CONGENITAL ADRENAL HYPERPLASIA:  
THE INFLUENCE OF PUBERTY ON  
CORTISOL PHARMACOKINETICS

EVANGELIA CHARMANDARI

MRCP (UK), MSc (UCL)

A thesis submitted to the University of London
for the degree of Doctor of Medicine
ABSTRACT

In Congenital Adrenal Hyperplasia (CAH) due to 21-hydroxylase deficiency, appropriate glucocorticoid substitution does not often result in adequate suppression of the hypothalamic-pituitary-adrenal (HPA) axis. Suboptimal control is especially observed in pubertal patients, despite adherence to medical treatment. The clinical studies described in this thesis were performed to address the effect of puberty on cortisol pharmacokinetics and HPA axis activity in patients with CAH due to 21-hydroxylase deficiency. Six studies were conducted on forty patients (14 prepubertal, 20 pubertal and 6 postpubertal) with classical 21-hydroxylase deficiency. They revealed:

i) An alteration in the pharmacokinetic parameters of total and free cortisol at puberty: There was an increase in cortisol clearance and volume of distribution but no change in half life. The half life of free cortisol was shorter in females compared to males.

ii) An increase in cortisol clearance and a decrease in half life in association with specific alterations in the endocrine milieu at puberty, including alterations in the growth hormone (GH) ~ insulin-like growth factors and 11β-hydroxysteroid dehydrogenase activity. Also, a decrease in cortisol half life in association with elevated adrenocorticotropic hormone (ACTH) concentrations.

iii) Essentially complete bioavailability of oral hydrocortisone tablets used as substitution therapy in these patients.

iv) A significant negative correlation between cortisol and 17-hydroxyprogesterone concentrations. Also, a difference in the activity of HPA axis between daytime and night-time, with the minimal activity observed between 1600h and 0400h.

v) No alteration in the negative feedback effect of cortisol at the pituitary level.

vi) A significant positive correlation between serum cortisol and GH concentrations.

The above findings may explain the difficulties encountered in the management of CAH patients at puberty and suggest that replacement therapy should be with frequent doses of glucocorticoid substitution in all pubertal patients, particularly females. Management should also aim at providing adequate HPA axis suppression between 0400h and 1600h, as well as preventing and/or treating hyperandrogenism.
For all patients with
congenital adrenal hyperplasia
CONTENTS

ABSTRACT .........................................................................................................................2
CONTENTS .........................................................................................................................4
LIST OF FIGURES ............................................................................................................9
LIST OF TABLES ............................................................................................................12
LIST OF ABBREVIATIONS ..........................................................................................13

INTRODUCTION .............................................................................................................16

CHAPTER I: ADRENAL STEROIDOGENESIS & GLUCOCORTICOID ACTION ............19
A. Pathways of steroid biosynthesis .................................................................20
B. Regulation of adrenal steroid secretion .......................................................24
   i) Cortisol secretion .................................................................24
   ii) Aldosterone secretion ..........................................................32
   iii) Adrenal androgen secretion ......................................................33
C. Molecular mechanisms of glucocorticoid action ........................................35
   i) The glucocorticoid receptor (GR) .............................................35
   ii) Glucocorticoid responsiveness and sensitivity .........................38
      iii) Molecular determinants of glucocorticoid sensitivity ...........39

CHAPTER II: 21-HYDROXYLASE DEFICIENCY: AN OVERVIEW .........................43
A. Clinical spectrum ............................................................................................44
B. Clinical manifestations of classical CAH ......................................................46
   i) Prenatal virilization ..................................................................46
   ii) Salt wasting ..............................................................................47
   iii) Postnatal signs of androgen excess ..........................................49
   iv) Reproductive function .............................................................50
   v) Neuropsychological aspects ......................................................52
   vi) Tumours ..................................................................................53
C. Diagnosis of 21-hydroxylase deficiency .......................................................... 54
   i) Evaluation of ambiguous genitalia ............................................................... 54
   ii) Newborn screening .................................................................................. 55
   iii) Further biochemical evaluation ............................................................. 58
   iv) Molecular genetic analysis ..................................................................... 61
D. Treatment ....................................................................................................... 65
   i) Glucocorticoid replacement ...................................................................... 65
   ii) Mineralocorticoid replacement ................................................................ 67
   iii) Other therapeutic approaches ................................................................ 68
   iv) Corrective surgery .................................................................................. 70
   v) Psychological counselling ........................................................................ 71
E. Limitations of standard replacement therapy .............................................. 72
   i) Control of hyperandrogenism and hypercortisolism ................................. 72
   ii) Hyperresponsiveness of the adrenal glands to ACTH ............................ 72
   iii) Resistance to replacement therapy ....................................................... 73
   iv) Redundancy of mechanisms governing ACTH secretion ....................... 73

CHAPTER III: INFLUENCE OF PUBERTY ON CAH CONTROL ........................................... 75
A. Alterations in the endocrine milieu at puberty ............................................ 76
B. Effect of puberty on CAH control ............................................................... 77
   i) Role of GH and IGFs ............................................................................... 77
      (a) Effect of GH and IGF-I on 11β-HSD activity ..................................... 77
      (b) Effect of GH and IGF-I on glomerular filtration rate ................. 80
      (c) Effect of GH on adrenal steroidogenesis .................................... 82
      (d) Effect of IGF-I and IGF-II on adrenal steroidogenesis ............. 83
   ii) Role of sex steroids .............................................................................. 85
      (a) Effect of sex steroids on 11β-HSD activity ................................... 85
      (b) Effect of sex steroids on corticosteroid binding globulin ........... 86
   iii) Role of insulin ..................................................................................... 88
      (a) Effect of insulin on IGFBP-1 ............................................................ 88
      (b) Effect of insulin on adrenal steroidogenesis ............................... 89
      (c) Effect of insulin on adrenal androgen production ...................... 92
C. Summary ...................................................................................................... 94
METHODOLOGY ............................................................................................................96

A. Population ..............................................................................................................97

i) Inclusion criteria .................................................................................................100

ii) Exclusion criteria ...............................................................................................100

iii) Ethical considerations .......................................................................................100

B. Methods ..................................................................................................................102

C. Studies ....................................................................................................................106

STUDY 1: PHARMACOKINETIC PARAMETERS OF TOTAL AND FREE CORTISOL ..............................................................................................................107

STUDY 2: CORTISOL PHARMACOKINETICS AND PUBERTY ......................................110

STUDY 3: THE BIOAVAILABILITY OF ORAL HYDROCORTISONE .........................112

STUDY 4: SERUM CORTISOL AND 17-OHP INTERRELATIONSHIP .......................116

STUDY 5: TESTING ALTERATIONS IN FEEDBACK MECHANISMS .......................121

STUDY 6: SERUM CORTISOL – GH INTERRELATIONSHIP .....................................123

D. Assays ....................................................................................................................125

i) Cortisol assay ........................................................................................................125

ii) CBG assay ............................................................................................................125

iii) Free cortisol estimation ......................................................................................125

iv) GH assay .............................................................................................................126

v) 17-OHP assay .......................................................................................................126

vi) Androstendione assay .......................................................................................126

vii) Testosterone assay ............................................................................................127

viii) Insulin assay .......................................................................................................127

ix) Plasma glucose assay .........................................................................................127

x) Leptin assay ..........................................................................................................127

xi) ACTH assay .........................................................................................................128

xii) IGF-I assay .........................................................................................................128

xiii) IGF-II assay .......................................................................................................128

xiv) IGFBP-3 assay ....................................................................................................128
E. Pharmacokinetics .................................................................130
  i) Clearance ...........................................................................130
  ii) Volume of distribution ...................................................131
  iii) Half life ..........................................................................132
  iv) Bioavailability ...............................................................133

F. Statistical analyses ............................................................135
  i) Unpaired t-test .................................................................135
  ii) One-way analysis of variance ..........................................135
  iii) Linear regression ..........................................................136
  iv) Stepwise multiple linear regression analysis ...................137

G. Time series analysis methods .............................................138
  Cross-correlation ...............................................................139

RESULTS ..................................................................................141

STUDY 1: PHARMACOKINETIC PARAMETERS OF TOTAL AND FREE
CORTISOL ..................................................................................142
STUDY 2: CORTISOL PHARMACOKINETICS AND PUBERTY ...........157
STUDY 3: THE BIOAVAILABILITY OF ORAL HYDROCORTISONE ....163
STUDY 4: SERUM CORTISOL AND 17-OHP INTERRELATIONSHIP ......166
STUDY 5: TESTING ALTERATIONS IN FEEDBACK MECHANISMS ......175
STUDY 6: SERUM CORTISOL – GH INTERRELATIONSHIP ..............178

Summary ....................................................................................182
LIST OF FIGURES

INTRODUCTION

CHAPTER I: ADRENAL STEROIDOGENESIS & GLUCOCORTICOID ACTION

Figure I1: Pathways of adrenal steroidogenesis .................................................................21
Figure I2: Pathway of biosynthesis of cortisol .................................................................23
Figure I3: Acute effects of ACTH on cholesterol metabolism in the zona fasciculata of the adrenal cortex .................................................................................................26
Figure I4: Feedback mechanisms regulating cortisol secretion .............................................28
Figure I5: Plasma ACTH concentrations in a normal subject ................................................29
Figure I6: ACTH- and non-ACTH-mediated regulation of the adrenal cortex: neural and immune inputs ..............................................................................................................31
Figure I7: Functional map of a glucocorticoid receptor (GR) ..............................................36
Figure I8: Model of GR activation and ATP-dependent cycling ............................................37

CHAPTER II: 21-HYDROXYLASE DEFICIENCY: AN OVERVIEW

Figure II1: Schematic representation of the clinical spectrum of 21- hydroxylase deficiency .................................................................................................................................45
Figure II2: Normal and abnormal differentiation of the urogenital sinus and external genitalia (Prader scale) ..............................................................................................................48
Figure II3: Simplified flowchart for initial evaluation of ambiguous genitalia .................56
Figure II4: Nomogram for comparing 17-OHP concentrations before and 60 min after a 250 µg of ACTH in subjects with or without 21-hydroxylase deficiency .................60
Figure II5: A: CYP21B and CYP21A gene mapping with the HLA complex. B. 21B gene structure and reported mutations in the CYP21B gene from alleles of patients from five populations with 21-hydroxylase deficiency CAH ........................................62
METHODOLOGY

Figure: Schematic representation of the investigation protocol .....................104

RESULTS

Figure 1: Serum total cortisol concentrations achieved following intravenous administration of hydrocortisone in a dose of 15 mg/m² body SA in a prepubertal patient .......................................................................................................................................144

Figure 2a: Total cortisol clearance in all patients with classical 21-hydroxylase deficiency ...........................................................................................................................................145

Figure 2b: Volume of distribution of total cortisol in all patients with classical 21-hydroxylase deficiency ...........................................................................................................................................147

Figure 2c: Half life of total cortisol ................................................................148

Figure 3: Serum free cortisol concentrations achieved following intravenous administration of hydrocortisone in a dose of 15 mg/m² body SA in a prepubertal patient .......................................................................................................................................151

Figure 4a: Free cortisol clearance in all patients with classical 21-hydroxylase deficiency ...........................................................................................................................................152

Figure 4b: Volume of distribution of free cortisol ...........................................................................................................................................153

Figure 4c: Half life of free cortisol ................................................................154

Figure 5: Half life of free cortisol in male and female patients ...........................................................................................................................................156

Figure 6: Linear correlation between total cortisol clearance corrected for body mass index (CL/BMI) and (a) IGF-I concentrations and (b) GH concentrations ..........159

Figure 7: Linear correlation between total cortisol half life and the (THF + allo-THF)/THE ratio ...........................................................................................................................................161

Figure 8: Median serum total cortisol concentrations following oral and intravenous administration of hydrocortisone in the morning ...........................................................................................................................................165

10
Figure 9: Mean serum (a) cortisol and (b) 17-OHP concentrations in adequately controlled patients .................................................................168

Figure 10: Mean serum (a) cortisol and (b) 17-OHP concentrations in inadequately controlled patients .................................................................170

Figure 11: Cortisol ~ 17-OHP interrelationship in inadequately controlled CYP21 deficient patients .................................................................171

Figure 12: Cross-correlation analyses between cortisol and 17-OHP values in the inadequately controlled patients (a) over a 24h period, (b) during daytime and (c) during night-time .........................................................................172-3

Figure 13a: 0800h ACTH concentrations in all patients with classical 21-hydroxylase deficiency before and after overnight suppression with two different doses of dexamethasone administered following randomisation.................................176

Figure 13b: 0800h ACTH concentrations following single dose dexamethasone suppression in adequately controlled patients .........................................................176

Figure 13c: 0800h ACTH concentrations following single dose dexamethasone suppression in inadequately controlled patients .........................................................177

Figure 14: Cross-correlation analyses between cortisol and GH values in (a) all patients participated in the study, (b) adequately controlled patients and (c) inadequately controlled patients .................................................................179-80

Figure 15: Cross-correlation analyses between cortisol and GH values in (a) males and (b) females with CYP21 deficiency ...........................................................................181
LIST OF TABLES

METHODOLOGY

Table 1: Clinical characteristics of CYP21 deficient patients .........................98
Table 2: Replacement therapy regimens in CYP21 deficient patients ..................99
Table 3: Clinical characteristics of patients participated in study 3 .....................113
Table 4: Clinical characteristics of patients participated in study 4 ......................118
Table 5: Replacement therapy regimens in patients participated in study 4 ..........119

RESULTS

Table 1: Pharmacokinetic parameters of total and free cortisol .......................143
Table 2: Biochemical parameters in the three groups of patients .....................158
Table 3: Stepwise multiple linear regression model for independent predictors of total and free cortisol CL/BMI .................................................................160
Table 4: Stepwise multiple linear regression model for independent predictors of total and free cortisol half life .................................................................162
Table 5: Pharmacokinetic parameters of oral and intravenous hydrocortisone ......164
Table 6: Biochemical parameters in patients participated in study 4 ....................167
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>ADH</td>
<td>Antidiuretic hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activating protein – 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the drug concentration versus time curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>C</td>
<td>Concentration</td>
</tr>
<tr>
<td>CAH</td>
<td>Congenital adrenal hyperplasia</td>
</tr>
<tr>
<td>CAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>C4</td>
<td>Fourth component of serum complement</td>
</tr>
<tr>
<td>CBG</td>
<td>Corticosteroid binding globulin</td>
</tr>
<tr>
<td>CL</td>
<td>Clearance</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin releasing hormone</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>CYP21</td>
<td>21-hydroxylase</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHEAS</td>
<td>Dehydroepiandrosterone sulphate</td>
</tr>
<tr>
<td>DOC</td>
<td>Deoxycorticosterone</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Em</td>
<td>Cortisone metabolites</td>
</tr>
<tr>
<td>F</td>
<td>Bioavailability</td>
</tr>
<tr>
<td>FBC</td>
<td>Full blood count</td>
</tr>
<tr>
<td>FE</td>
<td>Free cortisone</td>
</tr>
<tr>
<td>FF</td>
<td>Free cortisol</td>
</tr>
<tr>
<td>Fm</td>
<td>Cortisol metabolites</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
</tbody>
</table>
GFR  Glomerular filtration rate
GH   Growth hormone
GHRH Growth hormone releasing hormone
GnRH Gonadotrophin releasing hormone
GR   Glucocorticoid receptor
GREs Glucocorticoid response elements
h    Hour
HPA  Hypothalamic-pituitary-adrenal axis
HSP  Heat shock protein
3β-HSD 3β-hydroxysteroid dehydrogenase
11β-HSD 11β-hydroxysteroid dehydrogenase
IGF  Insulin-like growth factor
IGFBP Insulin-like growth factor-binding protein
IL   Interleukin
iv   Intravenous
k    Elimination rate constant
kDa  Kilodalton
kg   Kilogram
L    Litre/es
LDL  Low density lipoprotein
LH   Luteinising hormone
μg   Microgram
mg   Milligram
min  Minutes
ml   Millilitre
mU   Milliunit
MHC  Major histocompatibility complex
MRI  Magnetic resonance imaging
MSH  Melanocyte stimulating hormone
ng   Nanogram
NS   Not significant
Nt   Nucleotide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-OHP</td>
<td>17-Hydroxyprogesterone</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase-activating polypeptide</td>
</tr>
<tr>
<td>PCO</td>
<td>Polycystic ovary/ies</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>17-PGN</td>
<td>17-hydroxyprogrenolone</td>
</tr>
<tr>
<td>POMC</td>
<td>Propiomelanocortin</td>
</tr>
<tr>
<td>PRA</td>
<td>Plasma renin activity</td>
</tr>
<tr>
<td>PVN</td>
<td>Periventricular nucleus</td>
</tr>
<tr>
<td>RPF</td>
<td>Renal plasma flow</td>
</tr>
<tr>
<td>rhGH</td>
<td>Recombinant human growth hormone</td>
</tr>
<tr>
<td>SA</td>
<td>Surface area</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex hormone binding globulin</td>
</tr>
<tr>
<td>SNK</td>
<td>Student-Newman-Keuls</td>
</tr>
<tr>
<td>SS</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
</tr>
<tr>
<td>t¹/₂</td>
<td>Half life</td>
</tr>
<tr>
<td>THE</td>
<td>Tetrahydrocortisone</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrocortisol</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>V</td>
<td>Volume of distribution</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal polypeptide</td>
</tr>
<tr>
<td>yrs</td>
<td>Years</td>
</tr>
</tbody>
</table>
INTRODUCTION
Congenital adrenal hyperplasia (CAH) refers to a family of inherited disorders in which defects occur in one of the five enzymatic steps required to synthesise cortisol from cholesterol in the adrenal gland. The impaired cortisol secretion results in increased adrenocorticotropic hormone (ACTH) secretion by the anterior pituitary in an attempt to normalise circulating cortisol concentrations, hyperplasia of the adrenal cortex and accumulation of steroid precursors prior to the enzymatic defect. In 21-hydroxylation deficiency, which is responsible for 90–95% of all CAH cases, there is accumulation of the precursors immediately proximal to the 21-hydroxylation step and increased production of androgens for which 21-hydroxylation is not necessary (New MI, 1994; New MI et al., 1999; White PC et al., 2000).

The earliest documented description of CAH was in 1865 by a Neapolitan anatomist, Luigi de Crecchio, in which he described a cadaver as having a penis with the urethral opening on the ventral surface and undescended testes (De Crecchio L, 1865). To the surprise of De Crecchio, the post mortem dissection also revealed a vagina, a uterus, fallopian tubes and ovaries as well as markedly enlarged adrenal glands. The patient had had sex reassignment, having been declared a female at birth and a male four years later. As an adult, he conducted himself as a male socially and sexually. He died in his 40s after the last of several episodes of vomiting, diarrhoea and prostration. This was almost certainly a case of a genetic female, with masculinisation of the external genitalia caused by androgen excess, who had symptoms consistent with adrenal insufficiency. Early in this century, a number of other investigators reported similar
cases involving precocious puberty, hirsutism, pseudohermaphroditism and obesity (Fibiger J, 1905; Apert A, 1910). The term 'androgenital syndrome' was used for many years to describe conditions characterised by elevated adrenal androgens due to either virilizing adrenal tumours or CAH.

Despite this first description of CAH 135 years ago, a more thorough understanding was not forthcoming for almost another century, when the recessive nature of the genetic trait and identification of hormonal abnormalities were recognised (Bongiovanni AM et al., 1963). Medical therapy was also not instituted until the mid-20th century, when Wilkins et al. and Bartter et al. showed that the elevated 17-ketosteroids in children with CAH could be dramatically reduced by suppressing the adrenal cortex with exogenous administration of glucocorticoids (Wilkins L et al., 1950; Bartter FC et al., 1950). At the time, this demonstration gave rise to a universally shared optimism that replacement therapy with a glucocorticoid, and if necessary, a mineralocorticoid hormone would be a simple and effective treatment for this condition. After half a century, however, although there is no doubt that the medical management of CAH has saved the lives of many patients and spared most from progressive virilization, in many cases management is still far from optimal and calls for further advances and new therapeutic approaches.
Chapter I

Adrenal Steroidogenesis
& Glucocorticoid Action
A. Pathways of steroid biosynthesis

The glucocorticoid, cortisol and the mineralocorticoid, aldosterone, are synthesised by the adrenal cortex under the control of regulatory systems that largely function independently. The cortex is divided into three distinct zones – the outer zona glomerulosa, the middle zona fasciculata and the inner zona reticularis – which are defined by different cellular arrangements. These three zones are functionally distinct: mineralocorticoids are synthesised in the zona glomerulosa, glucocorticoids in the zona fasciculata and androgens are synthesised in the zona reticularis.

All steroid hormones produced by the adrenocortical tissue are derived from cholesterol. Low density lipoprotein (LDL)-cholesterol is the major source of cholesterol for adrenal steroidogenesis. Proteolytic and lipolytic enzymes act on LDL to release cholesterol esters for storage in lipid droplets in the adrenal cells (Carr BR et al., 1984; Boggaram V et al., 1985). In order for the adrenal cortex to synthesise active steroid hormones, a number of changes are required in the structure of cholesterol. Several of these reactions are catalysed by the steroid hydroxylases, which are members of a superfamily of genes known collectively as cytochrome P450 (CYP). Adrenal steroidogenesis follows three distinct routes, which reflect the zonal differences in function and regulation (Figure I.1).

The rate-limiting step in steroid biosynthesis is importation of cholesterol from cellular stores to the matrix side of the mitochondria-inner membrane, where the cholesterol side chain cleavage system is located. This is controlled by the steroidogenic acute regulatory (StAR) protein, the synthesis of which is increased within minutes by trophic
CHOLESTEROL

StAR protein

\[ \text{17α-hydroxylase}^* \quad \text{17,20-lyase} \quad \text{17β-HSD} \]

\[ \text{Pregnenolone} \rightarrow \text{17-OH Pregnenolone} \rightarrow \text{DHEA} \rightarrow \text{Androstenediol} \]

\[ \text{3β-HSD}^* \quad \text{3β-HSD}^* \quad \text{3β-HSD}^* \quad \text{3β-HSD}^* \]

\[ \text{Progesterone} \rightarrow \text{17-OH Progesterone} \rightarrow \text{A} \rightarrow \text{Testosterone} \]

\[ \text{21-hydroxylase}^* \quad \text{21-hydroxylase}^* \quad \text{Aromatase} \quad \text{Aromatase} \]

\[ \text{Deoxycorticosterone} \rightarrow \text{11-deoxycortisol} \quad \text{17β-HSD} \quad \text{Oestrone} \rightarrow \text{Oestradiol} \]

\[ \text{11β-hydroxylase}^* \quad \text{11β-hydroxylase}^* \]

\[ \text{Corticosterone} \rightarrow \text{Cortisol} \]

\[ \text{18-hydroxylase} \]

\[ \text{18-OH Corticosterone} \]

\[ \text{18-OH dehydrogenase} \]

\[ \text{Aldosterone} \]

**Figure 1.1. Adrenal steroidogenesis. Solid line: major pathway. Dotted line: major pathway in ovaries and minor in adrenals. *: deficient enzymatic activity results in CAH. StAR: steroidogenic regulatory protein. SCC: cholesterol side-chain cleavage enzyme. 3β-HSD: 3β-hydroxysteroid dehydrogenase. 17β-HSD: 17β-hydroxysteroid dehydrogenase. DHEA: dehydroepiandrosterone. A: androstenedione.**
stimuli such as ACTH (Stocco DM et al., 1996; Arakane F et al., 1998). The mechanism by which StAR mediates cholesterol transport across the mitochondrial membrane is not yet known. In addition to StAR protein, cholesterol transfer is also mediated by another protein that appears necessary for this process, the so-called peripheral benzodiazepine receptor (Papadopoulos V, 1998). This protein does not appear to be directly regulated by trophic stimuli but it is stimulated by endozepines, peptide hormones also called diazepam-binding inhibitors. Endozepins may be regulated by ACTH to some extent. Thus far, it is not yet clear whether there is a direct interaction between StAR and the peripheral benzodiazepine receptor.

The first enzymatic step in steroid biosynthesis common to all steroidogenic pathways takes place in the mitochondrion and leads to the cleavage of six carbon atoms from the side chain of cholesterol, converting this C_{27} compound to the C_{21} steroid pregnenolone. This reaction is known as cholesterol side-chain cleavage and is catalysed by the cytochrome P450 enzyme CYP11A (P450scc, cholesterol desmolase, side-chain cleavage enzyme), which is an integral protein of the inner mitochondrial membrane (Nebert DW et al., 1991). Pregnenolone, the common precursor for all other steroids, then presumably passes by diffusion from the mitochondrion to the endoplasmic reticulum where it undergoes further metabolism by several other enzymes (Figure I.2).

To synthesise mineralocorticoids in the zona glomerulosa, 3β-hydroxysteroid dehydrogenase (3β-HSD) in the endoplasmic reticulum and mitochondria converts pregnenolone to progesterone (Cherradi N et al., 1997). This is 21-hydroxylated in the endoplasmic reticulum by CYP21 (P450c21, 21-hydroxylase) to produce deoxycorticosterone (DOC). Aldosterone, the most potent 17-deoxysteroid with
mineralocorticoid activity, is produced by the 11β-hydroxylation of DOC to corticosterone, followed by 18-hydroxylation and 18-oxidation of corticosterone (Figure 1.1). The final three steps in aldosterone synthesis are accomplished by a single mitochondrial P450 enzyme, CYP11B2 (P450aldo, aldosterone synthase) (White PC et al., 1994).

To produce cortisol, the main glucocorticoid in man, CYP17 (P450c17, 17α-hydroxylase/17,20-lyase) in the endoplasmic reticulum of the zona fasciculata and zona reticularis converts pregnenolone to 17α-hydroxypregnenolone (Yanase T et al., 1991). 3β-hydroxysteroid dehydrogenase in the zona fasciculata utilises 17α-hydroxy-pregnenolone as a substrate, producing 17α-hydroxyprogesterone. The latter is 21-hydroxylated by CYP21 to form 11-deoxycortisol, which is further converted to cortisol by CYP11B1 (P450c11, 11β-hydroxylase) in mitochondria (Figure 1.2).

Figure 1.2. Pathway of biosynthesis of cortisol in the adrenal cortex (Reproduced from Simpson ER et al., 1995).
In the zona reticularis of the adrenal cortex and in the gonads, the 17, 20-lyase activity of CYP17 converts 17α-hydroxypregnenolone to dehydroepiandrosterone (DHEA), a C₉ steroid and sex hormone precursor. DHEA is further converted by 3β-HSD to androstendione. In the gonads, this is reduced by 17β-hydroxysteroid dehydrogenase (Penning TM, 1997). In pubertal ovaries, aromatase (CYP19, P450c₁₉) can convert androstendione and testosterone to oestrone and oestradiol respectively (Simpson ER et al., 1994). Testosterone may be further metabolised to dihydrotestosterone by steroid 5α-reductase in androgen target tissues (Wilson JD et al., 1993).

The enzymatic differences between the zona glomerulosa and the zona fasciculata can be summarised by stating that P450c₁₇α is absent from the zona glomerulosa, which additionally has the ability to catalyse the 18-oxidation of corticosterone, the precursor of aldosterone. Interestingly, this activity has been shown to be a property of the 11β-hydroxylase, which appears to be capable of 18-hydroxylation. Human and rat adrenals appear to have two distinct forms of P450c₁₁β, one of which can preferentially catalyse the synthesis of aldosterone (Ogishima T et al., 1989).

B. Regulation of adrenal steroid secretion

i) Cortisol secretion
Glucocorticoid concentrations in blood are regulated in ways that reflect both the varying physiological needs for the hormones under basal and stressed conditions as well as the vulnerability of the organism to damage from overexposure to the hormones. Basal levels follow a circadian rhythm and exert permissive and other actions required
to maintain or normalise functions of many homeostatic mechanisms and protect against severe stress.

Cortisol secretion is primarily regulated by ACTH, a 39-aminoacid peptide that is released by the anterior pituitary. ACTH is synthesised as part of a larger molecular weight precursor peptide, pro-opiomelanocortin (POMC). The POMC precursor peptide is also found in a variety of extrahypothalamic tissues, including the gastrointestinal tract, numerous tumours and the testis. ACTH is secreted in regular pulses of variable amplitude over 24 hours with most activity in the early morning hours (0400 – 0900h), which forms the basis of the circadian rhythm of plasma cortisol concentrations (Wallace WH et al., 1991).

The acute action of ACTH is to increase the flux of cholesterol through the steroidogenic pathway, resulting in rapid production of steroids. Two loci of ACTH action are envisaged (Figure 1.3). One involves the activation of cholesterol ester hydrolase, probably as a result of phosphorylation by way of cyclic adenosine monophosphate (cAMP)-dependent protein kinase. This is sufficient to ensure an increased supply of cholesterol to the mitochondria but it is inadequate to allow increased metabolism of this cholesterol by the mitochondria. In addition, ACTH initiates a sequence of events within the mitochondria, which results in a redistribution of mitochondrial cholesterol, such that more cholesterol binds to the cytochrome P450scc.

ACTH acts through a specific G protein-coupled receptor to increase cAMP levels (Mountjoy KG et al., 1992). cAMP has short term (minutes to hours) effects on chol-
Figure 1.3. Acute effects of ACTH on cholesterol metabolism in the zona fasciculata of the adrenal cortex. The plus signs (+) indicate activation and the minus signs (-) inhibition. LDL, low density lipoproteins; C.E., cholesterol esters; Chol., cholesterol; FFA, free fatty acids; AA, aminoacids; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; pr., protein; ACAT, acetyl CoA (cholesterol acyl transferase); P.G., prostaglandins (Reproduced from Simpson ER et al., 1995).
esterol transport into mitochondria (Stocco DM et al., 1996) but longer term (hours to
days) effects on transcription of genes encoding the enzymes required to synthesise
cortisol (Waterman MR et al., 1997). The transcriptional effects occur, at least in part,
through increased activity of protein kinase A but it is not known whether the targets of
this kinase act directly or indirectly on CYP21. ACTH also influences the remaining
steps of steroidogenesis as well as the uptake of cholesterol from plasma lipoproteins,
thus ensuring a continuous supply of cholesterol to the mitochondria to meet the
demands of activated pregnenolone biosynthesis. It also maintains the size of the
adrenal glands, it stimulates melanocytes and results in hyperpigmentation when
secreted in excess, as occurs in Addison’s disease.

Corticotrophin releasing hormone (CRH) is the principal hypothalamic factor that
stimulates the pituitary production of ACTH (Orth DN, 1995; Itoi K et al., 1998)
(Figure 1.4). It is produced in the paraventricular nuclei (PVN) of the hypothalamus and
is also found in other parts of the central nervous system and in non central locations,
such as peripheral leukocytes. Paracrine action of hypothalamic peptides, e.g.,
vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating
polypeptide (PACAP), plays a role in CRH release (Nussdorfer GG et al., 1998).
Hypothalamic CRH is transported to the anterior pituitary cells by the hypophyseal
portal vessels and activates ACTH secretion via a specific receptor coupled to cAMP-
dependent signalling. CRH is secreted in a pulsatile fashion that results in the episodic
secretion of ACTH and the circadian variation of cortisol secretion (Figures 1.4 & 1.5).
The magnitude of the cortisol response to each ACTH burst remains relatively constant.
It is therefore the number of secretory periods, rather than the magnitude of each pulse
Figure I.4. Feedback mechanisms regulating cortisol secretion from the adrenal cortex (Reproduced from Honour JW, 1993).
Figure 1.5. Plasma ACTH concentrations at 15 min intervals over 24h in a normal subject (Reproduced from Honour JW, 1993).

of CRH and ACTH, that determines the total daily cortisol secretion. In addition to CRH, vasopressin, a peptide product of the posterior pituitary gland, also stimulates ACTH release by acting synergistically with CRH and is an important physiological regulator of ACTH (Scott LV et al., 1998). Whereas CRH increases the amount of ACTH secreted from each responsive corticotroph, vasopressin appears to increase the number of CRH-responsive corticotrophs (Canny BJ et al., 1992; Schwartz J et al., 1992).
Numerous factors, such as metabolic, physical or emotional stress, influence levels of glucocorticoid secretion, mediated by ACTH secreted in response to hypothalamic secretion of CRH and vasopressin. In addition to ACTH-mediated regulation of the adrenal cortex, however, there is considerable and compelling evidence to suggest that other factors may also play an important role in adrenal responses to a number of stimuli. The regulation and central interactions of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system and immune system have been extensively studied (Reichlin S, 1993; Besedovsky HO et al., 1996), and in many clinical situations a dissociation between plasma ACTH concentrations and cortisol secretion has been described, which can not be explained by the kinetics of these hormones (Ehrhart-Bornstein M et al, 1998; Bornstein SR et al., 1999) (Figure I.6).

The paracrine action of various peptides may contribute to modulation of hormone production in the hypothalamus, pituitary and adrenal. Cortisol is the primary negative regulator of resting activity of the HPA axis through negative feedback on ACTH and CRH secretion (Keller-Wood ME et al., 1984) (Figure I.4). Furthermore, it may inhibit some of the higher cortical activities that lead to CRH stimulation. The negative feedback effects of cortisol are exerted at the level of both the hypothalamus and the pituitary and are mediated by Type II corticosteroid receptors (Evans RM et al., 1989). Whether and to what extent direct glucocorticoid feedback on the adrenal cortex itself regulates cortisol synthesis is not clear. In vitro studies using rat adrenocortical cells suggest that corticosterone may act to inhibit steroidogenesis (Carsia RV et al., 1983). Northern blotting demonstrates that glucocorticoid receptors are expressed in human adrenals (Reincke M et al., 1998) but a physiological role in direct negative regulation of cortisol secretion has not been demonstrated.
Figure 1.6: ACTH- and non-ACTH-mediated regulation of the adrenal cortex: neural and immune inputs (Reproduced from Bornstein SR et al., 1999).
ii) Aldosterone secretion

The rate of aldosterone synthesis, which is usually 100- to 1000-fold less than that of cortisol synthesis, is regulated mainly by the renin-angiotensin system and potassium concentrations, with ACTH having only short term effects (Rainey WE et al., 1998).

Renin is a proteolytic enzyme secreted by the juxtaglomerular apparatus of the kidney in response to low sodium concentrations or decreased intravascular volume. The substrate for renin is a plasma $\alpha_2$-globulin called angiotensinogen. Angiotensinogen is secreted by the liver and cleaved by renin to yield a decapeptide product, angiotensin I. Angiotensin I is, in turn, acted upon by a converting enzyme found mainly in the lungs, which cleaves the terminal leucine and histidine to yield the octapeptide angiotensin II. Angiotensin II occupies a G protein-coupled receptor-activating phospholipase C (Matsusaka T et al., 1997). The latter protein hydrolyses phosphatidylinositol biphosphate to produce inositol triphosphate and diacylglycerol, which raise intracellular calcium levels and activate protein kinase C and calmodulin dependent protein kinases. Similarly, increased levels of extracellular potassium depolarise the cell membrane and increase calcium influx through voltage-gated L-type calcium channels. Phosphorylation of as yet unidentified factor by CaM kinases increases transcription of aldosterone synthase (CYP11B2) enzyme required for aldosterone synthesis (Barrett PQ et al., 1989).

The pituitary is also involved in the regulation of aldosterone secretion and hypophysectomised subjects show diminished responses to sodium depletion. Infused ACTH causes a rise in circulating aldosterone concentrations by acting directly on the glomerulosa cells. However, since the increase in ACTH concentrations is invariably
associated with a concomitant increase in glucocorticoid secretion, the physiological importance of ACTH in the regulation of aldosterone secretion remains unclear.

Several other factors have been found to stimulate aldosterone secretion and zona glomerulosa function under experimental conditions but the physiological importance of their function remains to be clarified. These include serotonin, catecholamines, acetylcholine, vasoactive intestinal peptide, vasopressin and prostaglandin E. Inhibitors of aldosterone synthesis include atrial natriuretic peptides and dopamine (Mortensen RM et al., 1995).

### iii) Adrenal androgen secretion

Of the control mechanisms for the three classes of steroids secreted by the adrenal cortex, the mechanism for modulation of glucocorticoid secretion is the best understood. For adrenal androgens, ACTH is an important controlling factor under certain circumstances. At present, however, there is lack of general agreement on what constitutes a specific stimulus for adrenal androgen secretion.

Although it is generally accepted that ACTH is a regulating factor for adrenal androgen secretion under certain physiological and pharmacological conditions and plays a permissive role in the modulation of their secretion, there are additional mechanisms that act either alone or in conjunction with ACTH. Evidence to support this concept comes in part from observations made in a number of physiological or pathological situations in which there is a divergence of adrenal androgen and ACTH secretion as measured directly or as reflected by cortisol concentrations (Parker LN, 1995). One of the most striking examples of this dissociation occurs during adrenarche, when several
years before any increase in gonadotrophin secretion, adrenal androgen plasma concentrations and urinary excretion begin to rise. This occurs while cortisol concentrations, secretion rates and urinary excretion remain constant. Other examples of similar dissociation include obesity, anorexia nervosa, stress and critical illness.

In addition to ACTH, other regulators of adrenal androgen secretion have been suggested and those may be intra- or extra-adrenal. Intra-adrenal factors that have been investigated in animal studies include autocrine effects of locally produced steroids on steroidogenic enzyme activities, possibly via adrenal steroid receptors (Provencher PH et al., 1992; Hirst JJ et al., 1992). Extra-adrenal factors include primarily known hormones and growth factors. Insulin-like growth factor-I (IGF-I) has been found to stimulate both DHEA and cortisol secretion in human adrenal cells (Pham-Huu-Trung MT et al., 1991). The relationship between insulin and adrenal androgens is complex and may be influenced by additional factors, such as insulin or IGF-I receptor status, post-receptor mechanisms and ACTH concentrations (Poretsky L, 1991). Epidermal growth factor (EGF) has been shown to stimulate DHEA sulphate (DHEAS) secretion by human fetal adrenal cells but its physiological role is unclear, since its concentrations decrease in children beyond the age of 2 years. POMC derivatives, such as β-lipoprotein and γ-3 melanocyte-stimulating hormone (MSH), have also been studied with respect to their influence on adrenal androgen secretion. Concentrations of β-lipoprotein and β-endorphin have been reported to increase during adrenarche and puberty (Genazzani AR et al., 1983). It is of interest that testosterone regulates brain POMC gene expression, and DHEA has been shown to inhibit hypothalamic β-endorphin release, suggesting an adrenal androgen-POMC negative feedback loop. The possibility of multiple regulatory
controls exists, which would involve different mechanisms of adrenal androgen modulation (Parker LN, 1995).

C. Molecular mechanisms of glucocorticoid action

Glucocorticoids modulate a large number of metabolic, cardiovascular, immune and behavioural functions and influence most cells. They mainly initiate their effects by binding to intracellular receptors that regulate transcription of particular genes in a cell-specific manner. Glucocorticoid receptors, originally identified in thymocytes, are found in almost all nucleated cells.

i) The glucocorticoid receptor (GR)

At the cellular level, most known effects of glucocorticoids are mediated by a ~94kDa intracellular protein, the glucocorticoid receptor (GR). GR belongs to the phylogenetically conserved superfamily of nuclear hormone receptors, which includes receptors for mineralocorticoids, androgens, progestins, oestrogens, vitamin D, thyroid hormone, retinoic acid and a growing number of so-called ‘orphan’ nuclear receptors for which no specific ligand has yet been identified. In the hormone-bound state, these receptors specifically bind to and modulate the activity of target gene promoters and are, therefore, also known as ligand-dependent transcription factors (Bamberger CM et al., 1996).

Structure of GR: All members of the nuclear hormone receptor family share a characteristic three domain structure that was first described for the GR: The N-terminal
domain, which contains sequences responsible for activation of target genes, the DNA-binding domain, which also participates in receptor dimerisation, nuclear translocation and transactivation, and the C-terminal or ligand-binding domain, which has a more complex role (Figure 1.7). In addition to specifically binding the hormonal ligand, it contains sequences important for heat shock protein (hsp) binding, nuclear translocation, dimerisation, transactivation and silencing of the receptor in the absence of hormone.

**DOMAINS:**

<table>
<thead>
<tr>
<th>Amino-Terminal</th>
<th>DNA binding</th>
<th>Hormone binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>440</td>
<td>505</td>
</tr>
</tbody>
</table>

*Transactivation*  

*Hsp90 binding*  

*Nuclear localisation*  

*Dimerisation*  

**Figure 1.7:** Functional map of a glucocorticoid receptor (GR) based on composite information from studies of rat, mouse and human GR's. Three domains have been identified by partial proteolysis and found to correspond to the amino-terminal transcriptional transactivational domain, the DNA-binding domain and the hormone-binding domain. Site-directed mutagenesis and deletion mapping of the approximately 800 amino acid GR has revealed sequences specifying transactivation, dimerisation, nuclear localisation and hsp 90 interactions (Reproduced from Bodwell JE et al., 1996).
**GR-mediated transcription regulation**: Our understanding of how the GR is transformed from a silent to an active transcription factor can be described by the following model (Figure 1.8): The unliganded GR is part of a multiprotein complex that consists of the receptor, two molecules of hsp90, one molecule of hsp70, one molecule of hsp56 and often other less well characterised proteins. When glucocorticoids cross the cell membrane, they interact with the intracellular GR and induce a number of functional consequences: the hormone-bound GR is transformed to the activated hormone-bound GR complex after dissociation from the hsp complex. The activated complex then binds reversibly to the nucleus to give the nuclear bound form.

![Figure 1.8. Model of GR activation and ATP-dependent cycling. H: hormone; R: unliganded GR; HR and HR': non-activated and activated hormone-receptor complexes respectively. HR'n: nuclear-bound activated hormone-receptor complex. R': activated unliganded GR, which cannot bind hormone. Hsp90: 90 kDa heat shock protein. Other receptor-associated heat shock proteins (Hsp70, Hsp56) are presumed to accompany Hsp90 (Reproduced from Bodwell JE et al., 1996).](image-url)
When the hormone dissociates from either the non-activated or activated hormone-GR complex, the receptor is converted to a short lived unliganded form, which cannot bind hormone. The cycle closes with an adenosine triphosphate (ATP)-dependent reaction that re-associates the activated GR with hsp90 and other components, thus reconstituting the unliganded, oligomeric receptor to which hormone can bind again and initiate a new round of activation (Bodwell JE et al., 1996) (Figure I.8).

Within the nucleus, the hormone-activated GR can act in two ways, referred to as type 1 and type 2 mechanism of action. The type 1 mechanism is characterised by the GR interacting with specific DNA sequences, whereas the type 2 involves interaction of the GR with other transcription factors in the absence of specific DNA binding. The type 1 mechanism represents the classical model of GR action. In this model, a receptor homodimer binds to glucocorticoid response elements (GREs) in the promoter region of glucocorticoid responsive genes and – via interaction with components of the basic transcription machinery – it enhances transcription by RNA polymerase II. On the other hand, many effects of glucocorticoids are achieved by inhibition rather than by activation of target genes. This is especially true for the anti-inflammatory/immunosuppressive effects of glucocorticoids that involve negative transcriptional regulation of immune genes, such as the interleukin-2 (IL-2) genes. It is now well established that these genes are regulated by the type 2 mechanism of GR action (Bamberger CM et al., 1996).

ii) Glucocorticoid responsiveness and sensitivity

The response of a single cell exposed to glucocorticoids is the result of the interplay between the following three parameters: the concentration of free hormone, the relative
potency of the hormone and the ability of the cell to receive and transduce the hormonal signal. The concentration of the free hormone is primarily under the control of HPA axis but is also influenced by the plasma and tissue levels of corticosteroid binding globulin (CBG). The relative potency of any endogenous or exogenous glucocorticoid is influenced by its bioavailability, affinity for GR and ability to retain the GR in the nucleus. Although the third parameter, i.e. the ability of cells to respond to a given concentration of a defined glucocorticoid, has been recognised as a variable, it is still not clear how frequently and to what extent it is involved in pathophysiological processes.

At this point, it is important to introduce the two distinct concepts of glucocorticoid responsiveness and sensitivity. Glucocorticoid responsiveness is defined as the ability of a system to exhibit measurable changes in response to glucocorticoids. In practical terms, this definition refers to situations in which it is possible to establish a dose-response curve. In contrast, glucocorticoid sensitivity refers to the extent to which an a priori glucocorticoid-responsive system responds to glucocorticoids (i.e. to the position, the slope and the plateau level of the dose-response curve).

iii) Molecular determinants of glucocorticoid sensitivity

Factors that influence glucocorticoid sensitivity may be endogenous or exogenous and include the following:

*Intracellular hormone availability:* Glucocorticoids that have diffused through the cell membrane must gain access to the GR to exert their effects. Alterations in the
activity/expression of certain enzymes, for example, 11β-hydroxysteroid dehydrogenase (11β-HSD) could modulate glucocorticoid sensitivity (Whorwood CB et al., 1995).

**GR expression level:** It is well established that hormone-binding capacity, i.e. the level of cellular GR expression, is closely correlated with the magnitude of the GR-mediated response. Among the factors that alter GR expression, glucocorticoids themselves appear to be the most potent regulators and have been shown to cause down-regulation of the receptor in many cell lines and in tissues or cells from intact animals and healthy human subjects (Miller AH et al., 1990; Burnstein KL et al., 1992; Silva CM et al., 1994). This negative feedback of glucocorticoids on GR expression represents a short-loop feedback mechanism protecting tissues from excessive glucocorticoid levels in hypercortisolaemic states. Also, the half life of GR protein may be decreased in the presence of glucocorticoids (McIntyre WR et al., 1985). In addition to glucocorticoids, the effects of other steroids and neurotransmitters add to the complexity of GR transcription and expression.

**Hormone-binding affinity:** The potency of the GR as a transcriptional regulator also correlates with its hormone-binding affinity, which is determined by a number of factors, the most common being point mutations within the carboxy-terminal ligand-binding domain and factors that interfere with the GR/Hsp complex function.

**GR phosphorylation:** Concomitantly with or shortly after dissociation from the hsp complex, the basally phosphorylated GR becomes hyperphosphorylated, mostly on serine residues but it is not clearly established whether phosphorylation of GR alter its activity.
**Nuclear translocation:** The GR/Hsp complex is constantly shuttling between the cytosol and the nucleus of the cells and – depending on the predominant direction – the receptor may appear as a nuclear or a cytoplasmic protein. Nuclear translocation appears to be accelerated in response to hormone, which may unmask a nuclear localisation signal. It has also been demonstrated that heat shock also enhances GR-mediated transcription by further stimulating the hormone-induced nuclear transfer of the receptor (Sanchez ER et al., 1994).

**DNA / GRE binding:** Within the nucleus, the activated GR becomes associated with DNA and with specific GREs or other DNA sequences. A number of proteins, many of which are not well characterised, have been shown to enhance the binding of activated GR complexes to naked DNA in vitro. Stimulation of the cAMP/protein kinase A pathway has been reported to augment transcription in response to glucocorticoids (Reisfeld S et al., 1994). The ability of the hormone-activated GR complex to bind to DNA can also be negatively regulated by a number of factors, such as the active form of vitamin B₆, pyridoxical phosphate.

**Interaction with other nuclear factors:** The last step of GR-mediated transactivation involves complex interactions of the receptor with components of the basal transcription machinery and/or with other transcriptional activators or repressors (Bamberger CM et al., 1996). Synergism with GR has been demonstrated for the activating protein-1, a transcription factor, and many DNA-binding proteins, such as the activated thyroid hormone receptor and the GR homodimer itself.
From the above it is inferred that each step in the chain of events leading to activation of GR can be subject to modulating influences that either decrease or increase the glucocorticoid sensitivity of the responsive tissue and hence the activity of the HPA axis.
Chapter II

21-Hydroxylase Deficiency:
An Overview
A. Clinical spectrum

The hormonal imbalances in CAH result from the combination of impaired enzymatic activity and subsequent impaired cortisol synthesis. Clinical syndromes reflect the resultant increased concentrations of steroids proximal to the non-functioning enzymatic step and hyperstimulation of the adrenal gland by ACTH. In 21-hydroxylase deficiency, the conversion of 17α-hydroxyprogesterone (17-OHP), the main substrate of the 21-hydroxylase enzyme, to 11-deoxycortisol in the pathway of cortisol synthesis is impaired (Figure I.1). The enzymatic defect also impairs the conversion of progesterone to 11-deoxycorticosterone in the pathway of aldosterone biosynthesis. The decreased production of cortisol results in increased secretion of ACTH by the anterior pituitary and accumulation of precursors prior to the enzymatic block, i.e. 17-OHP, pregnenolone, 17-hydroxyprogrenolone and progesterone. These steroid precursors can serve as substrates for androgen biosynthesis and are diverted in the adrenals to androgen pathways, resulting in excess secretion of DHEA, Δ⁴-Androstendione and testosterone. In classical CAH, the production of these androgens early in gestation results in virilization of the external genitalia in the genetic female fetus (New MI, 1994; New MI et al., 1999; White PC et al., 2000).

The clinical spectrum of CAH due to 21-hydroxylase deficiency ranges from most severe to moderately severe to mild forms reflecting varying degrees of 21-hydroxylase deficiency (Figure II.1). Depending on the degree of enzymatic activity 21-hydroxylase, CAH can be divided into three main forms: (i) classical salt wasting, (ii) classical simple virilizing and (iii) non-classical, a mild form of the disease.
The most severe classical salt wasting form is characterised by both cortisol and aldosterone deficiency, excess adrenal androgen production from early fetal life and virilization of the external genitalia in affected female infants. It accounts for three-fourths of all classical CAH cases. The aldosterone deficiency results in renal salt
wasting, hyponatraemia, hyperkalaemia, high plasma renin levels and intravascular volume depletion, a constellation that may result in life-threatening hypovolaemic shock. The *classical simple virilizing* form is characterised by progressive virilization with accelerated growth velocity and advanced bone age but no evidence of mineralocorticoid deficiency.

The *non-classical* form of CAH represents an attenuated late-onset form of adrenal hyperplasia, in which partial deficiencies of 21-hydroxylation result in postnatal androgen excess and milder symptoms. The severity of signs and symptoms varies widely and probably many affected individuals are asymptomatic. Females do not have genital ambiguity at birth, though both males and females may manifest signs of androgen excess at any stage of postnatal development. In pubertal-age girls, menarche may be delayed and in adolescent and young women, secondary amenorrhoea is common. In women, hirsutism, male-pattern baldness, oligomenorrhoea or amenorrhoea and/or polycystic ovary syndrome may occur (Kohn B et al., 1982; New MI et al., 1999). In males, oligospermia has been reported in some cases (Augarten A et al., 1991). For both men and women, compromised final height, severe cystic acne and reduced fertility can be seen in untreated groups (White PC et al., 2000).

**B. Clinical manifestations of classical CAH**

*i) Prenatal virilization*

Adrenal secretion of excess androgen precursors does not affect male sexual differentiation significantly. In females affected with CAH, however, the urogenital
sinus is in the process of septation when the fetal adrenal begins to produce excess androgens and levels of circulating adrenal androgens are sufficiently high to prevent formation of separate vaginal and urethral canals. Further interference with normal female genital anatomy occurs as adrenal-derived androgens interact with genital skin androgen receptors and induce clitoral enlargement, promote fusion of the labial folds and cause rostral migration of the urethral/vaginal perineal orifice.

The typical manifestation, therefore, in severely affected females is ambiguous or male-appearing external genitalia with perineal hypospadias, chordee and undescended testes (Grumbach MM et al., 1960). The severity of virilization is often quantitated using a five-point scale developed by Prader (Figures II.2) (Prader A, 1954). The degree of genital ambiguity varies considerably among females with classical CAH. The physical signs of androgen excess in these patients are dependent not only on direct adrenal secretion of androgen precursors but also on the efficiency with which such hormones are converted to more potent products, such as dihydrotestosterone, by peripheral enzymes, such as 5α-reductase (Wilson JD et al., 1993). In addition, the concentration and transcriptional activity of androgen receptors may play a further role in determining genital phenotype (Choong CS et al., 1996; Chamberlain NL et al., 1994).

**ii) Salt wasting**

Three fourths of patients with classical CAH cannot synthesise adequate amounts of aldosterone due to severely impaired 21-hydroxylation of progesterone. Severely affected patients invariably have concomitant cortisol deficiency that exacerbates further the effects of aldosterone deficiency (Funder JW, 1993) and increases the likelihood of hypovolaemic shock and severe hyponatraemia. Furthermore, accumulated
Figure II.2: Upper panel: Normal and abnormal differentiation of the urogenital sinus and external genitalia (cross-sectional view). Lower panel: normal and abnormal differentiation of the external genitalia (external view). Diagrams of normal female and male anatomy flank a series of schematic representations of different degrees of virilization, graded using the scale developed by Prader (Reproduced from White PC et al., 2000).
steroid precursors may antagonise the mineralocorticoid receptor directly and may exacerbate mineralocorticoid deficiency, particularly in untreated patients (Kuhnle U et al., 1986). Progesterone is well known to have antimineralocorticoid effects (Rafestin-Oblin ME et al., 1991; Oelkers WK, 1996) and it is a likely culprit in this phenomenon. However, there is no yet evidence to suggest that 17-OHP has direct or indirect antimineralocorticoid effects.

Salt wasting may manifest with non-specific symptoms, such as poor appetite, vomiting, lethargy and failure to thrive. Severely affected individuals usually present during the first 4 – 6 weeks of life with adrenal crises that are potentially fatal. This problem is particularly critical in male infants who have no genital ambiguity to alert physicians to the possibility of CAH.

***iii) Postnatal signs of androgen excess***

Ongoing adrenal sex steroid production in the untreated or incompletely treated patients results in accelerated somatic growth with advancement of epiphyseal maturation but compromised final height compared with the parentally determined target height (Jaaskelainen J et al., 1997; New MI et al., 1988), premature pubarche and apocrine body odour, increase in penile size without testicular enlargement in boys and progressive clitoral enlargement in girls. In adolescence, poorly controlled females manifest acne, hirsutism and ovarian dysfunction. There is considerable variation in signs of androgen excess for reasons similar to those that account for the variation in symptoms and signs of prenatal androgen excess.
iv) Reproductive function

Females: In women with classical CAH, reproductive problems become apparent in adolescence. In inadequately treated patients, menarche occurs later compared to their healthy counterparts (Helleday J et al., 1993). Such patients often have a clinical picture similar to polycystic ovarian syndrome with ultrasonographic evidence of multiple cysts, anovulation, irregular menstrual cycles and hyperandrogenic symptoms (London DR, 1987). Moreover, a significant reduction in insulin sensitivity is found among young women with non-classical CAH as compared with controls of similar age and weight (Speiser PW et al., 1992).

The precise mechanism of the above problems is not yet clear. Several hypotheses have been advanced: a) Hypothalamic aromatisation of excess adrenal androstendione might interfere with LH-releasing hormone secretion (Bonaccorsi AC et al., 1987). b) Excess adrenal progesterone may act as a ‘mini-pill’ to inhibit normal cycles (Helleday J et al., 1993) or it might antagonise oestrogen effects (Helleday J et al., 1993; Holmes-Walker DJ et al., 1995). c) Elevated progestins or sex steroids could induce abnormal ovarian function by programming the hypothalamus early in development (Barnes RB et al., 1994). d) Androgen excess may directly damage the ovaries. e) Adrenal rest tissue might displace normal gonadal parenchyma (White PC et al., 2000).

The majority of women with CAH eventually undergo menarche. In general, the regularity of menstrual cycles depends on the adequacy of treatment. A small proportion of women do not undergo menarche and are unable to suppress progesterone levels, even when 17-OHP is adequately suppressed (Holmes-Walker DJ, et al., 1995). Furthermore, breast development is suppressed in female patients. Evidence from
animal studies suggests that testosterone exposure in utero may also suppress the breast anlage, resulting in poor breast development in adolescence (Imperato-McGinley J et al., 1986).

Female patients are also known to have poor fertility rates and a number of factors may contribute to this. In addition to the above mentioned causes of amenorrhea/oligomenorrhea, it is now well established that: a) Patients with classical CAH, particularly with the salt-wasting form, have a greater degree of genital virilization and an inadequate introitus, even after reconstructive surgery (Qazi QH et al., 1972; Azziz R et al., 1986). b) Many patients with inadequate introitus are heterosexually inactive (Mulaikal RM et al., 1987). c) The psychosexual effects of prenatal and postnatal virilization prevent patients from establishing heterosexual relationships. Many authors quote low rates of marriage, heterosexual activity and sexual experience in general (Mulaikal RM et al., 1987; Doerner G et al., 1991; Kuhnle U et al., 1993; Kuhnle U et al., 1995).

It should be recognised that during pregnancy treatment is directed at the mother and not at the fetus, because both hydrocortisone and prednisone do not effectively cross the placenta. Despite elevated maternal androgen concentrations, unaffected female offspring appear to have no genital virilization (Lo JC et al., 1999). This is mainly due to the fact that placental aromatase effectively prevents maternal androgens from reaching the fetus. In addition, elevated maternal sex hormone-binding globulin (SHBG) (Kerlan V et al., 1994) and androgen antagonism by progesterone (Dorfman RL, 1999) may also restrict transplacental passage of androgens. There is no evidence
of increased prevalence of congenital malformations in the offspring of females with CAH.

**Males:** Men with CAH have impaired gonadal function less frequently than affected females. Most affected males are able to father children or at least have normal sperm counts (Urban MD et al., 1978). Low sperm counts, when they occur, do not always preclude fertility (Prader A et al., 1973). Patients with the simple virilizing form may have normal gonadal function even in the absence of treatment (Willi U et al., 1991).

**v) Neuropsychological aspects**

Psychosexual identification has been the main focus of psychological research and a major area that has been studied in female CAH patients. Psychosexual identification is evaluated by studying gender identity, gender role, sexual orientation and parenting.

Gender identity, the awareness of oneself as male, female or ambiguous, has been uniformly reported to be female in early-treated girls with CAH. Gender role, however, which refers to gender-stereotyped behaviours, has been found by most investigators to be more masculine as suggested by an increased tomboyish behaviour and more outdoor activities in girls or a more career-oriented outlook in young women (Fedderman DD, 1987; Doerner G et al., 1991; Dittman RW et al., 1990). Parenting rehearsal, such as doll play and baby care is decreased, whereas homosexuality in female patients appears to be increased compared to normal females (Doerner G et al., 1991). More recent studies also showed that patients with CAH differ from their healthy counterparts in all aspects of psychosexual identification. Many had a disturbed image, felt less feminine and attractive, had a more passive sexual life, as indicated by the fact that were unable
to establish a partnership or to marry, and had significantly less children (Kulne U et al., 1993; Kulne U et al., 1995).

It is interesting, however, that the overall quality of life in CAH compared to a control population is not reduced. This is consistent with the findings of studies that evaluated various patient populations and showed that there was not a significant decrease in quality of life despite impaired health status (Bullinger M et al., 1991). Accumulating evidence now suggests that these patients develop coping strategies and cognitive appraisals that enable them to accept their life and view it as very satisfactory. These forms of compensatory efforts are well documented in psychological research studies of adaptation (Lazarus B, 1978; Marcus H et al., 1986). Thus female patients with CAH accept their physical handicap and its impact on their female self but do not allow the impairment to interfere with their general quality of life.

**vi) Tumours**

**Adrenal:** The incidence of adrenal masses appears to be higher in CAH patients and in heterozygotes than in the general population (Jaresch S et al., 1992). Histological types of these tumours include adenoma, myelolipoma and haemangioma. Steroid-responsive hyperplastic adrenal nodules can present in previously undiagnosed patients late in life and can potentially be confused with virilizing adrenal adenomas. Since the tumours may regress with glucocorticoid therapy, it may not be necessary to resect them as long as they are followed carefully. Most adrenal masses in children with CAH are benign (Lightner ES et al., 1993).
**Testicular:** Although seen most often in inadequately controlled patients, testicular adrenal rests accompanied by deficient spermatogenesis may occur despite treatment, particularly in males with the salt wasting form of 21-hydroxylase deficiency (Avila NA et al., 1999). The preferred mode of treatment consists of effective adrenal suppression with dexamethasone, since most of these tumours are ACTH responsive. When they do not respond to dexamethasone, testis-sparing surgery may be performed after imaging of the tumour by ultrasonography and/or magnetic resonance imaging (MRI) scan. Testicular masses have been detected in patients as young as 3 years (Srikanth MS et al., 1992), indicating that boys with CAH should undergo a baseline testicular ultrasonogram during childhood and certainly before adolescence.

**Pituitary:** Although glucocorticoid replacement doses exceed physiological cortisol production, CRH and ACTH often are not suppressed fully by treatment, as evidenced by basal and stimulated concentrations after testing with ovine CRH. In one study, four out of seven CAH patients undergone brain MRI scan were found to have pituitary abnormalities (three had microadenomas and one empty sella) (Speiser PW et al., 1995). However, symptomatic pituitary tumours have not been reported.

**C. Diagnosis of 21-hydroxylase deficiency**

**i) Evaluation of ambiguous genitalia**

In the evaluation of ambiguous genitalia, physical examination should identify the urethral meatus and should include careful palpation for gonads in the inguinal canals and labia or scrotum. Standard diagnostic tests include basal serum 17-OHP
concentrations, a rapid karyotype and a pelvic and abdominal ultrasound scan. Also, a complete profile of adrenocortical hormones before and 1 hour after ACTH stimulation is recommended but should be performed beyond the first 24 – 48 hours of life. This will not only confirm the diagnosis but will also exclude other defects of adrenal steroidogenesis. Plasma renin activity (PRA) and aldosterone are elevated in many normal infants and do not usually add much useful information within the first days of life (Grumbach MM et al., 1998) (Figure II.3).

**ii) Newborn screening**

CAH is a condition suitable for newborn screening because it is a potentially fatal childhood disease, it is diagnosed easily by a simple hormonal measurement in blood and its early recognition and treatment can, in principle, prevent serious morbidity and mortality. The diagnosis is suspected when one finds a markedly elevated filter paper blood 17-OHP level by radioimmunoassay (Pang S et al., 1997). Normative values for filter paper assays vary in different laboratories. These assays use the same ‘Guthrie’ cards that are used for screening of phenylketonuria and hypothyroidism. Subsequent measurement of serum 17-OHP is performed to confirm the diagnosis. Ill or stressed newborns tend to have higher concentrations of 17-OHP and may generate many false positive results unless higher normal cut-off values are used. Similar problems with interpretation arise as a result of screening of preterm neonates, who also have elevated 17-OHP concentrations compared to term infants, particularly around the 29th week of gestation, when the function of several adrenal steroidogenic enzymes reaches its nadir (Nomura S, 1997).
Figure II.3. Simplified flowchart for initial evaluation of ambiguous genitalia. Decision points are denoted by circles and endpoints by rectangles. Karyotype should always be performed, although palpation of the gonads and a pelvic ultrasound scan (USS) permit a tentative sex assignment in many cases (Reproduced from White PC et al., 2000).
It is now well established that neonatal screening reduces the time to diagnosis of infants with CAH (Brosnan PG et al., 1999; White PC et al., 2000). The main putative benefit of this is the reduced morbidity and mortality, since infants with the salt wasting form of disease are diagnosed more promptly. As undiagnosed infants who die suddenly may not be ascertained, it is difficult to demonstrate a benefit of screening by direct comparison of death rates from CAH in non-screened and screened populations. However, males with salt wasting CAH are more likely than females to suffer from delayed or incorrect diagnosis because there is no genital ambiguity to alert the clinician. Thus, a relative paucity of salt wasting males in a patient population may be taken as indirect evidence of unreported deaths from salt wasting crises. Indeed, females outnumbered males in some (Thompson R et al., 1989) but not all (Thilen A et al., 1990) retrospective studies in which CAH was diagnosed clinically. By contrast, cases with salt wasting CAH ascertained through screening programmes are equally or more likely to be males rather than females (Balsamo A et al., 1996; Brosnan PG et al., 1999).

As for morbidity, infants ascertained through screening have less severe hyponatraemia and tend to be hospitalised for shorter periods of time (Brosnan PG et al., 1999).

Although salt wasting males would seem to derive the greatest benefit from screening programmes, the delay before correct sex assignment of severely virilized females is also reduced markedly. Moreover, males with simple virilizing disease may otherwise not be diagnosed until rapid growth and accelerated skeletal maturation are detected later in childhood, at which time final height may already be adversely affected. However, it is debatable whether this last benefit itself justifies the costs of a screening programme.
The estimated cost of screening each newborn infant was $2.70 in Sweden. Given the disease incidence in this population (1: 9,800), the cost for each male salt wasting infant expected to be detected by screening is $71,000. The cost of neonatal screening in Texas was higher at $87,000 per CAH case, as separate hormonal assays were performed on each infant at birth and again at 1-2 weeks of age (Therrell BL Jr et al., 1998). Nevertheless, the cost of screening is within the general range estimated for other newborn disease detection programmes.

Much of the expense required for the follow up of positive (including false positive) screening tests could be avoided with a second level of screening based on detection of actual mutations. This could be accomplished on DNA extracted from the same dried blood spots as are used for hormonal screening. Since 90 – 95% of mutant alleles carry one or more of a discrete number of mutations, samples that carry none of these mutations may be presumed with more than 99% confidence to be unaffected. Heterozygous carriers of a mutation for classical 21-hydroxylase deficiency would still need to be followed up due to the chance that the other allele might carry a novel mutation. Two large-scale studies that evaluated the effectiveness of genotyping as part of neonatal screening have shown that this is a useful adjunct to hormonal measurements (Fitness J et al., 1999; Nordenstrom A et al., 1999). The cost was estimated to be $5 per sample analysed. At present, however, there are few laboratories equipped to do rapid, accurate and large-scale CYP21 genotyping.

iii) Further biochemical evaluation

A basal serum or filter paper 17-OHP is not fully informative and it is necessary to evaluate suspected patients further. In cases where there is no neonatal screening
programme but 21-hydroxylase deficiency is suspected on clinical grounds, ACTH stimulation test with measurements of cortisol, 17-OHP and androgens before and 1 hour after exogenous administration of ACTH should be performed after the first 24–48 hours of life (Figure II.4), because there is a high incidence of both false-positive and false-negative results when samples are obtained immediately after birth. Ideally, to fully differentiate between various enzymatic defects of adrenal steroidogenesis measurements should also include DOC, 11-deoxycortisol, 17-hydroxypregnenolone, DHEAS and androstendione both before and 1 hour after stimulation.

Elevated PRA values and particularly the ratio of PRA to 24h urinary aldosterone, are often used as markers of impaired aldosterone synthesis. They can also be increased in patients with normal aldosterone secretion who have high circulating concentrations of ACTH, 17-OHP and progesterone, making poorly controlled simple virilizers biochemically resemble salt wasters. Conversely, mineralocorticoid therapy may aid adrenal suppression in these patients. In interpreting renin levels, it should be kept in mind that they are normally higher in neonates than in older children and age specific reference values vary by laboratory.

Several other diagnostic biochemical assays have been proposed but few are widely available. The main urinary metabolite of 17-OHP, pregnanetriol, can be also used to diagnose 21-hydroxylase deficiency. As an alternative to enzyme-linked immunoassays, urinary steroid metabolites can be analysed by urine gas chromatography/mass spectrometry, in which case several relevant markers for CAH and other disorders of steroid metabolism can be assayed simultaneously (Malunowicz EM et al., 1997).
**Figure 11.4.** Nomogram for comparing 17-OHP concentrations before and 60 min after a 250 µg intravenous bolus of ACTH in subjects with or without 21-hydroxylase deficiency. Note that values for normals and heterozygotes overlap (Reproduced from New MI et al., 1999).
iv) Molecular genetic analysis

21-Hydroxylase deficiency is inherited as a monogenic autosomal recessive trait that is closely linked to the HLA major histocompatibility complex (MHC) located on the short arm of chromosome 6 (Dupont B et al., 1977). The HLA complex is an assembly of genes coding for cell-surface antigens that are the major barriers for allogenic transplantation. HLA-A, HLA-B and HLA-C are structurally related and are referred to as ‘class I’ antigens, while HLA-DR is the main ‘class II’ antigen (Bodmer WF, 1984). The ‘class III’ genes are located between the class I and II antigens. Data on intra-HLA recombination indicated a gene locus for 21-hydroxylase between HLA-B and HLA-DR (Levine LS et al., 1978) (Figure II.5a).

The structural gene encoding the adrenal cytochrome P450 specific for 21-hydroxylation (P450c21) is named \(\text{CYP21}\) or \(\text{CYP21B}\). \(\text{CYP21}\) and its homologue, the pseudogene \(\text{CYP21P}\) or \(\text{CYP21A}\), alternate with two genes called C4B and C4A that encode the two isoforms of the fourth component (C4) of serum complement (White PC et al., 1985; White PC et al., 1986) (Figure II.5a). \(\text{CYP21}\) and \(\text{CYP21P}\), which each contain 10 exons, share 98% sequence homology in exons and approximately 96% sequence homology in introns (White PC et al., 1986). The pseudogene \(\text{CYP21P}\) does not produce mRNA or a protein due to several deleterious mutations.

Most mutations causing 21-hydroxylase deficiency that have been described so far are the result of either of two types of combinations between \(\text{CYP21}\), the normally active gene, and the \(\text{CYP21P}\) pseudogene. These two mechanisms are unequal crossing over during meiosis, resulting in a complete deletion of C4B and a net deletion of \(\text{CYP21}\) (Werkmeister et al., 1986; White PC et al., 1988) or apparent gene conversion events.
Figure 11.5: A: CYP21B and CYP21A gene mapping with the HLA complex. B: 21B gene structure and reported mutations in the CYP21B gene from the alleles of patients from five populations with severe salt wasting (SW), moderately severe simple virilizing (SV) and mild non-classical (NC) spectrum of 21-hydroxylase deficiency CAH. Numbers in the mutant allele boxes indicate codon numbers. Numbers in the shaded boxes indicate exon numbers (Reproduced from Pang S, 1997).
that transfer deleterious mutations normally present in CYP21P to CYP21 (Urabe K et al., 1990).

The deleterious mutations in CYP21P include an A → G substitution 13 nucleotides (nt) before the end of intron 2 (Figure II.5b) that results in aberrant splicing of pre-mRNA, an 8-nt deletion in exon 3 and a 1-nt insertion in exon 7, each of which shifts the reading frame of translation, and a nonsense mutation in codon 318 of exon 8. There are also 8 missense mutations in CYP21P, 7 of which have been observed in patients with 21-hydroxylase deficiency (White PC et al., 2000).

The functional effects of missense mutations have been assessed *in vitro* by recreating them in CYP21 cDNA and expressing the mutant cDNA using an appropriate expression vector. Several systems have been used including transfection of plasmids in mammalian cells, infection of mammalian cells with recombinant vaccinia virus or expression in yeast or bacteria (Lajic S et al., 1997; Nikoshkov A et al., 1997; Nikoshkov A et al., 1998). In general, these systems have yielded similar results regarding the effects of particular mutations on enzymatic activity. The simplest way to compare these studies is to consider whether a particular mutation destroys, drastically reduces or partially reduces the enzymatic activity. A more quantitative way is to express mutant enzymatic activity as a percentage of wild-type activity.

The classification of 21-hydroxylase deficiency into salt wasting, simple virilizing and non-classical forms is a useful way to roughly grade the severity of the disease and to predict the therapeutic interventions that will likely be required. If molecular diagnosis could predict this classification, it would increase the utility of prenatal diagnosis and
neonatal screening and would serve as an additional to ACTH stimulation test
diagnostic tool. The simplest way to correlate genotype and phenotype is to determine
which mutations are characteristically found in each type of 21-hydroxylase deficiency.
This is most informative for frequently occurring mutations. Deletions and large
conversions are most often found in salt wasting patients, the intron 2 nt 656G mutation
is found in both salt wasting and simple virilizing patients, I172N is characteristically
found in simple virilizing patients and V281L and P30L are found in non-classical
patients (Higashi Y et al., 1991; Mornet E et al., 1991). This distribution is consistent
with the compromise in enzymatic activity conferred by each mutation.

However, patients are usually compound heterozygotes for different mutations and so
this approach has little predictive value in itself. A useful analytical strategy is to
consider that, since 21-hydroxylase deficiency is a recessive disease, the phenotype of
each patient is likely to reflect his or her less severely impaired allele. If mutations are
provisionally classified by the degree of enzymatic compromise – severe, moderate or
mild – then one might hypothesise that salt wasting patients would have severe/severe
genotypes, simple virilizing patients would have severe/moderate or moderate/moderate
genotypes and non-classical patients would have severe/mild, moderate/mild or
mild/mild genotypes. In one study of 88 families these three predictions were correct in
90%, 67% and 59% respectively (Speiser PW et al., 1992b). An expanded follow up
study of the same population showed even better results, with 88% of patients being
correctly classified in this manner (Wilson RC et al., 1995). Similar results were
obtained in other studies using the same approach (Wedell A et al., 1994; Jaaskelainen J
et al., 1997).
Since the distinction between simple virilizing and non-classical forms is very difficult in males, attempts have been made to correlate genotype with quantitative measures of disease severity, such as basal and ACTH-stimulated 17-OHP concentrations, PRA to urinary aldosterone ratio and Prader genital virilization scores. In general, these are no better correlated with genotype than the broader clinical categories. There is excellent discrimination between severe and mild genotypes but a high degree of overlap between moderate genotypes and those either more or less affected (Speiser PW et al., 1992b).

D. Treatment

i) Glucocorticoid replacement

Patients with classical 21-hydroxylase deficiency and symptomatic patients with non-classical disease are treated with glucocorticoids in an attempt to suppress the excessive secretion of CRH and ACTH by the hypothalamus and pituitary and to reduce the circulating concentrations of adrenal androgens. The preferred glucocorticoid employed for replacement is hydrocortisone because its short half life minimises growth suppression and other adverse side effects of more potent, longer acting glucocorticoids, such as prednisone and dexamethasone.

 Neonates are treated with a minimum of 6 mg/day of hydrocortisone given in three divided doses whereas children and adolescents are given hydrocortisone doses of 10 – 20 mg/m²/day in two or three divided doses. These doses exceed physiological levels of cortisol secretion, which are 6 – 7 mg/m²/day in children and adolescents (Linder BL et al., 1990; Kerrigan JR et al., 1993). These supraphysiological doses are required to
suppress adrenal androgens adequately and to minimise the possibility of developing adrenal insufficiency. If control cannot be achieved with hydrocortisone, it is reasonable to use either prednisone or dexamethasone for 3 – 4 days before resuming hydrocortisone. Older adolescents and adults may be treated with modest doses of prednisone or dexamethasone that do not exceed the equivalent of 20 mg/m² of hydrocortisone daily, and should be monitored carefully for signs of iatrogenic Cushing’s syndrome, such as rapid weight gain, hypertension, pigmented striae and osteopaenia. Males with testicular adrenal rests may require higher doses of dexamethasone to suppress ACTH.

Treatment efficacy, i.e. adequate suppression of adrenal androgens, is assessed by monitoring annualised growth velocity as well as 17-OHP and androstendione concentrations. Testosterone is also useful in females and prepubertal males. Hormones should be measured at a consistent time in relation to medication dosing, preferably at 0800h at the physiological peak of ACTH secretion or, at least, at the nadir of serum cortisol concentrations just before the next dose of hydrocortisone is to be given.

Excessive glucocorticoid treatment with hydrocortisone doses higher than the individual’s requirements and certainly in excess of 20 mg/m²/day is potentially detrimental to growth (Jaaskelainen J et al., 1997). A randomised prospective crossover trial showed that patients treated with 15 mg/m²/day of hydrocortisone were less likely to show growth suppression compared to those taking doses of 25 mg/m²/day (Silva IN et al., 1997). High body mass index in childhood also correlates with poor final height and may be a surrogate marker of overtreatment (Jaaskelainen J et al., 1997; Yu AC et al., 1995). In general, and despite linear growth averaging approximately 1 standard
deviation (SD) below the mean, bone mineral density does not appear to be compromised in CAH patients treated with standard glucocorticoid doses (Cameron FJ et al., 1995; Gussinye M et al., 1997). Only one study showed low bone density in the femoral neck and lumbar spine regions but those findings were attributed to excessive doses in some of the patients (Guo CY et al., 1996).

Since patients with classical CAH are not able to mount a satisfactory cortisol response to stress, pharmacological doses of hydrocortisone are required in situations such as febrile illness and surgery under anaesthesia. Such treatment should approximate typical endogenous adrenal secretion in critically ill and perioperative patients (Lamberts SW et al., 1997). Dose guidelines include tripling the maintenance dose of oral hydrocortisone and administering in three divided doses in minor febrile illnesses. For major surgery, intravenous hydrocortisone up to 100 mg/m²/day divided in four doses is warranted for at least 24 hours peri- and postoperatively before tapering over several days to a maintenance dose. Intravenous hydrocortisone is preferred over equivalent doses of methylprednisolone or dexamethasone because its mineralocorticoid activity is able to substitute for oral fludrocortisone.

**ii) Mineralocorticoid replacement**

Patients with the salt wasting form of classical CAH require mineralocorticoid (9α-fludrocortisone) in addition to glucocorticoid replacement. During infancy sodium chloride supplements are also added because the sodium content of breast milk as well as most infant formulae (~ 8 mEq/L) is only sufficient for maintenance sodium requirements in healthy infants. Although patients with the simple virilizing form of the disease by definition secrete adequate amounts of aldosterone, they are nevertheless
often treated with fludrocortisone, which can aid in adrenocortical suppression, thus reducing the maintenance dose of glucocorticoid.

PRA may be used to monitor adequacy of mineralocorticoid replacement. Hypertension, tachycardia and suppressed PRA are signs of overtreatment with mineralocorticoids (Biglieri EG et al., 1991). Excessive doses of fludrocortisone may also result in growth retardation (Lopes LA et al., 1998).

**iii) Other therapeutic approaches**

*Pharmacological:* A number of new medical approaches have been suggested that may be expected to offer the prospect of an improved outcome. The study that showed the most promising results after a two year trial proposes a four drug regimen with flutamide (an androgen receptor-blocking drug), testolactone (an aromatase inhibitor) and reduced doses of hydrocortisone and fludrocortisone. After 2 years of treatment, the children in the experimental group showed higher concentrations of 17-OHP, androstendione, DHEA, DHEAS and testosterone but more appropriate linear growth, slower rate of skeletal maturation and improved predicted height compared to children on standard therapy. However, central precocious puberty occurred and required treatment with a gonadotrophin-releasing hormone (GnRH) analogue in 3 out of 8 males in the experimental group and none of the 9 males in the control group (Merke DP et al., 2000). It remains to be seen whether longer term studies will show a favourable outcome in patients treated with this regimen.

*Adrenalectomy:* Laparoscopic adrenalectomy has been suggested as an alternative to medical therapy in patients with severe salt wasting disease who had ongoing...
virilization despite adequate doses of standard replacement regimens (Van Wyk JJ et al., 1996). Severely affected patients, particularly females, may be more easily managed as Addisonians with low doses of glucocorticoids and mineralocorticoids than with adrenal glands secreting excessive amounts of sex steroids. Opponents of surgical treatment feel that it is too radical a step that places patients at risk from the surgical procedure and incurs further risks from iatrogenic adrenal insufficiency. In addition, the beneficial effects of adrenalectomy may be confounded by the development of gonadal adrenal rests that can secrete androgen precursors (Zachmann M et al., 1984). Finally, the beneficial effect of physiological adrenal DHEA production on lipid profile and quality of life will be lost following adrenalectomy. Although there are a few reports of paediatric patients undergone adrenalectomy (Zachmann M et al., 1984; Gunther DF et al., 1997), further data are required before deciding whether this is a viable alternative to medical therapy. Meanwhile, it may be considered in patients with severe 21-hydroxylase deficiency refractory to medical management.

Gene therapy: Although 21-hydroxylase deficient mice have been rescued by transgenesis with a murine CYP21 gene, the disorder does not seem a promising system for human gene therapy (Gotoh H et al., 1994; Stratakis CA et al., 1999). High level expression would need to be targeted to the adrenal cortex for normal concentrations of cortisol under both normal and stress conditions to be produced. Also, such levels of expression would need to be maintained indefinitely and to be cost effective compared to standard medical therapy, which – albeit not perfect – is effective and relatively inexpensive. These criteria seem unlikely to be met in the foreseeable future.
iv) Corrective surgery

In congenital adrenal hyperplasia and regardless of the appearance of the external genitalia in females, the recommended sex assignment and rearing should be that of the genetic/gonadal sex, so that the patient could retain the possibility of reproductive function. This is particularly true for the female patients, who have normal internal genital structures and potential for childbearing.

Whether, how and when to intervene surgically for correction of the genital anomalies and feminisation of the external genitalia in females has been the subject of controversy (Wilson BE et al., 1998; Schober JM, 1998). Adult patients with CAH and other intersex disorders who are unhappy with their sex assignment, as well as some physicians, suggest that genital surgery should be postponed until the affected individual is able to provide informed consent for cosmetic genital surgery and select the gender with which she/he is more comfortable (Wilson BE et al., 1998; Howe EG, 1998; Kipnis K et al., 1998). On the other hand, however, it is not certain whether families would readily accept the idea of raising a child with indeterminate gender and/or ambiguous genitalia, whether children would psychologically traumatised due to lack of societal acceptance of such conditions and whether these children would be able to develop an unambiguous gender identity at all. It must also be recognised that recommendations for sex assignment are to some extend culture specific. In cultures that value boys more than girls, parents may strongly resist the idea of rearing a female with ambiguous genitalia as a girl and a number of the severely virilized females will be reared as males (Abdullah MA et al., 1991; Chatterjee S et al., 1992).
Surgical correction of the external genitalia mainly involves clitoroplasty, which is performed in infancy (Newman K et al., 1992). Vaginal reconstruction is usually postponed until the age of expected sexual activity (Alizai NK et al., 1999), although single-stage corrective surgery has been successfully performed in some cases. Correction in infancy may be more successful for cases of simple labial fusion than in cases where the distal vagina must be reconstructed (Powell DM et al., 1995; Alizai NK et al., 1999). Newer modifications in vaginoplasty procedures are expected to improve the outcome in patients with urogenital sinus for whom alternative methods have not been useful.

v) Psychological counselling

Parents of patients with congenital adrenal hyperplasia should be offered psychological counselling as soon as the diagnosis is confirmed, particularly if the patient involved is a female requiring genital reconstruction. Intermittent assessment of family functioning may be a useful tool in predicting future problems. Children should be repeatedly informed about their condition by both their parents and their physicians in a sensitive and age-appropriate manner. Although the psychosexual development of females with classical CAH is not fully understood, it is reasonable to prepare parents of such children that their affected daughters will be more likely to exhibit tomboyish behaviour, masculine play preferences and, when older, a preference for a career over domestic activities (Dittmann RW et al., 1990). Parents should also be reassured that the majority of females function heterosexually, although they may require genital reconstruction before becoming sexually active. Finally, health care professionals involved with the management of these patients should accept that a minority of females
with CAH may be more comfortable as homosexuals and that such individuals should be helped to come to terms with their sexual orientation.

E. Limitations of standard replacement therapy

It is now well recognised that suboptimal control of CYP21 deficient patients can be observed despite adequate replacement therapy and adherence to treatment, indicating that the problems encountered in the management of these patients are inherent in our ability to control ACTH secretion and suppress the adrenal cortex effectively (Van Wyk JJ et al., 1996).

i) Control of hyperandrogenism and hypercortisolism

Achieving and maintaining adrenal suppression in CAH is far more challenging than preventing adrenal crises and in a fair number of patients it has proven impossible to control androgen overproduction without employing supraphysiological doses of glucocorticoids and therefore producing an unacceptable and undesirable degree of hypercortisolism. This will further contribute to a compromised final height and in addition will result in obesity and a decrease in bone mineral density.

ii) Hyperresponsiveness of the adrenal glands to ACTH

Prolonged exposure of the adrenal glands to elevated ACTH concentrations results in hypertrophy of the glands and an exaggerated response with increased production of androgens following a small ACTH challenge; conversely, pharmacological doses of glucocorticoids eventually induce adrenal atrophy and the glands respond only
minimally to either endogenous or exogenous ACTH. Accordingly, CYP21 deficient patients with longstanding suboptimal control will require higher doses of glucocorticoids for a period of time to induce adrenal suppression and atrophy before instituting maintenance doses.

**iii) Resistance to replacement therapy**

The androgens and androgen precursors produced in CYP21 deficient patients in the face of ACTH hypersecretion compete with the exogenously administered glucocorticoids and mineralocorticoids for the same receptors resulting in a degree of resistance to therapy (Janowski A, 1977; Duncan M et al., 1979). Thus, CAH patients are more prone to responding to stress with rapid salt loss and collapse compared to their healthy or Addisonian counterparts.

**iv) Redundancy of mechanisms governing ACTH secretion**

A final source of difficulty in suppressing the pituitary with glucocorticoids relates to the fact that the glucocorticoid feedback is only one of the mechanisms governing ACTH secretion. Although cortisol inhibits ACTH release by blocking the release of CRH in the hypothalamus as well as its action in the pituitary (Keller-Wood ME et al., 1984), there are a number of other neural peptides that are capable of releasing ACTH from the anterior pituitary. The antidiuretic hormone (ADH) is probably the most important of these. ADH rises in response to stress, hypovolaemia and many other stimuli, and ADH concentrations can be elevated in children with CAH because of water and electrolyte imbalance. Other ACTH secretagogues that have been recognised over the last decades include interleukin (IL)-1, IL-2, IL-6, tumour necrosis factor-α (TNF-α) and prostaglandins. These alternative secretagogues may explain why
individuals who, as a result of long-term steroid therapy may not be able to respond to normal feedback mechanisms, can often still secrete ACTH in response to stress or dehydration. In view of the redundancy of mechanisms leading to ACTH release, it is clear that appropriate glucocorticoid substitution therapy and adequate adrenocortical suppression may not be the only answer to medical management in a number of patients with classical 21-hydroxylase deficiency.
Chapter III

Influence of Puberty on CAH Control
A. Alterations in the endocrine milieu at puberty

Puberty results from increased gonadotrophin secretion from the anterior pituitary in response to increased GnRH release from the hypothalamus. GnRH is released in a pulsatile fashion into the hypothalamic-pituitary portal system, whereby it reaches the pituitary gonadotrophs (Styne DM, 1993). GnRH then binds to cell surface receptors of gonadotrophs and stimulates the production of follicle stimulating hormone (FSH) and luteinising hormone (LH) (Hazum E et al., 1988).

Gonadotrophins are released into the circulation in a pulsatile manner due to the pulsatile nature of GnRH secretion and lead to secretion of oestradiol and testosterone by the gonads. In males, Leidig cells respond when LH binds to their membrane receptors. The ligand-receptor complex stimulates membrane-bound adenylyl cyclase to increase cAMP, which then stimulates protein kinase, which in turn stimulates the conversion of cholesterol to pregnenolone by P450scc, the first step in the production of testosterone. FSH, on the other hand, binds to specific receptors on the cell surface of Sertoli cells and causes a sequence of events that culminates in increased protein kinase in a manner similar to the stimulatory effect of LH on Leidig cells (Doody KJ et al., 1990). FSH results in an increase in the mass of seminiferous tubules and supports the development of sperm. In females, LH binds to membrane receptors of ovarian cells and stimulates the activity of adenylyl cyclase to produce cAMP, which in turn stimulates the production of pregnenolone from cholesterol, thus initiating steroidogenesis. FSH binds to its own cell-surface receptors on the glomerulosa cells and stimulates the conversion of testosterone to oestrogen (Ross GT, 1990).
At puberty, rising sex steroid concentrations are associated with increased growth hormone (GH) secretion from the anterior pituitary leading to increased IGF-I concentrations and the pubertal growth spurt (Miller JD et al., 1982; Martha PM Jr et al., 1989; Rose SR et al., 1991; Blum WF et al., 1993). Sex steroids appear to stimulate IGF-I secretion not only indirectly but also directly from the cartilage and independent of changes in GH secretion (Attie KM et al., 1990).

The rise in serum GH concentrations is associated with a marked decrease in insulin sensitivity and a parallel elevation in insulin concentrations (Amiel SA et al., 1986; Cutfield WS et al., 1990). At the tissue level, insulin reduces insulin-like growth factor binding protein-1 (IGFBP-1) concentrations, thus further increasing IGF-I concentrations and enhancing its mitogenic effects (Conover CA et al., 1990; Travers SH et al., 1998). These changes in the endocrine milieu at puberty may be responsible for a decrease in the concentrations of serum cortisol and may enhance steroidogenesis in a number of ways, thus imposing greater difficulty in achieving adequate CAH control during pubertal years.

B. Effect of puberty on CAH control

i) Role of GH and IGFs

a) Effect of GH and IGF-I on 11β-HSD activity

A number of cytosolic and microsomal enzymes, including cytochrome P450, 5α/5β-reductase, 3α/3β-oxidoreductase and 11β-hydroxysteroid dehydrogenase (11β-HSD)
play a key role in the hepatic metabolism of cortisol (Gower DB, 1984; Iyer RB et al., 1990; Abel SM et al., 1992). Of those, particularly important at puberty is the role of 11β-HSD, which is responsible for the interconversion of active glucocorticoids, cortisol and corticosterone to their hormonally inactive metabolites cortisone and 11-dehydrocorticosterone respectively. Two isoforms of 11β-HSD have been identified. Type 1 (11β-HSD1) is a NADP(H)-dependent enzyme expressed in the liver, gonads, adipose tissue and central nervous system tissues and acts predominantly as an oxo-reductase, generating cortisol from cortisone. Type 2 (11β-HSD2) isoform is a NAD-dependent dehydrogenase expressed in the mineralocorticoid target tissues, kidney and colon and catalyses the conversion of active cortisol to inactive cortisone, thus protecting the mineralocorticoid receptor from illicit occupancy by cortisol (Edwards CRW et al., 1988; Tannin GM et al., 1991; Albiston AL et al., 1994; Stewart PM, 1996; White PC et al., 1997; Rickets ML et al., 1998).

It is now well documented that inhibition of 11β-HSD1 reductase activity is observed in association with an increase in GH and IGF-I concentrations (Gelding SV et al., 1998; Moore JS et al., 1999; Toogood AA et al., 2000). The decrease in 11β-HSD1 activity is evidenced by a decrease in the ratio of urinary cortisol to cortisone metabolites (Fm/Em) (index of overall 11β-HSD activity) in the presence of normal ratio of urinary free cortisol to free cortisone (FF/FE) (index of 11β-HSD2 activity) (Palermo M et al., 1996). This inhibition of type 1 isoform without any alteration in type 2 isoform activity is expected to result in decreased conversion of inactive cortisone to active cortisol and, therefore, lower serum cortisol concentrations in patients who are on hydrocortisone replacement therapy, such as patients with enzymatic defects of adrenal steroidogenesis, adrenal insufficiency or hypopituitarism.
Studies of hypopituitary adults showed a significant, dose-independent, persistent decrease in the urinary Fm/Em ratio with no concomitant change in the urinary FF/FE ratio following treatment with GH (Gelding SV et al., 1998; Toogood AA et al., 2000). These findings provide evidence for a direct or indirect modulation of cortisol to cortisone interconversion and thus of cortisol metabolism by GH, and suggest that this occurs via an alteration in the activity of the hepatic isoform of 11β-HSD. Similar were the findings of studies on acromegalic patients: four months after withdrawal from medical therapy, GH and IGF-I concentrations rose significantly, whereas the urinary [tetrahydrocortisol (THF) + allo-THF] / tetrahydrocortisone (THE) ratio decreased markedly in the presence of an unaltered urinary FF/FE ratio (Moore JS et al., 1999), suggestive of an inhibition of 11β-HSD1 activity. Conversely, when acromegalic patients underwent transphenoidal surgery, the fall in GH concentrations was associated with a significant increase in the urinary (THF + allo-THF)/THE ratio, once again, in the presence of unaltered urinary FF/FE ratio. Acromegalic patients from either group, who failed to demonstrate a change in GH concentrations, showed no significant alteration in the (THF + allo-THF)/THE ratio (Moore JS et al., 1999).

The above findings have been further confirmed by in vitro studies of 11β-HSD activity. Incubation of both primary cultures of omental adipose stromal cells and 293T1 cells with IGF-I resulted in a dose-dependent inhibition of type 1 isozyme activity, with the most marked effect being observed after 48 hours of incubation. In contrast, GH had no effect on 11β-HSD1 activity, even at high concentrations and at incubation times of 48 hours, indicating that the GH effects observed in clinical studies are probably mediated by IGF-I (Moore JS et al., 1999). Furthermore, the effect on 11β-HSD1 activity was specific in that neither IGF-I nor GH had any effect on 11β-HSD2.
activity in 293T1 cells and other cell lines that express 11β-HSD2 but not 11β-HSD1 (Moore JS et al., 1999). Similar were the findings of Jamieson et al., who examined the in vitro activity of 11β-HSD1 in cultured intact rat primary hepatocytes and found that GH had no effect on the enzyme activity (Jamieson PM et al., 1995). By contrast, other studies have shown that GH itself causes inhibition of 11β-HSD1 in cultured rat hepatocytes, as evidenced by a decrease in the isozyme activity and mRNA expression. This effect was present when GH was administered as a continuous infusion (female pattern) but not when it was given intermittently (pulsatile, male pattern) (Low SC et al., 1994; Albiston AL et al., 1995), suggesting that the activity of type 1 isozyme is highly dependant on sexually dimorphic patterns of GH secretion.

b) Effect of GH and IGF-I on glomerular filtration rate

Since the primary site of cortisol metabolism in humans is the liver and only less than 1% of cortisol is excreted unchanged in the urine, the metabolic clearance of cortisol would be influenced primarily by factors altering hepatic clearance and to a much lesser degree by factors affecting renal excretion. Renal clearance, therefore, represents a small part of cortisol clearance, however, factors that could potentially influence renal excretion at puberty would have an effect, albeit small, on cortisol clearance.

The renal clearance of a drug is the net result of three different processes: filtration, secretion and reabsorption and the renal clearance by filtration increases with an increase in glomerular filtration rate (GFR) (Birkett DJ, 1998e; Rowland M et al., 1980a).
Many studies have described the effects of GH and IGF-I on renal function (Hammerman MR, 1999). Chronically elevated GH concentrations, such as in acromegaly, are associated with an increase in renal plasma flow (RPF), GFR and kidney size whereas decrease in GH concentrations in these individuals following hypophysectomy or introduction of a somatostatin analogue has the opposite effects (Ikkos D et al., 1956; Falkheden T, 1963; Falkheden T et al., 1965; Dullaart RP et al., 1992). Also, a rapid increase in plasma GH concentrations following GH administration in normal or GH deficient adults results in an increase in both PRF and GFR (Christiansen JS et al., 1981; Hirschberg R et al., 1989). Similar were the findings of more recent studies (Tonshoff B et al., 1993; Riedl M et al., 1995). Tonshoff et al., showed that administration of recombinant human (rh) GH to healthy normotensive subjects resulted in a 50% increase in serum IGF-I concentrations and a 10% rise in GFR after three days of treatment. When the study was repeated under co-administration of indomethacin, which was introduced two days prior to rhGH, a similar rise in serum IGF-I concentrations was noted, however, the increase in GFR was completely blocked by indomethacin, suggesting that the rise in GFR during GH treatment requires the presence of vasodilating prostanoids (Tonshoff B et al., 1993).

These actions of GH are likely to be mediated by IGF-I rather than being direct. IGF-I increases GFR via a direct effect on the glomerular vasculature. In rats, infusion of IGF-I decreases renal glomerular afferent and efferent arteriolar resistances and increases the glomerular ultrafiltration coefficient (Hirschberg R et al., 1991; Krieg RL et al., 1995). The action of IGF-I in dilating pre-glomerular vessels is probably mediated by local production of nitric oxide and vasodilatory prostaglandins, since, in blood-perfused rat juxtamedullary nephron preparations: a) IGF-I induces a rapid increase of nitric oxide
concentration in intact renal microvessels; b) the vasodilatory action of IGF-I is abrogated by nitric oxide synthase inhibitors; and c) IGF-I induced vasodilation is inhibited by indomethacin (Tonshoff B et al., 1998).

c) Effect of GH on adrenal steroidogenesis

The effect of GH and IGFs on adrenal steroidogenesis has long been recognised. Initial studies suggested a synergism between GH and ACTH that were thought to act primarily on the mitochondrial hydroxylases and enhance the activity of 11-hydroxylase, thus facilitating the conversion of 11-deoxycortisol to cortisol (Castromagana M et al., 1983; Merola B et al., 1992). This was demonstrated by performing an ACTH stimulation test in children with isolated GH deficiency before and after treatment with rhGH. A more recent study investigated the GH effect on adrenal steroidogenesis in non-GH-deficient subjects with Turner syndrome (Balducci R et al., 1998). In all subjects an ACTH test was performed before and 6 months after treatment with rhGH and the results were compared with controls. The authors reported a significant rise in DHEA concentrations post treatment with rhGH compared to the control group but no differences in 17-hydroxyprogrenolone (17-PGN), 17-OHP and androstendione concentrations. Comparison of the stimulated 17-PGN/17-OHP ratio (index of 3β-HSD activity) and 17-OHP/androstendione ratio (index of 17,20-lyase activity) suggested a decrease in 3β-HSD activity and/or an increase in 17,20-lyase activity following treatment with rhGH, which would result in increased androgen production. In addition to the above clinical studies, a number of in vitro studies have also shown that GH and IGF-I enhance adrenal steroidogenesis by enhancing responsiveness to ACTH (Penhoat A et al., 1992; Bianchi P et al., 1994).
d) Effect of IGF-I and IGF-II on adrenal steroidogenesis

Insulin-like growth factors IGF-I and IGF-II are involved in the regulation of cell growth and differentiation. Besides their mitogenic effects, they maintain differentiated functions of various organs, such as in steroidogenic ovarian and testicular cells (Adashi EY et al., 1985; Saez JM, 1994). While IGF-I plays a major role postnatally by mediating most of the somatotropic effects of GH, IGF-II has been implicated as an important regulator of fetal growth. It is known to enhance steroidogenesis in human fetal adrenal cells and an auto- or paracrine, ACTH-stimulated formation of IGF-II has been demonstrated in human fetal adrenal cells (Voutilainen R et al., 1987; Mesiano S et al., 1993).

L’Allemand D et al. examined the long term effects of IGF-I and IGF-II on cultured human adrenal fasciculata-reticularis cells and compared them with other growth promoting factors, such as GH, insulin and ACTH (L’Allamand D et al., 1996). Treatment for three days with IGF-I or -II slightly increased the production of androstendione, cortisol and DHEA approximately 1.5-fold over that of control cells. Moreover, the acute steroidogenic response to ACTH of cells pretreated with IGF-I, IGF-II or insulin was 3- to 6-fold higher than that of control cells. For each hormone, these effects of IGF-I and IGF-II were dose-dependent. The secretion of androstendione was more potently stimulated than the secretion of DHEA or cortisol and this effect was more clearly yielded by pretreatment with IGF-II than with IGF-I. In addition, the same investigators showed that in cells pretreated with IGF-I or –IGF-II, the mRNA levels of cytochrome P450 17α-hydroxylase and of 3β-HSD were increased but the mRNA levels of cholesterol side-chain cleavage enzyme were unchanged (L’Allemand D et al.,
1996). These findings suggested that both IGF-I and IGF-II contribute significantly to clinical states with hyperandrogenaemia.

One year later, Kristiansen et al. reported on the regulation of the steroidogenic enzyme gene expression by IGF-I and insulin in primary cultures of human adrenocortical cells from donors aged 19 - 72 years (Kristiansen SB et al., 1997). Insulin and IGF-I at physiological concentrations increased mRNA levels for 17α-hydroxylase and type II 3β-HSD in the absence of cAMP or ACTH, whereas they had lesser effects on 21-hydroxylase and cholesterol side-chain cleavage enzyme mRNA levels and no effect on the 11β-hydroxylase mRNA. The mRNA levels of all steroidogenic enzymes were strongly increased by cAMP or ACTH, indicating increased activity of the above enzymes in clinical conditions characterised by elevated ACTH concentrations.

Subsequent studies confirmed the above findings and showed that both IGFs predominantly stimulate androgen biosynthesis, however, IGF-II is more potent than IGF-I in enhancing basal as well as ACTH-induced DHEAS and cortisol production (Mesiano S et al., 1997; Fottner C et al., 1998). The same studies also demonstrated that, although both IGF receptors are present in adult human adrenocortical cells, the steroidogenic effects of IGF-I and IGF-II are mediated through the IGF-I receptor. These data provide further evidence of an IGF-I / IGF-II-mediated regulation of the differentiated function of adult human adrenocortical cells and also suggest that in patients with classical 21-hydroxylase deficiency the GH~IGF axis may play an important role in enhancing adrenal androgen production at puberty.
ii) Role of sex steroids

a) Effect of sex steroids on 11β-HSD activity

In addition to GH and IGFs, there is considerable evidence to suggest that gonadal steroids influence 11β-HSD1 activity. In rats, a sexually dimorphic pattern favouring males in the activity of 11β-HSD1 has been shown by most investigators (Monder C et al., 1984; Low SC et al., 1993; Albiston AL et al., 1995; Nwe KH et al., 2000). Low et al. found the 11β-HSD1 activity in female liver to be 50% lower than that of male liver, whereas Albiston et al. determined that hepatic 11β-HSD1 mRNA levels in female rats were 18-fold lower than in male rats. Furthermore, the above investigators showed that in male rat liver, gonadectomy and oestradiol treatment leads to a dramatic decrease in both 11β-HSD1 activity (69 +/- 8% decrease) and mRNA expression (97 +/- 1% decrease), whereas gonadectomy and testosterone replacement has no effect on type 1 isozyme activity. However, in female rat liver, gonadectomy results in a marked increase in 11β-HSD1 activity (120 +/- 37% rise), which can be reversed by oestradiol replacement therapy but not testosterone treatment (Low SC et al., 1993). Similar were the findings of subsequent investigators (Albiston AL et al., 1995; Jamieson PM et al., 1995; Nwe KH et al., 2000), which confirmed that oestrogens decrease the activity of type I isoform, thus resulting in lower serum cortisol concentrations in patients who are on hydrocortisone replacement therapy.

Sexual dimorphism in the activity of 11β-HSD has also been documented in healthy subjects (Raven PW et al., 1995) as well as hypopituitary patients on optimal replacement therapy (Weaver JU et al., 1998). Weaver et al., demonstrated that in hypopituitary patients receiving optimal replacement therapy, the 11-OH/11-oxo
cortisol metabolite ratio (index of overall 11β-HSD activity) was lower in females as compared to males, whereas the urinary FF/FE ratio was similar in both groups. In addition, the former ratio was inversely related to body weight, percentage of fat and total fat, and directly related to insulin sensitivity, thus suggesting that a decrease in insulin sensitivity is associated with decreased activity of 11β-HSD1.

b) Effect of sex steroids on corticosteroid binding globulin

It is well documented that oestrogens result in significant increases in the concentration of corticosteroid binding globulin (CBG) in plasma (Brien TG, 1981). The original observation that administration of oestrogens caused a substantial rise in the levels of total serum cortisol was made in subjects receiving high doses of stilboestrol for carcinoma of the breast or prostate (Taliaferro I et al., 1956; Robertson ME et al., 1959; Mills IH et al., 1960). This increase in serum total cortisol concentrations was shown to be due to a rise in the CBG-bound fraction of cortisol (Mills IH et al., 1960).

A few years later attention shifted to the effects of combined oral contraceptives and a number of reports suggested that oestrogens, either alone or combined with progesterone, increased the protein binding of cortisol in a dose-dependent manner but that progesterone alone had no effect (Doe RP et al., 1964; Musa BU et al., 1967a; Darj E et al., 1993; Van den Ende A et al., 1997). Although the association between oestrogen and CBG-bound cortisol concentrations was well established, the effect of oestrogen on the non-bound, free and metabolically active fraction of cortisol remained the subject of controversy, probably because of the different effects exerted by different doses (Musa BU et al., 1967b; Burke CW, 1970; Brien TG, 1975).
Animal studies provided evidence for a sexually dimorphic pattern in CBG concentrations and implicated sex steroids in the programming of CBG levels at different stages of ontogenesis. In female rats, CBG concentrations were 2.5-fold higher than that in male rats and castration led to a decrease in CBG concentrations. In mature female rats or castrated male rats treated with testosterone, CBG concentrations decreased by 40-50%, whereas administration of oestradiol had no effect on CBG concentrations in males or ovariectomised females (Mataradge GD et al., 1992). Other investigators also demonstrated a sexually dimorphic pattern in CBG secretion in rats, however, they attributed the difference in serum CBG concentrations between sexes to the different growth hormone secretory patterns (Jansson JO et al., 1989). They showed that in hypophysectomised rats, neither androgen nor oestrogen had an effect on serum CBG concentrations. However, administration of continuous infusions of human or bovine GH resulted in increased CBG concentrations, whereas intermittent GH administration subcutaneously at 12 hourly intervals had no effect. The sex difference in serum CBG concentrations in rats was, therefore, attributed to the more continuous pattern of GH secretion observed in females.

A sexually dimorphic pattern in CBG concentrations has also been established in healthy subjects. Stolk RP et al., determined serum CBG concentrations in healthy adults and found that women had significantly higher concentrations than men (Stolk RP et al., 1996).

Despite the extensive investigation of the effect of physiological or pharmacological doses of oestrogen on CBG concentrations, reports on alterations in CBG concentrations at puberty and in association with the alterations in the endocrine milieu
are lacking. If there was an increase in CBG concentrations at puberty, it would be expected to result in an increase in the CBG-bound moiety of cortisol. Under physiological circumstances this would be associated with a parallel elevation of the total cortisol concentrations but when a fixed dose of glucocorticoid is administered and the HPA axis is suppressed, it is expected to result in a concomitant reduction in the free, metabolically active fraction of cortisol.

**iii) Role of insulin**

*a) Effect of insulin on IGFBP-1*

The metabolic and mitogenic effects of insulin-like growth factors, IGF-I and IGF-II, are modulated by a family of six IGF binding proteins (IGFBPs). These binding proteins, although structurally similar, have unique biological properties and are regulated independently (Baxter RC et al., 1989). Whereas IGFBP-3, the major circulating form, is GH dependent (Blum WF et al., 1993), IGFBP-1 is GH independent and is primarily influenced by insulin (Suikkari AM et al., 1988; Conover CA et al., 1990; Lewitt MS et al., 1989). Circulating IGFBP-1 concentrations have been shown to correlate inversely with plasma insulin concentrations; thus, elevated IGFBP-1 concentrations are seen in low-insulin states such as fasting, exercise and insulin-dependent diabetes mellitus (Cotterill AM et al., 1988; Suikkari AM et al., 1989; Batch JA et al., 1991). On the other hand, in high-insulin conditions, such as hyperinsulinaemic clamps, insulinomas and postprandial states IGFBP-1 concentrations are reduced (Lee PD et al., 1993).
It has been demonstrated that conditions associated with insulin resistance, including obesity, polycystic ovary syndrome and non-insulin dependent diabetes mellitus, are also associated with decreased IGFBP-1 concentrations (Buyalos Rp et al., 1995; Mogul HR et al., 1996). In children, serum IGFBP-1 concentrations have been found to correlate inversely with pubertal stage (Ibáñez L et al., 1997; Travers SH et al., 1998), a relationship previously explained by the increased insulin concentrations that accompany puberty (Holly JMP et al., 1989).

IGFBP-1 is known to inhibit the actions of IGF-I by preventing the binding of IGF-I to receptors on cell membranes (Ritvos O et al., 1989). Consequently, at puberty, when insulin secretion is high, suppressed IGFBP-1 concentrations enhance the insulin-like actions of IGF-I and potentiate its effects on 11β-HSD1 activity and adrenal steroidogenesis described above, thus resulting in decreased conversion of cortisone to cortisol and increased production of adrenal androgens.

**b) Effect of insulin on adrenal steroidogenesis**

Insulin and the insulin-like growth factors have a multiple role in gene expression in steroidogenic cells. In human adrenocortical cells, physiological concentrations of insulin increase mRNA levels for 17α-hydroxylase and type II 3β-HSD in the absence of cAMP or ACTH. They appear, however, to have lesser effects on 21-hydroxylase and cholesterol side-chain cleavage enzyme mRNA levels and no effect on the 11β-hydroxylase mRNA (Kristiansen SB et al., 1997). Similar findings were reported by L’Allemand et al., who showed that insulin enhances adrenal androgen production in human cultured adrenal cells. The acute steroidogenic response to ACTH of cells pretreated with insulin was 3- to 6-fold higher than that of control cells and the secretion of
androstendione was more potently stimulated than DHEA or cortisol. The authors suggested that this action of insulin was mediated by alterations in the 17α-hydroxylase and 3β-HSD activity, since the mRNA levels of both steroidogenic enzymes were found to be increased (L’Allamand D et al., 1996). These data provide additional evidence that may explain why conditions associated with a fall in insulin sensitivity and, consequently, hyperinsulinaemia may also be associated with elevated adrenal androgen concentrations.

In addition to the above, insulin has been found to decrease the activity 11β-HSD1 and mRNA expression in cultured rat hepatocytes, and antagonise the effect of glucocorticoids on the enzymatic activity of type I isoform (Jamieson PM et al., 1995). This would compound the effect of GH and IGFs on 11β-HSD1 activity and further decrease the conversion of cortisone to cortisol.

To assess the in vivo effects of insulin on adrenal steroidogenesis, Moghetti et al., carried out an ACTH stimulation test in hyperandrogenaemic women during a three hour hyperinsulinaemic euglycaemic clamp (Moghetti et al., 1996). All patients had normal glucose tolerance and preliminary screening excluded known causes of androgen excess. In all these women the procedure was repeated during saline infusion and data were compared. Serum cortisol, 17-PGN, 17-OHP, DHEA and androstendione were measured after 2 hours of insulin or saline infusion and, subsequently, 30 min and 60 min after an intravenous bolus of ACTH. Although no difference was found between groups prior to stimulation with ACTH, the ACTH-stimulated serum 17-PGN and 17-OHP concentrations were significantly higher during insulin than during saline infusion. Also, ACTH-stimulated 17-PGN/DHEA and 17-OHP/androstendione molar ratios,
which represent index of apparent 17,20-lyase activity, were significantly higher during clamp studies than during saline infusion, suggesting an impaired enzyme activity. Conversely, 17α-hydroxylase activity, which is shared by the same enzyme, CYP450c17α, but is independently regulated (Miller WL, 1988), was increased during hyperinsulinaemia. No difference was found in the apparent activity of 3β-HSD during insulin and saline infusions. These observations are also in support of the hypothesis that insulin potentiates ACTH-stimulated steroidogenesis and highlight the importance of preventing hyperinsulinaemic states in patients with hyperandrogenaemia, activated HPA axis or both, such as patients with congenital adrenal hyperplasia.

Evidence that insulin stimulates primarily adrenal and to a much lesser extent ovarian steroidogenesis was provided by the work of Martikainen et al., who studied obese hyperandrogenaemic women prospectively (Martikainen et al., 1996). All patients underwent an ACTH stimulation test and blood samples for insulin, cortisol, androgens and androgen precursors were collected from both the left adrenal and ovarian vein following selective catheterisation. The secretory gradients of testosterone and its precursors, progesterone, 17-OHP, DHEA and androstendione in the selective catheterisations showed the adrenal gland to be the main source of excessive steroid production in these patients. Also, the concentrations of progesterone, 17-OHP, DHEA, androstendione, testosterone and cortisol in the adrenal vein correlated strongly with insulin measured from same samples, further suggesting that insulin constitutes an important component of the pathogenetic mechanism of hyperandrogenism by preferentially stimulating adrenal androgen biosynthesis.
c) Effect of insulin on ovarian androgen production

Insulin has a stimulatory effect on steroidogenesis by granulosa cells of normal and polycystic ovaries and interacts with gonadotrophins in an additive or, as in the case of LH, a synergistic manner. Insulin also appears to have a role in amplifying LH-induced androgen production by theca cells, which may explain the prominence of symptoms of hyperandrogenism in patients with insulin resistance. Recent data from studies of dispersed thecal cells in culture have confirmed that insulin acts as co-gonadotrophin in steroidogenesis, such as in the granulosa compartment of the follicle, and that is equipotent to IGF-I and IGF-II in stimulating androstendione production by isolated theca cells (Bergh C et al., 1993; Nahum R et al., 1995; Franks S et al., 1999).

Incubation conditions can significantly influence the magnitude of the insulin effects on androgen production, since pre-incubation for 12 hours has been shown to result in a more than fivefold amplification in androstendione production by insulin, which further increases with the addition of LH. Theca cells isolated from women with polycystic ovary syndrome may show increased production of androgens in response to insulin as compared to theca cells obtained from normal ovaries (Nestler JE et al., 1998).

Blockade of the above described insulin effects by an insulin receptor antibody suggests that insulin action in both normal and polycystic ovaries is mediated by way of its own receptor rather than the type I IGF receptor (Franks S et al., 1999). These findings indicate that the physiological rise in insulin concentrations observed at puberty, in conjunction with the rise in LH concentrations may potentiate ovarian androgen production, especially in patients with polycystic ovaries.

The relationship between elevated androgen concentrations and insulin dynamics is a complex one. Most work has dealt with the polycystic ovaries (PCO), since this is a
common condition in which hyperandrogenism and insulin resistance coexist, even in subjects who are not obese (Dunaif A, 1997). The observation that insulin is capable of stimulating ovarian androgen secretion may account for the persistence of syndromes of excess androgen and insulin secretion but does not explain how they arise in the first place. A number of reports provide contradicting evidence as to which is the primary phenomenon. Thus, Dunaif et al., showed that suppression of hyperandrogenism for up to 12 weeks did not decrease hyperinsulinism in women with PCO (Dunaif et al., 1990). In contrast, the administration of exogenous insulin to women with PCO failed to demonstrate the primacy of insulin in driving the androgen-insulin cycle, since androgen concentrations in these patients actually fell during the insulin infusion (Dunaif A et al., 1989).

It is possible, however, that androgens may produce mild insulin resistance. Women receiving oral contraceptives containing ‘androgenic’ progestins can experience decompensations in glucose tolerance, as can individuals receiving synthetic anabolic steroids (Godsland IF et al., 1992). Prolonged testosterone administration to female-to-male transsexuals, which resulted in circulating testosterone concentrations being within the normal male adult range, caused significant decreases in insulin-mediated glucose uptake in euglycaemic clamp studies (Polderman KH et al., 1994). More recent studies in testosterone-treated castrated female rats have suggested that androgen-mediated insulin resistance may be the result of an increase in the number of less insulin-sensitive type II b skeletal muscle fibres and an inhibition of muscle glycogen synthase activity (Rincon J et al., 1996). Future studies to assess insulin sensitivity in adequately and inadequately prepubertal and pubertal patients with classical congenital adrenal hyperplasia may be enlightening.
C. Summary

In patients with classical 21-hydroxylase deficiency, alterations in the endocrine milieu at puberty may result in inadequate suppression of HPA axis despite optimal substitution therapy and adherence to treatment. The increase in GH secretion and the rise in IGF-I, IGF-II and oestradiol concentrations may result in decreased conversion of cortisone to cortisol and, therefore, hypocortisolaemia. In addition, these alterations in association with the elevated insulin concentrations may lead to increased adrenal and ovarian androgen production. Since androgens and androgen precursors are known to compete with the exogenously administered glucocorticoids for the same receptors (Janowski A, 1977; Duncan M et al., 1979), both hypocortisolaemia and hyperandrogenaemia are independent factors of hypothalamic and pituitary stimulation and may result in increased ACTH secretion. ACTH hypersecretion, in turn, may further potentiate hypocortisolism by increasing the metabolic clearance rate of cortisol (Zisper RD et al., 1976) and further activating the HPA axis, thus establishing a vicious cycle.

It is worth noting that the above alterations are expected to have a greater effect on female pubertal patients as compared to male patients, mainly because of the sexually dimorphic patterns in GH secretion, the activity of 11β-HSD1 and the CBG concentrations. An additional factor that may account for poor control in female patients is the association of polycystic ovaries, a result of hyperandrogenism per se, and obesity, most often the result of short- or long-term treatment with supraphysiological doses of glucocorticoid in an attempt to suppress the HPA axis. The association of hyperandrogenism and obesity may result in hyperinsulinaemia and insulin resistance,
which may further stimulate adrenal and ovarian steroidogenesis and aggravate the already encountered problems. Furthermore, hyperandrogenism may be amplified in hyperinsulinaemic states because insulin suppresses the synthesis of sex hormone binding globulin (SHBG) by the liver (Plymate SR et al., 1988; Singh A et al., 1990). Hyperandrogenaemic patients with the non-classical form of 21-hydroxylase deficiency have been found to have significantly lower values of insulin sensitivity as compared to normal controls (Speiser PW et al., 1992) but similar reports on patients with the classical forms of CAH are lacking.

It is therefore evident that the management of pubertal patients with congenital adrenal hyperplasia should be based on the understanding of all the above pathophysiological alterations and should aim at providing adequate and frequent glucocorticoid substitution as well as preventing and/or treating hyperandrogenism and insulin resistance.
METHODOLOGY
A. Population

All patients participated in the studies were children with classical 21-hydroxylase deficiency. Forty patients (M:14, F:26; median age: 12.6 years (yrs), range: 6.1 – 20.3 yrs) attending the London Centre for Paediatric Endocrinology were studied prospectively. The patients were consecutive attendees at the Outpatient Clinics at The Middlesex Hospital and Great Ormond Street Hospital for Children, London. They consisted of 14 prepubertal (M/F: 5/9; median age: 9.4 yrs, range: 6.1 – 11.0 yrs), 20 pubertal (M/F: 7/13; median age: 13.5 yrs, range: 10.6 – 16.8 yrs) and 6 postpubertal (M/F: 2/4; median age: 18.2 yrs, range: 17.2 – 20.3 yrs) patients. Each group of patients was further subdivided into adequately and inadequately controlled depending on the suppression of HPA axis, as defined by a) 0800h ACTH concentrations (ACTH < 71 pg/ml), b) serial measurements of 17-OHP concentrations (17-OHP < 20 nmol/L) and c) the plasma renin activity (recumbent PRA: 1.1 – 2.7 nmol/h/L). The clinical characteristics of all CYP21 deficient patients are summarised in Table 1.

Patients were receiving standard dose regimens for replacement therapy in the form of oral hydrocortisone given twice or thrice daily and 9α-fludrocortisone given once or twice daily. Two patients had simple virilizing congenital adrenal hyperplasia and did not require mineralocorticoid substitution. Median daily hydrocortisone and 9α-fludrocortisone replacement doses were 17.4 mg/m² body surface area (SA) and 126.1 μg/m² body SA respectively in the prepubertal group, 17.8 mg/m² and 123.8 μg/m² respectively in the pubertal group and 17.2 mg/m² and 73.4 μg/m² respectively in the postpubertal group (Table 2).
Table 1: Clinical characteristics of CYP21 deficient patients

<table>
<thead>
<tr>
<th></th>
<th>No</th>
<th>M/F</th>
<th>Age (yrs)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prepubertal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>5 / 9</td>
<td>9.4 (6.1 – 11.0)</td>
<td>19.4 (14.8 – 23.4)</td>
</tr>
<tr>
<td>Adequately controlled</td>
<td>10</td>
<td>3 / 7</td>
<td>9.4 (6.1 – 10.8)</td>
<td>19.7 (14.8 – 23.4)</td>
</tr>
<tr>
<td>Inadequately controlled</td>
<td>4</td>
<td>2 / 2</td>
<td>8.9 (6.2 – 10.0)</td>
<td>18.6 (17.9 – 23.0)</td>
</tr>
<tr>
<td><strong>Pubertal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>7 / 13</td>
<td>13.4 (10.6 – 16.8)</td>
<td>25.7 (15.8 – 42.5)</td>
</tr>
<tr>
<td>Adequately controlled</td>
<td>10</td>
<td>3 / 7</td>
<td>13.7 (11.4 – 16.4)</td>
<td>25.5 (15.8 – 38.8)</td>
</tr>
<tr>
<td>Inadequately controlled</td>
<td>10</td>
<td>4 / 6</td>
<td>13.1 (10.6 – 16.8)</td>
<td>26.6 (15.8 – 42.5)</td>
</tr>
<tr>
<td><strong>Postpubertal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>2 / 4</td>
<td>18.2 (17.1 – 20.3)</td>
<td>26.0 (22.5 – 31.5)</td>
</tr>
</tbody>
</table>

Data shown are medians with range in parentheses

*BMI: Body Mass Index

*M: males, F: females*
Table 2: Replacement therapy regimens in CYP21 deficient patients

<table>
<thead>
<tr>
<th></th>
<th>Hydrocortisone (mg/m²/day)</th>
<th>Fludrocortisone (µg/m²/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prepubertal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17.4 (14.4 - 22.6)</td>
<td>126.1 (72.0 - 145.8)</td>
</tr>
<tr>
<td>Adequately controlled</td>
<td>17.4 (14.4 - 22.6)</td>
<td>129.9 (72.0 - 145.8)</td>
</tr>
<tr>
<td>Inadequately controlled</td>
<td>16.5 (14.4 - 21.6)</td>
<td>121.7 (88.9 - 138.3)</td>
</tr>
<tr>
<td><strong>Pubertal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17.8 (10.2 - 19.9)</td>
<td>123.8 (58.2 - 160.0)</td>
</tr>
<tr>
<td>Adequately controlled</td>
<td>17.1 (10.2 - 19.9)</td>
<td>117.1 (58.2 - 160.0)</td>
</tr>
<tr>
<td>Inadequately controlled</td>
<td>18.3 (12.5 - 19.3)</td>
<td>124.1 (80.8 - 141.6)</td>
</tr>
<tr>
<td><strong>Postpubertal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17.2 (14.5 - 18.9)</td>
<td>73.4 (44.3 - 128.6)</td>
</tr>
</tbody>
</table>

Data shown are medians with range in parentheses
i) Inclusion criteria

Patients were invited to participate in the study if:

- The diagnosis of classical 21-hydroxylase deficiency had been confirmed following detailed biochemical investigations and there was no possibility of an alternative enzymatic defect of adrenal steroidogenesis.
- They were older than 5 years.
- They were reported to adhere to medical therapy.

ii) Exclusion criteria

Patients were excluded from the study if there was evidence of:

- Central precocious puberty or other associated endocrine disorder.
- Clinical or biochemical evidence of hepatic or renal disease.
- History of the patient being on drugs that are known to alter CBG concentrations, such as oestrogen, or to induce mixed function oxidase enzymes, such as certain anti-epileptics.

iii) Ethical considerations

All studies performed were approved by the University College London Hospitals Committee on the Ethics of Human Research and the Great Ormond Street Hospital for Children NHS Trust / Institute of Child Health Research Ethics Committee. Informed written consent was obtained in all cases from one of the parents and assent from children older than 9 – 10 years. Detailed information on the purpose and aims of the study as well as the investigations protocol was given to parents and patients and any questions or concerns surrounding the study were discussed before admission to the
hospital. All patients recruited and their parents were aware of the fact that they were free to withdraw at any stage during the study.
B. Methods

Patients were admitted to the Endocrine Unit, The Middlesex Hospital, one day prior to the investigations and a complete physical examination, including Tanner pubertal staging and anthropometry, was performed by the same observer. Two indwelling venous catheters were inserted at least 12 hours before sampling to allow a period of adaptation. Patients were allowed normal ambulatory activity and received their oral hydrocortisone tablets at 0900h and 2100h or at 0800h, 1500h and 2200h depending on whether they were on twice or thrice daily regimen. 9α-fludrocortisone was given with the morning and/or the evening hydrocortisone tablets. The studies performed are outlined in the Figure.

**Day 1:** On the first day of the study baseline investigations, including full blood count (FBC), renal, liver and thyroid function tests, testosterone and/or oestradiol, CBG, fasting insulin and glucose, leptin, ACTH, IGF-I, IGF-II, IGFBP-3 concentrations and recumbent PRA were performed at 0800h. Blood samples for serum total cortisol, GH and 17-OHP concentrations were collected at 20 min intervals for a total of 24 hours from 0800h to 0800h the following morning. Blood samples were spun and separated within six hours after collection. Plasma or serum samples were stored at −20°C prior to assay. During the first 24 hours all urine specimens were collected for urinary steroid profile analysis.

**Day 2:** On the second day patients were given their usual dose of mineralocorticoid substitution at 0800h and one hour later intravenous (iv) hydrocortisone sodium succinate was administered as a bolus in a dose of 15 mg/m² body SA through the 1st
cannula. Blood samples for cortisol concentrations were collected through the 2nd cannula at 10 min intervals for a total of 6 hours following the injection of hydrocortisone. Blood samples were centrifuged, separated and stored at – 20° C prior to assay.

On the second day of the study patients were also randomised to two different small doses of Dexamethasone (0.3 mg/m² or 0.5 mg/m²) that they received on the third and fourth night of their stay in the hospital and instead of the evening dose of hydrocortisone. There was no alteration in the administration schedule of 9α-fludrocortisone.

**Day 3:** On the third day a blood sample for measurement of ACTH concentrations was taken at 0800h before the morning doses of hydrocortisone and 9α-fludrocortisone were administered. The evening hydrocortisone dose was omitted and patients received the second dose of Dexamethasone at 2200h.

**Day 4:** On the fourth day blood samples for ACTH concentrations and repeat FBC were taken at 0800h and prior to administration of the morning replacement therapy. The two indwelling venous catheters were removed and patients were allowed home. All patients continued to attend at Outpatient Clinics and their management was modified according to the results of the above investigations, a number of which represented part of their regular assessment.
**Figure: Schematic representation of the investigation protocol**

**DAY 1:**

20 min interval sampling commenced sampling completed

↓

0800h 2400h 0800h

*Day 1:* 20 min interval sampling for GH, cortisol and 17-OHP commenced at 0800h and continued for 24 hours. Urine specimens were collected for 24 hours. Patients received their usual doses of hydrocortisone and 9α-fludrocortisone during this period.

**DAY 2:**

iv HC given & 10 min interval sampling commenced sampling completed

↓

0900h 1500h 2200h

*Day 2:* The morning dose of 9α-fludrocortisone was given at 0800h whereas the oral dose of hydrocortisone (HC) was omitted and a bolus of intravenous hydrocortisone (15 mg/m²) was given at 0900h. Blood samples for total cortisol were collected at 10 min intervals for 6 hours after the hydrocortisone injection. The first dose of Dexamethasone (0.3 mg/m² or 0.5 mg/m²) was administered at 2200h. Patients received their usual dose of fludrocortisone in the evening.
DAY 3:

ACTH measurement Dexamethasone

\[ \downarrow \quad \downarrow \]

0800h \quad 2200h

Day 3: A blood sample for measurement of ACTH concentration following the first dose of Dexamethasone was collected at 0800h and before the morning doses of hydrocortisone and 9α-fludrocortisone were administered. The second dose of Dexamethasone (0.5 mg/m² or 0.3 mg/m² respectively) was given at 2200h.

DAY 4:

ACTH measurement

\[ \downarrow \]

0800h \quad 1200h

Day 4: A blood sample for measurement of ACTH concentration following the second dose of Dexamethasone was collected at 0800h and before the morning doses of hydrocortisone and fludrocortisone were administered. Patients were advised to continue with their usual replacement therapy regimens and discharged home.
C. Studies

The studies performed fall into four main groups. The first examines the pharmacokinetic parameters of total and free cortisol. The second describes the interrelationship between cortisol and 17-OHP and evaluates current therapeutic regimens in the light of the estimated cortisol pharmacokinetic parameters and the relationship between cortisol and 17-OHP. The third study examines possible alterations in the feedback mechanisms regulating ACTH secretion whereas the fourth determines the relationship between serum GH and cortisol concentrations.

The subcomponents of those study groups are as follows:

Study 1: Pharmacokinetic parameters of total and free cortisol.

Study 2: Cortisol pharmacokinetics and puberty: independent predictors of total and free cortisol clearance and half life.

Study 3: The bioavailability of oral hydrocortisone.

Study 4: Serum cortisol and 17-hydroxyprogesterone interrelationship: Is current replacement therapy satisfactory?

Study 5: Testing alterations in feedback mechanisms regulating ACTH secretion.

Study 6: Serum cortisol and GH interrelationship.
STUDY 1: PHARMACOKINETIC PARAMETERS OF TOTAL AND FREE CORTISOL

Aim

The aim of this study was to determine the main pharmacokinetic parameters of total and free cortisol and to investigate whether the pubertal process is associated with an alteration in cortisol pharmacokinetics resulting in a loss of control of HPA axis and, consequently, a significant increase in the concentrations of androgens and androgen precursors. In addition, this study examined whether gender or control of 21-hydroxylase deficiency influences total and free cortisol pharmacokinetics.

Patients

All forty patients (M: 14, F: 26; median age: 12.6 yrs, range: 6.1 – 20.3 yrs) were studied. They consisted of 14 prepubertal (M/F: 5/9; median age: 9.4 yrs, range: 6.1 – 11.0 yrs), 20 pubertal (M/F: 7/13; median age: 13.5 yrs, range: 10.6 – 16.8 yrs) and 6 postpubertal (M/F: 2/4; median age: 18.2 yrs, range: 17.2 – 20.3 yrs) patients. Alternatively, patients were divided into adequately controlled (M/F: 6/17; median age: 11.8 yrs, range: 6.1 – 18.4 yrs) and inadequately controlled (M/F: 8/9; median age: 12.8 yrs, range: 6.2 – 20.3 yrs) depending on the suppression of HPA axis and the PRA levels (Table 1). The doses of hydrocortisone and 9α-fludrocortisone replacement are summarised in Table 2.
Study design

The pharmacokinetic parameters of total and free cortisol were determined following the intravenous administration of hydrocortisone (15 mg/m$^2$) in the morning of the second day of the study and subsequent measurement of total cortisol concentrations at 10 min intervals for a total of 6 hours.

Pharmacokinetics

The pharmacokinetic parameters examined included clearance (CL), volume of distribution (V) and half life (t$^{1/2}$). Clearance was calculated following estimation of the area under the drug concentration versus time curve (AUC) from time (t) 0 min to infinite time (Rowland M et al., 1980a; Birkett DJ, 1998a): $CL = \frac{dose (iv)}{AUC_{0-\text{inf}}}.$

The elimination rate constant (k) was calculated from the slope of the regression line of the natural log (log$_e$) transformed cortisol data versus time.

The volume of distribution was estimated by dividing cortisol clearance by the elimination rate constant (Birkett DJ, 1998b): $V = \frac{CL}{k}.$

Finally, half life was estimated by dividing 0.693 (log$_e$2) by the elimination rate constant (Birkett DJ, 1998c): $t^{1/2} = \frac{0.693}{k}.$ Half life is a composite pharmacokinetic parameter determined by both clearance and volume of distribution: $t^{1/2} = \frac{0.693 \times V}{CL}.$
and it is therefore increased by an increase in volume of distribution or a decrease in clearance and vice versa.

The maximum plasma concentration ($C_{\text{max}}$) is the experimental value with the highest plasma concentration. The time of the maximum plasma concentration ($t_{\text{max}}$) is the experimental data point when $C_{\text{max}}$ was measured.

**Statistical analysis**

All data were log$_e$ transformed prior to statistical analysis. Clearance, volume of distribution and half life of total and free cortisol were estimated as described above. One-way analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) test were used to compare the mean clearance, volume of distribution and half life of total and free cortisol in the three groups of patients. In the adequately controlled group student’s t-test was used for comparisons between prepubertal and pubertal CYP21 deficient patients. Student’s t-test was also used for comparisons between males and females as well as between adequately and not adequately controlled patients.
Aim

The aim of this study was to relate biochemical changes observed at puberty as well as adequacy of control to cortisol pharmacokinetic parameters determined in all patients. The study also investigated independent predictors of total and free cortisol clearance and half life.

Patients

All forty patients (M: 14, F: 26; median age: 12.6 yrs, range: 6.1 – 20.3 yrs) participated in the study. The clinical characteristics of patients are shown in Tables 1 & 2.

Study design

Biochemical parameters and measurements reflecting adequacy of control of classical 21-hydroxylase deficiency were determined at the beginning of the study, at 0800h on day 1. Also, on the first day of the study blood samples for total cortisol, GH and 17-OHP concentrations were collected at 20 min intervals for a total of 24 hours in all but
three postpubertal patients. Urine steroid profile analysis was performed on 24 hour collections in all but three postpubertal patients.

**Statistical analysis**

All data were log (log_{10}) transformed prior to statistical analysis. One-way analysis of variance and Student-Newman-Keuls (SNK) test were used to compare the mean values of the transformed parameters between the three groups of patients. Stepwise multiple linear regression analysis was used to investigate independent predictors of serum cortisol clearance corrected for BMI and half life. Independent variables tested included ACTH, integrated 17-OHP and GH concentrations, IGF-I, IGF-II, IGFBP-3, fasting insulin, glucose and leptin, CBG, (THF+alloTHF)/THE ratio, THF/alloTHF (5α/5β) ratio, gender and pubertal status.

Integrated GH and 17-OHP concentrations were calculated as the area under the serum concentration versus time curve using the trapezoid method. This was performed for the time period 0800h – 0800h (total of 24 hours) and was calculated as follows:

\[ AUC = (\text{Sum of concentrations from } t = 0 \text{ min to } t = 24 \text{ h}) \times 20 - 0.5 \times (C_0 + C_{24}) \times 20 \]

where \( C_0 \) is the concentration at time \( t = 0 \) min, \( C_{24} \) is the concentration at time \( t = 24\) h and number 20 represents the time interval between consecutive samples expressed in minutes.
STUDY 3: THE BIOAVAILABILITY OF ORAL HYDROCORTISONE

Aim

The aim of this study was to determine the morning and evening bioavailability of oral hydrocortisone used in the treatment of patients with congenital adrenal hyperplasia (Hydrocortone tablets, Merck Sharp & Dohme Limited).

Patients

Sixteen patients (M/F: 4/12; median age: 10.9 yrs, range: 6.0 – 18.4 yrs) were recruited to participated in this study. They consisted of 9 prepubertal, 5 pubertal and 2 postpubertal children and their clinical characteristics are shown in Table 3. All patients were adequately controlled and had minimal endogenous production of cortisol, as evidenced by the undetectable cortisol concentrations before the oral and intravenous hydrocortisone doses were given. They were therefore reminiscent of patients with dexamethasone induced HPA axis suppression in terms of endogenous cortisol production. All patients were on standard dose replacement therapy with oral hydrocortisone (median dose: 17.4 mg/m^2 body SA) and 9α-fludrocortisone (median dose: 123 μg/m^2 body SA). The median hydrocortisone morning dose was 10.2 mg/m^2 body SA (range: 4.8 – 12.9 mg/m^2) whereas the median hydrocortisone evening dose was 6.1 mg/m^2 body SA (range: 3.4 – 9.7 mg/m^2).
Table 3: Clinical characteristics of patients participated in study 3

<table>
<thead>
<tr>
<th>No</th>
<th>M/F</th>
<th>Age (yrs)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>4 / 12</td>
<td>10.9 (6.1 – 18.4)</td>
</tr>
<tr>
<td>Prepubertal</td>
<td>9</td>
<td>2 / 7</td>
<td>9.5 (6.1 – 11.0)</td>
</tr>
<tr>
<td>Pubertal</td>
<td>5</td>
<td>2 / 3</td>
<td>14.5 (11.4 – 16.4)</td>
</tr>
<tr>
<td>Postpubertal</td>
<td>2</td>
<td>0 / 2</td>
<td>18.1, 18.4</td>
</tr>
</tbody>
</table>

Data shown are medians with range in parentheses. All patients were adequately controlled. Median (range) daily hydrocortisone and 9α-fludrocortisone doses were 17.4 mg/m² (14.4 – 22.6) and 122.9 µg/m² (62.5 – 145.8) respectively.

**BMI: Body Mass Index**
Study design

The bioavailability of oral hydrocortisone was estimated following measurement of serum total cortisol concentrations after the morning and evening doses of hydrocortisone were given on the first day of the study as well as the serum cortisol concentrations determined at 10 min intervals after administration of the intravenous hydrocortisone bolus on the second day.

Pharmacokinetics

The area under the serum cortisol concentration versus time curve (AUC) was calculated using the trapezoid method. The area under cortisol concentration versus time curve following oral administration of hydrocortisone (AUC_{oral}) was calculated from cortisol concentrations obtained at 20 min intervals following the morning and evening dose of oral hydrocortisone until cortisol concentrations were undetectable. The area under cortisol concentration versus time (t) curve from point t = 0 min to infinite time following intravenous administration of hydrocortisone (AUC_{iv}) was calculated from cortisol concentrations measured at 10 min intervals after the intravenous hydrocortisone bolus for a total of 6 hours. Both AUC_{oral} and AUC_{iv} were corrected for the hydrocortisone doses given orally and intravenously respectively after all doses were standardised in molar terms (1 mg = 10^6/362.46 nmol/L, where 362.47 is the molecular weight of cortisol).
The absolute bioavailability in the morning was calculated by dividing the corrected for hydrocortisone dose $\text{AUC}_{\text{oral}}$ following administration of the morning oral dose by the corrected for dose $\text{AUC}_{\text{iv}}$ and exemplified as a percentage. The apparent bioavailability of oral hydrocortisone in the evening was estimated by dividing the corrected for dose $\text{AUC}_{\text{oral}}$ following administration of the evening dose by the corrected for dose $\text{AUC}_{\text{iv}}$ and exemplified as a percentage (Birkett DJ, 1998a; Birkett DJ, 1998d; Rowland M et al., 1980c).

**Statistical analysis**

All data were log transformed prior to statistical analysis. The bioavailability was expressed as a 90% CI calculated using the two one-sided test procedure (Schuirmann DJ, 1987; Nation RL et al., 1994).
STUDY 4: SERUM CORTISOL AND 17-HYDROXYPROGESTERONE INTERRELATIONSHIP: IS CURRENT REPLACEMENT THERAPY SATISFACTORY?

Aim

The aim of this study was to determine the relationship between serum total cortisol and 17-OHP concentrations and to evaluate current therapeutic regimens in the light of the estimated cortisol pharmacokinetic parameters and the findings of cortisol – 17-OHP cross-correlation studies.

Patients

Thirty six patients (M: 13, F: 23; median age: 12.3 yrs, range: 6.1 – 18.8 yrs) were recruited to participate in this study. They consisted of 14 prepubertal (M/F: 5/9; median age: 9.6 yrs, range: 6.1 – 11.0 yrs), 19 pubertal (M/F: 7/12; median age: 13.3 yrs, range: 10.6 – 16.8 yrs) and 3 postpubertal (M/F: 1/2; median age: 18.4 yrs, range: 18.1 – 18.8 yrs) patients.

Patients were divided into two groups depending on the adequacy of HPA axis suppression, as defined by the 0800h ACTH concentrations (ACTH < 75pg/ml) determined on the first day of the study. The first group consisted of 17 adequately controlled (M/F: 3/14; median age: 10.8 yrs, range: 6.1 – 18.4 yrs) patients whereas the second group consisted of 19 inadequately controlled (M/F: 10/9; median age: 12.8 yrs,
range: 6.2 – 18.8 yrs) patients. The clinical characteristics of the patients participated in this study are shown in Table 4. Median daily hydrocortisone and 9α-fludrocortisone replacement doses were 18.0 mg/m² body SA and 130.0 μg/m² body SA respectively in the prepubertal group, 17.5 mg/m² and 123.5 μg/m² respectively in the pubertal group and 18.7 mg/m² and 62.5 μg/m² respectively in the postpubertal group (Table 5).

Study design

Baseline investigations, including androgens and androgen precursors were performed at 0800h on the first day of the study. Serum cortisol and 17-OHP concentrations were established in all patients following 20 min interval sampling for a total of 24 hours (Day 1). During this period patients received their usual doses of hydrocortisone and 9α-fludrocortisone. Blood samples were centrifuged, separated and stored at -20°C prior to assay.

We arbitrarily divided the 24 hour period into two 12 hourly intervals, from 0400h to 1600h (daytime) and from 1600h to 0400h (nighttime) because we expected maximal increases in 17-OHP concentrations in the early morning hours. In addition, cortisol concentrations were expected to be maximal during the same period (0400h – 1600h), since all patients had the largest hydrocortisone dose given in the morning. In this way we could examine the main peaks of both cortisol and 17-OHP in parallel.
Table 4: Clinical characteristics of patients participated in study 4

<table>
<thead>
<tr>
<th></th>
<th>No</th>
<th>M/F</th>
<th>Age (yrs)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>36</td>
<td>13/23</td>
<td>12.3 (6.1-18.8)</td>
<td>22.8 (14.8-38.8)</td>
</tr>
<tr>
<td>Prepubertal</td>
<td>14</td>
<td>5/9</td>
<td>9.6 (6.1-11.0)</td>
<td>19.6 (14.8-23.4)</td>
</tr>
<tr>
<td>Pubertal</td>
<td>19</td>
<td>7/12</td>
<td>13.3 (10.6-16.8)</td>
<td>25.2 (15.8-38.8)</td>
</tr>
<tr>
<td>Postpubertal</td>
<td>3</td>
<td>1/2</td>
<td>18.4 (18.1-18.8)</td>
<td>25.3 (22.8-26.6)</td>
</tr>
</tbody>
</table>

### Adequately controlled

<table>
<thead>
<tr>
<th></th>
<th>No</th>
<th>M/F</th>
<th>Age (yrs)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>17</td>
<td>3/14</td>
<td>10.8 (6.1-18.4)</td>
<td>22.6 (14.8-38.3)</td>
</tr>
<tr>
<td>Prepubertal</td>
<td>9</td>
<td>2/7</td>
<td>9.6 (6.1-11.0)</td>
<td>19.7 (14.8-23.4)</td>
</tr>
<tr>
<td>Pubertal</td>
<td>6</td>
<td>1/5</td>
<td>13.9 (10.6-16.0)</td>
<td>28.0 (15.8-38.8)</td>
</tr>
<tr>
<td>Postpubertal</td>
<td>2</td>
<td>0/2</td>
<td>18.1, 18.4</td>
<td>22.8, 25.3</td>
</tr>
</tbody>
</table>

### Inadequately controlled

<table>
<thead>
<tr>
<th></th>
<th>No</th>
<th>M/F</th>
<th>Age (yrs)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>19</td>
<td>10/9</td>
<td>12.8 (6.2-18.8)</td>
<td>23.0 (15.8-35.3)</td>
</tr>
<tr>
<td>Prepubertal</td>
<td>5</td>
<td>2/3</td>
<td>9.2 (6.2-10.0)</td>
<td>19.0 (16.9-23.0)</td>
</tr>
<tr>
<td>Pubertal</td>
<td>13</td>
<td>7/6</td>
<td>13.2 (10.8-16.8)</td>
<td>23.9 (15.8-35.3)</td>
</tr>
<tr>
<td>Postpubertal</td>
<td>1</td>
<td>1/0</td>
<td>18.2</td>
<td>26.8</td>
</tr>
</tbody>
</table>

Data shown are medians with range in parentheses

*BMI: Body Mass Index*
Table 5: Replacement therapy regimens in patients participated in study 4

<table>
<thead>
<tr>
<th></th>
<th>No</th>
<th>M/F</th>
<th>HC (mg/ m²/day)</th>
<th>FC (µg/ m²/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>36</td>
<td>13 / 23</td>
<td>17.9 (10.2 – 22.6)</td>
<td>123.8 (54.0 – 160.0)</td>
</tr>
<tr>
<td>Prepubertal</td>
<td>14</td>
<td>5 / 9</td>
<td>18.0 (14.4 – 22.6)</td>
<td>130.0 (72.0 – 145.8)</td>
</tr>
<tr>
<td>Pubertal</td>
<td>19</td>
<td>7 / 12</td>
<td>17.5 (10.2 – 19.9)</td>
<td>123.5 (58.2 – 160.0)</td>
</tr>
<tr>
<td>Postpubertal</td>
<td>3</td>
<td>1 / 2</td>
<td>18.7 (15.6 – 18.9)</td>
<td>62.5 (54.0 – 126.9)</td>
</tr>
</tbody>
</table>

**Adequately controlled**

<table>
<thead>
<tr>
<th></th>
<th>No</th>
<th>M/F</th>
<th>HC (mg/ m²/day)</th>
<th>FC (µg/ m²/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>17</td>
<td>3 / 14</td>
<td>17.5 (12.6 – 22.6)</td>
<td>124.1 (62.5 – 145.8)</td>
</tr>
<tr>
<td>Prepubertal</td>
<td>9</td>
<td>2 / 7</td>
<td>16.8 (14.4 – 22.6)</td>
<td>129.4 (72.0 – 145.8)</td>
</tr>
<tr>
<td>Pubertal</td>
<td>6</td>
<td>1 / 5</td>
<td>18.2 (12.6 – 19.9)</td>
<td>113.4 (87.6 – 132.5)</td>
</tr>
<tr>
<td>Postpubertal</td>
<td>2</td>
<td>0 / 2</td>
<td>15.6, 18.7</td>
<td>62.5, 126.9</td>
</tr>
</tbody>
</table>

**Inadequately controlled**

<table>
<thead>
<tr>
<th></th>
<th>No</th>
<th>M/F</th>
<th>HC (mg/ m²/day)</th>
<th>FC (µg/ m²/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>19</td>
<td>10 / 9</td>
<td>18.1 (10.2 – 20.0)</td>
<td>123.5 (54.0 – 160.0)</td>
</tr>
<tr>
<td>Prepubertal</td>
<td>5</td>
<td>2 / 3</td>
<td>19.8 (14.5 – 20.0)</td>
<td>132.6 (120.7 – 143.0)</td>
</tr>
<tr>
<td>Pubertal</td>
<td>13</td>
<td>7 / 6</td>
<td>17.0 (10.2 – 19.3)</td>
<td>123.5 (58.2 – 160.0)</td>
</tr>
<tr>
<td>Postpubertal</td>
<td>1</td>
<td>1 / 0</td>
<td>18.9</td>
<td>54.0</td>
</tr>
</tbody>
</table>

Data shown are medians with range in parentheses

HC: Hydrocortisone. FC: 9α-fludrocortisone
Statistical analysis

The relationship between baseline 0800h androstendione concentrations and integrated 17-OHP and cortisol concentrations was investigated by linear regression and the calculation of Pearson's correlation coefficient. Integrated 17-OHP and cortisol concentrations were calculated as the area under the serum concentration versus time curve using the trapezoid method.

To search for a time ordered relationship between cortisol and 17-OHP, analysis of correlations between the absolute values of the time series of each hormone was computed at 20 min intervals at various time lags covering the 24 hour period of the study. Only data from the group of inadequately controlled CYP21 deficient patients were used for cross-correlation studies because they demonstrated significant variation in 17-OHP concentrations to allow such an analysis. All data were stationarized and processed as a 3 point moving average to reduce assay noise as previously described (Matthews DR, 1988; Diggle PJ, 1990). Cross-correlation was computed after lagging (shifting) the concentration time-series of 17-OHP relative to the concentration time-series of cortisol. If $r_t$ is the coefficient of correlation between cortisol and 17-OHP at a lag time $t$ for one patient, then the mean $r_t$ for all patients was considered significant when it exceeded zero by more than two standard errors (SE) of the mean. The SE was calculated from the individual values of $r_t$ for all patients at the lag time $t$. 
STUDY 5: TESTING ALTERATIONS IN FEEDBACK MECHANISMS REGULATING ACTH SECRETION

Aim

This study was designed to examine whether there is an alteration in the negative feedback effects of cortisol at the pituitary level and to determine the lowest possible dose of dexamethasone required to suppress the 0800h serum ACTH concentrations when administered as a single dose the night before.

Patients

Thirty six patients (M: 13, F: 23; median age: 12.3 yrs, range: 6.1 – 18.8 yrs) were recruited to participate in the study. They consisted of 14 prepubertal (M/F: 5/9; median age: 9.6 yrs, range: 6.1 – 11.0 yrs), 19 pubertal (M/F: 7/12; median age: 13.3 yrs, range: 10.6 – 16.8 yrs) and 3 postpubertal (M/F: 1/2; median age: 18.4 yrs, range: 18.1 – 18.8 yrs) patients.

Patients were further subdivided into adequately and inadequately controlled depending on the 0800h ACTH concentrations determined on day 1. Their clinical characteristics and daily glucocorticoid and mineralocorticoid requirements are summarised in Tables 4 & 5.
Study design

Following randomisation to two different doses of dexamethasone (0.3 mg/m$^2$ and 0.5 mg/m$^2$) an overnight single dose dexamethasone suppression test was performed on the second and third day of the study. The dexamethasone dose was administered instead of the evening hydrocortisone dose at 2200h on days 2 and 3 (Figure). There was no alteration in the administration schedule of 9α-fludrocortisone. A blood sample for measurement of ACTH concentrations was obtained the following morning (Days 3 and 4 respectively). Blood samples were centrifuged and separated immediately after collection, and stored at −20°C until assayed.

Statistical analysis

The proportion of patients who achieved suppression of 0800h ACTH concentrations following administration of the two different dexamethasone doses was determined and compared between adequately and inadequately controlled patients.
STUDY 6: SERUM CORTISOL AND GH INTERRELATIONSHIP

Aim

The aim of this study was to determine the relationship between cortisol concentrations achieved following administration of oral hydrocortisone and spontaneous GH secretion in patients with classical 21-hydroxylase deficiency.

Patients

Thirty six patients (M: 13, F: 23; median age: 12.3 yrs, range: 6.1 – 18.8 yrs) were recruited to participate in this study. They consisted of 14 prepubertal, 19 pubertal and 3 postpubertal children and were characterised as adequately or inadequately controlled depending on the 0800h ACTH concentrations determined on the first day of the study. Their clinical characteristics and daily glucocorticoid and mineralocorticoid requirements are summarised in Tables 4 & 5.

Study design

Blood samples for determination of serum cortisol and GH concentrations were collected at 20 min intervals for a total of 24 hours in all patients (day 1). Samples were centrifuged, separated and stored at – 20°C until assayed. During this period patients received their usual replacement therapy with hydrocortisone and 9α-fludrocortisone.
Statistical analysis

To search for a time ordered relationship between cortisol and GH, analysis of correlations between the absolute values of the time series of each hormone was computed at 20 min intervals at various time lags throughout the 24 hour period of the study. All data were stationarized and processed as a 3 point moving average as previously described (Matthews DR, 1988; Diggle PJ, 1990). Cross-correlation was computed after lagging the concentration time-series of GH relative to the concentration time-series of cortisol. If $r_t$ is the coefficient of correlation between cortisol and GH at a lag time $t$ for one patient, then the mean $r_t$ for all patients was considered significant when it exceeded zero by more than two standard errors (SE) of the mean. The SE was calculated from the individual values of $r_t$ for all patients at the lag time $t$. 
D. Assays

i) Cortisol assay
Serum total cortisol was measured using the Coat-A-Count radioimmunoassay (Coat-A-Count, DPC, Los Angeles, U.S.A). This is a solid phase radioimmunoassay with a sensitivity of 6 nmol/L. The within assay coefficients of variation (CsV) were 5.7% and 2.6% at serum concentrations of 28 nmol/L and 552 nmol/L respectively and the between assay CsV were 6.3% and 4.5% at serum concentrations of 138 nmol/L and 276 nmol/L respectively.

ii) CBG assay
Corticosteroid binding globulin was assayed with the CBG-RIA-100 Diagnostic kit (Biosource, Belgium). The minimum detectable concentration of transcortin (CBG) was 0.25 μg/ml. The within assay CsV were 7.7% and 3.3% at serum concentrations of 33.1 μg/ml and 109.4 μg/ml respectively. The between assay CsV were 5.0% and 4.5% at serum concentrations of 31.9 and 105.0 μg/ml respectively.

iii) Free cortisol estimation
Free cortisol was calculated according to Biosource Diagnostics’ protocol using the formula:

$$U = (Z^2 + 0.0122C)^{1/2} - Z \mu M$$

where $$Z = \frac{1}{2K + (T-C)2(1+N)} = 0.0167 + 0.182(T-C) \mu M$$. In this equation U represents the molar concentration of unbound cortisol, C the molar concentration of total cortisol and T the concentration of CBG. K corresponds to the affinity of
transcortin for cortisol at 37°C and N to the proportion of albumin bound to non-CBG bound cortisol.

**iv) GH assay**

GH was measured using the Immunotech immunoradiometric assay (Immunotech, Marseille, France). This assay utilises mouse monoclonal antibodies that recognise the 22kDa GH monomer. The sensitivity of the assay was 0.1mIU/L. The intra-assay CV were 0.7%, 1.5% and 1.3% at serum concentrations of 7.3 mIU/L, 13.6 mIU/L and 56.0 mIU/L respectively. The inter-assay CV were 13.4%, 14.0% and 13.1% at serum concentrations of 6.5 mIU/L, 12.1 mIU/L and 51.4 mIU/L respectively.

**v) 17-OHP assay**

17-OHP was measured using the 17α-hydroxyprogesterone Coat-A-Count assay (Coat-A-Count, DPC, Los Angeles, U.S.A.). This is a solid phase radioimmunoassay with a sensitivity of 0.21 nmol/L. The intra-assay CV were 6.7% and 3.5% at serum concentrations of 0.9 nmol/L and 19.7 nmol/L respectively. The inter-assay CV were 11% and 8.5% at serum concentrations of 1.1 nmol/L and 18.5 nmol/L.

**vi) Androstendione assay**

Androstendione was measured using a radioimmunoassay (Ortho Clinical Diagnostics, Amersham, U.K.) with a sensitivity of 0.35 nmol/L. The intra-assay coefficients of variation were 8.3% and 4.9% at serum concentrations of 2.2 nmol/L and 21.8 nmol/L respectively.
vii) **Testosterone assay**

Serum total testosterone was measured using the Coat-A-Count radioimmunoassay (Coat-A-Count, DPC, Los Angeles, U.S.A.). The sensitivity of the assay was 0.14 nmol/L. The intra-assay CsV were 9.1% and 6.9% at serum concentrations of 2.2 nmol/L and 19.0 nmol/L.

viii) **Insulin assay**

Insulin was assayed using the Diagnostic Systems Laboratories kit (Insulin RIA, DSL-1600, Webster, Texas, U.S.A.). This radioimmunoassay has a sensitivity of 1.3 µIU/ml. The within assay CsV were 8.3% at serum concentration of 4.8 µIU/ml and 6.4% at serum concentration of 54.6 µIU/ml. The between assay CsV were 12.2% at 4.9 µIU/ml and 4.7% at 52.9 µIU/ml.

ix) **Plasma glucose assay**

Whole blood glucose concentrations were measured using the hexokinase method (Cobas Integra Analyser, Roche, Switzerland). The sensitivity of the assay was 0.1 mmol/L. The intra-assay variations were 2.2% and 1.9% at concentrations of 4.4 mmol/L and 12.1 mmol/L respectively.

x) **Leptin assay**

Serum leptin concentrations were measured using a Diagnostic Systems laboratory kit (Human Leptin RIA, DSL-53100, Webster, Texas, U.S.A.). The sensitivity of the assay was 0.18 ng/ml. The intra-assay CsV were 8.7% and 5.5% at serum concentrations of 1.6 ng/ml and 24.7 ng/ml respectively. The inter-assay CsV were 5.2% and 6.3% at 1.6 ng/ml and 25.8 ng/ml respectively.
xi) ACTH assay

The assay used to determine ACTH concentrations (ACTH ¹²⁵I RIA kit, DiaSorin, Minnesota, U.S.A.) was a radioimmunoassay with a sensitivity of 15 pg/ml. The within assay variations were 12.5% and 8.1% at serum concentrations of 69 pg/ml and 600 pg/ml respectively whereas the between assay variations were 6.0% and 6.7% at serum concentrations of 23 pg/ml and 218 pg/ml respectively.

xii) IGF-I assay

IGF-I concentrations were measured using an immunoradiometric assay (IGF-I IRMA, Nichols Institute Diagnostics, California, U.S.A.) with a sensitivity of 6 ng/ml, intra-assay variations of 4.6% and 4.1% at serum concentrations of 61.0 ng/ml and 547.9 ng/ml respectively, and inter-assay variations of 15.8% and 9.3% at serum concentrations of 60.1 ng/ml and 594.3 ng/ml respectively.

xiii) IGF-II assay

IGF-II concentrations were determined using an immunoradiometric assay (DSL-2600 ACTIVE™ IGF-II IRMA kit, Diagnostic Systems Laboratories, Webster, Texas, U.S.A.) with a sensitivity of 12 ng/ml. The intra-assay CsV were 6.5% and 4.7% at serum concentrations of 245 ng/ml and 1432 ng/ml respectively. The inter-assay CsV were 5.3% and 4.5% at serum concentrations of 245 ng/ml and 1383 ng/ml respectively.

xiv) IGFBP-3 assay

IGFBP-3 was measured using an immunoradiometric assay (DSL-6600 ACTIVE™ IGFBP-3 IRMA kit, Diagnostic Systems Laboratories, Webster, Texas, U.S.A.) with a sensitivity of 0.5 ng/ml, intra-assay CsV of 3.9% and 1.8% at serum concentrations of
7.4 ng/ml and 82.7 ng/ml respectively, and inter-assay CsV of 0.6% and 1.9% at 8.0 ng/ml and 76.9 ng/ml respectively.

xv) **PRA assay**

PRA was measured as previously described (Menard J et al., 1972). The assay estimates the amount of angiotensin I generated at 37°C and pH: 5.5 by the action of renin on angiotensinogen and has a sensitivity of 0.1 nmol/h/L. The intra-assay CsV were 5.4%, 5.6% and 7.5% at serum concentrations of 1.2 nmol/h/L, 8.2 nmol/h/L and 13.8 nmol/h/L respectively. The inter-assay CsV were 6.0%, 6.2% and 8.0% at 1.2 nmol/h/L, 8.2 nmol/h/L and 13.8 nmol/h/L respectively.

xvi) **Urinary steroid profile analysis**

Urinary steroid profile analysis was performed by gas chromatography and mass spectrometry as previously described (Honour JW, 1997; Honour JW et al., 1997). Steroid metabolites measured included androsterone, aetiocholanolone, 17-hydroxy-pregnanolone, pregnanetriol, THF, allo-THF, α-cortol, THE, α-cortolone and β-cortol + β-cortolone.
E. Pharmacokinetics

Clearance and volume of distribution are the two primary pharmacokinetic parameters in terms of fundamental physiological processes, whereas the half life is a composite parameter derived from clearance and volume of distribution.

i) Clearance

Clearance (CL) describes the efficiency of irreversible elimination of a drug from the body and is defined as ‘the volume of blood cleared of the drug per unit time’ (Rowland M et al., 1980a; Birkett DJ, 1998a). Another definition of clearance is that ‘it is the constant relating the concentration of drug in the plasma to the elimination rate’:

\[
\text{elimination rate (mg/h)} = \text{clearance (L/h)} \times \text{plasma drug concentration (mg/L)}
\]

It is apparent that for a given clearance, which is a constant characteristic of a particular drug and a particular patient, the elimination rate varies directly with the plasma drug concentration.

Clearance is the one parameter that determines the maintenance dose rate required to achieve a target plasma concentration and therefore effect at steady state. Steady state is defined as the situation at which the rate of drug administration equals the rate of drug elimination so that the amount of drug in the body, and therefore the plasma drug concentration, remains constant (Birkett DJ, 1998a). When frequent blood samples are taken following a single intravenous dose of a drug, clearance can be calculated
following estimation of the area under the drug concentration versus time curve from
time zero to infinite time: \( CL = \frac{dose\ (iv)}{AUC_{0-\inf}} \)

**ii) Volume of distribution**

The volume of distribution (\( V \)) is the parameter relating the concentration of a drug in the plasma to the total amount of drug in the body. It is an imaginary volume that is mainly determined by the relative strength of binding of the drug to tissue components as compared with plasma proteins (Birkett DJ, 1998b). If a drug is tightly bound by the tissues, most of the drug in the body will be held in the tissues and very little in the plasma, so that the drug will appear to be dissolved in a large volume and the volume of distribution will be large. Conversely, if the drug is tightly bound to plasma proteins and not to tissues, the volume of distribution will be small and very close to blood volume.

The simplest method of calculating volume of distribution is by dividing the amount of drug in the body by the extrapolated plasma drug concentration (\( C \)) at \( t = 0 \) min: \( V = \frac{dose\ (iv)}{C_0} \). Alternatively, the volume of distribution can be calculated by dividing clearance by the elimination rate constant: \( V = \frac{CL}{k} \). The volume of distribution is the pharmacokinetic parameter used to calculate the loading dose of a drug required to get close to steady state more quickly:

\[
\text{Loading dose (mg)} = V (L) \times \text{Target plasma concentration (mg/L)}
\]

The rate of distribution from or to the site of action of a drug may determine the onset of adverse effects attributed to the given drug.
### iii) Half life

Half life \((t^{1/2})\) is the time taken for the amount of the drug in the body or the plasma concentration to fall by half. The elimination of a drug is usually an exponential (logarithmic) process so that a constant proportion of the drug is eliminated per unit time (Rowland M et al., 1980b; Birkett DJ, 1998c). The fall in plasma drug concentration after a single dose is described by the expression: 

\[ C_t = C_0 \cdot e^{-kt} \]

where \(C_t\) is the concentration at various times \((t)\) after the dose, \(C_0\) is the initial concentration at time zero and \(k\) is the elimination rate constant. The elimination rate constant is a proportionality constant expressing the proportion of drug in the body eliminated per unit time. Solving the above equation when the plasma concentration of the drug has fallen by half gives:

\[
C_{t/2} = \frac{C_0}{2} = C_0 \cdot e^{-kt/2} \quad \Rightarrow \quad 1/2 = e^{-kt/2} \quad \Rightarrow \\

k \frac{t^{1/2}}{2} = 0.693 = \frac{1}{k} \quad \Rightarrow \quad \frac{k t^{1/2}}{2} = 0.693 \Rightarrow \quad t^{1/2} = \frac{0.693 \cdot V}{CL} \quad \Rightarrow \quad k = \frac{CL}{V}
\]

Alternatively, since half life is the time taken for the concentration of a drug to fall by half, for example from 4 to 2, the slope of the \(\log_e\) concentration-time line can be calculated as follows:

\[
k = \frac{(\log_e 4 - \log_e 2)}{t^{1/2}} = \frac{(1.386 - 0.693)}{t^{1/2}} \Rightarrow
\]
\[ k = \frac{0.693}{t^{\frac{1}{2}}} \Rightarrow t^{\frac{1}{2}} = 0.693 \cdot \frac{V}{CL} \]

Half life is a composite pharmacokinetic parameter determined by both clearance and volume of distribution: \(t^{\frac{1}{2}} = 0.693 \cdot \frac{V}{CL}\) and it is therefore increased by an increase in volume of distribution or a decrease in clearance and vice versa. It is not a good measure, however, of changes in the efficiency of elimination of drugs, since in disease states such as renal or hepatic failure, clearance and volume of distribution may change in the same direction, exerting opposing effects on half life which may, therefore not change.

**iv) Bioavailability**

Bioavailability (F) of a drug is the fraction of the dose which reaches the systemic circulation as intact drug and is dependent upon drug absorption from the gastrointestinal tract into the portal circulation and first-pass hepatic clearance:

\[ F = f_g \cdot f_H = f_g \cdot (1 - E_H) \]

where \(f_g\) is the fraction absorbed, \(f_H\) is the fraction that escapes removal by the liver at first-pass clearance and \(E_H\) is the hepatic extraction ratio (Rowland M et al., 1980b; Birkett DJ, 1998d). Low hepatic extraction ratio drugs are poorly extracted by the liver, nearly all the dose gets through the liver first pass and their bioavailability is essentially complete as long as they are well absorbed from the gut. On the contrary, high hepatic extraction ratio drugs are efficiently extracted by the liver on the first pass through the liver and only a minor proportion of the administered dose reaches the systemic
circulation intact. The bioavailability of these drugs is therefore low even when absorption is complete (Rowland M et al., 1980b).

Absolute bioavailability is the bioavailability measured against an intravenous reference dose, since the bioavailability of an intravenous dose is 100% by definition. Absolute bioavailability can be calculated following estimation of the AUC following oral and intravenous administration of the same dose of the drug: \( F = \frac{AUC_{oral}}{AUC_{iv}} \) where \( AUC_{oral} \) is the AUC following oral administration and \( AUC_{iv} \) is the AUC following intravenous administration of the drug. Alternatively, when different oral and intravenous doses are used bioavailability can be calculated following correction of the AUC for the dose given:

\[
F = \frac{AUC_{oral} \times Dose_{iv}}{AUC_{iv} \times Dose_{oral}} \Rightarrow
\]

\[
F = \frac{CL_{iv} \times AUC_{oral}}{Dose_{oral}} \Rightarrow
\]

\[
F = \frac{CL_{iv}}{CL_{oral}}
\]

where \( CL_{iv} = \frac{Dose_{iv}}{AUC_{iv}} \) is the true clearance of the drug estimated following intravenous administration of the drug and \( CL_{oral} = \frac{Dose_{oral}}{AUC_{oral}} \) is the apparent clearance of the drug estimated after oral administration (Rowland M et al., 1980c; Birkett DJ, 1998a; Birkett DJ, 1998d). The bioavailability of an oral formulation (test formulation) which is assessed against a second oral (reference) formulation is referred to as relative bioavailability (Birkett DJ, 1998d).
F. Statistical analyses

Parametric methods were used to compare data between groups following logarithmic transformation. All analyses were performed using the Statistical Package for Social Sciences (SPSS) (Norusis MJ, 1990). The main principles of the statistical methods (Campbell M et al., 1993; Kirkwood BR, 1988) used for the analysis of the data are outlined below:

i) Unpaired t-test

This test was used to compare data between two independent groups. It requires that the population distribution is normal and its validity depends on the equality of the two population standard deviations. When the population standard deviations of the two groups are different it is recommended that the first approach should be to seek a suitable change of scale to remedy this, so that the t test can be used. For example, if the standard deviations seem to be proportional in size to the means, then taking logarithms of the individual values may help. Alternatively, a non-parametric test may be used.

ii) One-way analysis of variance

One-way analysis of variance (ANOVA) was used to compare the means of several groups. The method is based on assessing how much of the overall variation in the data is attributable to differences between the group means, and comparing this with the amount attributable to differences between individuals in the same group.
Initially, the variance of all observations is calculated, ignoring their subdivision into groups. Variance is the square of the standard deviation and equals the sum of squared deviations of the observations about the overall mean, divided by the degrees of freedom. One-way analysis of variance partitions this sum of squares into two distinct components:

(a) The sum of squares due to differences between the group means.
(b) The sum of squares due to differences between the observations within each group – the residual sum of squares.

The total degrees of freedom are similarly divided. The amount of variation per degree of freedom is called the mean square. The significance test for differences between the groups is based on a comparison of the between-groups and within-groups mean squares.

One-way analysis of variance also requires that the data are normally distributed and that the population value for the standard deviation between individuals is the same for each group. This is estimated by the square root of the within-groups mean square. If the standard deviations are unequal the data need to be transformed.

**iii) Linear regression**

This method is used to investigate the linear association between two continuous variables. The correlation coefficient provides a measure of closeness of an association, while linear regression gives the equation of the straight line that describes how the $y$ variable increases or decreases with an increase in $x$ variable. The equation of the regression is: $y = a + bx$, where $a$ is the intercept and $b$ the slope of the line. The slope $b$
is sometimes called the regression coefficient and has the same sign as the correlation coefficient.

**iv) Stepwise multiple regression analysis**

Stepwise multiple regression analysis is used to explore situations were the y variable might depend on more than one independent variables/factors. In that case the corresponding model is:

\[ y = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \ldots + \beta_k x_k \]

where \( x_1 \) is the first independent variable, \( x_2 \) is the second, and so on up to the \( k^{th} \) independent variable \( x_k \). Although these variables are called independent, they need not be independent of one another. The term \( \alpha \) is the intercept or constant term and is the value of \( y \) when all the independent variables are zero.

In stepwise multiple linear regression simple linear regressions are carried out on each of the explanatory independent variables. The one accounting for the largest percentage of variation is chosen and kept as the first variable. Multiple regressions with two variables are then carried out adding each of the other explanatory variables in turn. The two-variable regression accounting for the largest percentage is chosen. The process continues, an additional variable being selected at each stage. It stops when the extra variation accounting for by adding a further variable is non-significant for all the remaining variables. Data are reported as the regression equation, the correlation coefficient of the relationship and the standard error of every parameter estimate.
G. Time Series Analysis Methods

Adequate description of the secretion of a hormone known to be released in pulsations requires a number of measurements performed at regular and frequent intervals - 'a time series'. The dissection into attributes of pulsatility patterns of certain hormones using time series data derived from several subjects is known as 'time series analysis' (Matthews DR, 1988; Diggle PJ, 1990). A number of factors need to be taken into consideration when collecting data and/or prior to such analysis:

Signal to noise ratio: Within any data array there is a signal – the ‘real’ hormone oscillation – and ‘noise’ (e.g. errors due to sampling technique or assay). The signal to noise ratio must be sufficient to allow detection of the peak. This means that the trough – to – peak height needs to exceed three times the standard error (SEM) of the assay estimation of either the peak or trough. The SEM of the estimate can be improved by assaying the samples repeatedly or by improving the coefficient of variation (precision) of the assay.

Sampling interval: A sufficiently frequent sampling interval is particularly important since mismatching of infrequent sampling intervals to the predominant true period of pulsatility of the hormone under observation may lead to erroneous conclusions.

Duration of time series: The total duration of sampling is also important because it affects the precision of the estimate of the frequency of pulsatility. The greater the duration of the sampling the more precise the estimate.

Discrete and integrated sampling: Compared to discrete sampling that may miss a peak or trough, integrated sampling (continuous withdrawal of blood into aliquots) is a more
reliable way to assess oscillations as peaks are always embraced, though their amplitude may be underestimated.

**Stationarization:** This is a technique required to remove the trend from the data before analysing the oscillatory characteristics of a hormone. A stationary data array is one in which there is no progressive shift in the baseline with time. There are two main types of stationarization, ‘linear’, which removes the trend by subtracting the regression slope and ‘differencing’ which uses the difference between successive data points instead of the original data.

**Cross-correlation**

This function determines whether there is a non-random relationship between values of two different hormones. If the release of one hormone is regulated by the other then one would expect the concentration time-series of the former hormone to follow in time the concentration time-series of the latter. For this reason, the data array of one hormone is correlated with the data array of the other hormone which is progressively lagged in time.

The cross correlation function is based on principles similar to those applied in the linear association between two continuous variables. When the lag is such that the peaks and troughs of one hormone coincide with the peaks and troughs respectively of the other hormone the correlation coefficient is +1, whereas when the peaks of one hormone coincide with troughs of the other hormone and vice versa the correlation coefficient is −1. Results that are not significantly different from zero indicate that there
is no systematic relationship between the two hormones and that any observed alterations in hormone concentrations are random.

The following section (Results) presents the results of all investigations performed. Results are presented under the same six studies described in the Methodology section.
RESULTS
STUDY 1: PHARMACOKINETIC PARAMETERS OF TOTAL AND FREE CORTISOL

Pharmacokinetics of total cortisol

Initial analysis included all prepubertal, pubertal and postpubertal patients with classical 21-hydroxylase deficiency and was followed by comparisons between adequately and inadequately controlled prepubertal and pubertal patients. The pharmacokinetic parameters of total and free cortisol in all patients are summarised in Table 1.

All CYP21 deficient patients

The serum total cortisol clearance curve was monoexponential (Figure 1). A maximum serum total cortisol concentration ($C_{\text{max}}$) of 2700 nmol/L was achieved at a maximum time of ($t_{\text{max}}$) 10 min. Mean peak total cortisol concentration was 1844.7 nmol/L (SD: 592.7). The mean clearance of total cortisol was 248.7 ml/min (SD: 100.6) in the prepubertal, 427.0 ml/min (SD: 133.4) in the pubertal and 292.4 ml/min (SD: 106.3) in the postpubertal patients (one-way ANOVA, $F = 9.8$, $P<0.001$). Mean total cortisol clearance was significantly higher in the pubertal group as compared to the prepubertal and postpubertal groups (SNK, $P<0.05$) but there was no significant difference between prepubertal and postpubertal groups (Figure 2a). Similarly, mean total cortisol clearance corrected for body mass index (BMI) was higher in the pubertal than the prepubertal and postpubertal patients (ANOVA, $F = 5.0$, $P = 0.012$; SNK, $P<0.05$) with no significant difference between prepubertal and postpubertal groups.
Table 1: Pharmacokinetic parameters of total and free cortisol

<table>
<thead>
<tr>
<th></th>
<th>Prepubertal</th>
<th>Pubertal</th>
<th>Postpubertal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Cortisol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL (ml/min)</td>
<td>248.7 (100.6)</td>
<td>427.0 (133.4)</td>
<td>292.4 (106.3)</td>
</tr>
<tr>
<td>CL/BMI (ml<em>m^2/kg</em>min)</td>
<td>12.5 (4.0)</td>
<td>16.8 (5.8)</td>
<td>11.0 (3.5)</td>
</tr>
<tr>
<td>V (L)</td>
<td>27.1 (8.4)</td>
<td>49.5 (12.2)</td>
<td>40.8 (16.0)</td>
</tr>
<tr>
<td>V/BMI (L*m^2/kg)</td>
<td>1.4 (0.3)</td>
<td>1.9 (0.4)</td>
<td>1.5 (0.5)</td>
</tr>
<tr>
<td>Half life</td>
<td>80.2 (19.4)</td>
<td>84.5 (24.9)</td>
<td>96.7 (10.0)</td>
</tr>
</tbody>
</table>

| **Free Cortisol**    |             |             |              |
| CL (ml/min)          | 2477.4 (988.6) | 4787.7 (2386.8) | 3001.8 (1090.4) |
| CL/BMI (ml*m^2/kg*min) | 127.1 (50.8)   | 190.2 (95.6)  | 11.0 (3.5)   |
| V (L)                | 237.0 (95.3)  | 540.7 (493.3) | 276.6 (116.4) |
| V/BMI (L*m^2/kg)     | 12.1 (4.5)    | 21.6 (17.8)   | 10.1 (3.2)   |
| Half life            | 67.2 (9.4)    | 77.1 (44.4)   | 62.5 (6.6)   |

Data shown are means with standard deviation (SD) values in parentheses

*F: Cortisol*

*CL: Clearance*

*V: Volume of distribution*

*BMI: Body mass index*
Figure 1: Serum total cortisol concentrations achieved following intravenous bolus injection of hydrocortisone (15 mg/m² body SA) in a prepubertal patient. The total cortisol clearance curve is best described as monoexponential (lower panel: logarithmic scale).
Figure 2a: Clearance of total cortisol in all CYP21 deficient patients. There was a significant increase in cortisol clearance at puberty.
The mean volume of distribution was 27.1 L (SD: 8.4) in the prepubertal, 49.5 L (SD: 12.2) in the pubertal and 40.8 L (SD: 16) in the postpubertal (ANOVA, $F = 15.2$, $P < 0.001$) (Figure 2b) patients. The mean volume of distribution in the pubertal patients was significantly greater than the mean volume of distribution in the prepubertal ones (SNK, $P < 0.05$), whereas comparison between prepubertal and postpubertal as well as pubertal and postpubertal groups did not reach statistical significance. Similar observations were made when mean volume of distribution was corrected for BMI (ANOVA, $F = 7.0$, $P = 0.003$).

The mean total cortisol half life was 80.2 min (SD: 19.4) in the prepubertal group of patients, 84.5 min (SD: 24.9) in the pubertal and 96.7 min (SD: 10.0) in the postpubertal group. Comparison between groups showed no significant difference in total cortisol half life (ANOVA, $F = 1.2$, $P = 0.3$) (Figure 2c).

**Adequately controlled patients**

To exclude a possible effect of ACTH on cortisol metabolic clearance rate, data were analysed including the adequately controlled patients only. The postpubertal patients have been excluded from analysis because of the small sample size.

The mean total cortisol clearance was 232.9 ml/min (SD: 99.5) in the prepubertal and 409.3 ml/min (SD: 122.5) in the pubertal adequately controlled patients ($P = 0.002$). Total cortisol clearance corrected for BMI was also significantly higher in the pubertal group ($P < 0.05$). The mean volume of distribution was 25.1 L (SD: 7.3) in prepubertal patients and 49.2 L (SD: 11.6) in pubertal patients ($P < 0.001$) and the significance
Figure 2b: Volume of distribution of total cortisol in prepubertal, pubertal and postpubertal patients. A significant increase at puberty was noted.
Figure 2c: Half life of total cortisol in prepubertal, pubertal and postpubertal patients. There was no significant difference between groups of patients.
remained after correction for BMI (P = 0.004). There was no difference in the mean half life of total cortisol between prepubertal (mean: 80.6 min, SD: 22.0) and pubertal (mean: 86.3 min, SD: 22.1) patients.

**Effect of control on total cortisol pharmacokinetics**
There was no significant difference in mean serum total cortisol clearance, volume of distribution and half life between adequately and inadequately controlled prepubertal or pubertal patients. This effect persisted when cortisol clearance and volume of distribution were corrected for BMI.

**Effect of gender on total cortisol pharmacokinetics**
There was no significant difference in total cortisol clearance between males (mean: 386.5 ml/min, SD: 138.5) and females (mean: 321.8 ml/min, SD: 28.3). However, when corrected for BMI, cortisol clearance was found to be higher in males than females (P = 0.04). Similarly, although there was no significant difference in the volume of distribution between males (mean: 45.7 L, SD: 3.9) and females (mean: 37.5 L, SD: 15.2), when adjusted for BMI, the volume of distribution was found to be greater in males than females (P = 0.01). There was no difference in the half life of total cortisol between males (mean: 84.9 min, SD: 25.8) and females (mean: 84.7 min, SD: 19.6).
Pharmacokinetics of free cortisol

All CYP21 deficient patients

The free cortisol clearance curve was also monoexponential (Figure 3). Mean peak free cortisol concentration was 379.6 nmol/L (SD: 205.6). The mean clearance of free cortisol was significantly higher in the pubertal patients (mean: 4787.7 ml/min, SD: 2386.8) compared to the prepubertal (mean: 2477.4, SD: 988.6) and postpubertal (3001.8, SD: 1090.4) patients (one-way ANOVA, $F = 6.9$, $P = 0.003$; SNK, $P<0.05$) (Figure 4a). Free cortisol clearance corrected for BMI was higher in the pubertal than the prepubertal (ANOVA, $F = 4.2$, $P = 0.023$; SKN, $P<0.05$) but not than the postpubertal group.

The mean volume of distribution was significantly higher in the pubertal patients (mean: 540.7 L, SD: 493.3) than the prepubertal (mean: 237.0 L, SD: 95.3) but not than the postpubertal (mean: 276.6 L, SD: 116.4) ones (ANOVA, $F = 3.3$, $P = 0.048$; SNK, $P<0.05$) (Figure 4b). However, when the volume of distribution was corrected for BMI, comparison between groups did not reach statistical significance (ANOVA, $F = 3.0$, $P = 0.06$).

Finally, the half life of free cortisol was 67.2 min (SD: 9.4) in the prepubertal, 77.1 min (SD: 44.4) in the pubertal and 62.5 min (SD: 6.6) in the postpubertal patients. Comparison between groups suggested no significant difference in half life (ANOVA, $F = 0.6$, $P = 0.52$) (Figure 4c).
Figure 3: Serum free cortisol concentrations achieved following an intravenous bolus of hydrocortisone (15 mg/m² body SA) in a prepubertal patient. The free cortisol clearance curve is best described as monoexponential (lower panel: logarithmic scale).
Figure 4a: Free cortisol clearance in prepubertal, pubertal and postpubertal CYP21 deficient patients. There was a significant increase in cortisol clearance at puberty.
Figure 4b: Volume of distribution of free cortisol in prepubertal, pubertal and postpubertal CYP21 deficient patients. There was a significant increase in the volume of distribution at puberty.
Figure 4c: Half life of free cortisol in prepubertal, pubertal and postpubertal CYP21 deficient patients. There was no significant difference between groups of patients.
**Adequately controlled patients**

In adequately controlled patients the mean free cortisol clearance was significantly higher in the pubertal (mean: 4832.7 ml/min, SD: 1638.7) compared to the prepubertal (mean: 2405.5 ml/min, SD: 1039.8) group (P = 0.001) and the difference persisted after correction for BMI (P<0.02). The mean volume of distribution was also higher in the pubertal (mean: 414.2 L, SD: 110.1) than the prepubertal patients (mean: 238.3 L, SD: 107.0) (P = 0.002) but not so when corrected for BMI. No significant difference in the mean half life of free cortisol between prepubertal (mean: 69.1 min, SD: 10.0) and pubertal (mean: 61.0 min, SD: 9.9) patients was noted.

**Effect of control on free cortisol pharmacokinetics**

The mean clearance and volume of distribution of free cortisol did not differ between adequately and inadequately controlled prepubertal or pubertal patients, even when corrected for BMI. No significant difference was noted in half life of free cortisol between adequately and inadequately controlled patients.

**Effect of gender on free cortisol pharmacokinetics**

Free cortisol clearance did not differ significantly between males (mean: 4148.8 ml/min, SD: 1676.6) and females (Mean: 3475.6 ml/min, SD: 2312.9), even when corrected for BMI. The volume of distribution of free cortisol was not different between male (mean: 550.1 L, SD: 567.2) and female (mean: 311.1 L, SD: 196.5) patients. However, when adjusted for BMI, the volume of distribution was higher in males than females (P = 0.02). The half life of free cortisol was significantly shorter in females (mean: 64.0 min, SD: 15.1) compared to males (mean: 85.3 min, SD: 48.3) (P = 0.04) (Figure 5).
Figure 5: Half life of free cortisol in male and female patients with classical 21-hydroxylase deficiency. Half life was significantly shorter in females compared to males.
STUDY 2: CORTISOL PHARMACOKINETICS AND PUBERTY: INDEPENDENT PREDICTORS OF TOTAL AND FREE CORTISOL CLEARANCE AND HALF LIFE

**Descriptive statistics:** The pharmacokinetic parameters determined in all patients are summarised in Table 1. The biochemical parameters of the patients are outlined in Table 2. IGF-I concentrations were significantly higher in the pubertal than prepubertal and postpubertal patients (one-way ANOVA: F = 12.1, p = 0.001; SNK, p<0.05). IGF-II concentrations were found to be significantly raised in the postpubertal group compared to the prepubertal and pubertal groups (ANOVA: F = 6.7, p = 0.003; SNK, p<0.05). No difference between groups was noted in IGFBP-3 concentrations. Tetrahydrocortisone (THE) concentrations were higher in the pubertal than the prepubertal but not than the postpubertal patients (ANOVA: F = 4.8, p = 0.014, SNK, p<0.05). ACTH concentrations, integrated 17-OHP and GH concentrations, CBG, fasting insulin and glucose concentrations, leptin, tetrahydrocortisol (THF) + allo-THF, (THF + allo-THF)/THE ratio and allo-THF/THF ratio did not differ between the three groups of patients.

**Stepwise multiple linear regression analysis:** When all biochemical parameters were considered, stepwise multiple linear regression analysis indicated that IGF-I, gender and integrated GH concentrations were independently related to total cortisol clearance corrected for BMI ($r^2 = 0.53$, p<0.001) (Table 3, Figure 6). Free cortisol clearance corrected for BMI was found to be independently related to IGFBP-3, integrated GH concentrations, gender and leptin concentrations ($r^2 = 0.64$, p<0.001) (Table 3).
<table>
<thead>
<tr>
<th></th>
<th>Prepubertal</th>
<th>Pubertal</th>
<th>Postpubertal</th>
<th>One-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F</td>
</tr>
<tr>
<td>CBG (µg/ml)</td>
<td>46.3 (30.0-60.0)</td>
<td>41.3 (32.5-60.0)</td>
<td>45.0 (35.0-115)</td>
<td>2.2</td>
</tr>
<tr>
<td>ACTH (pg/ml)</td>
<td>53.5 (24.9-228.0)</td>
<td>123.3 (17.3-856)</td>
<td>92.9 (32.4-624)</td>
<td>2.1</td>
</tr>
<tr>
<td>24h 17-OHP AUC (nmol*min/L)</td>
<td>6460.5 (450-501000)</td>
<td>49605 (482-923690)</td>
<td>2670.6 (1831-161500)</td>
<td>2.6</td>
</tr>
<tr>
<td>24h GH AUC (mIU*min/L)</td>
<td>5556 (2710-20825)</td>
<td>6257 (1736-29967)</td>
<td>6237 (4056-7514)</td>
<td>0.001</td>
</tr>
<tr>
<td>IGF-I (ng/ml)</td>
<td>196.8 (91.9-484.7)</td>
<td>346.6 (212.1-628.4)</td>
<td>243.4 (176.1-336.7)</td>
<td>12.1</td>
</tr>
<tr>
<td>IGF-II (ng/ml)</td>
<td>650.9 (486.7-891.5)</td>
<td>626.6 (431.2-821.6)</td>
<td>796.0 (658.0-1512.7)</td>
<td>6.7</td>
</tr>
<tr>
<td>IGFBP-3 (ng/ml)</td>
<td>3315.0 (2260.4-4813.4)</td>
<td>4038.9 (1476.4-5352.8)</td>
<td>4279.5 (3064.3-6212.2)</td>
<td>2.8</td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>8.5 (3.2-34.9)</td>
<td>10.9 (3.7-23.7)</td>
<td>17.4 (8.5-23.3)</td>
<td>2.2</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.2 (3.8-6.0)</td>
<td>4.2 (3.5-5.3)</td>
<td>4.3 (4.1-5.7)</td>
<td>0.4</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>10.5 (3.1-35)</td>
<td>20.1 (1.6-71.6)</td>
<td>19.2 (9.4-27.9)</td>
<td>1.4</td>
</tr>
<tr>
<td>THF + allo-THF (µg/day)</td>
<td>2380 (200-5170)</td>
<td>3790 (30-9130)</td>
<td>2010 (1590-2930)</td>
<td>2.7</td>
</tr>
<tr>
<td>THE (µg/day)</td>
<td>2575 (210-4480)</td>
<td>3920 (1040-11780)</td>
<td>1980 (1510-2090)</td>
<td>4.8</td>
</tr>
<tr>
<td>(THF + allo-THF) / THE ratio</td>
<td>1.0 (0.9-1.6)</td>
<td>0.8 (0.6-2.0)</td>
<td>1.0 (1.0-1.4)</td>
<td>1.5</td>
</tr>
<tr>
<td>Allo-THF(5α) / THF(5β) ratio</td>
<td>1.2 (0.2-3.0)</td>
<td>0.9 (0.4-2.0)</td>
<td>1.2 (0.8-3.9)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Data shown are medians with range in parentheses

NS: not significant
Figure 6: Linear correlation between total cortisol clearance corrected for body mass index (CL/BMI) and (a) IGF-I concentrations and (b) GH concentrations.
Table 3: Stepwise multiple linear regression model for independent predictors of total and free cortisol CL/BMI

<table>
<thead>
<tr>
<th>Variables</th>
<th>Beta</th>
<th>SE</th>
<th>Adjusted $r^2$</th>
<th>P value</th>
</tr>
</thead>
</table>

**DEPENDENT VARIABLE: Total cortisol CL/BMI**

*Independent variables:*

- IGF-I 0.469 0.128 0.39 < 0.001
- Gender (M=0, F=1) -0.281 0.041 0.46 < 0.001
- 24h GH AUC 0.320 0.219 0.53 < 0.001

**DEPENDENT VARIABLE: Free cortisol CL/BMI**

*Independent variables:*

- IGFBP-3 0.378 0.229 0.29 0.001
- 24h GH AUC 0.637 0.105 0.40 < 0.001
- Gender (M=0, F=1) -0.444 0.049 0.50 < 0.001
- Leptin 0.470 0.064 0.64 < 0.001

CL: Clearance
BMI: Body mass index

Beta: Standardised coefficients
SE: Standard error of the estimate
$r^2$: Adjusted R square
Integrated 17-OHP concentrations were related to ACTH concentrations and fasting glucose concentrations ($r^2 = 0.49$, $p<0.001$). Leptin concentrations correlated positively with BMI and integrated 17-OHP concentrations ($r^2=0.66$, $p<0.001$).

When stepwise multiple linear regression was used to identify independent predictors of total cortisol half life it became evident that $(\text{THF} + \text{allo-THF})/\text{THE}$ ratio, integrated 17-OHP concentrations, ACTH and IGFBP-3 were related to half life ($r^2 = 0.55$, $p<0.001$) (Figure 7). Free cortisol half life was best predicted by CBG concentrations ($r^2 = 0.14$, $p = 0.02$) (Table 4).

**Figure 7**: Linear correlation between total cortisol half life and the $(\text{THF}+\text{allo-THF})/\text{THE}$ ratio, which represents an index of overall activity of $11\beta$-hydroxysteroid dehydrogenase.
Table 4: Stepwise multiple linear regression model for independent predictors of total and free cortisol half life

<table>
<thead>
<tr>
<th>Variables</th>
<th>Beta</th>
<th>SE</th>
<th>Adjusted $r^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DEPENDENT VARIABLE: Total cortisol half life</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Independent variables:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(THF + allo-THF) / THE</td>
<td>0.471</td>
<td>0.108</td>
<td>0.27</td>
<td>0.001</td>
</tr>
<tr>
<td>24h 17-OHP AUC</td>
<td>0.692</td>
<td>0.017</td>
<td>0.34</td>
<td>0.001</td>
</tr>
<tr>
<td>ACTH</td>
<td>-0.519</td>
<td>0.041</td>
<td>0.48</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>-0.290</td>
<td>0.128</td>
<td>0.55</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>DEPENDENT VARIABLE: Free cortisol half life</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Independent variables:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBG</td>
<td>-0.411</td>
<td>0.307</td>
<td>0.14</td>
<td>0.017</td>
</tr>
</tbody>
</table>

*F*: Cortisol  
*CL*: Clearance  
*V*: Volume of distribution  
*Beta*: Standardised coefficients  
*SE*: Standard error of the estimate  
*$r^2$: Adjusted R square
STUDY 3: THE BIOAVAILABILITY OF ORAL HYDROCORTISONE

All pharmacokinetic parameters estimated in the sixteen patients participated in this study are summarised in Table 5. After oral administration of the morning hydrocortisone dose, maximum total cortisol concentration ($C_{\text{max}}$) of 2520 nmol/L was achieved at $t_{\text{max}}$ of 20 min. Serum total cortisol concentrations reached a median peak of 729.5 nmol/L (range: 492 - 2520 nmol/L) at 1.2h (range: 0.3 - 3.3h) and declined monoexponentially thereafter to reach undetectable concentrations 7h (5 - 12h) after administration of oral hydrocortisone (Figure 8).

After oral administration of hydrocortisone in the evening, $C_{\text{max}}$ of 736 nmol/L was observed at $t_{\text{max}}$ of 180 min. Serum total cortisol concentrations reached a median peak of 499 nmol/L (range: 333 - 736 nmol/L) also at 1.2h (range: 0.3 - 3.0h) and subsequently declined gradually to reach undetectable concentrations at 9h (range: 5 - 12h) after administration of oral hydrocortisone.

Following intravenous administration of hydrocortisone, $C_{\text{max}}$ of 2700 nmol/L was achieved at $t_{\text{max}}$ of 10 min. Median peak cortisol concentration was 1930 nmol/L (range: 1124-2700 nmol/L) and was observed at 10 min (range: 10-20 min). Serum cortisol concentrations fell rapidly thereafter and reached undetectable levels 6h after intravenous hydrocortisone administration (Figure 8). Cortisol concentrations before oral and intravenous administration of hydrocortisone were undetectable in all patients.
Table 5: Pharmacokinetic parameters of oral and intravenous hydrocortisone

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>Oral</th>
<th>Intravenous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (range)</td>
<td>Median (range)</td>
</tr>
<tr>
<td><strong>Morning</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak F concentration (nmol/L)</td>
<td>729.5 (492 - 2520)</td>
<td>1930 (1124-2700)</td>
</tr>
<tr>
<td>Time of peak concentration</td>
<td>1.2 h (0.3 – 3.3 h)</td>
<td>10 min (10-20 min)</td>
</tr>
<tr>
<td>AUC (nmol/L*h)</td>
<td>279627 (167254 - 462796)</td>
<td>209551.5(158821- 337737)</td>
</tr>
<tr>
<td>Cmax (nmol/L)</td>
<td>2520</td>
<td>2700</td>
</tr>
<tr>
<td>tmax (min)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Absolute Bioavailability (%)</td>
<td>94.2 (90%CI: 82.8 – 105.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Evening</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak F concentration (nmol/L)</td>
<td>499 (333 – 736)</td>
<td></td>
</tr>
<tr>
<td>Time of peak concentration</td>
<td>1.2h (0.3 – 3.0h)</td>
<td></td>
</tr>
<tr>
<td>AUC (nmol/L*h)</td>
<td>112775 (64000 – 250540)</td>
<td></td>
</tr>
<tr>
<td>Cmax (nmol/L)</td>
<td>736</td>
<td></td>
</tr>
<tr>
<td>tmax (min)</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Apparent Bioavailability (%)</td>
<td>128 (90% CI: 119 – 138)</td>
<td></td>
</tr>
</tbody>
</table>

F: Cortisol
AUC: Area under the curve
Cmax: Maximum concentration of cortisol
tmax: Time that Cmax was observed
Figure 8: Median serum total cortisol concentrations following oral and intravenous administration of hydrocortisone in the morning. Cortisol concentrations decline monoexponentially and reach undetectable levels 6 – 7 hours after administration.

The absolute bioavailability of oral hydrocortisone in the morning (F_m) was 94.2% (90% CI: 82.8 – 105.5%) whereas the apparent bioavailability in the evening (F_ev) was estimated to be 128% (90% CI: 119 – 138%). Since F_m = CL_{iv} / CL_{oralm} and F_ev = CL_{iv} / CL_{oralev}, it is evident that: F_m/F_ev = CL_{oralev}/CL_{oralm} \Rightarrow CL_{oralev} = F_m * CL_{oralm} / F_ev \Rightarrow CL_{oralev} = 0.74 * CL_{oralm} and therefore the clearance of hydrocortisone in the evening is 26% lower than the hydrocortisone clearance in the morning.
STUDY 4: SERUM CORTISOL AND 17-HYDROXYPROGESTERONE INTERRELATIONSHIP: IS CURRENT REPLACEMENT THERAPY SATISFACTORY?

24 hour cortisol and 17-OHP profiles

The 24 hour, daytime and night-time mean concentrations of cortisol and 17-OHP concentrations in adequately and inadequately controlled patients are listed in Table 6 (Figures 9 & 10). In adequately controlled patients the 24h mean cortisol concentration was 107 nmol/L (SD: 59). Daytime (0400 – 1600h) and night-time (1600 – 0400h) mean concentrations were 206 nmol/L (SD: 163) and 164 nmol/L (SD: 121) respectively. In inadequately controlled patients the 24h mean cortisol concentration was 112 nmol/L (SD: 70) and the daytime and night-time mean concentrations were 185 nmol/L (SD: 138) and 138 nmol/L (SD: 84) respectively.

In the adequately controlled patients, mean 24h 17-OHP concentration was 2 nmol/L (SD: 2.8) and daytime and night-time mean concentrations were 2.5 nmol/L (SD:3.8) and 1.1 nmol/L (SD: 1.2) respectively. 17-OHP concentrations remained suppressed (<20 nmol/L) throughout the 24 hour period, with no variation in response to alterations in cortisol concentrations and reached their peak values between 0400h and 0900h (Figure 9b).

In the inadequately controlled patients, mean 17-OHP concentration over the 24 hour period was 147 nmol/L (SD: 210). Daytime and night-time mean concentrations were
### Table 6: Biochemical parameters in CYP21 deficient patients

<table>
<thead>
<tr>
<th></th>
<th>Cortisol (nmol/L)</th>
<th>17-OHP (nmol/L)</th>
<th>Androstendione (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adequately controlled</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hour concentrations</td>
<td>107 (59)</td>
<td>2.0 (2.8)</td>
<td></td>
</tr>
<tr>
<td>Daytime concentrations</td>
<td>206 (163)</td>
<td>2.5 (3.8)</td>
<td></td>
</tr>
<tr>
<td>Night-time concentrations</td>
<td>164 (121)</td>
<td>1.1 (1.2)</td>
<td></td>
</tr>
<tr>
<td>Integrated concentrations</td>
<td>276,779 (69,060)</td>
<td>3104 (3237)</td>
<td></td>
</tr>
<tr>
<td>0800h concentration</td>
<td>0.7 (0.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inadequately controlled</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hour concentrations</td>
<td>112 (70)</td>
<td>147 (210)</td>
<td></td>
</tr>
<tr>
<td>Daytime concentrations</td>
<td>185 (138)</td>
<td>201 (286)</td>
<td></td>
</tr>
<tr>
<td>Night-time concentrations</td>
<td>138 (84)</td>
<td>88 (129)</td>
<td></td>
</tr>
<tr>
<td>Integrated concentrations</td>
<td>237,542 (89,886)</td>
<td>160367 (213093)</td>
<td></td>
</tr>
<tr>
<td>0800h concentration</td>
<td>20.4 (30.8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data shown are means with standard deviations (SD) in parentheses.
Figure 9: Mean serum (a) cortisol and (b) 17-hydroxyprogesterone concentrations in adequately controlled patients. 17-OHP concentrations remained suppressed during the 24 hour period. Y bars represent standard deviation (SD) scores.
201 nmol/L (SD: 286) and 88 nmol/L (SD: 129) respectively. As in the first group, 17-OHP concentrations reached peak values in the early morning hours (0400h – 0900h) and decreased gradually in response to the rise in cortisol concentrations. Mean 17-OHP concentrations remained low between 1600h and 0400h and showed a precipitous rise after 0400h, when cortisol concentrations were undetectable (Figures 10b). During the course of the day (0400h – 1600h) 17-OHP concentrations decreased only in response to elevated cortisol concentrations, whereas during night-time (1600h – 0400h) they remained very low regardless of the level of circulating cortisol concentrations (Figure 11a & 11b).

Androstendione and integrated 17-OHP and cortisol concentrations

Mean 0800h androstendione concentrations were 0.7 nmol/L in the adequately controlled and 20.4 nmol/L in the inadequately controlled patients. Mean integrated 17-OHP and cortisol concentrations were 3,104 nmol*min/L and 276,779 nmol*min/L respectively in the adequately controlled patients and 160,367 nmol*min/L and 237,542 nmol*min/L respectively in the inadequately controlled patients. There was a strong positive correlation between 0800h androstendione concentrations and integrated 17-OHP concentrations (r = 0.81, P < 0.0001). No significant correlation between 0800h androstendione and integrated cortisol concentrations was noted.
Figure 10: Mean serum (a) cortisol and (b) 17-hydroxyprogesterone concentrations in inadequately controlled patients. Peak 17-OHP concentrations reflect increased HPA activity and are observed between 0400h and 0900h. Y bars represent SD values (Different scale used for 17-OHP concentrations).
Figure 11: Mean serum cortisol and 17-OHP concentrations in the inadequately controlled patients. Serum 17-OHP concentrations remained low between 1600h and 0400h regardless of circulating serum cortisol concentrations.
Cross-correlation studies

The graphs depicting the minimum, 1\textsuperscript{st}, 2\textsuperscript{nd} (median), 3\textsuperscript{rd} quartile and maximum coefficients of correlation from the cross-correlation analyses over the 24 hour period between cortisol and 17-OHP values in the inadequately controlled CYP21 deficient patients are shown in Figure 12. The analysis showed a significant negative correlation between cortisol and 17-OHP at lag time 0 min ($r = -0.187$, $P<0.0001$), peaking at lag time 60 min ($r = -0.302$, $P<0.0001$). In addition, a significant positive correlation was observed over time between cortisol and 17-OHP, peaking at lag time 6h 40 min ($r = 0.359$, $P<0.0001$), with cortisol leading 17-OHP by these time intervals (Figure 12a).

![Figure 12](image)

**Figure 12:** Cross-correlation analyses between cortisol and 17-OHP values in the inadequately controlled patients (a) over the 24 hour period.
Figure 12: Cross-correlation analyses between cortisol and 17-hydroxyprogesterone values in the inadequately controlled patients (b) during daytime and (c) during nighttime.
When data were analysed after dividing the 24h profiles into daytime and night-time 12h profiles the following results were obtained: There was a significant negative correlation between daytime cortisol and 17-OHP concentrations (r = -0.586, P<0.001) at lag time 0 min, peaking at lag time 60 min (r = -0.730, P<0.001) (Figure 12b), with cortisol leading 17-OHP at these times. Also, there was a significant negative correlation between night-time cortisol and 17-OHP concentrations (r = -0.436, P<0.001), which reached a peak at lag time 20 min (r = -0.447, P<0.001) (Figure 12c). Finally, a significant positive correlation was noted over time between daytime and night-time cortisol and 17-OHP concentrations, which reached a peak at lag times 2h 20 min and 3h respectively.
Single dose dexamethasone suppression test

Mean 0800h ACTH concentrations in the groups of adequately and inadequately controlled patients were 45.8 pg/ml (SD: 16.8) and 239.6 pg/ml (SD: 228.2) respectively. In the adequately controlled group, all patients achieved sufficient suppression of 0800h ACTH concentrations (<71 pg/ml) following either dose of dexamethasone (Figures 13a & 13b). Mean ACTH concentrations were 29.0 pg/ml and 33.1 pg/ml following administration of dexamethasone in a dose of 0.3 mg/m\(^2\) and 0.5 mg/m\(^2\) respectively.

In the group of inadequately controlled patients, three (15.8%) did not demonstrate sufficient suppression of the 0800h ACTH concentrations following 0.3 mg/m\(^2\) of dexamethasone and one (5.3%) did not show sufficient suppression even after the higher 0.5 mg/m\(^2\) dexamethasone dose was given (Figures 13a & 13c). Two of those patients had extremely high baseline ACTH concentrations (856 pg/ml and 506 pg/ml). Mean ACTH concentrations were 43.5 pg/ml and 39.9 pg/ml following 0.3 mg/m\(^2\) and 0.5 mg/m\(^2\) of dexamethasone respectively.

No significant difference in ACTH concentrations was observed between males (mean: 248.4 pg/ml, SD: 261.1) and females (mean: 113.2 pg/ml, SD: 118.4).
Figure 13: (a) 0800h ACTH concentrations in all patients with classical 21-hydroxylase deficiency before and after overnight suppression with two different doses of dexamethasone (0.3 mg/m$^2$ and 0.5 mg/m$^2$ body SA) administered following randomisation; (b) 0800h ACTH concentrations following single dose dexamethasone suppression in adequately controlled patients; (c) 0800h ACTH concentrations following single dose dexamethasone suppression in inadequately controlled patients with classical congenital adrenal hyperplasia. All adequately controlled and 84.2% of the inadequately controlled patients achieved sufficient suppression following 0.3 mg/m$^2$ of dexamethasone, whereas the remaining 15.8% of inadequately controlled patients required a higher dose to suppress the 0800h ACTH concentrations.
STUDY 6: SERUM CORTISOL AND GH INTERRELATIONSHIP

Cross-correlation studies

The graphs depicting the minimum, 1st, 2nd (median), 3rd quartile and maximum coefficients of correlation from the cross-correlation analysis over the 24 hour period between cortisol and GH concentrations in all thirty six patients participated in this study are shown in Figure 14. The analysis showed a significant positive correlation between cortisol and GH at lag time 0 min \( (r = 0.148, P<0.0001) \), peaking at 1h 40 min \( (r = 0.251, P<0.0001) \). Also, a significant negative correlation between cortisol and GH was observed over time, peaking at lag time 6h 20 min \( (r = -0.302, P<0.0001) \), with cortisol leading GH by these intervals (Figure 14a).

When data were analysed in the adequately and inadequately controlled patients a very similar pattern of cortisol-GH interrelationship was noted. In the group of adequately controlled patients the significant positive correlation reached a peak at lag time 40 min \( (r = 0.252, P<0.0001) \), whereas the peak of negative correlation was observed at lag time 6h 20 min \( (r = -0.316, P<0.001) \), with cortisol leading GH by these time intervals (Figure 14b). In the inadequately controlled patients there was a significant positive correlation between cortisol and GH at lag time 0 min \( (r = 0.133, P<0.0001) \) which reached a peak at 1h 40 min \( (r = 0.285, P<0.0001) \). In addition, a negative correlation was observed over time, peaking at lag time 7h 40 min \( (r = -0.329, P<0.0001) \), with cortisol leading GH (Figure 14c).
(a) Correlation Coefficient (r) vs Time (hours)

- MINIMUM
- 1st Quart
- MEDIAN
- 3rd Quart
- MAXIMUM

(b) Correlation Coefficient (r) vs Time (hours)

- MINIMUM
- 1st Quart
- MEDIAN
- 3rd Quart
- MAXIMUM
**Figure 14:** Cross-correlation analyses between cortisol and GH values in (a) all patients participated in the study, (b) adequately controlled patients and (c) inadequately controlled patients over a 24 hour period. There was a significant positive correlation between the two hormones, with cortisol leading GH by 1h 40 min. This time interval was noted to be shorter in the adequately controlled patients.

Finally, when data were analysed in male and female patients, a positive cross-correlation was noted at 0 min in both sexes. The significant positive correlation reached a peak at 20 min ($r = 0.28, P<0.0001$) in males and at 2h ($r = 0.23, P<0.0001$) in females (Figure 15).
Figure 15: Cross-correlation analyses between cortisol and GH values in (a) males and (b) females participated in the study. There was a significant positive correlation between the two hormones, with cortisol leading GH by 20 min in males and 2h in females.
Summary

The results of the above studies can be summarised as follows:

Study 1: Puberty was associated with alterations in the pharmacokinetic parameters of total and free cortisol. There was an increase in cortisol clearance and volume of distribution but no change in half life. The half life of free cortisol was shorter in females compared to males.

Study 2: IGF-I and tetrahydrocortisone (THE) concentrations were significantly higher in the pubertal group than the prepubertal group of patients. Stepwise multiple linear regression analysis showed that integrated GH concentrations, IGF-I and gender were independent predictors of total cortisol clearance corrected for BMI. Total cortisol half life was best predicted by the (THF + allo-THF)/THE ratio, which represents an index of 11β-HSD activity.

Study 3: The bioavailability of oral hydrocortisone (Hydrocortone tablets, Merck, Sharp & Dohme Ltd) is almost complete (94.2%, 90% CI: 82.8 – 105.5) and peak cortisol concentrations are attained 1.2 hours after administration. The increase in the apparent bioavailability in the evening (128%) is most likely due to a decrease in cortisol clearance.

Study 4: In the adequately controlled patients, 17-OHP concentrations remained low during the 24 hour period of the study. In the inadequately controlled patients, 17-OHP concentrations were elevated during daytime (0400h – 1600h) and decreased only in
response to sufficiently high cortisol concentrations. During night-time, however, 17-OHP concentrations remained low, despite the undetectable cortisol concentrations, indicating decreased activity of the HPA axis. There was a significant negative correlation between cortisol and 17-OHP at lag time 0 min, peaking at lag time 60 min, which suggests that maximal suppression of 17-OHP concentrations is achieved one hour after cortisol concentrations reach their peak. 0800h androstendione concentrations showed a significant positive correlation with integrated 17-OHP but not cortisol concentrations.

Study 5: 84.2% of inadequately controlled patients showed sufficient suppression of the 0800h ACTH concentrations (ACTH < 71 pg/ml) following 0.3 mg/m² of dexamethasone, whereas the remaining 15.8% required a higher dose (0.5 mg/m²).

Study 6: There was a significant positive correlation between cortisol and GH, with cortisol leading GH by 1h 40 min. This time interval was noted to be shorter in the adequately controlled patients compared to the inadequately controlled ones.

The following section (Discussion) presents the discussion of the results reported in this section. Discussion of the results is presented under the same six studies described in the Methodology section.
DISCUSSION
STUDY 1: PHARMACOKINETIC PARAMETERS OF TOTAL AND FREE CORTISOL

This study demonstrates differences in the pharmacokinetic parameters of total and free cortisol in patients with classical 21-hydroxylase deficiency and provides evidence of a significant rise in cortisol clearance at puberty, which is associated with an increase in volume of distribution but no change in half life.

The primary site of cortisol metabolism in humans is the liver and a number of cytosolic and microsomal enzymes, including cytochrome P450, 5α/5β-reductase, 3α/3β-oxidoreductase and 11β-hydroxysteroid dehydrogenase (11β-HSD) play an important role in the hepatic metabolism of cortisol (Iyer RB et al., 1990; Gower DB, 1984; Stewart PM, 1996). The major routes of hepatic metabolism determined from both urinary analysis and in vitro studies involve A-ring and side-chain reduction followed in vivo by conjugation with glucuronic acid and sulphate (Abel SM et al., 1992). The inactive glucuronide and sulphate metabolites are excreted by the kidneys whereas only less than 1% of cortisol is excreted unchanged in the urine. The metabolic clearance of cortisol, therefore, is influenced primarily by factors altering hepatic clearance and to a much lesser degree by factors affecting renal excretion.

Changes in the endocrine milieu at puberty may affect cortisol clearance in a number of ways. Puberty results from increased secretion of sex steroids by the gonads in response to gonadotrophin secretion from the anterior pituitary. Rising sex steroid concentrations are associated with increased pulse amplitude of GH secretion resulting in increased insulin-like growth factor–I (IGF-I) concentrations (Miller JD et al., 1982; Blum WF et
The rise in serum GH concentration is also associated with a marked
decrease in insulin sensitivity and a parallel elevation in serum insulin concentrations
(Amiel SA et al., 1986; Cutfield WS et al., 1990). At the tissue level, insulin reduces
insulin-like growth factor binding protein-1 (IGFBP-1) concentrations, further
increasing IGF-I concentrations and enhancing its mitogenic effects (Conover CA et al.,
1990; Travers SH et al., 1998).

The increase in cortisol clearance observed in the pubertal patients may be explained by
an alteration in 11β-HSD activity secondary to the rise in GH and IGF-I concentrations.
It is now well documented that inhibition of the activity of type 1 isoform (11β-HSD1),
which acts predominantly as an oxo-reductase converting inactive cortisone to active
cortisol, is observed in association with an increase in GH and IGF-I concentrations
(Low SC et al., 1994; Gelding SV et al., 1998; Moore JS et al., 1999; Toogood AA et
al., 2000). Studies of hypopituitary adults showed a significant, dose-independent,
persistent decrease in the activity of 11β-HSD1 following treatment with GH (Gelding
SV et al., 1998; Toogood AA et al., 2000). Similar were the findings of recent studies
on acromegalic patients: withdrawal from medical therapy resulted in a significant rise
in GH and IGF-I concentrations with a concomitant decrease in 11β-HSD1 activity,
whereas transphenoidal surgery and excision of the pituitary adenoma resulted in a
decrease in GH concentrations and a parallel increase in 11β-HSD1 activity (Moore JS
et al., 1999). The above findings have been further confirmed by in vitro studies, which
also suggested that the GH effects are probably mediated by IGF-I (Moore JS et al.,
1999) and that the effect on 11β-HSD1 activity was specific, in that neither IGF-I nor
GH had any effect on 11β-HSD2 activity. By inhibiting 11β-HSD1 activity, GH
effectively increases the metabolic clearance rate of cortisol (Moore JS et al., 1999).
In addition to GH and IGF-I, there is considerable evidence to suggest that gonadal steroids also influence 11β-HSD1 activity. In rats, a sexually dimorphic pattern favouring males in the activity of 11β-HSD1 has been documented in a number of studies (Low SC et al., 1993; Albinston AL et al., 1995; Jamieson P et al., 1995; Nwe KH et al., 2000). In male rat liver, gonadectomy and oestradiol treatment led to a dramatic decrease in both 11β-HSD1 activity and mRNA expression, whereas gonadectomy and testosterone replacement had no effect on type 1 isoform activity. However, in female rat liver gonadectomy resulted in a marked increase in 11β-HSD1 activity, which was subsequently reversed by oestradiol replacement therapy but not testosterone treatment (Low SC et al., 1993). In addition, sexual dimorphism has been documented in healthy (Raven PW et al., 1995) and hypopituitary subjects on optimal replacement therapy (Weaver JU et al., 1998). Weaver et al., demonstrated that in hypopituitary adults receiving optimal replacement therapy, the activity of type 1 isoform was lower in females as compared to males and was directly related to insulin sensitivity, thus suggesting that a fall in insulin sensitivity may be associated with a decrease in 11β-HSD1 activity (Weaver JU et al., 1998). Although caution must be exercised in extrapolating inferences about 11β-HSD1 regulation from rodents to man, the above observations may suggest a similar role of gonadal steroids in humans with oestrogen inhibiting 11β-HSD1 activity and, hence, increasing cortisol clearance, and testosterone exerting no effect on reductase activity.

Except for alterations in the activity of 11βHSD1, hormonal changes at puberty may result in alterations in the activity of other enzymes that play an important role in cortisol metabolism. The effects of IGF-I, IGF-II and insulin on the regulation of 17α-hydroxylase and of 3β-hydroxysteroid dehydrogenase (3β-HSD) have long been
recognised. Studies that examined the long term effects of IGF-I and IGF-II on cultured human adrenal fasciculata/reticularis cells demonstrated that, in cells pre-treated with IGF-I or IGF-II, the mRNA levels of cytochrome P450 17α-hydroxylase and of 3β-HSD were increased (L’Allamand D et al., 1996). Similar studies demonstrated that both insulin and IGF-I at physiological concentrations increased mRNA levels of both 17α-hydroxylase and 3β-HSD in the absence of cAMP or ACTH (Kristiansen SB et al., 1997). Subsequent reports confirmed the above findings and also showed that IGF-II was more potent than IGF-I in modulating the activity of steroidogenic enzymes (Mesiano S et al., 1997; Fottner C et al., 1998).

The increase in cortisol clearance observed in pubertal patients may also be due to an increase in renal clearance of cortisol secondary to an increase in glomerular filtration rate (GFR) (Birkett DJ, 1998a; Birkett DJ, 1998d). Many studies have described the effects of GH and IGF-I on renal function and demonstrated that chronically elevated GH concentrations are associated with an increase in renal plasma flow (RPF), GFR and kidney size, whereas decrease in GH concentrations has the opposite effects (Christiansen JS et al., 1981; Hammerman MR, 1999). These actions of GH are likely to be mediated by IGF-I rather than being direct. IGF-I has been shown to increase GFR via a direct effect on the glomerular vasculature and a decrease in renal glomerular afferent and efferent arteriolar resistances (Krieg RL et al., 1995; Hirschberg R et al., 1991; Tonshoff B et al., 1998).

The increase in the volume of distribution in the pubertal and postpubertal patients is probably due to the increase in body surface area in these two groups as compared to the prepubertal group (Birkett DJ, 1998b). Except for body size, however, the volume of
distribution is also determined by the relative strength of binding of the drug to tissue components as compared with plasma proteins. Drugs that are tightly bound to plasma proteins and not to tissues have volume of distribution that is very close to blood volume. Conversely, drugs that are very tightly bound by tissues and not by plasma proteins are held mostly in the tissues, they appear to be dissolved in a large volume and their volume of distribution is large. The dependency of volume of distribution on both plasma protein binding and body surface area is illustrated by the fact that the volume of distribution of free cortisol, which takes into consideration plasma protein binding, adjusted for BMI is not significantly different between the three groups of patients.

Despite the increase in cortisol clearance at puberty, no difference in half life of total and free cortisol is observed between prepubertal, pubertal and postpubertal patients. This is because the elimination of a drug from the body does not depend on clearance only. It is a function of both clearance and volume of distribution as is the half life (Rowland M et al., 1980; Birkett DJ 1998c). Therefore, a concomitant rise in both cortisol clearance and volume of distribution may not result in a significant change in half life. However, the half life of free cortisol is significantly shorter in females as compared to males, indicating that female patients would probably benefit from more frequent than twice daily hydrocortisone replacement.

In summary, this study demonstrates that, in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency, puberty is associated with alterations in cortisol pharmacokinetics that primarily reflect alterations in the endocrine milieu. The increase in cortisol clearance is associated with an increase in volume of distribution but no change in half life. The above observations may have implications for therapy and
may indicate glucocorticoid replacement with more frequent daily doses at puberty, especially in female patients.
STUDY 2: CORTISOL PHARMACOKINETICS AND PUBERTY: INDEPENDENT PREDICTORS OF TOTAL AND FREE CORTISOL CLEARANCE AND HALF LIFE

The previous study (study 1) revealed an alteration in cortisol pharmacokinetics at puberty and the candidate factors that might influence these changes were considered. To explore this concept further, we examined whether there is an association between cortisol clearance and a number of biological parameters. The relationships were explored using linear modelling and stepwise multiple regression analysis. Although these are robust statistical methods, it is important to emphasise that they are statistical methods with their own inherent assumptions that may not necessarily reflect the biological reality.

The results of the present study showed that a number of alterations in the endocrine milieu at puberty are independently related to cortisol clearance and half life. IGF-I and GH concentrations were found to be independently related to total cortisol clearance and this effect was probably the result of inhibition of 11β-HSD1 activity. This is supported by the observation that there was an increase in urinary cortisone metabolites (THE) in the pubertal group of patients compared to the prepubertal group with no concomitant rise in (THF + allo-THF)/THE ratio (index of overall activity of 11βHSD). This would indicate a decreased conversion of cortisone to cortisol (Stewart PM, 1996). Although it is difficult to determine whether this is a direct GH effect or it is mediated by IGF-I, it is worth noting that IGF-I in this model exerts a major effect on cortisol metabolic clearance ($r^2 = 0.39$, $P<0.001$). The decrease in 11β-HSD1 activity observed in association with an increase in GH and IGF-I concentrations is now well established.
and has been confirmed by in vitro studies of enzyme activity (Low SC et al., 1994; Jamieson P et al., 1995; Gelding SV et al., 1998; Moore JS et al., 1999).

A greater number of independent variables are related to free cortisol clearance corrected for BMI. The positive correlation between IGFBP-3 and cortisol clearance ($r^2 = 0.29$, $P = 0.001$) is likely to reflect the IGFBP-3 dependency on GH state, since there is no evidence to suggest that IGFBP-3 influences cortisol metabolism. IGFBP-3 is the major IGFBP in normal human serum and its concentrations correlate with GH sufficiency and sensitivity. Blum et al., examined the relationship between 24h GH secretion, IGF-I concentrations and IGFBP-3 concentrations in a large cohort of 114 healthy children and adolescents and showed that IGF-I and IGFBP-3 concentrations reflect spontaneous GH secretion (Blum WF et al., 1993).

Serum leptin concentration is another variable that correlates positively with free cortisol clearance. Leptin is a peptide hormone, secreted by the white adipocytes and encoded by the obese (ob) gene, that exerts a regulatory effect on food intake and energy expenditure. Plasma leptin concentrations are known to correlate positively with many indices of obesity, such as body mass index, waist circumference and fasting insulin concentrations and are higher in women than in men (Zimmet P et al., 1996; Pasquali R et al., 2000; Panarotto D et al., 2000). The positive correlation between leptin concentrations and BMI ($r^2 = 0.49$, $P<0.001$) in our patients supports these findings. On the other hand, an increase in total fat and percent of body fat has been shown to result in a decrease in 11β-HSD1 activity that could explain the positive correlation between cortisol clearance and leptin concentrations (Weaver JU et al., 1998).
Total cortisol clearance adjusted for BMI was found to be higher in males than females and gender was an independent predictor of both total and free cortisol clearance. This, however, was not associated with any significant difference between sexes in all other biochemical parameters examined. Since most reports on sexual dimorphism of 11β-HSD1 activity suggest that oestrogen concentrations are responsible for the decreased enzyme activity documented in females (Albiston AL et al., 1995; Weaver JU et al., 1998; Nwe KH et al., 2000), it is likely that the increase in cortisol clearance in male patients reflects primarily a sex steroid effect on the GH ~ IGF-I axis. Previous studies have demonstrated that testosterone increases GH secretion by increasing both GH pulse frequency and amplitude and that it exerts its effect via aromatisation to oestradiol and inhibition of somatostatin secretion (Devesa J et al., 1991). The testosterone effect on the GH ~ IGF-I axis is consistent with the more pronounced age-related fall in GH secretion in men, which results in a sexually dimorphic pattern of growth hormone secretion in older individuals (Hindmarsh PC et al., 1999). Although not significantly different between groups, integrated GH concentrations were higher in males (median: 7350 mIU*min/L, range: 1844 – 29967) than females (median: 5838 mIU*min/L, range: 1736 – 20825), whereas IGF-I concentrations were comparable (Males: median: 283.1 ng/ml, range: 175.2 – 528.1; Females: median: 296.5 ng/ml, range: 91.9 – 628.4).

The half life of serum total cortisol was independently predicted by (THF + allo-THF)/THE, integrated 17-OHP concentrations, ACTH and IGFBP-3 concentrations. The half life is a composite pharmacokinetic parameter determined by both clearance and volume of distribution \((\text{half life} = 0.693*V/CL)\), and is therefore influenced by factors affecting both clearance and volume of distribution (Birkett DJ, 1998c). The positive correlation between (THF + allo-THF)/THE ratio and total cortisol half life
suggests that a decrease in cortisol/cortisone metabolite ratio is associated with a shorter half life. Since the (THF + allo-THF)/THE ratio represents an index of overall 11ß-HSD activity, factors decreasing 11ß-HSD activity by inhibiting the activity of 11ß-HSD1 isoform will result in increased cortisol clearance and hence a shorter half life. Such factors have been described above and the fact that they all operate at puberty may explain the association between pubertal process and alterations in cortisol pharmacokinetics.

A weak positive correlation between integrated 17-OHP concentrations and total cortisol half life was also observed. Elevated 24h 17-OHP concentrations imply adrenal androgen excess, which is usually associated with polycystic ovaries and decreased CBG concentrations (Invitti C et al., 1991). The decrease in CBG concentrations results in an increase in the apparent volume of distribution and therefore an increase in half life (Birkett DJ, 1998b). It is important to underline that it is unlikely that there is a true positive correlation between ACTH/17-OHP concentrations and cortisol clearance. On the contrary, previous studies have demonstrated that elevated ACTH concentrations result in increased cortisol metabolic clearance rate by increasing its hepatic extraction (Zisper et al., 1976). Furthermore, hyperandrogenism is likely to be associated with a fall in insulin sensitivity and a parallel elevation of insulin concentrations (Dunaif A, 1997). Although there was no significant difference in fasting insulin and glucose concentrations between adequately and inadequately controlled patients, stepwise linear regression indicated that there was a positive correlation between integrated 17-OHP concentrations and fasting glucose concentrations. The rise in insulin concentrations reduces IGFBP-1 concentrations and enhances the action of IGF-I (Ritvos O et al., 1989; Conover CA et al., 1990). This would compound the IGF-I effects described
above and would further support the concept of increased cortisol clearance in the inadequately controlled patients.

The negative correlation between ACTH and half life is consistent with previous reports (Zisper RD et al., 1976) and suggests that inadequately controlled CYP21 deficient patients clear cortisol faster and, hence, have a shorter cortisol half life. These patients are therefore likely to require more frequent daily glucocorticoid substitution to maintain cortisol concentrations within the expected range. The negative correlation between IGFBP-3 and half life accords with the positive correlation between IGFBP-3 and cortisol clearance and most likely reflects a similar underlying mechanism.

Free cortisol half life was only related to CBG concentrations and the negative correlation between them ($r^2 = 0.14, P = 0.02$) can be explained by the inverse relationship between CBG concentrations and volume of distribution (Birkett DJ, 1998b). The significantly shorter half life of free cortisol in females compared to males indicates that, like inadequately controlled CYP21 deficient patients, female patients would probably benefit from more frequent than twice daily hydrocortisone replacement therapy.

In summary, this study shows that alterations in the endocrine milieu at puberty, including alterations in GH ~ IGFs/IGFBP-3 axis, $11\beta$-HSD1 activity and insulin sensitivity, are associated with increased clearance and shorter half life of total and free cortisol in patients with classical 21-hydroxylase deficiency. These alterations in cortisol pharmacokinetics will result in a loss of control of HPA axis and inadequate suppression of androgens and androgen precursors. It is therefore appropriate to
recommend more frequent doses of glucocorticoid substitution in all pubertal patients, especially females. Alternatively, a sustained release formulation of hydrocortisone may provide adequate adrenocortical suppression and may prove to be a more effective therapeutic modality in the management of pubertal as well as inadequately controlled prepubertal patients. Antiandrogen treatment – in addition to standard replacement therapy – will diminish the adverse effects of androgen excess and will offer the prospect of an improved outcome.
STUDY 3: THE BIOAVAILABILITY OF ORAL HYDROCORTISONE

The previous studies demonstrated that puberty is associated with increased cortisol clearance and shorter half life, indicating a need for an alternative therapeutic approach. Alterations in replacement therapy and decisions on the schedule and frequency of glucocorticoid substitution should be based on the knowledge of the pharmacokinetic parameters of hydrocortisone formulations used in the treatment of patients with classical 21-hydroxylase deficiency. Having determined the basic pharmacokinetic parameters of total cortisol (clearance, volume of distribution and half life), we performed this study to address the bioavailability (F) of oral hydrocortisone tablets (Hydrocortone, Merck Sharp & Dohme Limited) (British National Formulary, 1998a).

Although hydrocortisone was designated as a drug whose different brands and dosage forms should be examined for bioequivalence (Fed Register, 1976), very few studies have been performed to determine its bioavailability. Most intravenous pharmacokinetic studies used hydrocortisone hemisuccinate as a water soluble pro-drug (Toothaker RD et al., 1982). However, after intravenous hemisuccinate administration of other corticosteroids substantial amounts of the pro-drug could be found in the urine making it a less than optimum standard for the assessment of bioavailability (Möllman H et al., 1988; Derendorf H et al., 1985).

The pharmacokinetic parameters determined following intravenous administration of hydrocortisone sodium succinate (Solu-Cortef, Pharmacia & Upjohn) in the present study (94.2%, 90%CI: 82.8 – 105.5) are consistent with those previously reported after oral and intravenous administration of 20 mg of hydrocortisone (Hoechst®) (Derendorf
H et al., 1991). The latter study was performed in healthy adult volunteers following suppression of the HPA axis by dexamethasone tablets given at 2200h one day prior to the study. Our study was performed in much younger (median age: 10.9 yrs) patients with classical CYP21 deficiency who had minimal endogenous cortisol production, as evidenced by the undetectable cortisol concentrations before the oral and intravenous hydrocortisone doses were given. They were therefore reminiscent of patients with dexamethasone induced HPA axis suppression in terms of endogenous cortisol production. The fact that the pharmacokinetic parameters of hydrocortisone derived from two different studies and using two different oral formulations are similar indicates that the two formulations used are bioequivalent, i.e. the extents and rates of absorption of the two formulations are so similar that there is likely to be no clinically important difference between their effects, either therapeutic or adverse.

Initial studies conducted to examine the bioavailability of hydrocortisone reported that approximately 50 – 60% of the drug was available to the systemic circulation following 10-20 mg tablet or suspension doses (Toothaker RD et al., 1982; Patel RB et al., 1984). Heazelwood et al. performed a similar study in adults with primary or secondary adrenal insufficiency and used the same formulation of oral hydrocortisone as we did (Heazelwood VJ et al., 1984). The bioavailability of oral cortisol (Hydrocortone, Merck Sharp & Dohme Ltd) was calculated against an intravenous injection of hydrocortisone sodium succinate (Efcortelan, Glaxo) given as a bolus in a dose of 50 mg. In contradistinction to our findings, however, the authors reported a wide variation in cortisol bioavailability (range: 26-91%, mean 54.0 ± 6.9%) secondary to considerable inter-individual variation in the AUC of cortisol derived from intravenous and oral hydrocortisone administration. This variation in bioavailability was thought to be due to
incomplete absorption secondary to intrinsic absorption effects, first-pass clearance at the liver, or both (Patel RB et al., 1984).

Our observations are consistent with those of Derendorf et al., who estimated the absolute bioavailability of hydrocortisone to be 95.9% (SD: 19.6) (Derendorf H et al., 1991). The high oral bioavailability of hydrocortisone indicates that the drug is almost completely absorbed by the gastrointestinal tract and that the fraction of drug escaping first pass hepatic clearance is high, thus implying a low hepatic extraction ratio. For low hepatic extraction ratio drugs, hepatic enzyme activity is not a major determinant of first-pass metabolism and oral bioavailability, although it plays an important role in hepatic clearance (Rowland M et al., 1980b). Therefore, factors such as gender, age or pubertal process, that influence hepatic enzyme activity and in particular activity of 11β-hydroxysteroid dehydrogenase would have little or no effect on cortisol bioavailability. In addition, the bioavailability of low hepatic extraction ratio drugs is not influenced significantly by the presence of liver disease, adverse effects are not observed and a modification of the dose is not required (British National Formulary, 1998b).

The high oral bioavailability of hydrocortisone also indicates that, if absorption from the gastrointestinal tract is not impaired, an oral dose needs to be only 6% higher than an intravenous dose to achieve similar plasma concentrations and glucocorticoid effect. The oral route of administration of hydrocortisone is therefore almost as effective as the intravenous route and can be used safely in patients requiring systemic corticosteroid treatment (Birkett DJ, 1998f). Furthermore, such high bioavailability suggests that the decision about the biggest dose of hydrocortisone in patients with adrenocortical
insufficiency should be made with caution, since relatively lower doses may also result in peak cortisol concentrations within or above the effective range. Also, in the presence of an intercurrent illness, when doubling of the total daily hydrocortisone dose is recommended, it may be preferable to administer it in three or four rather than two divided doses.

The bioavailability of oral hydrocortisone in the evening was calculated using as reference the true clearance of hydrocortisone that had been estimated following the intravenous bolus of hydrocortisone in the morning. Since the bioavailability of a drug can never exceed 100%, the increase in bioavailability in the evening is likely to reflect a decrease in the absolute clearance of hydrocortisone in the evening compared to the clearance in the morning. Since the $F_m/F_{ev} = 0.74$, it is inferred that the hydrocortisone clearance in the evening is 26% slower than the clearance in the morning. These observations may explain why the median peak cortisol concentration after oral administration of the morning dose (median: 7h, range: 5 – 12h) was observed 2 hours earlier than the median peak concentration attained after the evening oral dose was given (median: 9h, range: 5 – 12h) despite the fact that the higher hydrocortisone dose had been given in the morning. However, the clearance of hydrocortisone in the evening can only be estimated reliably following intravenous administration of a hydrocortisone bolus after suppression of the HPA axis and measurement of serum total cortisol concentrations at regular (10 min) intervals (Birkett DJ, 1998a; Rowland M et al., 1980c).

A decrease in cortisol clearance in the evening may be due to a circadian variation in CBG concentrations and therefore alterations in the pharmacokinetic parameters of
cortisol or a circadian variation in CBG binding capacity. Animal studies have demonstrated that there is a circadian variation in CBG concentrations with a gradual increase throughout the day, peak concentrations being attained at 1800h, and a subsequent decline during the night hours, nadir concentration being observed at 0600h (Hsu BR et al., 1988; Meaney MJ et al., 1992). Although similar studies are lacking in humans, a circadian variation of CBG binding capacity for prednisolone with maximum binding observed at midnight and minimum at 0800h has been reported in healthy subjects (Angeli A et al., 1978). Alternatively, the decrease in cortisol clearance in the evening may relate to the circadian variation in ACTH secretion. Previous studies have demonstrated that elevated ACTH concentrations result in increased cortisol clearance by increasing its hepatic extraction (Zisper RD et al., 1976). Although these studies did not determine the plasma ACTH concentrations following administration of ACTH infusion, the physiological rise in ACTH concentrations early in the morning may be sufficient to result in increased cortisol clearance in the morning. These findings clearly have implications for replacement therapy with hydrocortisone regimens, since it is likely that the evening oral hydrocortisone dose will provide glucocorticoid cover for a greater length of time than an equal dose administered in the morning.

In summary, this study shows that the bioavailability of oral hydrocortisone is essentially complete and may result in supraphysiological cortisol concentrations within 1–2 hours after administration of high doses. Decisions on the schedule and frequency of administration need to be made with caution and should be based on the knowledge of the pharmacokinetic parameters of hydrocortisone formulations currently used in the treatment of patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Further studies are required to examine the pharmacokinetic parameters of
total cortisol in the evening after suppression of the HPA axis and to determine whether there is a circadian variation of CBG concentrations and binding capacity for cortisol.
STUDY 4: SERUM CORTISOL AND 17-HYDROXYPROGESTERONE INTERRELATIONSHIP: IS CURRENT REPLACEMENT THERAPY SATISFACTORY?

In Congenital Adrenal Hyperplasia due to 21-hydroxylase deficiency, glucocorticoid treatment is offered in an attempt to suppress the excessive secretion of CRH and ACTH by the hypothalamus and anterior pituitary respectively, and to reduce the circulating concentrations of adrenal androgens and androgen precursors. The preferred glucocorticoid option is hydrocortisone because its short half life minimises growth suppression as well as other adverse side effects of more potent, longer acting glucocorticoids (White PC et al., 2000). Achieving and maintaining adrenal suppression is far more challenging than preventing adrenal crises and often requires increased doses of glucocorticoid substitution that may produce an unacceptable and undesirable degree of hypercortisolism (Van Wyk JJ, 1996). Treatment efficacy reflects the adequacy of adrenocortical suppression and is assessed by monitoring annualised growth velocity, rate of skeletal maturation and serum concentrations of 17-hydroxyprogesterone (17-OHP) and androstendione (White PC et al., 2000). It is important, therefore, to determine the relationship between cortisol and 17-OHP in these patients and to evaluate current replacement therapy in the light of these findings.

It has long been recognised that, in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency, 17-OHP concentrations demonstrate circadian variation. Peak values have been documented early in the morning and trough values in the evening in patients before treatment, during inadequate treatment or after withdrawal from treatment (Atherden SM et al., 1972; Frisch H et al., 1981; Solyom J, 1984; Young
MC et al., 1988). The circadian variation persists after introduction of glucocorticoid therapy — however, the magnitude varies according to the degree of therapeutic control — and is abolished by excessive glucocorticoid treatment.

The findings of this study illustrate the relationship between cortisol and 17-OHP in patients with classical congenital adrenal hyperplasia and suggest that this is primarily dependent upon the adequacy of adrenocortical suppression. In the adequately controlled CYP21 deficient patients, mean 17-OHP concentrations remained suppressed (< 20 nmol/L) during the 24 hour period, even between 0400h and 0900h, when peak concentrations were attained in response to the increased pulsatile secretion of ACTH. By contrast, in the inadequately controlled patients mean 17-OHP concentrations were elevated for most of the 24 hour period, with the peak concentrations also observed in the early morning hours.

The pattern of 17-OHP secretion in relation to circulating cortisol concentrations in the second group of patients is more informative, in that conclusions can be drawn about variations in 17-OHP concentrations in response to the oral administration of hydrocortisone. The extremely high 17-OHP concentrations early in the morning gradually declined as cortisol concentrations began to rise, after the morning dose of hydrocortisone was given, and remained suppressed for as long as the cortisol concentrations remained elevated. The decrease in cortisol concentrations in the early afternoon hours was associated with a parallel but of lesser magnitude rise in 17-OHP concentrations. Mean 17-OHP concentrations remained undetectable between 1600h and 2000h, despite the very low cortisol concentrations, indicating a period of minimal activity of the HPA axis. Finally, they demonstrated a precipitous rise beyond 0400h,
when increased activity of HPA axis was observed and by which time a significant reduction in circulating cortisol concentration had taken place. These observations support previous reports on the circadian rhythm of 17-OHP secretion and also suggest that administration of hydrocortisone late in the evening may not be necessary and it may be more appropriate to administer a dose just before the time of the rapid rise in 17-OHP concentrations.

The difference in the activity of HPA axis between daytime and night-time implies a different threshold effect; i.e. higher concentrations of cortisol are required to result in adequate suppression of 17-OHP and androgens during daytime compared to cortisol concentrations required to have a similar effect during night-time. This is illustrated in the graphical representation of 17-OHP concentrations in relation to cortisol concentrations during the two 12h intervals. During the course of the day (0400h – 1600h) 17-OHP concentrations decreased only in response to elevated cortisol concentrations and cortisol concentrations had to exceed 200 nmol/L before any decrease in 17-OHP concentrations was observed. By contrast, during night-time (1600h – 0400h) 17-OHP concentrations remained very low regardless of the level of circulating cortisol concentration. This observation further supports the concept of minimal activity of HPA axis between 1600h and 0400h and underlines the fact that the administration of oral hydrocortisone in the evening would only be useful for substitution and should not be expected to contribute significantly towards achieving and/or maintaining adrenocortical suppression.

The cortisol – 17-OHP interrelationship also provides useful information about monitoring patients with classical 21-hydroxylase deficiency. By observing the pattern
of 17-OHP secretion throughout the 24 hour period, it becomes clear that single measurements of 17-OHP and/or androgen concentrations are unreliable and can be particularly misleading if blood specimens are obtained beyond 1600h, during the period of decreased activity of HPA axis. Serum androstendione concentrations, on the other hand, obtained early in the morning before the administration of oral hydrocortisone, showed a strong positive correlation with integrated 17-OHP concentrations and can, therefore, be used as a more reliable marker of HPA axis activity and adequacy of adrenocortical suppression, if a single blood sample is obtained. Alternatively, patients may undergo serial measurements of 17-OHP and androgen concentrations, which should be preferred over single measurements because they will give a more accurate profile of control status and will enable physicians to re-adjust replacement therapy accordingly. For example, measurement of cortisol and 17-OHP concentrations every hour for the first four hours after hydrocortisone administration and 2 hourly thereafter until the next dose is due will provide information on both the overall exposure to glucocorticoid and the adequacy of adrenocortical suppression.

The cross-correlation analysis performed in the inadequately controlled patients showed that 17-OHP concentrations were sufficiently suppressed one hour after cortisol concentrations reached their peak and this effect was reversed 6h 40 min later, when 17-OHP reached their peak just before the next hydrocortisone dose was administered. When we examined the cortisol – 17-OHP interrelationship during daytime and nighttime, it appeared that the peak negative correlation was observed much earlier during nighttime (at lag time 20 min) than during daytime (at lag time 60 min), indicating a greater sensitivity of HPA axis during the second part of the 24h period. These findings
suggest that maximal suppression of 17-OHP concentrations is achieved one hour after cortisol concentrations reach their peak if the hydrocortisone dose is given during the course of the day (0400h – 1600h) and 20 min after peak cortisol concentrations are attained if hydrocortisone is given between 1600h and 0400h.

In summary, hydrocortisone treatment in classical 21-hydroxylase deficiency should aim at providing adequate suppression of the HPA axis between 0400h and 1600h. The biggest hydrocortisone dose should be given in the morning because circulating cortisol concentrations attained following evening doses are likely to be undetectable by the time of the rapid rise in 17-OHP concentrations at 0400h. Single measurements of androgens and androgen precursors performed as part of monitoring CYP21 deficient patients should include androstendione and 17-OHP concentrations obtained in the morning, before the oral hydrocortisone dose is given. However, it should be emphasised that serial measurements of 17-OHP and/or androgens are likely to provide more detailed information on the adequacy of adrenocortical suppression and help clinicians reach more appropriate decisions in the management of these patients.
STUDY 5: TESTING ALTERATIONS IN FEEDBACK MECHANISMS REGULATING ACTH SECRETION

In the previous study (study 4) we examined the relationship between cortisol and 17-OHP in an attempt to evaluate therapeutic regimens currently used in the management of patients with classical 21-hydroxylase deficiency. The present study was designed to investigate whether there is an alteration in the negative feedback effects of cortisol at the pituitary level that may partly explain the difficulty in achieving adrenocortical suppression in inadequately controlled patients, despite using optimal glucocorticoid substitution.

The results of this study showed that, although baseline mean ACTH concentrations were much lower in the adequately controlled patients (mean: 45.8 pg/ml, SD: 16.8) compared to the inadequately controlled ones (mean: 239.6 pg/ml, SD: 228.2), almost all patients achieved sufficient suppression of HPA axis following either dose of dexamethasone. The smaller dose of 0.3 mg/m², recommended for the single dose dexamethasone suppression test (Hindmarsh PC et al., 1985), sufficiently suppressed 0800h ACTH concentrations in all adequately controlled and in the majority (84.2%) of inadequately controlled patients, whereas the higher dose of 0.5 mg/m² was required only for a small proportion (15.8%) of inadequately controlled patients.

These data suggest that the negative feedback of cortisol on ACTH secretion in CYP21 deficient patients is not altered significantly, and that the difficulty in attaining optimal control relates to alterations in cortisol pharmacokinetics and the effects of hypocortisolism and/or hyperandrogenism (studies 1 & 2). Based on these findings one
may suggest that, when a short course of dexamethasone is required to achieve adequate suppression of HPA axis, the dose of choice should be 0.5mg/m² on the first day and 0.3 mg/m² on subsequent days. It should also be stated that this represents the dose required for overnight suppression of the HPA axis and not the daily requirements in terms of glucocorticoid substitution.
STUDY 6: SERUM CORTISOL AND GH INTERRELATIONSHIP

In classical 21-hydroxylase deficiency, conventional therapy with twice or thrice daily hydrocortisone substitution does not mimic normal adrenal responses and results in less than physiological cortisol concentrations. In view of the well established interaction between the HPA and GH axes, we investigated the cortisol – GH interrelationship in an attempt to evaluate the effect of exogenously administered hydrocortisone on the pattern of GH secretion in patients with CYP21 deficiency.

At the pituitary level, GH secretion is regulated by GH-releasing hormone (GHRH) and a GH release-inhibiting hormone, somatostatin (SS). Several neuropeptides can, in turn, modify GH secretion by acting on the central nervous system to modulate the hypothalamic secretion of GHRH and SS. Although a number of studies have investigated the interaction between HPA and GH axes, the GH – cortisol interrelationship has not been fully elucidated yet.

The temporal relationship between circulating cortisol and GH concentrations under physiological conditions is a complex one. While chronic exogenous or endogenous hypercortisolism results in reduced GH secretion and growth suppression (McArthur RG et al., 1972; Magiakou MA et al., 1994a; Magiakou MA et al., 1994b; Allen DB, 1996) as well as attenuation of GH response to exogenous stimuli (Kaufmann S et al., 1988; Miell JP et al, 1991; Bozzola M et al., 1991), patients with idiopathic ACTH deficiency need appropriate glucocorticoid replacement to re-establish the normal pattern of GH release in response to provocation, suggesting that normal glucocorticoid concentrations are required to ensure adequate GH secretion (Giustina A et al., 1989).
On the other hand, GH deficient patients have been shown to have low basal and stimulated cortisol concentrations, which are subsequently normalised after treatment with exogenous GH administration (Castro-Magana M et al., 1983).

To elucidate further the mechanism of glucocorticoid action on GH axis, Senaris et al, evaluated the effect of dexamethasone on hypothalamic SS and GHRH mRNA levels by in situ hybridisation (Senaris RM et al., 1996). The authors found a significant decrease of SS mRNA content in the periventricular nucleus (PVN) of the hypothalamus after 3, 8 and 15 days of treatment with dexamethasone and a reduction in GHRH mRNA levels in the arcuate nucleus after 8 and 15 days of treatment with the same steroid. They also found a significant decrease in GH receptor mRNA levels in the periventricular and arcuate nucleus after 1, 3, 8 and 15 days of treatment. Finally, in hypophysectomised rats, dexamethasone treatment for 15 days did not reduce SS mRNA levels in the PVN but decreased GHRH mRNA content in the arcuate nucleus significantly. Thus, their results suggested an inhibitory GH-mediated effect of dexamethasone on SS mRNA levels in the periventricular nucleus and an inhibitory direct effect of dexamethasone on GHRH neurones in the arcuate nucleus (Senaris RM et al., 1996).

The results of this study revealed a significant positive correlation between cortisol and GH concentrations at lag time 0 min, peaking at 1h 40 min. Also, a significant negative correlation between cortisol and GH was observed over time, peaking at lag time 6h 20 min, with cortisol leading GH by these time intervals. These findings are in agreement with previous studies, which documented a positive correlation between mean 24h cortisol concentration and mean 24h GH concentration, the sum of 24h GH pulse amplitudes and the number of GH pulses over a 24h period (Martinelli C et al., 1994),
and which confirmed that pre-treatment with hydrocortisone significantly augments the GH response to GHRH (Raza J et al., 1997). The negative correlation between cortisol and GH observed over time is probably due to the fact that circulating cortisol concentrations are undetectable and ACTH/CRH concentrations are therefore elevated approximately 4-5 hours after administration of hydrocortisone. It is worth noting, however, that the peak of the significant positive correlation in our patients was observed earlier (lag time 1h 40 min) than in short normal children (Ghizzoni L et al., 1996b; Hermida RC et al., 1999). This indicates that the supraphysiological cortisol concentrations attained following administration of oral hydrocortisone and/or the non-physiological pattern of circulating cortisol concentrations during the 24 hour period may alter the pattern of GH secretion in patients with CYP21 deficiency.

When data were analysed according to adequacy of adrenocortical suppression, a very similar pattern of cortisol-GH interrelationship was noted, however, the significant positive correlation reached a peak at lag time 40 min in the adequately controlled group and at lag time 1h and 40 min in the inadequately controlled group. This lag time difference between the two groups of patients most probably reflects and can be explained by differences in CRH concentrations. Inadequately controlled patients have higher CRH concentrations and hence increased release of SS, which may account for the delay in GH secretion observed in them as compared to the adequately controlled patients (Katakami H et al., 1985; Rivier C et al., 1985; Giustina A et al., 1990; Thomas GB et al., 1997).

Corticotrophin releasing hormone, although initially reported to be a potent and specific stimulus for ACTH release with no effect on other pituitary hormones (Grossman A et
al., 1982), was subsequently found to exert an effect on GH secretion. Animal studies provided considerable evidence that endogenous CRH can serve as a chemical mediator in the stress-induced inhibition of GH secretion (Rivier C et al., 1984; Ono N et al., 1984): intracerebroventricular injection of CRH inhibited pulsatile GH secretion and prevented GHRH-stimulated GH release in rats (Rivier C et al., 1984; Ono N et al., 1984). The suppressive action of CRH on GH secretion was attributed to increased release of SS because firstly, it was noted to be abolished by previous treatment with anti-SS serum (Katakami H et al., 1985; Rivier C et al., 1985) and secondly, prior administration of pyridostigmine, an agent which is reported to reduce somatostatine tone, was shown to enhance the GH response to dexamethasone administration (Giustina A et al., 1990). More recent studies in non-stressed conscious rats also showed that intravenous administration of CRH inhibits GH secretion (Thomas GB et al., 1997).

Similar findings were reported by investigators who examined the interaction between HPA and GH axis in man (Holsboer F et al., 1988; Barbarino A et al., 1990; Ghizzoni L et al., 1996a). Barbarino et al., investigated the effect of CRH on GHRH-induced GH secretion in healthy non-stressed individuals by using variable doses of CRH (100 µg, 50 µg and 25 µg) – which were administered intravenously and simultaneously with GHRH – and noted that the different doses of CRH had a different inhibitory effect on GH secretion in the two sexes, when the same subject was studied repeatedly. In women, GHRH-induced GH secretion was significantly inhibited after all doses of CRH, whereas in men CRH induced GH inhibition only after administration of a 100 µg dose. In addition, a dose of 100 µg CRH injected one hour before the administration of GHRH also resulted in inhibition of GHRH-induced GH release in both men and
women. These results supported previous reports and indicated that CRH exerts an inhibitory influence on pituitary hormone secretion for up to two hours after its administration. Subsequent studies confirmed those findings and also showed that the inhibitory effect of CRH on GHRH-induced GH release is not the result of ACTH or cortisol release but probably reflects a direct action of CRH on GH secretion, and that the acute rise in GH following ACTH or glucocorticoid administration can be partly explained by a rapid suppression of endogenous CRH (Raza J et al., 1997).

In female subjects CRH was effective at doses much lower than those required in males, even when the difference in body weight between two sexes had been taken into consideration. These findings most probably reflect gender differences in the neuroregulation of GH secretion. A sexual dimorphism in the secretory pattern of GH has been shown in rats and has been attributed to a higher somatostatinergic tone in male animals (Jansson OJ et al., 1985; Chowen-Breed JA et al., 1989). It is possible that, analogously, men have a higher somatostatinergic tone than women and thus, small doses of peripherally injected CRH are unable to induce a variation in GH secretion in male subjects. On the contrary, women may show an inhibition of GH secretion after small doses of CRH by virtue of a lower somatostatinergic tone.

In the present study, a lag time difference in the peak positive correlation was noted between males and females, with males demonstrating a more rapid release of GH as compared to females. Although not significantly different between the two groups, ACTH concentrations were higher in males (mean: 248.4 pg/ml, SD: 261.1) compared to females (mean: 113.2 pg/ml, SD: 118.4) and it is likely that this difference accounts for an increase in GHRH-induced GH secretion in males, in whom the significant
positive correlation between cortisol and GH reaches a peak 1h 40 min earlier than females (Raza J et al., 1997).

In summary, this study provides evidence of a positive correlation between circulating cortisol concentrations achieved following hydrocortisone administration and spontaneous GH secretion in patients with classical 21-hydroxylase deficiency, which reaches a peak earlier in the adequately controlled than the inadequately controlled patients. The negative correlation observed over time most probably reflects an alteration in the activity of HPA axis in the presence of low circulating cortisol concentrations. Further analysis using more robust mathematical models is required to evaluate the pattern of GH secretion and the relationship between cortisol and GH in these patients.
Summary

In patients with classical 21-hydroxylase deficiency, puberty is associated with alterations in cortisol pharmacokinetics. The rise in sex steroid concentrations and the concurrent alterations in the GH ~ IGFs / IGFBP-3 axis, 11β-HSD1 activity and insulin sensitivity are associated with increased clearance and shorter half life of total and free cortisol. Hypocortisolism, in turn, results in a loss of control of HPA axis and inadequate suppression of androgens and androgen precursors, which would further aggravate ovarian androgen production and insulin resistance. Hypocortisolism and hyperandrogenism lead to hypothalamic and pituitary stimulation and increased ACTH secretion. Raised ACTH concentrations further potentiate hypocortisolism by increasing cortisol clearance and decreasing its half life. The above alterations in cortisol clearance and CAH control affect females more than males, probably because of the sexually dimorphic patterns in GH secretion, 11β-HSD activity and CBG concentrations.

The increased activity of HPA axis between 0400h – 1600h suggests that glucocorticoid treatment should provide adequate suppression of the ACTH concentrations during this time. The hydrocortisone formulations currently used (Hydrocortone tablets) have essentially complete bioavailability but short half life, resulting in supraphysiological cortisol concentrations within one hour after administration of the oral dose but undetectable cortisol concentrations 4 – 5 hours later. It appears, therefore, that the limitations of current replacement therapy, which are inherent in our ability to control ACTH hypersecretion and suppress the adrenal cortex effectively, relate to the pharmacokinetic properties of the formulations used and the non-physiological cortisol concentrations attained. Consequently, sustained release formulations or constant
infusions (pump therapy), which would result in sufficient adrenocortical suppression, might be the only option of effective medical therapy. Further studies are required to explore whether these are viable alternatives to current replacement therapy. Meanwhile, administration of frequent (three or four) daily doses of oral hydrocortisone to provide adequate suppression of the HPA axis during daytime (0400h – 1600) and prevention or treatment of hyperandrogenism and insulin resistance appear to be the only options of medical treatment that are expected to offer the prospect of an improved outcome.
REFERENCES


Canny BJ, Jia LG, Leong DA. 1992. Corticotropin-releasing factor, but not arginine vasopressin, stimulates concentration-dependent increases in ACTH secretion from a


**Carsia RV, Malamed S.** 1983. Glucocorticoid control of steroidogenesis in isolated rat adrenocortical cells. *Biochim Biophys Acta** **763**: 83 – 89.


De Crecchio L. 1865. Supra un caso di apparenze virili in una donna. Il *Morgagni* 7: 154 – 188.


Grossman A, Kruseman AC, Perry L, Tomlin S, Schally AV, Coy DH, Rees LH, Comaru-Schally AM, Besser GM. 1982. New hypothalamic hormone, corticotropin-


Merke DP, Keil MF, Jones JV, Fields J, Hill S, Cutler GB Jr. 2000. Flutamide, testolactone, and reduced hydrocortisone dose maintain normal growth velocity and


Norusis MJ. 1990. SPSS PC+ 4.0 for the IBM PC/XT/AT and PS/2. Chicago, USA.


DECLARATION OF INVOLVEMENT

The studies presented in this thesis were performed at the London Centre for Paediatric Endocrinology following approval by the University College London Hospitals Committee on the Ethics of Human Research and the Great Ormond Street Hospital NHS Trust / Institute of Child Health Research Ethics Committees. The patients participated in the studies were consecutive attendees at the Outpatient Clinics at The Middlesex Hospital and Great Ormond Street Hospital for Children.

All patients were recruited by myself and detailed information on the purpose and aims of the studies was given to both patients and parents. The studies were undertaken on Carousel Ward, The Middlesex Hospital, and a single trained observer performed the anthropometric measurements on the day of admission to hospital. I co-ordinated all patients’ admission to the Ward and I was responsible for their care, including the administration of all intravenous and oral medication. I performed the 32 hour hormone profiles (a nurse performed the overnight sampling on most occasions) and the 0800h ACTH measurements on days 3 and 4. I was also responsible for spinning, separating and storing all samples.

Because of the large number of samples generated (10,600 samples) and the intensive sampling requirements, the hormone assays were performed by Ms B Leonard and supervised by Ms PJ Pringle in the Cobbold Laboratories, The Middlesex Hospital.

Determination of the pharmacokinetic parameters of total and free cortisol described in studies 1, 2 and 3 was carried out in collaboration with Dr A Johnston, Senior Lecturer in Clinical Pharmacology, St Bartholomew’s Medical School, London. Cross-correlation analyses described in studies 4 and 6 were performed in collaboration with Dr DR Matthews, Radcliffe General Infirmary, Oxford, who provided assistance in the mathematical processing of the data. Further data analysis, interpretation and presentation were carried out by myself. This work was supervised by Professor CGD Brook and Dr PC Hindmarsh, London Centre for Paediatric Endocrinology.
The studies described in this thesis gave rise to the following manuscripts:


2. E Charmandari, PC Hindmarsh, A Johnston, JW Honour, CGD Brook. Alterations in the endocrine milieu at puberty are associated with increased cortisol clearance (submitted for publication).


Two additional studies based mainly but not solely on this work gave rise to the following manuscripts:

ACKNOWLEDGEMENTS

I would like to start by telling you a story that I read recently. “One day, a little boy went to a parade with his father. In order for the boy to be able to see, his father hoisted him up onto his shoulders. As the parade was passing by, the little boy kept telling his father how well he could see and how spectacular the view was. Unfortunately, he became so arrogant about his wonderful view that he challenged those around him, who could not see very well: ‘If you could see what I can see’. But what the boy did not see was why he could see, and that he was tall enough to see only because he was sitting on someone else’s shoulders........”. The story continues and the author concludes by suggesting that in daily life, it is not happiness that makes us grateful but gratefulness that makes us happy.

First, I would like to express my gratitude to my supervisors, Professor CGD Brook and Dr PC Hindmarsh for encouraging me to undertake such an interesting research project and for providing the financial support for it. I am most grateful for their constant support, encouragement and guidance throughout these years, without which this work would not have reached its conclusion. I have spent three highly enjoyable and intellectually stimulating years working in their Department and I am indebted to them for giving me the opportunity to undertake clinical and research training in Paediatric Endocrinology under their supervision.

Also, I owe a great deal to Dr Atholl Johnston, Senior Lecturer in Clinical Pharmacology, St Bartholomew’s Medical School, who essentially played the role of a third supervisor and spent several hours with me facilitating the pharmacokinetic analysis of my data. Atholl introduced me to the basic concepts of pharmacokinetics and biopharmaceutics and encouraged me to discuss a number of issues surrounding the statistical analysis of my data. I am most grateful for his meticulous and expert work, for his scientific advice and for undertaking this work with a lot of enthusiasm and a great sense of humour.
I wish to acknowledge Dr DR Matthews at the Diabetes Research Centre, Radcliffe Infirmary, Oxford, who facilitated the mathematical processing of cross-correlation studies and assisted me in the interpretation of the results.

I am most grateful to Mrs Brankica Leonard and Mrs Nadia Payne for their technical support and Ms Jane Pringle for co-ordinating and supervising all hormonal analyses performed in the Cobbold Laboratories and for providing answers to a number of practical problems.

Special thanks go to Ms Jane McLean and the staff on Carousel Ward, for accommodating all patients and for making their stay in hospital as pleasant as possible. Special thanks also go to Mrs Susan Clark, for performing the anthropometric measurements on all patients on the day of their admission to hospital.

None of this work would have been possible without the participation of the forty patients with classical 21-hydroxylase deficiency in these studies. I am immensely grateful to all of the children and their parents.

Since I am submitting this thesis at the end of an eight-year period of training in England and just before leaving the United Kingdom, I would like to acknowledge a number of Consultants, who supervised my training in Paediatrics during the first five years and played a special role in my so far career by providing invaluable help and support when it was most needed. They are: Dr John Alexander, Consultant Paediatrician, Pontefract General Infirmary, Pontefract; Dr Anthony Robinson, Consultant Paediatrician, Withington Hospital, Manchester; Dr Paula Bolton-Maggs, Consultant Paediatric Haematologist, Alder Hey Children’s Hospital, Liverpool; Dr Kevin Walsh, Consultant Paediatric Cardiologist, Alder Hey Children’s Hospital, Liverpool; and Dr David Easton, Consultant Paediatrician, Basildon General Hospital, Basildon.

Finally, I would like to thank three close friends, Julia and Panos, who witnessed the ebbs and flows of this research work and provided much needed support throughout the last two years, and Paul, who awakened my interest in research and medical education and has always encouraged me to give everything I have to everything I do. I cannot
submit this work without also acknowledging my parents and my brother, to whom I owe a great deal.

Ευαγγελία Χαμάνδαρη

October 21, 2000
APPENDICES
### APPENDIX I

**Clinical characteristics of the 40 patients with classical 21-hydroxylase deficiency**

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>Age (yrs)</th>
<th>BMI (kg/m$^2$)</th>
<th>HC (mg/m$^2$/day)</th>
<th>FC (μg/m$^2$/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>JM</td>
<td>6.1</td>
<td>14.8</td>
<td>22.6</td>
<td>129.4</td>
</tr>
<tr>
<td>2.</td>
<td>IL</td>
<td>6.2</td>
<td>23.0</td>
<td>20.0</td>
<td>122.8</td>
</tr>
<tr>
<td>3.</td>
<td>JK</td>
<td>6.8</td>
<td>16.9</td>
<td>20.0</td>
<td>143.0</td>
</tr>
<tr>
<td>4.</td>
<td>KG</td>
<td>7.4</td>
<td>19.2</td>
<td>15.9</td>
<td>105.8</td>
</tr>
<tr>
<td>5.</td>
<td>TL</td>
<td>8.0</td>
<td>17.9</td>
<td>15.6</td>
<td>88.9</td>
</tr>
<tr>
<td>6.</td>
<td>HT</td>
<td>9.2</td>
<td>19.0</td>
<td>17.7</td>
<td>132.6</td>
</tr>
<tr>
<td>7.</td>
<td>AH</td>
<td>9.3</td>
<td>23.4</td>
<td>16.8</td>
<td>130.6</td>
</tr>
<tr>
<td>8.</td>
<td>LW</td>
<td>9.5</td>
<td>19.7</td>
<td>21.6</td>
<td>72.0</td>
</tr>
<tr>
<td>9.</td>
<td>CG</td>
<td>9.6</td>
<td>20.0</td>
<td>14.4</td>
<td>143.8</td>
</tr>
<tr>
<td>10.</td>
<td>SH</td>
<td>9.9</td>
<td>18.0</td>
<td>14.5</td>
<td>120.7</td>
</tr>
<tr>
<td>11.</td>
<td>AR</td>
<td>9.9</td>
<td>22.6</td>
<td>18.2</td>
<td>145.8</td>
</tr>
<tr>
<td>12.</td>
<td>DR</td>
<td>10.0</td>
<td>19.2</td>
<td>19.8</td>
<td>138.3</td>
</tr>
<tr>
<td>13.</td>
<td>CC</td>
<td>10.6</td>
<td>28.8</td>
<td>12.6</td>
<td>93.9</td>
</tr>
<tr>
<td>14.</td>
<td>GH</td>
<td>10.8</td>
<td>19.7</td>
<td>14.8</td>
<td>84.6</td>
</tr>
<tr>
<td>15.</td>
<td>SB</td>
<td>10.8</td>
<td>25.2</td>
<td>18.5</td>
<td>123.5</td>
</tr>
<tr>
<td>16.</td>
<td>AP</td>
<td>11.0</td>
<td>19.9</td>
<td>20.3</td>
<td>121.9</td>
</tr>
<tr>
<td>17.</td>
<td>EBE</td>
<td>11.4</td>
<td>27.9</td>
<td>18.8</td>
<td>102.7</td>
</tr>
<tr>
<td>18.</td>
<td>HBR</td>
<td>12.2</td>
<td>35.3</td>
<td>16.5</td>
<td>110.1</td>
</tr>
<tr>
<td>19.</td>
<td>LS</td>
<td>12.4</td>
<td>23.5</td>
<td>18.7</td>
<td>124.7</td>
</tr>
<tr>
<td>20.</td>
<td>LH</td>
<td>12.5</td>
<td>15.8</td>
<td>19.3</td>
<td>135.0</td>
</tr>
<tr>
<td>21.</td>
<td>AW</td>
<td>12.8</td>
<td>28.6</td>
<td>18.1</td>
<td>103.0</td>
</tr>
<tr>
<td>22.</td>
<td>MR</td>
<td>13.1</td>
<td>19.5</td>
<td>16.9</td>
<td>---</td>
</tr>
<tr>
<td>23.</td>
<td>EB</td>
<td>13.2</td>
<td>22.8</td>
<td>10.2</td>
<td>58.2</td>
</tr>
<tr>
<td>24.</td>
<td>NP</td>
<td>13.3</td>
<td>15.8</td>
<td>17.3</td>
<td>129.5</td>
</tr>
<tr>
<td>25.</td>
<td>VS</td>
<td>13.6</td>
<td>22.7</td>
<td>19.2</td>
<td>96.2</td>
</tr>
</tbody>
</table>
Clinical characteristics of the 40 patients with classical 21-hydroxylase deficiency (cont…)

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>Age (yrs)</th>
<th>BMI (kg/m²)</th>
<th>HC (mg/m²/day)</th>
<th>FC (μg/m²/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>MS</td>
<td>14.0</td>
<td>21.6</td>
<td>16.0</td>
<td>160.0</td>
</tr>
<tr>
<td>27</td>
<td>GM</td>
<td>14.3</td>
<td>30.1</td>
<td>16.2</td>
<td>80.8</td>
</tr>
<tr>
<td>28</td>
<td>HB</td>
<td>14.5</td>
<td>38.8</td>
<td>19.9</td>
<td>124.1</td>
</tr>
<tr>
<td>29</td>
<td>KB</td>
<td>14.5</td>
<td>28.1</td>
<td>19.3</td>
<td>132.5</td>
</tr>
<tr>
<td>30</td>
<td>JS</td>
<td>14.5</td>
<td>42.5</td>
<td>18.0</td>
<td>135.0</td>
</tr>
<tr>
<td>31</td>
<td>SHK</td>
<td>15.0</td>
<td>23.9</td>
<td>18.4</td>
<td>138.0</td>
</tr>
<tr>
<td>32</td>
<td>AM</td>
<td>16.0</td>
<td>24.9</td>
<td>17.5</td>
<td>87.6</td>
</tr>
<tr>
<td>33</td>
<td>MIR</td>
<td>16.4</td>
<td>26.2</td>
<td>14.8</td>
<td>---</td>
</tr>
<tr>
<td>34</td>
<td>WL</td>
<td>16.8</td>
<td>28.0</td>
<td>17.0</td>
<td>141.6</td>
</tr>
<tr>
<td>35</td>
<td>SG</td>
<td>17.1</td>
<td>22.5</td>
<td>14.5</td>
<td>128.6</td>
</tr>
<tr>
<td>36</td>
<td>EBI</td>
<td>18.1</td>
<td>22.8</td>
<td>18.7</td>
<td>62.5</td>
</tr>
<tr>
<td>37</td>
<td>KS</td>
<td>18.3</td>
<td>31.1</td>
<td>18.8</td>
<td>84.4</td>
</tr>
<tr>
<td>38</td>
<td>EBU</td>
<td>18.4</td>
<td>25.3</td>
<td>15.6</td>
<td>126.9</td>
</tr>
<tr>
<td>39</td>
<td>SK</td>
<td>18.8</td>
<td>26.6</td>
<td>18.9</td>
<td>54.0</td>
</tr>
<tr>
<td>40</td>
<td>KG</td>
<td>20.3</td>
<td>31.5</td>
<td>15.0</td>
<td>44.3</td>
</tr>
</tbody>
</table>
### APPENDIX II

Pharmacokinetic parameters of total cortisol in the 40 patients

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>Clearance (ml/min)</th>
<th>Volume of distribution (L)</th>
<th>Half life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>JK</td>
<td>107.4</td>
<td>20.7</td>
<td>70.1</td>
</tr>
<tr>
<td>2.</td>
<td>CG</td>
<td>237.9</td>
<td>22.0</td>
<td>70.3</td>
</tr>
<tr>
<td>3.</td>
<td>KG</td>
<td>116.0</td>
<td>15.5</td>
<td>67.1</td>
</tr>
<tr>
<td>4.</td>
<td>GH</td>
<td>239.2</td>
<td>28.1</td>
<td>74.4</td>
</tr>
<tr>
<td>5.</td>
<td>AH</td>
<td>443.3</td>
<td>38.1</td>
<td>91.6</td>
</tr>
<tr>
<td>6.</td>
<td>SH</td>
<td>279.5</td>
<td>26.0</td>
<td>66.8</td>
</tr>
<tr>
<td>7.</td>
<td>TL</td>
<td>258.3</td>
<td>30.0</td>
<td>53.9</td>
</tr>
<tr>
<td>8.</td>
<td>JM</td>
<td>181.2</td>
<td>19.1</td>
<td>69.5</td>
</tr>
<tr>
<td>9.</td>
<td>AP</td>
<td>322.3</td>
<td>30.0</td>
<td>62.3</td>
</tr>
<tr>
<td>10.</td>
<td>AR</td>
<td>267.8</td>
<td>35.0</td>
<td>65.8</td>
</tr>
<tr>
<td>11.</td>
<td>DR</td>
<td>181.1</td>
<td>25.21</td>
<td>66.7</td>
</tr>
<tr>
<td>12.</td>
<td>HT</td>
<td>239.3</td>
<td>22.0</td>
<td>51.3</td>
</tr>
<tr>
<td>13.</td>
<td>LW</td>
<td>174.0</td>
<td>20.8</td>
<td>68.8</td>
</tr>
<tr>
<td>14.</td>
<td>IL</td>
<td>434.3</td>
<td>46.8</td>
<td>61.9</td>
</tr>
<tr>
<td>15.</td>
<td>EB</td>
<td>275.5</td>
<td>33.4</td>
<td>59.3</td>
</tr>
<tr>
<td>16.</td>
<td>HB</td>
<td>329.0</td>
<td>51.8</td>
<td>63.1</td>
</tr>
<tr>
<td>17.</td>
<td>EBE</td>
<td>413.3</td>
<td>46.1</td>
<td>51.0</td>
</tr>
<tr>
<td>18.</td>
<td>HBR</td>
<td>416.6</td>
<td>47.5</td>
<td>54.5</td>
</tr>
<tr>
<td>19.</td>
<td>SB</td>
<td>428.2</td>
<td>35.2</td>
<td>42.4</td>
</tr>
<tr>
<td>20.</td>
<td>KB</td>
<td>722.1</td>
<td>61.3</td>
<td>47.1</td>
</tr>
<tr>
<td>21.</td>
<td>CC</td>
<td>462.6</td>
<td>42.6</td>
<td>50.9</td>
</tr>
<tr>
<td>22.</td>
<td>LH</td>
<td>384.2</td>
<td>40.5</td>
<td>160.8</td>
</tr>
<tr>
<td>23.</td>
<td>SHK</td>
<td>223.5</td>
<td>48.4</td>
<td>83.9</td>
</tr>
<tr>
<td>24.</td>
<td>GM</td>
<td>779.5</td>
<td>70.2</td>
<td>225.3</td>
</tr>
<tr>
<td>25.</td>
<td>AM</td>
<td>348.0</td>
<td>61.4</td>
<td>81.8</td>
</tr>
</tbody>
</table>
Pharmacokinetic parameters of total cortisol in the 40 patients (cont...)

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>Clearance (ml/min)</th>
<th>Volume of distribution (L)</th>
<th>Half life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>NP</td>
<td>358.2</td>
<td>30.6</td>
<td>63.7</td>
</tr>
<tr>
<td>27</td>
<td>VS</td>
<td>487.8</td>
<td>47.3</td>
<td>117.3</td>
</tr>
<tr>
<td>28</td>
<td>MS</td>
<td>384.9</td>
<td>41.2</td>
<td>58.5</td>
</tr>
<tr>
<td>29</td>
<td>LS</td>
<td>293.2</td>
<td>37.6</td>
<td>51.6</td>
</tr>
<tr>
<td>30</td>
<td>AW</td>
<td>443.1</td>
<td>41.4</td>
<td>83.2</td>
</tr>
<tr>
<td>31</td>
<td>WL</td>
<td>422.8</td>
<td>64.9</td>
<td>62.2</td>
</tr>
<tr>
<td>32</td>
<td>JS</td>
<td>523.6</td>
<td>69.9</td>
<td>53.6</td>
</tr>
<tr>
<td>33</td>
<td>MIR</td>
<td>370.7</td>
<td>62.1</td>
<td>71.1</td>
</tr>
<tr>
<td>34</td>
<td>MR</td>
<td>474.4</td>
<td>57.8</td>
<td>60.2</td>
</tr>
<tr>
<td>35</td>
<td>EBI</td>
<td>229.8</td>
<td>28.2</td>
<td>84.9</td>
</tr>
<tr>
<td>36</td>
<td>EBU</td>
<td>298.8</td>
<td>43.9</td>
<td>101.7</td>
</tr>
<tr>
<td>37</td>
<td>KG</td>
<td>432.1</td>
<td>59.6</td>
<td>95.6</td>
</tr>
<tr>
<td>38</td>
<td>SG</td>
<td>243.3</td>
<td>29.9</td>
<td>85.1</td>
</tr>
<tr>
<td>39</td>
<td>SK</td>
<td>397.8</td>
<td>59.5</td>
<td>103.7</td>
</tr>
<tr>
<td>40</td>
<td>KS</td>
<td>152.9</td>
<td>24.0</td>
<td>108.9</td>
</tr>
</tbody>
</table>
### APPENDIX III

Pharmacokinetic parameters of free cortisol in the 40 patients

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>Clearance (ml/min)</th>
<th>Volume of distribution (L)</th>
<th>Half life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>JK</td>
<td>925</td>
<td>93.6</td>
<td>70.1</td>
</tr>
<tr>
<td>2.</td>
<td>CG</td>
<td>2210</td>
<td>224</td>
<td>70.3</td>
</tr>
<tr>
<td>3.</td>
<td>KG</td>
<td>738</td>
<td>71.5</td>
<td>67.1</td>
</tr>
<tr>
<td>4.</td>
<td>GH</td>
<td>2403</td>
<td>257.8</td>
<td>74.4</td>
</tr>
<tr>
<td>5.</td>
<td>AH</td>
<td>3266</td>
<td>431.9</td>
<td>91.7</td>
</tr>
<tr>
<td>6.</td>
<td>SH</td>
<td>2470</td>
<td>237.6</td>
<td>66.9</td>
</tr>
<tr>
<td>7.</td>
<td>TL</td>
<td>3571</td>
<td>277.8</td>
<td>53.9</td>
</tr>
<tr>
<td>8.</td>
<td>JM</td>
<td>2535</td>
<td>254.1</td>
<td>69.5</td>
</tr>
<tr>
<td>9.</td>
<td>AP</td>
<td>3859</td>
<td>346.8</td>
<td>62.3</td>
</tr>
<tr>
<td>10.</td>
<td>AR</td>
<td>2681</td>
<td>254.5</td>
<td>65.8</td>
</tr>
<tr>
<td>11.</td>
<td>DR</td>
<td>1383</td>
<td>133.2</td>
<td>66.7</td>
</tr>
<tr>
<td>12.</td>
<td>HT</td>
<td>3608</td>
<td>267.2</td>
<td>51.3</td>
</tr>
<tr>
<td>13.</td>
<td>LW</td>
<td>1831</td>
<td>181.7</td>
<td>68.8</td>
</tr>
<tr>
<td>14.</td>
<td>IL</td>
<td>3204</td>
<td>286.1</td>
<td>61.9</td>
</tr>
<tr>
<td>15.</td>
<td>EB</td>
<td>3984</td>
<td>340.7</td>
<td>59.3</td>
</tr>
<tr>
<td>16.</td>
<td>HB</td>
<td>3106</td>
<td>282.7</td>
<td>63.1</td>
</tr>
<tr>
<td>17.</td>
<td>EBE</td>
<td>4588</td>
<td>337.2</td>
<td>51.0</td>
</tr>
<tr>
<td>18.</td>
<td>HBR</td>
<td>3854</td>
<td>303.1</td>
<td>54.5</td>
</tr>
<tr>
<td>19.</td>
<td>SB</td>
<td>4265</td>
<td>260.7</td>
<td>42.4</td>
</tr>
<tr>
<td>20.</td>
<td>KB</td>
<td>7980</td>
<td>542.5</td>
<td>47.1</td>
</tr>
<tr>
<td>21.</td>
<td>CC</td>
<td>12128</td>
<td>891.1</td>
<td>50.9</td>
</tr>
<tr>
<td>22.</td>
<td>LH</td>
<td>3155</td>
<td>731.8</td>
<td>160.8</td>
</tr>
<tr>
<td>23.</td>
<td>SHK</td>
<td>1962</td>
<td>237.4</td>
<td>83.9</td>
</tr>
<tr>
<td>24.</td>
<td>GM</td>
<td>7535</td>
<td>2448.8</td>
<td>225.2</td>
</tr>
<tr>
<td>25.</td>
<td>AM</td>
<td>3801</td>
<td>448.7</td>
<td>81.8</td>
</tr>
</tbody>
</table>
### Pharmacokinetic parameters of free cortisol in the 40 patients (cont...)

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>Clearance (ml/min)</th>
<th>Volume of distribution (L)</th>
<th>Half life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>NP</td>
<td>4126</td>
<td>379.3</td>
<td>63.7</td>
</tr>
<tr>
<td>27</td>
<td>VS</td>
<td>5026</td>
<td>850.8</td>
<td>117.3</td>
</tr>
<tr>
<td>28</td>
<td>MS</td>
<td>6686</td>
<td>564.7</td>
<td>58.5</td>
</tr>
<tr>
<td>29</td>
<td>LS</td>
<td>2430</td>
<td>181.0</td>
<td>51.6</td>
</tr>
<tr>
<td>30</td>
<td>AW</td>
<td>4556</td>
<td>546.9</td>
<td>83.2</td>
</tr>
<tr>
<td>31</td>
<td>WL</td>
<td>2518</td>
<td>225.9</td>
<td>62.2</td>
</tr>
<tr>
<td>32</td>
<td>JS</td>
<td>3851</td>
<td>297.6</td>
<td>53.6</td>
</tr>
<tr>
<td>33</td>
<td>MIR</td>
<td>3617</td>
<td>370.9</td>
<td>71.1</td>
</tr>
<tr>
<td>34</td>
<td>MR</td>
<td>6585</td>
<td>571.8</td>
<td>60.2</td>
</tr>
<tr>
<td>35</td>
<td>EBI</td>
<td>1664</td>
<td>147.3</td>
<td>61.3</td>
</tr>
<tr>
<td>36</td>
<td>EBU</td>
<td>2857</td>
<td>147.3</td>
<td>61.5</td>
</tr>
<tr>
<td>37</td>
<td>KG</td>
<td>4411</td>
<td>253.6</td>
<td>63.5</td>
</tr>
<tr>
<td>38</td>
<td>SG</td>
<td>1831</td>
<td>403.9</td>
<td>50.9</td>
</tr>
<tr>
<td>39</td>
<td>SK</td>
<td>3571</td>
<td>134.4</td>
<td>69.0</td>
</tr>
<tr>
<td>40</td>
<td>KS</td>
<td>3677</td>
<td>135.3</td>
<td>68.8</td>
</tr>
</tbody>
</table>
APPENDIX IV

Mean 24h GH concentrations and 0800h IGF-I, IGF-II and IGFBP-3 concentrations

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>GH (mU/L)</th>
<th>IGF-I (ng/ml)</th>
<th>IGF-II (ng/ml)</th>
<th>IGFBP-3 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>JK</td>
<td>4.2</td>
<td>91.9</td>
<td>496.7</td>
<td>2260.4</td>
</tr>
<tr>
<td>2.</td>
<td>CG</td>
<td>2.9</td>
<td>201.0</td>
<td>513.4</td>
<td>2456.3</td>
</tr>
<tr>
<td>3.</td>
<td>KG</td>
<td>2.5</td>
<td>190.9</td>
<td>486.7</td>
<td>2737.8</td>
</tr>
<tr>
<td>4.</td>
<td>GH</td>
<td>5.6</td>
<td>258.3</td>
<td>767.2</td>
<td>3970.2</td>
</tr>
<tr>
<td>5.</td>
<td>AH</td>
<td>3.3</td>
<td>484.7</td>
<td>816.6</td>
<td>4813.4</td>
</tr>
<tr>
<td>6.</td>
<td>SH</td>
<td>5.7</td>
<td>175.2</td>
<td>643.6</td>
<td>2989.2</td>
</tr>
<tr>
<td>7.</td>
<td>TL</td>
<td>13.5</td>
<td>190.7</td>
<td>891.5</td>
<td>4601.8</td>
</tr>
<tr>
<td>8.</td>
<td>JM</td>
<td>6.0</td>
<td>189.9</td>
<td>658.2</td>
<td>3321.9</td>
</tr>
<tr>
<td>9.</td>
<td>AP</td>
<td>2.5</td>
<td>190.5</td>
<td>609.5</td>
<td>3436.9</td>
</tr>
<tr>
<td>10.</td>
<td>AR</td>
<td>1.8</td>
<td>216.1</td>
<td>551.2</td>
<td>2774.9</td>
</tr>
<tr>
<td>11.</td>
<td>DR</td>
<td>5.6</td>
<td>225.8</td>
<td>526.2</td>
<td>2690.9</td>
</tr>
<tr>
<td>12.</td>
<td>HT</td>
<td>5.1</td>
<td>201.8</td>
<td>731.2</td>
<td>3308.1</td>
</tr>
<tr>
<td>13.</td>
<td>LW</td>
<td>2.2</td>
<td>192.5</td>
<td>711.4</td>
<td>3339.2</td>
</tr>
<tr>
<td>14.</td>
<td>IL</td>
<td>3.2</td>
<td>373.4</td>
<td>668.8</td>
<td>3585.9</td>
</tr>
<tr>
<td>15.</td>
<td>EB</td>
<td>2.3</td>
<td>264.4</td>
<td>771.3</td>
<td>3362.1</td>
</tr>
<tr>
<td>16.</td>
<td>HB</td>
<td>1.2</td>
<td>319.3</td>
<td>628.3</td>
<td>3225.3</td>
</tr>
<tr>
<td>17.</td>
<td>EBE</td>
<td>7.3</td>
<td>426.1</td>
<td>598.3</td>
<td>3951.0</td>
</tr>
<tr>
<td>18.</td>
<td>HBR</td>
<td>5.4</td>
<td>381.1</td>
<td>751.0</td>
<td>1476.4</td>
</tr>
<tr>
<td>19.</td>
<td>SB</td>
<td>3.7</td>
<td>508.5</td>
<td>636.2</td>
<td>4329.6</td>
</tr>
<tr>
<td>20.</td>
<td>KB</td>
<td>5.5</td>
<td>479.6</td>
<td>629.0</td>
<td>4128.2</td>
</tr>
<tr>
<td>21.</td>
<td>CC</td>
<td>1.6</td>
<td>363.4</td>
<td>821.6</td>
<td>5352.8</td>
</tr>
<tr>
<td>22.</td>
<td>LH</td>
<td>7.3</td>
<td>463.8</td>
<td>676.8</td>
<td>4042.1</td>
</tr>
<tr>
<td>23.</td>
<td>SHK</td>
<td>5.0</td>
<td>344.4</td>
<td>578.5</td>
<td>3105.5</td>
</tr>
<tr>
<td>24.</td>
<td>GM</td>
<td>2.5</td>
<td>270.8</td>
<td>481.0</td>
<td>3294.1</td>
</tr>
<tr>
<td>25.</td>
<td>AM</td>
<td>1.2</td>
<td>280.4</td>
<td>624.8</td>
<td>3473.6</td>
</tr>
</tbody>
</table>
Mean 24h GH concentrations and 0800h IGF-I, IGF-II and IGFBP-3 concentrations (cont...)

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>GH (mU/L)</th>
<th>IGF-I (ng/ml)</th>
<th>IGF-II (ng/ml)</th>
<th>IGFBP-3 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>NP</td>
<td>8.5</td>
<td>303.8</td>
<td>644.5</td>
<td>4276.4</td>
</tr>
<tr>
<td>27</td>
<td>VS</td>
<td>5.5</td>
<td>628.4</td>
<td>559.4</td>
<td>4446.1</td>
</tr>
<tr>
<td>28</td>
<td>MS</td>
<td>6.3</td>
<td>323.4</td>
<td>789.6</td>
<td>4069.8</td>
</tr>
<tr>
<td>29</td>
<td>LS</td>
<td>4.7</td>
<td>322.7</td>
<td>431.2</td>
<td>3775.5</td>
</tr>
<tr>
<td>30</td>
<td>AW</td>
<td>3.2</td>
<td>363.3</td>
<td>617.1</td>
<td>4316.8</td>
</tr>
<tr>
<td>31</td>
<td>WL</td>
<td>3.8</td>
<td>212.1</td>
<td>500.2</td>
<td>3071.5</td>
</tr>
<tr>
<td>32</td>
<td>JS</td>
<td>2.7</td>
<td>289.2</td>
<td>821.6</td>
<td>4035.7</td>
</tr>
<tr>
<td>33</td>
<td>MIR</td>
<td>3.7</td>
<td>348.8</td>
<td>463.0</td>
<td>4103.3</td>
</tr>
<tr>
<td>34</td>
<td>MR</td>
<td>20.2</td>
<td>528.1</td>
<td>509.7</td>
<td>4131.3</td>
</tr>
<tr>
<td>35</td>
<td>EBI</td>
<td>2.9</td>
<td>200.5</td>
<td>658.0</td>
<td>3371.2</td>
</tr>
<tr>
<td>36</td>
<td>EBU</td>
<td>4.0</td>
<td>324.3</td>
<td>1090.8</td>
<td>5040.1</td>
</tr>
<tr>
<td>37</td>
<td>KG</td>
<td>---</td>
<td>285.9</td>
<td>698.8</td>
<td>3518.9</td>
</tr>
<tr>
<td>38</td>
<td>SG</td>
<td>---</td>
<td>336.7</td>
<td>888.2</td>
<td>5346.5</td>
</tr>
<tr>
<td>39</td>
<td>SK</td>
<td>5.1</td>
<td>180.1</td>
<td>703.8</td>
<td>3064.3</td>
</tr>
<tr>
<td>40</td>
<td>KS</td>
<td>---</td>
<td>176.1</td>
<td>1512.7</td>
<td>6212.2</td>
</tr>
</tbody>
</table>
### APPENDIX V

Mean 24h 17-hydroxyprogesterone (17-OHP) concentrations and 0800h ACTH, Androstendione (A) and PRA concentrations

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>17-OHP (nmol/L)</th>
<th>ACTH (pg/ml)</th>
<th>A (nmol/L)</th>
<th>PRA (nmol/h/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>JK</td>
<td>23.5</td>
<td>26.5</td>
<td>0.5</td>
<td>1.69</td>
</tr>
<tr>
<td>2.</td>
<td>CG</td>
<td>0.3</td>
<td>66.8</td>
<td>0.35</td>
<td>1.69</td>
</tr>
<tr>
<td>3.</td>
<td>KG</td>
<td>0.5</td>
<td>37.4</td>
<td>0.35</td>
<td>1.42</td>
</tr>
<tr>
<td>4.</td>
<td>GH</td>
<td>2.6</td>
<td>46.6</td>
<td>0.35</td>
<td>2.29</td>
</tr>
<tr>
<td>5.</td>
<td>AH</td>
<td>6.3</td>
<td>43.3</td>
<td>1.2</td>
<td>3.08</td>
</tr>
<tr>
<td>6.</td>
<td>SH</td>
<td>65.3</td>
<td>198.8</td>
<td>2.1</td>
<td>1.55</td>
</tr>
<tr>
<td>7.</td>
<td>TL</td>
<td>115.0</td>
<td>204.0</td>
<td>0.35</td>
<td>1.45</td>
</tr>
<tr>
<td>8.</td>
<td>JM</td>
<td>0.3</td>
<td>33.0</td>
<td>0.35</td>
<td>1.83</td>
</tr>
<tr>
<td>9.</td>
<td>AP</td>
<td>0.3</td>
<td>60.3</td>
<td>0.35</td>
<td>1.23</td>
</tr>
<tr>
<td>10.</td>
<td>AR</td>
<td>0.3</td>
<td>29.1</td>
<td>0.35</td>
<td>1.04</td>
</tr>
<tr>
<td>11.</td>
<td>DR</td>
<td>344.1</td>
<td>228.0</td>
<td>108.8</td>
<td>16.1</td>
</tr>
<tr>
<td>12.</td>
<td>HT</td>
<td>28.9</td>
<td>97.0</td>
<td>0.35</td>
<td>2.83</td>
</tr>
<tr>
<td>13.</td>
<td>LW</td>
<td>0.3</td>
<td>24.9</td>
<td>0.35</td>
<td>8.9</td>
</tr>
<tr>
<td>14.</td>
<td>IL</td>
<td>106.9</td>
<td>209.8</td>
<td>27.9</td>
<td>1.22</td>
</tr>
<tr>
<td>15.</td>
<td>EB</td>
<td>5.5</td>
<td>62.0</td>
<td>4.0</td>
<td>4.16</td>
</tr>
<tr>
<td>16.</td>
<td>HB</td>
<td>2.2</td>
<td>58.0</td>
<td>0.9</td>
<td>3.74</td>
</tr>
<tr>
<td>17.</td>
<td>EBE</td>
<td>39.2</td>
<td>93.6</td>
<td>6.4</td>
<td>2.17</td>
</tr>
<tr>
<td>18.</td>
<td>HBR</td>
<td>4.5</td>
<td>85.3</td>
<td>1.0</td>
<td>1.16</td>
</tr>
<tr>
<td>19.</td>
<td>SB</td>
<td>71.9</td>
<td>153.0</td>
<td>11.8</td>
<td>1.18</td>
</tr>
<tr>
<td>20.</td>
<td>KB</td>
<td>0.3</td>
<td>76.4</td>
<td>0.35</td>
<td>6.74</td>
</tr>
<tr>
<td>21.</td>
<td>CC</td>
<td>82.3</td>
<td>188.4</td>
<td>---</td>
<td>2.78</td>
</tr>
<tr>
<td>22.</td>
<td>LH</td>
<td>213.9</td>
<td>245.2</td>
<td>27.4</td>
<td>2.09</td>
</tr>
<tr>
<td>23.</td>
<td>SHK</td>
<td>580.1</td>
<td>856.0</td>
<td>---</td>
<td>6.55</td>
</tr>
<tr>
<td>24.</td>
<td>GM</td>
<td>22.1</td>
<td>535.1</td>
<td>0.35</td>
<td>1.41</td>
</tr>
<tr>
<td>25.</td>
<td>AM</td>
<td>3.7</td>
<td>29.3</td>
<td>0.35</td>
<td>2.95</td>
</tr>
</tbody>
</table>
Mean 24h 17-hydroxyprogesterone concentrations (17-OHP) and 0800h ACTH, Androstendione (A) and PRA concentrations (cont...)

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>17-OHP (nmol/L)</th>
<th>ACTH (pg/ml)</th>
<th>A (nmol/L)</th>
<th>PRA (nmol/h/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>NP</td>
<td>4.9</td>
<td>312.8</td>
<td>0.35</td>
<td>1.03</td>
</tr>
<tr>
<td>27</td>
<td>VS</td>
<td>145.8</td>
<td>502.5</td>
<td>20.8</td>
<td>2.28</td>
</tr>
<tr>
<td>28</td>
<td>MS</td>
<td>12.4</td>
<td>92.6</td>
<td>1.6</td>
<td>2.54</td>
</tr>
<tr>
<td>29</td>
<td>LS</td>
<td>60.4</td>
<td>244.5</td>
<td>9.0</td>
<td>3.12</td>
</tr>
<tr>
<td>30</td>
<td>AW</td>
<td>57.5</td>
<td>208.0</td>
<td>10.4</td>
<td>1.39</td>
</tr>
<tr>
<td>31</td>
<td>WL</td>
<td>136.5</td>
<td>90.0</td>
<td>84.6</td>
<td>1.48</td>
</tr>
<tr>
<td>32</td>
<td>JS</td>
<td>584.8</td>
<td>172.0</td>
<td>48.0</td>
<td>7.4</td>
</tr>
<tr>
<td>33</td>
<td>MIR</td>
<td>23.3</td>
<td>17.3</td>
<td>4.7</td>
<td>2.17</td>
</tr>
<tr>
<td>34</td>
<td>MR</td>
<td>7.4</td>
<td>45.4</td>
<td>0.35</td>
<td>4.26</td>
</tr>
<tr>
<td>35</td>
<td>EBI</td>
<td>1.2</td>
<td>32.4</td>
<td>0.4</td>
<td>6.92</td>
</tr>
<tr>
<td>36</td>
<td>EBU</td>
<td>1.8</td>
<td>67.8</td>
<td>2.1</td>
<td>7.51</td>
</tr>
<tr>
<td>37</td>
<td>KG</td>
<td>---</td>
<td>372.2</td>
<td>---</td>
<td>9.29</td>
</tr>
<tr>
<td>38</td>
<td>SG</td>
<td>---</td>
<td>78.0</td>
<td>---</td>
<td>12.8</td>
</tr>
<tr>
<td>39</td>
<td>SK</td>
<td>107.4</td>
<td>624.0</td>
<td>2.1</td>
<td>2.05</td>
</tr>
<tr>
<td>40</td>
<td>KS</td>
<td>---</td>
<td>107.8</td>
<td>1.8</td>
<td>11.1</td>
</tr>
</tbody>
</table>
APPENDIX VI

0800h Fasting Glucose, Insulin, Leptin and CBG concentrations

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>Glucose (mmol/L)</th>
<th>Insulin (mU/L)</th>
<th>Leptin (ng/ml)</th>
<th>CBG (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>JK</td>
<td>4.0</td>
<td>7.1</td>
<td>3.1</td>
<td>45.0</td>
</tr>
<tr>
<td>2.</td>
<td>CG</td>
<td>3.8</td>
<td>7.4</td>
<td>12.5</td>
<td>47.5</td>
</tr>
<tr>
<td>3.</td>
<td>KG</td>
<td>4.0</td>
<td>3.2</td>
<td>9.1</td>
<td>47.5</td>
</tr>
<tr>
<td>4.</td>
<td>GH</td>
<td>4.2</td>
<td>8.0</td>
<td>11.8</td>
<td>45.0</td>
</tr>
<tr>
<td>5.</td>
<td>AH</td>
<td>4.0</td>
<td>9.5</td>
<td>21.2</td>
<td>30.0</td>
</tr>
<tr>
<td>6.</td>
<td>SH</td>
<td>6.0</td>
<td>19.3</td>
<td>6.0</td>
<td>37.5</td>
</tr>
<tr>
<td>7.</td>
<td>TL</td>
<td>4.9</td>
<td>9.0</td>
<td>3.8</td>
<td>52.5</td>
</tr>
<tr>
<td>8.</td>
<td>JM</td>
<td>4.0</td>
<td>11.0</td>
<td>14.0</td>
<td>55.0</td>
</tr>
<tr>
<td>9.</td>
<td>AP</td>
<td>4.1</td>
<td>6.8</td>
<td>23.2</td>
<td>50.0</td>
</tr>
<tr>
<td>10.</td>
<td>AR</td>
<td>4.5</td>
<td>6.4</td>
<td>26.0</td>
<td>45.0</td>
</tr>
<tr>
<td>11.</td>
<td>DR</td>
<td>5.2</td>
<td>34.9</td>
<td>4.0</td>
<td>40.0</td>
</tr>
<tr>
<td>12.</td>
<td>HT</td>
<td>3.9</td>
<td>3.3</td>
<td>3.3</td>
<td>60.0</td>
</tr>
<tr>
<td>13.</td>
<td>LW</td>
<td>4.9</td>
<td>10.6</td>
<td>8.6</td>
<td>57.5</td>
</tr>
<tr>
<td>14.</td>
<td>IL</td>
<td>4.4</td>
<td>11.3</td>
<td>35.0</td>
<td>45.0</td>
</tr>
<tr>
<td>15.</td>
<td>EB</td>
<td>3.9</td>
<td>5.5</td>
<td>50.7</td>
<td>60.0</td>
</tr>
<tr>
<td>16.</td>
<td>HB</td>
<td>3.5</td>
<td>18.2</td>
<td>71.6</td>
<td>42.5</td>
</tr>
<tr>
<td>17.</td>
<td>EBE</td>
<td>4.8</td>
<td>9.4</td>
<td>33.0</td>
<td>42.5</td>
</tr>
<tr>
<td>18.</td>
<td>HBR</td>
<td>4.0</td>
<td>11.3</td>
<td>36.8</td>
<td>37.5</td>
</tr>
<tr>
<td>19.</td>
<td>SB</td>
<td>3.9</td>
<td>8.5</td>
<td>24.6</td>
<td>47.5</td>
</tr>
<tr>
<td>20.</td>
<td>KB</td>
<td>4.1</td>
<td>15.2</td>
<td>51.4</td>
<td>40.0</td>
</tr>
<tr>
<td>21.</td>
<td>CC</td>
<td>3.7</td>
<td>11.7</td>
<td>22.3</td>
<td>35.0</td>
</tr>
<tr>
<td>22.</td>
<td>LH</td>
<td>4.1</td>
<td>3.9</td>
<td>1.6</td>
<td>32.5</td>
</tr>
<tr>
<td>23.</td>
<td>SHK</td>
<td>4.2</td>
<td>4.7</td>
<td>2.4</td>
<td>37.5</td>
</tr>
<tr>
<td>24.</td>
<td>GM</td>
<td>4.0</td>
<td>8.4</td>
<td>33.7</td>
<td>35.0</td>
</tr>
<tr>
<td>25.</td>
<td>AM</td>
<td>4.8</td>
<td>12.3</td>
<td>7.0</td>
<td>37.5</td>
</tr>
</tbody>
</table>
### 0800h Fasting Glucose, Insulin, Leptin and CBG concentrations (cont...)

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>Glucose (mmol/L)</th>
<th>Insulin (mU/L)</th>
<th>Leptin (ng/ml)</th>
<th>CBG (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>NP</td>
<td>4.1</td>
<td>3.7</td>
<td>4.8</td>
<td>45.0</td>
</tr>
<tr>
<td>27</td>
<td>VS</td>
<td>4.8</td>
<td>17.0</td>
<td>11.0</td>
<td>45.0</td>
</tr>
<tr>
<td>28</td>
<td>MS</td>
<td>4.4</td>
<td>8.6</td>
<td>16.3</td>
<td>57.5</td>
</tr>
<tr>
<td>29</td>
<td>LS</td>
<td>4.6</td>
<td>12.9</td>
<td>11.0</td>
<td>37.5</td>
</tr>
<tr>
<td>30</td>
<td>AW</td>
<td>4.3</td>
<td>11.3</td>
<td>26.9</td>
<td>45.0</td>
</tr>
<tr>
<td>31</td>
<td>WL</td>
<td>4.5</td>
<td>10.4</td>
<td>17.9</td>
<td>32.5</td>
</tr>
<tr>
<td>32</td>
<td>JS</td>
<td>5.3</td>
<td>23.7</td>
<td>39.8</td>
<td>42.5</td>
</tr>
<tr>
<td>33</td>
<td>MIR</td>
<td>4.2</td>
<td>16.2</td>
<td>12.7</td>
<td>37.5</td>
</tr>
<tr>
<td>34</td>
<td>MR</td>
<td>4.7</td>
<td>8.6</td>
<td>2.5</td>
<td>42.5</td>
</tr>
<tr>
<td>35</td>
<td>EBI</td>
<td>4.3</td>
<td>23.3</td>
<td>27.0</td>
<td>45.0</td>
</tr>
<tr>
<td>36</td>
<td>EBU</td>
<td>4.2</td>
<td>8.5</td>
<td>27.9</td>
<td>47.5</td>
</tr>
<tr>
<td>37</td>
<td>KG</td>
<td>4.1</td>
<td>18.4</td>
<td>19.3</td>
<td>45.0</td>
</tr>
<tr>
<td>38</td>
<td>SG</td>
<td>4.1</td>
<td>20.2</td>
<td>19.1</td>
<td>40.0</td>
</tr>
<tr>
<td>39</td>
<td>SK</td>
<td>4.5</td>
<td>9.9</td>
<td>14.4</td>
<td>35.0</td>
</tr>
<tr>
<td>40</td>
<td>KS</td>
<td>5.7</td>
<td>16.3</td>
<td>9.4</td>
<td>115.0</td>
</tr>
</tbody>
</table>
### APPENDIX VII

**24h Urinary Cortisol (THF + allo-THF) and Cortisone (THE) metabolites**

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>THF + allo-THF (μg/day)</th>
<th>THE (μg/day)</th>
<th>(THF +allo-THF)/THE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>JK</td>
<td>1890</td>
<td>1580</td>
<td>1.20</td>
</tr>
<tr>
<td>2.</td>
<td>CG</td>
<td>2090</td>
<td>2550</td>
<td>0.82</td>
</tr>
<tr>
<td>3.</td>
<td>KG</td>
<td>1480</td>
<td>1140</td>
<td>1.30</td>
</tr>
<tr>
<td>4.</td>
<td>GH</td>
<td>3030</td>
<td>2460</td>
<td>1.23</td>
</tr>
<tr>
<td>5.</td>
<td>AH</td>
<td>3010</td>
<td>2930</td>
<td>1.03</td>
</tr>
<tr>
<td>6.</td>
<td>SH</td>
<td>2220</td>
<td>2860</td>
<td>0.78</td>
</tr>
<tr>
<td>7.</td>
<td>TL</td>
<td>200</td>
<td>210</td>
<td>0.95</td>
</tr>
<tr>
<td>8.</td>
<td>JM</td>
<td>1840</td>
<td>2360</td>
<td>0.78</td>
</tr>
<tr>
<td>9.</td>
<td>AP</td>
<td>2870</td>
<td>2780</td>
<td>1.03</td>
</tr>
<tr>
<td>10.</td>
<td>AR</td>
<td>5170</td>
<td>3150</td>
<td>1.64</td>
</tr>
<tr>
<td>11.</td>
<td>DR</td>
<td>3080</td>
<td>2460</td>
<td>1.25</td>
</tr>
<tr>
<td>12.</td>
<td>HT</td>
<td>1990</td>
<td>2910</td>
<td>0.68</td>
</tr>
<tr>
<td>13.</td>
<td>LW</td>
<td>2540</td>
<td>2600</td>
<td>0.98</td>
</tr>
<tr>
<td>14.</td>
<td>IL</td>
<td>3330</td>
<td>4480</td>
<td>0.74</td>
</tr>
<tr>
<td>15.</td>
<td>EB</td>
<td>3380</td>
<td>3310</td>
<td>1.02</td>
</tr>
<tr>
<td>16.</td>
<td>HB</td>
<td>4000</td>
<td>3920</td>
<td>1.02</td>
</tr>
<tr>
<td>17.</td>
<td>EBE</td>
<td>1930</td>
<td>2650</td>
<td>0.73</td>
</tr>
<tr>
<td>18.</td>
<td>HBR</td>
<td>2340</td>
<td>3450</td>
<td>0.68</td>
</tr>
<tr>
<td>19.</td>
<td>SB</td>
<td>4150</td>
<td>6260</td>
<td>0.66</td>
</tr>
<tr>
<td>20.</td>
<td>KB</td>
<td>930</td>
<td>1040</td>
<td>0.89</td>
</tr>
<tr>
<td>21.</td>
<td>CC</td>
<td>suppressed</td>
<td>suppressed</td>
<td>---</td>
</tr>
<tr>
<td>22.</td>
<td>LH</td>
<td>1630</td>
<td>2270</td>
<td>0.72</td>
</tr>
<tr>
<td>23.</td>
<td>SHK</td>
<td>6970</td>
<td>3580</td>
<td>1.95</td>
</tr>
<tr>
<td>24.</td>
<td>GM</td>
<td>5580</td>
<td>5510</td>
<td>1.01</td>
</tr>
<tr>
<td>25.</td>
<td>AM</td>
<td>3870</td>
<td>4840</td>
<td>0.80</td>
</tr>
</tbody>
</table>
### 24h Urinary Cortisol (THF + allo-THF) and Cortisone (THE) metabolites (cont...)

<table>
<thead>
<tr>
<th>No</th>
<th>Patients</th>
<th>THF + allo-THF (µg/day)</th>
<th>THE (µg/day)</th>
<th>(THF + allo-THF)/THE</th>
</tr>
</thead>
<tbody>
<tr>
<td>26.</td>
<td>NP</td>
<td>1640</td>
<td>2020</td>
<td>0.81</td>
</tr>
<tr>
<td>27.</td>
<td>VS</td>
<td>6370</td>
<td>10120</td>
<td>0.63</td>
</tr>
<tr>
<td>28.</td>
<td>MS</td>
<td>3260</td>
<td>2950</td>
<td>1.11</td>
</tr>
<tr>
<td>29.</td>
<td>LS</td>
<td>2840</td>
<td>5010</td>
<td>0.57</td>
</tr>
<tr>
<td>30.</td>
<td>AW</td>
<td>2700</td>
<td>3960</td>
<td>0.68</td>
</tr>
<tr>
<td>31.</td>
<td>WL</td>
<td>5500</td>
<td>4560</td>
<td>1.21</td>
</tr>
<tr>
<td>32.</td>
<td>JS</td>
<td>9130</td>
<td>11780</td>
<td>0.78</td>
</tr>
<tr>
<td>33.</td>
<td>MIR</td>
<td>5780</td>
<td>5340</td>
<td>1.08</td>
</tr>
<tr>
<td>34.</td>
<td>MR</td>
<td>3790</td>
<td>2950</td>
<td>1.28</td>
</tr>
<tr>
<td>35.</td>
<td>EBI</td>
<td>2930</td>
<td>2090</td>
<td>1.40</td>
</tr>
<tr>
<td>36.</td>
<td>EBU</td>
<td>1590</td>
<td>1510</td>
<td>1.05</td>
</tr>
<tr>
<td>37.</td>
<td>KG</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>38.</td>
<td>SG</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>39.</td>
<td>SK</td>
<td>2010</td>
<td>1980</td>
<td>1.02</td>
</tr>
<tr>
<td>40.</td>
<td>KS</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>