steroidogenesis pathway catalysed by the affected enzyme is interrupted and there is impaired biosynthesis of steroid hormones by the adrenal cortex ²⁹⁵. Patients who develop the disease inherit two mutated genes, one from each parent (homozygotes) and usually carry a combination of two different mutations (compound heterozygotes). Carriers of the disease have one mutated and one normal allele (heterozygotes) and are often asymptomatic or have mild disease and usually do not need regular treatment.

CAH is one of the most common inherited metabolic conditions with incidence of the commonest mild forms (non-classic CAH due to 21-hydroxylase deficiency) 1:1000 live births ²⁹⁶. Data from national screening programmes from the 1980s show higher incidence in some ethnic groups such as Yupik Eskimos in Alaska (1:282) and La Reunion island (1:214). The worldwide incidence of the severe classic form of 21-hydroxylase deficiency is about 1:13000 to 1:16000 live births for homozygotes, and 1:60 for heterozygotes and a gene frequency of 0.01 ²⁹⁷⁻³⁰⁰. The most commonly affected enzyme is 21-hydroxylase, which accounts for up to 90-95% of cases in the UK. The second most common cause is 11 β -hydroxylase deficiency caused by gene defects in *CYP11B1*. Rarer causes affect the enzymes 3 β -hydroxysteroid dehydrogenase, 17 α -hydroxylase/ 17,20-lyase deficiency, electron donor enzyme P450 oxidoreductase (Table 6). Rare CAH cases occur more commonly in consanguineous families ³⁰¹.

Table 6: Causes of Congenital adrenal hyperplasia ^{69, 78, 299, 302-307}

Gene defect	Enzyme affected	Steroids	Biochemical diagnosis
CYP21A2	21-hydroxylase	SS ↑ MC ↓ GC ↓	<ol style="list-style-type: none"> 1. High 17OHP and adrenal androgens (DHEA, A4, T) 2. High renin and low aldosterone 3. Hyperkalaemia, hyponatraemia 4. Low cortisol
CYP11B1	11β-hydroxylase	SS ↑ MC: Aldo ↓, precursors ↑ GC ↓	<ol style="list-style-type: none"> 1. Raised DOC, 11-deoxycortisol, androgens 2. Hypokalaemia 3. Suppressed renin 4. Low cortisol
HSD3B2	3β-Hydroxysteroid dehydrogenase	SS ↓ MC ↓ GC ↓	<ul style="list-style-type: none"> • High 17-pregnenolone and DHEA, low A4 and T • Classical form: Low aldosterone Low cortisol Hyperkalaemia, hyponatraemia and metabolic alkalosis
CYP17A1	17α-Hydroxylase/ 17,20-lyase	SS ↓ MC ↑ GC ↓	<ul style="list-style-type: none"> • Raised precursors: progesterone, DOC, corticosterone and DOC metabolites • Hypokalaemia and metabolic alkalosis
STAR	Steroidogenic acute regulatory protein deficiency	SS ↓ MC ↓ GC ↓	<ol style="list-style-type: none"> 1. Low androgens: DHEA, A4, T 2. High renin, low aldosterone <p>High ACTH, low cortisol</p>
CYP11A1	Cholesterol side-chain cleavage enzyme	SS ↓ MC ↓ GC ↓	<ol style="list-style-type: none"> 1. Low androgens: DHEA, A4, T 2. High renin, low aldosterone 3. High ACTH, low cortisol
POR	POR deficiency	SS ↓ GC ↓	<ul style="list-style-type: none"> • Low androgens • Suboptimal SST
CYB5A	Cytochrome b5	SS ↓ MC/ GC Normal	<p>Isolated 17, 20 lyase deficiency:</p> <ul style="list-style-type: none"> • Low DHEA, A4, no response to HCG test for T and A4 • Normal cortisol and aldosterone • Methemoglobinemia

SS: sex steroids, MC: mineralocorticoids, GC: glucocorticoids, Aldo: aldosterone, DOC: 11-deoxycorticosterone, T: testosterone, A4: androstenedione, SST: short synacthen test, HCG: human chorionic gonadotropin

Most patients with CAH have a significant reduction in cortisol secretion leading to reduced negative cortisol feedback to the pituitary gland, increased ACTH secretion that drives continued stimulation of steroidogenesis in the adrenocortical cells and overproduction of substrates from earlier steps of steroidogenesis⁵⁴. Precursor steroids could be biologically active and if they accumulate may override the deficiency of the more potent end-steroid products. Accumulation of androgen precursors may feed production of potent sex steroids and lead to virilisation in females. These effects are seen early in life in infancy in the severe forms of enzymatic deficiency but may present later in childhood or early adulthood in milder forms of the disease.

The phenotype in CAH is varied because of the number of hormones that can be affected and their varied effects from salt and water regulation (mineralocorticoids), stress response and glucose homeostasis (glucocorticoids) and sex differentiation and reproduction (Table 7). In general, the enzymatic deficiency causes a block in the production of subsequent steroid hormones and an over-availability of substrates prior to the block and there is a mixed biochemical picture of deficiency of some steroid hormones and excess of others and careful assessment and interpretation of biochemistry points to the enzymatic defect. The clinical presentation depends on the enzyme affected and its residual functionality and different mutations result in different levels of residual enzyme functionality²⁹⁶. There is usually some residual enzymatic activity that allows low-grade steroid synthesis through the enzymatic block. Since the phenotype is primarily depended on the enzyme affected we will explore each defect separately.

21-hydroxylase deficiency

The most common cause of CAH is deficiency of the enzyme 21-hydroxylase which is encoded by the *CYP21A2* gene that accounts for 95% of all CAH cases. The enzyme catalyses the penultimate step for the production of cortisol, which is the production of 11-deoxycortisol from 17-hydroxyprogesterone (Figure 2). The non-classic form of 21-hydroxylase deficiency affects 1:1000 live births and is one of the most common autosomal recessive conditions²⁹⁹. Carrier frequency is high in the general population for 'classic' mutations 1:62 and for non-classic between 1:5 and 1:16^{308, 309}.

The *CYP21A2* gene is the functional gene and there is a pseudogene *CYP21A1P* in close proximity in the genome that shares 98% homology with the functional gene but has accumulated a number of mutations that render it non-functional. The majority of inactivating mutations that cause 21-hydroxylase deficiency are deletions and conversions. *CYP21A2* undergoes high genetic recombination with parts of the pseudogene inserted into the functional gene because of misalignment of sister chromatids during meiosis. This allows transfer of pseudogene mutations to the functional gene and causes the majority of inactivating mutations³¹⁰. Large genetic studies have shown that there is good genotype-phenotype association and the genetic defects can help predict the functionality of the enzyme and the clinical features especially in the severe forms of the disease³¹¹.

Most patients inherit one mutation from each parent and have two different *CYP21A2* mutations that affect the functionality of the enzyme differently (compound heterozygous). It is usually the mutation with the mildest effect on enzyme functionality that defines the phenotype⁵⁴. In complete enzyme function loss or minimal activity (up to 2%) there is deficiency of aldosterone and cortisol and the patients present with the most severe form of the disease, the salt-wasting classic form. When the functionality is up to 10%, aldosterone is usually normal but there is cortisol deficiency and patients present with the simple virilising form²⁹⁶. Mutations that allow higher enzyme functionality are associated with the mild, non-classic form of CAH with either normal cortisol levels or partial cortisol deficiency which may be undiagnosed clinically.

The clinical features and age of presentation are determined by the individuals' genetic makeup. Patients with genetic defects that allow enough 21-hydroxylase residual functionality have the mild form of the disease and present primarily with androgen excess; in these cases, the partial defect of cortisol synthesis leads to ACTH overstimulation of the steroidogenesis pathway that can overcome the glucocorticoid and mineralocorticoid deficiency and cause androgen excess due to increase precursor substrate supply. Simple virilising occurs in 25-30% of patients with classical CAH and salt-wasting in approximately 70% of patients³⁰². Adult patients with congenital adrenal hyperplasia exposed to high sex steroid levels may have short stature and men and women of the severe forms of the disease commonly have subfertility due to a

combination of hormonal factors, anatomical and psychological issues relating to abnormal production of sex steroids^{308, 312, 313}.

Males and females have differences in their clinical presentation as androgen excess affects them differently. In newborn females with the classic form, the presence of ambiguous genitalia at birth is usually the first clinical sign and leads to relevant investigations and diagnosis of cortisol and aldosterone deficiency whereas newborn boys usually present with glucocorticoid deficiency, adrenal crisis and a salt-wasting crisis (low serum sodium, hypovolaemia, hyperkalaemia) or CAH may be unrecognised until later presentation with crisis in infancy³⁰². Ambiguous genitalia in females with classic CAH is variable and spans from clitoral enlargement to fused labioscrotal folds and formation of penile urethra with normal development of female internal genitals which is controlled by the unaffected anti-Mullerian hormone, AMH³⁰². Some females with severe virilisation at birth are assigned male sex and reared as males and diagnosed with CAH later in childhood. Ideally sex assignment at birth in 46XX babies with CAH should follow the biological sex and this approach preserves fertility, however some studies report adult male gender identity and male gender role compatible to gender assignment in patients with simple virilising classic form^{314, 315}.

Females with the non-classical form have less hyperandrogenaemia and milder symptoms than females with the classical form. They are born with normal genitalia however they may develop late menarche and secondary amenorrhoea in adolescence or as young adults, and may develop hirsutism, oligomenorrhoea, features consistent with polycystic ovary syndrome and infertility³⁰². Men with the non-classic form also do not have symptoms at birth but may develop hyperandrogenism later in life with oligospermia in some cases. Following birth there may be progressive virilisation in both sexes including precocious puberty, advanced bone age and epiphyseal development and patients may have no apparent clinical symptoms but may have lower final adult height than their genetic potential, insulin resistance and subfertility^{302, 316-318}.

Subfertility in women is frequent and induced by elevated sex steroids. The mechanisms include anovulation due to interference with the hypothalamic-pituitary-gonadal axis and gonadotropin (LH) release, menstrual irregularities, and inhibition of

implantation^{308, 319, 320}. Furthermore, over two-thirds of women with virilisation at birth have had genital reconstruction surgery and have structural abnormalities in the genital tract making intercourse difficult, have low numbers of sexual partners and don't pursue fertility^{312, 317}. In poor disease control, ovarian tissue damage can occur due to the development of adrenal rest tissue and high 17-hydroxyprogesterone and progesterone levels have a contraceptive-type effect on the endometrium increasing cervical mucous thickness and inhibiting implantation. Subfertility in men may be due to elevated adrenal androgens and secondary gonadal failure either as a direct effect or following aromatisation of androgens to oestrogens^{308, 313, 321}. Additionally, the presence of testicular adrenal rest tissue, which are benign lesions found in 69% of men with CAH can impair fertility; progression of TARTs causes blockage of seminiferous tubules and obstructive azoospermia and subsequent Leydig cell failure³¹³.

11 β -hydroxylase deficiency

This is the second most common cause of CAH affecting about 5% of patients with CAH or 1 in 100,000 births and is more common in some populations such as Moroccan Jews due to the presence of founder mutations^{299, 308}. The genetic mutation affects the gene *CYP11B1* and its gene product enzyme 11 β -hydroxylase which is located in the inner mitochondrial membrane and catalyses the final step of cortisol production which is the hydroxylation of 11-deoxycortisol to form cortisol. It also affects mineralocorticoid synthesis blocking the conversion of 11-deoxycorticosterone to corticosterone but does not interfere with androgen production. As a result, the precursors 11-deoxycorticosterone and 11-deoxycortisol accumulate, aldosterone and cortisol production is reduced, and there is an increase of synthesis of adrenal androgens due to substrate availability and ACTH drive of steroidogenesis due to low cortisol.

The majority of genetic mutations involved are missense, nonsense, and small deletions and insertions and affect electron transfer, binding to adrenodoxin and substrate binding^{301, 308, 322-325}. Very rarely unequal crossing-over of *CYP11B1* and *CYP11B2*, which encodes for aldosterone synthase or P450c11A5, creates a chimeric *CYP11B2/CYP11B1* gene that causes 11 β -hydroxylase deficiency²⁹⁹.

The condition clinically presents with symptoms and signs of mineralocorticoid and androgen excess and cortisol deficiency but the phenotype is variable ³⁰³. The mineralocorticoid effects, are due to the accumulation of the aldosterone precursor 11-deoxycorticosterone that has significant mineralocorticoid activity and causes volume expansion, hypertension and hypokalaemia in the majority of patients and usually from early age in childhood ³²⁶. Androgen excess causes ambiguous genitalia in the newborn female and postnatal virilisation in both females and males with premature development of secondary sexual characteristics in the severe classic form ^{301, 303, 325, 326}. Due to the accumulation of corticosterone which has glucocorticoid effects patients may not have pronounced glucocorticoid deficiency ²⁹⁹. Milder forms (non-classic) rarely present later in life with virilisation and infertility in women and precocious puberty in men ^{322, 326}.

3 β -hydroxysteroid deficiency

This is a rare cause of CAH due to mutations of the *HSD3B2* gene, which encodes for the 3 β -HSD isoenzymes type 1 and 3 that are expressed in gonadal and adrenal tissues. Steroidogenesis in both adrenal and gonadal tissues is affected and the enzymatic block is early in the steroidogenesis pathway and affects the production of mineralocorticoid, glucocorticoid precursors and androgens. There is accumulation of pregnenolone, 17 α -hydroxypregnenolone and DHEAS which is converted peripherally to more potent androgens ³⁰². The clinical presentation is of mineralocorticoid and glucocorticoid deficiency and sex steroid excess or deficiency. In severe deficiency both women and men present with ambiguous genitalia; in men this is due to incomplete prenatal differentiation of the external genitalia ³⁰².

Table 7: Summary of clinical features of Congenital adrenal hyperplasia ^{69, 78, 299, 302-307}

CAH	Clinical features Women	Men	Sex reversal
21-Hydroxylase deficiency	<p><i>Severe form, salt wasting:</i> Ambiguous genitalia, precocious puberty, infertility, hyperandrogenism Salt-wasting crisis Adrenal crisis, hyperpigmentation</p> <p><i>Severe form, simple virilising:</i> Ambiguous genitalia, short stature, precocious puberty, hyperandrogenism, infertility Hyperpigmentation</p> <p><i>Mild form:</i> Normal genitalia at birth, amenorrhea, late menarche, clitoromegaly, PCO, precocious puberty, infertility</p>	<p><i>Severe form, salt wasting:</i> Normal genitalia or phallic enlargement, short stature, precocious puberty, testicular adrenal rest tumours, infertility Salt-wasting crisis Adrenal crisis, hyperpigmentation</p> <p><i>Severe form, simple virilising:</i> Normal genitalia or phallic enlargement, short stature, precocious puberty, testicular adrenal rest tumours, infertility Hyperpigmentation</p> <p><i>Mild form:</i> Normal genitalia at birth, precocious puberty, occasionally oligospermia, possible oligospermia</p>	Female to male
11β-Hydroxylase deficiency	Ambiguous genitalia, precocious puberty, menstrual irregularity, infertility Salt retention and HTN Hyperpigmentation	Acne, infertility, phallic enlargement Salt retention and HTN Hyperpigmentation	Female to male
3β-Hydroxysteroid dehydrogenase deficiency ^{g/a}	Phallic enlargement at puberty if genetically male, partial virilisation/ ambiguous genitalia, infertility, precocious puberty Salt-wasting and adrenal crisis (classical)	Ambiguous genitalia, hypospadias, infertility Salt-wasting and adrenal crisis (classical)	Male to female and female to male

CAH	Clinical features		Sex reversal
	Women	Men	
17α-Hydroxylase/17,20-lyase deficiency ^{g/a}	Delayed or absent pubertal development HNT Puberty disorder with normal GC and MC in isolated 17,20-lyase deficiency	Ambiguous genitalia, infertility HTN Ambiguous genitalia and puberty disorder with normal GC and MC in isolated 17,20-lyase deficiency	Male to female
Steroidogenic acute regulatory protein (StAR) deficiency (Lipoid CAH) ^{g/a}	Normal genitalia at birth Absent or delayed pubertal development Low cortisol Salt wasting Hyperpigmentation	Ambiguous genitalia, under-virilisation at birth and no pubertal development Salt wasting Low cortisol Hyperpigmentation	Male to female
Cholesterol side-chain cleavage enzyme deficiency ^{g/a}	Prematurity Normal genitalia at birth Delayed pubertal development Salt wasting Adrenal crisis Hyperpigmentation	Prematurity Ambiguous genitalia, under-virilisation at birth and no pubertal development Salt wasting Adrenal crisis Hyperpigmentation	Male to female
POR deficiency ^{g/a}	Ambiguous genitalia and disturbed pubertal development Possible maternal virilization during pregnancy Possible skeletal malformations (Antley-Bixler syndrome)	Ambiguous genital and disturbed pubertal development Possible maternal virilization during pregnancy Possible skeletal malformations (Antley-Bixler syndrome)	Male to female and female to male
Cytochrome b5 deficiency ^{g/a}	Female genitalia at birth Absent or disturbed pubertal development	Ambiguous genitalia Absent or disturbed pubertal development	Male to female

MC: mineralocorticoids, GC: glucocorticoids, PCO: polycystic ovaries, SST: short synacthen test, HCG: human chorionic gonadotropin, ^{g/a} steroidogenesis is affected in the gonads and the adrenals

17 α -hydroxylase/ 17,20-lyase deficiency

This is the cause of CAH in 1% of cases and affects steroidogenesis in the gonads and the adrenals. The enzyme P450c17 encoded by the *CYP17A1* gene has a combined 17-hydroxylase and 17,20-lyase activity. Reduced 17-hydroxylase functionality causes a reduction in the production of glucocorticoids and androgens but the production of mineralocorticoids is unaffected; in this situation the cortisol deficiency causes an elevated ACTH that drives the accumulation of mineralocorticoids especially 11-deoxycorticosterone and corticosterone that cause hypokalaemia, hypertension, suppressed renin, and metabolic alkalosis ²⁹⁹. Isolated 17,20-lyase deficiency causes reduction in sex-steroids only and cortisol and mineralocorticoid production is preserved ³²⁷.

There is usually deficiency of both the 17-hydroxylase and 17, 20-lyase activity and this is caused by mutations of the gene encoding P450 oxidoreductase (POR) or the *CYP17A1* gene. POR is a protein involved in electron transfer to P450c17 and in addition to the steroidogenesis deficiency clinical presentation includes dysmorphic skeletal features (Antley-Bixler phenotype) ⁷⁸. Very rarely some mutations of the *CYP17A1* gene selectively affect the 17,20-lyase activity and in this case the mutations affect the area of interaction of P450c17 with cytochrome b5 and POR and patients present with isolated sex steroid deficiency and preserved 17-hydroxylase activity. ^{328, 329}.

The classic presentation is with female external genitalia in 46XX and 46XY patients, hypertension, and absence of development of secondary sexual characteristics ²⁹⁹. In the partial forms women are born with normal genitalia but there is failure of development of secondary sexual characteristics, delayed puberty, and hypergonadotropic hypogonadism ^{330, 331} and men are born with under-masculinisation; they have ambiguous or predominantly female genitalia at birth but no internal female organs as AMH from testicles inhibits the development of Mullerian structures, and delayed or absent pubertal development ^{302, 306, 329, 331}.

Steroidogenic acute regulatory protein (StAR) deficiency (Lipoid CAH)

Lipoid congenital adrenal hyperplasia is the most severe form of CAH and is caused by loss-of function mutations of the *StAR* gene. *StAR* is essential for the initiation of steroidogenesis and is responsible for the rapid delivery of cholesterol to the inner mitochondrial membrane to initiate the first step of steroidogenesis, the conversion of cholesterol to pregnenolone catalysed by P450_{scc}^{68, 70, 332}. Mutations of the *StAR* gene block conversion of cholesterol to pregnenolone and significantly impair steroidogenesis of all steroids; there is deficiency of mineralocorticoids, glucocorticoids and sex steroids. The adrenocortical cells develop characteristic histological features due to lipid droplet accumulation. Glucocorticoid deficiency leads to continuous stimulation by ACTH and intracellular accumulation of cholesterol. Only low-level steroidogenesis may continue due to *StAR*-independent cholesterol transfer to the mitochondria and in some cases, this can produce sufficient oestrogens in the ovary to allow development of secondary female sexual characteristics during puberty^{68, 333, 334}. Cholesterol has a toxic effect to the cell and intracellular accumulation eventually destroys all steroidogenic capacity^{335, 336}.

Lipoid CAH is more common in Japanese and Palestinian populations likely due to a founder effect as some mutations occur repeatedly⁷⁵. Genetic defects include deletions, frameshift, missense and nonsense mutations that cause a truncated protein^{75, 304, 334-336}. Mutations resulting in defects to the C-terminal (cholesterol binding site) cause significant loss of enzyme functionality whereas mutations affecting the N-terminal (mitochondrial targeting) enable the retention of some protein functionality and steroidogenic capacity^{73, 103, 332-334}. Mutations of *CYP11A1* were initially thought to be incompatible with life due to the lack of progesterone production from the placenta, which is necessary for the maintenance of pregnancy, however, there have been a few reports of CAH due to mutations of the *CYP11A1* gene causing a truncated non-functional P450_{scc} protein^{305, 337-339}. Patients have similar biochemical and clinical characteristics to Lipoid CAH without adrenal enlargement.

Clinical presentation is with glucocorticoid and mineralocorticoid deficiency within the first postnatal weeks, and hyperpigmentation⁷⁵. Men have ambiguous genitalia at birth due to testosterone deficiency during fetal sexual differentiation, and 46XY individuals may be phenotypically women³⁰⁴. Women are born with female genitalia and may or

may not progress through puberty; as ovarian steroidogenesis starts in the pre-pubertal period; the ovarian tissue is initially spared from damage from the excess intracellular cholesterol^{75, 334, 335, 340}. Some patients have learning difficulties, encephalopathy and developmental delay³⁰⁴.

Diagnosis of CAH

The diagnosis of CAH is made at birth for severe forms and later in childhood or adulthood in milder forms. Usually there is clinical suspicion due to clinical features or a history of gene defect in a sibling or parent, which is then confirmed with biochemical testing. Many countries introduced testing for 21-hydroxylase CAH in national newborn screening programmes aiming for early diagnosis, prevention of adrenal and salt-wasting crisis and correct sex assignment, however, this is not performed in the UK³⁰⁰. These screening programmes collect heel-prick dry blood on day3 of life and analyse for 17-hydroxyprogesterone²⁹⁷. The downside of national screening is picking up premature or ill babies with normal steroidogenesis who have elevated 17-hydroxyprogesterone without a genetic defect and possibly treating them with steroids while awaiting confirmatory testing.

The biochemical diagnosis is based on demonstration of elevated precursors and steroids not affected by the enzymatic block and deficiency of steroids affected by the enzymatic block in the blood or their metabolites in the urine. Stimulation testing with ACTH is particularly helpful in diagnosing mild forms of CAH as random basal hormonal levels in these patients may overlap with physiological outliers which can be genetically unaffected individuals or heterozygotes carriers of one mutation known to cause functional defect in a steroidogenesis enzyme³⁰².

In 21-hydroxylase deficiency the biochemical diagnosis is confirmed with elevated 17-hydroxyprogesterone in the follicular phase or after ACTH challenge. There is an increase of 17-hydroxyprogesterone levels post ACTH challenge and results can be interpreted against a diagnostic normogram³⁴¹. The more severe the loss of enzymatic deficiency the higher the baseline levels of 17-hydroxyprogesterone are and levels above 20,000 ng/dl are found in the classic salt-wasting form, levels between 10,000-

20,000 ng/dl are found in the classic simple virilising form, and lower levels in the nonclassic form (1,500-10,000 ng/dl) ¹⁴⁵. There is also raised progesterone, androgens (DHEA, A4 and testosterone), and renin with low aldosterone, cortisol, and metabolic alkalosis ³⁰².

In 11 β -hydroxylase deficiency the intermediates 11-deoxycorticosterone and 11-deoxycortisol are raised both at baseline and post response to ACTH challenge with high adrenal androgens and low cortisol, aldosterone and renin ^{302, 308}. In urine samples there are high levels of the metabolites of the above precursors, tetrahydro-11-deoxycortisol and tetrahydrodeoxycorticosterone, and high levels of urinary 17-ketosteroids reflecting the raised adrenal androgens in the blood.

Genetic testing is becoming more available and is used to confirm biochemical diagnosis or as first line in cases of screening. Genetic tests are useful for patients with classic and non-classic conditions and their partners. They can also be used for counselling in the context of fertility as the risk of CAH in the offspring can be estimated ³⁰⁸. The genetic diagnosis is complex due to high variability of the genomic region and whole gene sequencing is usually needed to determine accurate genotype ²⁹⁹. Prenatal genetic diagnosis is available when there is a risk of the fetus having the disease.

3.2. Medical treatment and monitoring of disorders of cortisol and corticotrophin excess

3.2.1. Cushing's syndrome

3.2.1.1. Medical Treatment

The only curative treatment in CS is surgical resection of the tissue that is causing the hormonal excess. The result of successful treatment is the reversal of the phenotype and metabolic disturbances however some features take a long time to improve. In CD the first line treatment is surgical resection of the pituitary tumour, usually performed through a trans-nasal approach. Immediate remission rates following pituitary surgery vary and can be up to 80%, however up to 20-50% of patients experience a recurrence of disease even after several years of treatment³⁴²⁻³⁴⁴. In recurrent disease the options for treatment are further pituitary surgery, radiotherapy if there is a suitable anatomic target or medical therapy³⁴⁵. Bilateral adrenalectomy is an effective treatment but with risks and therefore reserved in CD if surgery or radiotherapy have failed or are not possible, in EAS if there is no other treatment and in bilateral adrenal disease causing cortisol excess³⁴⁵.

Medical treatment for CS aims to reduce the hypercortisolism and this is achieved by medications that target the adrenal steroidogenesis (steroidogenesis inhibitors) and medications that target the GR (mifepristone). In CD medications that target ACTH release from the pituitary are also indicated (D2 receptor agonists bromocriptine and cabergoline and somatostatin agonists such as pasireotide that targets SSTR5). In malignant disease the options of medical therapy include the steroidogenesis inhibitor and adrenolytic agent Mitotane in adrenocortical carcinoma and chemotherapy or radiation therapy in EAS^{346, 347}.

Adrenal-targeted therapy for Cushing's syndrome

Steroidogenesis enzyme inhibitors are a group of agents that inhibit various steps of steroidogenesis and cause a reduction in cortisol synthesis. The most commonly used inhibitors metyrapone and ketoconazole have been used for decades in the medical therapy of CS and are still the mainstay of medical therapy however there is lack of evidence from prospective studies on their efficacy and mostly single-site

experience has been reported in retrospective studies. Other agents used for this indication are mitotane and etomidate and there are new steroidogenesis inhibitors in development.

Metyrapone is a potent inhibitor of P450c11 β which catalyses the final step to cortisol biosynthesis and inhibits P450cAS which catalyses the final step leading to aldosterone production to a lesser degree. As a result, the synthesis of cortisol and aldosterone is significantly reduced and there is an increase in cortisol precursors such as 11-deoxycortisol, 11-deoxycorticosterone and androgens which can manifest clinically with hirsutism and virilisation in women³⁴⁸. It has a short action, is absorbed quickly following oral administration and is very effective in reducing hypercortisolaemia³⁴⁹. The starting dose is usually 750-100 mg in divided doses and doses up to 6000 mg can be used although high doses are not well tolerated⁶. A large retrospective single-centre study reported 75% response based on normalisation of cortisol day-curves in 91 patients with CS, predominantly CD³⁴⁹. Overall in the literature there are 200 patients treated with metyrapone monotherapy for hypercortisolaemia due to CS, small studies and case reports, and the overall response rate is 75%^{6, 350, 351}. Metyrapone is the most commonly used medical therapy for hypercortisolaemia occurring in pregnancy.

Side effects of metyrapone are common but usually mild and well tolerated, most frequently gastrointestinal. Accumulation of mineralocorticoid precursors causes fluid retention and hypertension and androgen excess with hirsutism and acne is due to ongoing stimulation of steroidogenesis by ACTH^{6, 350}.

Ketoconazole is an imidazole derivative that was developed as an oral antifungal agent as it inhibits the synthesis of cell membranes sterols^{352, 353}. Ketoconazole causes reduction of cortisol and sex steroids due to inhibition of multiple steroidogenesis enzymes in the adrenals and the gonads; P450c17, P450cAS, P450c11 β , P450scc³⁵⁴⁻³⁵⁸. A large retrospective multi-centre study reported response rates based on normalisation of UFC in 50% of 200 patients with CS and improvement in clinical signs in 40-60% of patients³⁵⁹. Overall in the literature 456 patients with CS received ketoconazole monotherapy and 60% achieved complete biochemical response with normalisation of UFC^{6, 360-364}. Starting dose is 400 mg and this can increase up to 1600

mg in 2-3 divided doses ⁶. Co-administration with medications that increase the gastric acidity such as antacids and proton pump inhibitors reduce absorption and should be avoided ^{365, 366}.

Ketoconazole has been approved for use in hypercortisolaemia due to CS in Europe, however its use as an antifungal agent for skin and nail infections is restricted over concerns of hepatotoxicity including fatal hepatitis ³⁶⁷⁻³⁷⁰. In the largest retrospective study of 200 patients with CS treated with ketoconazole, 20% developed mild to severe liver dysfunction and 8.5% of CS patients who started treatment with ketoconazole in a prospective safety monitoring study; therefore, close monitoring of liver function tests is essential on starting treatment and upon dose titration and some patients may eventually need to discontinue treatment ^{359, 371}. Other side effects include hypogonadism due to inhibition of gonadal steroidogenesis with symptoms reported in men taking ketoconazole ³⁷². Due to the inhibition of gonadal steroidogenesis ketoconazole should be avoided in pregnancy as it can affect the development of male fetuses.

Mitotane (o,p'-DDD) is synthetic derivative of the pesticide DDD and inhibits multiple steroidogenesis enzymes mainly P450scc and also P450c11 β , P450AS and 5 α -reductase and particularly affects production of glucocorticoids and androgens with less effect on mineralocorticoids ³⁷³⁻³⁷⁷. It is a lipophilic agent with slow onset of action and a half-life of several weeks. It accumulates in the adipose tissue causing persistent effects for several months following discontinuation or dose change ³⁷⁸. Its active metabolites cause mitochondrial death and adrenocortical cell necrosis that affects the zona fascicularis and zona reticularis of the adrenal cortex and at high doses it acts as an adrenolytic agent causing non-reversible chemical adrenalectomy ^{379, 380}. For this reason, it has been licensed and used in the treatment of adrenocortical cancer with or without hypercortisolaemia ^{346, 379, 381}. Mitotane causes neurotoxicity and to minimise this, therapeutic levels are monitored during treatment aiming at levels between 8-20 mg/L ^{347, 382}. The anti-tumor effects require higher doses than the steroidogenesis inhibition effects ^{382, 383}. Common starting doses are 1-1.5 g in divided doses that can increase up to 12g however high doses are poorly tolerated ³⁴⁷. High remission rates of 70-80% have been described for patients with hypercortisolaemia due to CD and this response may be achieved after several months of treatment (5-8 months) ^{382, 384, 385}. While the effect of mitotane takes place concurrent treatment with another agent

will provide short-term control of hypercortisolaemia. It is usually given in a block and replace regimen. It causes an increase of CBG levels therefore higher steroid replacement doses are needed ³⁷⁷. Common side effects include gastrointestinal symptoms and hypogonadism due to inhibition of 5 α -reductase and symptoms may improve with dose reduction ^{347, 377, 384, 386}. Severe neurotoxicity is less common but may be poorly tolerated and lead to cessation of treatment ³⁸².

Etomidate is a parenteral anaesthetic agent that has been used in emergency intubations and cardiac surgery due to good anaesthetic safety profile. It has a rapid-onset and short duration of action and the lethal dose is 12 times the dose needed for hypnosis ³⁸⁷. It causes reduction of cortisol production due to reversible inhibition of steroidogenesis mainly due to inhibition of P40c11 β and to a lesser degree P450scc ³⁸⁷⁻³⁹³. As it is a sedative agent it is only administered in secondary care in specialist units with appropriate monitoring when rapid control of hypercortisolaemia or intravenous administration is essential. The dose of the 24-hour infusion is around 1.2-2.5 mg/h and this is adjusted daily based on sedation and serum cortisol ³⁹⁴. The steroidogenic effect is evident in doses lower than those required for the hypnotic effect and more prolonged causing adrenal suppression for several days following prolonged treatment ³⁹⁴⁻³⁹⁶. With prolonged use at high doses there is a risk of propylene glycol toxicity when the aqueous solution containing propylene glycol is used ³⁹⁷.

New steroidogenesis inhibitors are being developed for treatment of hypercortisolaemia and prospective studies underway will provide much needed safety and efficacy profiles. Osilodrostat (LCI699) is a potent inhibitor of P45011 β developed originally as an aldosterone synthase inhibitor for the treatment of hypertension. In pilot studies evaluating its efficacy as anti-hypertensive agent there was clinically significant reduction of cortisol and is being evaluated in patients with CS in a prospective phase III multicentre study ^{398, 399}. In a proof-of-concept study in 12 patients with CD and previous pituitary surgery, Osilodrostat induced normalisation of UFC in 92% of patients at doses 4-100 mg daily with fluid retention and hypokalaemia being the most significant adverse effects due to accumulation of 11-deoxycorticosterone ⁴⁰⁰. The ketoconazole enantiomer 2S,4R (levoketoconazole, COR-003) inhibits steroidogenesis and could have a better safety profile ⁴⁰¹⁻⁴⁰³. It was evaluated as a medical therapy for 94 patients with CS in a prospective phase 3 study administered twice daily to a maximum total daily dose of 600 mg. It was successful in normalising

UFC in 31% of patients with 11% showing biochemical evidence of liver toxicity and was discontinued due to poor tolerance in 13%⁴⁰⁴. Additional to inhibition of steroidogenesis enzymes new molecular targets that reduce steroidogenesis in the adrenals have been discovered and are under investigation. ALD1613 is a long-acting specific monoclonal antibody against ACTH that blocks ACTH-MC2R signalling in the adrenal and reduces glucocorticoid production in two animal models⁴⁰⁵. ATR-101 is a selective inhibitor of Acyl-CoA: cholesterol acyltransferase (ACAT1) that catalyses the formation of cholesterol esters which form the intracellular reservoir of cholesterol in adrenocortical cells, and acts by reducing cholesterol availability in the adrenocortical cells⁴⁰⁶. ATR-101 has been found to induce adrenocortical cell apoptosis, and reduce adrenocortical steroid production and ACTH-stimulated cortisol in an animal model with CS and may provide a therapeutic target for CS, ACC and CAH^{407, 408}.

Pituitary-targeted therapy for Cushing's Disease

These are medical therapies that reduce ACTH production and release from the corticotroph adenoma cells and are effective in CD. The medications act on receptors expressed by the corticotroph adenoma cells such as the dopaminergic (D2) and somatostatin receptors. Overall the efficacy of pituitary-directed treatment is less than the efficacy reported for steroidogenesis inhibitors with reported response rates in about 30% of patients.

Pasireotide is a somatostatin analogue that acts on multiple somatostatin receptor subtypes and especially the corticotroph-expressed SSTR type 5 to reduce ACTH secretion⁴⁰⁹. It has been shown to reduce cortisol secretion in 25-40% of patients with Cushing's disease with improvement of clinical features and is a useful agent in CD with mild hypercortisolaemia⁴¹⁰⁻⁴¹². It has been licensed for CD by the European Medicines Agency for the treatment of hypercortisolaemia in CD. A significant side effect is worsening of glycaemic control or new hyperglycaemia, which occurs in up to 75% of patients and is due to reduction in secretion of insulin from the pancreas and incretin hormones^{411, 413, 414}.

Cabergoline, a dopamine agonist that acts on D2 receptors present on corticotroph cells have been successful in inducing remission in 5-40% of patients with CD in clinical trials, however, some patients eventually escape control ⁴¹⁵⁻⁴²⁰. Cabergoline has been used in doses 1-7 mg/week, lower than the doses used in Parkinson's disease however with long-term high-dose treatment there is the risk of cardiac valvular disease.

Pituitary directed treatments under development include retinoic acid that has been shown to reduce *pomc* transcription and cortisol levels in animal corticotroph tumour models and at doses of 10-80 mg daily was well tolerated and induced biochemical response in 40% of patients with CD in a pilot study ⁴²¹⁻⁴²³. Gefitinib, an inhibitor of EGFR kinase and EGFR proliferation inducing signalling, inhibits *pomc* expression in mice and corticotroph cell proliferation in cell cultures, decreasing tumour growth and cortisol levels with improvement of clinical features ²⁵⁶. Silibinin, a plant extract, is a HSP90 inhibitor and targets GR signalling in the corticotroph tumour cells. It has been found to increase the transcriptional activity of GR by inducing its release from HSP90 in a stable and high-affinity state for ligand binding in murine corticotroph cells restoring glucocorticoid sensitivity. Silibinin has a favourable safety profile and has been effective in improving clinical features and biochemistry in an animal CD model and clinical trials using this agent are underway ^{424, 425}.

Glucocorticoid receptor targeted therapy for Cushing's syndrome

Mifepristone is a progesterone agonist and glucocorticoid receptor antagonist that binds to the GR with higher affinity than cortisol and blocks cortisol-GR α activation. It improves clinical features of CS in 40-87% of patients including hyperglycaemia, weight, insulin resistance, depression and cognition at doses 300-1200 mg/day ^{426, 427}. As it blocks the effects of cortisol at the receptor level, biochemical monitoring with cortisol levels does not correlate with efficacy and objective assessment of response is not possible. Monitoring of glycaemic control in CS-induced hyperglycaemia is an indirect method of monitoring efficacy and it has been used for the management of adrenal and pituitary CS related hyperglycaemia by the U.S. Food and Drug Administration Agency. The main adverse effects are worsening of hypertension and hypokalaemia and unrecognised adrenal insufficiency due to overtreatment ⁴²⁸, and

the effects of anti-progesterone activity with uterine hyperplasia in women seen on long-term treatment.

New glucocorticoid receptor antagonists are under development and relacorilant (CORT-125134), a selective glucocorticoid receptor modulator has improved blood pressure control and Hba1c in patients with CS in a phase 2 study and is currently evaluated in a phase 3 multicentre clinical trial in patients with CS and glucose intolerance and/or hypertension ⁴²⁹.

3.2.1.2. Monitoring of medical treatment

Patients with active CS receiving medical therapy to improve hypercortisolism need close monitoring to establish the effectiveness of treatment, identify any side effects and make appropriate titration decisions. Not all patients respond to the same treatment and to the same dose and some patients may develop side-effects and others may tolerate the same treatment very well. Side effects such as hyperglycaemia may need new medications or modification of medications to address this.

The monitoring is clinical and biochemical and the aim is to try to achieve the balance between clinical improvement in the features of cortisol excess and avoid any symptoms or signs of overtreatment causing cortisol deficiency. The improvement of hypercortisolaemia needs biochemical monitoring and this aids titration decisions. Biochemical monitoring is usually with UFC aiming for normalisation or improvement and cortisol day-curves (CDCs) aiming for mean cortisol levels between 150-300 nmol/L ^{146, 156}. Accurate analytical methods for quantification of cortisol in patients treated with steroidogenesis enzyme inhibitors are essential to minimise cross reactivity with cortisol precursors and cortisol overestimation. This is particularly important for patients treated with metyrapone as high levels of 11-deoxycortisol due to inhibition of P450c11 β cross-react in many commonly used immunoassays and there is documented overestimation of cortisol levels in these patients ⁴³⁰. This could

result in unnecessary up-titration of treatment and hypoadrenalism, which manifests with symptoms overlapping commonly reported side-effects of metyrapone therapy. Mitotane causes an increase in CBG and monitoring response with total cortisol levels is not reliable and UFC collections should be used. Biochemical monitoring with UFCs may miss overtreatment and development of adrenal insufficiency as UFC levels may still lie within the normal range therefore clinical correlation is essential. Patients that receive medical therapy following pituitary radiotherapy may require dose down-titration and eventually stopping therapy after several months once radiotherapy has taken full effect.

Safety blood test monitoring is usually necessary. Patients treated with pasireotide need glucose monitoring and treatment if hyperglycaemia develops, patients on ketoconazole need monitoring of their liver function tests closely at the initiation of treatment and after every dose change. Mitotane treatment needs close monitoring of therapeutic levels to avoid toxicity or inadequate therapeutic effect from undertreatment and thyroid function tests to look for hypothyroidism ^{6, 431}.

Patients with CD patient who have been treated with bilateral adrenalectomy are cured from hypercortisolaemia but need long-term replacement of adrenal steroids with glucocorticoids and mineralocorticoids and clinical and imaging monitoring for progression of any residual corticotroph tumour and development of NS ¹⁴⁶.

3.2.2. Nelson's syndrome

3.2.2.1. Medical Treatment

The first line treatment for Nelson's syndrome is pituitary surgery and radiotherapy or radiosurgery to reduce the tumour burden and activity ^{273, 432, 433}. These treatments offer the possibility of cure but are only suitable where the pituitary tumour is accessible to

surgery or radiotherapy and anatomically distinct from surrounding neurological structures such as the optic chiasm, cranial nerves and the cavernous sinus and this is often not the case²⁷⁴. Patients usually have invasive corticotroph tumours and pituitary surgery in these cases is a palliative procedure for controlling tumour volume^{434, 435}. In suitable patients surgery and radiotherapy are highly effective however there are significant peri-operative complications including a risk of hypopituitarism in 70% which requires long-term hormone replacement and affects the quality of life adversely despite hormone replacement^{274, 435}. Furthermore, some patients may not be candidates for surgery because of medical comorbidities that increase their peri-operative risk.

The remaining option for patients who need to be treated but are not suitable candidates for surgical treatment or radiotherapy is medical treatment. There is no standard medical treatment for NS and various medications have been tested in the past with mixed and frequently negative results. Due to the rarity of the condition most reports are either case studies or small series and frequently retrospective with limited follow-up. The antiepileptic sodium valproate has been tried in the past and is generally not effective⁴³⁶⁻⁴⁴¹. The dopamine agonist cabergoline showed more positive results and has been reported to induce remission of NS in case reports however this effect is inconsistent and there are only occasional responses in the literature⁴⁴²⁻⁴⁴⁵.

The peroxisome proliferator-activated receptor (PPAR) gamma is a nuclear receptor associated with differentiation of the adipocytes that was found to be abundantly expressed in ACTH-secreting corticotroph tumour cells. Agonists of PPAR-gamma are in clinical use as antidiabetic therapeutic agents and one of these agents, rosiglitazone, has been trialled in patients with NS. A case series using rosiglitazone in three patients with NS showed positive biochemical response in one and an initial biochemical response with subsequent relapse in another⁴⁴⁶. Two prospective clinical trials subsequently examined the effectiveness of rosiglitazone in patients with NS and both found no change in biochemical control; 6 patients received 8 mg/day for 12 weeks and 5 patients received 12 mg/day for 8 weeks which are higher doses than those used for hyperglycaemia in type 2 diabetic patients^{267, 447}. Rosiglitazone is therefore not recommended for use in NS. Temozolomide, an alkylating chemotherapeutic agent that crosses the blood-brain barrier, has anti-neoplastic effects through DNA damage and induction of apoptosis and is used to treat

aggressive pituitary tumours and there are case reports of successful tumour and biochemical control in patients with NS and aggressive corticotroph tumour however most patients experience side-effects such as fatigue, bone-marrow toxicity and gastrointestinal symptoms which need to be balanced against benefit and generally limit its use ⁴⁴⁸⁻⁴⁵⁰.

Pasireotide is somatostatin receptor analogue and activates multiple somatostatin receptor subtypes (1, 2, 3, and has high affinity for 5). SSTR5 is selectively expressed in human corticotroph tumour cells and has been found to regulate basal and CRH-induced ACTH release from corticotroph tumour cells in cultures ^{451, 452}. Somatostatin and somatostatin analogues have been found to reduce ACTH and cortisol levels in patients with CD and NS ^{410, 411, 453-455}. In *in vitro* studies treatment of human corticotroph tumour cells with pasireotide reduced ACTH release and cell proliferation ^{451, 456}. It is now licensed for the medical treatment of hypercortisolism associated with CD and but not Nelson's syndrome ⁴⁵⁷.

Pasireotide at a dose 900mcg-1200mcg twice daily s/c has been reported to induce biochemical response and most importantly control tumour volume in a de novo patient with CD due to a pituitary macroadenoma who declined surgery ⁴⁵⁸. A case report described the positive effects of pasireotide treatment in a patient with NS who had multiple previous surgical and radiation treatments for an invasive pituitary tumour who presented with cranial nerve palsy caused by the growing tumour. The patient was treated with monthly long-acting i.m. injections and showed significant improvement of ACTH levels, improvement of skin pigmentation and MRI showed reduction of tumour volume ⁴⁵⁹.

3.2.2.2. Monitoring of medical treatment

The therapeutic target in NS is to replace the adrenal steroids following bilateral adrenalectomy, improve ACTH levels, reduce or stabilise corticotroph tumour volume, and improve skin hyperpigmentation. Therefore, following bilateral adrenalectomy and documented progression of the corticotroph tumour volume with rising ACTH levels patients should have life-long clinical, biochemical, and radiological assessment. Replacement of glucocorticoids and mineralocorticoids is identical to patients with endogenous primary adrenal insufficiency (see section 3.3.1.).

ACTH levels are elevated in NS and are the biomarker used to monitor corticotroph tumour activity and response to treatment⁴⁶⁰. ACTH levels maintain diurnal variation and also fall post glucocorticoid administration therefore for monitoring of disease activity an early morning (08:00H) level 20 minutes before the morning glucocorticoid replacement dose should be taken and a two-hour post glucocorticoid morning dose is also recommended²⁷⁴. There is intra-individual variability of ACTH levels therefore careful consideration is needed before deciding about response to treatment²⁶⁷. Some patients treated with radiotherapy may have some lasting effects on ACTH reduction especially in the first few years after treatment therefore administration of medical treatment during this time needs careful consideration before any decision on response to treatment.

Clinical monitoring includes routine assessment of adequacy of glucocorticoid and mineralocorticoid hormone replacement post adrenalectomy (see section 3.1.1.), assessment for pituitary tumour growth with visual assessment for visual field defects and cranial nerve palsies, and assessment of skin pigmentation. Radiological surveillance for tumour volume is with magnetic resonance imaging and intravenous gadolinium contrast and MRIs is performed every 3-6 months for two years following adrenalectomy and annually thereafter and ideally the scans should be interpreted by a specialist radiologist and images compared with previous examinations²⁷⁴. The size of the adenoma could be measured by maximum diameter or equations that calculated tumour volume on cross-sectional imaging.

3.3. Medical treatment and monitoring of disorders of cortisol deficiency

3.3.1. Primary adrenal insufficiency

3.3.1.1. Medical treatment

The aim of treatment is replacement of the deficient hormones; mineralocorticoids, glucocorticoids, and adrenal androgens. Lifelong replacement with glucocorticoids is essential to restore well-being, reduce mortality and prevent life-threatening adrenal crisis. Mineralocorticoid replacement is essential to avoid salt-wasting crisis; in childhood salt is also essential but is not usually necessary in adulthood and MC dose requirements may decrease in adulthood.

Glucocorticoid replacement is essential to improve symptoms and avoid adrenal crisis during periods of intense physical or even mental stressors. There are various formulations of glucocorticoids that are used for replacement and we can divide these in short-acting formulations and long-acting formulations⁴⁶¹. Hydrocortisone is an immediate-release formulation that is identical to the cortisol. After oral administration it is absorbed within 15-30 min, reaches maximum levels in the blood after 60-70 min, and has a half-life of 60-100 min but there is significant inter-individual variability^{136, 462-465}. After intravenous administration peak cortisol level is at 10min and levels become undetectable by 6 hours⁴⁶⁶. Following an oral or intravenous dose of hydrocortisone at a dose used in clinical practice, supraphysiological peak levels are rapidly reached that decline to less than 100 nmol/L after 5 hours^{136, 462-464}. Liquid hydrocortisone for parenteral administration (intravenous or intramuscular) is used in emergency conditions, for example, when a patient is incapacitated or acutely unwell and for this reason is given during adrenal crisis²⁸⁶. The majority (80%) of patients on hydrocortisone receive a total daily dose of 15-30 mg⁴⁶⁷.

Long-acting synthetic glucocorticoid have also been used for replacement. Prednisolone is widely used for treatment of inflammatory conditions in high doses but also used at replacement doses in patients with glucocorticoid deficiency. Peak concentration following a dose of prednisolone is at 2 hours and half-life is 12-36 hours^{468, 469}. Prednisolone has about 80% of the mineralocorticoid effect of hydrocortisone.

It can be administered in once daily or twice daily regimens. Dexamethasone is a very long-acting glucocorticoid with potent anti-inflammatory and no mineralocorticoid action. It is absorbed quickly after oral intake and reaches maximum levels at 2 hours and has a long half-life 36-72 hours ^{468, 469}. For comparison purposes 20 mg of hydrocortisone are equivalent to 5 mg of prednisolone and 0.35-0.75 mg of dexamethasone in regards to their anti-inflammatory action ^{468, 470, 471}.

Glucocorticoid replacement aims to replace the amount of endogenous cortisol that is lacking and also deliver this in a manner that replicates the physiological pattern of cortisol production ⁴⁶⁷. Persistent symptoms despite adequate doses of glucocorticoid replacement and impact on quality of life are some reasons to suggest that replicating the pattern could improve patient care ⁴⁷². Furthermore, it has been shown that in patients with persistent symptoms simply up-titrating the dose of glucocorticoids doesn't improve symptoms and there are long-term effects with over-treatment ⁴⁷³. To achieve physiological replacement the considerations should be that the treatment regimen and formulation chosen should provide: 1. The appropriate amount of cortisol that the patient is lacking, 2. Cortisol levels that follow the circadian rhythm of cortisol production and match peaks and troughs in time. 3. Higher doses of readily absorbable glucocorticoid during stress.

The first step to physiological glucocorticoid replacement is administering a dose of that provides glucocorticoid cover equivalent to the endogenous cortisol production. In deciding which dose is appropriate one must consider the pharmacological characteristics of the compound, the absorption profile, and the bioequivalence ¹²⁷. For hydrocortisone, which has about 90-95% bioavailability, a total daily dose of 15 mg corresponds approximately to the calculated production cortisol rate of 5.7-6.1 mg/m²/day which should be divided in 2-3 doses in adults and 3-4 daily doses in children with half or two-thirds of the dose given on awakening in the morning ^{128, 129, 288, 462, 465, 474}. A total daily dose of 15 mg hydrocortisone in patients with severe secondary hypoadrenalism produced a 24-hour cortisol profile that matched the control's better than higher doses and some centres advocate this dose as the starting dose in patients recently diagnosed with adrenal insufficiency ^{119, 473}. Alternatively, the calculation of total daily hydrocortisone replacement dose can be done using weight adjusted (0.12 mg/kg) hydrocortisone ⁴⁷⁵. Although hydrocortisone is widely used for replacement especially in Europe, long-acting prednisolone and less frequently

dexamethasone are also extensively used in adults. The dose of prednisolone needed for physiological replacement is 3-5 mg/day^{469, 476}.

In the paediatric population hydrocortisone is the preferred glucocorticoid at doses of 8 mg/m² /day and is extensively used as there is evidence of benefits in growth compared with long-acting glucocorticoids^{286, 477-479}. In small children very small doses of hydrocortisone are needed as the dose is calculated according to surface area of the body. However, the strength of hydrocortisone tablets that are available are in the adult doses of 10 mg and 20 mg and further manipulation is needed to extract the appropriate dose for young children such as cutting, crushing the tablets and making up capsules from the powder at the desired dose. Most medications are studied and licensed in adults and there is often lack of evidence of pharmacokinetics in the paediatric population and medications are used unlicensed with no dose-appropriate formulations. Manipulations of hydrocortisone tablets is every day practice when treating children that need glucocorticoid replacement therapy, however there is evidence that such manipulations lead to variable and unpredictable doses which may have clinically significant adverse effects. Infacort is a new multi-particulate formulation of immediate release hydrocortisone that is available in small dose increments appropriate for suitable dose adjustment in the paediatric population. Pharmacokinetic studies in newborns and older children have been shown that it is bioequivalent to hydrocortisone.

The second step is mimicking the circadian rhythm. For the immediate-release hydrocortisone this is best achieved with thrice daily regimens where the higher dose of hydrocortisone is given in the morning, and two smaller ones one at midday and one before 6pm in the evening^{127, 157, 469, 480}. Twice daily hydrocortisone regimens (one early morning and 2 hours post lunch) provide the same total daily dose with two cortisol peaks and have the convenience of less doses during the day however do not replicate the circadian rhythm with low trough levels between doses in daytime and supraphysiologic peaks post dose^{52, 157}. Prednisolone is not absorbed rapidly and administration first thing in the morning will not provide enough glucocorticoid for the morning peak. Administration at bedtime will cause an increase in glucocorticoid cover during sleep at times when they are not needed and likely to exacerbate metabolic complications⁴⁸¹. Dexamethasone has a very long duration of action and it is not frequently used to treat cortisol deficiency; in primary adrenal insufficiency is given in

the morning with reducing levels in the afternoon that may manifest with tiredness and fatigue ⁴⁶⁹.

Newer glucocorticoid formulations and delivery methods have been specifically developed to mimic the physiological cortisol secretion pattern using modified-release or dual-release hydrocortisone or continuous infusions of hydrocortisone ^{117, 482}. Plenadren combines immediate and slow-release hydrocortisone with an immediate-release coating combined with a slow release core and when taken immediately after awakening it leads to peak cortisol levels after 50 min, has a half-life of 3.5 hours and mimics the physiological cortisol secretion during the day ^{483, 484}. Chronocort is a multiparticulate formulation where particles coated with hydrocortisone and a delayed-release coat allow hydrocortisone to be released when the particles reach the small bowel; taken in a toothbrush regimen in the evening and in the morning it aims to achieve the morning peak of cortisol from slow release of hydrocortisone from the evening administration and studies have shown that the 24hour profiles are similar to the physiologic cortisol secretion ^{116, 117}. Continuous infusions of hydrocortisone in the subcutaneous tissue through a pump, similar to the insulin delivery systems, can be planned to deliver variable doses and mimic the normal cortisol rhythm and this has had beneficial effects in some patients with primary adrenal deficiency due to Addison's disease and congenital adrenal hyperplasia ⁴⁸⁴⁻⁴⁸⁹.

The pulsatile manner of cortisol secretion may have extra benefits in improving symptoms (cognitive, behavioural) and reduce cardiovascular and metabolic adverse effects in patients receiving glucocorticoid replacement. There are currently no oral formulations that can mimic this feature of physiological cortisol secretion. Such a pattern, could be mimicked through portable infusion pumps programmed to deliver boluses of hydrocortisone; in a pilot study hydrocortisone delivered at 3hourly pulses or varying dose through a subcutaneous cannula reproduced the circadian cortisol production pattern with a total daily dose of 20 mg ⁴⁹⁰.

Higher doses of glucocorticoids are delivered during stress though immediate release hydrocortisone administered parenterally or orally. During periods of illness or during physical stress such as medical interventions and surgery, patients need to self-adjust their treatment and take extra hydrocortisone doses 3-4 times daily. All patients need

education to be able to recognise and apply these preventive measures (sick day rules). They also need to carry a steroid card for recognition of their medical condition in case they become incapacitated. When they are unable to take oral treatment, admission to hospital or a day ward and 24-hour hydrocortisone infusions or 6hourly intravenous or intramuscular injections are necessary for the duration of illness ⁴⁶⁶.

Overall, there are multiple regimens for glucocorticoid replacement in clinical use and treatment is tailored aiming at reducing life-threatening adrenal crises, minimising symptoms of cortisol deficiency, restoring quality of life and avoiding the risks of overtreatment ^{467, 469}. However, restoration of well-being is suboptimal and there is evidence that patients with primary adrenal insufficiency have persistent symptoms, low quality of life, 2-fold higher mortality, and reduced life expectancy and delivery of glucocorticoids in a non-physiological pattern has been implicated as a reason for this ^{282, 472, 491-495}. Over-exposure to glucocorticoids due to high replacement doses or regimens that result in non-physiological exposure at times when glucocorticoids are normally low in the circulation lead to accumulation of cardiovascular risk factors such as hypertension and obesity, higher incidence of cardiovascular events, reduced bone density and lower health-related quality of life scores ^{282, 467, 468, 493, 496-500}. For example, high hydrocortisone doses above 20-30 mg/day or above 0.3 mg/kg are associated with higher mortality in patients with secondary adrenal deficiency ⁵⁰¹⁻⁵⁰³. On the other hand, under-replacement is associated with persistent symptoms such as fatigue that impact on quality of life and higher risk of adrenal crisis ²⁸⁴. It is therefore essential that glucocorticoid replacement is carefully reviewed and adjusted to optimise long-term health and restore quality of life in this patient population and the total dose and circadian pattern of delivery need to be optimised for each patient for maximum benefit. Obstacles to this may be timely administration, absorption, appropriate dose and type of formulation used.

3.3.1.2. Monitoring of medical treatment

Hormone replacement in primary adrenal insufficiency needs regular monitoring because the dose may need adjustment, new medications may affect the metabolism of glucocorticoids, sick day rules may need re-discussion. The monitoring is clinical

and biochemical and addresses the mineralocorticoid and the glucocorticoid replacement separately and also screening for associated autoimmune conditions in autoimmune adrenalitis. For the mineralocorticoid replacement therapy monitoring includes clinical assessment of the deficiency with physical examination (presence of oedema) and history (postural symptoms and salt craving) and biochemical monitoring of sodium and potassium^{286, 288, 469}. The usual replacement is with fludrocortisone 50-200mcg daily and depending on the glucocorticoid formulation used and its mineralocorticoid potency there may be some added mineralocorticoid cover such as when hydrocortisone is used there is mineralocorticoid cover equivalent to 50mcg fludrocortisone for every 20 mg hydrocortisone²⁸⁴. Monitoring of renin levels which are increased when aldosterone is low has also been used to guide mineralocorticoid replacement and assessment of replacement dose adequacy aiming for renin levels in the high normal range²⁸⁹.

Glucocorticoid replacement is guided by clinical monitoring for evidence of symptoms and signs of glucocorticoid over or under replacement²⁸⁶. This is done through assessment of weight changes, presence of cushingoid features, postural symptoms, and subjective report of energy levels^{288, 469}. Special attention is placed in the identification of adrenal crisis and treatment regimens are always reviewed to try to develop an individualised regimen that best avoids adrenal crisis which can be life threatening. Although glucocorticoid replacement has improved mortality in patients with Primary adrenal insufficiency modern epidemiological studies still suggest an incidence of five and ten life-threatening adrenal crises per 100 patient years in patients on standard replacement therapy^{504, 505}.

There are no accurate biochemical markers that can be used to assess sufficiency of glucocorticoid dose replacement. Various biochemical tests have been used for this purpose but there is limited evidence of their support to optimising glucocorticoid dose replacement. Monitoring of ACTH levels during treatment is not helpful as ACTH levels reduce after the glucocorticoid dose and increase post dose and suppression of ACTH levels during treatment indicates overtreatment with glucocorticoids^{127, 506}. For patients on hydrocortisone the methods used in clinical practice involve cortisol day-curves that assess cortisol levels in relation to the timing of glucocorticoid replacement and meals¹⁶². This is helpful to establish the absorption of glucocorticoids with cortisol peaks measured after the morning dose aiming for levels in the normal range and detecting

under-replacement if pre-dose levels in the afternoon are low (less than 100 nmol/L). But it is time-consuming, expensive and needs to be done in a healthcare setting and therefore may not reflect the patients' routine and actual administration times and may not correlate accurately with clinical assessment^{157, 475, 507}. The protocols are variable but most centres propose sampling 1-2 hours after the morning dose (+/- after every other dose as well) with pre-dose samples for the midday and afternoon doses¹⁵⁷. Cortisol day-curves made of dry bloodspot sample from capillary blood are less invasive, correlate with plasma cortisol and could be developed in the near future for clinical use as an ambulatory and child-friendly test in place of serum cortisol⁵⁰⁸. Some studies have showed that timed cortisol curves following a single dose of cortisone acetate were not more sensitive in identifying over and under-replacement in hypo-adrenal patients than clinical monitoring and there is significant inter-individual variations of cortisol profiles after administration of glucocorticoids which adds to the complexity of deciding accurate cut-offs of target cortisol levels that ensure no overlap with well-replaced patients^{462, 509, 510}. A 2-hour post oral hydrocortisone administration plasma cortisol corresponds to peak cortisol achieved after a single dose and some authors recommend this is used for monitoring¹³⁶ and a single 4-hour post administration level helps monitor treatment using a published nomogram with levels outside the 10 and 90 percentile indicating absorption problems or over-replacement and requiring dose adjustment⁴⁷⁵.

Salivary cortisol levels are an attractive marker for the sufficiency of cortisol replacement as they reflect the biologically active free cortisol however studies have shown that they are not very reliable in monitoring patients on glucocorticoid replacement due to intra-individual variability of measurements and saliva contamination with the medication in patients on oral hydrocortisone^{136, 475}. Urinary free cortisol levels are not reliable for monitoring replacement adequacy in patients on hydrocortisone; following each bolus dose there is transient supraphysiologic cortisol levels and oversaturation of CBG levels that cause high excretion of free cortisol through the kidneys^{136, 473}. As urine free cortisol is calculated on a 24hour sample this obscures the assessment of cortisol sufficiency in other parts the day and may mask long periods of under-replacement⁵⁰⁷. Urine free cortisol estimations may be helpful in under-replacement due to non-compliance with treatment^{284, 511, 512}.

3.3.2. Congenital adrenal hyperplasia

3.3.2.1. Medical treatment

Treatment for CAH is primarily medical and the cornerstone is adequate replacement of the deficient hormones to ensure life-threatening adrenal crises are avoided. Additional treatment goals differ in children and adults and in childhood treatment focuses on gender assignment, genital surgery and optimisation of growth and pubertal development. In adults the focus moves to screening and treating metabolic complications due to long-term glucocorticoid replacement (obesity, metabolic syndrome and osteoporosis) to ensure long-term vascular health, treat troublesome androgen excess and addressing fertility problems⁵¹³. Patients with classic CAH have glucocorticoid and mineralocorticoid replacement from the age of diagnosis that continues lifelong and women with classic CAH and significant virilisation may need genital surgery at early childhood. Patients with non-classical CAH have the mild form of the disease and do not have mineralocorticoid deficiency. They usually do not need cortisol replacement and may not require any medical treatment unless there are specific indications; women with signs of virilization or to optimise fertility, and children who have early onset of disease and rapid progression of skeletal age/precocious puberty⁵¹⁴.

The goal of mineralocorticoid treatment is to replace mineralocorticoids and prevent salt-wasting crisis and electrolyte abnormalities while minimising side effects such as hypertension and water retention. Mineralocorticoids should be initiated in neonates and children with CAH with salt-wasting or raised plasma renin levels. The goals of glucocorticoid treatment is to treat the cortisol deficiency and prevent life-threatening adrenal crisis, prevent hyperandrogenaemia and control the overnight increase in adrenal androgens that is driven by the hypothalamic–pituitary–adrenal axis and the overnight increase in ACTH secretion due to activation of HPA axis and avoid long-term glucocorticoid excess⁵¹⁵.

The treatment aims are quite clear however getting the correct balance between over-treatment and undertreatment with glucocorticoids is difficult in practice. Undertreatment with lower glucocorticoid doses leads to inadequate

control of androgens excess with subsequent early puberty, hirsutism, and infertility later in life. When patients are overtreated with high GC doses, the androgens are well suppressed however there is cortisol excess with the associated long-term complications such as obesity, short stature, osteoporosis, insulin resistance, hypertension and adverse effects on quality of life ⁵¹³. Overtreatment with hydrocortisone in early childhood (up to 2 years) in 21-hydroxylase deficiency is associated with lower final height (248). Optimizing the treatment of CAH and glucocorticoid replacement is essential to improve clinical outcomes as in a national cross-sectional study (CaHASE) adult patients with CAH had higher mortality than the general population, were shorter and had adverse metabolic features and cardiovascular risk factors associated with GC over-replacement including higher BMI, hypercholesterolaemia, insulin resistance and osteopenia ^{318, 516}. The metabolic profile was worst for long-acting glucocorticoids such as dexamethasone ⁵¹⁷. In the CaHASE study the disease control measured by control of androgens and precursors in women was suboptimal. Ensuring there is good control of androgens and precursors is important for fertility in women.

Glucocorticoid replacement for the purposes of treating the cortisol deficiency in CAH is explored in the previous chapter along with other causes of primary adrenal failure (see chapter 3.3.1.). Treatment of pre-pubertal children with precocious puberty with growth hormone alone or in combination with gonadotropin-releasing hormone analogue improves growth rate and height prediction (254). If necessary, reconstructive surgery to external genitalia should be undertaken by expert surgeons (198).

For adults on glucocorticoid replacement there is no consensus for the type of glucocorticoid or regimen. Hydrocortisone at a daily total dose of 15–30 mg divided thrice daily, with the higher dose given in the morning is a regimen frequently used and treatment adjustment (dose titration) is based on clinical monitoring ⁵¹⁸. If there is suboptimal biochemical control on hydrocortisone then low dose prednisolone (0.5–2.5 mg) last thing at night with a reduction in the hydrocortisone dose first and then twice daily if there is good response to prednisolone ⁵¹³. Dexamethasone has the convenience of once daily administration and provides good control of androgen excess; however, it causes more insulin resistance ⁵¹⁷. Furthermore, it is not inactivated by placental 11 β -HSD2 and therefore is not recommended in women of

reproductive age who are not on contraception^{54, 517}. Hydrocortisone infusions have shown to reduce the early morning rise in ACTH and steroid precursors and could therefore be used to improve disease control⁴⁸⁵.

Some patients with CAH take glucocorticoids in a reverse circadian rhythm which involves administration of the highest dose of a long-acting glucocorticoid in the evening aiming to eliminate the early rise of ACTH and the subsequent drive for adrenal androgen production³¹⁸. There is no benefit of the reverse rhythm in improving biochemical control but reverse circadian rhythm may be helpful in women with significant symptoms due to hyperandrogenaemia or women of reproductive age that want to optimise their chances for fertility⁵¹⁹. Studies have shown that about 60% of adult patients with CAH receive long-acting glucocorticoids with treatment regimens not likely to replicate the physiological cortisol levels³¹⁸.

Current glucocorticoid regimens try to replicate the normal diurnal secretion of cortisol and ACTH and inhibit androgen excess which is important in women with CAH as the overnight increase in ACTH drives hyperandrogenaemia which has symptoms and adverse sequences on fertility⁵¹⁴. Studies have shown that prednisolone on twice daily reverse circadian regimen there is escape of ACTH control during the night with higher androgen levels during the day and high cortisol during the midnight. Dexamethasone on reverse regimen also shows escape of ACTH control in the afternoon with elevated androgens and high glucocorticoid exposure overnight. Current reverse circadian replacement regimens are likely exposing patients to too much glucocorticoids during the night, at a time where glucocorticoids are physiologically low and there is evidence this has more risk for long-term metabolic complications⁴⁸¹. Furthermore, a higher glucocorticoid dose does not always lead to better disease control and education on sick day rules should be provided⁵¹⁵.

To optimise fertility in patients, the glucocorticoid dose is optimised initially to ensure good control of androgen precursors and avoid secondary gonadal failure from the elevated sex steroids. In women hydrocortisone or prednisolone are used as they are inactivated by 11 β HSD and don't affect fetal adrenals. In women who fail to conceive an increase of the glucocorticoid dose to ensure biochemical markers (17-

hydroxyprogesterone, testosterone, androstenedione) are within normal range is first indicated and if this fails then long-acting steroid (prednisolone 2.5 – 3 mg 6 - 8 hourly) in multiple daily doses is used to ensure adequate androgen suppression throughout the day aiming for suppression of 17-OHP. Men with CAH have ultrasound screening to check for the presence of testicular adrenal rest tumours (TARTs). In men with TARTs who want fertility preservation the options are increasing the glucocorticoid dose and changing to a long-acting formulation such as dexamethasone, a sperm count and cryopreservation to avoid worsening of sperm count ^{313, 520-522}. Prenatal diagnosis is possible for couples at risk of having offspring with CAH and in these cases dexamethasone has been advocated for prenatal treatment until the sex determined to be male or throughout pregnancy to reduce risk of masculinisation of female fetuses but this is controversial ³⁰³.

Treatment of 11-hydroxylase deficiency is with glucocorticoids (hydrocortisone 10-15 mg/m² and antihypertensives (spironolactone, calcium channel blockers or amiloride) if necessary ³⁰³. Good compliance with glucocorticoid replacement normalises blood pressure ³⁰¹.

3.3.2.2. Monitoring of medical treatment

Patients have regular clinical reviews for evidence of over or under replacement with glucocorticoids and mineralocorticoids. Clinical assessment of fluid retention and blood pressure (mineralocorticoids), cushingoid features and virilisation (glucocorticoids) is essential in making decisions about dose titrations ⁵¹⁵. The replacement of glucocorticoids and mineralocorticoids is similar to patients with other causes of primary adrenal insufficiency however in patients with CAH there are additional biomarkers (steroid precursors) that help assess disease control and dose titration decisions and clinical evidence of androgen excess is also considered when assessing glucocorticoid dose and regimen adequacy.

Biochemical monitoring for glucocorticoid replacement is by monitoring the levels of precursors and androgens. For 21-hydroxylase deficiency the main biomarkers for

disease control are 17-hydroxyprogesterone and androstenedione; the target levels are serum 17-hydroxyprogesterone between 1-3 times the upper limit of normal and serum androstenedione within the reference range however the target for androgen control depends on the patient's needs and may change during life ⁵⁴. Levels above these ranges should trigger a review for possible undertreatment and levels of 17-hydroxyprogesterone within the normal range and low androstenedione should trigger a review for possible overtreatment and excessive glucocorticoid exposure. Ketotestosterone levels measured by sensitive analytical methods are increased in 21-hydroxylase deficiency due to adrenal overproduction and reflect clinical severity in studies, however their significance is emerging and there are no data on long-term monitoring of disease control and treatment targets ⁸⁶. For 11 β -hydroxylase deficiency the biochemical monitoring is with serum 11-deoxycortisol levels aiming for normalisation ³⁰³. In patients seeking fertility the targets may change to lower ones to improve chances of a pregnancy; this usually means a period of overtreatment with glucocorticoids to achieve this target.

When assessing response to treatment the timing of the blood test and the relationship to the administration of the medication is important and should be recorded to aid interpretation and comparison of values. Release of steroid precursors show circadian patterns and 17-hydroxyprogesterone is released in a circadian rhythm similar to cortisol with concentrations low at night, rising from 0200h, peak in the early morning (0800h), and decreasing throughout the day.

Biochemical monitoring for mineralocorticoid replacement is with sodium and potassium levels and renin levels aiming for the upper limit of normal ⁵⁴. In patients with hypertension a small reduction of the fludrocortisone dose and accepting a slightly raised level of renin in adult patients with hypertension is appropriate provided they are well and there is no evidence postural hypotension.

3.4. Hypothesis

This research was designed to test the hypothesis that it is possible to restore physiological cortisol and adrenocorticotrophin levels in patients with disorders of cortisol and adrenocorticotrophin production: cortisol deficiency (Congenital adrenal hyperplasia), cortisol excess (Cushing's syndrome) and adrenocorticotrophin excess (Nelson's syndrome) using medical therapy. The research will examine whether it is possible to:

1. Reduce cortisol levels by medical therapy in patients with overt cortisol excess
2. Reduce adrenocorticotrophin levels in patients with Nelson's syndrome
3. Deliver hydrocortisone for glucocorticoid replacement therapy accurately through nasogastric tubes and sprinkled on food
4. Use new biomarkers to monitor adequacy of glucocorticoid replacement and disease control in patients with Congenital adrenal hyperplasia

4. Methods

4.1. Overview

The specific methodology for each part is detailed in the method section of the published manuscripts included in the thesis. Further information on designing a data collection proforma for Study 1, biochemical analysis for Studies 1, 2, and 3 is provided in this section, as these areas could not be sufficiently extensive in the papers due to word number limitations.

4.2. Data collection

A multicentre national retrospective study was conducted in centres that made up the UK Endocrine Neoplasia Collaboration aiming to improve the understanding of the effectiveness of Metyrapone in restoring cortisol levels in patients with hypercortisolaemia due to Cushing's syndrome. The UK Endocrine Neoplasia Collaboration is a group of thirteen major UK tertiary referral centres in England and Wales spread over 9 cities. Each centre had a senior investigator who had oversight of the study locally and at least one centre sub-investigator who was trained in Endocrinology and had responsibility for data collection. Sheffield Teaching Hospitals NHS Trust was the central study site and the investigators in this centre co-ordinated data collection from the twelve peripheral study sites (King's College Hospital NHS Foundation Trust, London, Royal Victoria Infirmary, Newcastle, Queen Elizabeth Hospital, Birmingham, Aintree University Hospital and Royal Liverpool University Hospital, Liverpool, Manchester Royal Infirmary and The Christie NHS Foundation Trust, Manchester, Salford Royal Foundation Trust, Salford, St Bartholomew's Hospital, London, Addenbrookes Hospital, Cambridge, Oxford University Hospitals, Oxford, Imperial Healthcare, London, University Hospital of Wales, Cardiff).

Metyrapone is only available through hospital pharmacy therefore patients treated with Metyrapone were identified at each centre through pharmacy and electronic clinical records and patients treated between 1997 and 2013 were included in the study. To address the aims of the study it was necessary to conduct a review of the medical notes of all patients treated with Metyrapone and collect data on the diagnosis of Cushing's syndrome, indication and timing of metyrapone treatment in relation to other treatments, dose and regimen use, biochemical monitoring, assay information, and safety data.

To ensure the accuracy, reproducibility and quality of data a proforma was designed by the candidate who was also the central site sub-investigator. The data collection proforma included a combination of free text, selection of pre-defined choices, and results of specific investigations with the timing of results in relation to treatment given. The data collected included results of baseline and imaging tests at presentation and cause of Cushing's syndrome, clinical and demographic data (age, sex) at presentation and start of Metyrapone therapy, documented indication for metyrapone therapy (pre-defined options were routine practice, control of severe symptoms, delay for medical reasons or patient initiated delay), all therapeutic interventions undergone by each patient with dates (options were primary surgery, additional surgery, primary radiotherapy, adjuvant radiotherapy, chemotherapy, medical therapy). Multiple options were possible and a free text option was offered in all parts of the proforma (data collection proforma Appendix A.1.).

The proforma was piloted on five patients treated with Metyrapone in Sheffield Teaching Hospitals that fulfilled the inclusion criteria. The quality of data collected was reviewed and feedback to improve accuracy of data collected was invited from the senior investigators. Improvements were introduced and included designing a table for collecting long-term biochemical and clinical monitoring data following initiation of Metyrapone therapy and changing of date of birth to year of birth or age to minimise collection of patient identifiable information. The updated proforma was then piloted in 28 patients in the central site and these data were analysed to test if they provided answers to the study objectives. Written instructions were produced to guide completion and data entering from clinicians at the participating peripheral sites. The

instructions and the results of the pilot data collection were discussed with the study senior investigators and the proforma was approved for dissemination to the participating peripheral sites. Three case examples were also formulated accompanied with completed data collection proformas to serve as examples of satisfactory data entering. The sub-investigators at the participating centres all received and used the data collection proforma, written information for data entering, the three completed examples, and the contact information for the central site investigator (the student) who was available for help and guidance on data collection. The completed proformas were collected and analysed together at the central site by the candidate using excel spreadsheets and GraphPad Prism software.

4.3. Analytical biochemistry for cortisol quantification

4.3.1. Liquid chromatography tandem mass spectrometry

In Study 3, aqueous samples of hydrocortisone were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) at South Manchester University laboratories. LC-MS/MS is an accurate method of quantifying cortisol and is based on the principle of protein precipitation and fragmentation. It uses a combination of two techniques, liquid chromatography that allows separation of the analytes in a sample based on their polarity, and mass spectrometry which allows separation and accurate positive compound identification⁵²³. The combination of the two techniques increases the sensitivity and specificity of the mass spectrometer.

The main components of the LC-MS/MS are the liquid chromatography, the ion source, the mass analyser, the electron multiplier and the data processor. The preparation of the aqueous sample for analysis is shown in Appendix A.2.; this preparation involves use of Zinc sulphate and methanol to disrupt cells and precipitate all proteins in the serum and unbind compounds, and addition of internal standards to the sample. After preparation the sample initially undergoes separation by liquid chromatography, which

involves dissolution in a mobile phase, made up of water and an organic solvent (methanol). The fraction of water to organic solvent can be modified to allow better dissolution of the compound and the sample then travels through a stationary phase or column^{523, 524}. Analytes in the sample travel through the column at different speed and separate based on their hydrophobicity and by changing the polarity compounds such as steroids are extracted sequentially. This method of separation reduces any interference from prednisolone or cortisol metabolites.

The principle of mass spectrometry (MS) is to produce an ion from a neutral compound by removing an electron or by adding a proton, fragment the ion through collision with a gas and examine these fragments^{525, 526}. The fragments that each compound produces are characteristic of the compound therefore study of the fragments gives information for the compound and measuring fragments instead of cortisol adds specificity⁵²⁵. In tandem mass spectrometry (MS/MS) there are two mass spectrometers in a single instrument. In the first, the analyte is purified from the mixture using a magnetic field and then it is fragmented in a collision chamber. In the second mass spectrometer the fragments are analysed for identification and quantification⁵²⁷.

After separation by liquid chromatography, cortisol is diverted to the ion source that uses electrospray ionisation. Electrospray ionisation pumps the liquid sample through a metallic capillary that has a high voltage (3-5KV) applied to it and at the end of this process the analyte acquires an electric charge^{524, 526, 528}. Cortisol has a molecular mass of 362 and during ionisation a proton is added so the molecular ion of cortisol has a molecular mass of 363. The ionised molecules are led into an angle to enter the mass spectrometer and anything that is not following this route, for example non-ionised molecules, is dismissed and the voltage and angle can be altered to increase specificity.

After ionisation the compounds are diverted to the first chamber of the mass spectrometer where they travel through a magnetic field under vacuum and separate according to their mass-to-charge ratio (m/z)^{525, 526}. The magnetic field is created by six parallel rods (hexapole) that have a direct current (DC) or a radiofrequency potential applied to each opposite pair; the ions separate by mass as their m/z determines the curvature of the path taken by the ions⁵²⁶. Only ions with the target m/z values have

stable trajectories and exit to the next chamber and the selection of m/z depends on the DC and radiofrequency voltage applied. Ions with m/z values other than the preselected targets have unstable trajectories, collide with the rods or walls of the vacuum chamber and neutralise. On the other hand, different compound ions that have the same m/z will follow the same trajectory and will not separate.

After the first mass spectrometer the ions go into the collision cell where the ions get fragmented by colliding with a neutral target gas such as argon generating fragments that then pass through into the second mass analyser which contains a quadrupole and the ions are separated according to their m/z ratios again. The collision conditions (fragmentation) is controlled by changing the speed of the ions as they enter the collision cell and the number of collisions undertaken (collision gas pressure). Two fragments are produced from the cortisol molecular ion in the mass spectrometer; they have a molecular mass of 121 and 97. All the fragment ions (daughter ions cortisol-121 and cortisol-97) that enter the second mass spectrometer are derived from a single precursor ion (cortisol).

In the second mass spectrometer chamber we choose which target fragment is measured by predefining the target m/z ratio; the two cortisol fragments have different m/z therefore only the target fragment is measured. At the quadrupole exit the fragment with the target m/z will strike the detector and register a signal, which is detected by an electron multiplier, and the data are passed to a computer and processed⁵²⁹. The results are displayed as a mass spectrum, which is a graph with y-axis depicting the amount of the ions (relative abundance or signal strength) and their m/z values on the x-axis. Non-fragmented ions of the compound we want to measure usually have very low or even no signal. Mass spectrometers can be programmed to identify and quantify a selection of compound ions (parent ions) and their fragments (daughter ions) and this is called multiple reaction monitoring (MRM)⁵²⁸. There are different ways of presenting the results, they can be normalized using 100% as the biggest peak (base peak) of the spectrum (% relative abundance) or total ion current (=the sum of all the detector responses for each scan) against time⁵²⁵. Compounds that have been studied by mass spectrometry have a known spectrum and hydrocortisone measured as cortisol peaks on the graph (hydrocortisone % relative abundance versus time) has a retention time of about 1min. For plotting the standard curve (% response versus cortisol concentration), we used the cortisol-97 qualifier. For

data analysis the LC-MS/MS measures the response (MRM) as the ratio of cortisol-363 ion to the internal standard (molecular mass 367) which equals to the ratio of peak area of cortisol to the peak area of internal standard. Calibration lines of this ratio against cortisol concentration are plotted and the concentration of hydrocortisone (measured as cortisol) in the samples is calculated based on these lines.

4.3.2. Immunoassays

Quantification of cortisol by immunoassays was performed by the Biochemistry Department, Sheffield Teaching Hospitals NHS Trust for the Study 1 and Study 2 of this research, and the Biochemistry Departments for the thirteen participating centres in the Study 1.

The immunoassays used by the different departments were the following commercial immunoassays; Roche Cobas, Siemens Centaur, and Abbott Architect for serum and urine samples. The serum cortisol measuring range with Roche, Siemens and Abbott was 0.5 – 1750 nmol/L, 13.8 – 2069 nmol/L, and 27.6 -1649.8 nmol/L respectively, and CV% was 2.5-4.3, 4.9-6.0 and 1.6-3.9 respectively⁵³⁰. The assay analytic specificity from the product leaflet was reviewed for cross reactivity data and these data are shown in Table 8.

These are competitive binding chemiluminescence immunoassays and are based on the following principles: the sample containing the cortisol to be quantified is incubated with fixed amounts of anti-cortisol antibody and antigen labelled with a molecule that can be triggered to emit a photon of light⁵³¹. The labelled antigen competes with cortisol for the binding sites of the anti-cortisol antibody and the antigen-antibody complexes are bound to paramagnetic microparticles. The mixture is left to incubate to achieve equilibrium, washed to remove excess unbound cortisol and reagents, and

a trigger is added that interacts with the labelled antigen and this chemical reaction results in light emission. The light emission is quantified by photomultiplier and the signal is measured as Relative Light Units, plotted against a standard curve, and cortisol in the sample is quantified. The higher the chemiluminescent reaction, the lower the cortisol concentration in the sample. Acridinium and ruthenium esters are commonly used as labels in chemiluminescence assays and can be triggered by hydrogen peroxide to emit a photon of light ⁵³².

In the Roche assay the complex of anti-cortisol antibody with cortisol or labelled antigen is bound to microparticles that are magnetically captured on an electrode, application of voltage on the electrode induces a chemiluminescent emission, light is measured and the cortisol amount calculated ⁵³³. In the Siemens assay cortisol competes with acridinium ester-labelled antigen for binding to the anti-cortisol polyclonal antibody. The polyclonal anti-cortisol antibody is then bound to monoclonal mouse anti-rabbit antibody which is coupled to paramagnetic microparticles, the mixture is incubated and washed and then an acid and base trigger reagent is added which triggers the emission of light from the acridinium-labelled antigen ⁵³³. The Abbott assay has mouse monoclonal anti-cortisol coated paramagnetic microparticles. These are incubated with the sample containing cortisol (serum, plasma, or urine sample), acridinium-labelled conjugate is added and competes with cortisol for the binding sites of the anti-cortisol coated microparticles, the mixture is incubated and the microparticles are washed with a phosphate buffered saline solution containing an antimicrobial. Hydrogen peroxide and sodium hydroxide solutions are then added that cause a chemiluminescent reaction measured as relative light units ⁵³⁴.

Table 8: Cross reactivity of cortisol immunoassays

	Substance	Cross-reactivity (%)
Roche (Cobas e analyser)	Cortisone ¹	0.30
	Corticosterone ¹	5.8
	Cortisol-21-S ¹	0.04
	11-deoxycortisol ¹	4.1
	11-deoxycorticosterone ¹	0.69
	Dexamethasone ¹	0.08
	17 α -hydroxyprogesterone ¹	1.50
	Prednisolone ¹	0.35
	Progesterone ¹	0.35
	21-deoxycortisol ²	45.4
	6 β -hydroxycortisol ²	158
	Allotetrahydrocortisol ⁴	165
	Prednisolone ⁴	171
6a-methylprednisolone ⁴	389	
Siemens Centaur	11-deoxycorticosterone ¹	1.8
	11- deoxycortisol ²	23.3
	Prednisolone ³	109
	6-methyl-prednisolone ²	26.2
	Dexamethasone ¹	0.2
	Prednisone ²	34.0
Abbott Architect	Cortisone ¹	2.7
	Corticosterone ¹	0.9
	11-deoxycortisol ¹	1.9
	Fludrocortisone ²	36.6
	17-hydroxyprogesterone ¹	0.6
	Prednisolone ²	12.3

These three immunoassays were used for cortisol quantification in Studies 1 and 2.
Substance added per (1) 10 $\mu\text{g/ml}$, (2) 1 $\mu\text{g/ml}$, (3) 0.5 $\mu\text{g/ml}$, (4) 0.1 $\mu\text{g/ml}$

4.4. Ethical Approval

Ethical approval was sought for the four parts of this research that involved clinical data and patients; Study 3 did not involve any clinical data or animal experiments therefore an ethical approval was not required.

Studies 1 and 4 were approved as institutional case notes review projects by Sheffield Teaching Hospitals NHS Trust. The process included registration of the study as a clinical audit through the Clinical Governance Office. The process of applying to the Clinical Governance Office for clinical audit registration includes an application with details of the project proposal and the standards against which the data are compared, details of data analysis and plan for dissemination of results through presentation or publication. The data collection proforma was submitted with the application form and was approved before any data collection took place. The project registration submission was quality reviewed by the Clinical Effectiveness Project Panel who approved the registration of the project and the exact data that could be extracted. Once the project was approved then access to local medical and electronic records was allowed for data collection specified in the approved proforma. Study 1 was registered under the title 'Indications and monitoring of Metyrapone treatment in Cushing's Syndrome' with clinical audit project registration number 4947 and Study 4 under the title 'Monitoring hormonal replacement in congenital adrenal hyperplasia' with registration number 5384.

Study 1 was a national multicentre study therefore additional to the local registration at Sheffield Teaching Hospitals NHS Trust, it was also approved as an institutional case notes review at each participating centre. This process included local registration of the study as audit through the Clinical Governance Office at each participating NHS Trust through submission of an application and a list of the required data for pre-approval. For the data to be released externally a Caldicott Guardian approval had to be obtained by the participating NHS Trust. In Sheffield, the local Caldicott Guardian was identified through the local NHS Information Governance office and a request for data release was made through email with the purpose use of data, purpose of release and description of data to be released. The twelve peripheral sites applied for a Caldicott Guardian approval for data release to the central site (Sheffield Teaching Hospitals

NHS Trust) where all data were collated for central analysis and all centres applied for Caldicott approval for release of safety data to the funder (HRA Pharma).

Study 2 was a phase 2 clinical trial which was investigator initiated, led, and designed. It was sponsored by Sheffield Teaching Hospitals NHS Trust (STH Project reference number STH15164), registered as a clinical study (ClinicalTrials.gov ID: NCT01617733, EudraCT number 2009-014457-33), and approved by the UK Health Research Authority (ref 10/H1005/53). The principal investigator was Prof Newel-Price and funding was provided by the manufacturer of Pasireotide, Novartis Pharmaceuticals, UK.

Study 5 was a phase 1 clinical trial performed at a private clinical research facility (Simbec Research Ltd.) in Wales, UK. The protocol of the study, participant information leaflet and consent documents were reviewed and approved by the Wales Research Ethics Committee (reference number: 17/WA/0114) and Clinical Trials Authorisation was obtained from the Medicines and Healthcare Regulatory Agency according to UK and EU regulations. The ClinicalTrials.gov registration number was NCT03178214.

4.5. Data management and statistics

Clinical data including medical history, clinical evaluation, and laboratory values with dates were collected on specifically designed and pre-approved data collection forms for Studies 1, 2, and 4. The forms for each study were collected from all centres and kept together in secure and locked offices in the University of Sheffield accessible to the student, supervisors and the department's Research Co-ordinator and will be archived for 15 years post study completion. For Study 3 only laboratory data were collected.

The laboratory and anonymised clinical data were entered on excel files for analysis, one for each study part, and stored on the student's personal computer with regular back up files. The analyses were performed on the excel files and Graph Pad Prism and copies of all analyses, excel files with data and graphs are kept in USB and shared with supervisors. Details for further statistical analysis specific for each study are given in the individual published papers.

The results of the analyses and details on the statistics used have been published and the papers are available as open access research papers and deposited to the White Rose Research Online, a shared, open access research repository. The results were also presented in part or in full to National and International conferences as oral and poster communications (Studies 1, 2, 4, and 5). Two clinical study reports were prepared for Study 1 and Study 2 and shared with the funders (HRA Pharma and Novartis Pharmaceuticals UK Limited).

In Study 2 the anonymised Case report forms from all four centres were collected and stored as hardcopies in the Research Co-ordinator's office in the Royal Hallamshire Hospital/ University of Sheffield together with electrocardiographs, and CDs containing patient images and MRI scans.

For Study 4 a confidential clinical study report along with extensive tables and figures summarising the raw data was received by electronic mail and hardcopy and is stored in a locked office in the University of Sheffield Medical School.

5. Results

5.1. Study 1: Medical therapy in Cushing's syndrome

Effectiveness of metyrapone in treating Cushing's Syndrome: a retrospective multicenter study in 195 patients.

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Authors' accepted copy of the paper is included in this thesis, pages 109 to 129.

Authors' accepted copy

Effectiveness of metyrapone in treating Cushing's Syndrome: a retrospective multicenter study in 195 patients

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Abstract

Background: Cushing's syndrome is a severe condition with excess mortality and significant morbidity necessitating control of hypercortisolemia. There are few data documenting use of the steroidogenesis inhibitor metyrapone for this purpose.

Objective: To assess the effectiveness of metyrapone in controlling cortisol excess in a contemporary series of patients with Cushing's syndrome.

Design: Retrospective, multicenter.

Setting: Thirteen university hospitals.

Patients: 195 patients with proven Cushing's syndrome: 115 Cushing's disease (CD), 37 ectopic ACTH (EAS); 43 ACTH-independent disease (Adrenocortical Cancer [ACC] 10; adrenal adenoma [AA] 30; ACTH-independent adrenal hyperplasia (3)

Measurements: Biochemical parameters of activity of Cushing's syndrome: mean serum cortisol day-curve (CDC) (target 150-300nmol/L); 09.00h serum cortisol; 24h-UFC.

Results: 164/195 received metyrapone monotherapy. Mean age was 49.6 +/- 15.7 years; mean duration of therapy 8 months (median 3 months, range 3 days to 11.6 years). There were significant improvements on metyrapone - first evaluation to last review: CDC [91 patients, 722.9nmol/L (26.2µg/dl) vs. 348.6nmol/L (12.6µg/dl), $p<0.0001$]; 09.00h cortisol [123 patients, 882.9nmol/L (32.0µg/dl) vs. 491.1nmol/L (17.8µg/dl), $p<0.0001$]; UFC [37 patients, 1483nmol/24h (537µg/24h) vs. 452.6nmol/24h (164µg/24h), $p=0.003$]. Overall control at last review: 55%, 43%, 46% and 76% of patients who had CDCs, UFCs, 09.00h cortisol <331 nmol/L (12.0µg/dl) and 09.00h cortisol $<ULN/600$ nmol/L (21.7µg/dl). Median final dose: CD 1375mg; EAS 1500mg; benign adrenal disease 750mg; ACC 1250mg. Adverse events occurred in 25% of patients, mostly mild GI upset and dizziness, usually within 2 weeks of initiation or dose increase, all reversible.

Conclusions: Metyrapone is effective therapy for short- and long-term control of hypercortisolemia in Cushing's syndrome.

Introduction

Cushing's syndrome (CS) is a severe condition with excess mortality and significant morbidity necessitating effective biochemical control (1). Where a cause amenable to surgical intervention is identified, surgery at a center with appropriate expertise is the optimum management. Nevertheless, many patients need urgent control of severe or persisting hypercortisolemia. Options for medical treatment include steroidogenesis enzyme inhibitors suitable for all causes of CS (ketoconazole, metyrapone, mitotane), agents to suppress ACTH in Cushing's disease (CD), such as dopamine agonists and pasireotide, and the glucocorticoid receptor antagonist, mifepristone (2,3). The modern use of ketoconazole has recently been reported in a multicenter French Study (4), although its availability in the United States has been restricted following an FDA safety warning for hepatotoxicity in 2013 (5,6), but it is widely available in Europe in 2015 (7).

The cortisol-lowering effect of metyrapone was described as early as 1958 by Liddle and co-workers, with later reports confirming metyrapone as a potent inhibitor of the steroidogenesis enzyme 11β -hydroxylase (8,9). Since then, it has been used as a diagnostic test of adrenal reserve and to treat the hypercortisolism of CS. Despite its widespread use, data on metyrapone are scarce, with the largest study to date (including 91 patients) being published over 25 years ago (10). Here, we have assessed the effectiveness of metyrapone therapy in a contemporary series of patients with CS, by performing a retrospective study of patients treated in the UK.

Methods

A multicenter, retrospective study was performed across thirteen University Hospital centers in England and Wales, members of the UK Endocrine Neoplasia Collaboration. Patients treated with metyrapone were identified through pharmacy records and electronic databases. Patients with a diagnosis of CS and treated with metyrapone between 1997 and 2013 were included.

The same proforma was used in all centers to record anonymized data. Data were gathered from case records and electronic record systems. Baseline, demographic and safety data, the indication for treatment and dose of metyrapone therapy, any therapeutic intervention and any recorded adverse events were documented. Monitoring tests included early morning (09.00h) serum cortisol, 24-hour urinary free cortisol (UFC), serum potassium, plasma ACTH and serum cortisol 'day-curves' (CDC). In CDCs multiple samples for serum cortisol are collected across the day with the mean calculated (11). The majority (91%) of CDCs consisted of 4 or 5 serum cortisol samples (minimum 3, maximum 8, median 4). All tests performed during the monitoring period were collected and analyzed. All centers used immunoassay-based cortisol assays.

Patients were treated either with a dose titration regimen i.e. metyrapone dose was up-titrated according to response to achieve a biochemical target for cortisol, or a block-and-replace regimen where the dose of metyrapone was quickly up-titrated to achieve blockade of cortisol synthesis and a replacement dose of glucocorticoid was added to provide background physiological levels.

Biochemical targets for treatment (eucortisolemia) were defined as a mean CDC value of 150-300nmol/l (10.9µg/dl), which has been shown to equate to a normal cortisol production rate as assessed by stable isotopic methodology (11), a UFC level below the upper limit of normal (ULN) for the assay used or a 09.00h serum cortisol within target. Although 09.00h serum cortisol is occasionally being used as a sole test for evaluating patients' response to treatment there is currently no standardized agreement for what values of this test represent appropriate control. Two different levels of target 09.00h cortisol were therefore assessed; (i) below the upper limit of normal for the assay used, or less than 600nmol/l (21.7µg/dl) if the ULN was higher than this value, and (ii), a recommended value of 331nmol (12.0µg/dl) (12). Cortisol levels were reported in nmol/L and divided by 27.59 to calculate the equivalent value in µg/dl. There was a wide range of UFC assays used with variable reference range of normal values; therefore UFC values were converted to multiples of the upper limit of normal (ULN) for the assay and this value was used for statistical comparisons. Patients with sufficient monitoring data (i.e. at least one test as described above

repeated at least twice during the study period) were included in the efficacy analysis. For the efficacy analyses we compared the mean values at each monitoring test (CDC, 09.00h serum cortisol, UFC) before treatment to (i) the mean values on the last review on treatment (diagnosis vs. last review), and (ii) the mean of all tests performed in all patients during treatment (diagnosis vs. treatment), unless otherwise stated. The change of the biochemical markers between (i) baseline (at diagnosis/ pre-treatment) and the last review on treatment for 09.00h cortisol, and (ii) the first and the last biochemical review for CDCs (CDCs were not routinely performed prior to initiation of treatment) on treatment was also analyzed.

Statistical analysis was performed using the two-tailed Student's t test (GraphPad prism 6.0, GraphPad Software Inc., La Jolla, USA). Except where stated, values given are means +/- standard deviations. A p-value of less than 0.05 was considered significant. The study was approved as an institutional case notes review at each participating center.

Results

Baseline characteristics

One hundred and ninety-five patients were treated with metyrapone across the 13 centers. The majority of patients had CD (115 patients, 37 macroadenoma) with the remainder having ectopic ACTH syndrome (EAS, 37), adrenocortical carcinoma (ACC, 10), and benign adrenal disease [30 adrenal adenoma (AA), ACTH-independent macronodular adrenal hyperplasia (2) and primary pigmented nodular adrenal hyperplasia (1)] (Table 1). There was a female predominance in all causes of CS except EAS (female patients: 74% CD, 49% EAS, 86% AA, 80% ACC). Patients were treated with metyrapone between 1997 and 2013 (83% between 2007-2013). The average duration of treatment was eight months (median 3 months, range 3 days to 11.6 years). At initiation of treatment there was a wide age distribution, with 76% of patients aged 30-69 years (age range 1-81, median age 48, average age 49.6 +/- 15.7

years), and 32% of patients (n=63) were women in the reproductive ages 18 to 45 (Figure 1). Co-morbidities at presentation included hypertension (64.6%) and diabetes mellitus (35.3%). For patients with CD, baseline contrast-enhanced pituitary MRI was positive in all patients with a macroadenoma and in 53 out of 72 (73%) patients with a microadenoma.

The main indication for metyrapone therapy was the control of severe symptoms of CS (CD 58%, EAS 77%, benign adrenal disease 44% and ACC 80%). Medical therapy was initiated as part of routine local practice in 8 out of 13 centers for the management of patients after diagnosis and prior to definitive therapy (e.g. surgery) regardless of the level of hypercortisolemia in a smaller number of patients (CD 25%, EAS 11%, benign adrenal disease 37%, ACC 0%). Delay in definitive treatment for CS (either due to medical reasons or requested by the patient) was a reason for starting medical therapy in 19% of patients. 25/195 patients (12.8%) received only cortisol-lowering treatment for their CS because of either inconclusive surgical target, palliation of aggressive malignancy (ACC or lung carcinoma), patients' own preference, or high surgical risk.

Biochemical changes during metyrapone treatment

Monitoring data during metyrapone therapy were available for 193 patients. The frequency of the monitoring visits was variable with some centers opting for inpatient tests at the introduction of treatment and other centers using outpatient monitoring every few weeks. 81% of patients were treated with dose titration and 19% with 'block-and-replace'.

Metyrapone monotherapy

One hundred and sixty four patients received metyrapone monotherapy and all monitoring tests showed significant improvement during treatment (Table 2). At the last review, 55%, 43%, 46% and 76% of patients who had CDCs, UFCs, 09.00h cortisol <331nmol/L (12.0µg/dl) and 09.00h cortisol < ULN/600nmol/L (21.7µg/dl) were controlled.

Ninety-one patients were monitored with cortisol 'day-curves' during treatment; 47/91 (52%) patients achieved a mean CDC < 300nmol/L (10.9µg/dl) during treatment (i.e. normalized cortisol target) and 81% of those who did not normalize had an improvement between the first and the last assessment on treatment (Figure 2a). Patients on a block-and replace regimen were more likely to achieve have a mean CDC < 150nmol/L. A total of 123 patients had 09.00h serum cortisol levels monitored; during treatment 83% (102/ 123) had a 09.00h serum cortisol bellow 600nmol/L (21.7µg/dl) or the ULN for the assay used and 56% (69/ 123) had a 09.00h level bellow 331nmol/L (12.0µg/dl) with 86% of patients showing an improvement in cortisol levels (mean improvement 566nmol/L, median 467nmol/L) even if these biochemical targets were not achieved (Figure 2b).

Effectiveness of metyrapone monotherapy before surgery

The majority (124/164) of patients treated with metyrapone monotherapy received treatment before any surgical intervention (CD 81, EAS 11, benign adrenal disease 25, ACC 7) for an average of 4.0 months. There was a significant improvement in the biochemical targets during metyrapone therapy (Table 2). At the last review, 50%, 35%, 40% or 72% of patients who had CDCs, UFCs, 09.00h cortisol <331nmol/L (12µg/dl) or 09.00h cortisol < ULN/600nmol/L (21.7µg/dl) were controlled (for dose see Table 3).

At the time of the first normalization, 91% were treated with dose titration and 9% with block-and-replace. In ACTH-dependent disease plasma ACTH levels were measured too sporadically to allow meaningful analysis. 10/18 (56%) patients who did not achieve a biochemical target also had a reduction of cortisol levels.

Metyrapone monotherapy as secondary treatment

Thirty-one patients (29 CD, 1 EAS, 1 benign adrenal disease) received metyrapone as secondary treatment following either surgery (21) or pituitary radiotherapy (17): 21/31 as monotherapy; 10/31 as combination therapy. Of the patients who received metyrapone following primary surgery, 19 had pituitary surgery for CD (9 had a macroadenoma); one had a pancreatectomy for a neuroendocrine tumor; and one a

repeat adrenalectomy for an incomplete excision of an adrenal adenoma. Of the patients with CD 7/19 also received pituitary radiotherapy. For the patients on monotherapy (n=21), the mean starting dose of metyrapone was 1300mg (Table 3). Patients were treated for an average of 17.1 months. At the last review, 76%, 78% or 94% of patients who had CDCs, 09.00h cortisol <331nmol/L (12µg/dl) or 09.00h cortisol <ULN/600nmol/L (21.7µg/dl) were controlled. At normalization 35% (6/17) of patients were treated with block-and-replace, and 65% (11/17) with dose titration. Biochemical tests (mean CDC and 09.00h cortisol) improved during treatment (Table 2). Only four patients had UFCs during treatment, therefore the change in UFC for this group of patients was not analyzed.

Long-term treatment with metyrapone monotherapy

Monitoring data were available on 38 patients who received metyrapone monotherapy for longer than 6 months. The average duration of treatment was 18.6 months and 6 patients had block-and-replace at some point during their treatment. Biochemical tests improved during treatment (Table 2). Overall, eucortisolemia was achieved in 72% (18/25) of patients who had CDCs, 77% (24/31) and 94% (29/31) of patients who had 09.00h cortisols (based on <331nmol/L or <ULN/600nmol/L cut-offs) or 64% (9/14) of patients who had UFCs.

Starting and final dose (Table 3)

Mean, median and range of doses on metyrapone monotherapy at the initiation of treatment and at final review are shown in Table 3. On 'block-and-replace' the starting dose of metyrapone was higher (mean dose 1432mg vs. 939.2mg, $p<0.0001$). There were, however, no significant differences in the mean 09.00h serum cortisol levels during treatment or at the last review in the two groups [block-and-replace group during treatment 461.2nmol/L (16.7µg/dl) vs. dose titration group 507.8nmol/L (18.4µg/dl), $p=0.50$, last review 510.8nmol/L (18.5µg/dl) vs. 376.3nmol/L (13.6µg/dl), $p=0.26$].

Combination treatment

Twenty-nine patients were treated with a combination of metyrapone and other cortisol-lowering medication (mainly ketoconazole or mitotane, 7 patients had combination treatment from the start of therapy, whilst in 22 combination therapy was instigated after initial treatment with metyrapone). The CDC or 09.00h serum cortisol levels at diagnosis were not significantly different in the patients treated with combination compared with the patients treated with metyrapone monotherapy [CDC combination 830.8nmol/L (30.1µg/dl) vs. monotherapy 722.9nmol/L (26.2µg/dl), $p=0.558$, 09.00h cortisol, combination 1149nmol/L (41.6µg/dl), vs. monotherapy 882.9nmol/L (32.0µg/dl) $p=0.077$]. There was a significant improvement in CDC and 09.00h serum cortisol during treatment (Table 2). Only three patients on combination therapy had UFC monitoring, precluding analysis. At the last review, 47%, 52% or 75% of patients who had CDCs, 09.00h cortisol <331nmol/L (12µg/dl) or 09.00h cortisol <ULN/600nmol/L (21.7µg/dl) were controlled. Patients who at the last review were controlled on a dose titration regimen based on CDCs and UFCs received 1850mg mean total daily dose (median 1500mg, range 750-6000mg). No subgroup analysis for efficacy was performed for this group due to small numbers.

Safety considerations

Side effects were noted in 48/195 patients (25%): 88% were managed as outpatients, whereas 12% (7/57 events) required either admission for evaluation or prolongation of a current admission. The rate of adverse events in patients on therapy for >6months was 11% (4/38 patients). There were no pregnant women, and no deaths recorded due to an adverse event. The average dose of metyrapone at the time of an adverse event was 1600mg. Gastrointestinal upset (23%) and hypoadrenalism (7% - symptoms of dizziness, hypotension, with biochemical confirmation) were the most common side effects. Most adverse events (39/56) occurred within 15 days of initiation of metyrapone or after a dose increase. Gastrointestinal upset and dizziness were the main reasons for discontinuing treatment. Patients with confirmed hypoadrenalism were managed either by addition of glucocorticoid (regimen change to a block-and-replace) or temporary cover with glucocorticoid and simultaneous reduction of metyrapone dose. In 15% of cases the metyrapone dose was reduced. In 12 cases (23%) metyrapone was withdrawn temporarily or permanently, with 11/12

showing full resolution, and in one symptoms continued but became less severe - muscle aches at presentation worsened during metyrapone therapy but returned to pre-treatment levels after drug withdrawal. Symptoms of hyperandrogenism were not frequent; hirsutism was not reported and there was only one case of worsening acne during treatment. Similarly edema was only reported in one case but the causative drug was thought to be a calcium channel blocker. Hypoglycemia was reported in three patients on diabetic medications and was associated with improvement of hypercortisolism.

Potassium levels were monitored and actively treated at presentation and during therapy. In 138 patients on metyrapone monotherapy, with no other treatment interventions for their CS, mean potassium levels increased from 3.68nmol/L to 3.90nmol/L ($p=0.003$) during treatment (Figure 3).

Discussion

We report the effectiveness of metyrapone in clinical practice for the treatment of CS. To our knowledge this is the largest study of metyrapone use as either monotherapy or metyrapone in combination with other cortisol-lowering medications. Overall more than 80% of patients showed an improvement in levels of circulating cortisol with over 50% achieving biochemical eucortisolemia when on monotherapy when assessed by the stringent criterion of control on a CDC. It is likely that additional therapies were added because of the severity of disease and clinician preference, but the retrospective and multicenter nature of our study precludes a formal assessment of this. Furthermore, our data support that metyrapone monotherapy is an effective treatment for hypercortisolemia either before or after surgical intervention to the primary cause of CS.

Metyrapone is widely used in CS in the UK and other countries but less so in the USA. To date, the efficacy of metyrapone in reducing cortisol levels in CS has been described in case reports and small case series (13-16), with the largest series reported 25 years ago by Verhelst et al (10). In this single center experience, metyrapone was effective

in reducing cortisol levels in 75% of 91 patients with CD, EAS and ACC based on a mean CDC level <400nmol/L that is higher than the more stringent <300nmol/L level that we used in this study. Most patients in the Verhelst study received a short course of metyrapone except for 24 patients who had metyrapone for a median of 27 months following radiotherapy to the pituitary gland. Smaller studies have reported the efficacy of metyrapone in patients with CD undergoing radiotherapy (13-15) and EAS (17). Overall, in 200 cases of metyrapone monotherapy published in the English literature, biochemical control was achieved in 75% (18). We report similar efficacy. It is of note, however, that the majority of patients with CD in our study here were not treated in conjunction with pituitary radiotherapy, and there did not appear to be evidence of an escape of control phenomenon, although we cannot comment on plasma ACTH levels during monitoring.

Ketoconazole, an antifungal agent and inhibitor of adrenal steroidogenesis, has also been widely used as a cortisol-lowering agent in CS. In the largest report to date, Castinetti et al reported biochemical control in 50% of patients with CS treated with ketoconazole monotherapy with biochemical improvement in 75% and evidence of regression of clinical features in up to 60% (4). Overall, in 456 published cases treated with ketoconazole monotherapy, 60% achieved control (18). Combination treatment with metyrapone and ketoconazole is commonly used (19), especially for the rapid control of hypercortisolism prior to definite treatment. In 22 patients with severe hypercortisolism due to EAS (n=14) and ACC (n=8), combination treatment of metyrapone and ketoconazole dramatically improved UFC levels within a month of treatment, while half of the patients also started mitotane during this time (20). Kamenicky et al used a triple-medication protocol with simultaneous administration of ketoconazole, metyrapone and mitotane in 11 patients with hypercortisolism and life-threatening complications as an alternative to bilateral adrenalectomy; all patients showed rapid clinical and biochemical improvement (21). In both studies, the initial biochemical control is mainly due to the combination of ketoconazole and metyrapone as the onset of action of mitotane is usually delayed by several weeks due to accumulation in adipose tissue (22). In one of the few prospective studies of medical treatment of CD, Feelders et al used a stepwise approach to treat 17 patients

with CD with a combination of pituitary and adrenal-acting agents. Patients were initially treated with the somatostatin analog pasireotide, followed by cabergoline, and ketoconazole was later introduced if biochemical control was suboptimal. Nine out of 17 patients normalized with pasireotide/ cabergoline and ketoconazole induced biochemical control in 6/8 remaining patients (75%) within 20 days of treatment (23).

Metyrapone increases cortisol metabolites in the serum and urine due to the predominant inhibition of 11 β -hydroxylase, and to a lesser extent the other steroidogenesis enzymes (10,24). In particular, 11-deoxycortisol levels may become profoundly elevated in patients on metyrapone therapy, especially in patients with CD (25,26). 11-deoxycortisol is structurally very similar to cortisol and may cross-react with cortisol immunoassays resulting in an overestimation of serum cortisol values in patients on metyrapone (26). The importance of this is underscored by the fact that symptoms of adrenal insufficiency may overlap those of side effects of metyrapone. Thus, cortisol estimation by more accurate methods such as mass spectrometry is advisable, and should be used where available (27). Moreover, it is likely that our data may underestimate the efficacy of metyrapone therapy when assessing serum measurements of cortisol as the cross-reactivity in immunoassays results in approximate 20% elevated bias (25).

Hypokalemia has been described as a potential serious complication of metyrapone therapy (24,28) due to the increase in steroid precursors with mineralocorticoid activity (11-deoxycorticosterone). Our data suggest clinicians using metyrapone are well aware of the importance of monitoring and managing serum potassium levels since we found that these increase significantly with supportive measures during treatment. It is important to stress, however, that such active monitoring is required, as hypokalemia is also a potentially harmful feature of CS. The most common adverse effects observed were mild gastrointestinal symptoms and hypoadrenalism, the latter a positive response to treatment provided that it is recognized and managed early. Patients on long-term treatment are more likely those who tolerate metyrapone well, therefore the rate of adverse events was favorable in this subgroup. Interestingly, hirsutism was not reported.

This study carries the limitations imposed by its retrospective design. Furthermore, there is currently no standardized monitoring and dosing regimen for patients on metyrapone therapy. The monitoring of hypercortisolemia in patients with CS on medical treatment is important to ensure that patients are treated with the correct dose and that hypoadrenalism, if present, is recognized early; measurement of serum cortisol allows this. Even though the study was conducted in University centers with significant expertise in the management of CS, the choice of biochemical monitoring test and frequency of monitoring varied. This has affected the uniformity of the data presented. During the period of the study the common clinical practice was to aim for a 09.00h cortisol below the upper limit of normal for the assay used or less than 600nmol/L. Any results above these levels would prompt up-titration of the dose or addition of a second agent. Therefore we have reported these cut-offs as the criteria for normalization of hypercortisolemia. More stringent 09.00h serum cortisol levels to define control have been proposed recently (12), with suggested values below 331 nmol/L (12µg/dL). It is not possible to know whether clinicians would have up-titrated the dose of metyrapone had this criterion been used, and therefore we can only speculate that the overall control when using this criterion might have been better if applied in practice.

In conclusion, our data show that metyrapone is effective and safe in treating hypercortisolemia in patients with Cushing's syndrome.

Acknowledgements

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Conflict of interest

The study was supported in part by an unrestricted grant from HRA Pharma.

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Figures and Tables

Figure 1
Age of patients at initiation of metyrapone therapy and diagnosis of Cushing's syndrome

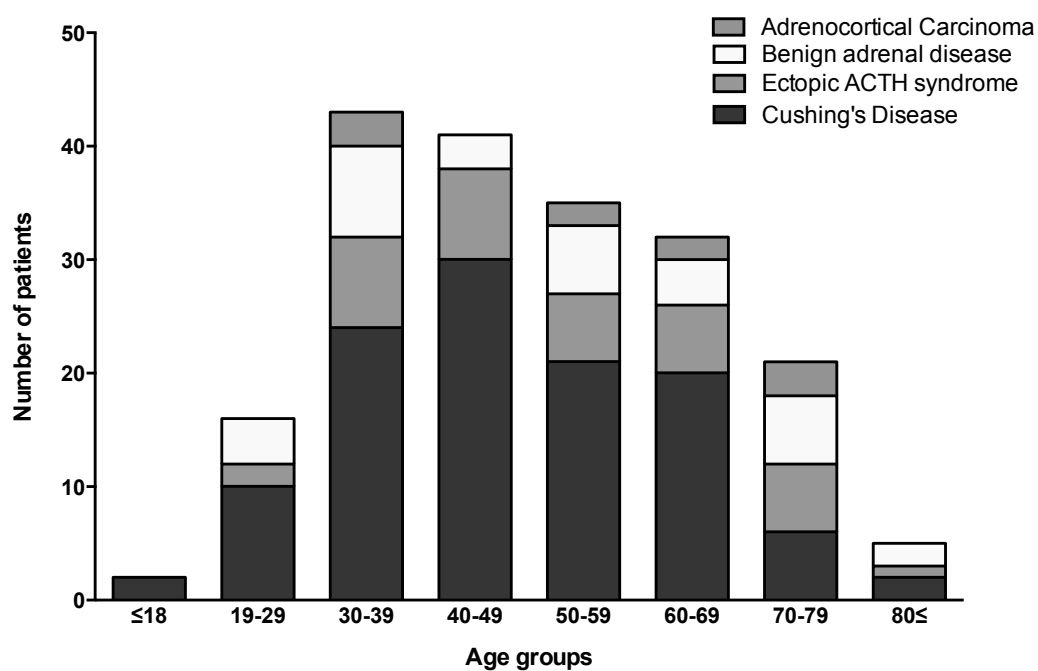


Figure 2

Mean serum cortisol day curve (CDC) and 09.00h serum cortisol levels during treatment with metyrapone monotherapy.

a), Change in mean CDC in 91 patients treated with metyrapone monotherapy between the 1st review *following* initiation of metyrapone and the last review on treatment: 52% (47/91) patients achieved biochemical normalization, 89% showed an improvement).

b), Change in the pre-treatment 09.00h cortisol level in 123 patients treated with metyrapone monotherapy and the last review on treatment: 86% showed an improvement; 102 (83%) patients had a 09.00h serum cortisol value below the ULN for the assay used or 600nmol/L (whichever was lowest) and 69 (56%) had a 09.00h level <331nmol/L.

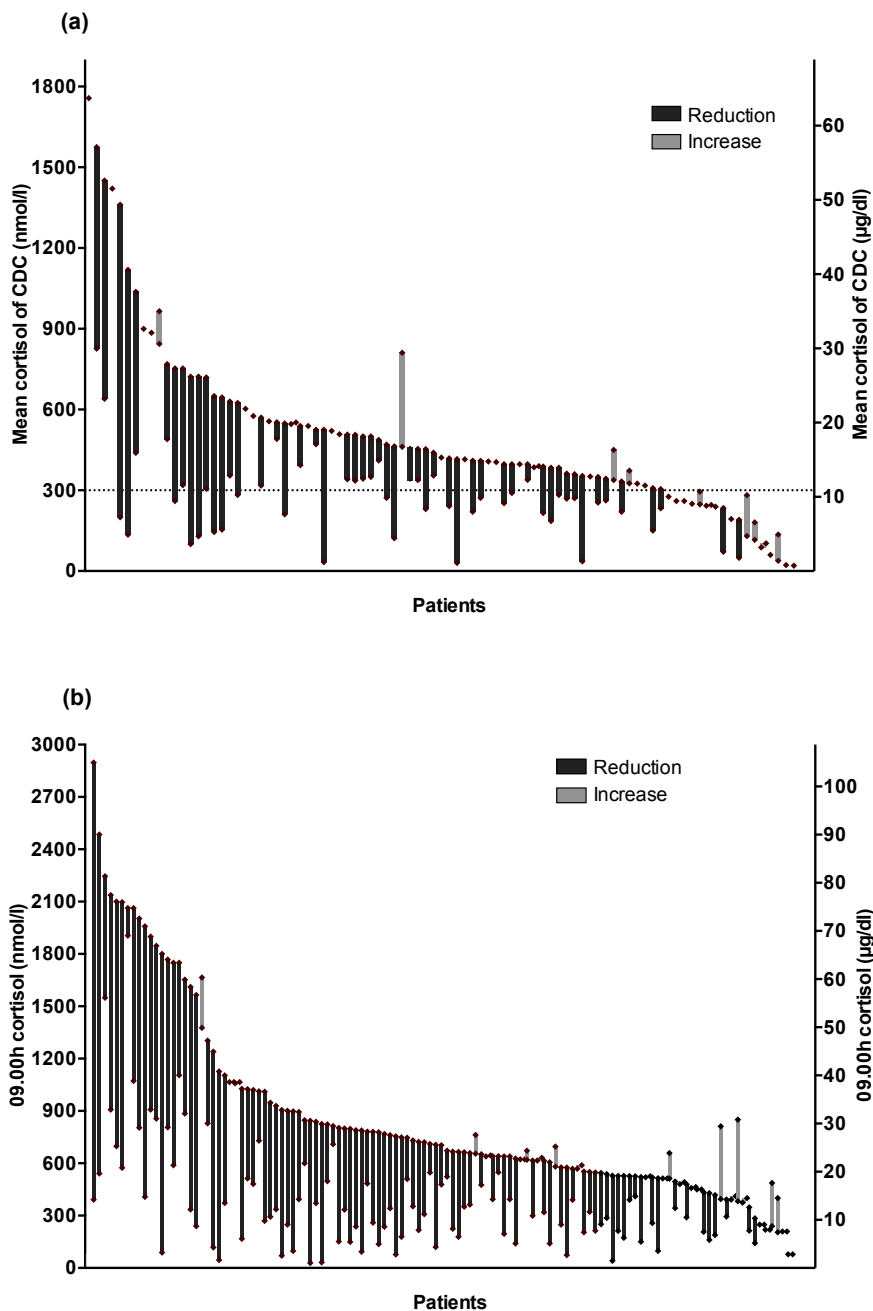


Figure 3
Potassium levels before and during metyrapone monotherapy in 138 patients.

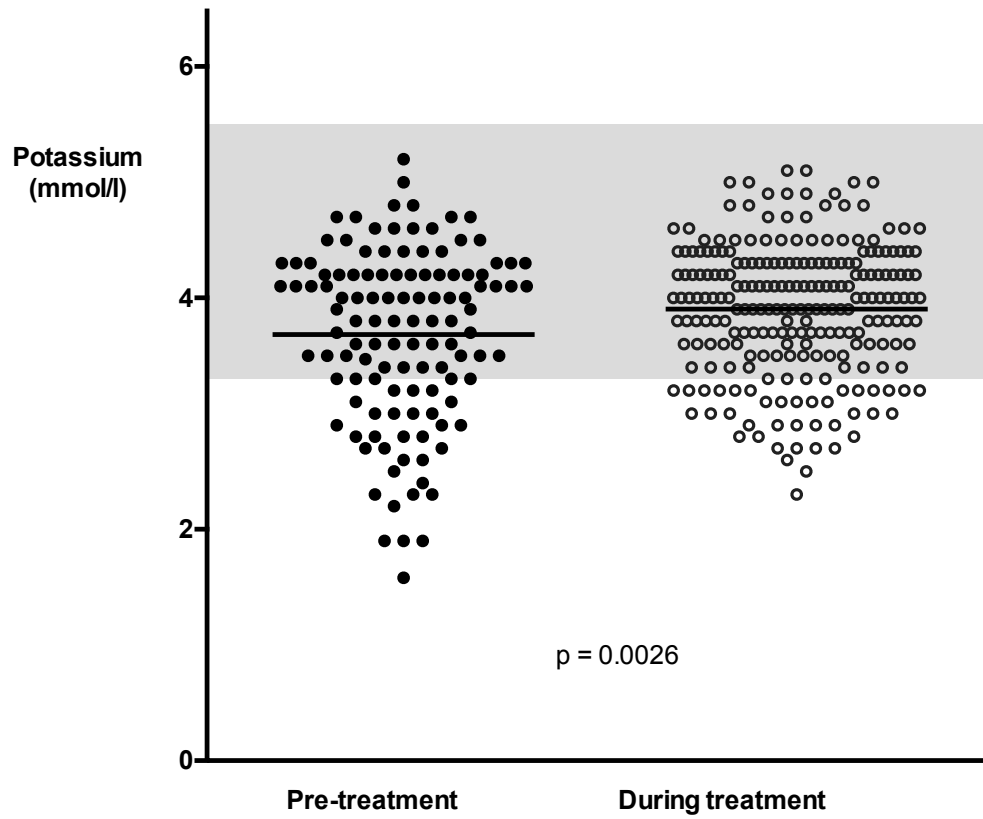


Table 1 Baseline patient characteristics

Etiology	No of patients	Female/ Male	Average age at diagnosis (years)	Average age at metyrapone onset (years)
Cushing's disease	115*	85/30	45.9	47.4
Macroadenoma	37			
Microadenoma	77			
Ectopic ACTH syndrome	37	18/19	52.6	52.9
Benign Adrenal Disease	33	27/6	50.3	51.2
Adrenal adenoma	30	26/4		
AIMH	2	1/1		
PPNAD	1	0/1		
Adrenocortical Carcinoma	10	8/2	56.0	56.4

*Size of adenoma not available in 1 patient

Table 2: Change in biochemical markers during metyrapone therapy (mean values)

	Number of patients	Pre-treatment	During treatment ¹	At the last review on treatment ¹
Monotherapy				
Overall	164			
Mean CDC ²	91	722.9nmol/L (26.2µg/dl)	396.4nmol/L (14.4µg/dl), p<0.0001	348.6nmol/L (12.6µg/dl), p<0.0001
0900h serum cortisol	123	882.9nmol/L (32.0µg/dl)	527.8nmol/L (19.1µg/dl), p<0.0001	491.1nmol/L (17.8µg/dl), p<0.0001
Urinary free cortisol	37	1483nmol/24h (537µg/24h)	1070nmol/24h (388µg/24h), p=0.588	453nmol/24h (164µg/24h), p=0.003
UFC:ULN ³	37	7.2	5.4, p=0.556	2.5, p=0.020
Before surgery	124			
Mean CDC	70	691.5nmol/L (25.1µg/dl)	407.7nmol/L (14.8µg/dl), p<0.0001	351.5nmol/L (12.7µg/dl), p<0.0001
0900h serum cortisol	82	779.7nmol/L (28.3µg/dl)	508.0nmol/L (18.4µg/dl), p<0.0001	495.6nmol/L (18.0µg/dl), p<0.0001
Urinary free cortisol	25	1318nmol/24h (478µg/24h)	1049nmol/24h (380µg/24h), p=0.704	525nmol/24h (190µg/24h), p=0.008
UFC:ULN	25	6.4	5.5, p=0.553	2.9, p=0.014
Secondary therapy	21			
Mean CDC	12	478.5nmol/L (17.3µg/dl)	311.0nmol/L (11.3µg/dl), p=0.001	248.9nmol/L (9.0µg/dl), p=0.001
0900h serum cortisol	17	659.6nmol/L (23.9µg/dl)	361.3nmol/L (13.1µg/dl), p=0.0001	281.3nmol/L (10.2µg/dl), p=0.002
Long-term treatment⁴	38			
Mean CDC	24	451.4nmol/L (16.4µg/dl)	339.5nmol/L (12.3µg/dl) p=0.07	366.2nmol/L (13.3µg/dl), p=0.35
0900h serum cortisol	31	734.2nmol/L (26.6µg/dl)	428.2nmol/L (15.5µg/dl), p<0.0001	384.5nmol/L (13.9µg/dl), p<0.0001
Combination therapy	29			
Mean CDC	17	830.8nmol/L (30.1µg/dl)	314.2nmol/L (11.4µg/dl), p<0.0001	278.7nmol/L (10.1µg/dl), p<0.0001
0900h serum cortisol	20	1149nmol/L (41.6µg/dl)	522.9nmol/L (19.0µg/dl), p<0.0001	471.9nmol/L (17.1µg/dl), p=0.003

¹ Statistical analysis is compared to the pre-treatment value

² Mean cortisol of a cortisol day-curve

³ Urinary free cortisol to the upper limit of normal for the assay used

⁴ More than 6 months

Table 3 Total daily dosage of metyrapone for patients treated with a dose titration regimen

	*Starting dose (mg)	*Final dose (mg)
Metyrapone monotherapy (n=164)	1040, 750, 250-3750	1425/ 1500/ 500-4000
CD (n=96)	1020, 750, 250-3000	1380/ 1375/ 500-3500
EAS (n=27)	1260, 1000, 500-3750	1990/ 1500/ 500-3750
Benign adrenal disease (n=31)	820, 1000, 250-2250	1210/ 750/ 500-4000
ACC (n=10)	1230, 1500, 750-2000	1190/ 1250/ 750-1500
Pre-surgery (n=124)	1000, 750, 500-2250	1440, 1500, 500-4000
CD (n=81)	980, 750, 500-2250	1400, 1500, 500-3500
EAS (n=11)	1200, 1500, 500-2000	2120, 2250, 500-3750
Benign adrenal disease (n=25)	880, 750, 500-2250	1230, 1000, 500-4000
ACC (n=7)	1250, 1500, 750-2000	1080, 1000, 750-1500
Secondary treatment (n=25)	1300, 1125, 500-3000	1400, 1500, 500-2250
Long-term treatment (>6months) (n=38)	940, 750, 500-3000	1560, 1500, 500-4000

**given in 2-4 divided doses; mean/ median/ range*

5.2. Study 2: Medical therapy in Nelson's syndrome

A prospective longitudinal study of Pasireotide in Nelson's syndrome.

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Authors' accepted copy of the paper is included in this thesis, pages 131 to 150.

Authors' accepted copy

A prospective longitudinal study of Pasireotide in Nelson's syndrome

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Keywords: Nelson's, pasireotide, medical therapy, corticotroph pituitary adenoma

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Abstract

Purpose: Nelson's syndrome is a challenging condition that can develop following bilateral adrenalectomy for Cushing's disease, with high circulating ACTH levels, pigmentation and an invasive pituitary tumor. There is no established medical therapy. The aim of the study was to assess the effects of pasireotide on plasma ACTH and tumor volume in Nelson's syndrome.

Methods: Open labeled multicenter longitudinal trial in three steps: 1) a placebo-controlled acute response test; 2) one month pasireotide 300-600 μ g s.c. twice-daily; 3) six months pasireotide long-acting-release (LAR) 40-60mg monthly.

Results: Seven patients had s.c. treatment and 5 proceeded to LAR treatment. There was a significant reduction in morning plasma ACTH during treatment (mean \pm -sd; 1823 \pm -1286ng/l vs. 888.0 \pm -812.8ng/l during the s.c. phase vs. 829.0 \pm -1171ng/l during the LAR phase, $P < 0.0001$). Analysis of ACTH levels using a random intercept linear mixed-random effects longitudinal model showed that ACTH (before the morning dose of glucocorticoids) declined significantly by 26.1ng/l per week during the 28-weeks of treatment (95% CI -45.2 to -7.1, $P < 0.01$). An acute response to a test dose predicted outcome in 4/5 patients. Overall, there was no significant change in tumor volumes (1.4 \pm -0.9 vs. 1.3 \pm -1.0, $P = 0.86$). Four patients withdrew during the study. Hyperglycemia occurred in 6 patients.

Conclusions: Pasireotide lowers plasma ACTH levels in patients with Nelson's syndrome. A longer period of treatment may be needed to assess the effects of pasireotide on tumor volume.

Introduction

Nelson's syndrome is a very challenging condition that can develop following bilateral adrenalectomy (BLA) for Cushing's disease (CD), and is due to the development of a progressive tumor of the corticotroph cells in the pituitary (1). It occurs in up to 30% of patients with CD undergoing bilateral adrenalectomy (2) although progression of the size of a corticotroph tumor as assessed by MRI is more common and is detected in 50% of patients within 10 years of BLA for CD (3,4). The corticotroph tumor may be small in some cases but may also be extensive and locally invasive in others; patients can present with mass effects, headache, visual field defects, and external ophthalmoplegia (5,6). The hallmarks of the syndrome are skin hyperpigmentation and high plasma adrenocorticotrophic hormone (ACTH) levels that reflect the activity of the tumor and are used for monitoring (7). Treatment of Nelson's is restricted to pituitary surgery and radiotherapy only when there is an amenable anatomic target and the patient's condition allows (8-10). In many patients with Nelson's syndrome these conditions are not met; the levels of ACTH continue to rise, the symptoms persist and there are limited treatment options.

There is currently no medical therapy that can consistently reduce plasma ACTH levels and corticotroph tumor growth, and there is a real need for an effective medical management for Nelson's syndrome. The anti-epileptic sodium valproate was frequently used in the past with disappointing or variable results (11-16) and dopamine agonists such as cabergoline only occasionally result in satisfactory response (17-20). Peroxisome proliferator-activated receptor gamma (PPAR γ) agonists such as rosiglitazone have also been studied: one report showed biochemical response in two out of three patients, but one of these subsequently escaped (21). Our group previously showed that even high doses of rosiglitazone (12mg/day) do not reduce plasma ACTH levels (5). Temozolomide is a medical treatment for aggressive pituitary tumors, but is associated with significant toxicity limiting its use (22).

Pasireotide exerts its pharmacologic effects by binding and activating multiple somatostatin receptor subtypes (1, 2, 3, and 5). In vitro experiments have shown that pasireotide inhibits ACTH secretion in cultured corticotroph adenoma cells (23) and prospective clinical trials have proved

effectiveness in lowering cortisol levels in patients with active Cushing's disease (24-26). More recently, pasireotide LAR was used to treat a patient with an invasive corticotroph tumor resulting in clinical improvement, and reductions in tumor size and plasma ACTH levels (27).

In light of these data we have performed a prospective multicenter clinical study aiming to investigate the effects of pasireotide on circulating plasma ACTH and tumor size in patients with Nelson's syndrome. In particular, the study was structured to assess: 1) the acute effects of pasireotide on circulating levels of plasma ACTH after a single 600µg s.c. injection, and whether this would allow prediction of individual longer-term response, 2) the effects of four-weeks of pasireotide s.c. on circulating plasma ACTH, 3) the effects of pasireotide LAR given monthly for 6 months on circulating plasma ACTH, and 4) the effect of pasireotide s.c. and LAR on tumor volume.

Methods

Study design

This was an open labeled longitudinal trial over a 31-week period conducted in four tertiary centers in England, UK. As there is currently no alternative treatment for Nelson's syndrome, no control group was used. There were three parts to the study. Initially, an acute response of plasma ACTH to pasireotide was assessed in a placebo-controlled randomized single-blinded crossover intervention where patients received either a test dose of 600µg pasireotide s.c. or an equivalent volume of saline s.c. whilst omitting their glucocorticoid treatment to establish if an acute response predicts future efficacy. In the second part of the study, patients received short-term (4-weeks) open label treatment with pasireotide twice-daily s.c. (600µg b.d. or 300µg b.d. if dose reduction due to tolerability was necessary). In the last part of the study patients had long-term open label treatment with pasireotide LAR 60mg (or 40mg if reduced for tolerability) every 28 days for 24 weeks (Figure 1).

Study endpoints

Primary endpoint: Early morning plasma ACTH sampled before (0 hours), and 2 hours after morning glucocorticoid (GC) replacement during 4 weeks of pasireotide s.c. 1200 µg/day (or 600 µg/day if reduced for tolerability issues) compared with levels at these respective time points found at baseline and after chronic depot pasireotide LAR i.m. every 28 days. The response criteria were defined according to the fall in plasma ACTH prior to first morning dose of GC or the fall in plasma ACTH 2 hours after the morning dose of GC. Complete success was defined as a fall in pre-GC plasma ACTH >400ng/l, or fall of >200ng/l 2 hours after GC; partial success a fall in pre-GC plasma ACTH <399ng/l >200ng/l, or 2 hours after GC <199ng/l >100ng/l; and not successful a fall in pre-GC plasma ACTH <199ng/l, or 2 hours after GC <99ng/l. The baseline value of the pre-GC ACTH was the mean of 4 values (from visits 1 to 4). The baseline of the post-GC ACTH was the value from visit 1 (screening visit).

Secondary endpoints: (1) Plasma ACTH before and at 2, 3, 4, 5, and 6 hours after an acute single dose of 600µg pasireotide or saline. (2) Changes in tumor volume at the end of the study determined by MRI. (3) Changes in skin pigmentation at the end of the study period compared with pre-treatment. (4) Change in HbA1c, fasting insulin and glucose levels during pasireotide s.c. and LAR treatment. (5) Tolerability and safety of pasireotide.

Patients

Patients with Nelson's syndrome were eligible to take part in this study. All patients gave informed consent and the study was approved by the UK Health Research Authority (ref 10/H1005/53). The inclusion criteria were: male or female patients aged 18-80 years with signs, symptoms and biochemistry consistent with Nelson's syndrome and a negative pregnancy test (where applicable). The exclusion criteria were: (1) pituitary radiotherapy within the last year prior to study entry, (2) recent significant deterioration in visual fields or other neurological signs related to tumor mass requiring surgery, (3) severe liver disease, (4) symptomatic cholelithiasis, (5) clinically significant abnormal laboratory values, (6) a QTcF interval measured on the EKG >480ms, (7) pregnancy or lactation, (8) recent (last 6 months) history of alcohol or drug abuse, (9) concurrent administration of investigational drug for another study, (10) history of non-compliance, or inability to complete

the entire study for any reason. Skin pigmentation was assessed at screening, at the start and at the end of pasireotide LAR treatment. Patients received no previous medical treatment for NS; patient characteristics are provided in the Supplementary Table.

Measurements

Imaging

Gadolinium-enhanced MRI of the pituitary was performed at the participating centers before and after treatment to assess the tumor volume. A blinded radiologist assessed the scans using standard volumetric techniques (28-30). Abdominal USS was performed at screening and at the end of the study to assess for the presence of cholelithiasis.

Skin pigmentation

An assessment of the pigmentation by the attending physicians and medical photographs of participants were performed at screening, at the start, and at the end of pasireotide LAR treatment. The photographs of all participants were collected and analyzed centrally.

Assays

Fasting insulin and ACTH samples were collected and analyzed at the central Clinical Chemistry laboratory. Insulin was measured with the Roche electrochemiluminescence immunoassay on a cobas e602 module (reference range 17.8-173pmol/L, CV 1.8% and 2.5% at values of 121 and 2062pmol/L). ACTH was measured by chemiluminescent immunometric assay on the Siemens Immulite 2000 analyzer (reference range <46ng/L at 9am and <15ng/L at midnight, CV 5.56% and 6.94% at values of 26.2 and 382ng/L).

Statistical Analysis

Sample size: A target of 17 patients was calculated taking into account the variability of ACTH levels and a 13% dropout rate which was recorded for a pasireotide phase 2 study (31). Accounting for a

within person variability of ACTH levels of approximately 400ng/l for the pre-GC dose and 250ng/l post-GC dose, 15 patients were needed to detect a clinically significant change of 200ng/l with a power of 80% with 5% significance and a further two patients to cover possible dropout (5).

Statistical analysis was performed using GraphPad (6.0d GraphPad Software, La Jolla California USA), SPSS v22 (IBM Corp., Armonk, NY) and STATA (StataCorp., College Station, TX: StataCorp LP). The main aim of the analysis was to establish whether ACTH levels change over time after pasireotide therapy. ACTH levels at 0h (before morning glucocorticoid dose) and 2-post GC dose, were compared before onset of pasireotide treatment ('baseline'), during s.c. and LAR pasireotide treatment using the Kruskal-Wallis non-parametric test; results are reported in mean+/-sd. Baseline ACTH levels at 0h were compared with baseline ACTH levels at 2h using a two tailed Mann-Whitney non-parametric test.' For the acute response test we calculated the relative decrease of ACTH levels at 2, 3, 4, 5, and 6 hours after a single s.c. pasireotide dose from the mean pre-dose ACTH levels (time points were at -1, -0.5 and 0 hours before dose) as well as a mean relative decrease. Comparison of safety blood tests was with one-way ANOVA. The longitudinal data (ACTH levels) were analyzed using a linear mixed-random effects model. We report estimates for the coefficient(s) from these regression models along with their associated 95% confidence interval (CI). Tumor volumes before and after treatment were compared by paired t-test, assuming a normal distribution; results are reported in mean+/-sd. A p value <0.05 was considered statistically significant.

Results

Patients

Eight patients were recruited, all females. Of the eight patients, two withdrew during the s.c. phase (1, 8) and two in the LAR phase of treatment (5, 7) and 4 patients (2, 3, 4, 6) completed all of the study visits (Table 1). In all patients any radiotherapy had been administered at least 5 years prior to study entry. Patient 1 withdrew after 11 days of s.c. pasireotide 1200 µg b.d. due to abdominal cramps (resolved after stopping pasireotide). Patient 5 withdrew after completing the s.c. phase. Patient 7 withdrew during the LAR phase due to significant hyperglycemia that persisted at the end

of the study visit 2 months after stopping pasireotide but improved to baseline on longer follow-up after study completion. Patient 8 withdrew during the first visit of the s.c. phase due to adverse events (felt unwell, drowsy, had a headache and was hypotensive during the visit); ACTH sampling from this visit was incomplete and therefore ACTH levels from this patient were not included in the statistical analysis.

Plasma ACTH levels improved during pasireotide treatment

ACTH levels at 0h prior to the morning glucocorticoid dose (ACTH 0h) at baseline were compared with ACTH 0h levels during the s.c. phase and ACTH 0h levels during the LAR phase. Overall, there was a significant reduction in ACTH 0h during treatment (mean baseline 1823 +/- 1286ng/l vs. 888.0 +/- 812.8ng/l during the s.c. phase vs. 829.0 +/- 1171ng/l during the LAR phase, $p < 0.0001$, $H = 20.93$ mean ranks 57.3 vs. 37.5 vs. 29.8) (Figure 2). Similarly, comparison of ACTH levels 2h after the morning glucocorticoid dose showed reduction of ACTH 2h levels during the two treatment phases (mean baseline 1100 +/- 987ng/l vs. 490.0 +/- 460.3ng/l during the s.c. phase vs. 262.2 +/- 219.4ng/l during the LAR phase, $p = 0.001$, $H = 13.38$ mean ranks 40.2 vs. 31.1 vs. 21.1). Baseline ACTH levels at 0h and 2h post glucocorticoid dose were significantly different ($p = 0.04$). In order to investigate the change of ACTH levels over time, plasma ACTH (0h pre-GC and 2h post-GC) at each study visit and for each individual patient were analyzed using a marginal Generalized Linear Model (GLM) for longitudinal data. There was a statistically significant decline in the ACTH 0h pre-GC levels throughout the study; ACTH 0h levels declined by 26.1 ng/l per week (95% CI -45.2 to -7.1; $P < 0.007$). GLM analysis showed that plasma ACTH 2h post-GC levels did not significantly change over time; ACTH 2h levels declined by 4.0ng/l per week, 95% CI -12.58 to 4.49, $p = 0.35$.

Applying the a priori ACTH response criteria at the end of 4-weeks of s.c. pasireotide (or at the last visit if patient withdrew prior to the end of this phase) 5/8 patients had a complete response, 2/8 had a partial response while one patient did not respond (Table 1). The patient who did not respond withdrew very early from the study after one s.c. dose of pasireotide (patient 8). At the end of 24-weeks of pasireotide LAR treatment or at the last visit, 3/5 patients had a complete response, 1/5 a partial response and 1/5 showed no response. Four patients completed the study; 3/4 had a complete response at the end of the study and 1/4 did not respond (Table 1). Overall, 6/8 patients

had complete or partial responses at their last biochemical assessment (either at the end of the study or last visit before withdrawal (Figure 3). There was no clear relationship between dose administered and effect.

Acute response to pasireotide test dose

Six patients received the pasireotide/placebo test dose while omitting their glucocorticoid treatment (Figure 4); 5/6 patients showed a consistent reduction in plasma ACTH levels and one (patient 6) did not respond (patient 4 received her usual glucocorticoid dose during the test and is excluded). The mean relative decrease in plasma ACTH levels before and 2-6hours after a pasireotide test dose in the five patients who showed a positive response to the test was between 25-84% (patient 2 79%, patient 3 25%, patient 4 84%, patient 5 53%, patient 7 70%); all patients with a positive acute response (i.e. reduction in ACTH levels post pasireotide test dose) showed a positive response at the s.c. phase of treatment. The maximum reduction was observed between 4 to 6h for all patients; those with a maximum relative decrease of at least 42% of their baseline ACTH levels following a test dose showed some response (complete or partial) to pasireotide treatment.

Change in tumor volume and skin pigmentation

Tumor volume: Five patients had MRIs at screening and at the end of the study; four patients completed the 28-weeks of the treatment protocol (patients 2, 3, 4, 6) and one patient (patient 7) withdrew during the LAR phase. Overall, there was no significant change in tumor volumes between the pre-treatment and post-treatment scans (1.4+/-0.9 vs. 1.3+/-1.0, p=0.86).

Skin pigmentation: There was no evidence of a change in skin pigmentation during the study as assessed by the independent assessor, although the attending physicians at the centers felt there was an improvement in 3 patients (patients 3, 6, 7).

Hyperglycemia during treatment

Fasting blood glucose and Hba1c increased during therapy and 6 patients developed hyperglycemia (Figure 5). Fasting glucose: mean at baseline 4.6 +/- 0.6mmol/l vs. 6.9 +/- 1.6mmol/l during s.c. phase vs. 9.6 +/- 2.9mmol/l during LAR phase, $p < 0.01$, Hba1c in mmol/mol: mean at baseline 42.9 +/- 7.8 vs. 45.6 +/- 8.5 during s.c. phase vs. 60.0 +/- 13.6 during LAR phase, $p < 0.01$. Patient 7 withdrew from the study due to significant hyperglycemia after 16 weeks of treatment. Fasting insulin levels reduced during s.c. and LAR pasireotide treatment (mean baseline 118.1 +/- 23.70 vs. mean during s.c. treatment 51.09 +/- 12.52 vs. 64.94 +/- 111.90 during LAR phase, $p = 0.04$).

Adverse events

During the study the majority of patients reported diarrhea (7 patients), nausea and headaches (6 patients), dizziness (5), abdominal cramps (4), flu-like symptoms (4) and symptoms of hyperglycemia (4). There were no events attributed to cholelithiasis, no clinically significant events relating to baseline blood tests (electrolytes, renal and liver function tests).

Discussion

Nelson's syndrome affects a significant number of patients treated with bilateral adrenalectomy for the management of hypercortisolism associated with Cushing's disease, and can be severely debilitating and life threatening. Although Nelson's syndrome is a condition that may be anticipated to occur after BLA, it poses a significant clinical management challenge, as there is currently no medical treatment that works consistently. In this prospective clinical study we have shown that pasireotide significantly reduces plasma ACTH levels in patients with Nelson's syndrome. All 7 patients treated with s.c. pasireotide (600 or 300µg b.d.) had a significant reduction in plasma ACTH levels and 4 out of 5 patients who progressed to receive monthly LAR pasireotide treatment continued to show a biochemical response. Pasireotide could, therefore, be considered for the treatment of patients with Nelson's syndrome especially if there is positive biochemical

response to s.c. pasireotide after a short 4-week treatment trial. Following this period patients that respond could continue on s.c. treatment or change to monthly LAR administration.

In this 28-week study there were no conclusive changes in either skin pigmentation or tumor volume, although there was an indication of possible improvement in pigmentation in 3/7 patients and there was at least one patient with minimal improvement in tumor volume. It is reasonable to anticipate that a biochemical response would be followed by a reduction in tumor volume on long-term treatment and our negative findings could be due to the small patient numbers or the short duration of treatment. A reduction of tumor volume with pasireotide treatment has been documented in patients with CD (24, 32) and a case report of a patient with Nelson's syndrome treated with pasireotide LAR (27); similar to our findings, the reduction in ACTH in this case report was evident early, within one month of treatment with improvement of skin hyperpigmentation. Tumor shrinkage is also well documented in patients with acromegaly treated with first and second generation somatostatin analogs (33). A longer period of treatment is needed to fully assess the effects of pasireotide on tumor volume in Nelson's syndrome.

In the advent of personalized medicine, predicting which patients are more likely to benefit from pasireotide treatment is extremely desirable as it could avoid expensive unnecessary treatment trials and exposure of patients to potential side effects. A positive acute response to pasireotide test dose (i.e. reduction in plasma ACTH levels following a single 600µg s.c. dose) may predict response to long-term treatment in the majority of patients, but a negative response does not exclude that a response will be seen; 5 out of 6 patients who had a consistent reduction in plasma ACTH after a test dose had a response to pasireotide treatment. Furthermore, patients that exhibited a decrease of plasma ACTH by at least 42% from baseline 4 to 6 hours after a pasireotide test dose showed some response (partial or complete) to pasireotide treatment. Histopathological analysis of tissue samples in patients with prior pituitary surgery looking specifically at the expression of somatostatin receptors (SSTR) could be assessed as a factor for predicting responsiveness. Unfortunately, the historical histological samples in this study were not available for re-examination but correlation of the SSTR expression patterns and biochemical response would have been interesting to examine and could help explain the differences in response between patients. However, it is also possible that SSTR expression from the original corticotroph

tumor is different than the active Nelson's tumor, and potentially this might be affected by other modalities of therapy, including radiotherapy. Furthermore, recent molecular studies in patients with CD suggest that there is enhanced SSTR5 mRNA expression in corticotroph adenomas harboring somatic mutations of the USP8 gene, and it is possible that the presence of USP8 mutations could help predict response to pasireotide treatment in Nelson's tumors (34). In our study no clear dose response relationship was observed on the effect on plasma ACTH. This may be due to varying expression of somatostatin receptors in the tumors or their signaling. Interestingly, there does not appear to be a dose response relationship for the effects of pasireotide in Cushing's disease.

The future place of pasireotide in patients with Nelson's syndrome needs to be balanced by its side effects, especially hyperglycemia. Hyperglycemia was a frequent adverse event associated with pasireotide treatment in this study with six out of seven patients developing abnormal fasting glucose and either new or worsening diabetes. Fasting glucose and HbA1c continued to increase during treatment in spite of the clinicians' attempts to treat this medically and one patient withdrew due to hyperglycemia. Similarly, high rates of hyperglycemia were reported in 49% of patients treated with LAR pasireotide (35) and 73% of patients treated with s.c. pasireotide (1200 or 1800 µg daily) for CD (24); in this study 6% of patients discontinued treatment due to a hyperglycemia related adverse event and 46% had to start a new anti-diabetic medication. The significant fall in insulin levels observed in our study is consistent with suppression of insulin secretion from beta cells of the pancreas, in keeping with the known action of pasireotide at the somatostatin subtype 5 receptors on these cells (36). The observed hyperglycemia following pasireotide treatment is due to the suppression of insulin and incretin response (glucagon-like peptide 1 and glucose-dependent insulinotropic polypeptide) (36). Greater physician awareness of the pasireotide-associated hyperglycemia and more aggressive management of glucose-related AEs may make pasireotide more acceptable for managing this challenging condition (37). Active monitoring and management of glucose homeostasis is needed and patients counseled about this prior to therapy.

The main limitations of this study are the small patient numbers, with this reflecting on overall generalizability, and the fact that half the patients did not complete the study. Although the

recruitment target was not met, the results confirm a statistically significant biochemical effect even in this small patient size. Plasma ACTH levels before the morning administration of glucocorticoid dose (ACTH 0h) are most commonly used for monitoring of patients with Nelson's syndrome and our results show statistically significant reductions during treatment with the robust GLM test. A non-significant trend of reduction of plasma ACTH levels 2h post glucocorticoid dose with GLM is likely due to lack of power and lower baseline levels of ACTH after glucocorticoid administration (mean baseline ACTH 0h 1823 +/- 1286ng/l vs. mean baseline ACTH 2h 1100 +/- 987ng/l). Three of the patients who showed response received radiation therapy 6-16 years prior to study entry and in the absence of historic ACTH levels a small lasting effect of radiation treatment on ACTH levels cannot be definitely excluded, but given the rapid fall in ACTH seen on treatment and the very long time period from radiation administration a significant contributing effect of radiation is unlikely. Treatment for periods longer than this study protocol (7 months) are likely needed to investigate the effect of treatment in tumor volume. The strengths of the study lie in the prospective design and the statistically significant evidence of biochemical response to medical therapy.

In conclusion, pasireotide treatment (s.c. and LAR) was effective in reducing ACTH levels in Nelson's syndrome and might represent a potential treatment on an individualized basis as treatment options are limited; the lack of complete consistency of response precludes making firm recommendations. If considered, active monitoring and management of glucose homeostasis is mandatory. The patients who responded did so soon after initiation of pasireotide, and thus it would be reasonable to consider a complete lack of response after two months of treatment as a failure of response and therapy be discontinued. The LAR preparation appears as effective as the s.c. preparation and is likely to be more acceptable to patients. It would seem reasonable to commence therapy at a lower dose and escalate if tolerated, as there appears to be no clear relationship between dose and effect. Our study is limited, however, by the small sample size and duration of therapy, precluding wide generalizability, and further studies are needed of longer duration (12-24 months) in greater numbers to formally assess the impact of pasireotide in Nelson's syndrome.

Conflict of Interest: JNP has research awards and consultancy from Novartis.

Ethical approval: All procedures performed in the study involving human participants were in accordance with the ethical standards of the UK Health Research Authority (reference 10/H1005/53) and with the 1964 Helsinki declaration and its later amendments. This article does not contain any studies with animals performed by any of the authors.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figures

Figure 1: Pasireotide treatment in Nelson's syndrome: study design

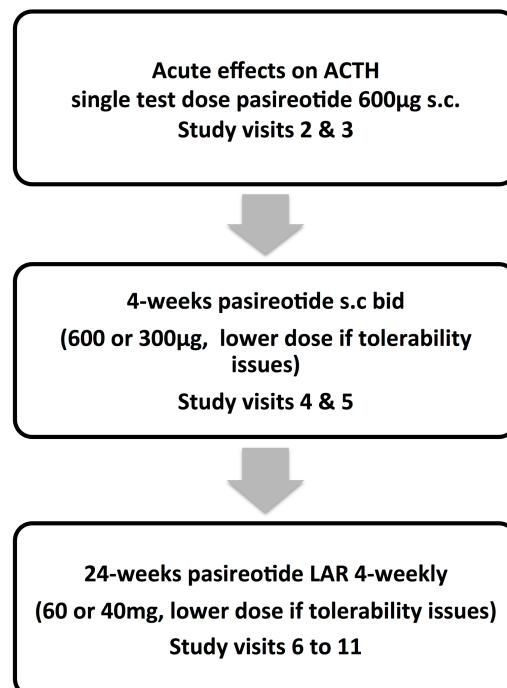


Figure 2: Mean plasma ACTH at 0 hours prior to the morning dose of glucocorticoids improved during pasireotide treatment (mean baseline 1823+/-1286ng/l vs. 888.0+/-812.8ng/l during the s.c. phase and vs. 829.0+/-1171ng/l during the LAR phase, p<0.0001)

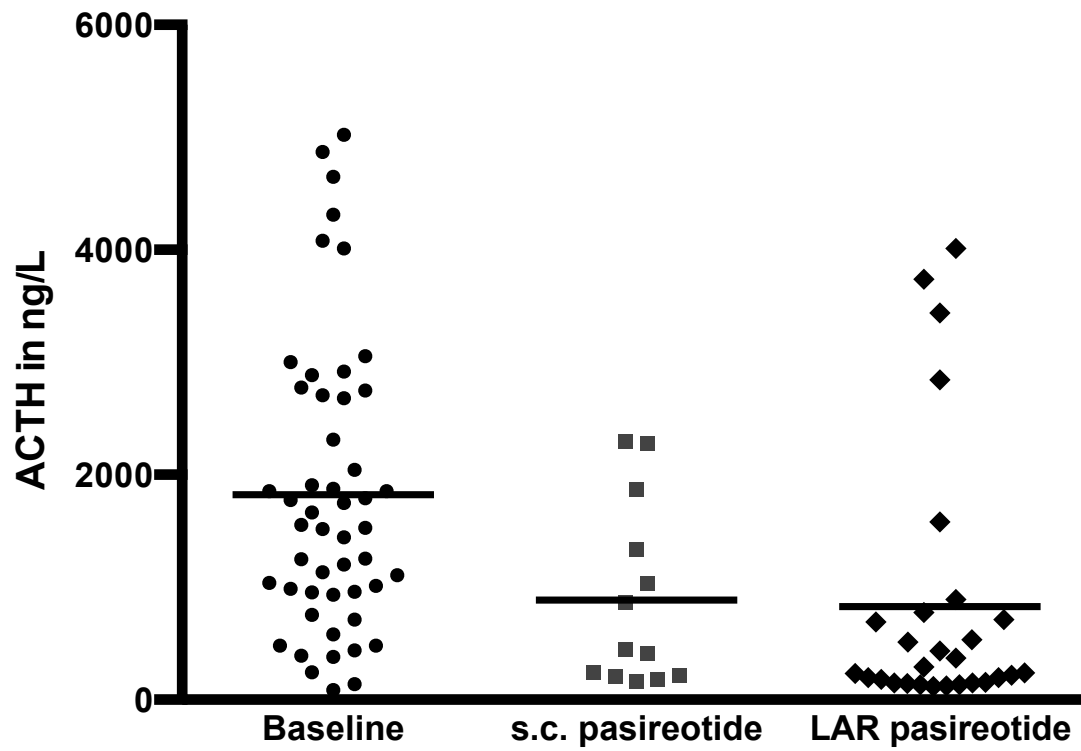


Figure 3: Individual plasma ACTH changes during the study in eight patients (ACTH levels before the morning dose of hydrocortisone)

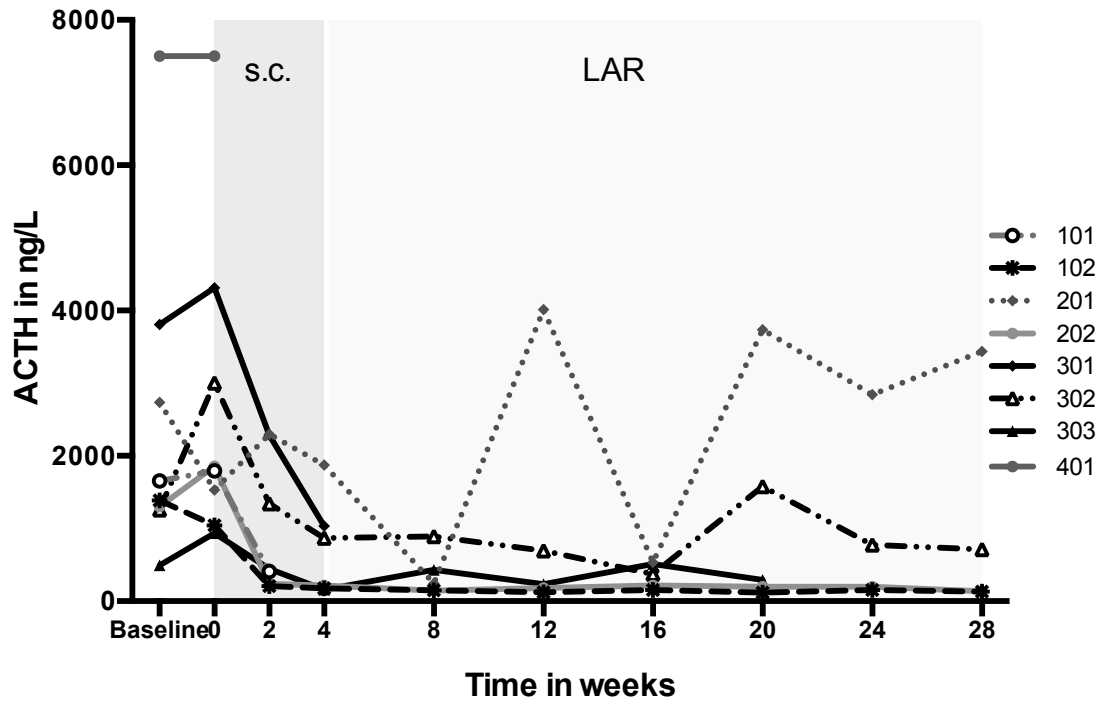


Figure 4: Acute response of plasma ACTH levels to a single dose of pasireotide 600µg s.c. in 7 patients [Patients (a) 2, (b) 3, (c) 4, (d) 5, (e) 6, (f) 7]

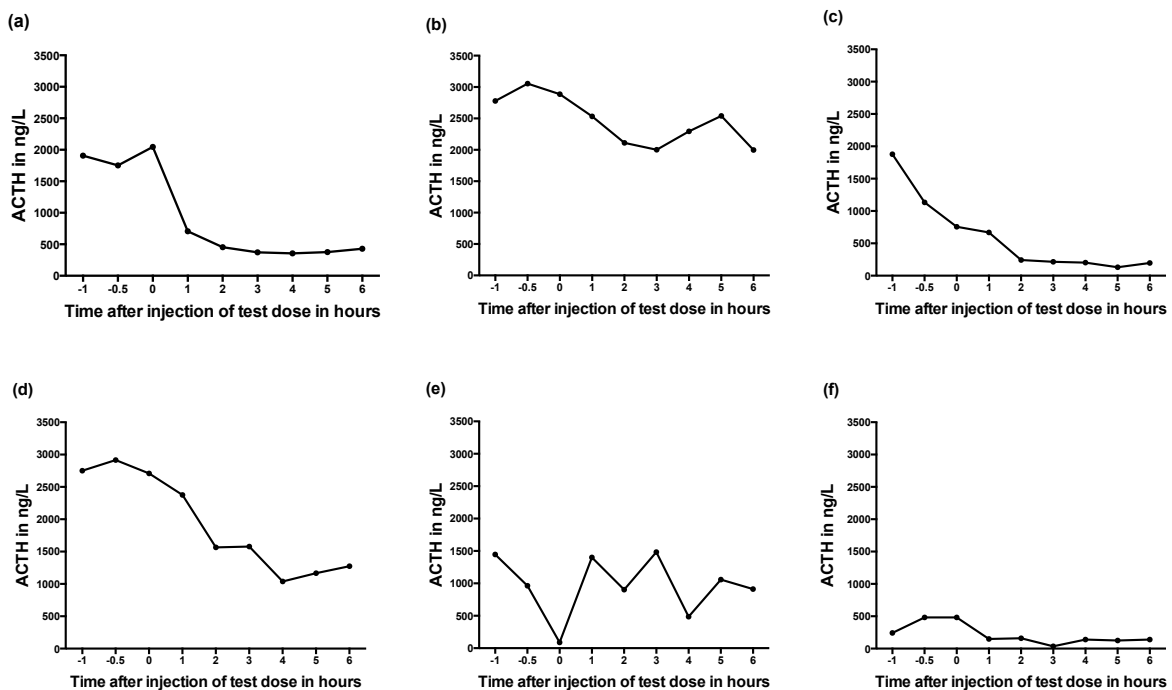


Figure 5a: Mean fasting glucose increased during pasireotide treatment (values from 7 patients included in the baseline mean value and s.c. phase, 5 patients for the LAR phase)

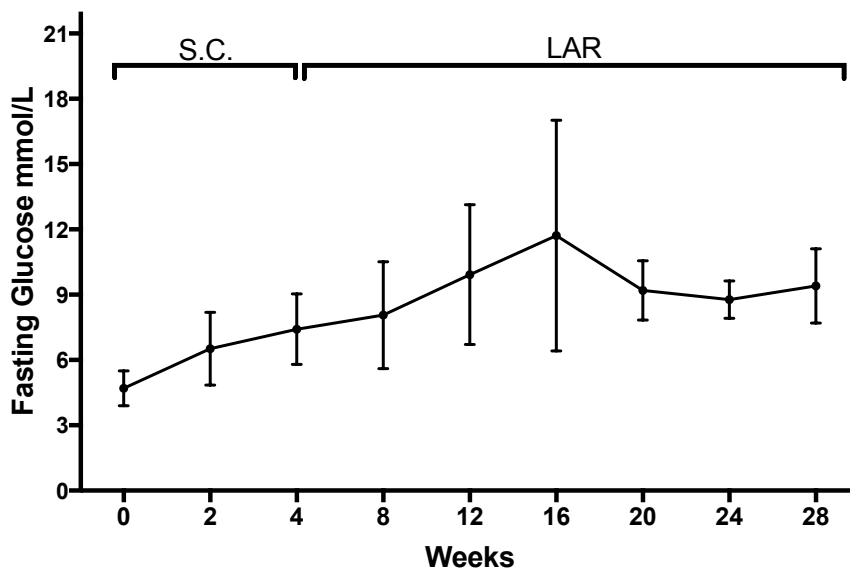
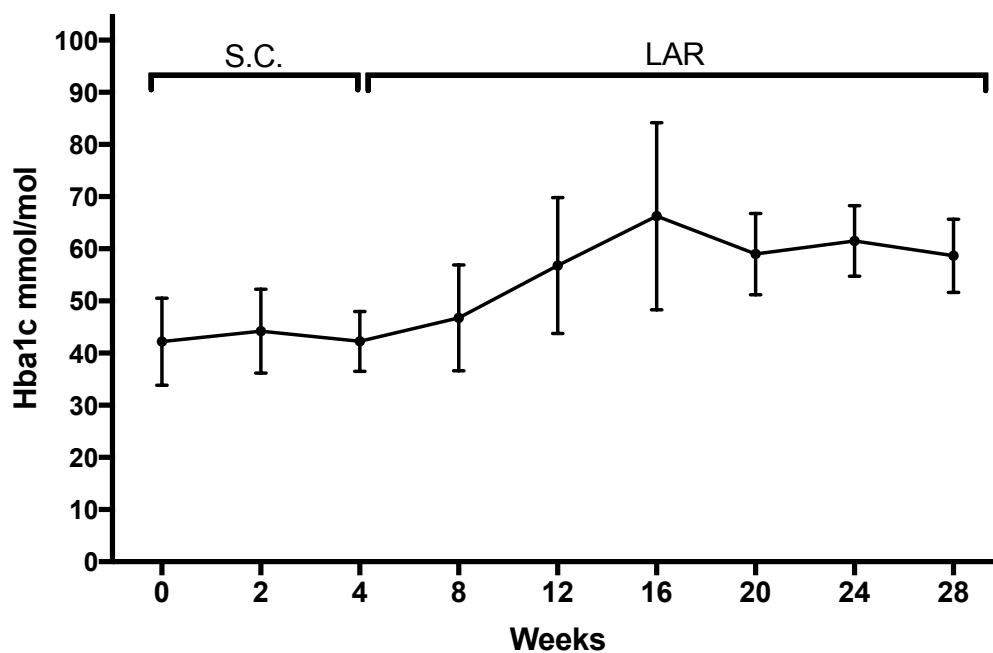


Figure 5b: Mean HbA1c levels increased during pasireotide treatment (values from 7 patients during s.c. phase and 5 patients during LAR phase)



5.3. Study 3: Administration of Hydrocortisone through nasogastric tubes

Accuracy of hydrocortisone dose administration via nasogastric tube.

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Authors' accepted copy of the paper is included in this thesis, pages 152 to 173.

Authors' accepted copy

Accuracy of hydrocortisone dose administration via nasogastric tube

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Abstract

Objective: Hydrocortisone via nasogastric (NG) tube is used in sick children with adrenal insufficiency; however, there is no licensed formulation for NG administration.

Methods: We investigated hydrocortisone recovery after passage through NG tubes *in vitro* for 3 formulations: liquid suspension, crushed tablets mixed with water, and hydrocortisone granules designed for oral administration to children. Cortisol was measured by LC-MS/MS.

Results: Hydrocortisone content was variable and recovery low after preparation in syringe and prior to passage through NG tubes. For doses 0.5mg & 2.0mg mean percentage recovery was: liquid suspension 57% & 58%; crushed tablets 46% & 30%; and hydrocortisone granules 78% & 71%. Flushing the administering syringe increased recovery. Hydrocortisone recovery after passage with flushing through 6-12Fr gauge NG tubes was variable: liquid suspension 61-92%, crushed tablets 40-174%, hydrocortisone granules 61-92%. Administration of hydrocortisone granules occluded 6 & 8Fr NG tubes however administration using a sampling needle to prevent granules being administered gave a recovery of 74-98%.

Conclusions: The administration of hydrocortisone through NG tubes is possible; however, current methods deliver a variable dose of hydrocortisone, generally less than that prescribed. Attention should be placed on the technique used to optimise drug delivery such as flushing of the administering syringe. Hydrocortisone granules block small NG tubes but behaved as well as the commonly used liquid suspension when prepared with a filtering needle that filters out granules.

Introduction

Long-term treatment with hydrocortisone is required in children with adrenal insufficiency and treatment starts from birth in neonates with congenital adrenal hyperplasia. Replacement therapy with oral hydrocortisone is generally given in 3-4 daily doses (1-3). The administration of oral hydrocortisone in young children may require a nasogastric (NG) tube during inter-current illness, and treatment with hydrocortisone to reduce bronchopulmonary dysplasia in premature infants is becoming more popular (4); however, there are no licensed formulations for administration via NG tube.

Hydrocortisone is poorly soluble in aqueous solutions and the suspension is viscous and therefore its delivery may be adversely affected when intervening equipment such as syringes and NG tubes are used (5). Inaccuracy in the hydrocortisone dose delivered leads to under- or over- replacement and is associated with poor disease control and potentially poor long-term health outcomes (6-8). For hydrocortisone administration via the NG route, the preparations most commonly used in paediatric practice are liquid suspensions (syrup) available as special unlicensed formulations and tablets crushed into a fine powder and mixed with water (9-12). A multi-particulate immediate-release formulation of hydrocortisone, has been specifically developed for oral administration to neonates, infants and young children (13,14). This study investigated the in vitro recovery of hydrocortisone after passage through NG tubes of varying bore for three different hydrocortisone preparations; a liquid suspension (Rosemont Pharmaceuticals Ltd, UK), crushed hydrocortisone tablets mixed with water

(Auden McKenzie (Pharma Division) Ltd, UK), and hydrocortisone granules (Alkindi, Diurnal Ltd, UK).

Methods

Protocol development and hydrocortisone formulations tested: The experimental protocol was developed following consultation with adult and paediatric endocrine specialist nurses, senior neonatal intensive care nurses, paediatric pharmacists and a review of current clinical practice (9,15-18). In children, oral hydrocortisone is usually given in 3-4 daily doses from 0.5mg upwards with the commonest dose being 2mg (2,3,13,19), so we chose to test doses of 0.5mg and 2.0mg (=target doses). Current practice in our institution is to use either liquid in suspension (100ml bottle at 5mg/5ml) or crushed 10mg hydrocortisone tablets. When using NG tubes in neonates the total drug administration volumes should be minimal with NG flushes up to 2ml (15,17), so we chose to give doses in maximum 2ml volume with 2ml flush in the NG tubes 6-8Fr that are used in this age group. The protocol was tested on the bench multiple times, timed and supervised by a paediatric endocrine nurse to ensure compliance with clinical practice. Two researchers performed the experiments and all stages were timed for standardisation.

Protocol for preparation of hydrocortisone formulations for administration:

- Liquid hydrocortisone suspension: the bottle (100ml bottle at 5mg/5ml) was shaken vigorously and the required amount drawn into a sterile 10ml syringe.
- Hydrocortisone tablets: one 10mg tablet was crushed using a tablet crusher into a fine powder, 10ml of sterile water were added and mixed and the required amount drawn into a sterile 10ml syringe.
- Hydrocortisone granules: the contents of one capsule (0.5mg or 2mg) were suspended in 2ml sterile water in a 10ml sterile syringe, the suspension was shaken vigorously for 30 seconds, left on the bench for 15 minutes and then shaken again for 30 seconds.

Hydrocortisone recovery at the nasal end of the NG tube: the experiment assessed the recovery of hydrocortisone in samples prepared for NG administration (but not administered) according to the above protocol. There were two parts in this experiment. In the first part two target doses, 0.5mg and 2mg were prepared as above and then expelled into bijoux tubes. Six repeats were performed. The second part assessed whether the suboptimal recovery of hydrocortisone was due to dose remnants in the administering syringe: a second set of samples for the liquid suspension formulation was collected that included flushing of the administering syringe with 2ml water. The flushing liquid was collected together with the sample for hydrocortisone quantification and three repeats were performed. The samples were stored at 4°C prior to analysis.

Hydrocortisone administration through NG tubes: NG tubes come in variable sizes and are measured using the French (Fr) scale, with smaller French values representing a narrower diameter and shorter length. A size 6Fr NG is used for long-term feeding in a small neonate and 12Fr is the adolescent and young adult size 16. Medicines and fluids are administered at the nasal end of the NG tube and exit through a small ovoid opening next to the gastric end. The administration of all three preparations was tested using transparent 6, 8, 10 and 12Fr NG tubes to cover the size range used across the paediatric population. Each NG tube was held in a ring stand, at a height of 30cm, with the lower end in a collecting tube. Each formulation was administered from the 10ml syringe used for preparation and using the same syringe each tube was then flushed with water (2ml for the 6Fr and 8Fr tubes, 5ml for the 10Fr tubes and 10ml for the 12Fr tubes). The NG tubes were left to drain all administered materials into the collecting tube at the gastric end of each NG for 3 minutes (NG-passage sample). The experiment was repeated 6 times. Following hydrocortisone granules administration only, the NG tubes were observed for the presence of granules intraluminally. If any granules were present, the tube was flushed once more 30 minutes later; the patency of the tube was recorded but the liquid was not added to the previously collected NG-passage sample.

Alternative method for preparation of hydrocortisone granules for administration: a second method of sample preparation for hydrocortisone granules was developed to test the feasibility of neonatal size (6Fr) NG administration and assess the recovery of hydrocortisone. In a bijoux tube, 2mg of hydrocortisone granules were suspended in 2ml sterile water, the suspension was shaken vigorously for 30 seconds and allowed to rest on the bench for 0, 15,

30, 45, or 60 minutes, then shaken vigorously again for 30 seconds. Immediately afterwards, 1ml of suspension (target dose 1mg) was aspirated into a 2.5ml sterile syringe through a metallic sampling needle used for aspirating drugs for oral administration that prevented any granules from entering the syringe (Nutrisafe2 sampling needle, external diameter 1.1mm and 52mm long, Vygon (UK) Ltd). The needle was then removed and the contents of the syringe were either pushed down a 6Fr NG tube or expelled into a small bijou tube (control sample). With the same syringe, 2ml sterile water were aspirated and then flushed into the NG tube (NG passage sample) or expelled into the control samples. The NG tubes were left to drain into the bijou tubes for 3 minutes and the experiment was repeated 5 times.

Quantification of hydrocortisone by Liquid chromatography tandem mass spectrometry (LC-MS/MS): All samples were labelled with a numerical code, stored at 4°C and transferred on ice for LC-MS/MS analysis of hydrocortisone at the Biochemistry Department, Manchester University NHS Foundation Trust. Prior to analysis the samples were warmed in a hot bath for 5min, shaken and a 1:10,000 dilution with water was made. The LC-MS/MS method has been described elsewhere (20) but briefly, standard, quality control or hydrocortisone sample (20 µL) was manually pipetted directly into the well of a 96-deep well block (Thermo, Hemel Hempstead, UK). To this, 40 µL of 0.1 mol/L zinc sulphate was added. This was vortexed for 10 s followed by the addition of 100 µL of internal standard. The block was heat-sealed (Thermo, Hemel Hempstead, UK) and vortexed for 1 min, then centrifuged at 8000 g for 5 min. Following centrifugation, the plate was transferred directly to the autosampler for analysis; 10 µL of sample was injected into the liquid chromatography (LC) system using partial loop mode. LC-MS/MS was performed using an Acquity I Class coupled to a XEVO TQ-

D detector (Waters, Wilmslow UK). The quantity of hydrocortisone in mg in each sample was calculated from the hydrocortisone concentration. The inter-assay imprecision (%CV) was 13%, 9% and 5% at concentrations of 100, 400 and 800 nmol/L, respectively. The intra-assay imprecision was 12%, 7% and 9%.

Data presentation and statistical analysis: Results are shown as mean \pm sd of the repeats. The data are expressed either as mean hydrocortisone content in mg or % hydrocortisone recovery i.e. the mean hydrocortisone content in each set expressed as a percentage of the dose administered (target dose). ANOVA with multiple comparisons was used for the analysis of differences between the 3 hydrocortisone formulations and between the bench time rest periods allowed for the alternative preparation method for hydrocortisone granules suspension (GraphPad 7, GraphPad Software, La Jolla California USA). Unpaired two-tailed t-tests were performed for comparison of pre and post NG administration recovery for each time-point in the alternative method of preparation. A p value of <0.05 was considered significant.

Results

Recovery of hydrocortisone prior to NG administration (Figure 1): The recovery of hydrocortisone from all three preparations at the nasal end of the NG tube prior to NG administration was low: mean \pm sd % recovery of target dose for doses 0.5mg and 2.0mg was;

liquid suspension $57\pm 7\%$ & $58\pm 18\%$, crushed hydrocortisone mixed with water $46\pm 18\%$ & $30\pm 5\%$, hydrocortisone granules $78\pm 15\%$ & $71\pm 4\%$. The delivery of hydrocortisone with hydrocortisone granules was significantly better than crushed hydrocortisone for the 0.5mg dose ($p<0.01$) and the 2mg dose ($p<0.01$), and the liquid suspension was better than the crushed hydrocortisone for the 2mg dose ($p<0.01$) (Figure 1).

The delivery of hydrocortisone in the pre-administration samples of the liquid suspension increased significantly following flushing of the administering syringe; mean \pm sd % recovery of target dose; 0.5mg dose without flushing $57\pm 7\%$ vs. with flushing $147\pm 31\%$ ($p<0.01$); 2mg dose without flushing $58\pm 18\%$ vs. with flushing $105\pm 8\%$ ($p<0.01$, Figure 2). Based on these results syringes for all formulations were flushed for the experiments using the NG tubes.

Hydrocortisone recovery after passage through NG tubes: In this in vitro setting, it was possible to administer hydrocortisone through neonatal, paediatric and adolescent size NG tubes using all three preparations, although the delivery was variable. Hydrocortisone granules and the liquid suspension showed similar results throughout the range of NG tube sizes whereas the crushed hydrocortisone tablets gave greater variability for both doses (Figure 3, Table 1). The delivery of hydrocortisone for the 0.5mg dose mean \pm sd % recovery of target dose for the 4 different size NG tubes was; liquid suspension $65\pm 32\%$ to $92\pm 34\%$, crushed hydrocortisone $59\pm 22\%$ to $174\pm 118\%$, hydrocortisone granules $66\pm 13\%$ to $83\pm 17\%$ and for the 2mg dose; liquid suspension $61\pm 14\%$ to $65\pm 6\%$, crushed hydrocortisone $40\pm 5\%$ to $96\pm 34\%$, hydrocortisone granules $61\pm 7\%$ to $92\pm 14\%$.

The possibility of mechanical tube occlusion due to administration of hydrocortisone granules was further explored. Following nasogastric administration of hydrocortisone granules the NG tubes were observed for granules; no remaining granules were visible in the 10Fr and 12Fr. However, hydrocortisone granules were trapped within 6Fr and 8Fr tubes and the water flush did not remove them completely (Figure 4). Flushing the NG tubes immediately after administration of hydrocortisone granules was difficult although there was no complete occlusion of the NG tube during the administration phase. When NG tubes were left to drain for 30min and a second flush was attempted complete occlusion was observed in 10% of 6Fr NG tubes and 50% of 8Fr NG tubes. Fewer granules were observed to enter the 6Fr NG tube compared to 8Fr tube.

Recovery of hydrocortisone from hydrocortisone granules using an alternative method of preparation: To avoid granules entering the NG tube from the administering syringe an alternative preparation method was developed and tested in the neonatal size (6Fr) NG tubes. Hydrocortisone granules were suspended in water for 0, 15, 30, 45 and 60 minutes to test if suspension time affected recovery. As shown in Figure 5, hydrocortisone recovery before and after administration down the NG tube was similar for each time point (p values 0.1 to 0.6). In the pre-administration control set, hydrocortisone recovery between the different time points significantly increased between time zero and 15 minutes of bench suspension ($p < 0.01$) and the same was found for the post NG passage samples ($p < 0.01$, Figure 5). For post NG tube passage the recovery was: 0 minutes $14 \pm 4\%$, 15 minutes $74 \pm 20\%$, 30 minutes

89±12%, 45 minutes 89±18%, 60 minutes 98±15%. No NG tube blockages were observed with this method.

Discussion

We have shown that it is possible to administer hydrocortisone via a nasogastric tube, however dose recovery at the gastric end of the nasogastric tube is very variable and generally less than that administered. Three hydrocortisone formulations were tested: a liquid suspension (Rosemont Pharmaceuticals Ltd, UK), crushed tablets mixed with water (Auden McKenzie (Pharma Division) Ltd, UK), and hydrocortisone granules (Alkindi, Diurnal Ltd, UK). At the nasal end of the NG tube recovery was poor for all three formulations, between 30-78% unless the administering syringe was flushed. The recovery after passage down NG tubes with flushing was variable (40-174%) and generally <80% of the dose administered with the greatest variability seen for crushed tablets where in some cases recovery was <50% of the dose administered. Variability was least with hydrocortisone granules with recovery between 61 and 92%. Recovery of the dose administered was not affected by tube size for the liquid suspension but for crushed tablets and hydrocortisone granules recovery was best with the largest tube (12Fr). Hydrocortisone granules blocked the smaller NG tubes but this was avoided by generating a hydrocortisone suspension from the granules by leaving in water for 15 minutes and then using a sampling needle for drug aspiration that didn't allow granules to be aspirated into the syringe.

Crushing oral medication to a fine powder is common practice for nasogastric administration in adults and children but it is an unlicensed use of the medication (21). Compounding from adult dose formulations is common in paediatrics when no dose appropriate formulation is available (13,22), it is undertaken by pharmacy as well as carers and can lead to therapeutic failure among other risks (23). Capsules prepared by pharmacy from compounded hydrocortisone tablets have been found to have unacceptably variable drug content in over 20% of batches, and have led to clinically and biochemically evident glucocorticoid overtreatment (7,22). In our study, crushed hydrocortisone tablets mixed with water showed significant variability in the recovery of the administered hydrocortisone dose, usually with significantly low recovery but occasionally the recovery was above 100% of the target dose meaning that higher amount of hydrocortisone than the target dose (0.5mg or 2mg) was recovered in the sample. This likely reflects problems with the current practice of preparing small doses from 10mg adult dose tablets. Another factor could be the loss of active pharmaceutical ingredient that could be up to 10% of the mass during hydrocortisone compounding because hydrocortisone sticks in the equipment used for compounding (24). Furthermore, hydrocortisone is relatively insoluble in water (5,25), which means most hydrocortisone is in suspension not solution.

Few studies have reported the administration of medications through NG tubes and none have reported on hydrocortisone (26-29). Our results show suboptimal recovery of hydrocortisone at the gastric end. High variability and low recovery of medications such as proton pump inhibitors administered through NG tubes was commonly observed in in vitro studies and recovery increased when higher volumes of solvent were used for drug

dissolution prior to NG administration and flushing of the equipment (17,26-28). Similar to our observation, formulations consisting of granules frequently cause NG tube obstructions (30,31).

It is important to follow appropriate techniques when administering medications down NG tubes and this applies to patients and carers who can be trained to give medications through NG tubes in the community. However, medicines are usually used out of license and there is lack of data on the accuracy of drug delivery through this method (9,16,30). We found that flushing the equipment (syringes) improves delivery for liquid suspension hydrocortisone. This has implications in children treated with hydrocortisone via the oral route when intervening equipment such as syringes are used; flushing of devices is important to maximize recovery for hydrocortisone, which is poorly soluble in water and sticks to plastics (5,24). Our in vitro results demonstrate that specific methods need to be followed for different formulations of hydrocortisone to maximize recovery and accurate dosing and that most methods lead to under dosing.

Hydrocortisone granules have been recently licensed in Europe for replacement therapy of paediatric adrenal insufficiency and according to the summary of product characteristics they are not suitable for administration through nasogastric tubes (13). Consistent with this we found that hydrocortisone granules blocked smaller NG tubes. Removing the granules by creating a suspension in a universal tube shaken and left for 15 min then aspirating using a sampling needle to avoid granules and administered down a NG tube resulted in a dose recovery of 74-98% which was comparable to and less variable than the other hydrocortisone

formulations; however, this is not a licensed method of administration for hydrocortisone granules.

The strengths of this study are the protocol design that was developed to reflect current clinical practice in the administration of hydrocortisone in young children and the accurate method for estimating hydrocortisone concentration by liquid chromatography tandem mass spectrometry. The methods for the preparation of the three hydrocortisone formulations were different because we were comparing a liquid solution, tablets and granules that are available in clinical practice in different dose strengths (liquid 1mg/ml vs. tablets 10mg vs. granules 0.5mg and 2mg). These differences could affect the results for example the accuracy of hydrocortisone administration from crushed tablets might be better if a 5mg tablet was used for the chosen target doses 0.5 and 2mg rather than a 10mg tablet however a 5mg tablet is not available in Europe and therefore not tested. Two researchers performed the experiments and although the data were reviewed to check for operator-dependent trends there was no formal statistical comparison between the two and this is a limitation of the study. This was an in vitro study and the concentration of hydrocortisone at the end of the NG tubes, however accurate, does not necessarily reflect the plasma concentrations in vivo and our results should be viewed in this light.

In conclusion, although delivery of hydrocortisone through NG tubes is possible, significant attention should be placed on the technique used to optimise drug delivery. The delivery of hydrocortisone with hydrocortisone granules was comparable with the currently used formulations and in fact granules seem to behave as well as the liquid suspension which is the

current standard and most optimal formulation for oral administration; however, it leads to tube occlusions in the smaller gauge NG tubes (6Fr and 8Fr). Using a sampling needle to prevent the administration of granules is an alternative technique that delivers 74-98% of the required target dose.

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Figures

Figure 1: Mean hydrocortisone content prior to NG tube administration. Three hydrocortisone formulations (liquid suspension, crushed 10mg tablets, and hydrocortisone granules) were prepared in syringes at 0.5 & 2.0 mg absolute dose and then expelled into a universal tube with hydrocortisone content in universal measured by LC-MS/MS. (a) 0.5 mg dose (b) 2.0 mg dose (*: $p=0.004$, **: $p=0.001$, ***: $p<0.001$).

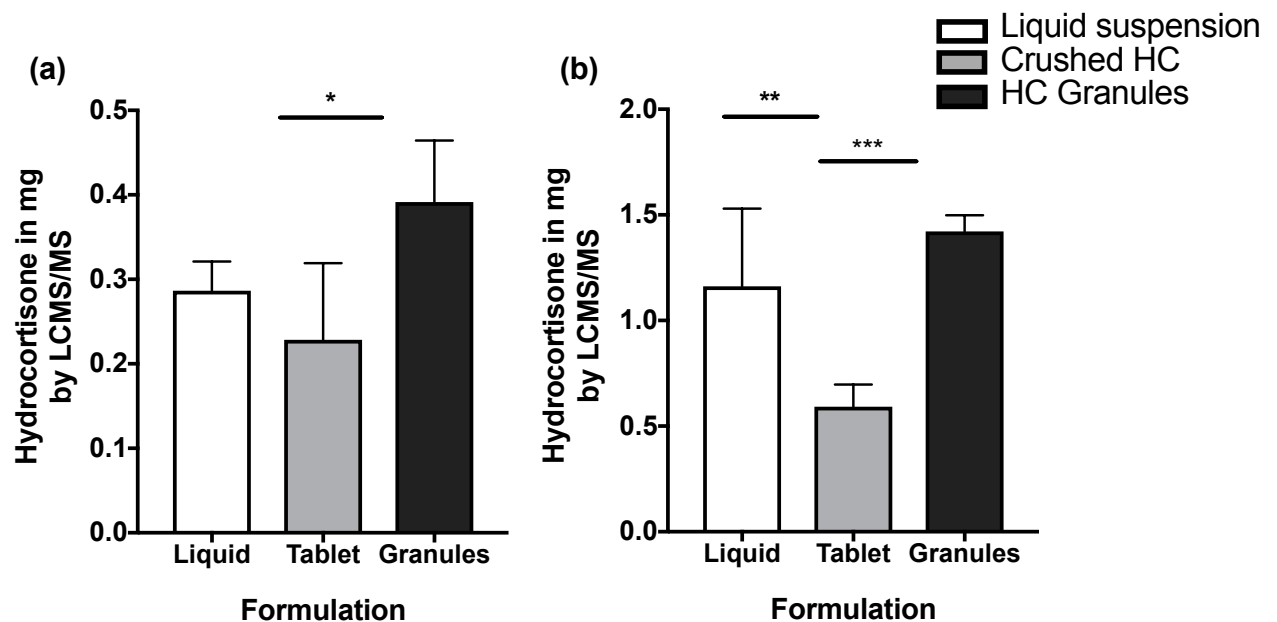


Figure 2: Mean hydrocortisone content prior to NG tube administration after flushing of the syringe used to draw up the dose (Flush: pre-administration samples with 2ml flushing of the administrating syringe, N: pre-administration samples without flushing of the syringe, *: $p < 0.001$, **: $p = 0.002$).

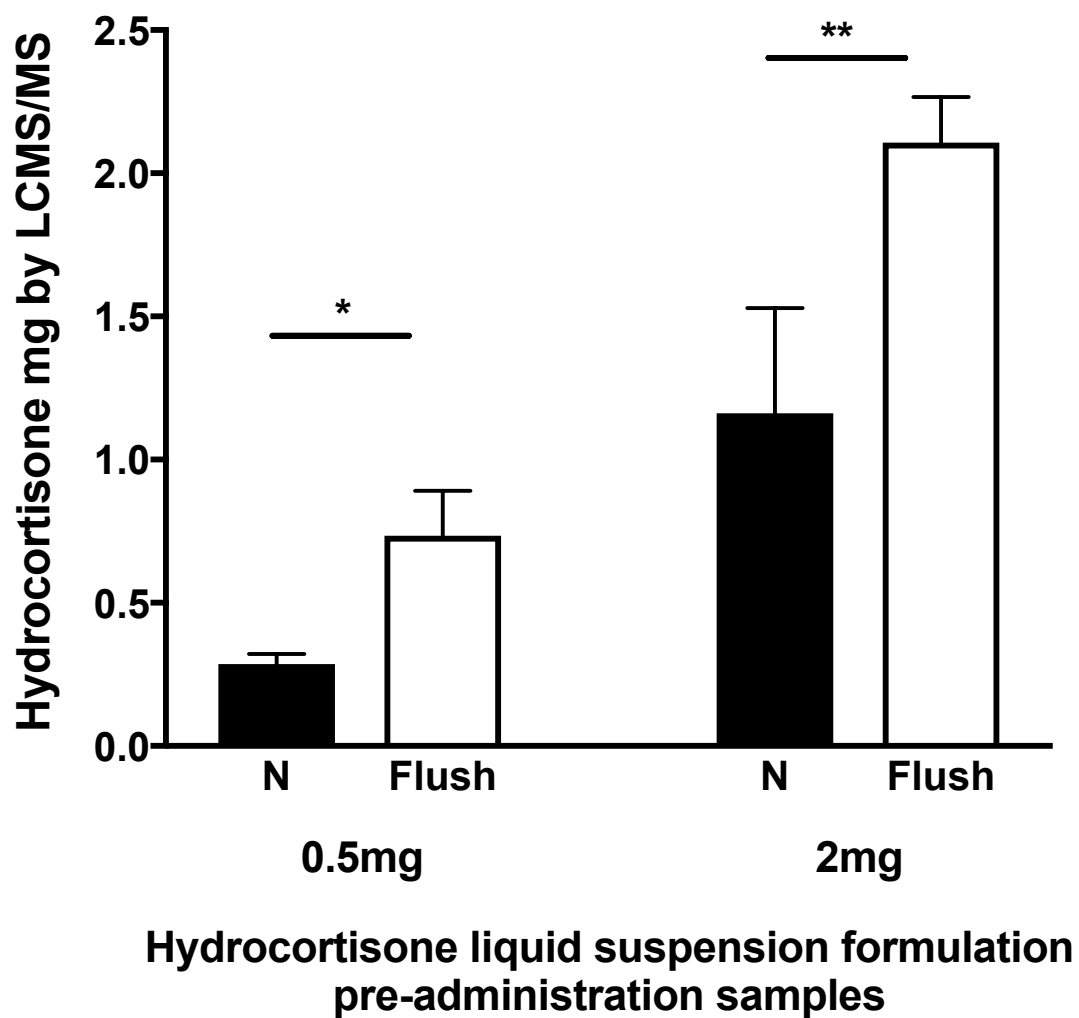
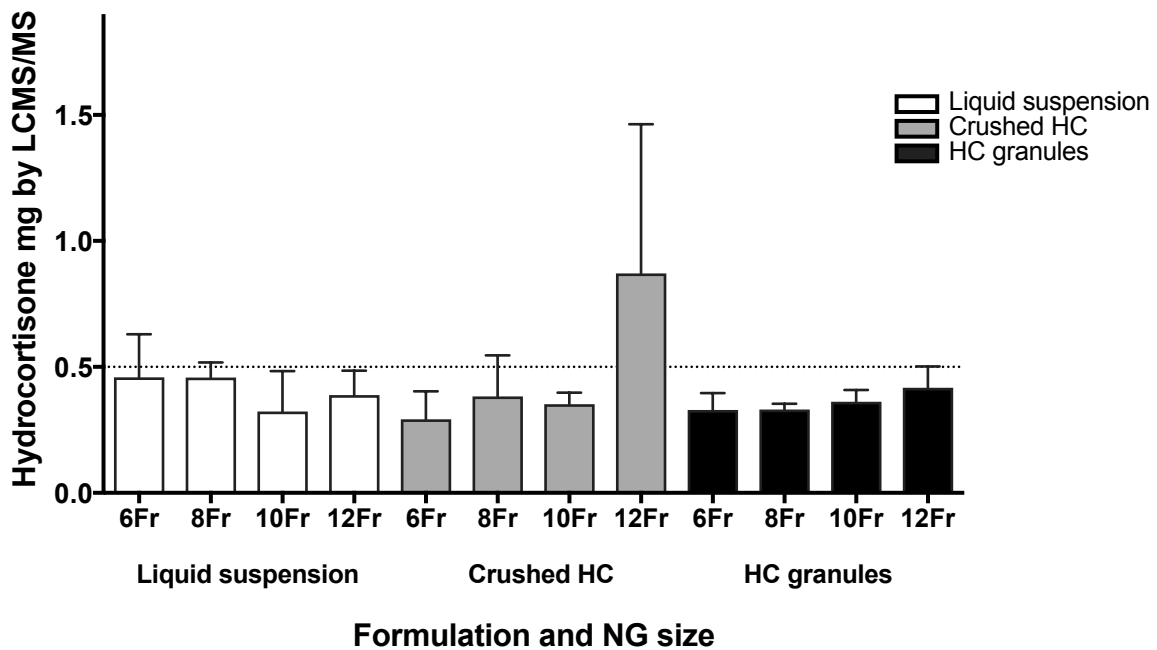


Figure 3: Mean hydrocortisone content after preparation in a syringe, administration through NG tubes gauge 6-12 Fr followed by flushing (a) Hydrocortisone dose 0.5mg and (b) Hydrocortisone dose 2.0mg.

(a)



(b)

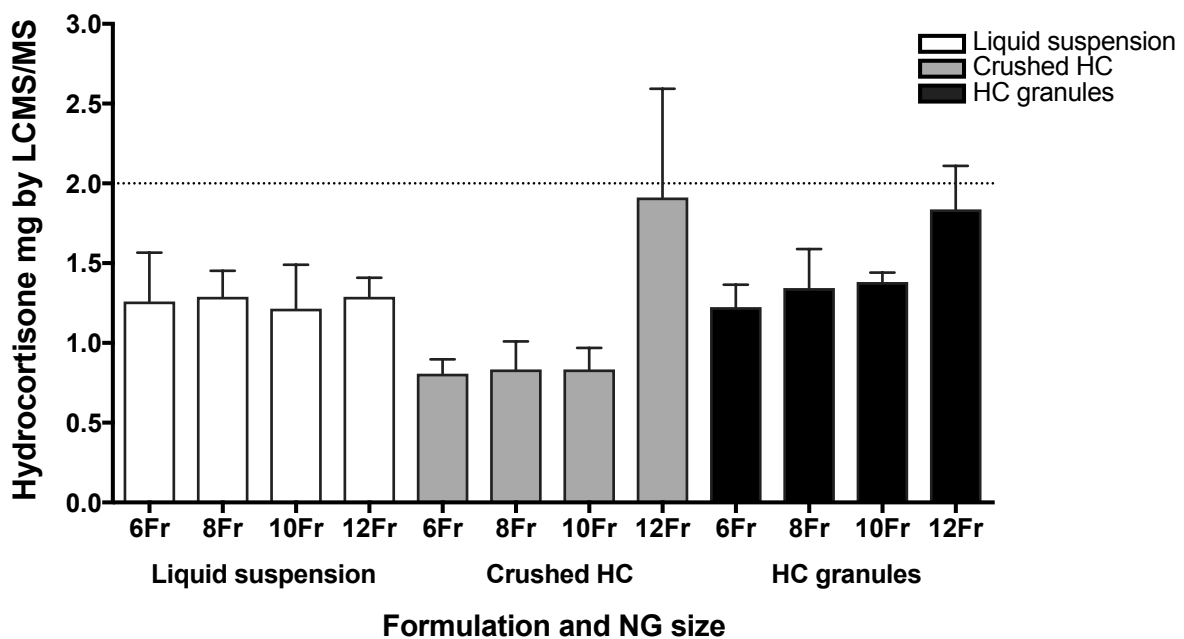


Figure 4: Hydrocortisone granules occluding 6Fr NG tube.

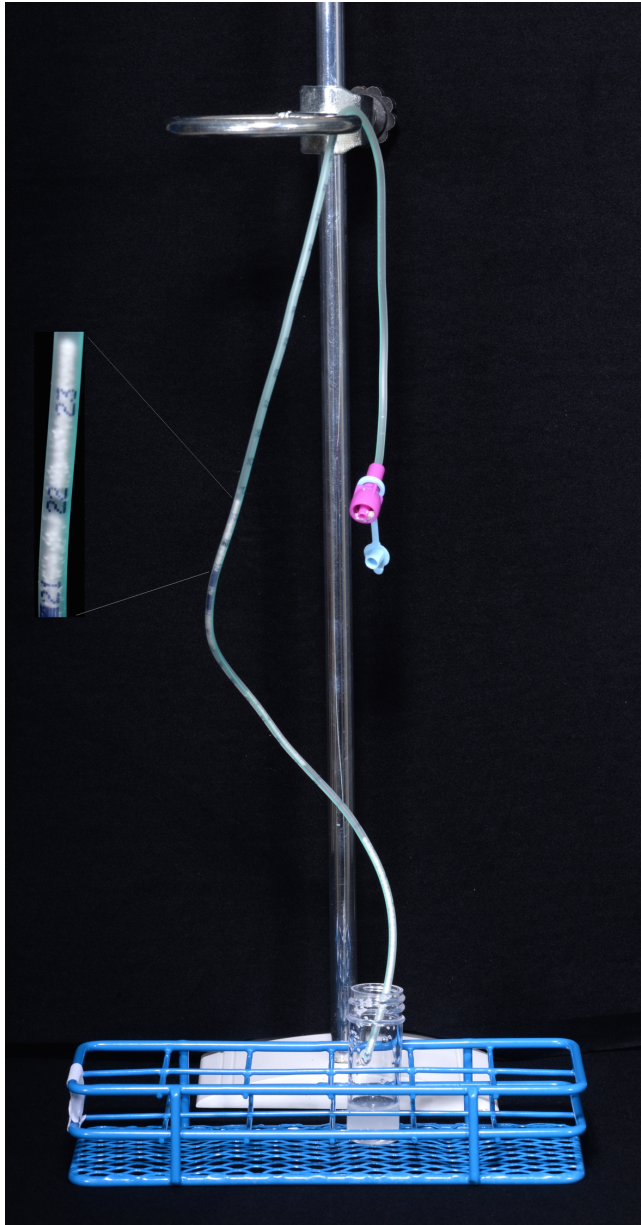
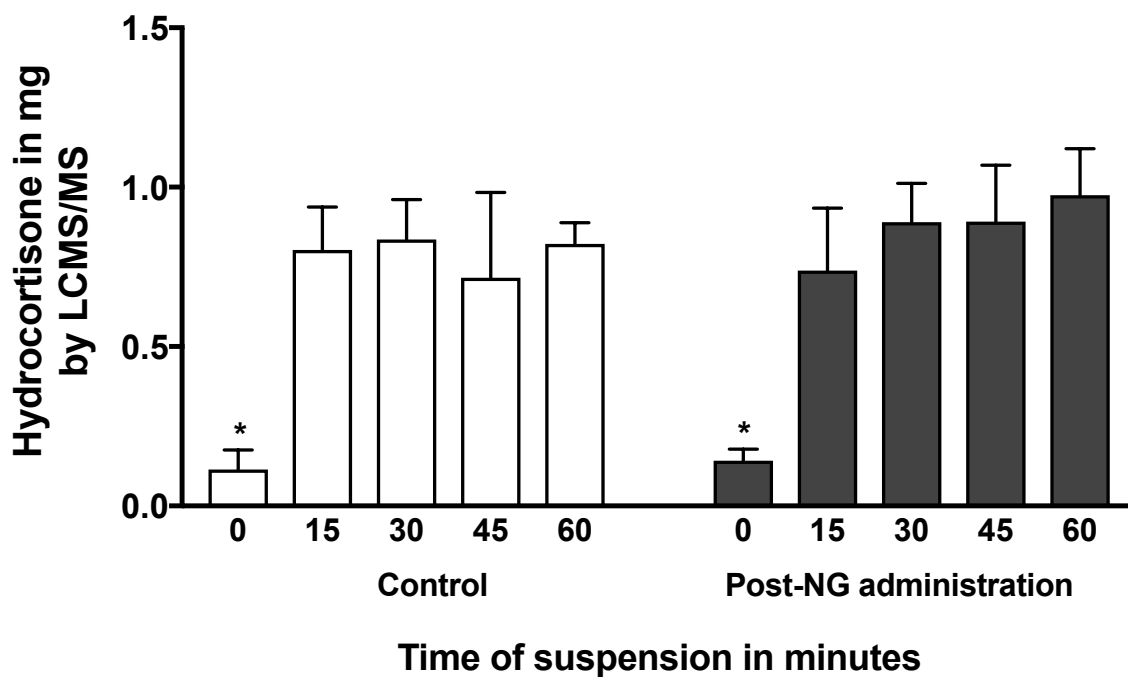


Figure 5: Recovery of hydrocortisone from hydrocortisone granule suspension in water (1mg/ml) pre- and post-administration through 6Fr gauge neonatal NG tube. Hydrocortisone granules was mixed with water and the samples were allowed 0, 15, 30, 45, and 60-minute bench rest before aspiration of the required dose using a syringe connected to a sampling needle that excluded aspiration of granules. (*: $p < 0.001$ ANOVA analysis, post-hoc analysis shows significant difference between time 0 to all other time points).



5.4. Study 4: Biomarkers for monitoring hormone replacement in CAH

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Androgens correlate with increased erythropoiesis in women with congenital adrenal hyperplasia

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ABSTRACT

Objective: Hyperandrogenism in congenital adrenal hyperplasia (CAH) provides an *in vivo* model for exploring the effect of androgens on erythropoiesis in women. We investigated the association of androgens with haemoglobin (Hb) and haematocrit (Hct) in women with CAH.

Design: Cross-validation study

Patients: Women with CAH from Sheffield Teaching Hospitals, UK (cohort 1, the training set: n=23) and National Institutes of Health, USA (cohort 2, the validation set: n=53).

Measurements: Androgens, full blood count and basic biochemistry, all measured on the same day. Demographic and anthropometric data.

Results: Significant age-adjusted correlations ($P<0.001$) were observed for Ln testosterone with Hb and Hct in cohorts 1 and 2 (Hb $r=0.712$ & 0.524 and Hct $r=0.705$ & 0.466), and remained significant after adjustments for CAH status, glucocorticoid treatment dose and serum creatinine. In the combined cohorts Hb correlated with androstenedione ($P=0.002$) and 17-hydroxyprogesterone ($P=0.008$). Hb and Hct were significantly higher in cohort 1 than those in cohort 2, while there were no group differences in androgen levels, glucocorticoid treatment dose or body mass index. In both cohorts women with Hb and Hct in the highest tertile had significantly higher testosterone levels than women with Hb and Hct in the lowest tertile.

Conclusions: In women with CAH, erythropoiesis may be driven by androgens and could be considered a biomarker for disease control.

INTRODUCTION

The effect of androgens on erythropoiesis is well described and initially came to light through the observation that men have higher levels of haemoglobin (Hb) than women (1). Pre-pubertal boys and girls have similar levels of Hb but boys acquire higher Hb levels following puberty that coincides with the surge in testosterone levels (2). Lower Hb levels in women are not due to chronic menstrual blood loss as this gender difference persists in non-menstruating women (3, 4). The evidence for an erythropoietic effect of testosterone led to its use as a treatment for anaemia in renal failure (4) and bone marrow failure (5) in the past before the invent of recombinant erythropoietin. In men intramuscular testosterone replacement, is often associated with polycythemia (6), which reverses with a dose reduction or discontinuation of therapy (7). Conversely, androgen deprivation therapy for prostate cancer leads to a reduction in Hb levels (8).

Congenital adrenal hyperplasia (CAH) is the commonest genetic endocrine disorder and 21-hydroxylase deficiency accounts for more than 95% of the cases (9). In this condition, defective cortisol synthesis in the adrenal glands leads to the loss of negative feedback inhibition of ACTH secretion by the pituitary. The elevated ACTH leads to hyperplasia of the adrenal glands and excess production of adrenal androgens (9). Treatment with glucocorticoids aims to control the androgen excess and replace the steroid deficiencies; however, it is challenging to achieve the correct balance

between over and under-treatment. When patients are under-replaced, adrenal androgens are elevated and women are affected by symptoms of hyperandrogenism. With over-replacement adrenal androgens are suppressed.

The effect of elevated adrenal androgens on erythropoietic markers in patients with CAH has been assumed but not studied in detail. Polycythaemia is seen in neonates with CAH (10) and there have been a few case studies reporting polycythemia in untreated men and women with CAH and androgen excess (11, 12). To the best of our knowledge, there are no studies examining the relationship of androgens and erythropoiesis in women with CAH. The present study investigates the association of androgens with Hb and haematocrit (Hct) in women with CAH in a cross-validated study.

METHODS

Study population

This was a retrospective analysis of data from two cohorts of CAH patients managed in two tertiary centers with expertise on the management of CAH. Cohort 1 comprised of patients from Sheffield Teaching Hospitals, UK and cohort 2 from National Institutes of Health, Bethesda, USA.

Data gathering

Demographic, anthropometric, biochemical, haematological and hormonal data measured on the same day were recorded. A total of 83 women (cohort 1: n = 30, cohort 2: n = 53) with CAH were eligible for recruitment. Seven women were excluded from cohort 1 prior to the analysis (four due to incomplete biochemical data and three due to medical conditions or medications known to affect the erythropoiesis or red cell parameters i.e. iron deficiency anaemia, vitamin B12 deficiency and methotrexate treatment). After screening for completeness, data of 76 women were used in the final analysis, 23 in cohort 1 and 53 in cohort 2.

Biochemical data for androgens [total testosterone, androstenedione and 17-hydroxyprogesterone (17-OHP)], full blood count, serum urea, creatinine and electrolytes were retrieved from electronic data systems. In cohort 1 the majority of samples were measured between 0800-1400hrs during clinic visits, after the morning dose of glucocorticoids, whereas for cohort 2 most samples were measured before the morning dose of glucocorticoids between 0700-0900hrs. The two laboratories had different reference ranges for Hb (cohort 1 110-147g/L, cohort 2 112g/L-157g/L). Hence, the tertiles were used for comparison between two cohorts in analysis. Age, height, weight, glucocorticoid treatment dose, CAH phenotype and smoking, medical and drug history were obtained from medical case notes. Body mass index (BMI) was calculated; weight (kg) divided by height (m) squared (kg/m²). Since patients were treated with different glucocorticoid regimens (hydrocortisone, prednisolone/prednisone and dexamethasone), those glucocorticoid doses were

converted to hydrocortisone equivalent dose using the ratio hydrocortisone: prednisolone: dexamethasone of 1:5:80 (13). The values used to calculate the hydrocortisone equivalent doses vary widely and we chose to use 5 times potency for prednisolone/prednisone, which is the widely accepted. For dexamethasone we chose that originally proposed by Wilkins in 1965 "The potency of this glucocorticoid in suppressing adrenal steroid biosynthesis relative to cortisol is about 80:1" and partially evaluated in CAH by Rivkees (13).

Hormonal assays

In cohort 1, 17-OHP was measured by the Siemens Coat-a-Count radioimmunoassay (RIA) [inter-assay coefficient of variance (CV) 5.0-11%] until October 2014 and thereafter with Diasource RIA (inter-assay CV 6.3-16%). Androstenedione was measured using the Siemens Immulite 2000 chemiluminescence immunoassay (CLIA) (inter-assay CV 8.5-12.0%) until February 2014 and using the Beckman Coulter Active RIA (inter-assay CV 4.5-16.9%) thereafter. Total Testosterone was measured using the Siemens Advia Centaur CLIA (inter-assay CV 6.8-13.3%) until January 2011 and by the Roche Cobas e602 electrochemiluminescence immunoassay (ECLIA) (inter-assay CV 3.5-7.3%).

In cohort 2 all the androgens were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). From 2005 to 2012 assays were performed at Mayo Medical Laboratories, Rochester, MN; The androstenedione assay had a sensitivity of

15ng/dl; inter-assay CV of 7.9, 7.2, 8.7%; intra-assay CV of 13.9, 5.9, 2.6 at mean concentration of 112, 916, and 2281ng/dl respectively, and normal range of 40-150ng/dl for males and 30-200ng/dl for females. The 17-OHP assay had an analytical sensitivity of 40ng/dl, inter-assay CV of 9.7, 8.7, 6.8%; intra-assay CV of 6.8, 2.9, 4.4% with a mean concentration of 111, 751, and 2006ng/dl, respectively, and normal range of less than or equal to 220ng/dl for males and less than or equal to 285ng/dl for females; 2012 onwards androstenedione and 17-OHP were measured by LC-MS/MS at National Institutes of Health, Bethesda MD; intra-assay CV ranged from 2.5-9.5% and inter-assay CV from 2.9 - 11.1%.

Statistical analysis:

Data were analyzed using SPSS v22. Group differences were determined by Student's t-tests. Relationships of Hb and Hct with androgens were assessed by partial correlations to enable adjustments for confounding factors including age, study cohorts, glucocorticoid treatment dose, CAH status and renal function. Data for androgens and glucocorticoid treatment dose were logarithmically transformed due to being positively skewed.

RESULTS

Characteristics of the study populations

Mean age of women in cohort 1 was 35.3 (SD \pm 14) years (Table 1). Among this cohort of women, 17 (73.9%) had classic CAH, in whom 13 (73%) were salt wasting and 4 (23%) simple virilizing subtypes, and 6 (26.1%) had non-classic CAH. The mean age of women in cohort 2 was 30.8 (SD \pm 11.4) years. This cohort comprised mostly of women with classic CAH (n = 51, 96.2%), of whom 33 (65%) had the salt wasting and 18 (35%) the simple virilizing type. There was one (1.9%) patient with non-classic CAH and one (1.9%) with 11- β hydroxylase deficiency.

In cohort 1 the majority received either hydrocortisone alone (n=10, 43.47%) administered twice or thrice daily, or prednisolone alone (n=9, 39.1%) administered once or twice daily. The remaining patients were treated with either dexamethasone once daily (n=2, 8.69%), or hydrocortisone and dexamethasone combined (n=2, 8.69%). In cohort 2, the majority was treated with prednisone (n=21, 39.6%) administered twice daily, followed by hydrocortisone (n=14, 26.4%) thrice daily, and dexamethasone once daily (n=12, 22.64%). Hydrocortisone combined with either prednisone or dexamethasone and prednisolone alone was given in one patient each (1.89%).

Correlations of androgens with erythropoiesis

The associations of testosterone with Hb and Hct in the two cohorts are shown in Figures 1 and 2. The regression slopes were similar in both cohorts but the intercepts were lower in cohort 2. In cohort 1, age adjusted Ln testosterone correlated positively with Hb and Hct ($P < 0.001$) (Table 2). These relationships remained significant ($P < 0.01$) after further adjustments for CAH status, glucocorticoid treatment dose and serum creatinine levels. The results from the cohort 2 confirmed these relationships but were less strong. These associations continued to persist after the two cohorts were analyzed together (Table 2). In both cohorts, the androgen precursors androstenedione and 17-OHP also correlated with Hb and Hct but the correlations were weaker than for testosterone.

Androgens, glucocorticoid treatment dose and anthropometry of women with erythropoietic markers in the highest tertile were compared with those of women in the lowest tertile (Table 3). Women with Hb or Hct in the highest tertile had significantly higher testosterone levels compared with women with Hb or Hct in the lowest tertile in both cohorts. The same was true for androstenedione and 17-OHP in cohort 1 but only for androstenedione and Hb in cohort 2. In cohort 2 women in the highest tertile of Hb and Hct had a higher BMI and higher glucocorticoid treatment dose.

Comparisons between cohort 1 and cohort 2

There were no group differences in age, anthropometric, BMI or glucocorticoid treatment dose between cohorts 1 and 2 (Table 1). Women in cohort 1 had significantly higher mean Hb ($P = 0.031$) and Hct ($P = 0.035$) levels than those in cohort 2 (Table 1). Similarly, substantially higher proportions of women had Hb and Hct above the upper limit of the reference range in cohort 1 (Hb: 30.4%, Hct: 47.8%) compared with cohort 2 (Hb, Hct <4%). The levels of total testosterone and its precursors, androstenedione and 17-OHP and creatinine levels did not differ significantly between the two study cohorts.

DISCUSSION

We have demonstrated that androgen levels in women with CAH are positively associated with Hb and Hct, suggesting that these markers of erythropoiesis are a potential biomarker of androgen control in women with CAH. The findings strengthen the evidence for an action of androgens on erythropoiesis in women.

The mechanism by which androgens promote erythropoiesis is not established (1, 14). There are conflicting results on the effect of testosterone on erythropoietin, the major regulator of erythropoiesis. Some studies have suggested that testosterone increases

erythropoietin production (1, 15), while others found no evidence to support these findings (6, 16). Other possible mechanisms by which testosterone might induce erythropoiesis include a direct effect on the bone marrow hematopoietic stem cells by stimulating insulin-like growth factor 1 and erythrocyte colony forming units (17), and increasing intestinal iron absorption and incorporation into erythrocytes (14).

Exogenous androgens have been associated with an increase in erythropoiesis. Supraphysiologic pharmaceutical doses of androgens cause an increase in Hb and Hct in men (18), which is dose-dependent and polycythaemia is a common but unwanted side-effect of testosterone therapy in hypogonadal men (6). Similarly in women, androgen therapy was associated with an increase in Hb and erythroid cell hyperplasia in bone marrow aspirates (19). In gender reassignment, hormone therapy raising testosterone levels in female-to-male reassignment leads to an increase in Hb levels while suppressed testosterone levels in male-to-female reassignment leads to a decrease in Hb levels (20). The levels of endogenous androgens has also been associated with erythropoiesis; healthy adult men with low free testosterone levels have a lower haematocrit than men with normal free testosterone (21) and Hb levels correlate with total and bioavailable testosterone in men and women older than 65 years (22).

Conditions associated with significant hyperandrogenism such as Cushing's disease and androgen producing ovarian tumors may present with polycythaemia (23, 24). We hypothesized that lower chronic elevations of androgens may be associated with more

subtle increases in erythropoietic markers. Women with CAH have elevated levels of adrenal androgens if inadequately treated with glucocorticoids (25) and provide a free-living model for exploring the effect of androgens on erythropoiesis. Cortisol has been implicated to play a mediating role in erythropoiesis (26, 27). Activation of the glucocorticoid receptor promotes 'stress erythropoiesis' and maturation of erythroid progenitors in vitro (28). It is well documented that anaemia occurs in patients with hypocortisolism, e.g. Sheehan's syndrome (29) and polycythemia in women with hypercortisolism (30). Correcting hypocortisolism with glucocorticoid replacement (31) or hypercortisolism by surgery (24) leads to normalization of Hb levels. Hypogonadal men with active Cushing's disease have low erythroid parameters that improve slowly after correction of hypercortisolism in parallel with improvements in testosterone levels. In our study, glucocorticoid equivalent doses did not differ between women with normal and those with elevated haematological parameters. A previous study of testosterone replacement in two men with aromatase deficiency has shown that the action of testosterone on erythropoiesis does not require its aromatization to oestrogen (32).

In our study, androgen precursors androstenedione and 17-OHP were weakly associated with erythropoietic markers compared with testosterone. Androgenic precursors exert their androgenic effect through conversion to testosterone and do not directly activate the androgen receptor, which may explain the weaker relationship with erythropoiesis. Free testosterone may have a stronger association with erythropoiesis but was not calculated in the present study because sex hormone

binding globulin (SHBG) was not measured. We have however adjusted our data for body mass index, which relates inversely to SHBG levels. It would be of interest to examine the association of Hb and Hct with dihydrotestosterone, which has tenfold greater affinity for androgen receptor than testosterone (33). However, dihydrotestosterone is not routinely measured in the clinical setting and therefore was not available in the present study. Chronic kidney disease is also associated with anaemia due to the reduction in renal production of erythropoietin (34). In the two cohorts presented here there were no subjects with chronic kidney disease and the relationship between androgens and markers of erythropoiesis continued to persist after adjusting for creatinine.

The two cohorts of women could potentially have differences in genotypes and exposure to lifestyle factors, which could affect the outcomes, but our results were reproducible in the two cohorts. This is evident by the parallel regression slopes for the association of testosterone with Hb and Hct in the two study cohorts. Interestingly, mean Hb and Hct were higher in the UK cohort than in the US cohort with no differences in androgen levels, body mass index or glucocorticoid treatment dose between the two cohorts. This may indicate underlying genetic differences between the two cohorts that could affect the action of testosterone on erythropoiesis e.g. differences in androgen receptor CAG repeat lengths. Lifestyle factors such as smoking and dietary iron intake and menstruation status may be some other factors to consider, however both cohorts had similar mean age. Compliance with glucocorticoid treatment or error in reporting of treatment dose may also explain this difference.

Strengths and limitations of the present study: The strengths of the present study lie in its robust cross-validation study design and adjustments for a number of major confounding factors. The study is limited by its retrospective nature and sampling bias might have been introduced as data collection spanned across approximately ten years. Different assays had been used during this period, which might have affected the accuracy of the biochemical data and also the two cohorts have used different assay techniques for androgens, which limits the comparison between the two cohorts. Another limitation of the study is wide variation of androgen levels observed in both cohorts. However, this reflects the previous observations with poor disease control on current therapeutic regimens (9) and potentially affected by differences in time of blood sampling. Lifestyle factors such as diet and smoking history were not available given this was a retrospective study.

In conclusion, the strong association of adrenal androgens with Hb and Hct in two cohorts of women with CAH suggests that these markers of erythropoiesis may be considered as biomarkers of disease control in women with CAH and in those with polycythaemia or anemia under or over suppression of adrenal androgens should be considered as a cause. Chronic over and under-treatment of CAH patients may have an effect on erythropoiesis, which can also potentially impact physical performance (35).

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Tables and Figures

Table 1. Characteristics of women with congenital adrenal hyperplasia in cohort 1, UK (n = 23) and cohort 2, US (n = 53).

	Cohort 1	Cohort 2	Group difference	
	(n = 23)	(n = 53)	(cohort 1 minus cohort 2)	
	Mean (SD)	Mean (SD)	Mean (95% CI)	<i>P</i>
Age (years)	35.3 (13.9)	30.8 (11.4)	4.4 (-1.6, 10.5)	0.148
Haemoglobin (g/L)	140.4 (13.3)	134.1 (10.5)	6.3 (0.6, 11.9)	0.031
Haematocrit (%)	41.7 (04.0)	39.9 (3.1)	1.8 (0.1, 3.5)	0.035
17-OHP (nmol/L)	98.3 (151.4)	127.1 (150.1)	-28.9 (-110.7, 53.0)	0.484
Androstenedione (nmol/L)	12.4 (13.3)	15.4 (19.6)	-3.0 (-12.3, 6.3)	0.519
Testosterone (nmol/L)	3.2(6.1)	2.7 (5.5)	0.5 (-2.4, 3.4)	0.748
Height (m)	1.58 (0.08)	1.57 (0.08)	0.00 (-0.03, 0.05)	0.687
Weight (kg)	86.4 (27.2)	78.2 (29.0)	8.1 (-8.8, 24.4)	0.323
Body mass index (kg/m ²)	34.6 (11.4)	31.7 (12.1)	2.9 (-3.9, 9.7)	0.396
Serum creatinine (μmol/L)	66.5 (13.1)	73.6 (14.4)	-7.1 (-14.3, 0.10)	0.053
Glucocorticoid treatment dose (mg/day)	28.2 (11.2)	29.4 (13.4)	-1.3 (-7.7, 5.1)	0.692

Table 2. Partial correlations of haemoglobin and haematocrit with androgens in women from two separate study cohorts. All analyses were adjusted for age. Further adjustments were made for glucocorticoid treatment dose, CAH status and serum creatinine.

	Ln 17-OHP		Ln Androstenedione		Ln Testosterone	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Cohort 1: Adjusted for age						
Haemoglobin	0.472	0.056	0.352	0.129	0.712	<0.001
Haematocrit	0.508	0.037	0.485	0.030	0.705	0.001
Cohort 2: Adjusted for age						
Haemoglobin	0.508	0.037	0.372	0.007	0.524	<0.001
Haematocrit	0.176	0.211	0.298	0.032	0.466	<0.001
Cohort 1: Adjusted for age + CAH status + Ln glucocorticoid treatment dose + serum creatinine						
Haemoglobin	0.524	0.066	0.555	0.032	0.797	<0.001
Haematocrit	0.570	0.042	0.724	0.002	0.778	0.001
Cohort 2: Adjusted for age + CAH status + Ln glucocorticoid treatment dose + serum creatinine						
Haemoglobin	0.301	0.038	0.363	0.011	0.491	<0.001
Haematocrit	0.168	0.253	0.259	0.075	0.415	0.003
Both cohorts: Adjusted for study group + age						
Haemoglobin	0.316	0.008	0.357	0.002	0.545	<0.001
Haematocrit	0.260	0.031	0.349	0.003	0.497	<0.001
Both cohorts: Adjusted for study group + age + CAH status + Ln glucocorticoid treatment dose + serum creatinine						
Haemoglobin	0.294	0.019	0.325	0.008	0.490	<0.001
Haematocrit	0.225	0.076	0.314	0.010	0.438	<0.001

Table 3. Independent t-tests to assess differences in androgens, glucocorticoid treatment dose and anthropometry of CAH women with Hb or Hct in the highest tertile compared with those in the lowest tertile (Hb cut-offs at 137 and 147 g/l in cohort 1 and at 130 and 138 g/l in cohort 2; Hct cut-offs at 41.0 and 43.7% in cohort 1 and at 38.8 and 41.1% in cohort 2).

	Hb: highest tertile minus lowest tertile		Hct: highest tertile minus lowest tertile	
	Mean difference (95% CI)	<i>P</i>	Mean difference (95% CI)	<i>P</i>
Cohort 1				
Ln 17-OHP (nmol/L)	2.79 (0.94, 4.64)	0.007	2.61 (0.82, 4.39)	0.006
Ln Androstenedione (nmol/L)	1.83 (0.36, 3.30)	0.018	2.15 (0.72, 3.57)	0.006
Ln Testosterone (nmol/L)	1.67 (0.20, 3.14)	0.029	1.59 (0.14, 3.03)	0.034
Ln Glucocorticoid treatment dose (mg/day)	0.04 (-0.43, 0.51)	0.848	0.08 (-0.41, 0.57)	0.781
Height (m)	0.01 (-0.08, 0.11)	0.755	0.03 (-0.07, 0.13)	0.509
Body mass index (kg/m ²)	1.9 (-16.7, 20.4)	0.824	-3.4 (-15.2, 8.4)	0.522
Cohort 2				
Ln 17-OHP (nmol/L)	1.44 (-0.12, 3.00)	0.069	0.54 (-0.97, 2.05)	0.472
Ln Androstenedione (nmol/L)	1.44 (0.54, 2.34)	0.003	0.76 (-0.17, 1.70)	0.105
Ln Testosterone (nmol/L)	1.75 (1.02, 2.48)	<0.001	1.27 (0.52, 2.02)	0.002
Ln Glucocorticoid treatment dose (mg/day)	0.19 (-0.09, 0.48)	0.181	0.25 (0.01, 0.50)	0.043
Height (m)	-0.04 (-0.10, 0.02)	0.193	-0.04 (-0.10, 0.01)	0.140
Body mass index (kg/m ²)	10.1 (3.7, 16.5)	0.003	10.0 (3.7, 16.2)	0.003

Figure 1. Relationship between haemoglobin and testosterone levels in women with CAH (● and solid line indicate cohort 1; △ and dashed line indicate cohort 2). Regression equations for cohort 1: Haemoglobin = 4.6 (95%CI: 1.5-7.8) x Ln Testosterone + 141 (95% CI: 137-145) ($r^2 = 31.5\%$) and for cohort 2: Haemoglobin = 4.4 (95%CI: 2.4-6.5) x Ln Testosterone + 133 (131-136) ($r^2 = 27.5\%$). The slopes of regression did not differ between the two cohorts.

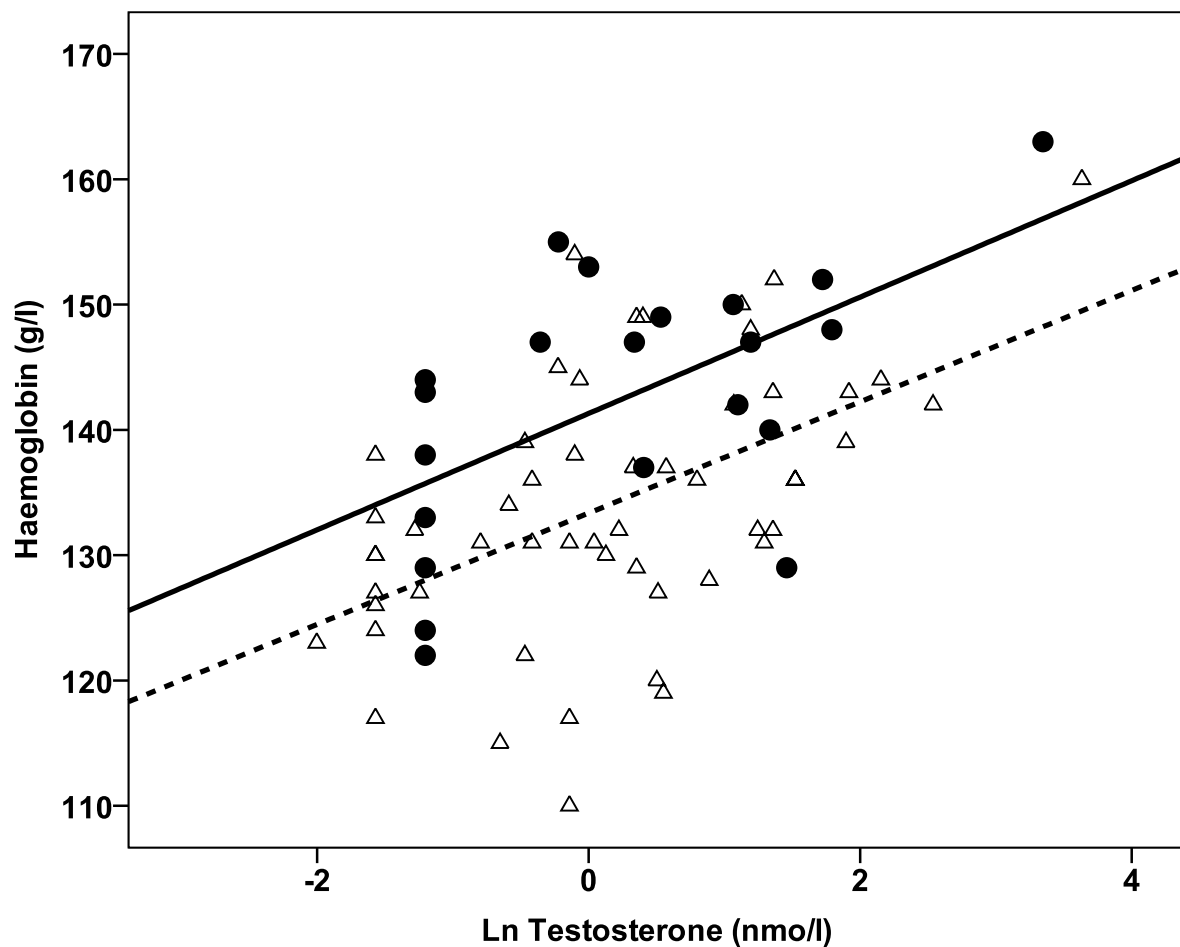
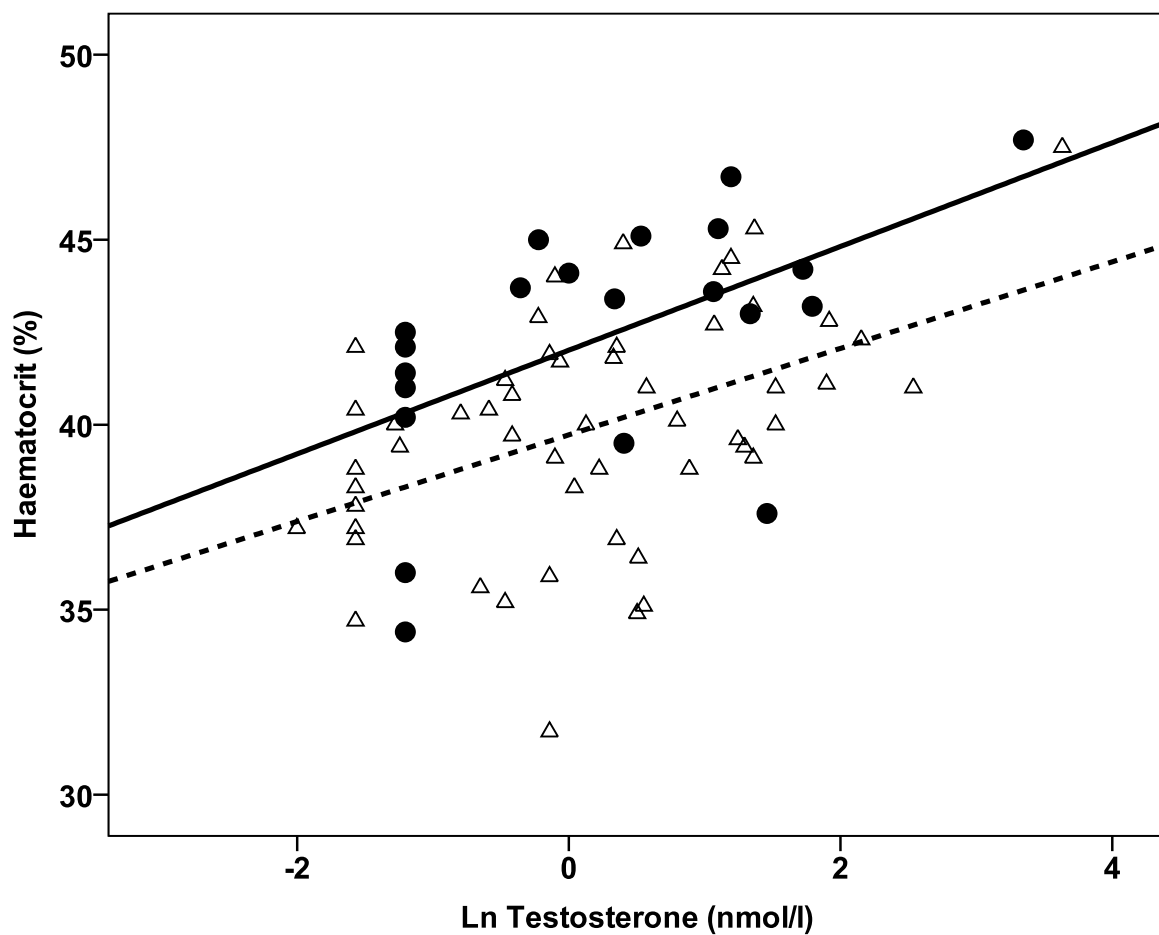


Figure 2. Relationship between haematocrit and testosterone levels in women with CAH (● and solid line indicate cohort 1; △ and dashed line indicate cohort 2). Regression equations for cohort 1: Haematocrit = 1.4 (95%CI: 0.4-2.4) x Ln Testosterone + 42.0 (95% CI: 40.7-43.4) ($r^2 = 30.3\%$) and for cohort 2: Haematocrit = 1.2 (95%CI: 0.5-1.8) x Ln Testosterone + 39.7 (39.0-40.5) ($r^2 = 21.9\%$). The slopes of regression did not differ between the two cohorts.



5.5. Study 5: Pharmacokinetic analysis of Hydrocortisone granules

Hydrocortisone Granules Designed for Children with Taste Masking and Age Appropriate Dosing are Bioequivalent When Sprinkled onto Food or Given Directly on the Tongue.

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Authors' accepted copy of the paper is included in this thesis, pages 198 to 221.

Authors' accepted copy

Hydrocortisone Granules are Bioequivalent When Sprinkled onto Food or Given Directly on the Tongue

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Short title: Hydrocortisone Granules sprinkled on food

Keywords: Hydrocortisone, paediatric, glucocorticoid replacement, adrenal insufficiency, congenital adrenal hyperplasia

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Abstract

Background: Immediate-release hydrocortisone granules in capsules for opening in paediatric appropriate doses have recently been licensed for children with adrenal insufficiency. This study evaluated the bioavailability of hydrocortisone granules administered as sprinkles onto soft food and yoghurt compared to direct administration to the back of the tongue.

Methods: Randomised, three-period crossover study in 18 dexamethasone-suppressed healthy men. In each period the fasted participants received hydrocortisone granules 5mg either directly to the back of the tongue, or sprinkled onto soft food (applesauce), or yoghurt, followed by 240mL of water. Serum cortisol was measured by LC-MS/MS.

Results: The cortisol geometric mean C_{max} and AUC for direct administration, sprinkles onto yoghurt, and sprinkles onto soft food were: C_{max} 428, 426, 427 nmol/L & AUC_{0-inf} 859, 886, 844 h*nmol/L, & AUC_{0-t} 853, 882, 838 h*nmol/L respectively. The 90% confidence intervals (CI) for the ratios of C_{max} , AUC_{0-inf} & AUC_{0-t} for administration with soft food or yoghurt to direct administration were well within the bioequivalent range, 80-125%. Median T_{max} was similar between methods of administration: 0.63h administered directly, 0.75h on soft food and 0.75h on yoghurt. No adverse events occurred during the study.

Conclusions: Hydrocortisone granules administered as sprinkles onto soft food or yoghurt but not mixed with are bioequivalent to those administered directly to the back of the tongue. Carers, parents or patients may choose to administer hydrocortisone granules either directly or sprinkled onto soft food or yoghurt.

Introduction

Hydrocortisone is the standard treatment for children with adrenal insufficiency who need life-long glucocorticoid hormone replacement (1,2). Congenital adrenal hyperplasia is the commonest cause of adrenal insufficiency in children and hydrocortisone replacement therapy needs to be initiated at diagnosis in the neonate to avoid death due to an adrenal crisis. Hydrocortisone doses are calculated according to body surface area and require careful adjustment as children grow to prevent under- or over-treatment. The total daily dose is usually 8-15mg/m² divided in 3-4 administrations with the highest level in the morning and doses as low as 0.5mg may be needed to appropriately titrate treatment (1-5).

Currently, children are medicated with compounded tablets prepared by pharmacists or carers to achieve paediatric appropriate doses (3). However, studies of compounding hydrocortisone reported that up to 25% of batches from pharmacies and 50% by parents were out of specification leading to clinically evident under- or over- treatment (6-8). Immediate-release hydrocortisone granules in paediatric-appropriate doses of 0.5, 1.0, 2.0 and 5.0mg have been shown to be well tolerated, easy to administer and to provide appropriate cortisol levels in neonates, infants and children with adrenal insufficiency (9). They have been designed for children with taste masking to cover the bitter taste of hydrocortisone. Administration is by opening the capsule and placing the granules onto a spoon or directly onto the child's tongue (4). The granules have been recently approved

in the European Union for replacement therapy of adrenal insufficiency in infants, children and adolescents, from birth to < 18 years old.

Co-administration or sprinkling of medications onto food is a commonly used practice that provides flexibility and ease of administration for caregivers, particularly of young children or children with difficulty swallowing medication (10-12). Sprinkling medication onto food could alter its pharmacokinetic characteristics and it is not known if co-administration of hydrocortisone granules with food affects its bioavailability. This clinical study was performed in dexamethasone-suppressed healthy men to investigate if hydrocortisone granules administered sprinkled onto soft food or yoghurt are bioequivalent to hydrocortisone granules administered directly to the back of the tongue.

Methods

Study population: The target sample size was 18 participants. Between June 2017 and July 2017 19 participants were enrolled. All participants signed an informed consent form and satisfied the inclusion and exclusion criteria. One participant withdrew for personal reasons after the second treatment period and was replaced. Serum cortisol concentration values from the 18 participants that completed all three treatment periods

were included in the pharmacokinetic analysis and safety and tolerability data from all 19 participants were collected and analysed (13).

The inclusion criteria were: healthy men aged 18-45 years with no significant medical history and a satisfactory baseline physical examination, body mass index (BMI) 18-30kg/m², normal baseline safety tests (biochemistry, haematology, electrocardiography, vital signs, urine analysis), negative urine drug screen, negative viral serology for HIV, Hepatitis B and C and use of effective contraception. The exclusion criteria were: use of concomitant medications other than acetaminophen within 14 days prior to dosing, vaccination within the previous month, any significant medical history including history of any gastrointestinal disorder likely to affect drug absorption, history of infections such as current or past tuberculosis, systemic fungal or viral infection and acute bacterial infection, sensitivity or contraindication to hydrocortisone or dexamethasone and/or any of the ingredients contained in soft food or yoghurt, clinically significant history of drug or alcohol abuse, positive alcohol screen prior to dosing, participation in another clinical trial or blood donation or transfusion $\geq 450\text{mL}$ within the previous 3 months, smoking within 6 months prior to the study, inability to communicate well with the Investigator and shift work.

Study design

Open label, randomised, single-dose, single-centre, three-period crossover study in dexamethasone-suppressed healthy men to determine the bioavailability of three

methods of administration of hydrocortisone granules (Alkindi® Diurnal Ltd, UK): 1. Hydrocortisone granules administered directly to the back of the tongue; 2. Hydrocortisone granules sprinkled onto 5mL soft food (applesauce) and swallowed within 3 minutes of preparation; 3. Hydrocortisone granules sprinkled onto 5mL yoghurt and swallowed within 3 minutes of preparation. All doses were followed by 240 mL of water. Primary endpoints were the pharmacokinetic parameters: C_{max} (peak cortisol concentration), AUC_{0-t} (area under the curve from the time of administration to the final time-point of serum cortisol measurement at 12h), AUC_{0-inf} (area under the curve from the time of administration projected to infinity) of hydrocortisone granules 5mg administered as sprinkles onto soft food and yoghurt compared to hydrocortisone granules 5mg administered as dry granules to the back of the tongue. Secondary endpoints were T_{max} (time to peak cortisol concentration), safety and tolerability. The study design was based on the European Medicines Agency and the United States Food and Drug Administration guidelines for the design, conduct and evaluation of bioavailability and bioequivalence studies and complied with the ethical standards laid by the Declaration of Helsinki and regulatory bodies (13-17). The study was reviewed and approved by the Wales Research Ethics Committee (reference number: 17/WA/0114). Clinical Trials Authorisation was obtained from the Medicines and Healthcare Regulatory Agency prior to the start of the study in accordance with Part 3, Regulation 12 of the United Kingdom (UK) Statutory Instrument.

The study was performed at Simbec Research Ltd. All participants underwent successful screening and eligibility checks. They were admitted to the research facility on the

afternoon of the first day (Day -1) and were discharged on the evening of the second day (Day 0) of each of the three treatment periods. Participants fasted from 22:00h on Day -1 to 12:00h on Day 0 and received three doses of dexamethasone 1mg with 240mL water at 22:00h on Day -1, 06:00h and 12:00h on Day 0 for suppression of their endogenous cortisol production. On Day 0 of each treatment period 5mg hydrocortisone granules were administered at 08:00h by one of the three administration methods. The sequence of administration methods for each participant was determined by a randomisation code generated by SAS® software version 9.3 (SAS Institute Inc., Cary, NC, USA). For each dosing one 5mg capsule was opened, the contents either poured out onto a spoon or sprinkled onto soft food or yoghurt, and the capsule inspected for residual granules. Participants remained seated upright for 4h after dosing. There was a 7 day washout between treatment periods which is longer than 5 elimination half-lives (the half-life of hydrocortisone is approximately 100min) (13). Post-study assessments were performed 7 days after the last dose of hydrocortisone granules. Safety and tolerability assessments (adverse events, laboratory safety, vital signs and 12-lead electrocardiography) were recorded throughout the study.

Sample collection and analysis

Three blood samples were taken 5 minutes apart starting at 0.5h pre-dose to monitor cortisol suppression. Further blood was collected pre-dose and up to 12h post dosing on Day 0 for quantification of serum cortisol concentration (a total of 20 samples for each

individual and treatment period with post-dose samples at 0 (-2mins), 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 11, 12 hours). The blood samples were processed and kept at -20°C and analysed for serum cortisol concentration by liquid chromatography tandem mass spectrometry (LC-MS/MS) at Seirian Laboratories, Simbec Research Ltd, Cardiff, UK with assay performance data as previously reported (4).

Pharmacokinetic parameters

All participants received dexamethasone for suppression of endogenous cortisol levels to <1.8µg/dL (<50nmol/L). The mean of three samples taken 5 minutes apart 30 minutes pre-dose confirmed suppression and this mean determined the individual endogenous baseline serum cortisol. All serum cortisol concentrations thereafter were corrected for endogenous baseline levels by subtraction. Any negative baseline adjusted values or original concentrations below the limit of quantification were set to zero. The pharmacokinetic parameters were calculated following baseline cortisol correction and therefore reflect the concentrations achieved by the administration of hydrocortisone granules and not endogenous cortisol production (13). The pharmacokinetic parameters C_{max} , T_{max} , AUC_{0-t} , AUC_{0-inf} , λ_z (elimination rate constant), $t_{1/2}$ (terminal half-life), CL/F (clearance), and V_z/F (apparent volume of distribution), were determined from the individual baseline adjusted serum cortisol concentration-time curve using WinNonlin Phoenix 6.3 (Certara L.P., St Louis, USA). The actual time of blood sampling was used in the calculation of the derived pharmacokinetic parameters.

Statistical analysis

Statistical analysis was performed using SAS® software version 9.3 (SAS Institute Inc., Cary, NC, USA). For the comparative pharmacokinetic analysis the reference administration method was hydrocortisone granules placed directly to the back of the tongue and the test administration methods were hydrocortisone granules sprinkled onto soft food or yoghurt. Following logarithmic transformation C_{max}, AUC_{0-t} and AUC_{0-inf} values were subjected to an analysis of variance (ANOVA) including fixed effects for sequence, period, treatment and subject nested within sequence. Point estimates and 90% two-sided confidence intervals (CI) for the difference between administration methods were obtained using the residual mean square error obtained from the ANOVA model and back-transformed to give the CI for the ratio on the original scale (13). The administration methods were confirmed to be bioequivalent if the 90% CI of the ratio of the test to the reference administration method was within the 80 to 125% range (13). T_{max} was compared between treatments using separate Wilcoxon Signed-Rank tests at the two-sided 5% significance level to test the differences and Hodges-Lehmann estimates of the median difference between treatments and corresponding 95% CIs were calculated.

Results

Participants and demographics

Nineteen male participants were randomised and received at least one dose of hydrocortisone granules and were eligible for the safety population. Of these, one participant withdrew from the study for personal reasons and was replaced. Eighteen participants completed the three sequences of this study and were eligible for the pharmacokinetic analysis population.

Mean age (standard deviation sd, range) for the 19 participants who enrolled into the study was 31.4 years (8.71, 21 - 44) and mean BMI (sd, range) was 25.96 (2.75, 20.7 - 29.7). All participants had adequate baseline cortisol suppression with mean pre-dose serum cortisol concentrations $<1.8\mu\text{g/dL}$ ($<50\text{nmol/L}$) at each of the three treatment days (Day 0) prior to administration of hydrocortisone granules. Overall median baseline cortisol for each administration method (direct/ yoghurt/ soft food) (range) was 15.3 (10.6, 72.4)/ 15.9 (12.5-26.6)/ 14.6 (9.85-81.8).

Pharmacokinetic analysis

Following a single 5mg dose of hydrocortisone granules the mean serum cortisol concentration over time curve was plotted for each of the three administration methods, to assess the rate and extent of absorption. Figure 1 shows the mean and the SD of the

serum cortisol concentration-time curves adjusted for baseline cortisol for administration as dry granules, sprinkles onto soft food, and sprinkles onto yoghurt. The curves were very similar between the 3 treatments; there was an initial rapid increase in cortisol concentration as expected for an immediate release formulation followed by a gradual decline.

Pharmacokinetic parameters were calculated from the baseline adjusted serum cortisol concentration for each administration method and are shown in Table 1. For direct administration, administration onto yoghurt, administration onto soft food the maximum cortisol concentration C_{max} nmol/L (geometric mean) was 428, 426, 427; AUC_{0-t} (nmol*h/L) was 853, 882, 838; AUC_{0-inf} (nmol*h/L) was 859, 886, 844. There was no statistical difference in C_{max} or AUC between methods of administration. T_{max} (median h, range) for dry granules was (0.625, 0.5-1.25), sprinkles onto soft food (0.75, 0.25-1.25), sprinkles onto yoghurt (0.75, 0.25-1.5) with no relevant difference between methods of administration.

Comparative bioavailability

The ratios of the geometric least square means of the pharmacokinetic parameters C_{max} , AUC_{0-t}, and AUC_{0-inf} for the test (soft food or yoghurt) to the reference (dry granules) administration methods were calculated to compare the bioavailability between the administration methods. The 90% CI of the ratio for C_{max} , AUC_{0-t}, and AUC_{0-inf} were

well within the 80-125% limits which confirmed that 5mg hydrocortisone granules administered as sprinkles onto soft food or yoghurt is bioequivalent to 5mg administered directly as dry granules (Table 2). Soft food to direct administration ratios and 90% CI were: C_{max} 99.68 (93.98-105.72), AUC_{0-t} 98.24 (94.42-102.21), AUC_{0-inf} 98.21 (94.24-102.36). Yoghurt to direct administration ratios and 90% CI were: C_{max} 99.43 (94.33-104.80), AUC_{0-t} 103.33 (94.80-112.62), AUC_{0-inf} 103.07 (94.55-112.35).

Safety and tolerability

Hydrocortisone granules were safe and well tolerated. There were no adverse events and no tolerability issues. Safety laboratory tests (biochemistry, haematology, urine analysis), vital signs, and 12-lead electrocardiography parameters were satisfactory at baseline and showed no relevant changes over time. There were no relevant physical examination findings during the study. All treatment periods exhibited similar safety profile and drug tolerability.

Discussion

These data show that hydrocortisone granules sprinkled onto soft food and yoghurt are bioequivalent to granules administered directly to the back of the tongue in dexamethasone-suppressed healthy men. Test-to-reference ratios of the pharmacokinetic parameters C_{max} , AUC_{0-t} , and AUC_{0-inf} were well within the 80-125% limits required to confirm bioequivalence. The peak and total cortisol exposure from hydrocortisone granules measured as C_{max} and AUC was the same for the three administration methods and there was no relevant difference in the rate of absorption measured by T_{max} . In this short study, hydrocortisone granules were safe and well tolerated, which confirms previous findings (4,9).

Administration of a medication mixed with food is a drug manipulation and could affect the absorption of the active ingredient; for example, due to exposure to different pH (18). The medicines regulatory agencies in the US and Europe, the FDA and EMA respectively, recommend that any such manipulation of drug administration should be studied and verified 'with respect to its potential impact on efficacy and safety' which may include bioavailability studies to confirm if medications sprinkled onto food have the same bioavailability as direct administration (18,19). In accordance with this advice several studies have assessed the bioequivalence of sprinkles versus the intact form of the medication in children and adults (20-25). This study was designed to compare the

bioavailability of sprinkling the hydrocortisone granules onto food compared to the approved use as dry granules to the back of the tongue and confirmed that sprinkling hydrocortisone granules onto food does not change its pharmacokinetics. Mixing or stirring of hydrocortisone granules with food is not recommended and was not assessed due to hydrocortisone granules having a taste-masking layer added to neutralise the bitter taste of hydrocortisone which could dissolve if granules are stirred into food. The results on direct administration of dry granules in this study mirror previous findings by Whitaker et al who tested the pharmacokinetics of single administration of hydrocortisone granules in varying doses (0.5, 2.0, 5.0 and 10mg) in 16 dexamethasone-suppressed healthy adult men (4). The dose tested in our study (5mg) is part of the dose range used to treat adrenal insufficiency both in paediatric and adult patients (4,26). In the paediatric population the pharmacokinetics of hydrocortisone granules have been studied in twenty-four young children with adrenal insufficiency (1month to 6 years old) with results comparable to the healthy adult men (9). The palatability of hydrocortisone granules was assessed in healthy men who found that the taste was neutral (neither good nor bad) (4).

Administering medications to children can be challenging and many children report problems swallowing solid and liquid medicines in the absence of underlying neurological disease (27). Compounding of medications to administer as powder and mixing medication with food, juice, and sweeteners is a common approach that parents and paediatric nurses take to improve compliance especially when there are problems

swallowing or bitter tasting medications (10,12,28,29) and joint administration of medicines with food or drink is an effective strategy to ensure swallowing in children (11). Liquid formulations are favoured by young children and contain sweeteners to mask any bitter taste. However, such hydrocortisone suspensions are not licensed, the hydrocortisone content may be inconsistent leading to treatment failures (30) and may contain sucrose that can have adverse effects on teeth with long-term use (31). Food is chewed to <2mm (32) therefore sprinkling beads of smaller size onto food should not cause problems swallowing. Furthermore, sprinkling of medication may have advantages in improving adherence and facilitate caregiving of patients with swallowing difficulties and this approach has been explored in children and elderly patients with potential swallowing and adherence difficulties such as in Alzheimer's disease, attention-deficit hyperactivity disorder, and epilepsy (21,22,24,25).

Dosing errors are common in young children and cause 20% of all medication errors in acute neonatal care (33). This is due to the lack of paediatric-appropriate dosage and the common use of unlicensed, 'off-label' and/or compounded medicines that don't have appropriate labelling, safety or dosing data (33,34). In children adverse drug reactions are more common with unlicensed medications (35) and international initiatives have tried to address these issues and proposed approaches to improve availability of paediatric-appropriate formulations and treatment outcomes (29,36). For children with adrenal insufficiency compounding hydrocortisone from adult tablets and splitting of adult tablets provides much needed flexibility in dosing however recent studies show significant

inaccuracy in the content of active ingredient leading to clinically significant consequences including Cushing's syndrome (6-8).

The FDA defines yoghurt products as having a pH of up to 4.6 (37) . The pH of fresh plain yoghurt is around 4.3-4.6 and this decreases rapidly with storage time to 4.0-4.2 (38-40). The pH of different yoghurt products vary within these ranges and is affected by the time since production, the initial dairy culture used, addition of fruit or fruit puree and the type of fruit added (38). The addition of sweeteners only slightly reduces pH (41) (range of pH 3.94-3.98 vs 4.09-3.94). For comparison, the pH of applesauce is lower than yoghurt and is between 3.1-3.6. Since the pharmacokinetic analysis in our study showed bioequivalence between sprinkles on yoghurt and applesauce we believe that any commercial yoghurt product with a pH in the above ranges could be used as a vehicle for the sprinkling of hydrocortisone granules.

The strengths of the study lie in the 3-period crossover design that ensures same within-participant control and thus less variability of the data obtained. A double-blind design was not required as the primary objective of the study was to compare the bioavailability of hydrocortisone granules administered via 3 different methods. The pharmacokinetic parameters investigated were objective, and the sequence of administration methods was randomly allocated for each individual therefore the open label design conferred minimal risk of introducing bias into the study. Further strengths of this study are the

accurate measurement of cortisol with LC-MS/MS and the complete suppression of endogenous cortisol levels in all participants ensuring that cortisol measured was the result of treatment and not endogenous production. The study population was healthy young men and this can be a potential limitation as hydrocortisone granules are designed for the paediatric population; however, the absorption of hydrocortisone granules was previously studied in twenty-four young children and the results were comparable to the adult population (9). Children may have differences in physiology and pharmacokinetics but clinical studies are performed in children only under exceptional circumstances and this approach is considered adequate by regulatory agencies (13,15,17). Dexamethasone has been reported in vivo and in vitro to induce CYP3A4 of which hydrocortisone is a substrate (42-44). It is possible that dexamethasone could alter the pharmacokinetics of hydrocortisone but as each limb of the trial was treated in the same way this shouldn't affect the comparative bioavailability under different modes of administration.

In conclusion it has been demonstrated that hydrocortisone granules can be administered either directly or sprinkled onto soft food (applesauce) or yoghurt which, when consumed within 3 minutes, did not result in any significant or clinically relevant change of overall drug exposure and rate of absorption. Based on the data shown patients have the flexibility of multiple administration methods and prescribers can safely recommend sprinkled administration of hydrocortisone granules. Carers and children may welcome the flexibility of different options for administering hydrocortisone to young children on

multiple-time daily dosing and it would be interesting to see if this flexibility improves adherence to treatment, disease management and clinical outcomes.

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Figures and Tables

Figure 1: Mean adjusted serum cortisol concentration and standard deviation over time after administration of hydrocortisone granules in 18 fasted, dexamethasone-suppressed healthy men. The serum cortisol concentrations for each participant were corrected for endogenous baseline cortisol by subtraction of the mean pre-dose value.

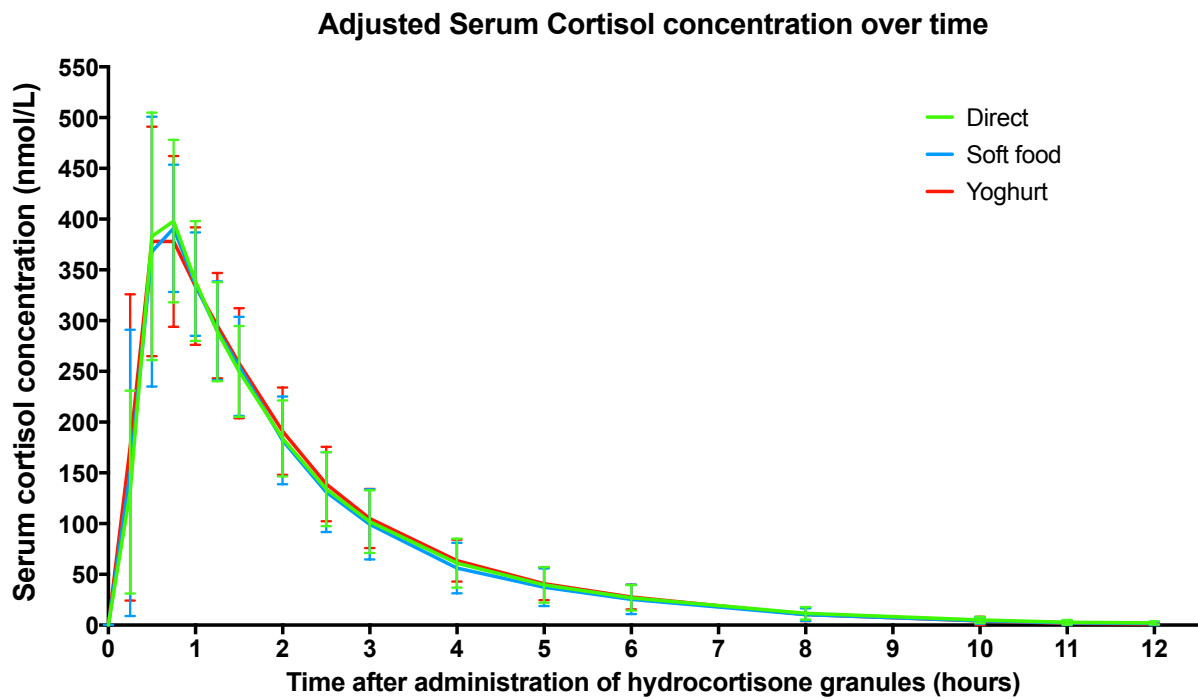


Table 1: Pharmacokinetic parameters calculated from baseline adjusted serum cortisol following a dose of 5mg hydrocortisone granules administered by three methods

	Statistic	Direct administration	Soft food	Yoghurt
C_{max} (nmol/L)	Geometric Mean	428	427	426
	SD	82	78	88
AUC_{0-t} (nmol/L*h)	Geometric Mean	853	838	882
	SD	203	198	161
AUC_{0-inf} (nmol/L*h)	Geometric Mean	859	844	886
	SD	204	197	162
T_{max} (h)	Median	0.63	0.75	0.75
	Range	0.5, 1.25	0.25, 1.25	0.25, 1.5
λ_z (1/h)	Geometric Mean	0.48	0.53	0.55
	SD	0.46	0.24	0.09
t_{1/2} (h)	Geometric Mean	1.4	1.3	1.3
	SD	0.6	0.4	0.2

sd: standard deviation, cv: coefficient of variation, Geom: geometric mean, C_{max}: maximum serum cortisol concentration after administration, T_{max}: time to C_{max}, AUC_{0-t}: area under the serum cortisol concentration time curve from administration to the end of the sampling at 12h, AUC_{0-inf}: area under the serum cortisol concentration time curve from administration extrapolated to infinite time, λ_z: terminal rate constant, t_{1/2}: serum cortisol concentration half-life

Table 2: Bioequivalence comparison between the reference administration method (direct administration of dry hydrocortisone granules to the back of the tongue) and the test administration methods (hydrocortisone granules sprinkled onto yoghurt and sprinkled onto soft food)

	Granules sprinkled onto soft food to direct administration of dry granules		Granules sprinkled onto yoghurt to direct administration of dry granules	
	Geometric LSmean ratio	90% CI	Geometric LSmean ratio	90% CI
Cmax (nmol/L)	99.68	93.98-105.72	99.43	94.33-104.80
AUC0-t (nmol/L*h)	98.24	94.42-102.21	103.33	94.80-112.62
AUC0-inf (nmol/L*h)	98.21	94.24-102.36	103.07	94.55-112.35

Cmax: maximum serum cortisol concentration after administration, Tmax: time to Cmax, AUC0-t: area under the serum cortisol concentration time curve from administration to the end of the sampling at 12h, AUC0-inf: area under the serum cortisol concentration time curve from administration extrapolated to infinite time.

6. Discussion

Conditions of cortisol excess and deficiency have significant mortality and morbidity when inadequately treated and medical therapy is an essential part of therapy. This research examined whether it is possible to use medical therapy to normalise cortisol levels in cortisol excess and deficiency and five studies were designed to explore this. Study 1 is the largest reported cohort of patients with CS treated with the steroidogenesis inhibitor metyrapone. Study 2 is the first prospective study that reports efficacy of medical treatment in NS. Study 3 is the only study in the literature to examine the accuracy of delivery of hydrocortisone through nasogastric tubes and study 5 examines whether co-administration with soft food affects the pharmacokinetic parameters of hydrocortisone granules. Study 4 investigates the correlation of haemoglobin and haematocrit with androgen levels in women with CAH.

6.1. Why are physiological cortisol levels important?

Abnormalities in cortisol secretion are associated with significant mortality and morbidity and there is evidence that the correction of hypercortisolism and hypocortisolism by medical or surgical methods significantly improves morbidity and mortality. Restoration of physiological cortisol levels is key to improving patients' self-perceived health status and cognitive function, which are impaired in patients with conditions of cortisol excess or deficiency. Patients experience on-going symptoms and disability and have adverse clinical outcomes when therapy leads to under- or over-exposure to glucocorticoid hormones.

At diagnosis, patients with cortisol excess have increased incidence of cardiovascular risk factors including hypertension and diabetes⁵³⁵. There is an increased risk of mortality, cardiovascular events, peptic ulcers, thromboembolic disease, fractures, and infections in patients with overt cortisol excess due to benign adrenal or pituitary disease and prior to the introduction of cortisol-reducing treatment the mean survival of patients with CS was 5 years^{216, 535-537}. This phenotype is linked to cortisol excess; higher levels of cortisol confer higher

risk for infections, mortality following surgical treatment for pituitary adenoma is higher in patients with CD than patients with non-functioning adenoma, and mortality in patients diagnosed with mild hypercortisolism due to adrenal adenoma improves after treatment for cortisol excess^{223, 538-542}. Patients with active cortisol excess have high mortality even after treatment of the glucocorticoid-associated complications with increased standardised mortality rate up to 5-fold higher than the general population^{535, 538} and patients with persistent or recurrent disease have higher than expected mortality^{538, 540, 543}.

Cortisol deficiency was associated with poor life expectancy prior to the introduction of life-saving glucocorticoid therapy to restore cortisol levels¹³. The introduction of adrenal gland extracts containing glucocorticoid steroids as treatment for patients with Addison's disease improved 1.5-year survival from 35% to 55% and introduction of the first generation of synthetic glucocorticoids improved survival further to 85%⁵⁴⁴. Prior to glucocorticoid replacement pregnancy was considered detrimental for female patients. Overall, most deaths occurred due to either an adrenal crisis caused by intercurrent illness or cardiovascular events⁵⁴⁵. Neonates with CAH invariably died in infancy and despite some improvement in survival following treatment with salt it was the introduction of cortisone therapy in the 1950s that improved survival and reduced androgen excess⁵⁴⁶. Glucocorticoid replacement also improved symptoms of hypoadrenalism including gastrointestinal and hypoglycaemia, reduced fatigability, and promoted weight gain.

Quality of life is reduced in active cortisol excess and hypoadrenalism. Persistent cortisol excess due to disease relapse or failure to control hypercortisolism is associated with worst QoL compared with biochemical cure^{547, 548}. Cortisol secretion abnormalities seem to be specifically associated with impairment in QoL; in patients with pituitary adenoma, those with cortisol excess had the worst QoL and least improvement following treatment compared with patients with GH or prolactin excess⁵⁴⁸. There are deficits in most measurable aspects of QoL and cognitive function assessment with the exception of pain. Improvement in QoL occurs after surgical treatment of cortisol excess irrespective of the specific operation; QoL scores following bilateral adrenalectomy for CD are comparable to scores following remission from pituitary surgery^{549, 550}. Cognitive deficit has also been reported in patients with PAI⁵⁵¹.

Treatment to reverse glucocorticoid deficiency or excess is therefore essential to improve prognosis and general health status. In cortisol excess this is best achieved with surgical

resection of the primary site of hormonal excess. After successful surgical treatment for cortisol excess the cardiovascular risk factors improve but remain high for years likely due to the metabolic sequelae of chronic exposure to glucocorticoid excess prior to definitive treatment^{223, 535, 540, 543, 552-554}. Restoration of eucortisolaemia improves glucocorticoid-associated cardiovascular and metabolic risk factors such as obesity, hypertension, glucose tolerance, dyslipidaemia as well as quality of life, osteoporosis and psychiatric conditions in patients with cortisol excess⁵⁵⁵. Left ventricular dysfunction and psychiatric morbidity also improve following restoration of eucortisolaemia in patients with CS⁵⁵⁶. Overall there is evidence from long-term follow-up cohorts that mortality and morbidity improve when cortisol excess is successfully treated but remain higher compared to the general population's and aggressive treatment of glucocorticoid-associated cardiac risk factors is necessary for patients with CS in remission^{535, 543, 557-559}. International guidelines state that for patients with overt cortisol excess there is benefit in giving medical treatment to normalise cortisol levels in the blood or at the receptor level to eliminate the signs and symptoms of CS³⁴⁵.

Similarly, correction of hypoadrenalism by glucocorticoid replacement therapy changed the prognosis and significantly improved survival in patients with adrenal insufficiency since its introduction in the 1940s^{544, 545, 560}. The total daily dose of replacement matters as under-replacement and over-replacement are associated with adverse symptoms and signs. Current estimates of appropriate hydrocortisone equivalent total daily dose is around 15-20 mg. Under-replacement is associated with persistent fatigue, low quality of life and higher risk of adrenal crisis²⁸⁴. Over-replacement is associated with high incidence of central adiposity, hypertension and dyslipidaemia and patients with CAH exposed to high glucocorticoid doses in childhood develop adverse metabolic features, cardiovascular risk factors and have higher than expected mortality as adults^{318, 515, 561-563}. Patients with primary or secondary hypoadrenalism on glucocorticoid replacement continue to have a 2-fold increase in the mortality compared to the general population with excess mortality due to infections, cardiovascular events and cancer and supra-physiological daily hydrocortisone doses above 30 mg/day or 0.3 mg/kg are associated with higher mortality^{493, 495, 501-503}. Reduction of glucocorticoid replacement in patients with secondary hypoadrenalism and high total doses may improve body composition, adiposity and lipid profile⁵⁶⁴. Non-physiological replacement with long-acting glucocorticoids that increase exposure to glucocorticoids at times when there is low physiological exposure also promotes an adverse metabolic profile⁴⁸¹.

Quality of life and cognitive function improve with treatment of cortisol excess and deficiency but do not normalise and recovery may take several years^{549, 555, 565-567}. QoL improves in CS patients in remission compared to patients with hypercortisolism independent of treatment or underlying cause of CS^{548, 550, 568}. However, many years following biochemical cure for CD, patients report worst mental, cognitive, and physical scores (including anxiety, depression, fatigue, social and physical functioning) compared with healthy matched adults⁵⁵⁰. In pituitary disease, QoL scores are worst in the presence of multiple hormone deficiencies, glucocorticoid deficiency, and history of cortisol excess with worst QoL impairment reported in GH-deficiency associated with CD compared to other causes of GH-deficiency^{569, 570}. There were cognitive deficits in patients cured from CD for many years (mean 13 years, 27% of patients received post-op radiotherapy and 58% had one or multiple pituitary hormone deficiencies) mainly in visual memory, verbal learning and executive function compared with matched controls. In the same study, patients cured from NFA had deficits in executive function compared with matched controls; post-op radiotherapy was given to 44% of patients cured from NFA and 93% had at least one pituitary hormone deficiency with 57% receiving glucocorticoid replacement. Overall patients with CD had higher cognitive impairment in memory and executive function than patients with NFA or controls and scores were better with longer duration of remission⁵⁷¹. In a different study, long-term residual QoL impairment was demonstrated in 23 patients with long-term remission from CD and this was not found to be associated with glucocorticoid replacement or previous radiotherapy⁵⁵⁵. In contrast, in 11 patients with GH deficiency and secondary hypoadrenalism a reduction of supra-physiological GC replacement dose was associated with improved QoL scores⁵⁶⁴.

Patients with cortisol deficiency due to PAI or CAH also have residual impairment in QoL despite adequate glucocorticoid replacement and this is not associated with the cause of AI or presence of concomitant disease^{469, 492, 572}. There were subtle cognitive function deficits related to verbal learning in 30 patients with long-term PAI on GC replacement (15-35 mg HC equivalent dose, mean 21 mg) compared to controls⁵⁷³. Cognitive deficits affecting memory and executive skills were also demonstrated in 31 patients with PAI on hydrocortisone replacement (HC dose 12.2 mg/m⁴ BSA or mean weight-adjusted 0.28 mg/Kg)⁵⁷⁴. Poor QoL in patients with disturbed diurnal secretion of cortisol or non-physiological replacement may be associated with effects of diurnal cortisol levels on sleep. The results of the above studies may indicate that a certain level of GC is essential for learning and normal cognitive function and that both cortisol excess and deficiency are related to cognitive defects⁵⁷⁵. Alternatively, the results may reflect periods of over-replacement and their long-lasting effects on cognitive function.

There are widespread effects of glucocorticoids in the brain and it is possible that cortisol excess or deficiency may have long-standing neurological effects despite cure or adequate hormone replacement ⁵⁷⁶. Cortisol affects cognitive function and behaviour in complex ways and is important for neurocognitive adaptation to stress ^{575, 577}. Cognitive deficits associated with cortisol excess include short-term memory, executive function, working memory and attention and these are associated with structural abnormalities and atrophy in the hippocampus, amygdala and frontal cortices of the brain ^{569, 576, 578-584}. The hippocampus is important for memory and learning. Radiological evidence of loss of hippocampal volume was associated with a deficit in verbal learning and recall and high levels of hypercortisolaemia in patients with CS and reversal of cortisol excess was followed by an increase in hippocampal volume ^{576, 583}. Generalised cerebral atrophy is prevalent in patients with CS of all ages and both endogenous and exogenous cortisol excess are associated with premature cerebral atrophy ⁵⁸⁴⁻⁵⁸⁶. Data on hypocortisolism are more limited but animal data suggest hippocampal neuronal death affecting the dentate gyrus following adrenalectomy which may suggest problems with learning and memory ⁵⁸⁷⁻⁵⁸⁹. Structural neurological changes are partially reversible after correction of cortisol excess however cognitive deficits persist and in children with CS cognitive function continued to decline for 1 year after biochemical cure ^{584, 585, 590, 591}. Cognitive deficits persist in patients with CD cured with surgery years after treatment despite restoration of physiological circadian pattern of cortisol secretion and it is likely that some consequences of brain exposure to cortisol excess are reversible following restoration of normal cortisol levels and some persist ⁵⁹².

The current glucocorticoid replacement regimens cannot replicate the ultradian rhythmicity of endogenous cortisol secretion and this may contribute to low self-perceived QoL in patients with hypoadrenalism. There is evidence that pulsatile cortisol secretion is important for normal brain functioning and behaviour under non-stressed conditions including sleep quality, memory performance, and regulation of emotional responses ⁵⁹³. A study of glucocorticoid administration in dexamethasone-suppressed healthy men showed that lack of pulses of cortisol in a subcutaneous continuous HC infusion delivery system was associated with poorer working memory performance at times of high cognitive demands and quality of sleep compared with pulsatile HC infusion ⁵⁹³.

6.2. Can medical therapies restore physiological cortisol secretion?

Restoration of physiological cortisol levels is central to improving survival and health status in patients with cortisol excess and deficiency. There are various treatment options to achieve this: surgical, radiation and medical therapy in CS and NS and medical therapy in primary adrenal insufficiency and Congenital adrenal hyperplasia. Medical therapy for these conditions has existed for decades however there are still unresolved issues of efficacy, safety, best treatment regimens and persistent symptoms.

Cortisol deficiency

The cornerstone of management of adrenal insufficiency is the replacement of glucocorticoids and there are various formulations with distinct pharmacokinetic properties and treatment regimens that set out to replace glucocorticoids according to the physiological pattern of cortisol secretion. Some regimens are more successful in doing this than others but overall the current treatment options that are available in clinical practice approximate the circadian pattern of cortisol secretion but do not replicate the ultradian pulsatility. The pulsatile pattern of cortisol secretion is considered important in cognitive function and lack of it may explain the low QoL scores in some patients with adequate glucocorticoid dose replacement.

Current knowledge suggests that the optimal glucocorticoid replacement dose in adults is 10-12 mg/m²/day, which is slightly higher than the calculated daily cortisol production of 6-8 mg/m²/day to allow for 90-95% bioavailability and first pass hepatic metabolism of oral treatment. These calculations equate to the usual adult daily replacement doses of hydrocortisone-equivalent 15-25 mg^{127, 475}. There is significant variability of cortisol concentration post oral administration between individuals due to variable metabolism, age, gender, and levels of cortisol binding proteins and some patients may be over- while others may be undertreated with these doses therefore clinical assessment is vital⁴⁷⁵.

In the absence of a perfect replacement option the optimisation and individualisation of treatment seems the only solution. Under-replacement is avoided with adequate dose up-titration driven by careful clinical assessment. Thrice-daily oral hydrocortisone regimens avoid dips in cortisol levels during the day that may cause fatigability. Short acting hydrocortisone-

based regimens avoid overexposure of glucocorticoids during the quiescent period that increases risk of metabolic and cardiovascular complications⁴⁸¹. These regimens are also preferable in children as long-acting glucocorticoids are associated with growth suppression. Long-acting glucocorticoids tighten control of ACTH secretion in women with CAH seeking fertility optimisation and they are indicated for short-term use accepting they provide glucocorticoid over-replacement. At the same time until a perfect regimen is available it is necessary to address treatment-related morbidity with patient education and proactive and reactive actions; treat and prevent cardiovascular risk factors, promote weight-control and healthy lifestyle.

New treatments set out to address the deficiencies of existing regimens; their theoretical and pharmacological advantages will be tested against time and clinical outcomes. There are high expectations as these treatments are designed to improve persistent symptoms with pharmacokinetic properties better suited to allow a better replication of circadian cortisol secretion, add convenience of administration and improve acceptance and compliance. Hydrocortisone pumps are being investigated to offer the closest replication of cortisol secretion and are the only treatment that can be programmed to replicate the pulses of cortisol secretion. The technology to optimise the pattern of glucocorticoid delivery is in sight with the compromise of an invasive mode of treatment delivery, demanding patient engagement and likely financial cost. New treatments are promising but will be more expensive and may drive an overall rise in the cost of glucocorticoid replacement.

Cortisol excess

There are specific indications for medical therapy in the treatment algorithm of cortisol excess. Overall medical therapy is frequently necessary to help control disease activity in patients with persistent disease or non-candidates for surgery, which is generally the first line therapy. The Endocrine Society guidelines recommend steroidogenesis inhibitors as second-line after surgery in CD, combined with radiotherapy for the same indication, first-line in ectopic ACTH-secretion when surgery is not an option, and as adjunct in adrenocortical carcinoma associated with cortisol excess³⁴⁵. New medications have been tested and approved in the last few years and are indicated in CD (pituitary-directed agents, pasireotide) and in cortisol excess-associated glucose intolerance (glucocorticoid receptor antagonist mifepristone).

Steroidogenesis enzyme inhibitors have been used to treat cortisol excess for many decades and are the first line agents. They are the most commonly used medications and are very effective in reducing cortisol levels as monotherapy or in combination with other agents. Ketoconazole and metyrapone show 50-75% effectiveness, have a short duration of action and need to be given in multiple doses daily. We have shown in the largest reported cohort that normalisation of cortisol levels with metyrapone is possible with CDCs achieving the target of 150-300 nmol/L that reflects normal cortisol production which agrees with previous reports ^{1, 6, 349}.

There has been no assessment of characteristics of cortisol secretion such as diurnal and pulsatile secretion pattern in this research ¹. Rhythmicity in cortisol secretion has been reported to recover after successful surgery for CD ⁵⁹². In 6 out of 12 patients with CD on medical therapy, the cortisol secretion pattern as grossly assessed by a 5-point serum CDCs showed recovering diurnal variation after 80-day treatment with pasireotide, cabergoline and ketoconazole ⁵⁹⁴. No differences in QoL existed between patients with rhythmicity improvement versus no improvement in this study. In patients with active CS there is no diurnal rhythmicity, nadir midnight cortisol, and quiescent period in the evening. An assessment of these characteristics of physiological secretion pattern would be useful to do in patients with cortisol excess who are likely to remain on medical therapy long-term and this is only a small subgroup of patients receiving treatment with steroidogenesis inhibitors. The short duration of action of metyrapone and ketoconazole provides in theory the opportunity to reduce cortisol using a fine-tuned dose titration regimen to mimic physiological production patterns. The highest dose of metyrapone can be given at bedtime with additional evening doses to achieve higher suppression of cortisol levels. Another option of restoring physiological pattern is to use a block and replace regimen with high doses of steroidogenesis enzyme inhibitors that block cortisol production completely and render the patient cortisol deficient and add glucocorticoid replacement similar to glucocorticoid replacement in endogenous adrenal insufficiency. The first approach is intensive and requires frequent clinical and biochemical assessments and the second tolerance of large doses of steroidogenesis inhibitors. In both cases access to accurate analytical methods for quantification of endogenous production such as LC-MS/MS is necessary. There are no previous studies in CS that examined if metyrapone with appropriate dosing could replicate physiological cortisol levels but the effects of such a fine-tuned approach are worth investigating in patients who are likely to remain on medical treatment long-term and who are to receive longer-term benefit to counteract the intensive monitoring requirements. Such approach has been tried in patients with autonomous cortisol production due to an adrenal adenoma and has shown that a single evening dose of

metyrapone improved cortisol secretion profiles ⁵⁹⁵. It is therefore feasible to mimic physiological cortisol production in patients on medical therapy for cortisol excess and the task is to identify those who are likely to benefit from this approach.

The quantification of efficacy of medical therapies depends on the definition of normalisation of cortisol levels and on the test chosen to assess this. Cortisol day-curves and UFC have been used to monitor therapy in cortisol excess but have disadvantages and they do not provide an accurate assessment of cortisol secretion pattern.

Compliance and adequate dose titration

The effectiveness of medical therapy is linked to correct and judicious administration. Medications work when patients take them as directed and there are many factors that affect compliance; dislike of medication form or size of tablets, complexity of regimen and high frequency of dosing, socioeconomic, individual health beliefs, and health behaviour ^{596, 597}. Patient education of indication and side effects is key however compliance has presented a persistent and underrecognized health problem affecting the management of many patients treated with medical therapy for chronic conditions ⁵⁹⁸. Non-compliance with essential medication can be as high as 60% in patients with long-term diabetes and hypertension ^{597, 599}. Moreover, 71% of patients with epilepsy reported missing medication doses and 40% of patients with chronic conditions report taking their medications later than prescribed ⁶⁰⁰. Every increase in the frequency of doses increased the likelihood of dose omissions by one third ^{597, 601}.

Children are a group of patients facing particular problems with compliance due to negative attitudes towards taking medications ⁶⁰². The problems are multiple and include swallowing difficulty in young children, unpleasant taste of medications, and behavioural obstacles especially in young children. Designing medications to address these issues is essential in improving acceptability, ease of administration and accuracy of dose delivery and improvement in compliance should be expected to improve effectiveness and likely clinical outcomes.

Medical treatments for cortisol excess and deficiency need individualisation and there is no regimen that is suitable for all. Decisions on dose titrations are key to achieving the best treatment results that each regimen can provide. Dose titrations require engagement with monitoring, availability of accurate methods for monitoring, and frequent assessment of dose adequacy. There is some evidence that patients on steroidogenesis inhibitors may not have sufficient dose up-titration and efficacy of medical therapy may be reduced because of this ^{1, 359}. Pre-agreed monitoring protocols may encourage sufficient up-titration and will allow more accurate assessment of tolerance problems, adequate dose titration and effectiveness.

Safety considerations

The benefit of medical therapy is always weighed against its adverse effects. Metyrapone has been shown to be safe and well tolerated with a side effect profile that likely overlaps with symptoms of overtreatment. This is likely true with other steroidogenesis inhibitors such as ketoconazole and mitotane. Accurate and reliable biochemical assessment of treatment response can minimise overtreatment and likely improve tolerability but such accurate assessments are not widely available. The management of these patients should be done in specialist centres where there is adequate clinical experience to assess and manage the intricacies of medical therapy and improve safe use of medical therapy.

Accurate characterisation of the safety and side-effect profile of medications is better done in prospective studies, which are not available for the most commonly used steroidogenesis inhibitors. Due to the mechanism of action metyrapone could cause accumulation of mineralocorticoid and androgen precursors and result in fluid retention and symptoms of hyperandrogenism in women, however, these concerns were not significantly reflected in the findings of Study 1. Ketoconazole causes hepatitis and very rarely acute liver failure and frequent monitoring of LFTs is essential. Because of the severity of these side effects, the use of Ketoconazole as an antifungal has been restricted and the dose for this indication was lower than the dose used in CS.

Pasireotide causes hyperglycaemia in a significant number of patients treated with CD due to inhibition of insulin secretion through SSTR1,2, and 5 mediated signalling. In the majority of

patients treated with pasireotide there is worsening of diabetes control, new onset of diabetes or anti-glycaemic medications and patients need to be well-informed and closely monitored.

6.3. Methodological challenges in studying conditions of cortisol excess and deficiency

6.3.1. Assessing treatment effectiveness

Medical therapies come with risks of side-effects and financial cost and should only be considered if there is reasonable expectation of benefit over no treatment. Documenting the effectiveness of treatment is important in clinical decision making such as weighting continued use over no treatment or change of treatment. There are various ways of assessing treatment effectiveness: biochemical monitoring of change in biomarkers, clinical monitoring of change in clinical features and symptoms, improvement in clinical outcomes in treated patients over time, improvement in self-perceived health status or quality of life. Ideally these measures must be validated and associated with long-term desirable patient outcomes in a representative population of patients. This high level of evidence requires prospective application of the measures of treatment effectiveness in a large patient sample, reflection and debate on their use, and confirmation in multiple cohorts. In practice, commonly used medical therapies have not been tested against all aspects of this vigorous process.

Metyrapone has been used as a medical therapy for CS for many decades however clinical experience was mainly restricted to a few patients in tertiary centres. This is not surprising as CS is a rare condition and only some patients receive treatment with metyrapone. In the literature there has been no prospective studies on its use in improving cortisol levels in CS; there have been case reports, expert opinions, and a large retrospective study of 91 patients. For this reason, its use was not standardised; multiple biochemical tests were used and the frequency of biochemical monitoring and dose titration varied or no dose titration was done. The selection of an appropriate biochemical biomarker to monitor treatment response is essential in assessing treatment effectiveness. Verhelst et al. reported a single centre

experience of metyrapone where all patients were monitored by CDCs aiming a mean level between 150-300 nmol³⁴⁹. Study 1, a multicentre retrospective study, included a large patient sample, almost equal in number to all previous cases reported in the literature, and showed that frequently other tests were used to assess efficacy; UFC and morning cortisol levels as well as CDCs. These three tests have not been compared directly in patients treated with metyrapone and it is not possible to say which one is preferable, performing all three is likely excessive. UFCs are commonly used to monitor treatment response in CS clinical trials aiming for normalisation but they are not useful in detecting adrenal insufficiency therefore they may not be useful in fine dose titration. Single morning cortisol levels are less time consuming than CDCs however the target range is not clear; is the aim of treatment the normalisation of levels or a specific value and is the sample pre or post morning dose? The cut-off levels proposed in Study 1 are based on expert opinion and reflect the clinical practice at the time but they are not validated against other biomarkers or patient outcomes.

On the other hand, the biochemical target of the mean cortisol level in CDCs corresponds to the daily cortisol production rate in healthy volunteers and was associated with clinical improvement in the series described by Verhelst et al. However, to have accurate biochemical results the method used to quantify cortisol must be specific due to high levels of 11-deoxycortisol, a cortisol precursor with molecular structure similar to cortisol that is significantly increased by metyrapone therapy and cross-reacts in cortisol immunoassays leading to overestimation of cortisol levels⁴³⁰. Another option is abolishing the approach of dose titration in favour of a block and replace regimen where higher doses on metyrapone are used for complete blockage of cortisol production and glucocorticoids are added for replacement. This negates the requirement of regular monitoring of treatment response with the following assumptions; patients tolerate high metyrapone doses and are meticulous in compliance for all (multiple) tablets.

In study 2, the use of ACTH as a biochemical biomarker of disease activity and response to treatment in NS was tested in a prospective study. The method of testing for ACTH was standardised allowing comparison between values over time; ACTH was measured before and after the morning glucocorticoid dose, at the same time intervals from the start of treatment and the response criteria and their interpretation were predetermined. This standardised application of a biochemical biomarker increases the confidence in assessing response to treatment. ACTH levels show intra-individual variability and generally fall after a glucocorticoid dose therefore it was useful to monitor pre and post dose levels and test at

frequent intervals during the study. In this study together with biochemistry there were clinical and imaging markers of response; skin pigmentation and tumour volume on magnetic resonance imaging are consistent and measurable features of NS and improvement is a desirable clinical outcome. The study showed that the medical therapy tested reduced ACTH levels significantly over time. Pasireotide was effective and this response reached statistical significance despite the small patient sample, however, because of the small number of patients reproduction of these results in further studies are necessary before any firm conclusions for a positive treatment effect is made.

In cortisol deficiency treated with glucocorticoid replacement there is no accepted biomarker to monitor disease response and usually patients receive a fixed dose, in some centres adjusted for weight and thereafter dose titrations depend primarily on clinical assessments. Quantification of the glucocorticoid used for replacement can be done with Hydrocortisone day-curves and prednisolone levels ¹⁶⁶. The former is useful in detecting poor absorption and overtreatment and the latter is not widely available and should be done using LC-MS/MS technique. Clinical features are mainly used to assess response to treatment and these include absence of Cushingoid and hypoadrenal symptoms and signs. Adrenal crisis is an extreme presentation which is life threatening and its prevention is a primary aim of treatment; they are usually caused by infections unrelated to the cause of adrenal insufficiency but the frequency over time can help assess response especially in a large patient sample. Furthermore, changes in quality of life are important for patient perceived health status but are not formally part of dose assessment. Bone markers could be used as biomarkers of overtreatment rather than efficacy. Hair cortisol has been used in the assessment of chronic stress as a marker of long-term cortisol exposure and there are no obvious applications in guiding management of hypoadrenal patients ⁶⁰³.

6.3.2. Analytical biochemistry for measuring steroids

The assessment of treatment response and on-going monitoring of conditions of cortisol excess and deficiency requires accurate analytical chemistry methods for measuring steroids and cortisol in particular. The three immunoassays used in Studies 1 and 2 were competitive binding chemiluminescence immunoassays (Roche Cobas, Siemens Centaur, and Abbott

Architect) with good specifications however they have cross-reactivity with steroid precursors (Table 8). A number of different techniques and methods are used for quantifying cortisol in biological samples and these are immunoassays, high performance liquid chromatography (HPLC) and gas or liquid chromatography coupled with mass spectrometry.

The main challenges in the quantification of small steroids such as cortisol in biological samples are the specificity of antibodies used in immune-based methods, the presence of numerous structurally similar endogenous or exogenous steroids and steroid metabolites that can interfere with measurement, the binding of steroids to carrier proteins in the circulation and sex-specific differences⁶⁰⁴. Analytical methods with positive identification overcome these issues at the expense of lengthier and more operator-intensive protocols but these techniques were not widely available in clinical laboratories. Growing concerns about the accuracy and precision of some steroid immunoassays, their effect in diagnosis and monitoring of patients, and the consequence in the reporting of studies in medical publications led to some publishers issuing guidance against the use of certain assays and a move towards harmonisation and standardisation of analytical methods. The Endocrine Society warned against the use of direct unextracted immunoassays for quantification of serum cortisol in manuscripts due to low sensitivity and possible cross-reactivity with exogenous glucocorticoids and the risk of cross reactivity of salivary cortisol with cortisone measured by immunoassays restricting their clinical application in the diagnosis of adrenal insufficiency or Cushing's syndrome⁶⁰⁵.

Immunoassays are the most commonly used assays to quantify serum cortisol in clinical laboratories being simple to use and automated to have high throughput. However, immunoassays suffer from low specificity due to antibody specificity problems and cross-reactivity from other steroid molecules. Endogenous steroid molecules and drugs that are structurally similar to the target hormone could cross react with the antibody in the immunoassay and an example is cortisol and its precursor molecule 11-deoxycortisol. Cross-reaction can cause a clinically significant increase in cortisol quantification especially when the cross-reacting compounds are found in high concentrations such as in samples from patients suffering from conditions causing elevated steroid precursors, for example some forms of CAH, and patients treated with steroidogenesis enzyme inhibitors such as metyrapone¹³⁷. Immunoassays may also be affected by variable separation of cortisol from CBG, which affects cortisol quantification in conditions where CBG is elevated such as women who are pregnant or taking the oral contraceptive pill, and in these cases under-recovery of cortisol from CBG leads to under-estimation of total cortisol^{28,530}. Furthermore, immunoassays

may have low sensitivity in low concentrations of circulating cortisol due to low-affinity of anti-cortisol antibodies and sensitivity in saliva is also not acceptable at the low concentrations needed for measuring nadir cortisol levels in patients investigated for hypercortisolism due to interference by salivary cortisone ⁶⁰⁶.

Radioimmunoassay used to be the gold standard for cortisol and steroid quantification but was labour-intensive and involved the use of radioactive isotopes. Modern chemiluminescent immunoassays are sensitive even to low concentrations of cortisol and have a linear relationship between the intensity of emitted light and amount of biological substance to be quantified. They are comparable to RAI in terms of sensitivity, specificity, precision and accuracy but more rapid and eliminating the need for radiation and have been the standard cortisol immunoassay in clinical practice ⁶⁰⁷. Direct cortisol immunoassays without extraction and pre-purification are not specific enough due to competition of plasma cortisol binding proteins with the assay antibody and interference with antisteroidogenic drugs. Immunoassays measuring cortisol in the urine have poorer performance due to dilution and the presence of large amounts of cortisol metabolites that cross-react with the assay and lead to overestimation of free cortisol concentration ⁶⁰⁸.

HPLC is specific but not sensitive enough to quantify cortisol at physiological levels and has limited use unless paired with mass spectrometry. Gas chromatography mass spectrometry (GS-MS) is the gold standard method for steroid analysis and is used extensively for urinary steroid metabolome analysis (506). It is an accurate method that involves sample preparation by extraction of steroids on a hydrophobic sorbent and purification by liquid-gel chromatography followed by computerised gas chromatography-mass spectrometry analysis ^{609, 610}. Gas chromatography separates organic compounds in a complex sample in the gas-phase over time and mass spectrometry analyses compounds based on their structural properties and m/z ratio ⁶¹¹. GS-MS was developed six decades ago but is not used routinely in clinical practice and is mainly a research tool as it is time-consuming with long sample preparation and analysis ^{523, 612}.

In contrast, LC-MS/MS has emerged as an accurate method for glucocorticoid analysis, which is practical enough to be applied in research studies and routine clinical practice. It is sensitive and specific with results comparable to GC-MS but has faster analytical times and good specificity, higher than HPLC and immunoassay ¹⁴⁰. The major advantages is that it can measure multiple forms of an analyte, multiple analytes, does not dependent on specialised

immunologic reagents and can be standardised across different laboratories ^{523, 613}. The protocol for sample preparation is simple and only basic preparation is necessary as the technique is good at cutting out interferences. It has a low limit of detection in serum for steroids and can measure cortisol in a small amount of sample ^{527, 528}. An LC-MS/MS methodology for quantification of cortisol has been standardised by the US National Institute of Standards and Technology and a number of candidate reference methods have been published that are accurate, specific and sensitive to low cortisol levels ⁶¹⁴⁻⁶¹⁶. Over the last decade there has been a development of improved LC-MS/MS protocols that allow rapid cortisol quantification and can be used in busy clinical laboratories ⁶¹⁷.

Compared with immunoassays, LC-MS/MS methods have lower intra- and inter-assay variability and are more accurate especially in extreme high or low values. Furthermore, serum samples from patients that receive synthetic steroids, as well as salivary and urinary samples have significant cross-reactivity in immunoassays and for accurate measurement should be analysed by LC-MS/MS ⁵²³. Commonly used immunoassays for cortisol quantification are not linear with LC-MS/MS and it is recommended that the results of immunoassays are validated against LC-MS/MS methods ^{523, 606, 613}. A comparison of serum cortisol quantification by a reference LC-MS/MS method and the immunoassays used for quantification of cortisol in Study 1 showed that the three immunoassays had a bias of -4.9 to 18% in control men and non-pregnant women and significant inter and intra-assay variability whereas a routine LC-MS/MS method used in clinical practice was more accurate ⁵³⁰. In this study, Hawley et al. found that compared to the reference method, the routine LC-MS/MS performed well but there was positive bias in all the immunoassays (mean bias 49.4, 182.2 and 161.5 nmol/L for the Abbott, Roche, and Siemens assay) which would lead to changes in clinical decisions, up-titration of metyrapone dose when not necessary in some cases and overtreatment of patients ⁵³⁰.

A study comparing UFC by LC-MS/MS, GC-MS, and two commercial immunoassays showed that cortisol was grossly overestimated up to 2-fold by the two immunoassays due to interference from cortisol metabolites and that LC-MS/MS results correlated and performed well compared with the reference GC-MS method ¹⁴⁰. Salivary cortisol levels measured by various commercial immunoassays and compared with LC-MS/MS showed that the values obtained by the different immunoassays were not comparable and had significant variation in absolute values proposing indirect comparison of values by conversion by an immunoassay specific factor score which is impractical as it requires frequent review ⁶⁰⁶. These results

suggest that when monitoring tests are analysed by the immunoassays there is a significant risk that the inaccuracy of the immunoassays due to cross-reactivity would change clinical decisions with possible implications on the patients, for example up-titration of dose when it is not needed.

Immunoassays have significant inter-laboratory variability and this affects the comparability of patients' biochemical monitoring between multiple laboratories or over-time within the same laboratory if there is an assay change ^{530, 618, 619}. This is particularly a problem when reporting retrospective studies or multicentre studies where cortisol cut-offs must be different in each centre to reflect the characteristics of the local assay although commonly cut-offs from literature are followed without validation from the local assay. Immunoassays also generally overestimate cortisol concentrations especially after ACTH stimulation in the context of a SST and this may have clinical implications in the diagnosis of adrenal insufficiency increasing false positive results unless assay-specific cut-offs are used ⁶¹⁹.

6.3.3. Challenges in designing clinical trials for rare diseases

Cortisol excess due to endogenous Cushing's syndrome, Nelson's syndrome, Primary adrenal insufficiency and CAH are rare diseases as classified by Public Health England ⁶²⁰. The Department of Health defines a rare disease as 'a life-threatening or chronically debilitating disease that affects 5 people or fewer in 10,000 and requires special, combined efforts to enable patients to be treated effectively' ⁶²¹. The majority of about 5,000 to 8,000 rare diseases have a genetic cause and about 3 million people in the UK suffer from a rare disease ⁶²¹.

There are many methodological challenges associated with assessing treatment effectiveness for rare diseases primarily because of the small patient numbers. Recruiting sufficient numbers of patients to clinical studies is difficult due to slow enrolment and geographic dispersion of patients as demonstrated by Study 2. The optimal design of clinical trials involves having a control arm but there are ethical concerns about using placebo treatment arms in clinical trials when a disease is life-threatening with significant morbidity if untreated such as

CS, NS and hypoadrenalism and there are practical problems deciding what is standard treatment in conditions such as NS where effective medical therapy does not exist. Biomarkers are often used as surrogates of clinical outcomes in rare diseases but they may not be fully validated and used with un-established reliability⁶²². For example, in interventional studies in CS, UFC is usually used as the biochemical end-point for response however there is no universal agreement on the use of UFC or serum cortisol as a marker of cortisol normalisation, UFC alone does not provide information on change in cortisol-associated comorbidities that is a strong indication for treatment, and it has methodological disadvantages such as high variability that may affect interpretation of response in patients with mild disease⁶²³.

Due to the small population of patients affected there are also problems attracting funding for translational research into rare diseases including the conduction of costly prospective clinical trials. Such clinical studies are more acceptable if they pick up large treatment effects compared with studies performed with bigger patient numbers, for example interventions with a low number needed to treat in the order of 2 or 3 may be more acceptable in rare conditions which is in vast contrast with how effectiveness of an intervention is interpreted in common conditions^{624, 625}. Slow recruitment may delay implementation of effective therapies and to deliver a timely study with substantial patient numbers there may be the need for multiple sites, often in multiple countries, which requires co-ordination, expertise and financial resources⁶²⁶.

Nevertheless, to make clinical decisions that benefit patients we need evidence that these decisions are safe and effective. For the patient to make an informed decision on their treatment we need to know and discuss the disadvantages of the treatment options, the side effects and what their expected benefit is. To be able to navigate through these decisions we need an understanding of the short and long-term clinical issues and evidence. High quality evidence to answer these questions comes from interventional studies.

Most of the evidence that guides the clinical management of rare diseases comes from observational studies. Observational studies are often retrospective and are useful in rare conditions where retrospective data collection is easier to perform and can be analysed to help determine the natural course of disease, draw associations, formulate hypotheses and

plan controlled trials to test them. Observational treatment studies have some inherent disadvantages affecting the quality of evidence they provide and suffer from selection bias (confounding by indication)⁶²². There are various statistical adjustments that can help reduce bias in observational studies such as multivariate analysis and matching of groups, however, not all confounding factors are known and overall the risk of bias is not eliminated.

In retrospective observational studies the data collected depend on the recording in medical notes and the validity of the analyses and conclusions rely completely on the quality of data extracted. Lack of harmonisation of the sampling protocols for biomarkers used as measurable outcomes in studies on cortisol excess, ACTH excess and cortisol deficiency can add variability and affect comparison between different centres. Documentation of sampling times and association with other medications is essential in some cases and this is commonly lost in retrospective data collection and may affect the homogeneity of data compared. In Study 1, the data collection was standardised by using clinically-trained researchers trained in data abstraction and a validated and standardised data collection tool to enhance the quality of data⁶²⁷. Retrospective observational studies in rare diseases may provide results generalisable to the patient population especially if it is possible to ensure all patients with a particular variable are included in the analysis; in Study 1, reviewing the records of all patients treated with metyrapone in thirteen UK centres was possible through pharmacy records as metyrapone is only available through secondary care pharmacies. In Study 3 all women with CAH treated in a specialist clinic were identified and included in the study as cohort 1.

There are other study designs that provide higher quality of evidence such as randomised controlled trials (RCTs) and systematic reviews of RCTs, which are the optimal studies to test treatment effectiveness. Parallel groups randomised double-blind controlled trials are the best way to generate unbiased evidence and avoid confounding; if there is bias then this is due to chance and by increasing the sample size you reduce the probability that this occurs. This trial design minimizes selection bias and distributes confounding factors between the treatment groups and this is achieved by using random allocation of a large number of participants-patients who are closely monitored over a long-period of time⁶²². These studies provide high-quality evidence and guide medical practice in most disciplines but are commonly unfeasible in rare diseases due to lack of large sample size and homogeneity of the patient population, funding, and importantly ethical concerns with study design such as blinding and use of

placebo for comparing treatment effects ⁶²⁸. Rarely clinical practice in rare diseases is guided by RCTs.

The quality of evidence for treatment effectiveness is likely to improve with access to more and better-quality data. Analysis and reporting of clinical data from multiple patients can help unravel clinical problems and deficits in care, design safe treatment protocols, and plan studies to provide answers ⁶²⁹. Although there are centres of expertise in the management of rare diseases, many patients are monitored and treated by clinicians with limited number of similar patients ⁶²⁸. Dissemination of information could help guide clinicians and improve care. Patient registries using data entry from patients, clinicians and researchers or imported from electronic health-records can help collect information on demographic data, diagnosis and initial investigations, current treatment and history of treatments in a systematic and uniform manner. Clinical data from prospective international registries that collect information through authorised clinicians for rare diseases such as PAI and CAH, facilitate assessment of current practice, identify research questions, help design studies by assessing preliminary data and can even contribute to harmonisation and reduce inequalities in care ^{467, 624, 630}. Prospective longitudinal studies with pre-defined protocols for treatment response and biochemical sampling can be based on existing registries of patients with CAH improving the quality of data collected. Registries for rare diseases can also be used for post-market safety-monitoring after introduction of new medical therapies which is required by regulatory bodies and helps improve long-term safety of therapeutic interventions, assess standardisation and quality of care, and collect data for longitudinal studies and epidemiological research ^{631, 632}. Contact registries can be used for engagement with patients disseminating information regarding clinical research activities, identify participants and enhance enrolment in studies ⁶³³. Registration of clinical studies may also improve the recruitment and dissemination of data answering the specific questions irrespective of publication in a medical journal.

Specific study designs are best suited to clinical research of rare diseases with small patient numbers. These study designs are unusual in the research protocols of common diseases but as recommended by the Institute of Medicine, the 'appropriate use and further development of trial design and analytic methods tailored to the special challenges of conducting research on small populations' is essential to accelerate research and product development for rare diseases ⁶²⁴. Examples of study designs that are more likely to be appropriate are cross-over studies, N-of-1 trials, trials with adaptive design, sequential design, or internal pilot design ⁶³⁴.

Study designs that avoid the use of placebo or no-treatment arm or reduce time on placebo arm are likely to be more acceptable to patients and clinicians due to ethical concerns of having placebo treatment in patients with high-morbidity disease such as cortisol excess. Randomisation in crossover studies is used for the selection of the sequence of treatment periods, which usually involve placebo or active comparator versus experimental treatment. There is a short washout period in-between the two treatment periods and measurement of outcomes after each treatment period. N-of-1 trials apply the cross-over design to a single patient alternating multiple periods of the active treatment versus control and offer exposure to active treatment to all participants and flexibility benefiting recruitment ⁶³⁴. Adaptive randomisation designs reduce time spent on control or placebo arms by changing the ratio of patient allocation between treatment groups during the trial. Other adaptive designs allow modifications of eligibility criteria, completion time, re-calculation of sample size, or treatment arms at pre-specified time-points during the trial based on a preliminary assessment of outcomes. Randomised withdrawal design are 'enriched' with responders; patients most likely to respond are selected early following exposure to experimental treatment, then randomised to experimental treatment or placebo and outcomes are monitored. In the randomised placebo-phase design patients are randomised to experimental treatment or control and spend a small period on control before change to experimental treatment, the measurable outcome is usually time to response or time to escape and it is based on the assumption that if the treatment is effective patients will respond sooner ^{622, 635}. Internal-pilot designs allow the retention of patients included in the initial, and successful, pilot study.

The quality of feasible clinical studies can be improved by paying careful attention to the study design. Different study designs offer different advantages and contribute the most when applied appropriately. The use of historical controls in observational studies could improve strength of evidence and has been used in studying inborn errors of metabolism however in cortisol excess and deficiency changes in cortisol assays and optimal glucocorticoid replacement doses make historical comparisons unhelpful ⁶²⁸. Use of active comparator group instead of placebo is more ethical and acceptable to clinicians and patients although the active comparator may be chosen as the only commonly accepted treatment without any previous rigorous assessment of its effectiveness. Cross-over and N-of-1 studies maximise data from small groups of patients, allow smaller participant size and always give exposure to the experimental treatment improving acceptability ⁶³⁴. They are suitable for chronic relatively stable and incurable diseases and therapies with rapid onset of action that disappears soon after cessation and short-term endpoints. The participants act as their own control minimising

variability and confounding but they are sensitive to dropouts, carryover and period effects^{629, 636, 637}. The primary outcome of N-of-1 trials is usually to determine the treatment preference for the individual patient and are best indicated when the effectiveness of the treatment is in doubt⁶²⁸. Data from patient registries, pharmacological characteristics, and results of previous clinical trials can help define response and measurable outcomes in randomised withdrawal studies and, in addition, duration of control phase in randomised placebo-phase studies⁶²². Finally, regulatory authorities offer advice in designing protocols for rare diseases to ensure best use of data and resources.

Fulfilling recruitment targets is essential for high-quality data. Under-recruitment is a common problem in clinical research and only 31% of RCTs funded by the UK Medical Research Council achieved their recruitment targets within the original time-frame with the majority requiring an extension or revision of their recruitment target⁶³⁸. In Study 2, the recruitment target was not met mainly due to the rarity of NS and the small number of patients at each of the four participating centres that could be enrolled to the study and this affected the generalisability of the positive treatment effect of pasireotide in patients with NS. Recruitment problems are complex and recruitment is usually slower than expected, eligible patients are usually fewer than expected and there are usually delays in the setting-up of trials⁶³⁸. This is a particular problem in rare diseases due to the small overall patient number and the small number of patients per centre. Multicentre international studies are usually necessary to achieve recruitment targets and these studies are costly and complicated due to different national regulations often requiring up to 2 years to set up⁶²⁶. Patient involvement at an early stage helps identify enrolment issues, protocol concerns, logistic problems with participation and improve acceptability. Engagement with patients and patient-support societies and input from clinicians with expertise in the management of patients in the clinic in an early stage can help improve acceptability of protocols and increase screening numbers and recruitment⁶³⁹. Homogeneity of the study population is important but in small overall patient numbers it may need to be weighed against achieving a larger sample size and the balance is reflected on the eligibility criteria⁶³⁶. Examples of homogeneity concerns may include; studying ACTH dependent and independent CS together, studying both sexes together, studying mild and severe disease together (CS and CAH), studying patients who have previously received different forms of permanent treatment (radiotherapy in CD). Finally, the option for an open-labelled extension of an interventional study ensures access to the treatment post trial when the medical therapy tested is under development and not otherwise available to participants and is likely to enhance acceptability and enrolment to a randomised prospective trial⁶²².

The correct use of statistical analysis can help interpret the outcome of clinical trials in rare diseases and make the most of the collected data. Conventional study designs use the p-value to conclude effectiveness of an intervention as a measure of the size of effect and sample size and a surrogate for precision. Alternatively, Bayesian statistics applied to clinical trial designs allow use of prior probability based on expert opinion or previous studies such as early phase or studies in other populations, and incorporate the data from the clinical trial to come up with a posterior probability distribution which is not dependent on the sample size⁶²². Statistical analysis of observational studies can help quantify and reduce selection bias through the use of a propensity score to create matched cohorts based on their baseline characteristics⁶²². Subgroup analysis of patients based for example on their response usually requires a larger patient sample to have statistical power to detect differences and this should be considered in the development of statistical methods⁶²⁹. Statistical meta-analysis of multiple N-of-1 trials can help estimate overall population effects and increase the generalisability of treatment effect in a population making the most of small patient numbers in each centre⁶²².

6.4. New biomarkers for monitoring treatment response

Restoration of physiological levels of cortisol requires individualisation of treatment and for this to be achieved close monitoring of biochemistry is required. Biomarkers that are consistent, easy to measure and accurate are essential to aid assessment of adequacy of treatment. Multiple biomarkers are in use for this reason and in Congenital adrenal hyperplasia steroid precursors affected by the enzymatic defect are used for diagnosis and also monitoring of disease control. Biomarkers used for CAH due to 21-hydroxylase deficiency are 21-hydroxyprogesterone, androstenedione, and renin. These biomarkers have a diurnal rhythm of secretion and are affected by the time of administration of glucocorticoids that needs to be considered during assessment of results. They require accurate quantification methods which usually takes time and commonly is not available at all the hospital sites.

Markers of erythropoiesis such as haemoglobin and haematocrit are widely available and their quantification is standardised and accurate. Many factors and hormones affect erythropoiesis and this effect takes time to reflect on haemoglobin levels therefore as a biomarker it reflects chronic control. The usefulness of a biomarker needs to be validated in relation to clinical outcomes and it would be interesting to assess the usefulness of haemoglobin for example in fertility related to CAH control where a biomarker reflecting chronic control may be particularly useful. Individual genetic determinants that affect individual haemoglobin levels may limit its usefulness.

The development of biomarkers to guide glucocorticoid replacement is urgently required in clinical practice. Such biomarkers don't exist and we rely on serum or salivary cortisol levels, which have disadvantages and do not reflect the glucocorticoid availability and activity in the tissues. There are no tissue-specific markers of glucocorticoid action that are validated and part of routine clinical practice yet, however, several have been identified. Some evidence exists for bone-formation markers. Osteocalcin, a peptide produced by osteoblasts, increased following glucocorticoid dose reduction in 19 patients with adrenal insufficiency who were initially on excessive glucocorticoid replacement doses and, in another study, osteocalcin levels correlated with glucocorticoid dose in prednisolone-treated asthmatic patients.^{507, 640, 641}. The effects of glucocorticoids on osteocalcin levels appear dose-related and rapid, however, for routine glucocorticoid replacement dose changes may lie within a wide normal reference range^{642, 643}. Thrombospondin-1 is a glucocorticoid-responsive extracellular matrix protein secreted by activated platelets and expressed by many other cells that is elevated in CS and increases rapidly with higher hydrocortisone doses in patients with hypoadrenalism^{644, 645}. Other potential targets for tissue-specific biomarkers of glucocorticoid sufficiency include factors that regulate tissue sensitivity to glucocorticoids and glucocorticoid-responsive proteins such as immune response or bone turnover proteins. Tissue-specific biomarkers have the potential advantage of reflecting the adversary effects of hypercortisolism on the tissues and clinical endpoints better rather than pre-tissue cortisol excess in the circulation.

7. Conclusions and recommendations for future research

Clinical studies and laboratory experiments were performed to test whether it is possible to restore physiological cortisol and adrenocorticotrophin hormone levels in patients with disorders of cortisol and adrenocorticotrophin production. We found that medical therapy was an effective treatment for hypercortisolaemia either before or after first line surgical treatment in the largest reported cohort of patients with CS treated with metyrapone, improving cortisol excess in 80%. As monotherapy, metyrapone normalised cortisol levels in over 50% of patients monitored with CDCs. Medical therapy with pasireotide reduced plasma ACTH levels in patients with Nelson's syndrome in a prospective clinical study and the response persisted after daily subcutaneous pasireotide was changed to long-acting monthly intramuscular injections. In optimising glucocorticoid replacement for conditions of cortisol deficiency, we found in a laboratory experiment that it is possible to deliver hydrocortisone through nasogastric tubes, however, there are variable drug losses due to interaction with the administering equipment that should be considered when treating patients. Administration protocols were developed and tested and showed that two hydrocortisone formulations performed better at delivering the intended dose; Hydrocortisone in liquid suspension and Hydrocortisone granules suspended in water delivered 61-92% of the drug dose at the gastric end of the nasogastric tube. We have also showed that Hydrocortisone granules can be administered sprinkled on soft food (apple sauce or yoghurt) or directly to the back of the tongue without change of the pharmacokinetic properties including peak drug concentration and rate of drug absorption, therefore, it is possible and safe for patients to take the medicine with any of these three administration methods. With regards to biochemical monitoring of medical therapy and disease control, we found that markers of erythropoiesis (haemoglobin and haematocrit) correlate positively with androgen and steroid precursor levels in women with CAH and could be used as easily accessible biomarkers to monitor adequacy of glucocorticoid replacement and disease control.

Successful medical therapy preconditions the existence of a compound with appropriate chemical characteristics, a satisfactory safety profile, that can be administered in a method acceptable to patients, have sufficient absorption and achieve a satisfactory pharmacokinetic profile and clinical efficacy. The findings from the five studies presented in this thesis make the case that medical therapy is safe and effective in restoring cortisol and ACTH levels in cortisol and ACTH excess, can deliver accurate glucocorticoid dose replacement through

nasogastric tubes or co-administration with soft food, and that new easily available biomarkers can help monitor cortisol replacement in women with a genetic form of cortisol deficiency.

The data presented in this thesis answer clinical questions and add knowledge that advances the management of patients with cortisol and adrenocorticotrophin excess and deficiency. During the studies and interpretation of results further questions were raised and areas of improvement were identified. It is proposed that future research should aim to expand knowledge and improve clinical practice in the following:

1. Examining the effectiveness of metyrapone in restoring physiological cortisol levels in patients with CS in a prospective study using a standardised protocol for monitoring with pre-defined time-points for biochemical tests and dose titration and documentation of changes in morbidity and quality of life.
2. Examining the long-term effects of treatment with pasireotide on corticotroph tumour volume in patients with Nelson's syndrome.
3. Exploring the role of haemoglobin and haematocrit as biomarkers of disease control in women with CAH seeking fertility. Could they be used to guide treatment titration in the preconception period?
4. Exploring any benefits occurring from the flexibility in the administration methods of hydrocortisone granules on short and long-term compliance, patient or carer satisfaction and quality of life and any long-term treatment benefits.

Abbreviations

17OHP	17-Hydroxyprogesterone
3 β HSD1	3 β -Hydroxysteroid dehydrogenase type 1
3 β HSD2	3 β -Hydroxysteroid dehydrogenase type 2
A4	Androstenedione
ACTH	Adrenocorticotrophin hormone
AI	Adrenal insufficiency
AMH	Anti-Mullerian hormone
<i>ARMC5</i>	Armadillo repeat containing 5 gene
BSA	Body surface area
CAH	Congenital adrenal hyperplasia
CBG	Cortisol binding globulin
CD	Cushing's disease
CDC	Cortisol day-curve
CS	Cushing's syndrome
CTNN1B	beta-Catenin
DHT	Dihydrotestosterone
EGFR	Epidermal growth factor receptor
EPO	Erythropoietin
FSH	Follicle-stimulating hormone
GC	Glucocorticoid
GC-MS	Gas chromatography-mass spectrometry
GH	Growth Hormone
<i>GNAS</i>	G stimulatory protein subunit alpha gene

GR	Glucocorticoid receptor
HC	Hydrocortisone
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High performance liquid chromatography
ITT	Insulin tolerance test
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LFT	Liver-function test
LH	Luteinizing hormone
MC	Mineralocorticoid
MRM	Multiple reaction monitoring
NFA	Non-functioning pituitary adenoma
NS	Nelson's syndrome
ONDST	Overnight dexamethasone suppression test
PAI	Primary adrenal insufficiency
<i>PDE11A</i>	Phosphodiesterase 11A gene
<i>PPAR</i>	Peroxisome proliferator-activated receptor gene
PPNAD	Primary pigmented nodular adrenocortical disease
<i>PRKACA</i>	Protein kinase cAMP-activated catalytic subunit alpha gene
<i>PRKAR1A</i>	Protein kinase cAMP-dependent type I regulatory subunit alpha gene
QoL	Quality of life
RAI	Radioimmunoassay
RCT	Randomised controlled trial
SST	Short synacthen test
SSTR	Somatostatin receptor
T	Testosterone

TART	Testicular adrenal rest tumour
<i>TR4</i>	Testicular orphan nuclear receptor 4
UFC	Urinary free cortisol
<i>USP8</i>	Ubiquitin-specific protease 8 gene

Summary of figures

Figure 1: Molecular structures of endogenous steroids	Page 18
Figure 2: The steroidogenesis pathway	Page 26

Summary of tables

Table 1: Tissue-specific glucocorticoid effects	Page 17
Table 2: Nomenclature of endogenous glucocorticoids and mineralocorticoids	Page 19
Table 3: Enzymes and genes involved in steroidogenesis in human	Page 27
Table 4: Causes of Cushing's syndrome	Page 50
Table 5: Causes of Primary adrenal insufficiency	Page 58
Table 6: Causes of Congenital adrenal hyperplasia	Page 61
Table 7: Summary of clinical features of Congenital adrenal hyperplasia	Page 67
Table 8: Cross reactivity of cortisol immunoassays	Page 105

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Appendix

A.1. Metyrapone data collection form

Use of Metyrapone in Cushing's syndrome: Data collection form																											
Centre ID:	Patient ID:	Gender: F / M	DOB:																								
Diagnosis: <input type="checkbox"/> Cushing's disease <input type="checkbox"/> Macroadenoma <input type="checkbox"/> Microadenoma Baseline pituitary MRI findings: <div style="background-color: #cccccc; height: 20px; width: 100%;"></div> <input type="checkbox"/> Ectopic ACTH Cause:..... <input type="checkbox"/> Adrenal Adenoma <input type="checkbox"/> Adrenal Carcinoma <input type="checkbox"/> PPNAD <input type="checkbox"/> Sporadic <input type="checkbox"/> Carney <input type="checkbox"/> AIMAH <input type="checkbox"/> McCune Albright Date of diagnosis:		Intervention (with dates, details): <input type="checkbox"/> Primary definitive surgical therapy: <input type="checkbox"/> Additional surgical therapy: <input type="checkbox"/> Primary Radiation therapy: <input type="checkbox"/> Adjuvant Radiation therapy: <input type="checkbox"/> Chemotherapy: <input type="checkbox"/> Medical therapy only: <input type="checkbox"/> Other:																									
Biochemical monitoring and pre-treatment values <table border="1"> <thead> <tr> <th>Biochemical marker</th> <th>Value(s) and units</th> <th>Date</th> <th>Normal range</th> </tr> </thead> <tbody> <tr> <td>ACTH</td> <td></td> <td></td> <td></td> </tr> <tr> <td>Potassium</td> <td></td> <td></td> <td></td> </tr> <tr> <td>UFC</td> <td></td> <td></td> <td></td> </tr> <tr> <td>9am cortisol</td> <td>Basal: Post LDDST: Post ONDST:</td> <td></td> <td></td> </tr> <tr> <td>Cortisol day curve <i>(please enter method according to local protocol and values if applicable)</i></td> <td>mean cortisol:</td> <td></td> <td></td> </tr> </tbody> </table> <p>Cortisol assay:</p> <p>Please indicate if there is cross-reactivity with steroid precursors:</p> <p><input type="checkbox"/> Pre-existing diabetes mellitus / IGT Hba1c.....</p> <p><input type="checkbox"/> Pre-existing Hypertension</p>				Biochemical marker	Value(s) and units	Date	Normal range	ACTH				Potassium				UFC				9am cortisol	Basal: Post LDDST: Post ONDST:			Cortisol day curve <i>(please enter method according to local protocol and values if applicable)</i>	mean cortisol:		
Biochemical marker	Value(s) and units	Date	Normal range																								
ACTH																											
Potassium																											
UFC																											
9am cortisol	Basal: Post LDDST: Post ONDST:																										
Cortisol day curve <i>(please enter method according to local protocol and values if applicable)</i>	mean cortisol:																										
<div style="border: 1px solid black; padding: 5px; width: fit-content;">Hospital number:</div>																											

Initiation of Metyrapone therapy	
Indication for Metyrapone therapy (with details if needed / if more than one applies)	
<input type="checkbox"/> 1. Pre-definitive surgery <input type="checkbox"/> 2. After definitive surgery <input type="checkbox"/> 3. In conjunction with pituitary radiotherapy <input type="checkbox"/> 4. Medical therapy without surgery (e.g. ectopic ACTH)	
Metyrapone was given:	
<input type="checkbox"/> As routine practice <input type="checkbox"/> To control severe Cushing's <input type="checkbox"/> Delay in surgery	
Medical factors for delay: <div style="background-color: #cccccc; height: 20px; width: 100%;"></div>	
Patient factors for delay: <div style="background-color: #cccccc; height: 20px; width: 100%;"></div>	
Drug interactions	
Co-administration of medications that could affect HPA axis / Metyrapone metabolism / steroid replacement/ oral oestrogens?	
Yes	No
Details: <div style="background-color: #cccccc; height: 20px; width: 100%;"></div>	

Please complete Page 3: Metyrapone therapy monitoring table. Use extra sheets if required (mark: Page 3.1, 3.2, 3.3 etc)

Hospital number:

Metyrapone therapy monitoring

	Pre-treatment	Monitoring		
	Date	Dates		
ACTH				
Potassium				
UFC				
9am cortisol				
Cortisol day curve (Please enter time, values and mean)	Mean:.....	Mean:.....	Mean:.....	Mean:.....
Metyrapone dose	Decision for treatment			
Reason for dose change (1) Lack of clinical effect (2) Incomplete biochemical control (3) Block and replace (4) Other (details)	Please enter appropriate number and comment if needed			
Response	Please comment on change in clinical features, BP and diabetes control/ Hba1c			
Adverse effects	* Please complete safety section	Yes* / No	Yes* / No	Yes* / No
Block and replace regimen	Please state type of replacement and dose	Yes / No	Yes / No	Yes / No
Concurrent treatment with Ketoconazole	If Yes, please enter dose of medication	Yes / No	Yes / No	Yes / No
Cessation of treatment	Please enter <u>date</u> and <u>reason</u> for stopping Metyrapone			
Follow-up imaging	Please enter repeat MRI findings for patients with Cushing's disease			

Hospital number:

Please complete Page 4 for every separate adverse effect observed. Please use extra sheets if required

Use of Metyrapone in Cushing's syndrome: Safety section	
Centre ID:	Patient ID:
DOB:	Gender: F / M
Past medical history	Concomitant medications
	Other suspected drugs:
Date of onset of adverse reaction:	Date Metyrapone was started:
Metyrapone dose:	Date current dose was started:
Description of adverse reaction	
Causality with Metyrapone Not related / Unlikely / Possible / Probable / Certain / Not assessable	
Seriousness criteria Please circle if any of the following is true about the adverse reaction: Y / N Life-threatening Y / N Required hospitalisation or extension of existing hospitalisation Y / N Resulted in persistent or significant disability or incapacity Y / N Is a congenital anomaly or a birth defect Y / N Is a medically important event, details:	
Action taken: None / Metyrapone withdrawn / dose reduction / other measures taken:	
Outcome: Recovered / recovered with sequelae / continued effects / aggravation / death / unknown:	
Other comments:	

Hospital number:

A.2. Hydrocortisone measurement with LC-MS/MS protocol

Protocol for 1:10000 dilution with water

1. Warm the samples in a hot bath for a few minutes
2. Centrifuge the samples for 2min
3. To make a 1:10,000 dilution of the samples:
 - Add water, methanol and water to 3 universal tubes
 - Use 1.5ml Eppendorf tubes in 2 rows
 - Add 1ml of water to all tubes (M1000 pipette)
 - In the first row, put 10 μ l of the sample and vortex the tubes (M10 pipette)
 - Take 10 μ l from the first-row tubes and add to the tubes in the second row. Vortex. This is the 1:10000 dilution to use for the MS plate
 - Use the same pipette tip for all. Dry pipette tip before adding the content to the Eppendorf tubes. Between different samples wash pipette with water-methanol-water from the universal tubes and dry tip before continuing
4. Place 20 μ l of samples in a new plate for MS analysis
 - For the first 7 wells use known concentrations of cortisol solutions (to draw the standard curve)
 - For the next 3 wells use the 3 known Quality Controls (labelled A, B, C)
 - Then use 20 μ l of a 1:10,000 dilution of the samples, in duplicates
5. Add 40 μ l of zinc sulphate (repeat pipette, 0.1M)
6. Add 100 μ l of a solution of pure methanol + internal standard (repeat pipette, 0.1M)
7. Seal plate, mix and spin at 25000rpm for 5 min
8. Add the plate to the MS machine

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