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**CONGENITAL ADRENAL HYPERPLASIA, CYP21A2 DEFICIENCY:  
CLINICAL AND PHYSIOLOGICAL ASPECTS OF  
PREGNANCY, SCREENING AND GROWTH**

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To Nisse and Ingrid

## ABSTRACT

The subjects dealt with in this thesis are clinical aspects of congenital adrenal hyperplasia (CAH), such as neonatal screening, growth and the incidence of CAH during the last century in Sweden. In addition, we have used CAH as a model system to study possible prenatal effects of androgen exposure on growth and gestational length.

Gestational age at birth correlated with *CYP21A2* genotype in girls ( $P < 0.01$ ), but not in boys with CAH ( $n = 109$ ; 62 females, 47 males) (Paper I). The exact number of gestational days was known in 66 patients (37 females, 29 males). The pregnancy was longer for females with the most severe form, null genotype, 285.7 days, than for I172N, 273.9 days ( $P < 0.01$ ) or V281L, 274.7 days ( $P < 0.05$ ), indicating that higher androgen levels in severe forms could explain this effect. No differences between genotypes were seen in CAH males, possibly because testicular androgen production is high in normal male foetuses and adrenal androgens therefore may not have an additional effect. The cortisol deficiency is equal in CAH girls and boys, making this deficiency a less likely explanation.

Birth weight standard deviation score (SDS) corrected for gestational age in children with CAH ( $n = 73$ ; 43 females, 30 males) did not differ from that of the reference population (mean, CI 95%: 0.0, -0.3 to 0.3, and 0.2, -0.2 to 0.6, for boys and girls, respectively) (Paper II). Nor did the birth weight differ between *CYP21A2* genotype groups ( $P > 0.05$ ). In 29 46,XY females with complete androgen insensitivity syndrome (CAIS), the mean birth weight SDS was similar to that of reference boys (mean, CI 95%: 0.1, -0.2 to 0.4) and higher than the reference of females (mean, CI 95%: 0.4, 0.1 to 0.7,  $P = 0.02$ ). Hence, these results indicate that gestational age at birth, but not prenatal growth, is affected by androgen exposure.

In a retrospective, population-based cohort study we investigated the apparent incidence of CAH in Sweden between 1910 and 2011 (Paper III). We identified 606 patients with known *CYP21A2* genotype in 490 cases (81%). The female:male ratio was 1.25:1 for the whole cohort, but close to 1 in patients detected in the screening. The number of diagnosed patients increased dramatically in the 1960s and 1970s. The proportion of salt-wasting (SW) CAH compared to milder forms increased in both sexes after the introduction of neonatal screening from 114/242 to 165/292 ( $P < 0.05$ ). The milder forms were diagnosed more often in females. This means that both boys and girls with SW CAH were missed before screening and that screening for CAH does not only increase the number of detected boys with SW CAH as previously thought, but also of girls.

The neonatal screening for CAH in Sweden was studied from the start in 1986 to 2011 (Paper IV). A total of 2 737 932 neonates (99.8% of all live births) had been screened. No cases with evident SW CAH had been missed, sensitivity 100%. The sensitivity was lower in the simple virilising form, 79%, and non-classical CAH, 32%. The positive predictive value was higher in full-term infants, 25.1%, than in pre-terms, 1.4% ( $P < 0.001$ ). The recall rate was lower in full-terms, 0.03%, than in pre-term infants, 0.57% ( $P < 0.001$ ). An analysis of all publications describing neonatal screening programmes since 1996 revealed that the screening sensitivity correlated negatively with the duration of follow-up ( $P = 0.034$ ). In contrast to current reports, our study shows that neonatal screening is effective in identifying SW CAH.

Growth in CAH was studied in a prospective, observational cohort study including all children born or diagnosed with CAH between 1989 and 1994, 80 patients (46 females, 34 males). Most children were treated with a glucocorticoid dose within the recommended 10–15 mg/m<sup>2</sup> body surface area. Corrected final height correlated with *CYP21A2* genotype ( $P = 0.012$ ). An important finding was that the corrected final height SDS was lower in patients who had been treated with the addition of prednisolone,  $-1.1 \pm 1.0$ , than in those who had been treated with cortisone acetate and/or hydrocortisone alone,  $-0.60 \pm 1.0$  ( $P < 0.05$ ). Furthermore, body mass index at 18 years of age was higher in patients treated with prednisolone,  $25.3 \pm 4.7$  kg/m<sup>2</sup>, compared to  $23.4 \pm 4.5$  kg/m<sup>2</sup> ( $P < 0.05$ ). Hence, the results suggest that treatment with prednisolone should be avoided in growing subjects with CAH.

## LIST OF PUBLICATIONS

- I. Sebastian Gidlöf, Anna Wedell, Anna Nordenström.

**Gestational age correlates to genotype in girls with CYP21 deficiency.**

J Clin Endocrinol Metab 2007;92:246-249

- II. Harriet L Miles, Sebastian Gidlöf, Anna Nordenström, Ken K Ong, Ieuan Hughes.

**The role of androgens in fetal growth: observational study in two genetic models of disordered androgen signalling.**

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- III. Sebastian Gidlöf, Henrik Falhammar, Astrid Thilén, Ulrika von Döbeln, Martin Ritzén, Anna Wedell, Anna Nordenström.

**One hundred years of congenital adrenal hyperplasia in Sweden: a retrospective, population-based cohort study.**

Lancet Diabetes & Endocrinol 2013;1:35-42.

- IV. Sebastian Gidlöf, Anna Wedell, Claes Guthenberg, Ulrika von Döbeln, Anna Nordenström.

**Nationwide Neonatal Screening for Congenital Adrenal Hyperplasia in Sweden: A Longitudinal Prospective Population-based Study Covering 26 Years**

*Submitted*

- V. Sebastian Gidlöf, Daniel Eriksson Hogling, David Olsson, Astrid Thilén, Martin Ritzén, Anna Wedell, Anna Nordenström

**Growth and treatment in congenital adrenal hyperplasia: a prospective observational study from diagnosis to final height**

*Submitted*

# TABLE OF CONTENTS

1	Introduction .....	1
2	Background.....	3
2.1	Epidemiology .....	3
2.2	Pathophysiology .....	3
2.2.1	Genetics.....	3
2.2.2	Biochemistry.....	7
2.3	Clinical features.....	9
2.3.1	Foetus.....	9
2.3.2	Growth .....	12
2.3.3	Weight development.....	18
2.3.4	Congenital adrenal hyperplasia in adults .....	19
2.3.5	Management of congenital adrenal hyperplasia.....	21
2.4	Neonatal screening .....	26
2.4.1	Introduction to screening .....	26
2.4.2	Evaluating screening programmes .....	28
2.4.3	Screening for congenital adrenal hyperplasia .....	30
3	Aims.....	35
4	Subjects and methods .....	36
4.1	Paper I: Gestational age correlates to genotype in girls with CYP21 deficiency ....	36
4.1.1	Study population and design .....	36
4.1.2	Statistical methods .....	37
4.2	Paper II: The role of androgens in fetal growth: observational study in two genetic models of disordered androgen signalling .....	37
4.2.1	Study population and design .....	37
4.2.2	Statistical methods .....	37
4.3	Paper III: One hundred years of congenital adrenal hyperplasia in Sweden: a retrospective, population-based cohort study .....	38
4.3.1	Study population and design .....	38
4.3.2	Statistical methods .....	39
4.4	Paper IV: Nationwide neonatal screening for congenital adrenal hyperplasia in Sweden: a longitudinal prospective population-based study covering 26 years.....	40
4.4.1	Study population and design .....	40
4.4.2	Statistical methods .....	40
4.5	Paper V: Growth and treatment in congenital adrenal hyperplasia: a prospective observational study from diagnosis to final height.....	41
4.5.1	Study population and design .....	41
4.5.2	Statistical methods .....	42
4.6	Ethical considerations.....	42
5	Results.....	44
5.1	Relationship between length of pregnancy and genotype in congenital adrenal hyperplasia.....	44
5.2	Foetal growth may be independent of androgens .....	45
5.3	Description of congenital adrenal hyperplasia in Sweden during the last century ..	46
5.4	Neonatal screening for congenital adrenal hyperplasia in Sweden .....	48
5.5	Growth and treatment in children with congenital adrenal hyperplasia.....	52
6	Discussion.....	58
6.1	Paper I: Gestational age correlates to genotype in girls with CYP21 deficiency ....	58

6.1.1	Findings and interpretations .....	58
6.1.2	Methodological considerations.....	59
6.2	Paper II: The role of androgens in fetal growth: observational study in two genetic models of disordered androgen signalling .....	60
6.2.1	Findings and interpretations .....	60
6.2.2	Methodological considerations.....	61
6.3	Paper III: One hundred years of congenital adrenal hyperplasia in Sweden: a retrospective, population-based cohort study .....	61
6.3.1	Findings and interpretations .....	61
6.3.2	Methodological considerations.....	62
6.4	Paper IV: Nationwide neonatal screening for congenital adrenal hyperplasia in Sweden: A longitudinal prospective population-based study covering 26 years.....	63
6.4.1	Findings and interpretations .....	63
6.4.2	Methodological considerations.....	63
6.5	Paper V: Growth and treatment in congenital adrenal hyperplasia: a prospective observational study from diagnosis to final height.....	64
6.5.1	Findings and interpretations .....	64
6.5.2	Methodological considerations.....	65
7	Conclusions .....	68
8	Clinical implications.....	69
9	Suggestions for future research.....	70
10	Acknowledgements .....	71
11	References .....	73

## LIST OF ABBREVIATIONS

17-OHP	17-hydroxyprogesterone
ACTH	Adrenocorticotrophic hormone
AIS	Androgen insensitivity syndrome
AMH	Anti-müllerian hormone
AR	Androgen receptor
BMD	Bone mineral density
BMI	Body mass index
BSA	Body surface area
<i>C4A</i>	Gene encoding for complement C4-A
<i>C4B</i>	Gene encoding for complement C4-B
CAH	Congenital adrenal hyperplasia
CAIS	Complete androgen insensitivity syndrome
CI 95%	95% Confidence interval
cm	centimetre/s
CoA	Coenzyme A
COX-2	Cyclooxygenase-2
CRH	Corticotropin-releasing hormone
CYP2C19	Cytochrome P450 2C19
<i>CYP21A1P</i>	<i>CYP21A1</i> pseudogene
<i>CYP21A2</i>	Gene encoding for 21 $\alpha$ -hydroxylase
CYP3A4	Cytochrome P450 3A4
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
Dnr	Registration number
GH	Growth hormone
GnRH	Gonadotrophin-releasing hormone
HLA	Human leukocyte antigen
HPA axis	Hypothalamic-pituitary-adrenal axis
IGF-1	Insulin-like growth factor
IMT	Intima-media thickness
LC-MS/MS	Liquid chromatography and tandem mass spectrometry
LH	Luteinising hormone

m <sup>2</sup>	Square metre/s
mg	Milligram/s
mmol	Millimole/s
MHC	Major histocompatibility complex
NC CAH	Non-classical congenital adrenal hyperplasia
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
P450scc	Cholesterol side-chain cleavage enzyme
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
POR	P450 oxidoreductase deficiency
PPV	Positive predictive value
<i>RPI</i>	Retinitis pigmentosa 1 protein
<i>RP2</i>	Retinitis pigmentosa 2 protein
SHBG	Sex hormone-binding globulin
StAR	Steroidogenic acute regulatory protein
SW CAH	Salt-wasting congenital adrenal hyperplasia
SV CAH	Simple virilising congenital adrenal hyperplasia
TART	Testicular adrenal rest tumour
<i>TNXA</i>	Gene encoding for truncated protein tenascin X
<i>TNXB</i>	Gene encoding for tenascin XB
SCB	Statistics Sweden (Statistiska centralbyrån)
SD	Standard deviation
SDS	Standard deviation score
µg	Microgram/s



# 1 INTRODUCTION

Congenital adrenal hyperplasia (CAH) constitutes a group of autosomal recessive diseases. The most common form, 21 $\alpha$ -hydroxylase deficiency, is caused by a defective *CYP21A2* (1-7). In this thesis, CAH will refer to 21 $\alpha$ -hydroxylase deficiency if not stated otherwise.

In CAH, glucocorticoid and mineralocorticoid synthesis is impaired and there is a concomitant overproduction of adrenal androgenic precursors. This may lead to potentially lethal salt loss in both sexes and prenatal genital virilisation in females (1, 2, 4-7).

There are different clinical forms of CAH. The salt-wasting form (SW CAH) is marked by both cortisol and mineralocorticoid deficiency and overproduction of androgens (4, 8). The simple virilising form (SV CAH) is not associated with salt loss, but with cortisol deficiency and overproduction of androgens (3). SW CAH and SV CAH are sometimes referred to as classical CAH and both have their onset before 5 years of age (1, 2). Non-classical CAH (NC CAH) is the mildest form and may sometimes remain undetected. It is diagnosed more often in females, probably owing to more obvious symptoms of androgen excess, such as hirsutism and menstrual disturbances (3).

Since severe forms of CAH may be fatal, especially in infancy, many countries have introduced newborn screening programmes to detect the disease at an early stage (9-11).

The treatment of CAH consists of substitution therapy with glucocorticoids and mineralocorticoids in doses large enough to reduce the androgen overproduction. Most patients with classical CAH require treatment with glucocorticoids and mineralocorticoids, as well as supplementation therapy with sodium during infancy and early childhood (1, 2, 4-7). Androgen blocking drugs have been used experimentally, but are not yet included in clinical routine treatment (12).

Despite thorough follow-ups and seemingly adequate treatment, short stature remains a clinical problem (1, 2). Development of overweight is thought to be attributable to excessive glucocorticoid treatment (13).

In adult patients with CAH, fertility is compromised in both females and males (14). Although the mechanisms has not been entirely elucidated, high level of progesterone may negatively

affect the endometrium and ovulation in females (15). In males with CAH, testicular adrenal rest tumours often develop. These benign tumours have been associated with reduced fertility (16).

The long-term effects on cardiovascular disease and osteoporosis have recently begun to be investigated. The increased production of androgens raises the concern that patients with CAH may be at increased risk of atherosclerosis and ischaemic heart disease (17, 18). In addition, long-term excessive glucocorticoid treatment may have negative effects on bone mass (16).

In untreated patients, there is a special endocrine situation with overproduction of androgens and decreased production of cortisol and aldosterone. CAH can thus be used as a model for studying effects of androgens on human physiology. In this thesis two papers address the potential effect of these hormones on birth weight and length of pregnancy.

The management of patients with CAH was first described in the 19<sup>th</sup> century (19, 20) and has changed remarkably during the last 100 years. Before 1950 no efficient therapy was available. Neonatal screening for CAH was first described in the late 1970s and introduced in Sweden in 1986 (9). Today, deaths due to CAH are rare in countries with well-functioning screening programmes (11).

## 2 BACKGROUND

### 2.1 EPIDEMIOLOGY

The most common aetiology of CAH is 21 $\alpha$ -hydroxylase deficiency causing about 90–95% of the cases (2). 11 $\beta$ -hydroxylase deficiency is less common and is the cause in about 5% of CAH cases (21). Other more rare causes of CAH are 3 $\beta$ -hydroxysteroid dehydrogenase II deficiency, lipoid CAH, caused by mutations in steroidogenic acute regulatory protein (StAR) or cholesterol side-chain cleavage enzyme (P450scc), and 17 $\alpha$ -hydroxylase deficiency (4).

SW CAH has been reported to occur with an incidence of 1:10 000–23 000. Some ethnic groups show a profoundly increased rate of SW CAH. The incidence of SW CAH in Yupik Inuits is 1:282 and in the French island of La Reunion, east of Madagascar, an incidence of 1:2141 has been reported (11).

In most populations the mildest form, NC CAH, is more frequent than the more severe forms. The highest frequency of NC CAH has been reported among Ashkenazi Jews in New York City, where it was found to affect 1:27 (22). Other small studies have suggested high frequencies in Hispanics (1:40) (3), Croatians (1:50) (23) and Italians (1:300) (3). The prevalence of NC CAH in Sweden seems to be lower than in other reported populations (24).

### 2.2 PATHOPHYSIOLOGY

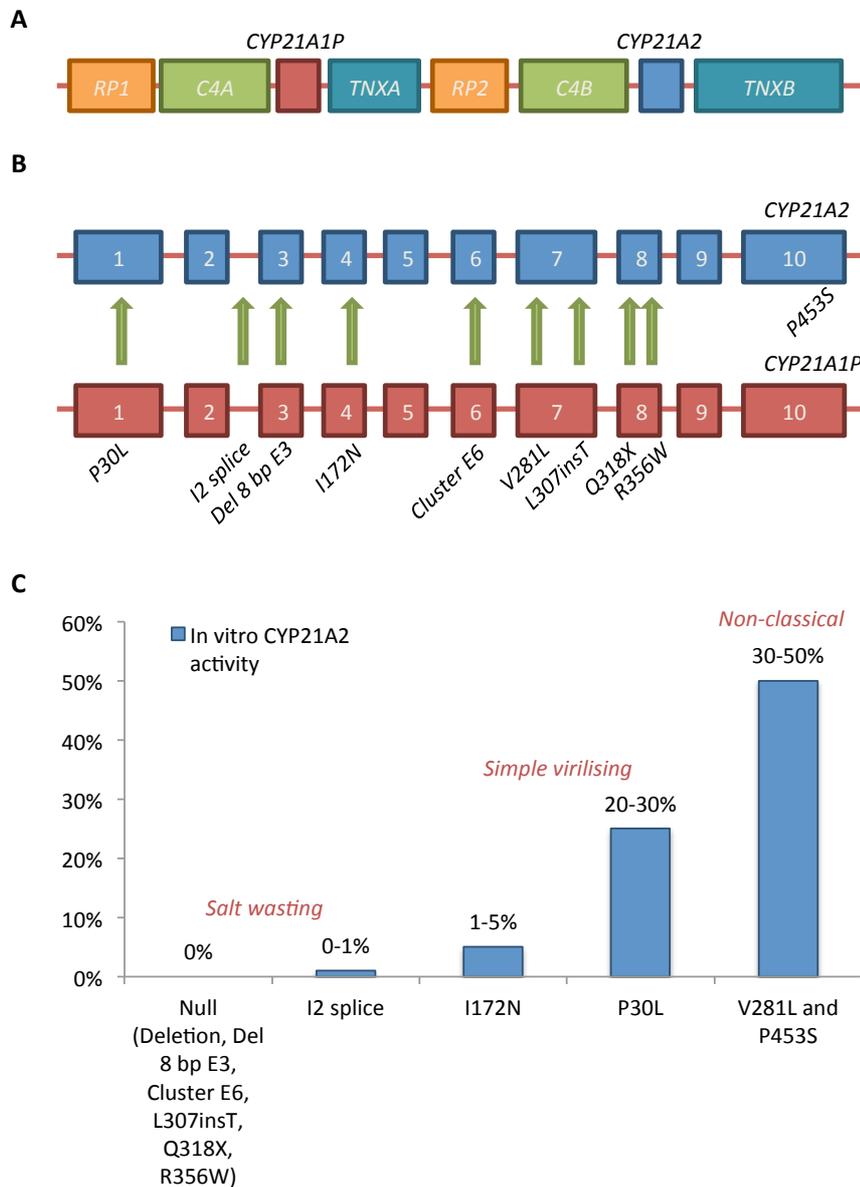
#### 2.2.1 Genetics

CAH due to 21 $\alpha$ -hydroxylase deficiency is caused by mutations in the *CYP21A2* gene, located on the short arm of chromosome 6 (band 6p21.3). The gene is located in the major histocompatibility complex (MHC) locus, known for a high degree of rearrangement leading to inter-individual variability (25).

A pseudogene, *CYP21A1P*, is located in tandem with the active gene, but is not expressed because of deleterious mutations. In fact, more genes in the same regions are highly homologous, with one gene being expressed to a functioning protein, whereas its counterpart will only be translated to a truncated protein. In the region, the following genes are arranged, from 5' to 3': *RP1*, *C4A*, *CYP21A1P*, *TNXA*, *RP2*, *C4B*, *CYP21A2* and *TNXB* (26, 27) (Figure 1A). The *RP1* gene encodes for a nuclear protein, whereas *RP2* forms a truncated protein just as *TNXA* forms a

truncated protein of *TNXB*, which encodes a functioning extracellular matrix protein. C4A and C4B both encode complement proteins in the innate immune system (25).

**Figure 1**



*A. Organisation of the CYP21A2 gene locus. Both the pseudogene and the functioning CYP21 gene are located in separate RCCX regions. B. Nine of the most common mutations are transferred from the CYP21A1P pseudogene by microconversion. C. Residual in vitro activity in different common mutations. The positive predictive value (PPV) for SW CAH with a null genotype is 96–100% (8, 28) and with I2 splice genotype 85–96% (28, 29). The PPV for SV CAH with I172N genotype is 53–74% (8, 29) and the PPV for NC CAH with V281L or P453S genotype is 63–100% (28, 29).*

As stated above, the repeated genes (*RP*, *C4*, *CYP21* and *TNX*) are referred to as the RCCX region. Since the RCCX region is highly homologous, misalignment may occur during meiosis causing a recombination of gene elements. Furthermore, small or large sequences from the pseudogene may be inserted into the functioning gene in a process termed gene conversion. These pseudogene-derived mutations result in impaired function of the encoded enzyme and are frequently found in patients with CAH (4).

Although nearly 100 disease-causing mutations in *CYP21A2* have been described, nine pseudogene-derived mutations are accountable for more than 95% of cases of CAH due to 21 $\alpha$ -hydroxylase deficiency (Figure 1B). Of these mutations, del 8 bp E3 (c.329\_336delGAGACTAC), Cluster E6 (c.707T>A+710T>A+716T>A), L307 frameshift (c.920\_921insT), Q318X (c.952C>T), R356W (c.1066C>T) result in no enzymatic activity. I2 splice (c.290-13A/C>G) leads to almost no enzymatic activity and is linked to severe forms of CAH. I172N (c.515T>A) has been linked to SV CAH, but generally not to SW CAH, and is characterised by less than 2% of *in vitro* residual enzymatic activity. Salt loss is seen in less than 10% of all cases with the I172N genotype (30). P30L (c.89C>T) and V281L (c.841G>T) generally lead to milder forms of CAH (25).

Most cases of CAH are compound heterozygous. The degree of severity is determined by the mildest affected allele (4).

In most cases of CAH, there is a reliable genotype phenotype correlation (30-32) (Figure 1C). Hence, a genetic analysis may facilitate decisions regarding the choice of treatment and frequency of follow-up in patients with CAH. Since males with SW CAH do not exhibit ambiguous genitalia, the distinction between SW CAH and SV CAH forms can often be facilitated by genetic analysis (8, 33).

Being an autosomal recessive disease with the affected gene closely linked to the class 3 *HLA* complex, the inheritance of CAH can be coupled to *HLA* markers. Before detailed analyses of *CYP21A2* were available, *HLA* linkage analysis was therefore used to diagnose foetal CAH in chorionic villus sampling/amniocentesis from subsequent pregnancies by comparison with *HLA* markers in the index sibling (34).

Southern blotting may be employed to detect gene deletion and large gene conversions. However, the method is time-consuming and has now been surpassed by more modern methods.

Real time quantitative polymerase chain reaction (PCR) is a more rapid method, which also detects deletions and can be used to estimate gene copy number. Multiplex ligation-dependent probe amplification is another commonly used method for gene copy number determination. Allele-specific PCR is designed to detect single point mutations, but it sometimes requires a knowledge of differences between the alleles; hence, parental DNA must be available (25).

Direct DNA sequencing remains the only alternative to reliably detect all possible mutations, except for larger rearrangements such as deletions. Lately, new techniques have made this approach faster; however, since most cases of CAH are caused by a limited number of mutations, it may not always be cost-effective (25).

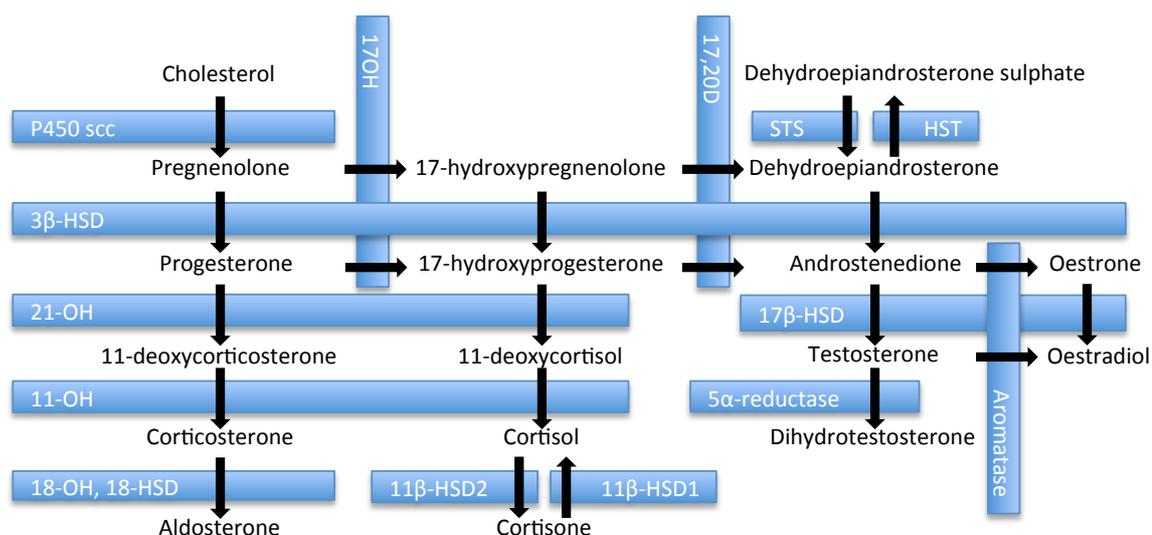
As mentioned before, CAH is a disease exhibiting clear genotype-phenotype correlations with few exceptions. This has been supported by enzyme activity measurements *in vitro*, which appear to be consistent with glucocorticoid and mineralocorticoid deficiency *in vivo* (1). However, compared to the correlation between genotype and the risk for salt loss, the degree of virilisation is not as dependent on the *CYP21A2* genotype (8, 28, 33, 35, 36). Female genital virilisation, defined as a Prader score, may vary even between patients with identical mutations. The reason for this is poorly understood, but it may be caused by variations in e.g., the androgen receptor (AR) or P450 oxidoreductase (POR) activity. The AR is known to be highly polymorphic in the number of CAG repeats at its 3' end, which is known to affect its activity (37). Initial results suggested a possible association between CAG repeats and virilisation in CAH patients (37), but these data were not supported by a later report (38). POR reduces cytochrome P450 enzymes including 21 $\alpha$ -hydroxylase, to reactivate them for further enzymatic activity (39). The POR gene has been shown to be polymorphic (40). Furthermore, the hepatic cytochrome P450 enzymes, CYP2C19 and CYP3A4 can 21-hydroxylate progesterone, but not 17-hydroxyprogesterone (17-OHP) *in vitro*, thus potentially reducing the deficiency of mineralocorticoids, but not of glucocorticoids *in vivo* (41). In addition, 17-OHP may be converted to dihydrotestosterone (DHT) via androsterone, according to the so called "back-door pathway" (42, 43). The possible importance of this pathway in humans (42), as well as its influence on prenatal virilisation in female fetuses affected with CAH, has attracted attention lately (44).

## 2.2.2 Biochemistry

All steroid hormones are synthesised from the same precursor, cholesterol. Cholesterol can be taken up from the intestine, either from the diet or from recirculation when secreted from the liver or synthesised *de novo*.

For further steroid biosynthesis (Figure 2), cholesterol needs to pass from the outer to the inner mitochondrial membrane. Although a detailed description of this process remains partly unknown, this transport is mainly facilitated by StAR, the rate-limiting step in steroid biosynthesis. P450<sub>scc</sub> then converts cholesterol to pregnenolone, the first step in all human steroid biosynthesis. The product, pregnenolone, is transported back into the cytosol for further steroid hormone biosynthesis (45).

**Figure 2**



*Human steroid synthesis. Aldosterone production occurs predominantly in the zona glomerulosa, whereas cortisol and androgenic precursor production occur predominantly in the zona fasciculata and zona reticularis of the adrenal gland. Testosterone is reduced to dihydrotestosterone in extra-adrenal tissue, as well as aromatised to oestrogens (52). P450<sub>scc</sub>, side-chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 21-OH, 21α-hydroxylase; 11-OH, 11β-hydroxylase; 18-OH, 18α-hydroxylase; 18-HSD, 18-hydroxysteroid dehydrogenase; 17-OH, 17α-hydroxylase; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; 17,20D, 17,20 lyase; STS, steroid-sulphatase; HST, hydroxysteroid sulphotransferase; 17β-HSD, 17β-hydroxysteroid dehydrogenase.*

The adrenal cortex consists of three anatomically and biochemically distinct layers, the outer zona glomerulosa, the middle zona fasciculata and the inner zona reticularis, closest to the adrenal medulla (46).

The zona glomerulosa differs from the other layers in that it does not express 17 $\alpha$ -hydroxylase, which is responsible for converting pregnenolone to 17-hydroxypregnenolone and further biosynthesis of glucocorticoids and androgens (47). Instead, zona glomerulosa cells express 3- $\beta$ -hydroxysteroid dehydrogenase which catalyses the conversion of pregnenolone to progesterone (48). Progesterone is then hydroxylated by 21 $\alpha$ -hydroxylase to 11-deoxycorticosterone. 11-hydroxylase and aldosterone synthase finish the biosynthesis of the most potent mineralocorticoid hormone, aldosterone, in the zona glomerulosa (49). Absence of a functioning 21 $\alpha$ -hydroxylase, due to mutations in *CYP21A2*, leads to an inability to produce aldosterone and an accumulation of precursors (2).

In contrast to the zona glomerulosa, the zona reticularis express 17 $\alpha$ -hydroxylase. Pregnenolone is therefore hydroxylated to 17-hydroxypregnenolone, which is further converted to dehydroepiandrosterone (DHEA) by 17,20-lyase (46). DHEA is a weak androgen and can be further metabolised into androstendione or dehydroepiandrosterone sulphate (DHEAS) in the adrenal cortex (50). These androgens are transported in the circulation bound to sex hormone-binding globulin (SHBG) and may be further metabolised to the more potent androgens testosterone and DHT in extra-adrenal tissue (51).

The zona fasciculata mainly contributes to the production of glucocorticoids. Pregnenolone may be converted to either 17-hydroxypregnenolone or progesterone by 17 $\alpha$ -hydroxylase or 3- $\beta$ -hydroxysteroid dehydrogenase, respectively. 17-hydroxypregnenolone is further converted into 17-OHP. Progesterone and 17-OHP are hydroxylated by 21 $\alpha$ -hydroxylase to 11-deoxycorticosterone and 11-deoxycortisol, respectively (46). 11 $\alpha$ -hydroxylase finalises the production of cortisol from 11-deoxycortisol and may hydroxylate 11-deoxycorticosterone to corticosterone, a weak mineralocorticoid hormone (49). Since both the zona fasciculata and the zona reticularis lack aldosterone synthase, corticosterone cannot be further converted to aldosterone in these layers. Although the zona fasciculata is the main site for the production of glucocorticoids and the zona reticularis is the main site for production of adrenal androgens, they both express the enzymes for both these processes (46, 50).

From the above description of adrenal steroid biosynthesis, it is clear that mutations causing decreased function in 21 $\alpha$ -hydroxylase will lead to an inability to produce adequate amounts of cortisol and aldosterone with a concomitant accumulation of precursors. Since androgen synthesis is independent of 21 $\alpha$ -hydroxylase, these precursors will be shuttled towards the biosynthesis of androgenic hormones (2, 4).

## **2.3 CLINICAL FEATURES**

21 $\alpha$ -hydroxylase deficiency results in decreased production of aldosterone and cortisol and concomitant overproduction of androgens. The symptoms of CAH are caused by these hormonal disturbances (2, 53, 54).

### **2.3.1 Foetus**

#### *2.3.1.1 Female virilisation*

The perhaps most prominent sign in severe forms of CAH is the prenatal virilisation of the external genitalia in females (53). There is a wide spectrum of degrees of virilisation that is related to the degree of 21 $\alpha$ -hydroxylase deficiency in that females with completely abolished enzyme function have pronounced virilisation. The correlation is not as strong in milder forms, perhaps allowing other factors, such as mentioned above, to contribute.

Normal sex differentiation is a complex embryonic process that partly remains elusive (44). Male and female embryos share the same internal and external appearances until the sixth gestational week (55). The gonads have the potential of developing either into testes or ovaries. In the presence of a Y chromosome, the Sertoli cells express the SRY gene product and thus activate the expression of further gene products, driving the gonads into testicular development (56). Leydig cells in the testes will form testosterone, which is converted into DHT, which is essential for the formation of the external genitalia (57). With the appearance of developing testes and testosterone production, the Wolffian ducts will develop into internal male genitalia. Anti-müllerian hormone (AMH), produced by the testicular Sertoli cells, enables the involution of the Müllerian ducts, which are the anlagen for internal female genitalia (58).

In the absence of adequate levels of testosterone, the Wolffian ducts will regress. Female development requires the absence of AMH and testosterone to facilitate the formation of the internal female genitalia from the Müllerian ducts (59).

Under the influence of DHT in the 46,XY embryo, the genital tubercle develops into a penis, the urethral folds into the urethra and the genital folds into the scrotum (58). However, in the absence of DHT, such as in the normal 46,XX embryo, the genital tubercle develops into the clitoris, the urethral folds into the labia minora and the genital folds into the labia majora (44).

Increased levels of androgens in 46,XX embryos during the first trimester may thus result in virilisation of the external, but not internal, genitalia (33, 60). In the presence of increased androgens in a 46,XX embryo, the genital folds that develop into the labia majora will fuse, partially or completely, resembling the development of the scrotum. Failure to develop the lower third of the vagina leads to the formation of a urogenital sinus. Increased androgens throughout pregnancy will lead to clitoral enlargement (44). The Prader score is used to categorise the degree of virilisation in CAH (Figure 3) (61).

**Figure 3**



*Prader stage. Increasing clitoromegaly from I to V and increasing posterior fusion from I to IV, with the formation of a sinus urogenitale in Prader III and IV. Prader V with complete fusion of the labioscrotal folds and the urethral opening at the tip of the glans. Originally published by Prader, A. et. al., 1955 (62).*

The phenotype of a virilised female infant with CAH may thus, in severe cases, resemble a male with hypospadias and undescended testes. Hence, sex assignment in females with virilising forms of CAH may be difficult since other rare conditions also present with ambiguous genitalia (44, 61).

### 2.3.1.2 Length of pregnancy

The physiological onset of parturition is a complex, not fully elucidated process. Foetal size, maternal and foetal endocrine factors and local inflammation are known to contribute (63). A detailed description of this area of research is not within the scope of this thesis.

Both preterm and term labour are, however, associated with an increased inflammatory state in the amniotic fluid, foetal membranes, myometrium and cervix (64). Throughout pregnancy, progesterone receptors are abundant in these tissues and are stimulated by high circulating levels of progesterone (63). The nuclear progesterone receptor acts in an anti-inflammatory way by inhibiting the pro-inflammatory transcription factor NF- $\kappa$ B (65). This inactivation of NF- $\kappa$ B is thought to contribute to the quiescent state of the myometrium throughout most of the pregnancy (63). It is noteworthy that progesterone production is increased in CAH.

In late pregnancy, uterine stretch (66), placental corticotropin-releasing hormone (CRH) production (67) and surfactant production (68) lead to the activation of macrophages that change the anti-inflammatory state to a more pro-inflammatory state of the amniotic fluid, foetal membranes, uterus and cervix by increasing the production of pro-inflammatory cytokines, resulting in NF- $\kappa$ B activation. This leads to down-regulation of the progesterone receptor function in the myometrium (63). The absence of the inhibitory action of the progesterone receptor and the consequently increased activation of NF- $\kappa$ B lead to enhanced prostaglandin production by up-regulation of COX-2 (69), increased expression of connexin 43 (70) and thus more gap-junctions and increased expression of oxytocin receptors in the myometrium (63, 71). This ultimately leads to a more contractile myometrium and the onset of contractions.

The human placenta has been suggested to increase the production of CRH at term. This CRH would then enhance the production of foetal ACTH, which stimulates cortisol production. Cortisol in the maternal-foetal circulation increases placental COX-2 production and hence prostaglandin synthesis (72). Furthermore, placental 17 $\alpha$ -hydroxylase is up-regulated by cortisol, leading to increased production of C-19 steroids that are aromatised into oestradiol, which inhibits the anti-inflammatory action of the progesterone receptor signalling (73). Oestradiol may also have pro-inflammatory effects on its own by activating COX-2 expression (Mendelson CR, personal communication, 2013). In addition, the physiological increase in foetal adrenal cortisol production may increase the production of surfactant-protein A, which increases uterine myometrial contractility (68). Cortisol production is decreased in CAH.

It has been suggested that males have been shown to have a prolonged gestation compared to females (74) and healthy male foetuses have been shown to produce more testosterone than female foetuses (75). Circulating testosterone in males is high at birth and rapidly falls after the

first post-natal week (76). However, the impact of testosterone on the length of pregnancy is not known.

#### *2.3.1.3 Foetal growth*

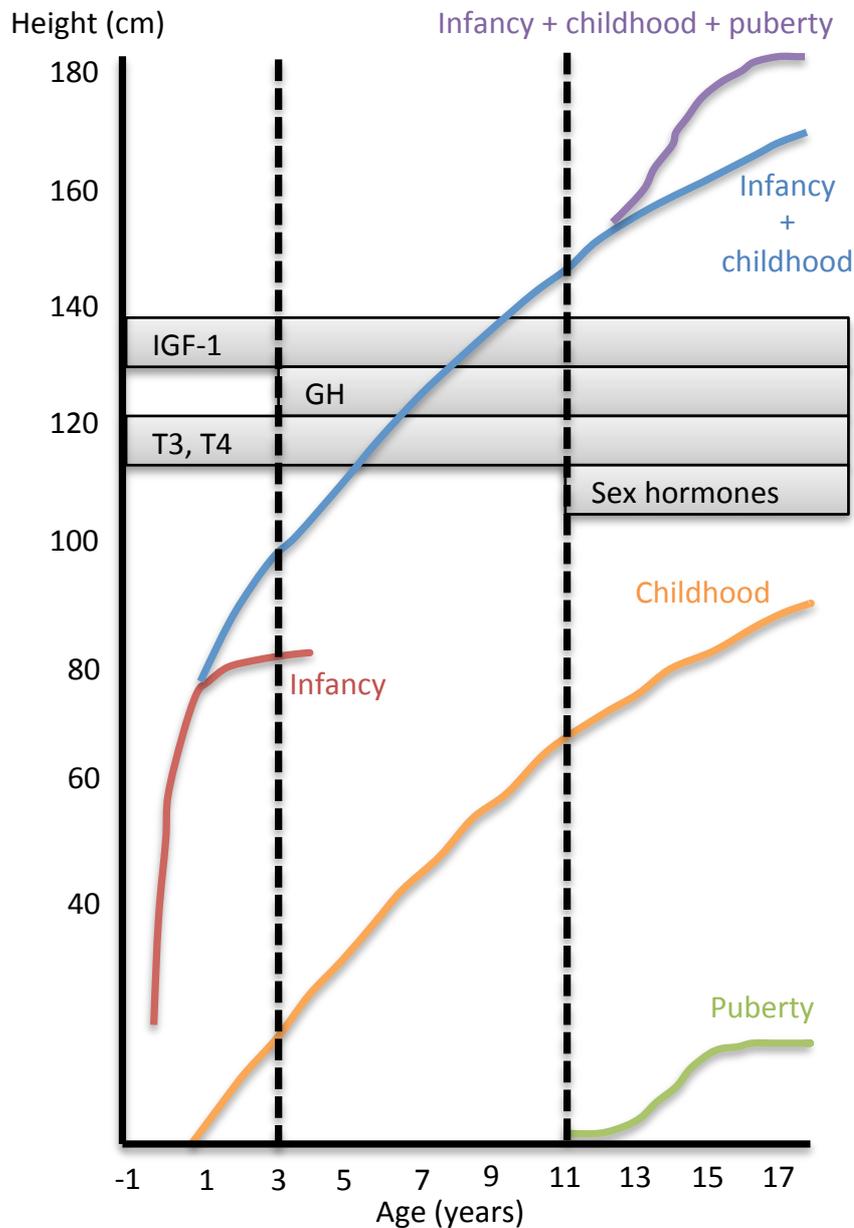
Healthy male foetuses are both slightly longer and heavier than females (77-79), raising the hypothesis that androgens may be responsible for this difference between the sexes. Both birth weights and birth lengths in infants with CAH have been reported to exceed the normal reference data, suggesting that increased androgen levels may increase growth (80, 81). However, administration of testosterone in pregnant sheep has actually resulted in reduced birth weights in the offspring (82). Birth weight is positively correlated with gestational age at birth (83).

### **2.3.2 Growth**

#### *2.3.2.1 Normal growth*

Normal longitudinal growth and weight development are regulated by complex mechanisms including hereditary factors, endocrine regulation and nutritional status (84, 85). Usually, growth in humans is divided into three distinct phases: infancy, childhood and puberty (Figure 4).

**Figure 4**



*Foetal and infant growth is dependent on thyroid hormones (T3, T4) and nutritional factors that increase hepatic production of IGF-1, but not growth hormone, which plays a more important role during childhood. Pubertal growth is dependent on testosterone and oestrogen while epiphyseal closure relies on oestrogen production. Infancy is marked by the fastest height velocity. The onset of the childhood growth phase actually occurs before the end of infancy. Similarly, childhood growth continues through puberty and adds to the pubertal growth spurt. The height given on the y-axis corresponds to males, but the growth pattern and endocrine regulation are similar in females.*

Longitudinal growth is a process that occurs in the growth plate as a result of endochondral ossification (86). Stem-like cells in the growth plate replicate and differentiate into proliferative chondrocytes which replicate rapidly (87). After several generations of chondrocyte replications, cell division ceases and the chondrocytes differentiate into hypertrophic cells, increasing 6- to 10-fold in cell height (88).

Even though there seems to be an intrinsic termination of cell division in the growth plate itself (89), the process is highly sensitive to regulatory mechanisms, including both endocrine signals (85) and nutritional state (84). Growth hormone (GH), insulin-like growth factor I (IGF-I) and thyroid hormone are definitely involved in the process of normal endochondral ossification (85, 86, 89) and increases longitudinal growth. Glucocorticoids exert negative effects on longitudinal growth, both by direct signalling to growth plate chondrocytes (90), by inducing chondrocyte apoptosis (91), and probably indirectly by lowering systemic GH concentrations (92).

Sex steroid hormone levels are high in both infants and healthy pubertal subjects (93). Although newborns seem to be insensitive to this 'biochemical mini puberty' (94), true pubertal growth acceleration is dependent on increased production of sex steroids (95). The positive effect on longitudinal growth is dependent on both oestrogens and androgens (95, 96). Although both androgens and oestrogens induce growth acceleration, oestrogen also triggers bone maturation and epiphyseal fusion, which marks the end of longitudinal growth (97).

During the infancy period, healthy children continue to grow at a dramatic rate and many researchers see this phase as a continuation of the foetal growth period. The average gain in length is about 25 cm for both sexes, although males are somewhat taller at 1 year of age (98). Typically, children born small for gestational age exhibit a catch-up in growth and children born large for gestational age exhibit a catch-down in growth, thereby diminishing the differences seen at birth (99). Normal growth development is dependent on nutritional status as well as physiological signalling of thyroid hormone, insulin and IGF-I (85). Androgens are not thought to play an important role in growth during infancy. High levels of androgens have been demonstrated at this period without signs of precocious puberty or growth acceleration. Transient physiological androgen insensitivity is therefore thought to be present during infancy (94).

The childhood growth phase begins when the high growth velocity in infancy abates (100). Growth during childhood is mostly linear with a stable growth rate of about 4–8 cm per year (85, 101) and there is almost no difference in height velocity between the sexes (101). The

hypothalamus-pituitary-gonad axis is inactive due to high sensitivity to negative feedback of oestrogen (102) and growth is dependent on the GH-IGF-1 axis (103).

In the later part of the childhood growth phase the adrenal gland increases its production of the weak androgens DHEA and DHEAS (104) in a process called adrenarche. Adrenarche typically begins at about 5–7 years of age (105, 106). Only humans and some great apes demonstrate this process in which pituitary adrenocorticotrophic hormone (ACTH) increases the production of not just DHEA and DHEAS, but also cortisol (51, 107).

Despite being a weak androgen, DHEA does not increase growth. On the contrary, the growth rate is decreased during adrenarche and the period immediately after (102). Rather than acting as an androgen, DHEA directly stimulates the oestrogen receptor in the growth plate and is aromatised into oestrogen, so as to reduce the proliferative rate in the growth plate (104). In fact, the growth rate before the onset of puberty is at its lowest since birth (108).

The onset of puberty is defined as the presence of two common pubertal signs in girls, thelarche and increased growth, and, in boys, increased testicular growth ( $\geq 4$  ml). In girls, the peak height velocity, the growth spurt, occurs at the beginning of puberty, whereas, in boys, the height velocity peaks later in puberty when the testicles are about 10 ml in size (109).

In the childhood phase, the amplitude of the pulsatile GnRH secretion is low, but adequate not to make the gonad completely quiescent. During puberty both the amplitude and frequency of these pulses increase (109). At the onset of puberty the amplitude, but not the frequency, of the nightly pulsatile secretion of GH is increased and leads to increased levels of IGF-1 (110, 111). In the absence of gonadotrophins or GH signalling, the pubertal growth spurts default. The two hormonal axes seem to be closely related as sex steroids, predominately oestrogens, increase production of GH (109).

The average gain in height during puberty is 20–30 cm but the interindividual differences are wide (112). Boys grow more during puberty than girls (113). If the age at onset of puberty is within the normal range, it does not seem to affect final height (114). However, an extremely early start of pubertal growth or a complete absence of puberty leads to short final stature (112, 115).

As chondrocyte proliferation decreases and they undergo apoptosis, longitudinal growth gradually ends (89). In a Swedish population-based study, girls reached their final height at a mean of 17.5 years of age and boys at a mean of 19.2 years (116).

Girls with the androgen insensitivity syndrome (AIS), and 46,XY, have a dysfunction in the AR and are thereby unable to respond to testosterone. They reach a final height nearly equivalent to the average for men (117), suggesting that androgens are not involved in the process of epiphyseal closure and that genetic influence from the Y-chromosome is important for the determination of final height.

In addition, the achieved final height is closely connected to parental height (118). The usual method for predicting a child's target height was proposed by Tanner in 1970 as the mid-parental height + 6.5 cm for boys and – 6.5 cm for girls (119).

Although final height seems to be genetically predetermined, environmental factors may contribute (120). Nutritional factors and intercurrent chronic disease modulate final height (121, 122). However, obese children who often exhibit increased childhood growth have an attenuated pubertal growth spurt leading to a final height not different from that of the normal population (123).

#### *2.3.2.2 Growth in congenital adrenal hyperplasia*

Children with CAH present a genuine challenge to the clinician, not least in trying to achieve a final height close to the predisposed target height and avoiding the development of overweight.

In CAH, growth during infancy has been shown to be impaired. Especially children with SW CAH have a markedly reduced growth velocity (124). High doses of glucocorticoids, which could potentially interfere with the endochondral ossification in the growth plate, are given to children with severe forms of CAH. In fact, impaired growth during the first year of life has been reported to be more frequent in children with SW CAH treated with hydrocortisone equivalent doses exceeding  $18 \text{ mg/m}^2 \text{ BSA/day}$  than in those with lower doses (125, 126). It has been noted that children with CAH on glucocorticoid treatment have a reduced height development compared to the normal population during the first 1–2 years of life (81) and that the height velocity correlated negatively with the glucocorticoid dose (127). Glucocorticoids suppress osteoblastogenesis and may increase osteoclastic bone resorption (128).

Untreated patients with CAH had a normal growth pattern until 18 months of age, suggesting that this growth period is androgen-insensitive (94). However, treatment is necessary in children with classical CAH who are at potential risk of an adrenal crisis and salt loss. Since children seem to be relatively androgen-insensitive during infancy, supra-physiological glucocorticoid

regimens to suppress androgen synthesis in children affected by CAH may not be needed during this phase (124). Overtreatment with glucocorticoids does not only affect final height (129), but also height velocity in all growth phases (130). The glucocorticoid dose is related to growth, but the effect of overtreatment appears to be strongest during infancy and puberty, whereas the effect during the childhood growth phase is not as evident (81).

Treatment with potent synthetic glucocorticoids such as prednisolone leads to reduced growth (131). Prednisolone needs to be recalculated into hydrocortisone equivalents to allow proper comparisons of dosing. Previous studies have calculated prednisolone to be 4 times (131), 5 times (132) or even 15 times (133) more potent than hydrocortisone in affecting growth in children with adrenal insufficiency.

Rivkees and co-workers (134) published their results on 26 children with CAH treated with the long-acting and potent glucocorticoid dexamethasone. In their study they saw normal growth and skeletal maturation during the 7 years of follow-up. However, they calculated dexamethasone to be 70 times more potent than hydrocortisone, rather than the manufacturer's suggestion of 30 times. The use of long-acting, potent glucocorticoids in growing patients with CAH must therefore be preceded by careful consideration concerning the aim of treatment and dosing.

Besides the deficiency in cortisol, a mineralocorticoid deficiency causes hyponatraemia. A deranged sodium balance has been connected with poor growth. Furthermore, adequate treatment with mineralocorticoids can lessen the need for glucocorticoids and thereby allow lower total doses, ultimately leading to an improved final height (135-137).

Poor compliance is hard to study, but still it is thought to contribute to a lower achieved final height (138).

The peak height velocity in puberty in patients with CAH develops about two years before expected for the normal population (139). Patients with CAH show reduced growth during puberty (127, 140, 141) and the magnitude of pubertal growth is related to the glucocorticoid dose (131). In addition, the total gain in height during puberty was significantly less for both males and females with classical CAH compared to a control group in the study by Bonfig and co-workers (131). It was suggested that to increase final height, unnecessary over-substitution of glucocorticoids needs to be especially avoided from 8 years of age and onwards (140).

In 2010 Muthusamy and co-workers published a meta-analysis concerning final height in classical CAH (142). The study included the results of 35 previous studies of children diagnosed before 5 years of age, treated and followed up to final height. They found a total final height SDS of -1.38 (CI 95%, -1.56 to -1.20). However, for that figure, studies as far back as 1966 contribute to the results. The corrected final height SDS (final height SDS – target height SDS) was -1.03 (CI 95%: -1.20 to -0.86) and was based on 17 studies also including target heights published between 1995 and 2007. In fact, on performing a regression analysis they found that the published achieved final height in patients with CAH correlated with the year of publication, meaning that older studies reported more impaired final height than recent ones.

Children with NC CAH often show a reduced final height. A contributing factor to this is probably the advanced skeletal maturation at diagnosis as these patients are often diagnosed later in life (143, 144). The achieved final height corresponds to the age of initiation of treatment, where an early start is associated with a better final height (138). Furthermore, a later diagnosis has been associated with a poor final height outcome (145).

Final height is negatively correlated with body mass index (BMI) during childhood in patients with CAH (81, 146). It has been interpreted as related to the glucocorticoid dose, or to an earlier pubertal onset and, in girls, an earlier menarche in the presence of obesity (147).

### 2.3.3 Weight development

#### 2.3.3.1 *Normal weight development*

As in the case of longitudinal growth, the development of childhood obesity is a multifactorial process including heredity, psychosocial factors, dietary intake, exercise and endocrine regulation (148-151). Weight development in healthy children is intense during infancy and BMI increases rapidly from about 14 kg/m<sup>2</sup> at birth to 17–18 kg/m<sup>2</sup> at about 1 year of age (152).

In the early childhood phase, healthy children grow in a linear fashion that is faster than the weight gain. This means that young children become physiologically leaner with a decreased BMI (152). At about 6–7 years of age, BMI increases again, the so-called adiposity rebound (153). The increase in BMI is thereafter relatively stable from adrenarche to the end of puberty (154).

Glucocorticoids increase energy intake and are linked to the pathophysiology of obesity (155).

### 2.3.3.2 *Weight development in congenital adrenal hyperplasia*

Similar to growth, weight development in children with severe forms of CAH has been reported to be impaired during the first year of life. Despite higher glucocorticoid dosages in children with severe forms of CAH, compromised weight development seems more pronounced in these children than in those with milder forms of CAH (124).

However, 75% of patients with CAH suffered from obesity in late childhood if high doses of hydrocortisone, defined as  $> 30 \text{ mg/m}^2$  BSA per day, had been given during the first two years of life, compared to only 11% if the doses were lower (13). BMI appeared to be higher in a cohort of children (2–8 years old) with classical CAH compared to a control group (156).

### 2.3.4 Congenital adrenal hyperplasia in adults

Adrenal crises are not as common in adults as they are in children, still they are feared and adjustments in treatment are necessary to reduce the risk of such events (14). Since salt-wasting is not as precarious in adults as it is in children, some researchers claim that mineralocorticoid substitution therapy may be discontinued in most adult patients (14). Others, on the other hand, believe in using mineralocorticoids in adults, not only in SW but also in SV and sometimes even in NC CAH, in an effort to be able to decrease the glucocorticoid doses. The doses of mineralocorticoids employed are however reduced with age due to side effects (157).

The primary objectives for treatment in adults with CAH, besides general well-being and reducing the risk for adrenal crisis, are to maintain fertility and reduce the risk of tumours in the adrenals and gonads. Since glucocorticoid treatment may provoke such side effects as iatrogenic Cushing syndrome and reduced bone mineral density (BMD), the doses have to be kept as low as possible (14, 158). Although a reduced BMD is common in CAH, osteoporosis is not (159). It may be that, although excess glucocorticoid treatment causes demineralisation of the bone, excess androgens may counteract this process (14).

Hypotension is uncommon in adults with CAH but, rather paradoxically, hypertension is sometimes seen (14).

Long-term excess ACTH stimulation of the adrenals may cause hyperplasia, which is associated with the development of such tumours as myelolipomas (160, 161).

#### 2.3.4.1 *Men*

Impaired fertility in men with CAH is common (162, 163). The development of testicular adrenal rest tumours (TARTs) has been linked to hyperandrogenaemia and they are found in most adult men with CAH (163, 164). TARTs increase the pressure within the testis, leading to reduced blood flow and, ultimately, compromised function (14). Furthermore, overproduction of sex steroids causes a down-regulation in gonadotrophin stimulation of the gonads, which may cause, reduced testosterone production from the Leydig cells, testicular atrophy and reduced fertility (14). If fertility is not important to the male patient with CAH, substitution therapy with hydrocortisone may be designed to mimic the physiological secretion of cortisol at a dose of about 8 mg/m<sup>2</sup> BSA per day (14). However, with low doses of glucocorticoids, there is an increased risk of TART development (165).

#### 2.3.4.2 *Women*

Excess androgens need to be suppressed more effectively in women to avoid androgen effects in the long-term perspective such as infertility, hirsutism and deepening of the voice. Women with CAH may require evening doses of glucocorticoids to reduce excessive androgen production (14); however, such side-effects as sleep disorder and weight-gain are common (14).

As in men, the fertility rate in women is reduced (166). However, this is due to endocrine factors as well as psychological ones, since women with severe forms, as a group, also express less interest in having children (166, 167). In order to establish ovulatory menstrual cycles and improved fertility, treatment with glucocorticoids may need to be supra-physiological in order to reduce both androgen and follicular phase progesterone excess (167).

Suboptimal glucocorticoid treatment in childhood and adolescence may cause adrenal hyperplasia and hence hyperandrogenism. This has been linked to the development of a polycystic ovarian syndrome (PCOS) phenotype in women with CAH. Ovarian androgen production is one of the hallmarks of PCOS. Thus, in women with both CAH and polycystic ovaries on ultrasound, androgen overproduction may originate from both the adrenals and the gonads (168). These patients may benefit from combined oral contraceptive pills to reduce the ovarian androgen production and to decrease free testosterone by inducing SHBG production (14).

#### 2.3.4.3 *Cardiovascular disease*

The risk for cardiovascular disease in CAH is still largely an unexplored field. Most studies have been conducted on rather young populations and with surrogate markers. However, it has been shown that the BMI in patients with CAH is often increased (169). This increase in BMI may correlate with excessive glucocorticoid treatment (170) and hypercortisolism has been linked to an increased risk of cardiovascular death (171).

Reduced insulin sensitivity has been reported to be more common in CAH, however most studies suggest that dyslipidaemia is not more common in patients with CAH than in the normal population (18, 172-174).

The results concerning whether hypertension is more common in adults with CAH than in the normal population are conflicting. One study found that the systolic blood pressure in a paediatric cohort of patients with CAH was elevated (175). However, these results have not been confirmed in larger populations of adults (174, 176).

Interestingly, Sartorato and co-workers found that the intima-media thickness (IMT) was increased in the common carotid and common femoral arteries, as well as in the abdominal aorta, in young adults with CAH, compared to healthy controls. IMT is measured with ultrasound and is a surrogate marker for atherosclerosis and it is considered to be a predictor of myocardial infarction and stroke (177).

### 2.3.5 Management of congenital adrenal hyperplasia

#### 2.3.5.1 *Children*

##### 2.3.5.1.1 **Aims of treatment**

The ideal aims of glucocorticoid treatment in children and adolescents with CAH should be to achieve a normal height velocity and normal bone maturation without developing overweight. By satisfying these criteria, treatment would be optimal, allowing no hyperandrogenism or hypo- or hypercortisolism (178). Keeping substitution therapy with glucocorticoids at a level where the HPA-axis is shut down without causing iatrogenic hypercortisolism is, however, a difficult task (1).

When the diagnosis has been demonstrated clinically and biochemically, genetic analysis is helpful not only for confirmation, but also for future genetic counselling, prognosis and optimising therapy (4, 33).

#### **2.3.5.1.2 Glucocorticoid substitution therapy**

The physiological endogenous production of cortisol is usually perceived to be about 6–8 mg/m<sup>2</sup> BSA per day (179-181). In order to mirror normal production, oral doses of 10–12 mg/m<sup>2</sup> BSA per day of hydrocortisone are usually needed to overcome the degradation in the enterohepatic circulation (182). However, to suppress the HPA axis, higher doses may be needed; doses of about 15 mg/m<sup>2</sup> BSA per day have been suggested (178). The joint ESPE/LWPES CAH working group recommended that the total dose of hydrocortisone equivalents per day in childhood should be 10–15 mg/m<sup>2</sup> BSA per day, and that the dose should be divided and administered at least three times per day, with the highest dose given in the morning (132).

Long-acting, potent glucocorticoids carry a higher risk of such adverse effects as compromised growth and development of obesity (136). However, successful careful treatment with dexamethasone, accompanied by close monitoring, has been reported by Rivkees and Crawford (134).

Since 9 $\alpha$ -fludrocortisone, besides acting on the aldosterone receptor, also has a strong affinity to the glucocorticoid receptor, this should be taken into account and added into the calculation of the total glucocorticoid dose (Table 1) (182).

**Table 1**

	Potency in relation to hydrocortisone		
	Anti-inflammatory	Mineralocorticoid	Growth inhibitory
<b>Hydrocortisone</b>	1	1	1
<b>Cortisone</b>	0.8	0.8	0.8
<b>Prednisolone</b>	5	0.8	5
<b>Fludrocortisone</b>	10	125	n/a

*Relative potency of the glucocorticoids most frequently used to treat CAH in Sweden. The relative growth inhibitory potency of fludrocortisone compared to hydrocortisone is not known. Modified from Gupta, et. al. 2008 (182).*

Glucocorticoid doses may need to be increased during puberty. There are several reasons for this. Firstly, the increased GH secretion inhibits reactivation of cortisone to cortisol (183). Secondly, increased oestradiol concentrations stimulate the production of cortisol binding globulin, thereby decreasing the free and biologically active cortisol fraction (178). Finally, increased GH secretion elevates the insulin levels that stimulate both the ovaries (184) and the adrenals (185) to produce androgens.

### **2.3.5.1.3 Mineralocorticoid substitution therapy and sodium supplementation**

In the neonatal period, electrolytes and blood glucose should be measured at diagnosis. In cases of salt loss, symptoms often occur in the second to third week of life (186) and potassium levels usually increase before sodium levels decline (187). If salt crisis occurs, intravenous fluid treatment with sodium chloride and glucose is necessary, in addition to glucocorticoid and mineralocorticoid treatment (52).

During the first 6 months of life, a salt crisis is particularly impending and patients with potential SW CAH require supplementation with sodium chloride consisting of 1–3 g/day (132). The risk of salt crisis and the need for supplementation is particularly important to consider in fully breast-fed infants, as breast milk contains very little sodium (188).

In SW CAH, substitution with the mineralocorticoid 9 $\alpha$ -fludrocortisone is needed. Doses may need to be higher during the first two years of life, i.e., about 50–300  $\mu$ g/day. Usually, the doses

can be lowered during childhood and at transition from paediatric care doses of 50–200 µg/day are often sufficient (132).

#### **2.3.5.1.4 Other adjuvant therapies**

If the control of disease is poor before final height is achieved, experimental treatment with aromatase inhibitor to reduce the conversion of androgens to oestrogens has been tried. By lowering the circulating levels of oestrogens, premature epiphyseal growth plate closure could be avoided or delayed (12).

Peripheral androgen blockade may be helpful for treating hyperandrogenism in CAH, especially in combination with an aromatase inhibitor, but the effect on growth and long-term effects in children still has not been fully studied (12).

The use of GH treatment to improve final height is still poorly investigated in CAH and the results are not convincing (126).

In case of evolving central precocious puberty, treatment with GnRH agonists may be introduced to halt this process and to reduce the risk of future short stature (189).

#### **2.3.5.1.5 Surgery**

Although bilateral adrenalectomy is an effective way to completely diminish hyperandrogenism in CAH and thereby reduce the risk of iatrogenic hypercortisolism, it leaves the patient with no residual ability for endogenous cortisol, mineralocorticoid or adrenaline production. It is therefore only recommended in experimental settings in patients with very poor disease control and where a long-term follow-up can be guaranteed (132).

Corrective genital surgery is a technically complicated and psychologically delicate matter. The aims of such interventions should be to see to it that the urinary tract function is good, without incontinence or recurrent infections, and to maintain good adult sexual and reproductive function and that the appearance of the external genitalia is congruent with the gender. Surgery has been reported to be technically easiest at 2–6 months of age; however, the level of the patient's own consent, rather than the parents', is of course limited at such an age (132). It is recommended that clitoroplasty, with reduction of clitoromegaly, and vaginoplasty is performed early in females with Prader IV-V in one stage. The reason for this is that clitoral tissue can then be used for the

vaginal construction (190). Presently, consensus has not been reached as to what degree of clitoromegaly that should indicate surgical intervention (191, 192).

#### **2.3.5.1.6 Monitoring**

In a review article, Hindmarsh suggests that the follow-up after first-discharge should be clinical check-ups at least every sixth week during the first 6 months and, after that, every third month up to 3 years of age. During childhood, check-ups can be done twice a year until puberty when more frequent clinical controls are again warranted, namely, at least every third month (178) .

Treatment with glucocorticoids is often based on clinical evaluation, laboratory markers such as 17-OHP, androstenedione and cortisol, auxological data such as height velocity and weight gain, and bone maturation (132).

When monitoring children with CAH it is important to evaluate growth, since disturbances may reflect both over- and undertreatment with glucocorticoids, as well as inadequate sodium supplementation in infancy (132). Increased weight development may mirror unnecessarily high doses of glucocorticoids (193). Some researchers advocate yearly radiological bone maturation evaluations to detect inadequately treated hyperandrogenism (5). Biochemical evaluations may give short-term information concerning the rationale for the current dose (6).

The risk for developing TARTs should be considered in adolescent boys. Regular ultrasound scans to detect these lesions early on are recommended (178). Increasing the glucocorticoid dose has been shown to reduce the risk for further development of TARTs (16).

Many adolescent girls with CAH develop polycystic ovaries, one of the features in PCOS. To what extent early detection of this syndrome influences the long-term consequences is not clear and at the moment the rationale for regular ultrasound screening remains unknown (178).

#### **2.3.5.2 Adults**

The adult patient with CAH presents other challenges to the physician than children. There is no need to adjust doses to allow for adequate growth and the risk of salt crisis is less significant (194). However, the concern about low BMD and overweight related to iatrogenic hypercortisolism remains the same (17). Furthermore, the adult patient often requires the physician to optimise treatment in order to increase fertility (14, 17).

Because of the decreased risk for salt crisis, mineralocorticoid substitution can be lowered according to the monitoring of plasma renin, sodium levels and blood pressure (14, 17, 194). Furthermore, sodium is excessive in the diet of the Western world (14).

The safest way to avoid iatrogenic cushingoid symptoms in adult patients with compromised adrenal steroid synthesis is probably to maintain the patients on a glucocorticoid substitution therapy based on hydrocortisone, divided as three doses daily (158). However, most adult patients are switched to two doses a day of either pure hydrocortisone, a combination therapy with prednisolone or pure prednisolone (14, 195-197). Prednisolone has the advantage of suppressing androgen production also during the night time and may be the preferred drug especially in women who are actively planning to become pregnant (14). Hyperandrogenism is often asymptomatic in men, but awareness of the development of TARTs is advised (14, 17).

#### *2.3.5.3 Prenatal treatment*

In the case of a previous sibling with classical CAH, the foetus in the next pregnancy to the same parents has a risk of 1:4 of being affected. Prenatal treatment with dexamethasone in subsequent pregnancies is an effective way to reduce the genital virilisation in girls with classical CAH, but it has no potential beneficial effects in boys. Therefore, only 1 in 8 fetuses would benefit from such treatment (7).

Lajic and co-workers in Sweden studied the psychological effects on children after prenatal treatment with dexamethasone and found a negative effect on verbal working memory in those treated during the first trimester. In addition, boys showed reduced masculine and more gender-neutral behaviour (198). A later study failed to reproduce these results; however, it was noted in that study that girls who had been treated throughout the pregnancy showed slower mental processing (199). As a result of these studies, prenatal treatment is not currently given in Sweden (200) and the recommendation is to administer prenatal treatment only as part of a clinical trial (7).

## **2.4 NEONATAL SCREENING**

### **2.4.1 Introduction to screening**

In 1951 the Commission on Chronic Illness Conference on Preventive Aspects of Chronic Disease, defined screening as:

*The presumptive identification of unrecognised disease or defect by the application of tests, examinations, or other procedures which can be applied rapidly. Screening tests sort out apparently well persons who probably have a disease from those who probably do not. A screening test is not intended to be diagnostic. Persons with positive or suspicious findings must be referred to their physicians for diagnosis and necessary treatment (201).*

This definition is still valid. In order to identify disease before actual symptoms develop, screening is employed for a wide spectrum of diseases and in various manners, such as the cervical Papanicolaou smear for early detection of cervical cancer (202), mammography for detection of breast cancer (203) and newborn screening for detection of inborn errors of metabolism (204) or congenital heart diseases (205). Some screening programmes employ histological analysis, radiography, laboratory markers or pulsoxymetry, as in the examples above, but clinical examination may also be utilised (206). Mass screening or universal screening sets out to detect disease in a whole population (207). Selective screening, sometimes referred to as case finding, is utilised in a high-risk group (208), for example, the screening for retinopathy in diabetic patients (209). Despite the differences in diseases or methods, all screening programmes have some common denominators.

#### *2.4.1.1 Criteria for screening according to Wilson and Junger (207)*

- 1. The condition should be an important health problem.*
- 2. There should be an accepted treatment for patients with recognised disease.*
- 3. Facilities for diagnosis and treatment should be available.*
- 4. There should be a recognisable latent or early symptomatic stage.*
- 5. There should be a suitable test or examination.*
- 6. The test should be acceptable to the population.*
- 7. The natural history of the condition, including development from latent to declared disease, should be adequately understood.*
- 8. There should be an agreed policy on whom to treat as patients.*
- 9. The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditures on medical care as a whole.*
- 10. Case-finding should be a continuing process and not a 'once and for all' project.*

## 2.4.2 Evaluating screening programmes

In any screening programme test results can come back as positive, negative or borderline (equivocal) (207). Positive test results can either be true, meaning that the individual actually has the disease, or false, meaning that the individual does not, despite the positive test result, have the disease. Similarly, negative test results can either be true, meaning that the individual does not have the disease, or false, meaning that the individual has the disease despite the negative test result (210).

Screening result	True classification	
	Has the disease	Does not have the disease
Positive	True positive	False positive
Negative	False negative	True negative
Total	Total cases with the disease	Total cases without the disease

From the contingency table (Table 2), the most common measurements in evaluating a screening programme can be calculated.

The *sensitivity*, meaning the proportion of individuals that actually have the disease who are detected by the test, is equal to the number of true positive cases divided by the total number of individuals with the disease (true positives and false negatives) (207, 210).

The *specificity* is the proportion of individuals without the disease who will actually have a negative test result and is equal to the number of true negatives divided by the total number of individuals without the disease (false positives and true negatives) (207, 210).

The *positive predictive value* (PPV) represents the proportion of positive tests that are in fact true positives. It is calculated as the number of true positives divided by the total number of positive tests (including false positive tests). Similarly, the *negative predictive value* is the proportion of true negatives divided by the total number of negative tests (including false negatives) and represents the proportion of negative tests that are in fact true negatives (207, 210).

All screening programmes struggle with similar difficulties. There will always be false test results. False positive results, when the screening test result is abnormal despite an absence of disease, may cause unnecessary worry and treatment of healthy individuals. False negative results, when the screening test result is normal despite the presence of disease, may cause unnecessary delay of treatment and further investigations. Furthermore, screening programmes result in economic costs and may cause adverse effects such as stress, anxiety and pain for the screened individuals and their families (208).

Research evaluating screening programmes is not trivial. As in all evidence-based medicine, the golden standard for assessing the efficiency of a clinical routine is to conduct a randomised clinical trial. However, as for many other areas in medicine, this may not be feasible for economic, ethical and practical reasons (208).

Case-control studies and, in particular, cohort studies are the most frequent study designs used to evaluate screening. Aside from other better known causes of bias, screening programmes are afflicted with some more specific causes of bias (208).

*Lead time bias* occurs when a disease is detected earlier with a screening programme than it would be with just clinical surveillance, but the earlier detection does not lead to increased survival. In that case, the survival time, from the detection of disease to death, will be increased although the actual survival time, from the onset of disease to death, is stable. Lead time bias may lead to the false assumption that survival time is increased (208).

*Overdiagnosis bias* occurs, for example, when a screening programme increases detection of diseases that would not normally lead to an adverse outcome in the near future. The classical example is prostate cancer, which can occur in different forms of severity. It is likely that a screening programme for prostate cancer would also detect milder, slow-growing forms of disease that would not normally lead to any adverse outcome for the patient; hence, the disease is detected early but does not increase survival time since the patient actually dies from something else (211).

*Selection bias* is a common problem in many other, particular epidemiological, research areas. It seems more likely that individuals with many close relatives who have died of colorectal cancer would be more prone to participate in a screening programme designed to detect this particular form of cancer than the general population would be. Since the risk of colorectal cancer

correlates with hereditary factors, this could influence the outcome of the screening programme (212).

*Length time bias* is often discussed in screening programmes setting out to detect different forms of cancer. Survival time is often longer with slow-growing tumours than fast-growing ones. Since slow-growing tumours exhibit a longer time before being symptomatic, they are more likely to be detected in a screening programme. By early detection of slow-growing tumours, compared to fast-growing ones, it may be that a screening programme could increase the apparent survival, but not the actual, survival time (212).

Cohort studies and case-control studies are often not designed to be robust against these types of bias. Furthermore, the control group in cohort studies may be from a different population, historically or geographically, leading to biased results that are difficult to generalise (208).

### 2.4.3 Screening for congenital adrenal hyperplasia

#### 2.4.3.1 *History*

In 1937 Butler and Marrian (213) described an abnormal excretion of hormonal rest products in the urine of patients with CAH. In the 1950s (19, 20) it was known that the disorder could be treated with glucocorticoids. However, a robust method for detecting CAH in screening was first developed in the late 1970s (9). It was based on measuring 17-OHP from micro-filter paper cuts using a radioimmunoassay, a technique developed by Rosalyn Yalow in the 1950s (214). In fact, Yalow was awarded the Nobel Prize for her development of the radioimmunoassay the same year as Pang and co-workers described its use for neonatal screening for CAH (215).

The first screening programme for CAH was developed in the U.S.A. in the 1970s. It was developed in Alaska and employed the method mentioned above to quantify 17-OHP (10). Since then, neonatal screening for CAH has been wide-spread and is currently used in more than 30 countries (11).

The original radioimmunoassay and the later introduced enzyme-linked immunosorbent assay have now been abandoned by most screening laboratories in favour of a direct solid-phase time-resolved fluoroimmunoassay (11, 216). This method is based on lanthanide-labelled antibodies that emit fluorescence. Compared to the previous methods, it is faster, automated and more precise.

The rate of false-positives is especially high in preterm infants. Possibly due to cross-reactivity with other steroids secreted by the immature adrenal gland in stressed infants (217).

Because of the high rate of false-positives in the screening for CAH, new strategies to reduce this rate have been developed (218). In 2004, Lacey and co-workers (219) reported the use of liquid chromatography, followed by tandem mass spectrometry (LC-MS/MS) as a method for second-tier testing after an initially positive test. This was developed to examine ratios between steroids before and after the hydroxylation step disturbed in CAH. Reports are promising and the method has been implemented as routine in some screening laboratories (11, 220, 221).

In 2005 genotyping was investigated as a second-tier test for the first time (222), but its use has not yet been investigated on a large scale (11).

#### *2.4.3.2 Methods*

All methods to determine 17-OHP in neonatal screening are based on preparing the samples from dried blood spots. This is usually done by eluting a paper disc of the filter paper, cut out from the blood spot, into a buffer (11).

##### **2.4.3.2.1 Radioimmunoassay**

The first method used to determine the concentration of 17-OHP in dried blood spots was, as mentioned above, radioimmunoassay. In brief, a known amount of radioactively labelled antigen, 17-OHP, is mixed with a known amount of a primary antibody with affinity for the antigen. The solution is mixed with the sample from the patient. Then the unlabelled antigen, 17-OHP, from the patient will compete with the labelled antigen and bind to the antibody. The unbound radioactive antigen is decanted from the solution and the radioactivity it is emitting can be measured in a gamma counter. The method has many different variations, but the basic idea is the same (214, 223).

##### **2.4.3.2.2 Enzyme-linked immunoassays**

As with radioimmunoassay, enzyme-linked immunoassays utilise the binding of an antibody to the antigen, 17-OHP, being measured. A primary antibody binds to the antigen. A secondary antibody with an attached enzyme is added. Any unbound antibodies are washed away and a substrate for the enzyme is added. When reacting with the enzyme the substrate often changes its colour. The colour change can be measured in a spectrometer (224, 225).

#### **2.4.3.2.3 Dissociation-enhanced lanthanide fluorescence immunoassay**

This method shares many similarities with enzyme-linked immunoassays; however, instead of the secondary antibody being attached to an enzyme, it is attached to a lanthanide chelate. After the unbound antibodies are washed away, an enhancement buffer dissociates lanthanide from the antibody. Lanthanide then produces a measurable fluorescent signal when stimulated with light of a certain wavelength (226). This method is by far the currently most frequently used for first-tier screening tests in neonatal screening for CAH (11, 216).

#### **2.4.3.2.4 Liquid chromatography and tandem mass spectrometry**

LC-MS/MS was implemented in newborn screening for inherited metabolic disease in the 1990s (227). The method is completely different from those mentioned above. The first step is a liquid phase chromatography that separates the chemicals included in the sample of interest by hydrophobic interactions in the presence of a hydrophilic solvent, such as water. The chemicals are then eluted in a more hydrophobic solvent, such as methanol, and released into the first of two mass spectrometers. The first mass spectrometer separates the chemicals based on their mass/charge ratio. The chemicals then enter a chamber called a collision cell in which the sample is broken down. The chemicals, now broken down into smaller fragments, are then analysed by the second mass spectrometer detector (228).

LC-MS/MS is a high-resolution technique in which the concentration of tiny fractions of a substance can be analysed. The advantages of LC-MS/MS are that it is a rapid and very accurate technique. In addition, it is possible to measure several substances at the same time. For some diseases included in screening programmes, such as analyses of acylcarnitines to detect medium chain acyl-CoA dehydrogenase deficiency, no other analytic methods are available (227). The first results concerning the potential use of LC-MS/MS in newborn screening for CAH were published in 2001 by Lai and co-workers (218). Since measuring the concentrations of different analytes simultaneously is very rapid and accurate with LC-MS/MS, it is possible to determine a ratio of steroid precursors before and after the enzymatic block in CAH due to a 21 $\alpha$ -hydroxylase deficiency, which would potentially lower the false positive rate in the screening (229).

#### **2.4.3.2.5 Genotyping**

Because of the high rate of false positives in neonatal screening for CAH, it is attractive to consider genotyping as a tool to assist in particularly equivocal results. As genotyping is costly

and time-consuming, it is not suitable for first-tier screening. However, its use has been described and suggested previously (222, 230-233) and recently described as a second-tier method (221).

#### **2.4.3.2.6 Screening for congenital adrenal hyperplasia in Sweden**

Newborn screening for CAH was introduced in Sweden in 1986. The results and experience from the screening programme are described in detail in Paper IV.

#### **2.4.3.2.7 Cost-effectiveness of screening for congenital adrenal hyperplasia**

A female preponderance is generally interpreted as missed male cases since girls with potentially lethal forms of CAH are often diagnosed before an actual salt crisis occurs and boys are at greater risk of dying before the diagnosis (234). Mortality in SW CAH in unscreened populations has been estimated to be to 4–10% (235).

It is difficult, however, to evaluate the effectiveness of screening by comparing screened and unscreened populations since children affected by SW CAH can die without a proper diagnosis being made (11). In a retrospective post mortem series, three out of 242 cases of sudden infant death syndrome had genetically verified classical CAH (236).

Boys with SV CAH who escape early detection present later on with accelerated growth and advanced bone age, which could negatively affect final height (11). Sometimes patients with NC CAH are detected in neonatal screening. However, the overall benefits for patients with milder forms remain uncertain (11).

Classical cost-benefit analyses are generally based on calculations concerning mortality and years of expected life. Thus, the analyses calculate the number of saved life-years in relation to the costs of a certain procedure, such as neonatal screening (11). Based on American screening programmes, it has been calculated that the cost of neonatal screening is between \$20 000 and \$250 000 per saved life-year (237, 238). A screening programme is generally considered worthwhile if the cost is less than \$50 000 per life-year (237). Besides the actual costs for the screening programme, additional costs, such as for further clinical examinations and laboratory investigations, are added subsequently.

In the case of a positive screening result, the family is obviously worried (239). However, the concern about the child's health seems to be reduced if the confirmatory test is negative (239). It

is, however, clearly important to keep the false positive rate as low as possible also for a number of other reasons.

An earlier diagnosis does not only lead to decreased mortality, but also to decreased morbidity. Boys with SW CAH diagnosed by neonatal screening have been shown to have higher mean sodium concentrations at diagnosis than those diagnosed by clinical surveillance: 134 mmol/l (range 115–148) versus 124 mmol/l (range 93–148) (187). Hence, they may escape neurological sequelae from salt-loss crises.

The clinical relevance of the finding that patients detected by screening tend to be hospitalised for a shorter period than patients detected clinically, without further considerations concerning morbidity, remains uncertain (11, 187, 240).

Overall, neonatal screening for CAH shortens the time to diagnosis (187, 241), which is especially important in SW CAH (11) since a salt crisis may be avoided. Furthermore, the time of uncertain sex in 46,XX individuals with classical CAH is shortened (187, 241).

### 3 AIMS

This thesis describes several aspects of CAH. One aim of the project was to describe and present the previous and present status of the clinical management for CAH in Sweden. In addition, CAH viewed as a model system for androgen exposure enabled us to investigate how androgens interfere with normal physiological phenomena such as foetal growth and duration of pregnancy.

The pre-specified hypotheses were:

1. Foetal growth is independent of androgen effects.
2. Pregnancy lengths are increased when the foetus is affected by severe CAH as compared to milder forms.
3. Neonatal screening for CAH increases survival compared to clinical surveillance alone.
4. Neonatal screening for CAH is effective in detecting SW CAH.
5. Growth and weight outcomes in children with CAH are dependent on treatment, sex and genotype.

The specific aims were:

1. To compare birth weight, as a measurement of foetal growth, between children with disordered androgen signalling and the normal reference population.
2. To compare lengths of pregnancy between children with different severities of CAH.
3. To investigate changes in the incidence of CAH over time in relation to improvements in treatment and the introduction of neonatal screening.
4. To describe the neonatal screening programme in Sweden.
5. To determine explanatory factors for growth and weight outcomes in children with CAH.

## 4 SUBJECTS AND METHODS

### 4.1 PAPER I: GESTATIONAL AGE CORRELATES TO GENOTYPE IN GIRLS WITH CYP21 DEFICIENCY

#### 4.1.1 Study population and design

Male sex has been associated with prolonged pregnancy (74). Male foetuses have higher levels of androgens compared to female foetuses (242). Androgens are particularly elevated in newborns with severe forms of CAH (232). The objective of this study was to investigate whether the length of pregnancy was prolonged for foetuses with severe forms of CAH, compared to milder forms.

The study retrospectively included patients with CAH detected through the national screening programme or included in a national prospective study (n = 165). Patients were excluded if their gestational age was unknown (n = 9), their sex was unknown (n = 1), genotyping had not been performed or their *CYP21A2* genotype group was not possible to determine (n = 33), prenatal treatment with dexamethasone was given during the whole pregnancy (n = 4) or delivered by elective caesarean section (n = 1). Patients who were born preterm (n = 8) were excluded from the statistical analyses that included a total of 109 patients.

Pregnancy lengths for children with CAH with different genotypes were compared. Furthermore, a comparison was made to the normal reference population born in singleton pregnancies lasting  $\geq 37$  weeks between 1987 and 1996 (74).

Data concerning gestational age were collected from the Guthrie cards used in the screening or, in patients who were born before the implementation of screening, from the medical records. The introduction of ultrasonographic determination of gestational age started in Sweden in the early 1980s (243).

*CYP21A2* mutation analyses had been carried out before this retrospective study. Patients were divided into four genotype groups depending on the severity of the mutation on the mildest allele, null, I2 splice, I172N, and V281L.

#### 4.1.2 Statistical methods

The Kruskal-Wallis test was used to analyse differences in gestational age between genotype groups and *post hoc* analyses were done between individual groups employing the Mann-Whitney test. Spearman's rank correlation coefficient was used to measure the statistical dependence between gestational age and genotype group for patients with CAH. Student's t-test was used to determine the difference in gestational age between patients with CAH and the normal population. The level of statistical significance was set at 0.05.

### **4.2 PAPER II: THE ROLE OF ANDROGENS IN FETAL GROWTH: OBSERVATIONAL STUDY IN TWO GENETIC MODELS OF DISORDERED ANDROGEN SIGNALLING**

#### 4.2.1 Study population and design

Similarly to the difference in gestational age, human males are heavier at birth than females (77-79). This difference seems to occur in most other primates as well (244). Since newborn males have higher androgen levels than females (242) and since testosterone is an anabolic hormone later in life, but does not seem to affect growth during the first 1.5–2 years of life, we wanted to investigate the relationship between birth weight in CAH, with foetal overexpression of androgens, and CAIS, with no foetal androgen effect.

*CYP21A2* genotype, length of pregnancy and birth weight were recorded for a total of 73 out of the 88 children diagnosed with CAH who were included in the prospective study. The study was a collaboration project with Professor Ieuan Hughes' group at the University of Cambridge. In their material, they identified 29 46,XY females with CAIS. The birth weight standard deviation score (SDS), adjusted for gestational age, was calculated from the normal population in Sweden and Great Britain, respectively.

#### 4.2.2 Statistical methods

Birth weights were calculated as the SDS, adjusted for gestational age, and compared with the relevant national normal population data using one-sample t-tests. This calculation is based on the fact that the normal population mean value for birth weight SDS is  $\pm 0$  and the test investigates whether the mean birth weight SDS in CAH or CAIS is significantly different from 0.

The non-parametric Kruskal-Wallis test was used to compare differences between CAH genotype groups. The level of statistical significance was set at 0.05.

### **4.3 PAPER III: ONE HUNDRED YEARS OF CONGENITAL ADRENAL HYPERPLASIA IN SWEDEN: A RETROSPECTIVE, POPULATION-BASED COHORT STUDY**

#### **4.3.1 Study population and design**

The study population consisted of the patients in the national CAH registry at the neonatal screening laboratory. This registry comprises all patients detected in the neonatal screening programme, missed cases who had been reported to the laboratory or who had been known to the laboratory through clinical contacts, patients for whom 17-OHP samples had been sent as clinical routine, patients included in previous Swedish studies of CAH and patients who had undergone *CYP21A2* analyses.

A total of 612 patients with CAH were included in the registry. The oldest patient was born in 1915. The *CYP21A2* genotype was known in 490 patients. Patients were divided into genotype groups according to the mildest mutated allele. Patients with no known genotype, but in whom the clinical form was known, were grouped solely based on the clinical classification. Null and I2 splice were combined in the group of clinically defined SW CAH. I172N and P30L were combined in the group of clinically defined SV CAH. Patients with V281L and other milder mutations were combined in the group with clinically defined NC CAH (Table 3). Six patients had other, more rare, causes of CAH apart from 21 $\alpha$ -hydroxylase deficiency and were excluded from the statistical analysis.

**Table 3**

n (%)	SW CAH		SV CAH		NC CAH		Unknown		Total
	M	F	M	F	M	F	M	F	
-1950	2 (67)	1 (33)	9 (60)	6 (40)	0 (0)	5 (100)	0 (0)	0 (0)	23 (4)
1950-1985	46 (41)	65 (59)	32 (41)	47 (59)	6 (21)	23 (79)	32 (62)	20 (38)	271 (45)
1986-2011*	73 (44)	92 (56)	41 (49)	43 (51)	15 (35)	28 (65)	13 (65)	7 (35)	312 (51)
<b>Total</b>	121 (43)	158 (57)	82 (46)	96 (54)	21 (27)	56 (73)	45 (63)	27 (37)	606 (100)

*SW CAH defined clinically as sodium <125 mmol/l or genetically, depending on the severity of the mildest allele as either null genotype group (deletion, R356W, Q318X, R483GGtoC, cluster E6, L308F, L307insT+Q318X, G291S, I7splice, W405X, R356P or V139E) or I2 splice genotype group (I2 splice, T52P or R356Q). SV CAH defined clinically as prenatal virilisation of external genitalia in females or symptoms before 5 years of age in males, but with no known signs of concomitant salt loss or genetically with I172N, P105L+P453S, H62L+P453S, P30L or G424S on the mildest allele. NC CAH defined clinically as onset of symptoms after 5 years of age or genetically with V281L, P453S, R233G or R341W. Unknown denotes cases with known CAH but no information on the severity of disease. Values represent the number of cases and, in parentheses, the percentage of males and females in each clinical group per time period.*

*\* The number of patients during 1986-2011 represents screened and unscreened individuals.*

Data on live births in Sweden were collected from the government agency Statistics Sweden (SCB), which has stored information on the number of live births per year since 1749 (245). Data on the number of males and females born alive in Sweden were obtained from the same source and was available from 1968 (246).

#### 4.3.2 Statistical methods

The proportions of males and females, genotype and clinical severity groups per decade and time period were compared using the  $\chi^2$  test. Statistical significance was set at  $P < 0.05$ .

#### **4.4 PAPER IV: NATIONWIDE NEONATAL SCREENING FOR CONGENITAL ADRENAL HYPERPLASIA IN SWEDEN: A LONGITUDINAL PROSPECTIVE POPULATION-BASED STUDY COVERING 26 YEARS**

##### **4.4.1 Study population and design**

Nationwide neonatal screening for CAH in Sweden has continued consecutively since its start in 1986. Data on the number of screened subjects and information concerning positive and false negative cases have been collected prospectively.

In Paper IV the results of the screening programme from 1986 to 2011 are described. During this period, 2 742 944 infants were born alive in Sweden and 2 737 932 (99.8%) were screened for CAH.

##### **4.4.2 Statistical methods**

Student's t-test was used for normally distributed continuous variables and the Mann-Whitney U test was used for non-parametric continuous variables. The  $\chi^2$  test was used for comparisons in contingency tables. For correlation analyses, Spearman's rank correlation was used for non-parametric variables and Pearson's correlation for normally distributed variables.

The marker for disease, 17-OHP, was not normally distributed and, furthermore, in 78 cases, the 17-OHP level was above the standard curve. For 42 of these cases, the exact value of 17-OHP was determined after dilution. However, for the remaining 36 cases, the exact 17-OHP values were not determined. In the statistical analysis, these 36 cases were given the mean 17-OHP value for the diluted tests. Mann-Whitney U and Kruskal-Wallis tests based on rank rather than the mean were therefore used to compare 17-OHP values between independent groups.

Wilcoxon's test was used to compare the 17-OHP levels between the first and second samples. Statistical significance was set at  $P < 0.05$ .

## **4.5 PAPER V: GROWTH AND TREATMENT IN CONGENITAL ADRENAL HYPERPLASIA: A PROSPECTIVE OBSERVATIONAL STUDY FROM DIAGNOSIS TO FINAL HEIGHT**

### **4.5.1 Study population and design**

Paper V describes a population-based observational cohort study. The study included all subjects born or diagnosed with CAH in Sweden between 1 January 1989 and 31 December 1994. A total of 88 children were eligible for inclusion. During the study period, two cases were found to be healthy and were thus excluded from further analysis. Six additional cases were excluded: two because of other intercurrent chronic disease and four due to loss to follow-up.

The diagnosis was confirmed by clinical examination, laboratory investigations and genetic analysis.

Cases diagnosed within the first month of age were considered early-diagnosed, whereas patients with a later diagnosis were regarded as late-diagnosed.

Patients were divided into genotype groups according to the mildest allele: null, I2 splice, I172N, P30L and V281L. Furthermore, because of the generally good concordance between genotype and phenotype, children with null and I2 splice were regarded as SW CAH, with I172N and P30L as SV CAH and with V281L as NC CAH.

The local paediatrician reported all changes in treatment and auxological findings continuously. The included subjects were followed prospectively until they achieved final height or 18 years of age. Auxological data were plotted onto a growth chart based on the Swedish reference population and extrapolated values of height and weight at 0, 0.25, 0.5, 0.75, 1, 1.5, 2 years and annually thereafter were read out manually. The standard deviation score (SDS) for height and weight was calculated based on the Swedish reference population. Body surface area (BSA) was calculated using the DuBois formula (247). Auxological data before diagnosis in late-diagnosed cases were collected retrospectively.

All complete growth charts were examined by a senior paediatric endocrinologist and classified as having a pubertal growth spurt or not (height velocity > 7 cm/year). Furthermore, it was noted whether the growth charts revealed a separate preceding growth spurt of > 7 cm/year before the

actual pubertal growth spurt (biphasic growth curve) or whether there was any occurrence of reduced growth before the pubertal growth spurt.

Every change in treatment was recorded and the mean doses of hydrocortisone equivalents were calculated for the periods of 0–0.25, 0.25–0.5, 0.5–0.75, 0.75–1, 1–1.5, 1.5–2 years of age and thereafter between all full years. All glucocorticoid treatments (hydrocortisone, cortisone acetate, prednisolone and 9 $\alpha$ -fludrocortisone) were converted into hydrocortisone equivalents. Patients who had undergone prednisolone treatment were compared with those who had not taken prednisolone.

The study aimed at examining growth and weight development in patients with different degrees of severity of CAH and *CYP21A2* genotypes and to correlate this with different treatment strategies used in clinical practice. Since treatment traditions differ between centres in Sweden, it was possible to compare the outcomes with hydrocortisone and/or cortisone acetate alone or with the addition of prednisolone.

#### 4.5.2 Statistical methods

The means of continuous variables were compared between two groups using Student's t-test if the data were normally distributed and the Mann-Whitney test if the data were not normally distributed. Wilcoxon's test was used to compare two paired samples with not normally distributed data. To compare repeatedly measured variables, such as height or dose, between groups, a two-way repeated measures analysis of variance was utilised. Since all paired data in the study violated the assumption of sphericity, the Greenhouse-Geisser correction was used. Bonferroni corrections were used in *post hoc* analyses. Given the nature of the SDS, comparisons with the normal population were calculated using the one-sample t-test. Spearman's correlation test was used for correlations since all data examined in that respect were not normally distributed. The  $\chi^2$  test for comparisons of proportions was used if all groups consisted of at least five cases; otherwise, Fisher's exact test was used. Statistical significance was set at  $P < 0.05$

## 4.6 ETHICAL CONSIDERATIONS

Committees on ethics in biomedical research have approved all the studies included in this thesis.

Approval numbers: Paper I (dnr: 89136, Uppsala University, and dnr: 95:137, Karolinska Institutet); Paper II (dnr: 89136, Uppsala University, and dnr: 95:137, Karolinska Institutet and

the Ethics Committee at Cambridgeshire Ethics 2); Paper III (2010/1869-31/1, Karolinska Institutet); Paper IV (2010/1869-31/1, Karolinska Institutet) and Paper V (dnr: 89136, Uppsala University and dnr: 95:137, Karolinska Institutet).

None of the studies in this thesis involves any medical risks to the patients, as they are all purely observational. The collection of data and publication of results have been carried out so as to guarantee the integrity and anonymity of the patients.

For the prospective study that recruited the patients described in Paper II, V and partly in Paper I, the informed consent of the parents was obtained at the start of the study. Papers III and IV are based on population-based registries in which informed consent was neither feasible nor required according to the Committee on Ethics in Biomedical Research at the Karolinska Institutet.

## 5 RESULTS

### 5.1 RELATIONSHIP BETWEEN LENGTH OF PREGNANCY AND GENOTYPE IN CONGENITAL ADRENAL HYPERPLASIA

Of the 165 patients eligible for the study, 109 met the criteria for inclusion (Table 4)

**Table 4**

Sex		Null	I2 splice	I172N	V281L	Total
<b>Male</b>	N	10	21	13	3	47
	GA	288.3 ± 7.6	286.3 ± 10.3	280.6 ± 12.2	-	284.8 ± 10.7
<b>Female</b>	N	18	15	22	7	62
	GA	285.7 ± 8.6	284.4 ± 7.1	273.9 ± 8.8	274.7 ± 10.5	280.8 ± 10.0
<b>Total</b>		28	36	35	10	109

*N*, number of patients that met the inclusion criteria. *GA*, mean gestational age at birth in days of patients with both exact numbers of days of pregnancy recorded ( $n = 66$ ), ± *SD*.

Gestational age correlated with the genotype group when the foetus had CAH (correlation - 0.362,  $P < 0.001$ ) in that patients with a more severe form had a longer pregnancy than those with milder forms. The correlation was significant in females with CAH ( $P = 0.003$ ), it but failed to reach significance when only males were examined.

The mean gestational age at birth was compared between the different genotype groups. A significant difference between genotype groups was noted in females ( $P = 0.002$ ). A *post hoc* analysis revealed that the gestational age in female patients with the most severe null form, 285.7 days, was higher than in those with genotypes I172N, 273.9 days ( $P = 0.003$ ) and V281L, 274.7 days ( $P = 0.04$ ), but did not differ from those with the almost as severe form, genotype I2 splice, 284.4 days ( $P > 0.05$ ). Female patients with I2 splice had a higher gestational age than both I172N and V218L patients ( $P = 0.011$  and  $P = 0.043$ , respectively). No significant difference was observed between females with I172N and V281L genotypes. The gestational age in different

genotype groups did not differ significantly in males ( $P > 0.05$ ) and therefore no *post hoc* analyses were carried out in males.

The mean length of pregnancy in the Swedish reference population was  $280.2 \pm 8.8$  days ( $\pm$  SD). The mean length for pregnancies in which the foetus was later diagnosed with CAH was  $282.5 \pm 10.4$  days and differed statistically significantly from the normal population ( $P < 0.05$ ). Males with CAH had a higher mean gestational age at birth,  $284.8 \pm 10.7$  days, compared to the normal male population,  $280.6 \pm 8.9$  days ( $P < 0.05$ ). In females with CAH, the length of pregnancy,  $280.8 \pm 10.0$  days, did not differ significantly from that of the normal female population,  $279.8 \pm 8.6$  days ( $P > 0.05$ ).

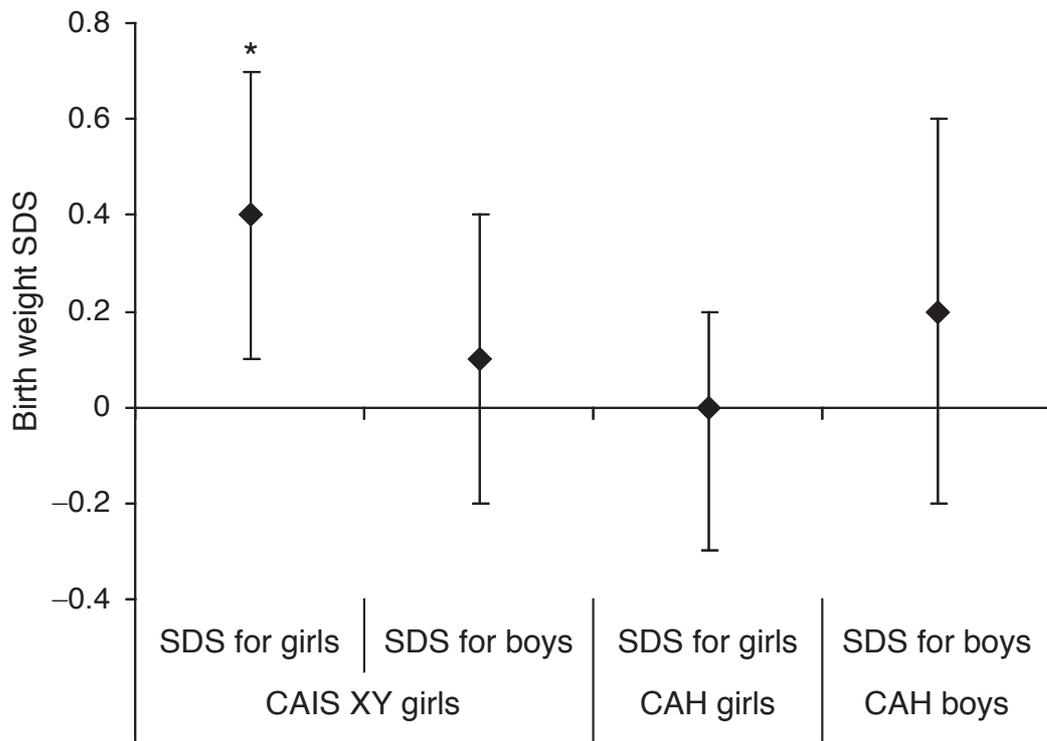
## **5.2 FOETAL GROWTH MAY BE INDEPENDENT OF ANDROGENS**

Birth weight SDS<sub>boys</sub> in CAIS girls was similar to that of reference boys (mean, CI 95%: 0.1, -0.2 to 0.4) and birth weight SDS<sub>girls</sub> was higher than the reference of females (mean, CI 95%: 0.4, 0.1 to 0.7,  $P = 0.02$ ).

Birth weight SDS in both girls and boys with CAH did not differ from that of the reference population (mean, CI 95%: 0.0, -0.3 to 0.3 and 0.2, -0.2 to 0.6, respectively). Birth weight SDS did not differ between genotype groups in children with CAH ( $P > 0.05$ ) (Figure 5). Birth length SDS was not different from the national reference in either sex.

No correlation between genotype and birth weight or birth length could be detected in children with CAH.

**Figure 5**



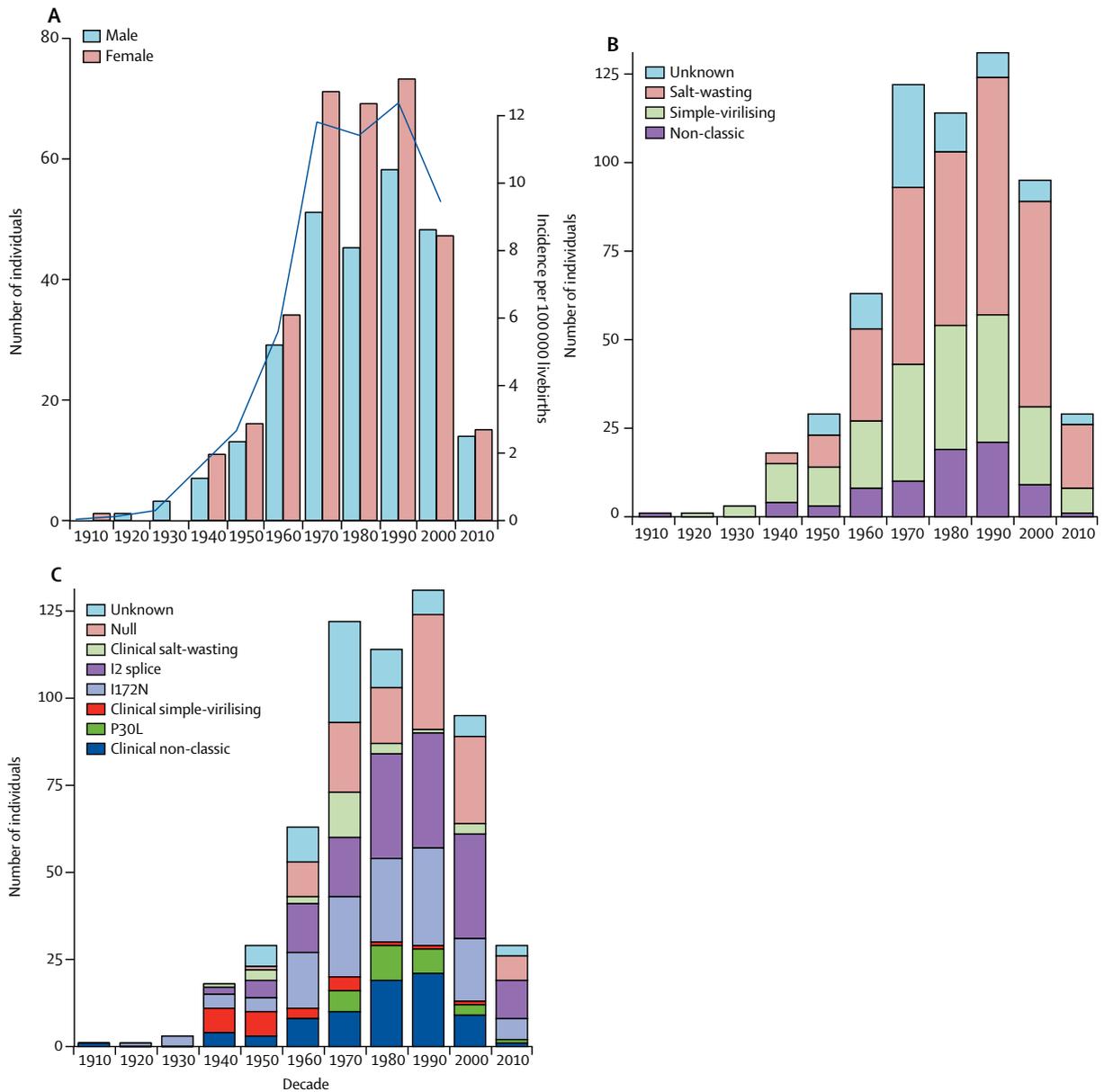
*Birth weight standard deviation score (SDS) adjusted for gestational age in complete androgen insensitivity syndrome (CAIS) and congenital adrenal hyperplasia (CAH).*

*\*  $P < 0.05$  compared to the national reference mean ( $SDS \pm 0$ ) using a one-sample  $t$  test.*

### **5.3 DESCRIPTION OF CONGENITAL ADRENAL HYPERPLASIA IN SWEDEN DURING THE LAST CENTURY**

Only 23 patients identified in the study were born before the introduction of glucocorticoid treatment in 1950 and the majority, 583 patients (96%), were born from 1950 onwards. The apparent incidence increased throughout the 20<sup>th</sup> century with a peak in the 1990s. The apparent incidence rose steeply, however, from the 1960s and 1970s (Figure 6A-C).

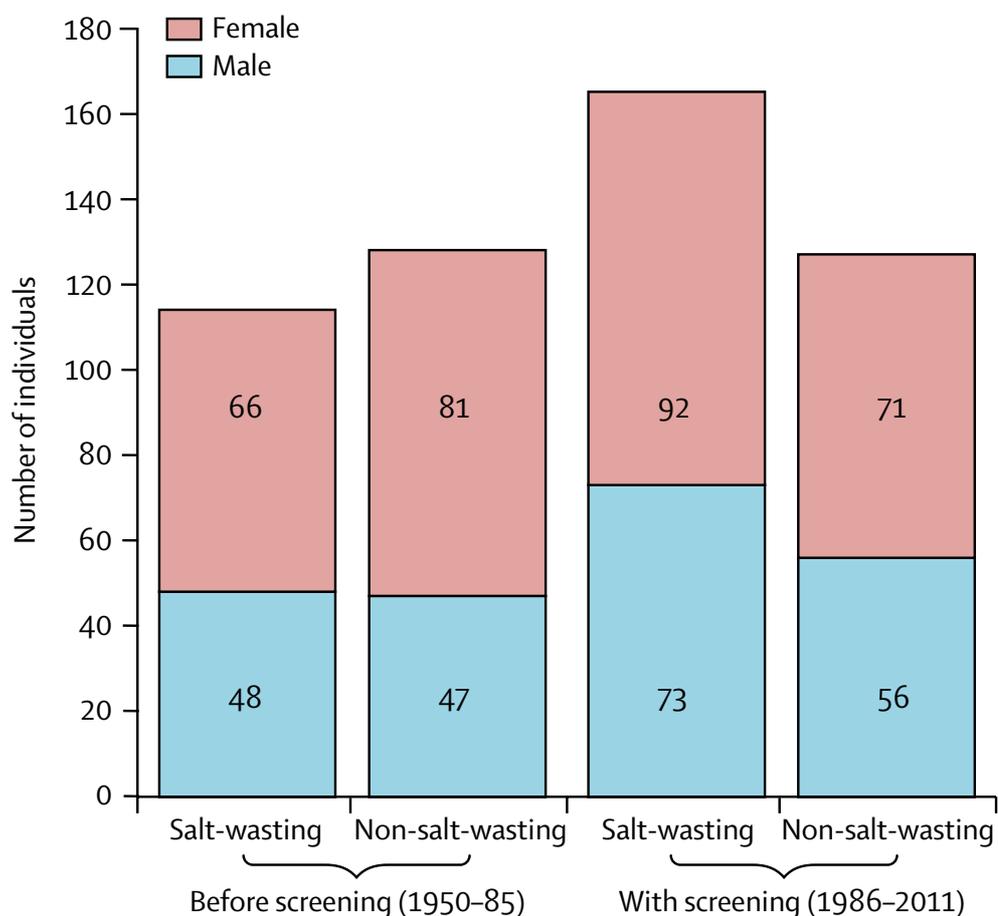
**Figure 6**



*A. The distribution of sex per decade and the apparent incidence per 100 000 live births per decade in Sweden during the last century. B. The distribution of SW CAH, SV CAH and NC CAH per decade. C. The distribution of severity of CAH based on the genetic or clinical diagnosis. Clinical SW and SV denote patients with a known severity of disease, but no information on genotype. NC denotes cases with a known mutation causing NC CAH and patients clinically classified as NC CAH. Neonatal screening for CAH was introduced in Sweden in 1986.*

Neonatal screening for CAH was implemented in Sweden in 1986. The overall sex ratio before and after the introduction of screening did not change significantly. However, the proportion of SW CAH among affected subjects increased significantly (Figure 7) ( $P = 0.038$ ).

**Figure 7**



*The proportion of SW CAH and non-SW CAH before (1950–1986) and after (1986–2011) the introduction of screening for both sexes. The sex ratio did not differ after the introduction of screening, but the proportion of cases diagnosed with SW CAH increased ( $P = 0.038$ ).*

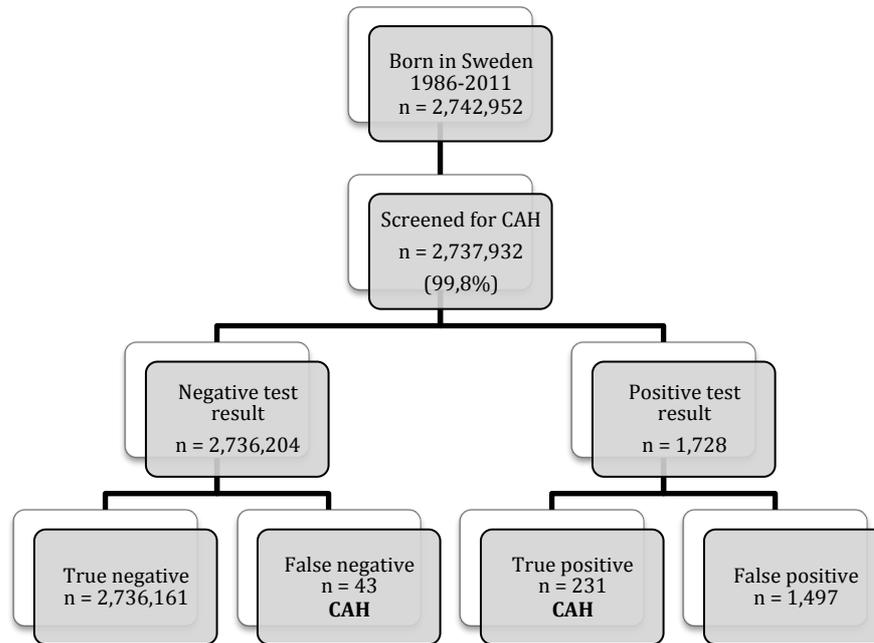
#### **5.4 NEONATAL SCREENING FOR CONGENITAL ADRENAL HYPERPLASIA IN SWEDEN**

Between 1986 and 2011, 2 742 944 infants were born alive in Sweden and 2 737 932 (99.8%) underwent neonatal screening for CAH. A total of 1728 tests were positive. Of these, 854 were from premature infants and 874 from infants born at term (Figure 8).

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**Figure 8**

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*Flow chart describing the study population in Paper IV.*

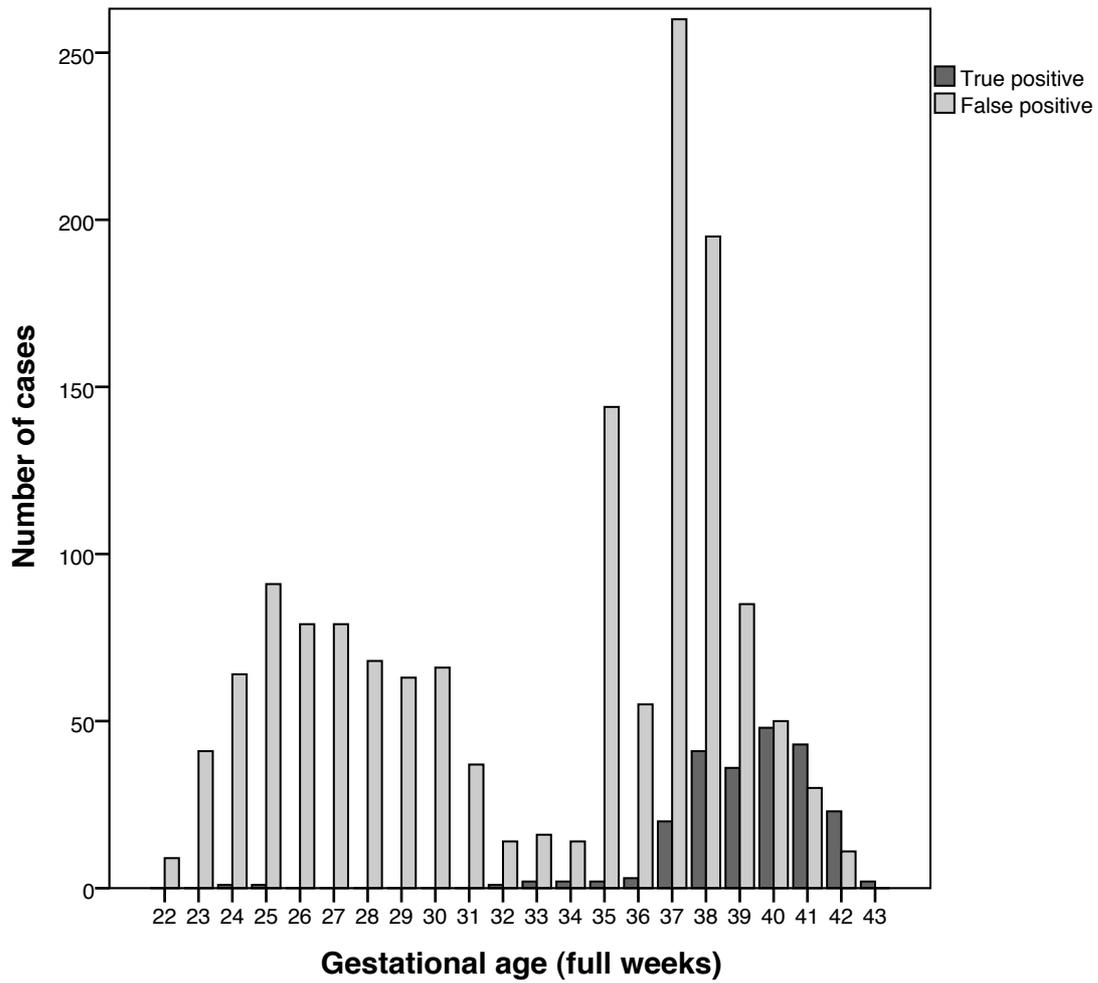
During the study period 274 subjects were diagnosed with CAH, 231 of which were detected in the screening. Thus, the overall sensitivity was 84.3%. There was no statistically significant difference between the sensitivity for males (87.2%) and that for females (81.6%) ( $P = 0.29$ ). The specificity was 99.9%.

There was no statistical skewness in the proportion of males in the CAH population compared to the general population, but more males (1063) had a positive test than females (665). The PPV was therefore higher in females (17.3%), compared to males (10.9%) ( $P = 0.001$ ).

The recall rate (proportion of positive tests) was lower in full-term infants (0.03%) than in pre-term infants (0.57%) ( $P < 0.001$ ). Furthermore, the PPV was higher in full-terms than in pre-terms, 25.1% and 1.4%, respectively ( $P < 0.001$ ). The PPV correlated positively with gestational age (correlation 0.98,  $P < 0.001$ ).

The number of false positives was high among extremely premature infants. Both true- and false-positive tests were low during weeks 32–34 and the proportion of true positives increased in infants born near term (Figure 9).

**Figure 9**



*Numbers of cases with a positive screening result per gestational age week.*

The sensitivity was higher in infants with severe forms of CAH compared to milder forms (Table 5).

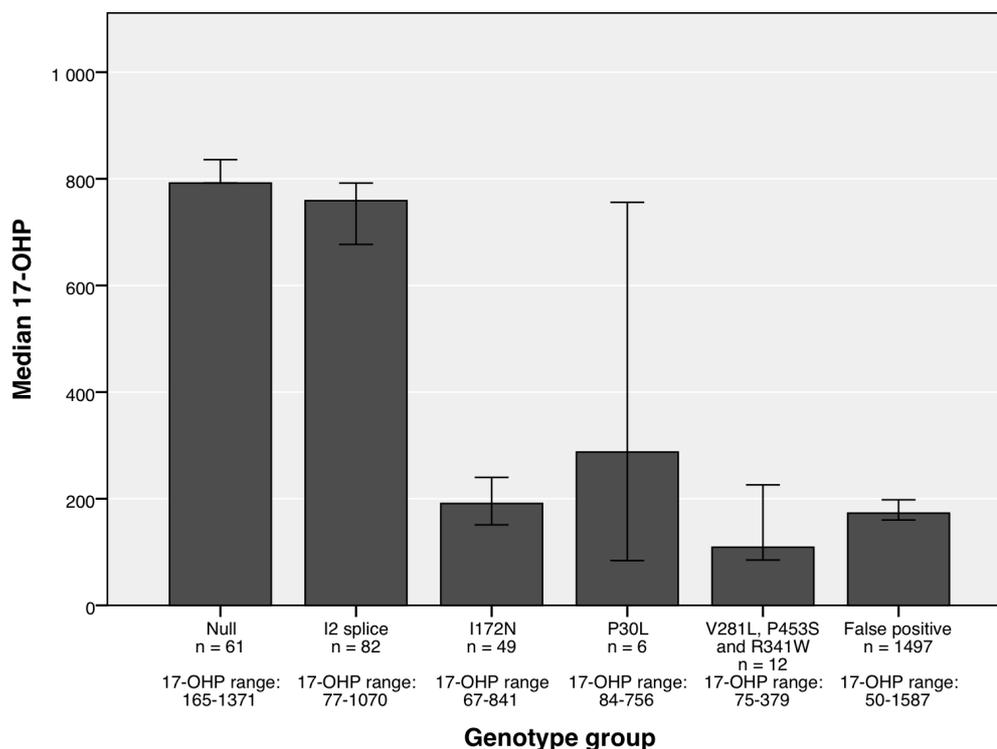
**Table 5**

<b>Genotype</b>	<b>Sensitivity</b>	<b>N</b>	<b>Median 17-OHP (95% CI)</b>
Null	100%	61	792 (792–836)
I2 splice	98.8% <sup>1</sup>	82	759 (677–792)
I172N	79.0%	49	191 (151–240)
P30L	85.7%	6	288 (84–756)
V281L, P453S and R341W	32.4%	12	109 (85–226)
False positives	-	1497	173 (160–198)
<b>Total (true positives)</b>	<b>84.3%</b>	<b>231<sup>2</sup></b>	<b>654 (524–740)</b>

*Sensitivity and 17-hydroxyprogesterone (17-OHP) for different genotype groups and false positives. <sup>1</sup> Excluding two cases that would have been picked up with the current cut-off level. <sup>2</sup> Seven cases had genotypes not possible to group, the genotype was unknown for 12 cases, one case had a 3 $\beta$ -hydroxysteroid dehydrogenase type II deficiency and one had a cytochrome P450 oxidoreductase deficiency.*

The 17-OHP level in the screening test correlated with the form of severity ( $P < 0.001$ ), but the range of the groups overlapped so it was not possible to determine the exact form of CAH from the screening sample alone (Figure 10).

**Figure 10**



*Median 17-hydroxyprogesterone (17-OHP) values for true-positive cases of congenital adrenal hyperplasia by genotype group. Bars represent medians and error bars 95% CIs for medians.*

Three of the 43 cases missed in the screening belonged to the I2 splice genotype group that is considered to be potentially salt wasting. However, none of these subjects showed any evidence of episodes of salt loss and probably represented rare, but previously known, cases of I2 splice where genotype and phenotype are not concordant. The other cases belonged to milder forms of the disease.

A review of all neonatal screening programmes published during the past 17 years revealed that the sensitivity correlated negatively with the published number of years of follow-up ( $P = 0.034$ , correlation  $-0.52$ ).

## **5.5 GROWTH AND TREATMENT IN CHILDREN WITH CONGENITAL ADRENAL HYPERPLASIA**

There were no significant differences in the average glucocorticoid dose in hydrocortisone equivalents between the sexes or different clinical or genotype groups, nor for any specific age

range (0–2 years, 2–11 years or 11–18 years) or overall from birth to 18 years of age. Doses were roughly within the recommended range of 10–15 mg hydrocortisone equivalents per m<sup>2</sup> BSA.

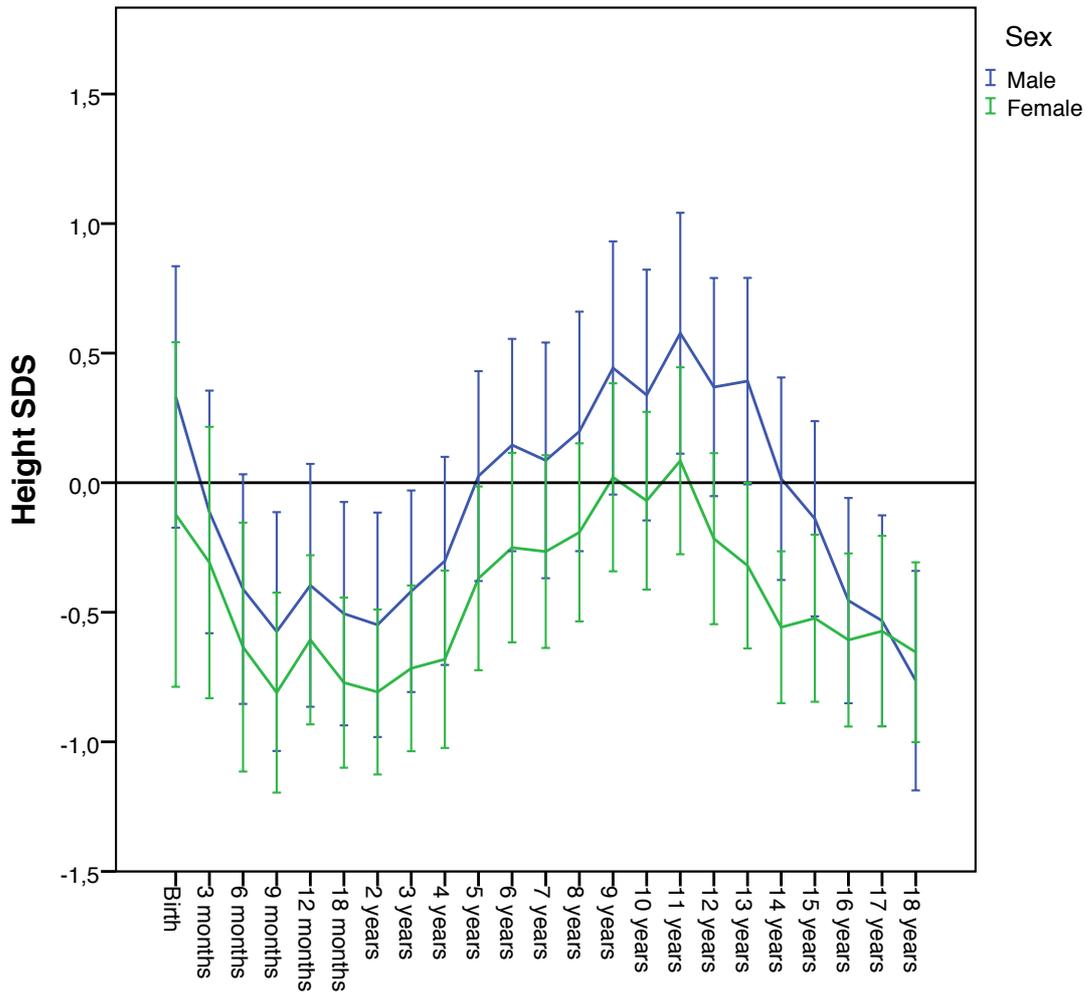
The glucocorticoid dose did not correlate with height velocity in any particular age range. Furthermore, the glucocorticoid dose in hydrocortisone equivalents for any specific age range (0–2 years, 2–11 years or 11–18 years) or overall from birth to 18 years of age did not correlate significantly with the corrected final height SDS or BMI at 18 years of age.

On the other hand, the corrected final height SDS was lower in subjects who had been treated with prednisolone,  $-1.1 \pm 1.0$ , than in those who had not been treated with prednisolone,  $-0.6 \pm 1.0$  ( $P = 0.047$ ).

In addition, BMI at 18 years of age was higher in the group that had been treated with prednisolone ( $25.3 \pm 4.7$ ) than in those not treated with prednisolone ( $23.4 \pm 4.5$ ) ( $P = 0.044$ ). BMI at 18 years of age correlated positively with the duration of the prednisolone treatment ( $P = 0.02$ , correlation 0.274), but not with the average dose per BSA per day ( $P = 0.129$ , correlation 0.326). For prednisolone-treated patients ( $n = 26$ ), the mean duration of treatment was 4.4 years (range 0.1 to 13.3 years) and therapy started at a mean age of 11.9 years (range 4.6 to 17.8 years). The mean dose during treatment was  $2.78 \pm 2.5$  mg per BSA per day. Prednisolone treatment was neither overrepresented in any genotype group ( $P = 0.462$ ), nor in males compared to females ( $P = 0.381$ ).

Children with early-diagnosed CAH exhibited impaired growth and weight development during infancy but displayed a catch-up growth in childhood (Figure 11).

**Figure 11**



*Height SDS from birth to 18 years. Please note that 3-month age intervals are shown from birth to 12 months, 6-months intervals from 12 months to 2 years and for each full year thereafter. Bars represent medians and error bars 95% CIs for medians.*

Complete growth charts were available for 75 patients, and only 11 of these did not show a pubertal growth spurt, defined as growth velocity of  $> 7$  cm/year. The presence of a pubertal growth spurt was not more prevalent in any *CYP21A2* genotype group or clinical group, sex or whether the child had been treated with prednisolone or not. Furthermore, it did not correlate with the average glucocorticoid dose at any specific age or overall.

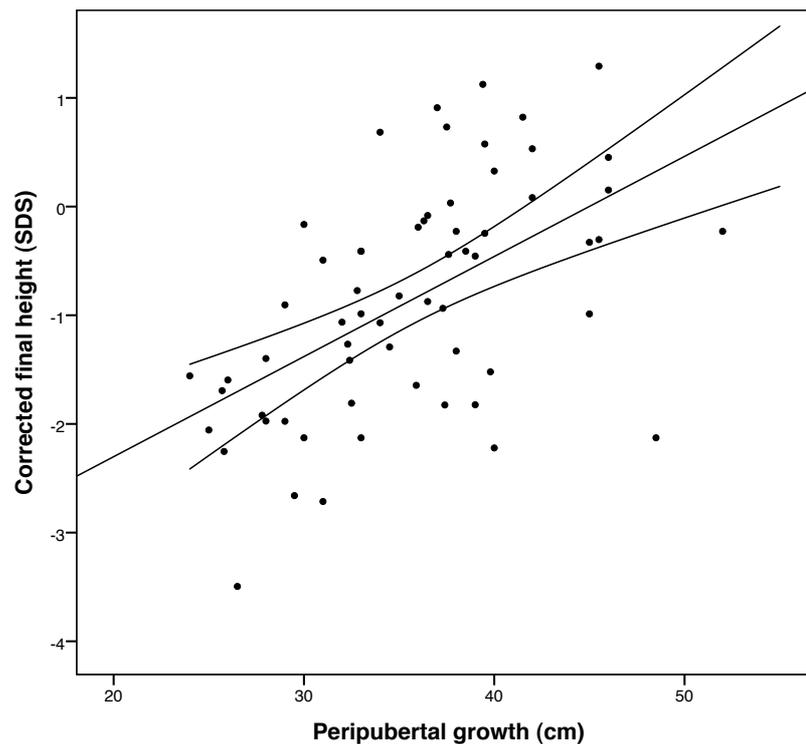
Onset of puberty was difficult to determine from the reported data. Instead, any growth after 8 years of age in females and 9 years of age in males was regarded as peripubertal. From these ages until final height, children with SV CAH grew more than children with SW CAH,  $39.3 \pm$

6.6 cm vs  $34.3 \pm 5.8$  cm ( $P = 0.012$ ). The magnitude of peripubertal growth correlated positively with the corrected final height SDS for both males ( $P = 0.009$ , correlation 0.495) and females ( $P < 0.001$ , correlation 0.664) (Figure 12) and negatively with BMI at 18 years of age ( $P = 0.002$ , correlation -0.398) (Figure 13). Early-diagnosed patients were more likely to exhibit a pubertal growth spurt than late-diagnosed patients ( $P < 0.001$ ).

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**Figure 12**

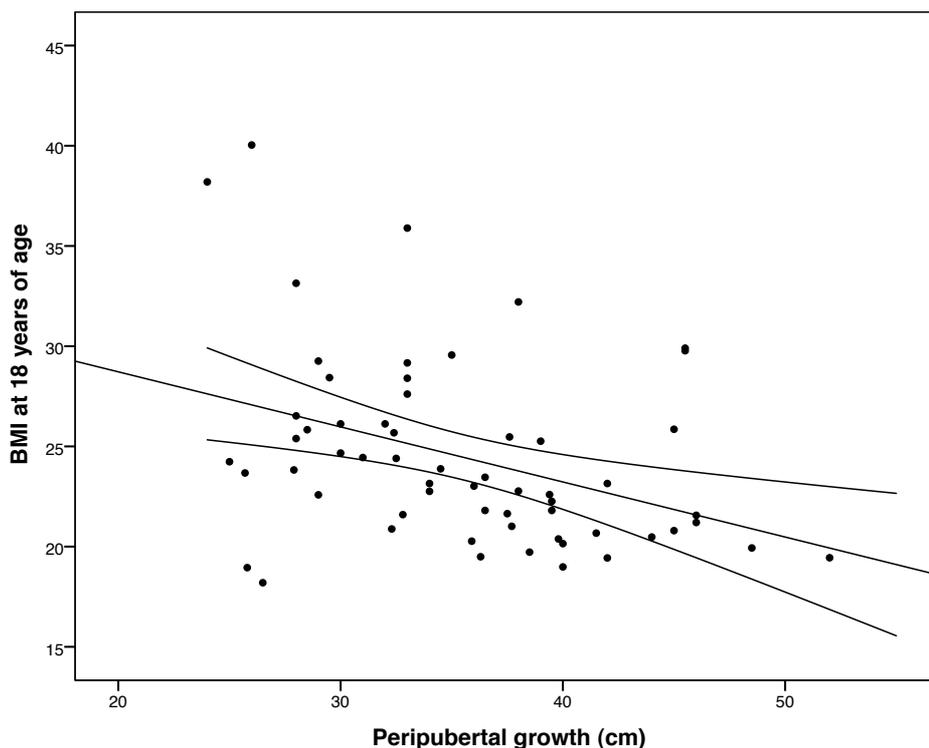
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*Height SDS corrected for parental heights in relation to peripubertal growth. The straight line represents the linear fit curve and the curved lines represent the 95% CI for the linear fit curve.*

**Figure 13**



*BMI at 18 years of age in relation to peripubertal growth. The straight line represents the linear fit curve and the curved lines represent the 95% CI for the linear fit curve.*

A growth spurt, defined as a height velocity of  $\geq 7$  cm per year after 8 and 9 years of age in girls and boys, respectively, was noted in 43 subjects. Ten of these subjects showed a reduced height velocity before the onset of a pubertal growth spurt, four of which had had a late diagnosis. Twenty-one subjects exhibited a biphasic growth pattern with a distinct separate period of increased growth velocity before the actual pubertal growth spurt. No pubertal spurt at all was seen in 11 subjects. Early-diagnosed patients were more likely to exhibit a pubertal growth spurt than late-diagnosed ones ( $P < 0.001$ ). The growth patterns, or the presence or absence of a pubertal growth spurt, were not associated with sex, clinical severity, genotype, or hydrocortisone or prednisolone treatment. However, among children who showed a biphasic growth curve, the glucocorticoid doses were increased after the period of the first growth acceleration by a mean of  $2.05 \pm 2.46$  mg HC-eq/BSA per day ( $P = 0.01$ ).

Although early-diagnosed patients were shorter than the normal population, their mean corrected final height SDS at 18 years of age was  $-0.78$  (CI 95%,  $-1.03$  to  $-0.54$ ) and thus was within -1

SD. No statistical difference in corrected final height SDS was seen between males (-0.81; CI 95%, -1.21 to -0.40) and females (-0.77; CI 95%, -1.08 to -0.45) ( $P = 0.737$ ).

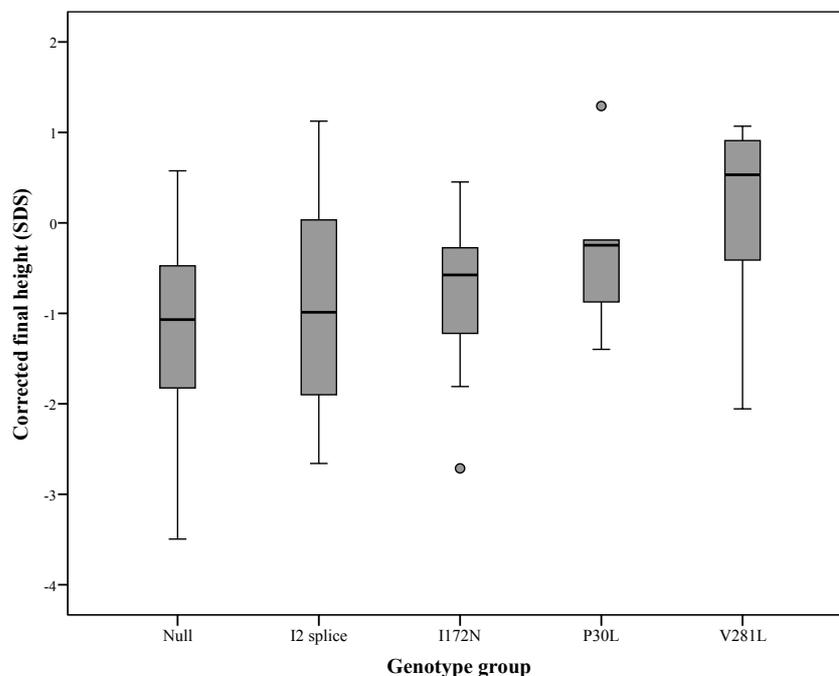
Final height was achieved (height velocity  $< 1$  cm/year) at  $16.5 \pm 1.3$  years of age in girls and at  $17.2 \pm 0.9$  years in boys. An older age at the achieved final height correlated positively with the corrected final height SDS ( $P = 0.011$ , correlation 0.32).

The corrected final height SDS correlated with genotype group in the sense that children with milder mutations achieved a higher final height than those with more severe mutations ( $P = 0.012$ , correlation 0.300) (Figure 14).

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**Figure 14**

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*Corrected final height (SDS) for different genotype groups.*

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Overweight and obesity were common in males; in fact, 52% of the males and 25% of the females had a BMI  $> 25$  kg/m<sup>2</sup> at 18 years of age ( $P = 0.031$ ). There were no significant differences in BMI between the genotype groups at 18 years of age. BMI at 18 years of age was higher in patients with an early start of treatment ( $24.6 \pm 4.7$  kg/m<sup>2</sup>) than in patients with a late diagnosis and start of treatment ( $21.5 \pm 2.9$ ) ( $P = 0.027$ ).

## 6 DISCUSSION

### 6.1 PAPER I: GESTATIONAL AGE CORRELATES TO GENOTYPE IN GIRLS WITH CYP21 DEFICIENCY

#### 6.1.1 Findings and interpretations

The results presented in Paper I suggest that gestational age correlates with the *CYP21A2* genotype in girls, but not in boys, with CAH. The length of pregnancy for boys with CAH was prolonged compared to the normal male population, although this comparison is accompanied by some methodological limitations as mentioned below.

The results are interesting since they suggest that androgens may be involved in prolonging gestation and hence may contribute to the difference in gestational age between the sexes seen in the normal population. However, not all investigators agree that there is a true difference in pregnancy length between the sexes, but that it is rather a systematic error when determining gestational length by ultrasonography (74). Taking these thoughts into account, it is biologically plausible for children with severe forms of CAH to be born post-term more often than milder forms. *Firstly*, in foetuses with CAH, there is increased production of 17-OHP. 17-OHP-caproate has been successfully used to delay threatening premature births in women at risk (248). *Secondly*, foetuses with CAH exhibit a decreased production of glucocorticoids. It has been shown that a surge in cortisol production, due to increased placental CRH production, precedes the onset of labour in normal births and may be one of the initiating processes of birth (249, 250). In our material, we saw a dose-response relationship between the severity of disease and the length of pregnancy for girls but not for boys with CAH. The hormonal disturbance in the adrenals is equal in foetuses with CAH, regardless of sex, regarding both androgen excess and cortisol and aldosterone deficiency. However, normal newborn males have high levels of testosterone due to testicular production. It may be that males with CAH do not differ as much regarding testosterone from healthy males as females with CAH do from healthy females. This hypothesis might then explain why the dose-response relationship is evident in females, but not in males.

Studying the length of pregnancy in CAH is interesting as pregnancy length is a predictor of birth weight. It has been shown by others that birth weight is increased in infants with CAH compared

to the normal population (80, 251), which may reflect a longer gestation rather than an increased foetal growth.

### 6.1.2 Methodological considerations

A total of 165 pregnancies were eligible for inclusion in the study and 109 were included for statistical analysis. Given the low incidence of CAH, this is a large cohort and thus provided the power to study the small differences in length of pregnancy. All included subjects were genotyped, rather than clinically classified. As shown before, the neonatal, and thus most probably also the prenatal, 17-OHP levels, which correspond to the level of adrenal insufficiency, correlate with the genotype (187).

This study suffers from some methodological limitations. *Firstly*, the study population consists of a cohort of patients with CAH whose data on gestational age at birth were collected in two different ways. For some patients, the information concerning length of pregnancy was collected from medical records and for some the information was obtained from the Guthrie card used in the screening. However, for the majority of the patients, both sources of information were available and they concurred with one another. *Secondly*, the normal population reference data were obtained from a separate study, meaning that we did not use a control group of our own. This poses a risk that the two groups, the CAH cohort and the normal population, were not measured equally. However, the data used to represent the control group in this study comprised children born contemporarily after full-term singleton pregnancies. The data had been obtained from the Medical Birth Registry in Sweden. This registry collects its data from medical files recorded by midwives, just as the information on the Guthrie cards and in the medical records. However, because of the methodological limitations, the results should be interpreted with caution. Furthermore, the retrospective observational design prevents us from additional investigations of such causative factors as hormonal samples from the foetus, amniotic fluid or the maternal circulation.

Despite the limitations associated with this study, a British research group reproduced the results shortly after its publication. Their report stated that post-term deliveries were more common in pregnancies with a foetus with SW CAH than in the normal population (252).

## **6.2 PAPER II: THE ROLE OF ANDROGENS IN FETAL GROWTH: OBSERVATIONAL STUDY IN TWO GENETIC MODELS OF DISORDERED ANDROGEN SIGNALLING**

### **6.2.1 Findings and interpretations**

We examined the birth weights in children with CAH and CAIS. Birth weight SDS did not differ from the reference for either boys or girls with CAH. Birth weight SDS in CAIS girls was higher than the reference for girls, but similar to the reference for boys.

The results are in contrast to those reported by Balsamo and co-workers (80) and Qazi and Thompson (251), both of whom demonstrated that birth weight was increased in CAH. In the work by Balsamo and co-workers (80), the CAH cohort, including children with gestational ages between 38 and 41 full weeks, was compared with a reference material consisting of girls born at week 39 and boys at week 40. These gestational ages corresponded to the mean gestational age for boys and girls with CAH, respectively, in their study. It is unclear why not all full-term infants from week 37 to 42 were included; the comparison between these groups may be inappropriate, as it does not really adjust for the differences in gestational age between the children with CAH and the controls. The work by Qazi and Thompson did not adjust for gestational age at all (251). Hence, the apparent difference in birth weight noted in these studies may be an effect of prolonged pregnancy for foetuses with CAH.

Girls with CAIS do not respond to androgens, as they have a completely abolished function of the AR. However, they have the 46,XY karyotype. Since their birth weight is higher than that of the reference girls, this suggests that other factors on the Y-chromosome, and not only androgen signalling, may influence foetal growth. Children with the 45,X0 karyotype (Turner's syndrome) that lack the Y-chromosome exhibit lower mean birth weights than other females (253), but no difference in birth weights between children with an increased number of Y-chromosomes and controls has been demonstrated (254, 255).

The results presented in Paper II do not provide any support for the hypothesis that there is a relationship between androgen signalling and birth weight. However, as it is decidedly impossible to statistically prove that there is no difference between groups, apart from showing the existence of such a difference, one has to interpret the results cautiously. Furthermore, the

study is observational and can only demonstrate the presence or absence of a relationship between androgen signalling and birth weight, and not causality.

In papers I and II CAH was used as a human model for studying the relationship between androgen excess and cortisol deficiency and length of pregnancy and birth weight.

### 6.2.2 Methodological considerations

The strength of this study is that it combines two different syndromes marked by disordered androgen signalling in an opposite fashion.

Gestational age at birth may be influenced by androgens and highly predicts birth weight. Therefore, birth weight SDS was adjusted for gestational age at birth. Birth weights were converted into SDS based on the reference material in the country in which each patient was born. This made the use of a specific control group less important.

## **6.3 PAPER III: ONE HUNDRED YEARS OF CONGENITAL ADRENAL HYPERPLASIA IN SWEDEN: A RETROSPECTIVE, POPULATION-BASED COHORT STUDY**

### 6.3.1 Findings and interpretations

The population of diagnosed cases of CAH during the past century was described. During the study period (1910 to 2011), the apparent incidence increased following the general improvements in diagnostics and care and particularly the introduction of an effective treatment for CAH. With the introduction of an effective treatment, it is likely that physicians increased their awareness concerning detecting cases of CAH, thereby leading to more patients being diagnosed. Furthermore, during the 1960s and 1970s in Sweden, national surveys of CAH probably increased awareness of the disease.

In this study it was noted that the female preponderance continued after the introduction of screening. However, before the introduction of screening, this can be attributed to higher survival among female cases with severe forms of disease, whereas, after the introduction of screening, the female preponderance is due to NC CAH being diagnosed more often in older girls and women than in men. In fact, the proportion of SW CAH increased in both sexes after the introduction of screening.

No difference in the sex ratio could be seen after the year 2000. This is in all probability because mild cases have not yet been diagnosed. Thus, there is a time lag before late onset cases contribute to the skewed sex ratio. This also explains why there is a reduction in the apparent incidence after the 1990s (Figure 6A).

### 6.3.2 Methodological considerations

The particular strengths of this study are that it is based on the whole population in Sweden and that it spans over a century. This reduces the problem of selection bias. The cohort is also quite large, especially for such a rare disease as CAH.

In order to calculate the apparent incidence for each decade, the exact number of individuals born each decade needs to be known. In Sweden there is the advantage of a centralised register that has collected this information since the 18<sup>th</sup> century (245). Furthermore, the sex ratio for live births is known since 1968 (246). In this study it was assumed that the overall sex ratio between 1968 and 2011 could also be applied from 1910 onwards.

The most obvious limitation of this study is that it reflects an historical cohort. Therefore, the finding that the proportion of SW CAH increased after the introduction of screening must be interpreted cautiously. It may be that the proportion of SW CAH cases would have increased independently of the introduction of neonatal screening from 1986 onwards. Furthermore, the inclusion of cases with CAH was based on all available sources. Although Sweden is a small country with centralised neonatal screening and *CYP21A2* investigations, there might be unknown cases that escaped inclusion in this study.

The classification of patients relied on both genotype and phenotype. However, for many cases, only one of these parameters was known. A small proportion of the included cases exhibited discordance between genotype and phenotype. This may be more common than could be investigated using the limited resource of data that was available for many patients.

## **6.4 PAPER IV: NATIONWIDE NEONATAL SCREENING FOR CONGENITAL ADRENAL HYPERPLASIA IN SWEDEN: A LONGITUDINAL PROSPECTIVE POPULATION-BASED STUDY COVERING 26 YEARS**

### **6.4.1 Findings and interpretations**

The neonatal screening programme for CAH is described in detail in Paper IV. The main finding is that the sensitivity for SW CAH is 100%. This finding is important and timely as a recent report published a considerably lower detection rate of SW CAH (256). In addition, the rationale for neonatal screening for CAH has been questioned owing to the high rate of false positives in premature infants (217). Also in the material reported in Paper IV the rate of false positives was high. However, screening in pre-terms may still be justified since it leads to earlier detection of patients and a second sample can easily be obtained from already admitted infants.

Apart from detection of potentially lethal forms of CAH, the secondary aim of the Swedish screening programme is to shorten the time of uncertain sex in virilised females. The present study did not address this issue, but a previous Swedish study has shown that the median time of uncertain sex in girls with CAH was lowered considerably from 23 to 3 days with the implementation of screening (187).

Paper IV included a comparison with recently reported screening programmes. The overall recall rate was low and the PPV was high compared to other studies. However, the overall sensitivity was low. This probably reflects the fact that, owing to a detailed collection of missed cases, a large proportion of late-diagnosed NC CAH was found. On including these cases in the calculation of sensitivity, the metrics dropped. In accordance with this, we found that the sensitivity in all compared studies correlated with the number of years of follow-up. The study described in Paper IV reported the longest follow-up yet published, and thus allowed identification of increasing numbers of false negative cases who presented with androgen symptoms as they grew older.

### **6.4.2 Methodological considerations**

This study is unique in many senses. It reports the longest follow-up of screening for CAH and continuously collected data on missed cases ever published. This allows a more accurate description of the true sensitivity of milder forms. Information concerning missed cases was reported by local clinicians to the laboratory. Furthermore, in the study described in Paper III, a

registry including all known cases of CAH was established. These cases were compared with patients detected through screening. Genotyping is a precise diagnostic tool and was carried out in 94.8% of cases, including all missed cases of CAH. The study was population-based, meaning that all (99.8%) infants born alive in Sweden during the study period were included.

Nevertheless, there are some limitations to the conclusions that can be drawn from the results. Despite the thorough procedures to collect missed cases, some cases of SW CAH might have been missed and escaped diagnosis completely; in which case, they would not be included in the data presented in Paper III either. If such cases exist, it would lower the sensitivity for severe forms.

Although the follow-up was carried out for a period of 26 years, newly screened and potentially missed very mild cases might not yet have been given an accurate diagnosis. Therefore, the actual sensitivity for mild forms of CAH may be even lower than reported here.

In spite of these drawbacks, the study is comprehensive and the approach to collecting data is methodical. A full guarantee against missing false negative cases in the follow-up of screening is obviously impossible.

## **6.5 PAPER V: GROWTH AND TREATMENT IN CONGENITAL ADRENAL HYPERPLASIA: A PROSPECTIVE OBSERVATIONAL STUDY FROM DIAGNOSIS TO FINAL HEIGHT**

### **6.5.1 Findings and interpretations**

The growth and treatment of a cohort of 80 out of 88 eligible children are described. Growth and weight development was impaired during infancy, but a catch-up in both growth and weight development was seen during childhood. This is in accord with previously reported data (94, 124). Others (257) have reported compromised pubertal growth in CAH. Since we could not uniformly state the exact onset of puberty in this cohort, it is not possible to make direct comparisons between the material in Paper V and those results. However, we could see that the size of the peripubertal growth in both males and females with CAH correlated positively with the corrected final height SDS and that an absence of the pubertal growth spurt was seen in only 11 out of 75 patients with complete growth charts.

The age at which children with CAH actually achieve their final height has not been reported before. In this material, girls grew until a mean of 16.5 years of age and boys until 17.2 years. It has previously been reported that the onset of the pubertal growth spurt is earlier in children with CAH than in the normal population (139). It is therefore somewhat surprising that the children in this cohort had continued growth for this long. In fact, the age at the achieved final height correlated positively with the corrected final height, suggesting that allowing for continued growth is both possible and important in children with CAH in order to avoid short adult stature.

Interestingly, the average glucocorticoid dose between 0 and 18 years of age did not correlate with the corrected final height SDS. Similarly, there were no correlations between the average dose during 0–2 years, 2–11 years or 11–18 years with the corrected final height. Since doses were roughly within the recommendations of 10–15 mg hydrocortisone equivalents per m<sup>2</sup> BSA per day in all groups of children, it may be that the differences were small or they were all optimally treated as to their biological need. However, the group of children who had been treated with the addition of prednisolone had a shorter corrected final height SDS ( $-1.1 \pm 1.0$ ) than those who had not been treated with prednisolone ( $-0.60 \pm 1.0$ ) ( $P = 0.047$ ). Furthermore, BMI at 18 years of age was higher in the prednisolone-treated group,  $25.3 \pm 4.7$ , compared to  $23.4 \pm 4.5$  ( $P = 0.044$ ), and BMI correlated with the duration of prednisolone treatment ( $P = 0.02$ , correlation 0.274), but not the average dose during treatment ( $P = 0.129$ , correlation 0.326). This suggests that prednisolone may be harmful to both growth and weight development and is in accord with previous results (131, 136). However, conclusions need to be drawn cautiously as this observational study does not allow analyses of causality. No randomisation concerning treatment was carried out and it cannot be ruled out that other factors, rather than prednisolone, influenced the higher BMI and shorter stature in this group of patients. It is, however, worth pointing out that prednisolone treatment was not more prevalent in any clinical or genotype group, or in males compared to females.

## 6.5.2 Methodological considerations

This study was designed to include children born or diagnosed with CAH between January 1989 and December 1994. In fact, all parents of children eligible for the study gave their informed consent for inclusion. Only eight patients were excluded from the statistical analysis, only four of which were due to loss to follow-up. Thus, the risk of a selection bias can be regarded as small.

The local clinician reported the data continuously. Every reported auxological measurement was plotted to produce a growth chart and the extrapolated value for the pre-specified ages was read off. The data concerning growth and weight development are therefore very reliable.

Similarly, every change in dose was recorded and the average glucocorticoid dose was calculated for the pre-specified age intervals. Since changes in doses are more radical during the first year of life, these age intervals were smaller at that age. This method of calculating the doses is time-consuming, but it gives a detailed description of the doses used in each patient. In addition, the glucocorticoids used in this study were converted into hydrocortisone equivalents. Generally, the conversion factors between different glucocorticoids are based on anti-inflammatory action or affinity to the glucocorticoid receptor (182). However, their growth-inhibitory action is not as well established. It may therefore be that hydrocortisone equivalents as calculated in this study do not actually correspond to the negative effect on growth exerted by the different drugs used. Similarly, different glucocorticoids may affect the appetite and the development of obesity differently than could be expected from their anti-inflammatory effects.

Each clinician oversaw only a few of the patients included in this study. The benefit is that it was possible to compare different strategies of treatment, such as the use of prednisolone. However, the risk for subjective decisions concerning treatment and individual evaluations of clinical findings, such as the onset of puberty, is inevitable. In this cohort, the onset of puberty was not defined uniformly and different clinicians surveyed its onset more or less scrupulously. It is therefore impossible to provide data on, for example, growth from the first sign of central puberty to final height.

The main objective of the study was to compare growth and BMI in different clinical and genetic forms of severity of CAH, as well as for different strategies of treatment, and therefore no control group was employed. Comparisons with the normal population on final height and BMI were based on one-sample tests of the height and weight SDS. There are two concerns regarding this method. *Firstly*, the method is vulnerable to secular trend and methodological differences in collecting the data. *Secondly*, one-sample tests are not statistically designed for repeated measures and there is an increased risk of type I errors.

As expected from previous studies concerning the distribution of forms of severity of CAH in Sweden (24, 33), the number of patients with NC CAH in this study was low. Therefore, this

group of patients had to be omitted from some statistical analyses and the risk of type II errors must therefore be regarded as increased.

As the study was observational, it is not possible to draw conclusions concerning causality.

## 7 CONCLUSIONS

This thesis describes five different studies designed to answer the pre-specified hypotheses.

The results from Paper I suggest that length of pregnancy may be prolonged in girls with severe forms of CAH. Another group later found that prolonged length of pregnancy was more common in children with CAH than in controls (252). Together with the findings that male foetuses have a slightly prolonged gestation (74), this may suggest that androgens, rather than the cortisol deficiency also seen in CAH, may be responsible for prolonging pregnancy.

Paper II addresses foetal growth and androgen signalling. Although it is notoriously statistically impossible to prove the absence of a difference, our results from two different syndromes, which in this respect may be said to mirror each other, suggest that androgens do not affect foetal growth. Children with CAH did not have increased birth weights and, furthermore, girls with CAIS had mean birth weights comparable to those of normal males, which is in accord with their genotype rather than their phenotype.

The historical medical improvements in diagnosis and care of patients with CAH over time were described in Paper III. We could see that the apparent incidence increased after treatment was available in 1950. There was a steep rise in the apparent incidence during the 1960s and 1970s. The exact reason for this is hard to state in this historical material but during the time paediatric endocrinology as a sub-speciality was developed in Sweden. Furthermore, the knowledge of an effective treatment may have encouraged physicians to detect cases.

The effects of neonatal screening were addressed in Paper III and described in Paper IV. The screening programme proved to be effective in detecting the potentially lethal SW CAH and the introduction of screening increased the detected incidence of SW CAH in both boys and girls.

The overall dose of hydrocortisone equivalent glucocorticoids in children with CAH in Sweden was within the recommended dose interval. The overall dose could not be correlated to the final height; however, the use of prednisolone was associated with both a compromised final height and a higher BMI at 18 years of age. It is probable that the hydrocortisone doses did not differ enough between subjects for us to be able to detect a correlation with final height and BMI, or the number of participants was too small. Genotype correlated with the final height in children with CAH.

## 8 CLINICAL IMPLICATIONS

The results in this thesis may directly influence the care and treatment for patients with CAH. The main findings concern screening and growth.

Screening for CAH is effective in detecting SW CAH and increases its incidence, saving lives in boys as well as in girls. The false positive rate can be kept acceptably low without losing in sensitivity for SW cases. The 17-OHP level in the screening gives a good indication of the severity of disease of the child which is a clinically useful information. A substantial proportion of all NC CAH cases are however not detected in the screening, but are diagnosed based on hyperandrogenic symptoms and signs during childhood, adolescence and even in adult life.

The observational study concerning growth and treatment in CAH described in Paper V suggests that prednisolone should be avoided in growing subjects with CAH and that special attention should be paid to growth in children with the severe forms.

This thesis also includes studies using CAH as a model system for prenatal androgen exposure, and the studies were designed to investigate important physiological issues, such as the influence of androgens on the length of pregnancy and birth weight. Although Paper I indicates that the degree of severity may statistically significant influence gestational age at birth in females with CAH, the differences between the groups *per se* are not clinically relevant. But the differences suggest that foetal androgen or 17-OHP over production may influence the timing of birth and may thus be important from a biological perspective. Moreover, the results from Paper II where birth weights were studied in two separate models of disordered androgen signalling do not suggest that androgens influence prenatal growth. This is in contrast to previous results concerning birth weights in CAH but is in accordance with the notion that androgens do not influence early postnatal growth, which is often seen as a continuation of foetal growth.

## 9 SUGGESTIONS FOR FUTURE RESEARCH

The patients included in Paper III are included in the Swedish CAH registry. Further epidemiological investigations of this cohort of 606 patients with CAH are initiated. Sweden is unique in that it holds records for the whole population concerning pregnancies and births dating from 1973. Within this database, it is possible to anonymise and retrospectively compare this cohort with the entire population since data has been collected uniformly for cases and controls.

Perinatal factors were collected for the study described in Paper V and we could see that 8% of mothers who gave birth to infants later diagnosed with CAH suffered from preeclampsia. The overall incidence of preeclampsia in the Swedish population is about 3% (258). However, data were not collected uniformly and did not allow statistical comparisons with the normal population. We now have a possibility to conduct epidemiological studies and investigate the incidence of preeclampsia in women who are obligate carriers of *CYP21A2* mutations, and to compare this to the normal population. This relationship has not been described previously and, it would be interesting to investigate if preeclampsia is more prevalent in these pregnancies since preeclampsia is more frequent in mothers who bear a male foetus (258), and higher levels of circulating androgens have been reported in mothers with preeclampsia (259).

Furthermore, because we have this large registry, it is possible for us to describe reproductive outcomes in women who themselves have CAH. In pregnancies of mothers affected by CAH, gestational diabetes may be more common (132) and elective caesarean section is recommended in women with prior genitoplasty (15). It has been suggested that women with CAH may have more android pelvic characteristics, suggesting an increased risk for cephalo-pelvic disproportion and thus labour dystocia (15). With this large series and uniformity in the collection of data, it is possible for us to provide a more definite epidemiological description of these and other reproductive outcomes in women with CAH.

Since fertility is reduced in women with CAH we have also initiated a study in which we compare the expression of endometrial steroid hormone receptors between women with CAH, PCOS and healthy controls. The expressions of steroid hormone receptors are known to be associated with endometrial receptivity for the early embryo. The results from this study may cast new light on reproductive pathophysiology in CAH.

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