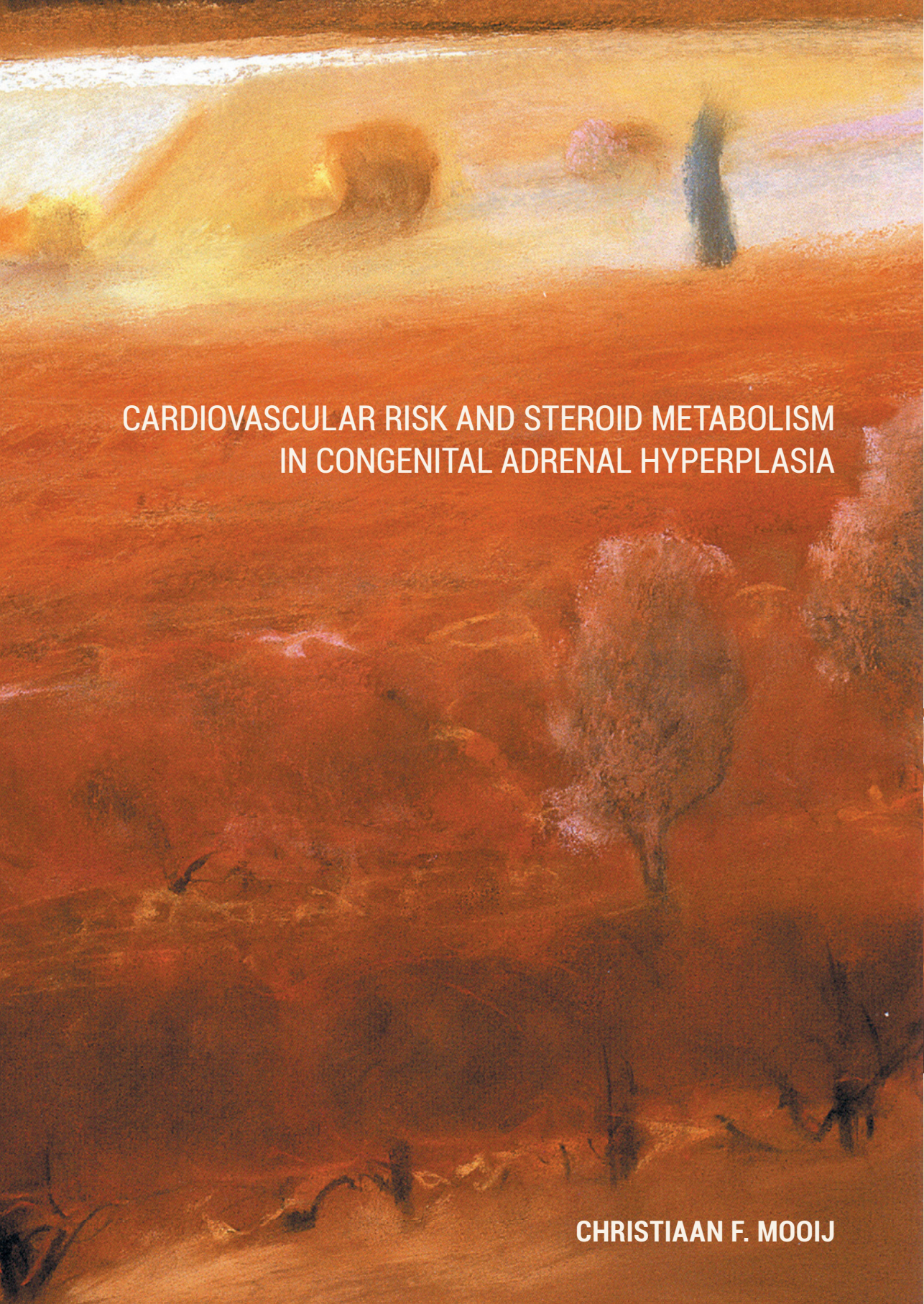

CARDIOVASCULAR RISK AND STEROID METABOLISM IN CONGENITAL ADRENAL HYPERPLASIA

CHRISTIAAN F. MOOIJ

CARDIOVASCULAR RISK AND STEROID METABOLISM IN CONGENITAL ADRENAL HYPERPLASIA

1. Bij patiënten met congenitale bijnierhyperplasie kunnen op de kinderleeftijd al ongunstige veranderingen in het cardiovasculaire risicoprofiel worden gezien. *(Dit proefschrift)*
2. Een verhoogde body mass index, toegenomen percentage lichaamsvet, verhoogde bloeddruk en insulineresistentie kunnen worden veroorzaakt door zowel de medicamenteuze behandeling als ook het hyperandrogenisme bij patiënten met congenitale bijnierhyperplasie. *(Dit proefschrift)*
3. Een verhoogde body mass index speelt een cruciale rol in het ontwikkelen van een ongunstig cardiovasculair risicoprofiel bij (pediatrische) patiënten met congenitale bijnierhyperplasie door 21-hydroxylase deficiëntie. *(Dit proefschrift)*
4. Reguliere evaluatie van minimaal bloeddruk en body mass index vanaf de kinderleeftijd zal leiden tot vroegtijdige detectie van ongunstige veranderingen in het cardiovasculaire risicoprofiel van patiënten met congenitale bijnierhyperplasie. *(Dit proefschrift)*
5. Vanwege verhoogde concentraties van steroidprecursors met glucocorticoïde activiteit voor het enzymatische blok, met name 21-deoxycortisol, is bij patiënten met milde 21-hydroxylase deficiëntie wellicht geen behandeling met glucocorticoïden noodzakelijk. *(Dit proefschrift)*
6. Reductie van cardiovasculaire morbiditeit bij patiënten met congenitale bijnierhyperplasie, door vroegtijdig signaleren en behandelen van ongunstige veranderingen in het cardiovasculaire risicoprofiel, zal resulteren in een verbetering van levenskwaliteit en verlaging van de zorgkosten. *(gebaseerd op Hummel S et al. Clin Endocrinol 2016; 85:361-368)*
7. Als je een speler ziet sprinten is hij te laat vertrokken. *(Johan Cruyff)*
8. Succes is een combinatie van geluk en kwaliteiten. *(Christiaan Mooij)*

Christiaan F. Mooij
Nijmegen, 12 oktober 2018

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Quality without results is pointless. Results without quality is boring.

- Johan Cruyff -

To Johannes

Contents

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1.1

Congenital adrenal hyperplasia – etiology and clinical aspects

Text is partially based on sections from "Disorders of Adrenal Steroidogenesis: Impact on Gonadal Function and Sex Development. Mooij CF, van Herwaarden AE, Claahsen-van der Grinten HL. Pediatric Endocrinology Reviews. 2016. 14(2):109-128"

Congenital adrenal hyperplasia (CAH) is a group of rare diseases affecting adrenal steroidogenesis (see chapter 1.3; figure 1) due to a defect in one of the enzymes involved in steroid synthesis. Disorders in adrenal steroidogenesis may affect glucocorticoid synthesis, mineralocorticoid synthesis and androgen synthesis leading to either lack of adrenal (and gonadal) androgen production or excess of androgen production (causing 46,XX Disorder of Sexual Differentiation (DSD)). In more than 90% of the cases CAH is caused by 21-hydroxylase (also termed CYP21A2 or P450c21) deficiency (21OHD). Deficiencies of e.g. 11-beta-hydroxylase, 3-beta-hydroxysteroid dehydrogenase, 17-alpha-hydroxylase can also cause disorders of adrenal steroidogenesis. In this thesis we discuss CAH due to 21OHD unless stated otherwise.

CAH is one of the most common inherited autosomal recessive disorders. Compound heterozygous or homozygous mutations in the genes encoding the enzymes involved in adrenal steroidogenesis are causative for the disorders. The clinical phenotype in these disorders depends on the mutation with the highest percentage of residual enzymatic activity with a more severe phenotype in patients with lower residual enzymatic activity. In all cases of CAH, the basic defect leads to a lack of cortisol synthesis resulting in stimulation and hyperplasia of the adrenals by ACTH and accumulation of the precursors before the enzymatic block due to the lack of negative feedback. Some of these precursors are diverted to the synthesis of androgens, causing ambiguous genitalia in newborn females and rapid postnatal growth in both sexes.

As an introduction we will briefly discuss the genetic background, the clinical phenotype, the diagnostic workup and current treatment of 21OHD.

21-hydroxylase deficiency

The defect in steroidogenesis

The *CYP21A2* gene is located on chromosome 6p21.3 consisting of 10 exons. Mutations in the *CYP21A2* gene lead to insufficient synthesis of the enzyme 21-hydroxylase. 21-hydroxylase converts progesterone to 11-deoxycorticosterone (DOC) in the zona glomerulosa and 17-OH progesterone (17OHP) to 11-deoxycortisol in the zona fasciulata of the adrenal. In case of complete absence of 21-hydroxylase these conversions are not facilitated leading to cortisol and aldosterone deficiency. Low (fetal) cortisol levels stimulate ACTH secretion *in utero* leading to adrenal hyperplasia and increased androgen production as the enzymes necessary for synthesis of adrenal androgens in the zona reticularis are not affected. Postnatally increased ACTH levels result in elevated levels of steroids before the enzymatic block: 17OHP and androstenedione. Figure 1 shows the effect of 21OHD on steroidogenesis schematically.

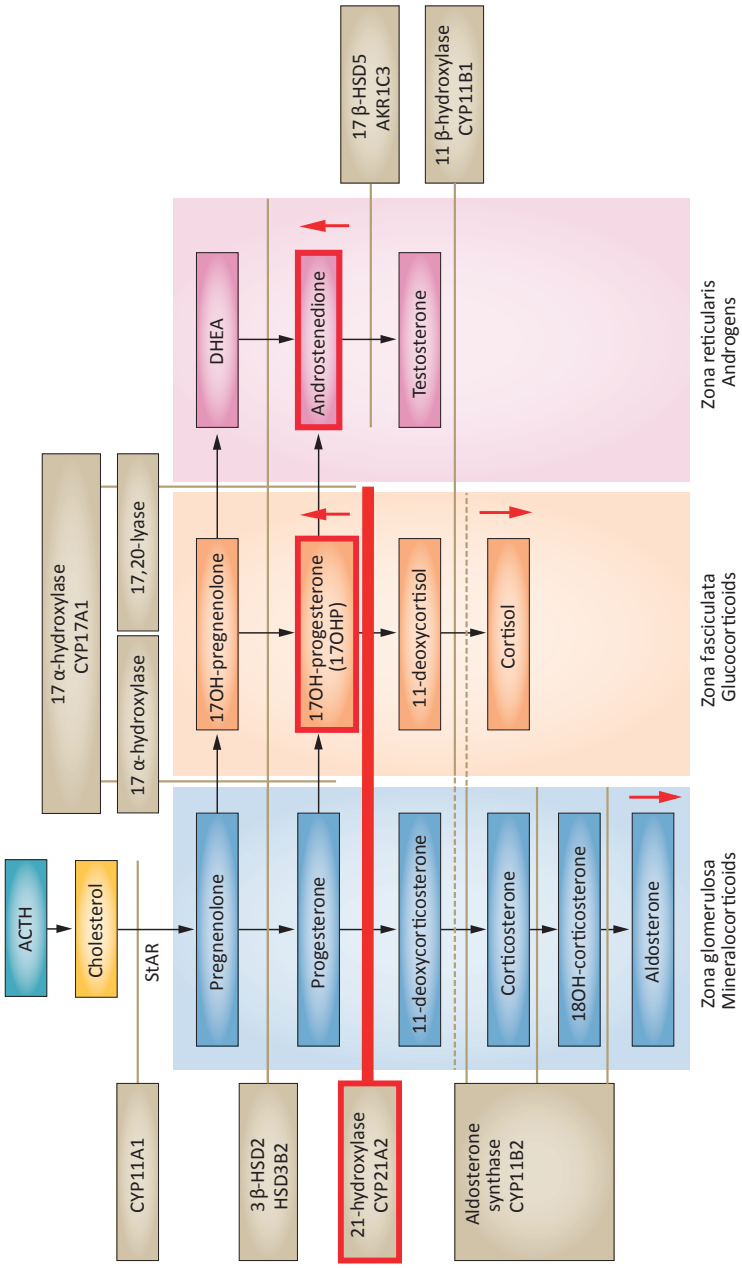

Figure 1 Schematic overview of CYP21A2 deficiency leading to impaired production of mineralocorticoids and glucocorticoids and increased production of adrenal androgens and precursors before the enzymatic block *(adapted from Han et al.[12] with permission)*.

The clinical phenotype of 21OHD is a continuum depending on the residual enzymatic activity. Traditionally, the clinical presentation of 21OHD is classified as classic salt-wasting (SW) CAH, classic simple virilizing (SV) CAH and non-classic (NC) CAH. Patients with the classic SW form have no residual enzymatic activity leading to severe salt losing typically after the first week of life and prenatal virilization of the female external genitalia (46,XX DSD). Patients with the classic SV form of CAH have a residual enzymatic activity of 1-2 % and usually have sufficient aldosterone production to prevent severe salt loss. The SV form leads to prenatal virilization of the female external genitalia and postnatal androgen excess in both sexes.[1,2] Classic 21OHD is detected in approximately 1 in 10.000 to 20.000 births in most populations, with an estimated carrier frequency of 1:55 based on newborn screening data.[3,4]

NC CAH is the mildest form of CAH and has a prevalence of about 0.1% in the general population.[5] In general NC CAH patients have a residual enzymatic activity of 20-50%. Clinical characteristics of NC CAH depend on the percentage of residual enzyme activity including premature pubarche and menstrual disturbances or mimic those found in polycystic ovary syndrome, but most male NC CAH patients are free of symptoms.[1,2,6]

The diagnostic workup of 21OHD patients

In several countries, such as The Netherlands, newborn screening, measuring 17OHP levels by immunoassay, has been introduced to screen for 21OHD to prevent severe salt wasting crisis in the neonatal period. In newly diagnosed CAH patients elevated ACTH and renin concentration are found as well as elevated concentrations of precursors 17OHP and androstenedione. Basal 17OHP values usually exceed 300 nmol/l in affected infants with the classic form of CAH, whereas the levels in unaffected newborns are below 3 nmol/l.[7] A cosyntropin stimulation test may be used to measure 17OHP and other elevated steroid precursors if basal 17OHP values do not prove or exclude the diagnosis of 21OHD. 21-deoxycortisol, a 11-hydroxylated product of 17OHP, has been described as a potential more sensitive marker for the diagnosis of 21OHD in premature neonates, NC patients and carriers.[8,9] Prenatal diagnosis of 21OHD is possible, and may be desirable if prenatal treatment with dexamethasone is considered in classic forms of 21OHD with virilization of the external genitalia. [10]

The treatment of 21OHD patients

Current treatment of CAH, caused by 21OHD, consists of lifelong replacement of glucocorticoids and, in cases with insufficient aldosterone production, also mineralocorticoids.[7] By treatment with glucocorticoids also suppression of adrenal androgen production can be achieved. However, in most patients supraphysiological doses of glucocorticoids are necessary to normalize androgen levels. Further treatment is

multidisciplinary, including surgical correction of ambiguous genitalia in affected female patients when desired.[7] Frequent follow-up of CAH patients is important to monitor mineralocorticoid and glucocorticoid replacement therapy. The follow up of CAH patients should also focus on long-term health problems that may be caused by androgen excess in CAH or by over-treatment or under-treatment (e.g. exogenous glucocorticoid excess). Therefore, evaluation of growth, reproductive health, cardiovascular and metabolic health and bone health should be incorporated in the follow up of CAH patients.[11] In Chapter 1.2b we will give more insights in long-term health consequences in young CAH patients.

CHAPTER 1.2

Cardiovascular and metabolic risk in congenital adrenal hyperplasia

Chapter 1.2a
Unfavorable trends in cardiovascular and metabolic risk in pediatric and adult patients with congenital adrenal hyperplasia?

Mooij CF, Kroese JM, Claahsen-van der Grinten HL, Tack CJ, Hermus AR.
Clinical Endocrinology. 2010; 73(2): 137-146

Summary

Context: As a result of the introduction of treatment with glucocorticoids and mineralocorticoids, now 60 years ago, congenital adrenal hyperplasia has become a lifelong chronic disease. Whether long-term treatment of the disease leads to long-term side effects remains unknown. In this respect, especially cardiovascular risk seems to be important.

Evidence synthesis: We reviewed the reported prevalence of conventional cardiovascular risk factors, i.e. obesity, insulin resistance, high blood pressure and dyslipidemia in patients with congenital adrenal hyperplasia. Overall, the studies suggest a tendency towards an increased body mass index and fat mass, the presence of insulin resistance and hypertension, although data are relatively scarce and obtained in heterogeneous populations.

Conclusions: Our findings suggest that adult CAH patients tend to have a cluster of metabolic risk factors, which are consistent with the metabolic syndrome. This notion may have consequences for the care for this group of patients.

Introduction

Congenital adrenal hyperplasia (CAH) is a disorder of adrenal steroidogenesis. Treatment with glucocorticoids and, if necessary, mineralocorticoids prevents adrenal crises and suppresses abnormal secretion of adrenal androgens. As a result of the introduction of treatment with glucocorticoids and mineralocorticoids, now 60 years ago, nearly all CAH patients currently reach adulthood. This renders it possible to unfold the clinical spectrum of CAH as a lifelong chronic disease, with the oldest salt-wasting patients now being in their 60s, and long-term consequences of therapy becoming increasingly important. Patients with CAH are at risk of developing signs and symptoms of iatrogenic Cushing's syndrome[1,2] as the therapeutic range of treatment with glucocorticoids is narrow and supraphysiological doses of glucocorticoids are used to suppress hyperandrogenism. As hypercortisolism is associated with cardiovascular morbidity,[3] it is suggested that patients with CAH may show an adverse cardiovascular risk profile.[4] Furthermore, other factors such as obesity and alterations in the leptin axis may contribute to the development of an unfavorable cardiovascular risk profile in CAH patients. In this review, we discuss cardiovascular risk factors in pediatric and adult CAH patients. Data reviewed in this paper are from patients suffering from CAH because of 21-hydroxylase deficiency unless stated otherwise.

Overview of CAH

In 95% of cases, CAH is caused by 21-hydroxylase deficiency.[2] Deficiency of 21-hydroxylase results in impaired adrenal synthesis of cortisol and often also of aldosterone, causing increased secretion of ACTH by the pituitary gland, adrenal hyperplasia and excessive production of adrenal androgens.

The symptoms of CAH depend on the degree of enzyme deficiency. Patients with the classic salt wasting (SW) type of CAH have no residual enzyme activity resulting in disturbed cortisol and aldosterone production typically leading to an Addisonian crisis 7–14 days after birth and prenatal virilization of the external genitalia in females. Patients with the classic simple virilizing (SV) type of CAH have a residual enzyme activity of 1–2% and are considered to have sufficient aldosterone production to prevent severe salt wasting. The simple virilizing form results in prenatal virilization in females and postnatal androgen excess in both sexes, when left untreated, leading to precocious pseudopuberty at a young age. The two classic forms of CAH have an incidence of approximately 1 in 15 000 live births worldwide.[5] The nonclassic or late onset form of CAH with a residual enzyme activity of 30–50% has a prevalence of 0.1% in the general white population,[6] a higher frequency being reported in Eastern European Jews (3–4%), and in Hispanics and Yugoslavs (1–2%).[6] Female patients may

be asymptomatic or are characterized by signs and symptoms of hyperandrogenism such as abnormalities of the menstrual cycle, acne, hirsutism and subfertility. Therefore, signs and symptoms in women with the late onset form of congenital adrenal hyperplasia are comparable with those seen in women with polycystic ovary syndrome.[2,7] Male patients are usually asymptomatic but occasionally, they present with subfertility caused by hypogonadotrophic hypogonadism because of suppression of gonadotrophins by elevated adrenal androgens.

Risk factors for atherosclerotic vascular disease

Atherosclerotic vascular disease is caused by a complex process, to which several risk factors contribute.[8] Traditional risk factors include age, male gender, familial predisposition, obesity, diabetes mellitus, dyslipidemia, hypertension, smoking and a sedentary lifestyle. In addition, several new nontraditional risk factors have been identified.[8] In the following part, some of these risk factors in CAH patients will be discussed in more detail.

Obesity

An increased fat mass and the presence of abdominal obesity are important factors in the development of cardiovascular disease. Many studies have investigated body mass index (BMI) in CAH patients and most,[9–18] but not all,[19,20] of them report an elevated BMI. Studies regarding body composition in patients with CAH are summarized in Table 1. Most studies used dual X-ray absorptiometry (DXA) scans to evaluate body composition, and one study used skinfold measurements.

In a cross-sectional study, Stikkelbroeck *et al.*[9] found an elevated BMI in young male and female adult CAH patients (*n* = 30, aged 17–25 years) compared with healthy controls. Increase in BMI was caused by an increase in fat mass. Fat mass adjusted for height and relative fat mass (i.e. fat mass divided by total body mass) was significantly higher in both males and females. Body fat distribution (measured by DXA) did not differ between patients and controls. Falhammar *et al.*[10] found a higher BMI in female CAH patients aged 30 years or older (*n* = 34) compared with controls. The elevated BMI in female CAH patients was not accompanied by elevation of total fat mass as found by Stikkelbroeck *et al.*[9] Falhammar *et al.*[10] did not find differences either in BMI or in body composition in female CAH patients younger than 30 years of age (*n* = 27) compared with controls. Bachelot *et al.*[11] reported a high BMI in adult male and female CAH patients (*n* = 45), but BMI in CAH patients was not compared with control subjects. BMI did not differ between the various clinical forms of CAH. BMI also showed no correlation with duration of treatment, dosage of hydrocortisone and 17alpha-hydroxyprogesterone (17-OHP) levels.

In a study by Hagenfeldt *et al.*[12] a significantly higher weight, BMI and absolute amount of body fat were found, but the fat/lean body mass ratio was not different between adult female CAH patients and age-matched healthy reference subjects. Cameron *et al.*[19] and Christiansen *et al.*[21] described an increased fat mass in male but not in female CAH patients. However, because different parameters were used in the different studies, i.e. relative fat mass and fat mass adjusted for height by Stikkelbroeck *et al.*,[9] fat/lean ratio by Cameron *et al.*[19] and fat mass percentage by Christiansen *et al.*,[21] it is difficult to compare these results.

Two studies have described BMI and body composition in children with CAH. Cornean *et al.*[13] reported an increased BMI in 21-hydroxylase-deficient children in a retrospective study. 'Adiposity rebound', defined as the age at which the decrease in BMI reverses, was assessed in a subset of 13 patients (nine boys, four girls) in which longitudinal data were available from birth. 'Adiposity rebound' took place at a mean age of 1.74 years in the patients compared with 5.5 years for controls from the normal UK population. Early 'adiposity rebound' is of importance as it increases the risk for the development of obesity in adolescence.[22] The increment in BMI was attributed to an increase in fat mass, skinfold thickness increasing significantly between 2.5 and 5.5 years. A limitation of this study is that data were collected retrospectively and that skinfold measurements to determine body composition were used. Völkl *et al.*[14] evaluated BMI in children in a retrospective cross-sectional study of 89 CAH patients (aged 0.2–17.9 years). BMI SDS of the whole group was significantly elevated. In addition, a significantly higher frequency of obesity, defined as BMI SDS >2.0, was observed. Glucocorticoid dosage was positively correlated with BMI SDS, although there was no significant difference in glucocorticoid and mineralocorticoid dosage between patients with a BMI SDS of >2.0 and <2.0.

In summary, CAH is associated with an increase in BMI and an increase in body fat in both adult and pediatric patients.[9,10,12,13,19,21] Further investigations are needed to define the time of onset of obesity especially in children with CAH. The cause of the excessive increase in body weight remains unclear, but several factors may contribute. It is suspected that obesity in CAH patients is related to glucocorticoid treatment. Obesity is reported in patients treated with both 'physiological' and supraphysiological doses of glucocorticoids. Furthermore, adrenomedullary dysfunction with decreased secretion of adrenaline may play a role in the development of obesity.[23] Catecholamines, such as adrenaline, contribute to lipolysis and inhibit insulin secretion, thereby preventing an increase in fat mass.[24] The decreased secretion of adrenaline in CAH is the result of prenatal adrenomedullary maldevelopment,[23] caused by low intra-adrenal levels of glucocorticoids.[25] Hypogonadism in men and hyperandrogenism in women are also associated with the development of obesity in CAH patients.[26] Another possible explanation for the presence of obesity in CAH patients is an altered leptin axis.[17]

Leptin is known to play a role in the regulation of body weight, with an increased leptin level suppressing appetite. The fact that most obese persons exhibit high leptin concentrations is often explained by the hypothesis that obesity is a state of leptin 'resistance'.[27] Changes in the leptin axis, for example a decrease in leptin receptor levels, may play a role in the development of obesity.[28–31] Both glucocorticoids and insulin increase leptin concentrations.[32,33] In contrast, androgens are known to decrease leptin concentrations.[34,35] Therefore, in CAH patients, an altered leptin axis may play a role in the development of obesity.

A decrease in soluble leptin receptor (sOB-R) serum levels has been described in CAH patients (aged 5.6–19.6 years, *n* = 51).[17] This observation suggests that also membrane leptin receptors are decreased leading to a state of 'leptin resistance'. In this study, sOB-R:leptin ratios and serum leptin levels were significantly correlated with insulin levels and insulin sensitivity. Serum leptin levels were not different in CAH patients compared with matched controls. In other studies, serum leptin levels have been described to be elevated[36] or normal[37] in CAH patients. Leptin levels are known to be correlated with fat mass. Two studies showed a positive correlation between BMI and serum leptin levels.[17,37] A positive correlation between the percentage of body fat and leptin levels was noted by Saygili *et al.*[37]

Dyslipidemia

Only five studies have reported on lipid profiles in CAH patients.[10,11,38–40] Bayraktar *et al.*[38] studied untreated adult female nonclassic CAH patients (*n* = 50, aged 22 ± 2.91 years) and found that lipid profiles were not different from controls (i.e. total cholesterol, 4.6 ± 0.83 *vs.* 4.7 ± 0.77 mmol/l; HDL-cholesterol, 1.50 ± 0.28 *vs*. 1.55 ± 0.26 mmol/l; LDL-cholesterol, 2.56 ± 0.56 *vs*. 2.58 ± 0.58 mmol/l; and triglycerides, 1.14 ± 0.13 *vs.* 1.01 ± 0.12 mmol/l). Sartorato *et al.*[39] found similar results in male and female adult patients with the classic form of CAH caused by 21-hydroxylase deficiency, all treated with glucocorticoids (*n* = 19, aged 28 ± 3.5 years). Lipid profiles were not significantly different compared with healthy controls (total cholesterol, 168 ± 49 *vs*. 189 ± 36 mg/dl; HDL-cholesterol, 65 ± 15 *vs*. 61 ± 32 mg/dl; and triglycerides, 83 ± 37 *vs*. 84 ± 56 mg/dl). Falhammar *et al.*[10] evaluated serum lipids in treated adult females with classic and nonclassic CAH caused by 21-hydroxylase deficiency (*n* = 61). Patients were divided into two groups: patients younger than 30 years and patients 30 years or older. All patients received treatment with prednisolone or hydrocortisone. Most patients (50 of 61) also received fludrocortisone. Total cholesterol, HDL-cholesterol, LDL-cholesterol, HDL to LDL ratio and triglycerides did not differ between younger patients and controls. Patients of 30 years of age or older had higher HDL to LDL ratios (*P* = 0.03) and a tendency towards higher HDL-cholesterol (*P* = 0.074) compared with healthy controls. This change in lipid profile is considered to be protective for cardiovascular disease. Falhammar *et al.*[10] did not show any

unfavorable changes in the lipid profile of adult female CAH patients. Bachelot *et al.*[11] found normal total cholesterol, triglycerides and LDL-cholesterol levels in adult patients with classic CAH, both with a BMI < 25 (*n* = 24) and with a BMI > 25 (*n* = 21). Only one study evaluated lipid profiles in children with CAH. Botero *et al.*[40] determined serum lipid profiles in 14 prepubertal children with CAH caused by 21-hydroxylase deficiency (four boys and 10 girls; aged 13 months–10 years) and in 14 healthy prepubertal children (eight boys and six girls; aged 21 months–9 years). All CAH patients were on glucocorticoid treatment with prednisone (dosage equivalent to 10–20 mg/m^2/day hydrocortisone). The study provided no information about the type of CAH and no matched control group was used. A statistically significant larger percentage of patients in the CAH group had serum triglycerides above 1.0 mmol/l compared with the control group (64.3%*vs*. 14.3%). Mean serum levels of triglycerides were also significantly elevated compared with the control group (1.32 ± 0.15 *vs*. 0.75 ± 0.07 mmol/l; *P* = 0.04). These findings were attributed to the use of glucocorticoids, especially prednisone. Furthermore, insulin resistance may also have contributed to the elevated levels of triglycerides. Mean serum levels of total cholesterol, LDL-cholesterol and HDL-cholesterol were not significantly different compared with controls.

In summary, studies evaluating the lipid profile in adult CAH patients[10,11,38,39] did not show unfavorable changes. Therefore, an altered lipid profile does not seem to be an important cardiovascular risk factor in adult CAH patients. Because obesity and insulin resistance (see further), both observed in CAH patients, are known to be associated with dyslipidaemia,[41] it is surprising that lipid profiles in CAH patients are normal. A possible explanation for this finding is that lipid profiles were mainly evaluated in younger CAH patients. Evaluation of dyslipidemia in older CAH patients, especially those older than 50 years of age, is needed.

Hypertension

Seven studies have evaluated blood pressure in CAH patients[10,39,42–46] as summarized in Table 2. Two studies[10,39] have evaluated blood pressure only in adult CAH patients, four studies[42–45] in both pediatric and young adult patients and one study[46] reports on blood pressure in pediatric CAH patients only.

Falhammar *et al.*[10] found supine and upright blood pressure values mostly within the normal range, both in female CAH patients younger than 30 years (*n* = 27) and 30 years or older (*n* = 34). No differences in supine and upright blood pressures were observed in female CAH patients and healthy controls. Three CAH patients older than 30 years received antihypertensive therapy, whereas controls did not receive anti-hypertensive medication. Defining hypertension as a supine blood pressure >140/90 mmHg, three CAH patients and four controls older than 30 years showed hypertension. Sartorato *et al.*[39] found blood pressure values within the normal range in both male and female classic CAH patients receiving glucocorticoid replacement therapy with

Table 1 Summary of studies addressing body composition in congenital adrenal hyperplasia due to 21-hydroxylase deficiency

Study	Study population (n)	Gender	Age (years), mean ± SD
Stikkelbroeck *et al*.[9]	Salt-wasting (24) Simple virilizing (3) Nonclassic (3)	12 ♀; 12 ♂ 3 ♂ 3 ♀	♂: 22 ± 2 ♀: 21 ± 3
	Controls (30)	15 ♀; 15 ♂	♂: 22 ± 2 ♀: 21 ± 2
Falhammar *et al*.[10]	Salt-wasting (27) Simple virilizing (28) Non-classic (6)	61 ♀	18-63 (range)
	Age-matched controls (61)	61 ♀	18-63 (range)
Hagenfeldt *et al*.[12]	Salt-wasting (12) Simple virilizing (1)	13 ♀	24 ± 3
	Controls (12)	12 ♀	22 ± 1
Cameron *et al*.[19]	Salt-wasting (18) Simple virilizing (3)	8 ♀; 10 ♂ 3 ♂	8 – 32 (range)
	Age-matched controls (21)	10 ♀; 11 ♂	not mentioned
Christiansen *et al*.[21]	Salt-wasting (17) Simple virilizing or nonclassic (1)	8 ♀; 10 ♂	18 – 33 (range)
	Age-matched controls (120)	80 ♀; 40 ♂	not mentioned
Cornean *et al*.[13]	Salt-wasting (19) Simple virilizing or nonclassic (3)	14 ♀; 8 ♂	12.6 (range 7.1-20.4)
	Controls: normal UK population		

[#] calculated from table 1 in Cameron *et al*.[19] using anti-inflammatory hydrocortisone equivalents for glucocorticoid doses[60]; SD, standard deviation; CAH, congenital adrenal hyperplasia; DXA, dual x-ray absorptiometry

Therapy CAH group	Methods	Outcome
Cumulative hydrocortisone dose 5 yr • Anti-inflammatory (g/m^2) ♂ 24 ± 5 ♀ 17 ± 5 • Growth retarding (g/m^2) ♂ 26 ± 6 ♀ 18 ± 5	DXA	↑Fat mass (adjusted for height) ↑Relative fat mass
All patients: glucocorticoids (±50% prednisolone, mean dosage 6.3±0.32 mg / day, ± 33% hydrocortisone 33.3 ± 2.1 mg / day, others cortisone acetate, dexamethasone or combination. 82% fludrocortisone (0.09±0.01 mg/day)	DXA	Similar percentage body fat and total and regional fat mass in patients and controls Higher total and regional fat mass in patients older than 30 years compared to younger patients
5: dexamethasone 0.5-0.75 mg/day 5: prednisolone 5.6 –12.5 mg/day 1: cortisone acetate 37.5 mg/day 1: triamcinolone 8 mg/day 1: cortisone acetate 15 mg & prednisolone 3.75 mg/day 12: fludrocortisone 0.075-0.15 mg/day	DXA	↑ Body fat
Mean hydrocortisone dose: 32.7 mg/day # Mean fludrocortisone dose: 0.17 mg/day #	DXA	↑Fat/lean ratio in ♂♂
All patients: glucocorticoids 17: mineralocorticoids Dose unknown	DXA	↑Fat mass percentage in ♂♂
Mean hydrocortisone dose: 18.9 mg/m^2/day Mean fludrocortisone dose: 0.13 mg/m^2/day	Skinfold measurement	↑Fat mass

Table 2 Summary of studies addressing blood pressure in congenital adrenal hyperplasia

Study	Study population (n)	Gender	Age (years), mean ± SD
Falhammar *et al.*[10]	Salt-wasting (27) Simple virilizing (28) Non-classic (6) Age-matched controls (61)	61 ♀ 61 ♀	18-63 (range) 18-63 (range)
Sartorato *et al.*[39]	Classic 21-OH deficiency (19) • Salt-wasting (12) • Simple virilizing (7) Controls (19)	10 ♀; 9 ♂ 10 ♀; 9 ♂	28 ± 3.5
De Silva *et al.*[42]	Classic 21-OH deficiency (9) • Salt-wasting (5) 11β-hydroxylase deficiency (1) Lipoid adrenal hyperplasia (1)	7 ♀; 4 ♂	14.5, range: 8.5-27.2
Roche *et al.*[43]	Classic 21-OH deficiency (38) •Salt-wasting (38)	23 ♀;15 ♂	11.2, range: 6.1-18.2
Völkl *et al.*[44]	Classic 21-OH-deficiency (55) • Salt-wasting (45) • Simple virilizing (10)	32 ♀;23 ♂	5.3-19.0 (range)
Hoepffner *et al.*[45]	Classic 21-OH deficiency (34) • Salt-wasting (28) • Simple virilizing (6)	21 ♀;13 ♂	6-17 years (n=23) 18-26 years (n=11)
Nebesio *et al.*[46]	Classic 21-OH deficiency (91)	49 ♀;42 ♂	Unknown

SD, standard deviation; OH, hydroxylase; BP, blood pressure; SBP, systolic blood pressure; DBP: diastolic blood pressure; ABPM: ambulatory blood pressure monitoring

Therapy CAH group	Methods	Blood pressure (mmHg) (± SD)
All patients: glucocorticoids (±50% prednisolone, ± 33% hydrocortisone, others cortisone acetate, dexamethasone or combination. 82% fludrocortisone	BP was registered supine and standing	♀<30 years: 110/71 (supine) ♀<30 years: 120/75 (upright) ♀>30 years: 110/75 (supine) ♀>30 years: 115/79 (upright)
All patients: glucocorticoid replacement with hydrocortisone or dexamethasone. 12 patients mineralocorticoid therapy.	Average of three BP measurements with a sphygmomanometer on separate occasions	♂: SBP 123.3 ± 13.2 ♂: DBP 83.3 ± 8.7 ♀: SBP 126.5 ± 7.5 ♀: DBP 83.9 ± 4.9
All patients were on glucocorticoid replacement at supraphysiological doses	24 hour ABPM	SBP: 115.1 ± 10.3 DBP: 68.3 ± 7.2
All patients received replacement therapy in the form of oral hydrocortisone and fludrocortisone	24-hour ABPM	Mean 24-hour BP: SBP: 124.9 ± 11.7 DBP: 76.2 ± 14.9
All patients received replacement therapy with glucocorticoids: hydrocortisone (40), prednisone (12) or dexamethasone (3). 53 patients received fludrocortisone.	24-hour ABPM	24-hour BP in SDS for age: ♂: SBP 0.23 ♂: DBP -0.65 ♀: SBP 0.47 ♀: DBP -0.65
All patients received glucocorticoid treatment with hydrocortisone	In outpatient clinic: oscillometric device (n=34), under hospitalisation: 24-hour BP measurement with portable oscillometric device (n=34) and 24-hour ABPM (n=11)	BP values in 11 children and adolescents who participated in all 3 measurements: Day-mean SBP in hospital:125.9 ± 9.6 Day-mean DBP in hospital:78.5 ± 7.0 SBP in outpatient clinic:117.1 ± 11.8 DBP in outpatient clinic:72.6 ± 6.3 Day-mean SBP of ABPM:114.8 ± 5.7 Day-mean DBP of ABPM:68.7 ± 4.6
All patients with hypertension were treated with both hydrocortisone and fludrocortisone (n=5)	Retrospective chart review, methods of BP measurement unknown	Peak recorded SBP for each patient with hypertension (n=4): 115 / 146 / 300 / 146

hydrocortisone or dexamethasone. In both male (*n* = 9) and female (*n* = 10) CAH patients, systolic and diastolic blood pressures did not differ from healthy controls.

De Silva *et al.*[42] performed 24-h ambulatory blood pressure monitoring in 11 treated CAH patients (M:F = 4:7) with a mean age of 14.5 years. Nine patients were 21-hydroxylase deficient (five salt losers), one patient had 11β-hydroxylase deficiency and one had lipoid adrenal hyperplasia. None of the patients had hypertension defined as a mean systolic and diastolic blood pressure >95th percentile. However, five patients had a high mean blood pressure (90th percentile) with elevated mean systolic and diastolic pressures during the awake period. Limitations of this study are the small sample size of the study population and the heterogeneity of patients, with inclusion of both children and adults, two of them not being 21-hydroxylase deficient. In addition, two different blood pressure references were used: 24-h ambulatory blood pressure specific references and office blood pressure references. When using specific ambulatory reference data, one patient showed both daytime and nocturnal hypertension, six patients (54%) had nocturnal hypertension and eight patients (73%) had nocturnal blood pressures above the 90th percentile.

Roche *et al.*[43] performed 24-h ambulatory blood pressure monitoring in 38 salt-wasting 21-hydroxylase-deficient patients (15 males, 23 females) with a mean age of 11.2 years. Using task force references for office blood pressure measurements[47] instead of 24-h ambulatory specific references, which would have been more appropriate, they found that 58% of the patients had systolic hypertension and 24% had diastolic hypertension. Mean daytime systolic blood pressure was significantly higher than in the reference population and 84% had no physiological nocturnal systolic dip. Mean systolic and diastolic blood pressures were not significantly different between males and females. In CAH patients, there was a positive relationship between systolic blood pressure and BMI, which was most marked in females.

Völkl *et al.*[44] performed 24-h ambulatory blood pressure monitoring in 55 treated patients with proven 21-hydroxylase deficiency (32 females, 23 males) aged between 5 and 19 years. Measured blood pressure values were transformed into SDS matched for age, height, sex and the same oscillometric method respectively. Their data showed a significantly elevated systolic blood pressure during both day- and night-time (daytime 0.67 SDS, nighttime 0.63 SDS). Surprisingly, diastolic blood pressure was significantly decreased during the day with normal values during the night. A normal nocturnal drop of systolic blood pressure was found. Blood pressure parameters positively correlated with BMI and skinfold thickness.

Hoepffner *et al.*[45] focused on differences in blood pressure measured in the outpatient clinic and in the ward in 23 children and adolescents and 11 adult CAH patients. All patients received glucocorticoid treatment with hydrocortisone. Blood pressure measured during hospital admission was significantly elevated in children and adolescents compared with that measured in the outpatient clinic. All blood

pressures measured were within the normal range. Measurement of 24-h ambulatory blood pressure showed blood pressure values in the normal range in all patients. No correlations were found between blood pressure on one hand, and sex, BMI or dosage of mineralocorticoids on the other hand.

Nebesio *et al.*[46] performed a retrospective chart review of children with 21-hydroxylase deficiency to evaluate the prevalence of hypertension, defined as blood pressure >95th percentile for age and gender, using published reference values. Six of 91 patients showed hypertension (6.6%). Patients were aged 58 days – 12.6 years at the time of hypertension diagnosis. No relation between blood pressure and BMI or dosage of hydrocortisone was found. Nebesio *et al.*[46] concluded that children with 21-hydroxylase deficiency have a higher prevalence of hypertension compared with historical reports on hypertension in a pediatric population.

In conclusion, in most studies performed in young CAH patients, there is a tendency towards an increased prevalence of hypertension. Further studies are required to investigate this relation in older CAH patients. It is well known that mineralocorticoid excess, like in primary aldosteronism or in forms of CAH with elevated levels of adrenal steroids with mineralocorticoid effect such as 11-hydroxylase deficiency, results in high blood pressure.[48] Therefore, treatment with too high doses of mineralocorticoids or unjustified treatment with mineralocorticoids in patients with the SV type of CAH with suppressed renin levels may cause high blood pressure. However, in the reviewed papers, no correlations between renin levels and blood pressure values were observed in CAH patients.[42–46] Glucocorticoid excess, as in Cushing's syndrome, is also known to result in high blood pressure.[49] Therefore, treatment with relatively high doses of glucocorticoids may also play a role in the development of high blood pressure in CAH patients.[50]

Insulin resistance

Nine studies have investigated insulin sensitivity in CAH patients, as summarized in Table 3.[11,17,27,36–39,51–53] Most studies have used the homeostasis model assessment method (HOMA-IR) or the oral glucose tolerance test (OGTT) to assess insulin sensitivity. Only the study of Paula *et al.*[51] has applied the forearm model combined with local indirect calorimetry, and showed that insulin sensitivity was decreased in adult females with CAH, compared with female controls. Thirty minutes after glucose ingestion, the serum insulin levels in their group of patients were significantly higher than in healthy controls. In a study by Speiser *et al.*,[52] using OGTT, insulin sensitivity was significantly lower than expected for BMI in patients compared with controls (4.1 ± 0.6 *vs*. 9.7 ± 1.2 [min/μU/ml]; $P < 0.05$). Saygili *et al.*[37] measured insulin sensitivity by HOMA-IR in untreated nonclassic 21-hydroxylase-deficient adult women, and found insulin resistance and hyperinsulinaemia. HOMA-IR was 3.2 ± 0.8 in patients and 1.8 ± 0.6 in controls. Serum fasting and nonfasting insulin levels in patients were

Table 3 Summary of studies addressing insulin resistance in congenital adrenal hyperplasia

Study (ref)	Study population (n)	Gender	Age (years), mean ± SD
Paula *et al*.[51]	Classic 21-OH deficiency (4) Nonclassic 21-OH deficiency (3) Controls (9)	7 ♀ 9 ♀	28 ± 3 27 ± 2
Speiser *et al*.[52]	Nonclassic 21-OH deficiency (6) Controls (12)	6 ♀ 12 ♀	27 27
Saygili *et al*.[37]	Nonclassic 21-OH deficiency (18) Controls (26)	18 ♀ 26 ♀	26 ± 9 29 ± 5
Sartorato *et al*.[39]	Classic 21-OH deficiency (19) • Salt-wasting (12) • Simple virilizing (7) Controls (19)	10 ♀; 9 ♂ 10 ♀; 9 ♂	28 ± 3.5
Bachelot *et al*.[11]	Classic 21-OH-deficiency (45) • Salt-wasting (23) • Simple virilizing (12) • Non-classical form (10)	36 ♀; 9 ♂	18-47 (range)
Bayraktar *et al*.[38]	Nonclassic 21-OH deficiency (50) Controls (25)	50 ♀ 25 ♀	22 ± 3 22 ± 3
Buffington *et al*.[53]	Nonclassic 21-OH deficiency (3) 3β-HSD deficiency (5) Controls (14) • Lean (6) • Obese (8)	8 ♀ 14 ♀	not mentioned not mentioned
Charmandari *et al*.[36]	- Classic 21-OH deficiency (16) • Salt-wasting (12) • Simple virilizing (4) - 11β-OH deficiency (2) Controls (28)	6 ♀; 12 ♂ 12 ♀; 16 ♂	7 ± 3 9 ± 2
Völkl 2009 *et al*.[17]	Classic 21-OH-deficiency (51) • Salt wasting (42) • Simple virilizing (9)	30 ♀; 21 ♂	5-19 (range)

SD, standard deviation; OH, hydroxylase; 3β-HSD, 3β-hydroxysteroiddehydrogenase; GnRH, gonadotropin-releasing hormone; iv GTT, intravenous glucose tolerance test; HOMA, homeostasis model assessment; OGTT, oral glucose tolerance test; ITT, intravenous insulin tolerance test; Si, insulin sensitivity

Therapy CAH group	Methods	Insulin resistant? (CAH patients vs controls)
Discontinuation of dexamethasone in classic 21-OH deficiency for 2-3 weeks prior to the study	Forearm model & local indirect calorimetry	Yes: Forearm glucose uptake (100.9 ± 10.0 vs 132.5 ± 21.2 mg/100 ml forearm), insulin response (98.6 ± 19.4 vs 59 ± 6.5 mU.ml-1/mg.100ml forearm) (P<0.05)
None	iv GTT	Yes: Si: 4.1 ± 0.6 vs 9.7 ± 1.2 (P<0.05)
None	HOMA	Yes: HOMA: 3.2 ± 0.8 vs 1.8 ± 0.6 (P<0.05)
All patients: glucocorticoid replacement with hydrocortisone or dexamethasone. 12 patients mineralocorticoid therapy.	OGTT HOMA	Yes: HOMA: 2.46 ± 1.92 vs 1.12 ± 0.58 (P=0.00)
All patients: long term glucocorticoids	OGTT HOMA	Yes: BMI < 25: HOMA 1.31 ± 0.23 BMI > 25: HOMA 2.05 ± 0.49
None	HOMA	No
None	OGTT ITT	No
All patients: Hydrocortisone & Fludrocortisone; 6 GnRH agonists Hydrocortisone	HOMA	Yes: HOMA: 2.2 ± 0.3 vs 0.7 ± 0.04 (P<0.001)
All patients: glucocorticoid substitution with hydrocortisone or dexamethasone	HOMA	Yes: HOMA: 2.7

significantly higher than in controls (14.6 ± 10.3 *vs.* 4.2 ± 0.5 IU/l, $P < 0.001$ and 85.4 ± 20.5 *vs*. 30.2 ± 3.5 IU/l, $P < 0.001$, respectively). Insulin resistance and hyperinsulinemia were associated with hyperandrogenism. Serum leptin levels did not differ between patients and controls.

Sartorato *et al.*[39] studied insulin sensitivity in 19 adult patients with CAH caused by 21-hydroxylase deficiency, using OGTT and HOMA-IR. Significantly elevated fasting plasma insulin levels (11.6 ± 3.20 µU/ml *vs*. 5.18 ± 2.4 µU/ml, $P < 0.0001$) and HOMA-IR (2.46 ± 1.9 *vs*. 1.12 ± 0.58, $P = 0.0033$) were found in CAH patients compared with controls.

An OGTT was also performed in adult CAH patients by Bachelot *et al.*[11] HOMA-IR was used to calculate insulin sensitivity. No control subjects were used in this study. During OGTT, two of 45 CAH-patients were identified with glucose intolerance. HOMA-IR was calculated in 40 patients: 1.58 ± 0.26 in salt wasting patients, 2.01 ± 0.91 in simple virilizing patients and 1.39 ± 0.19 in patients with the nonclassical form of CAH. HOMA-IR was significantly correlated with BMI and 17-OHP levels. Bachelot *et al.*[11] concluded, based on HOMA-IR, that insulin sensitivity tends to decrease when BMI and androgen levels increase.

In contrast to the studies discussed earlier, Bayraktar *et al.*,[38] using HOMA-IR, did not find a decrease in insulin sensitivity in CAH patients. HOMA-IR was 2.18 ± 0.11 for CAH patients and 2.16 ± 0.09 in controls. In addition, Buffington *et al.*[53] found no insulin resistance in CAH patients. Fasting and glucose-challenged insulin levels, glucose disappearance rates after insulin injection and *in vitro* insulin binding were similar to lean controls. However, the inclusion of both nonclassic 21-hydroxylase-deficient patients and 3β-hydroxysteroid dehydrogenase-deficient patients results in difficulty to arrive at solid conclusions. Furthermore, no data were provided about the age of the participating patients and controls.

Two studies have evaluated insulin sensitivity in pediatric CAH patients.[17,36] Charmandari *et al.*[36] found that HOMA-IR was significantly elevated in children with classic CAH, compared to healthy children (2.2 ± 0.3 *vs*. 0.7 ± 0.04, $P < 0.001$). CAH patients had significantly higher fasting serum insulin concentrations and significantly lower serum adrenaline and metanephrine levels. Völkl *et al.*[17] measured HOMA-IR in 51 patients, aged 5.1–19.6 years, with CAH caused by 21-hydroxylase deficiency. No control subjects were investigated. A HOMA-IR of 2.7 was found and the authors concluded that CAH patients were characterized by insulin resistance.

In summary, nine studies have explored insulin sensitivity either in adult or in pediatric CAH patients. Different methods were used to assess insulin sensitivity. Most studies suggest that CAH patients tend to develop insulin resistance, but studies using the gold standard technique to measure insulin sensitivity (euglycemic hyperinsulinemic clamp method) are lacking. In female CAH patients, hyperandrogenism may induce insulin resistance. In males, hypogonadism is associated with insulin resistance.

Most studies assessing insulin sensitivity in CAH patients contained only female patients.[37,38,51–53] Studies evaluating insulin sensitivity in a selected population with male CAH patients are lacking and a possible association between hypogonadism and insulin sensitivity has not been studied. No differences in insulin sensitivity between males and females were discussed in the studies containing both male *and* female CAH patients.[11,17,36,39] Unfortunately, glucocorticoids used to treat hyperandrogenism also induce insulin resistance.[3] Therefore, both treated and untreated CAH patients seem to be at risk to develop insulin resistance. In children, reference data are lacking for most parameters used to diagnose insulin resistance. Therefore, it is difficult to diagnose insulin resistance in children and predict future disease risk.[54]

Intima-media thickness

An increased intima-media thickness (IMT) has been described as a surrogate marker of atherosclerosis and consequently a higher cardiovascular risk.[55,56] Sartorato *et al.*[39] used the echo-Doppler method for evaluation of IMT of the abdominal aorta, right and left common carotid arteries, carotid bulbs and common femoral arteries in adult CAH patients, all treated with glucocorticoids (aged 28 ± 3.5 years, n = 19). Significantly increased values for IMT in all arteries were found compared with healthy controls. No correlation was found between IMT on the one hand and fasting glucose, insulin levels, cumulative doses of glucocorticoids, 17-OHP or androstenedione levels on the other hand. As increased IMT is an independent predictor of vascular events, Sartorato *et al.*[39] concluded that CAH patients might be at risk of developing coronary, cerebrovascular and peripheral vascular disease.

Conclusion

We have reviewed the current evidence regarding the presence of conventional cardiovascular risk factors, more specifically obesity, insulin resistance, high blood pressure and elevated lipid concentrations in CAH patients. Overall, these studies demonstrate the existence of an increased fat mass, insulin resistance and an elevated blood pressure but a normal lipid profile in CAH patients. Taken together, these findings suggest that adult CAH patients seem to have a high risk to cluster a number of risk factors, fitting within the metabolic syndrome. The finding of an increased IMT in these patients suggests that these risk factors may indeed translate to cardiovascular disease.

It has been suggested that CAH patients develop an unfavorable cardiovascular risk profile either because the existence of hyperandrogenism in untreated or undertreated patients or because of the (supra)physiological doses of glucocorticoids used to suppress androgen levels to normal values (graphically shown in Fig. 1). Many other

metabolic abnormalities, such as an altered leptin axis, may contribute to the development of cardiovascular disease. Further studies providing comprehensive information regarding cardiovascular risk profile are warranted, especially in older patients. Whether these risks are of a magnitude that justifies pharmacological intervention and at what age this should be started are further issues that need to be solved.

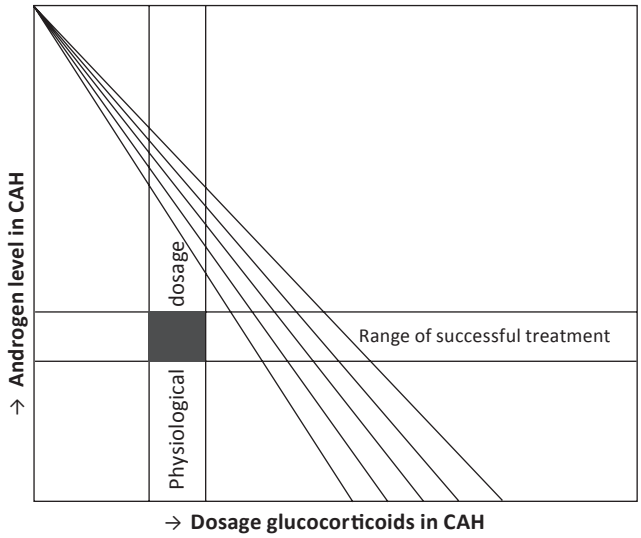

Figure 1 Hypothetical curves, showing the relation between androgen levels and glucocorticoid dosages in CAH patients. Untreated CAH patients have high androgen levels. Supraphysiological doses of glucocorticoids are needed to normalize androgen levels. Interindividual differences in glucocorticoid sensitivity (shown by the different curves), caused by different mechanisms,[57–59] may explain differences in glucocorticoid dosage between individual patients. Patients with impaired glucocorticoid sensitivity need higher glucocorticoid dosage to suppress androgen levels and these patients might have a higher chance to develop an unfavourable cardiovascular risk.

CHAPTER 1.2

Cardiovascular and metabolic risk in congenital adrenal hyperplasia

Chapter 1.2b
Cardiovascular health, growth and gonadal function in children and adolescents with congenital adrenal hyperplasia

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Abstract

After the introduction of replacement therapy with glucocorticoids and mineralocorticoids in the 1950s, congenital adrenal hyperplasia (CAH) is no longer a life-limiting condition. However, due to the successful introduction of medical steroid hormone replacement, CAH has become a chronic condition, with associated comorbidities and long-term health implications. The aim of treatment is the replacement of mineralocorticoids and glucocorticoids and the normalisation of elevated androgen concentrations. Long-term consequences of the condition and current treatment regimens include unfavorable changes in the cardiovascular risk profile, impaired growth, testicular adrenal rest tumors (TART) in male and subfertility in both male and female patients with CAH. Optimizing replacement therapy in patients with CAH remains challenging. On one hand, treatment with supraphysiological doses of glucocorticoids might be required to normalise androgen concentrations and decrease size or presence of TARTs. On the other hand, treatment with supraphysiological doses of glucocorticoids is associated with an increased prevalence of unfavorable cardiovascular and metabolic risk profiles as well as impaired longitudinal growth and gonadal function. Therefore, treatment of children and adults with CAH requires an individualized approach. Careful monitoring for early signs of complications is already warranted during pediatric healthcare provision to prevent and reduce the impact of comorbidities in later life.

Introduction

Congenital adrenal hyperplasia (CAH) is one of the most common inherited autosomal recessive disorders of metabolism. In 95% of cases, it is caused by 21-hydroxylase deficiency (21OHD).[1] Steroid 21OHD results in impaired adrenocortical synthesis of cortisol. Due to reduced negative feedback to the hypothalamus and pituitary gland, cortisol deficiency results in increased secretion of adrenocorticotropic hormone (ACTH) from the pituitary. This in turn leads to the accumulation of steroid hormone precursors and androgens. Depending on the severity of the enzymatic deficiency, 21OHD manifest as a continuum. Prenatal virilization in females depends on the degree of residual enzyme activity resulting in increased production of adrenal androgens, with a possible additional role for the production of androgens via the 'backdoor pathway'.[2]

The *CYP21A2* gene, encoding for 21-hydroxylase, is located on chromosome 6p21.3 within the human leucocyte antigen major histocompatibility complex.[3] Besides this active gene, humans also have a 98% homologous pseudogene (*CYP21A1P*), situated within 30 kb encoding a truncated, inactive enzyme.[3] Most of the mutations causing 21OHD derived from intergenic recombinations due to gene conversions between the two *CYP21A* genes. In most cases, the phenotype correlates with the milder affected allele, and consequently the residual 21-hydroxylase activity. For example, two severely affected alleles resulting in complete loss of 21-hydroxylase activity (such as complete deletions, chimeric genes and nonsense or frameshift mutations), typically result in the salt wasting (SW) form of 21OHD. In the case of a severely affected allele and a mildly affected allele, the mildly affected allele is responsible for the phenotype. In such cases, milder mutations have an in vitro residual 21-hydroxylase activity of approximately 20%–50%, which is sufficient for adequate production of cortisol and aldosterone. However, ACTH overstimulation leads to adrenal androgen excess and these variants are associated with non-classic 21OHD. The intron 2 splice site mutation is associated with both the SW and the simple virilizing (SV) form. The I172N mutation causes a variable phenotype, and is in most cases associated with a milder SV form. A strong genotype-phenotype correlation of 80%–90% have been described for 21OHD, but several studies showed discordance in individual cases.[4–9]

Historically, two clinical forms of 21OHD are distinguished: a classic form (commonly subdivided in a SW and SV form) and a non-classic form. The classic form has an incidence of between 1 in 10 000 and 1 in 15 000 live births in most Caucasian populations.[3] Patients with the classic SW form of CAH have no residual activity or hardly any residual activity when both alleles have the intron 2 splice site mutation. Females present most commonly at birth with virilization of the female external genitalia due to elevated adrenal androgen exposure in utero. Males present later in the neonatal period with SW. Patients with SV CAH have mutations with an in vitro

residual enzyme activity of 1%–2%. This enzyme activity is sufficient to produce aldosterone preventing clinically relevant SW; however, most patients will require cortisol replacement.[1,10] The non-classic form is the mildest form of CAH and has a prevalence of about 0.1% in the general white Caucasian population.[11] Patients with non-classic CAH have mutations causing an in vitro residual 21-hydroxylase activity of 20%–50%.[11] They frequently have elevated androgen concentrations resulting in a variable phenotype of premature pubarche, menstrual disturbances and polycystic ovary syndrome (PCOS).[1,10] Importantly, clinical phenotypic expression of CAH follows a continuum rather than a strict division in different forms. Thus, patient care needs to be individualized rather than following strict categorization in historic disease severities.

Treatment of classic CAH consists of lifelong glucocorticoid treatment and in patients with evident salt-loss mineralocorticoid replacement. In contrast to individuals with primary adrenal insufficiency, cortisol treatment also aims to reduce ACTH-driven adrenal androgen excess by reducing pituitary ACTH secretion. Unfortunately, with current cortisol replacement regimens, normalization of androgen concentrations frequently requires supraphysiological doses of glucocorticoids.

As a result of the treatment with glucocorticoids and mineralocorticoids, introduced in the 1950s, nearly all patients with CAH in Western countries currently reach adulthood.[12] Therefore, increased awareness of long-term complications and comorbidities in patients with CAH is becoming significantly more important to reduce long-term risks. Herein, we review unfavorable effects on the cardiovascular risk profile and gonadal function in children and young persons with 21OHD.

Cardiovascular and metabolic risk profile in children and young adults with CAH

The evidence for increased cardiovascular and metabolic risk in patients with CAH

There is clear evidence to show that adults with CAH have an increased cardiovascular and metabolic risk. Adults with CAH have been shown to may have an increased prevalence of insulin insensitivity, hypertension and obesity, with most body composition studies identifying an increase in overall and abdominal fat mass.[13–19] Increased cardiovascular and metabolic morbidity in patients with CAH has been described in a Swedish population-based cohort study with higher frequencies of hypertension, hyperlipidemia, atrial fibrillation, venous thromboembolism, obesity and type 2 diabetes.[20]

Data on cardiovascular and metabolic risk factors in children and young adults with CAH remain scarce with most studies including only small numbers of patients.

However, children and adolescents with CAH also show early signs of cardiovascular abnormalities. Increased intima-media thickness, a surrogate marker of atherosclerosis, has been described in adolescents and young adults with CAH.[21,22] Blood pressure within the first year of life has only been systematically assessed in two studies and was reported to be either normal or elevated.[23,24] Bonfig and Schwarz[23] described a correlation between hypertension and fludrocortisone as well as an inverse relationship with plasma renin activity (PRA), suggesting a role of over-treatment with fludrocortisone in the etiology of hypertension in infancy. Close monitoring of individual mineralocorticoid replacement is essential to reduce the potential negative side effects of fludrocortisone overexposure.[25] Furthermore, during childhood, a trend towards high blood pressure including absent nocturnal dipping and elevated body mass index (BMI) has been described,[13,15,19,26] which emphasizes the requirement of blood pressure monitoring in patients with CAH of all ages.

Glucocorticoid dose, advanced maturation of the bone age and parental obesity contribute to increased BMI-SD score (SDS) in children and adolescents with CAH.[27,28] An early 'adiposity rebound', defined as the age at which the physiological decrease in BMI reverses, has also been described in children with CAH.[29] The early 'adiposity rebound' increases the risk of developing obesity in adolescence.[30] Children and young adults with CAH do already show signs of impaired insulin sensitivity, which seems to be associated with glucocorticoid treatment.[15,26,31] There is no strong evidence for dyslipidemia in children, adolescents and adults with CAH.[15]

In summary, it is highly likely that overexposure to glucocorticoids, mineralocorticoids, and androgens contribute to the development of an unfavorable cardiovascular risk profile in patients with CAH. Since treatment with glucocorticoids and mineralocorticoids is ideally initiated within the first weeks of life, careful monitoring at close intervals of serum androgen precursors and renin concentrations is necessary to prevent overexposure to glucocorticoids and mineralocorticoids. Regular assessment of blood pressure is warranted in children and young adults with CAH to prevent iatrogenic hypertension due to mineralocorticoid overexposure.

Cardiovascular and metabolic risk profile—possible mechanisms

Glucocorticoid excess

The daily dose of glucocorticoids in the treatment of children with CAH has reduced over the last decades, with a current recommended daily hydrocortisone dose of 10–15 mg/m^2/day.[32] In most of the patients, supraphysiological glucocorticoid concentrations are needed to achieve sufficient normalization of adrenal androgens.[1,33] Hypercortisolism is associated with hypertension, obesity with abdominal fat accumulation and diabetes mellitus.[34] A Swedish population study described an increased risk ratio for all-cause mortality in adult patients with Addison's disease mainly due to cardiovascular death. This increase in cardiovascular morbidity and

mortality was hypothesized to be secondary to excess glucocorticoid exposure.[35] Similarly in patients with CAH the increased risk for cardiovascular mortality might well be caused by the exposure to supraphysiological doses of glucocorticoids from an early age.[36] Close individual tailoring of glucocorticoid treatment from an early age to limit overtreatment may reduce these adverse effects. Especially during puberty, steroid precursors increase significantly with the need of adaption of glucocorticoid medication. However, clear data on different glucocorticoid treatment regimens and their effects on the cardiovascular health in children are lacking. A small study in children with CAH showed that a hydrocortisone treatment regimen with the highest dose in the evening led to increased 24 hour blood pressure levels and did not improve biochemical control.[37] Population studies in adults do not show a clear superiority of a specific treatment regimen that warrants current clinical use.[38,39] Furthermore, the need of supraphysiological doses of glucocorticoids leads to loss of the cortisol circadian rhythm, which is associated with an increased incidence of obesity, diabetes mellitus and an increase in biomarkers of cardiovascular disease.[40] In adults with adrenal insufficiency, there is some evidence that mimicking the circadian rhythm of cortisol excretion with glucocorticoid replacement is associated with lower total daily doses of hydrocortisone and potentially reduces the prevalence of associated metabolic and cardiovascular complications.[41,42] Recently, novel approaches have been published to implement more physiological glucocorticoid replacement including continuous hydrocortisone infusion, once-daily modified-release hydrocortisone and delayed and sustained absorption hydrocortisone formulations.[40]

Hyperandrogenism

Many patients with CAH have persistently or intermittently elevated adrenal androgens despite treatment with supraphysiological glucocorticoid doses. Strong evidence suggests hyperandrogenism induces impaired insulin sensitivity;[43] thus contributing to an increased risk for cardiovascular disease in patients with CAH. An increased cardiovascular disease risk has also been described in PCOS, with hyperandrogenism being associated with visceral obesity and consequently impaired insulin sensitivity, hypertension, decreased leptin and increased adiponectin concentrations.[44] Therefore, elevated androgen concentrations are likely to increase the risk of early cardiovascular morbidity in patients with CAH with manifestations of its forerunners already in childhood.

Mineralocorticoid excess

High blood pressure is a characteristic and well-established feature in patients with mineralocorticoid excess syndromes such as primary aldosteronism and rare forms of CAH such as 11β-hydroxylase deficiency. Therefore, it has be suggested that

mineralocorticoid excess may also play an important role in the development of hypertension in CAH.[45] Mineralocorticoid treatment needs to be carefully monitored by evaluating renin concentrations every 3–6 months, and even more frequently during the first years of life. Regular evaluation of blood pressure is warranted to diagnose hypertension due to mineralocorticoid overexposure at an early stage. To prevent overtreatment with mineralocorticoids, especially together with salt supplementation in the first year of life, suppressed renin levels should be avoided. We, therefore, aim for renin concentrations in the upper normal or slightly elevated range in our clinical practice. Close monitoring is required in infants with CAH as mineralocorticoid sensitivity rapidly increases in the first few months and years of life. At birth there is a low expression of the mineralocorticoid receptor in the kidneys causing partial aldosterone resistance in newborns. The relative expression of the mineralocorticoid receptor increases within the first year of life causing an increased mineralocorticoid sensitivity.[46,47] Elevated 17-hydroxyprogesterone (17-OHP) concentrations, exhibiting anti-mineralocorticoid effects, can also influence the mineralocorticoid requirement due to antagonistic effects of 17-OHP on the mineralocorticoid receptor.[48] The Endocrine Society guidelines on CAH recommend treatment with mineralocorticoid in all patients with classic CAH during the newborn period and early infancy to avoid potential salt loss.[32] A recent report finding significant rates of hypertension despite adhering to the recommended fludrocortisone doses emphasizes the importance to closely monitor and titrate mineralocorticoid replacement to individual requirements to avoid iatrogenic mineralocorticoid excess.[23]

Bone health and linear growth in CAH

Chronic glucocorticoid excess leads to osteopenia and osteoporosis.[49] In patients with CAH aged over 40 years decreased bone mineral density has been described.[16,19,50,51] Similar abnormalities have not been identified in young adults with CAH.[19,52,53]

It is well established that glucocorticoid excess leads to impaired linear growth and consequently reduced final height. Continuous treatment with supraphysiological doses of glucocorticoids impairs growth by several mechanisms, including direct effects on the growth plate, resulting in disruption of the growth plate architecture and interference with the growth hormone—insulin-like growth factor-1 axis with long-term daily glucocorticoid treatment resulting in impaired growth hormone secretion and action.[54,55] On the other hand, hyperandrogenism results in advanced skeletal maturation and early epiphyseal fusion and has also a negative impact on final height. Several studies demonstrated a dose-dependent negative relationship between glucocorticoid dose and linear growth in patients with CAH during infancy, puberty and adolescence.[56–59] In contrast, a recent meta-analysis and a natural

history study showed that the cumulative glucocorticoid dose did not significantly influence the final height.[19,60,61] The meta-analysis showed a final height of –1.38 SDS in adult patients with CAH. All patients were treated with high doses of glucocorticoids in early life,[60] emphasizing the need to find the lowest glucocorticoid dose from early life onwards. It will be of great interest to evaluate the effect on linear growth of lower glucocorticoid doses used for treating patients with CAH after treatment regimens changed in the early 2000s.

Infancy is by far the most sensitive period of growth as the growth velocity is fastest. Higher glucocorticoid doses during infancy impair final height, and impaired linear growth during this time will not be recovered later in life.[56,57,60] Moreover, during the first 12–18 months of life androgen excess does not lead to a significant acceleration of growth and bone age maturation. Therefore, glucocorticoid doses are advised to be limited to a replacement dose during infancy, as growth in the first year of life is not impaired by hyperandrogenism. Overtreatment with glucocorticoids during puberty also negatively influences final height by suppressing growth hormone synthesis and consequently resulting in an impaired pubertal growth spurt. The hydrocortisone dose should not exceed 17 mg/m^2/day to improve final height to avoid suppression of pubertal growth.[62] Careful titration of the hydrocortisone dose reduces the risk of overtreatment and is likely to result in improved height outcomes in patients with CAH.

Male gonadal function in CAH

Fertility is decreased in males with classic CAH and lower child rate, abnormal semen analysis, decreased inhibin B and abnormal gonadotropin concentrations have been described. The most frequently reported causes of gonadal dysfunction and infertility in patients with CAH are the presence of testicular adrenal rest tumors (TART) and gonadotropin deficiency due to central hypogonadism.[63]

TART are benign lesions, typically localized within the rete testis and have adrenocortical features including the expression of adrenal-specific enzymes, adrenal steroid production, ACTH, luteinizing hormone (LH) and angiotensin II receptor type 2. Due to the common localization in the testes, TART often lead to compression of the seminiferous tubules consequently resulting in obstructive azoospermia and irreversible damage of the surrounding testicular tissue resulting ultimately in infertility.[64] The reported prevalence of TART in adults with CAH varies between 0% and 94%, depending on the selection of patients and the method of tumor detection.[64] Recent studies have shown that TART can already be found in childhood and infancy with a reported overall prevalence in children up to 39% and an increase in prevalence during puberty,[65] suggesting a role of LH in TART growth during puberty.[19,66–70]

Importantly, in most cases tumors were not detectable by palpation suggesting an important role for testicular ultrasound for diagnosing TART.[68] Tumors <2 cm are generally not palpable because of their central location. Therefore, without additional imaging such as ultrasound these tumors will be missed.[19,64,71,72] The situation regarding gonadal dysfunction during childhood and adolescence remains unclear.[68] A Dutch study investigating 34 patients aged 2–18 years showed normal testicular function,[68] whereas a study from Chile reported lower values for inhibin B, anti-Müllerian hormone-stimulated testosterone in 19 patients with CAH aged 2–10 years.[69]

The exact etiology of TART remains unclear. Since TART have been identified in young infants with severe 21OHD, it has been hypothesized that TART may have their origin in utero.[64] Interestingly, TART seems to be a characteristic feature in CAH as these cells are not found in unaffected newborn males.[73] Recent studies showed that TART express both adrenal and Leydig cell features, suggesting a more totipotent embryologic origin.[74] Therapeutic options remain limited. The first choice of treatment is optimizing glucocorticoid therapy to suppress ACTH as a probable growth factor for TART. However, due to the uncertain prognosis and the risk of irreversible damage of the testicular tissue, semen cryopreservation should be offered as soon as possible. Furthermore, regular ultrasound evaluation of TART might be beneficial from puberty to detect small non-palpable lesions to adjust treatment individually and subsequently prevent TART progression and ultimately improve male fertility.

Untreated or not well-controlled patients with CAH are exposed to elevated concentrations to androgens which are in part aromatized to estrogens. Exposure to long-term high concentrations of sex steroids leads to suppression of the hypothalamic-pituitary-gonadal (HPG) axis manifesting clinically as hypogonadotropic hypogonadism, but with normal production of testosterone from adrenal origin.[75–78] Therefore, inhibin B is superior marker for Sertoli cell function over follicle-stimulating hormone in adult males with CAH.[68,79] In addition, secondary hypogonadism in males with CAH can also be caused by suppression of the HPG axis due to glucocorticoid overtreatment, which is associated with reduced fecundity in males with CAH.[79]

Female gonadal function in CAH

Menstrual cycle

Disturbances of the menstrual cycle are regularly seen in females with CAH. Oligomenorrhea or amenorrhoea in patients with CAH is often associated with poor therapeutic control. In patients with non-classic CAH menstrual irregularity frequently is one of the presenting signs.[80] Even in the absence of adrenal androgen excess,

elevated progesterone concentrations of adrenal origin may cause menstrual cycle irregularity.[81] Therefore, it can be used as an important clinical parameter to monitor the degree of adrenal hormonal control in adolescent females with CAH.

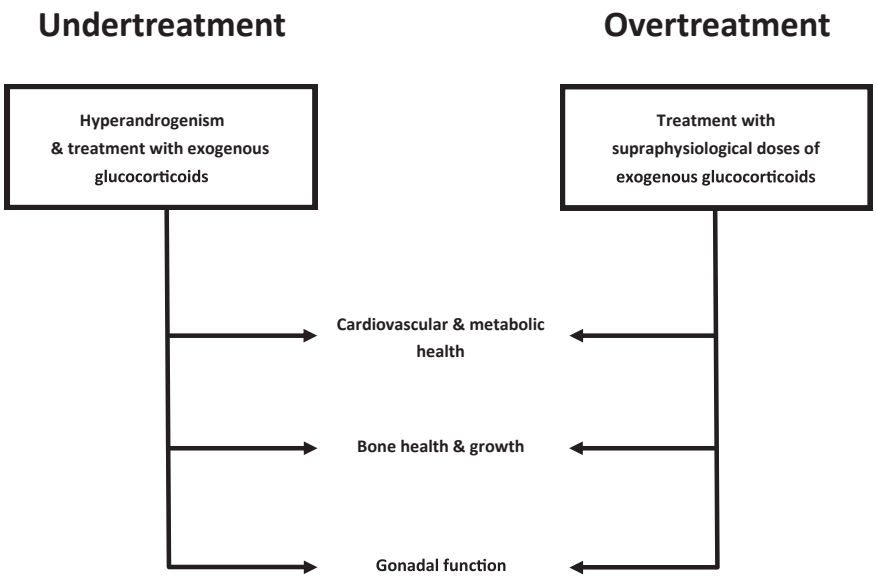
Fertility

Reduced fertility, but normal fecundity, has been described in adult females with CAH.[57,82–87] Females with CAH appear to be less sexually active and are less likely to have children compared with the general population. The more severely girls are affected the more pronounced is the reduced interest having children.[19,88] Impaired fertility in patients with CAH has several causes including adrenal overproduction of androgens and progestins, ovarian hyperandrogenism, polycystic ovaries, neuroendocrine factors (eg, suppression of the HPG axis due to glucocorticoid overtreatment), genital surgery and psychosocial factors such as delayed psychosexual development, reduced (hetero-)sexual activity and low maternal feelings.[80,89,90] Insufficient treatment with glucocorticoids, and consequently elevated adrenal androgen and progesterone concentrations, are therefore the most important medical cause of impaired fertility in females with CAH. The severity of 21OHD correlates with the severity of subfertility.[82] Treatment with increased doses of glucocorticoids is effective in improving fertility in almost all female patients.[91] On the other hand, overtreatment with glucocorticoids can cause hypogonadotropic hypogonadism via suppression of the HPG axis and consequently reduced fertility.

Besides hormonal causes, psychological causes seem to play an important role in the reduced female fertility in CAH. Impaired psychosexual development and sexual motivation may be caused by the stress due to genital surgery and following vaginal dilatation and repetitive genital examinations. Furthermore, early vaginoplasty may cause strictures leading to dyspareunia. The anatomical outcome of genital surgery is an important determinant of the sexual motivation in women with CAH.[80]

Conclusion

Unfavorable changes in the cardiovascular and metabolic risk profile and gonadal function can be commonly identified in children with CAH. Treatment with high doses of glucocorticoids might be necessary to suppress androgen concentrations and to decrease the prevalence and size of TARTs. However, supraphysiological doses of glucocorticoids increase the risk of patients with CAH developing an unfavorable cardiovascular risk profile and secondary hypogonadism (figure 1). In contrast, androgen excess may also affect cardiovascular health, growth and fertility in undertreated patients with CAH (figure 1). Therefore, treatment of children and adolescents with CAH needs to be personalized to the individual requirements



Undertreatment		Overtreatment
Insulin resistance with increased risk of hypertension, diabetes and dyslipidemia	**Cardiovascular & metabolic health**	Hypertension, insulin resistance, obesity with increased risk of diabetes and dyslipidemia
Impaired linear growth due to early epiphyseal closure as a result of hyperandrogenism	**Bone health & growth**	Osteopenia and osteoporosis Impaired linear growth due to effects of glucocorticoids on e.g. growth hormone regulation
Male subfertility TART Female subfertility due to hyper-androgenism Secondary hypogonadism	**Gonadal function**	Secondary hypogonadism

Figure 1 Balancing between the effects of hyperandrogenism and the effects of treatment with supraphysiological doses of glucocorticoids in congenital adrenal hyperplasia (CAH). Both undertreatment and overtreatment have effects on cardiovascular and metabolic health; bone health and gonadal function in patients with CAH. TART, testicular adrenal rest tumor.

balancing the use of supraphysiological glucocorticoid doses and hyperandrogenism. Optimization of care should focus on long-term aims of preserving fertility and preventing increased cardiovascular morbidity in adult life.

The current development of novel therapies mimicking a more physiological glucocorticoid replacement such as sustained-release hydrocortisone preparations and novel strategies towards anti-androgenic therapy may help in the long term to reduce the risk of overtreatment with glucocorticoids and may improve the balance between androgen levels and treatment with (supraphysiological of doses) glucocorticoids. Further improvements in the medical care and control of patients with CAH should lead to improved long-term health.

CHAPTER 1.3

The role of adrenal steroid precursors in the pathophysiology of congenital adrenal hyperplasia

Text is partially based on sections from "Disorders of Adrenal Steroidogenesis: Impact on Gonadal Function and Sex Development. Mooij CF, van Herwaarden AE, Claahsen-van der Grinten HL. Pediatric Endocrinology Reviews. 2016. 14(2):109-128"

Adrenal steroidogenesis in CAH is altered compared to healthy people due to its enzymatic defect. CAH due to 21-hydroxylase deficiency (21OHD) leads to cortisol deficiency and (in most cases) aldosterone deficiency. The compensatory increase in ACTH secretion by the pituitary gland results in stimulation of the adrenal cortex and consequently in accumulation of steroid hormone precursors before the enzymatic defect. In contrast to other types of adrenal insufficiency, the presence of increased concentrations of steroid precursors is a hallmark feature in patients with CAH and this can be used as a diagnostic marker.[1]

Adrenal steroidogenesis

The adrenal cortex consists of three different functional zones (zona glomerulosa, fasciculata and reticularis) which produce different steroid hormones. The zones differ in terms of presence of enzymes involved in specific steroid production. Mineralocorticoids (with its final product aldosterone) are mainly produced within the zona glomerulosa, glucocorticoids (with its final product cortisol) within the zona fasciculata and androgen precursors in the zona reticularis.

Cholesterol is the substrate for the synthesis of all steroid hormones within the gonad and adrenal cortex.[2] Most cholesterol used in steroidogenesis comes from plasma low density lipoproteins which contain dietary cholesterol.[3] Some cholesterol is synthesized within the adrenal cells by the endoplasmatic reticulum from acetate. The steroidogenic acute regulatory protein (StAR) facilitates the first step in the steroidogenesis by transporting cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane.[4,5] Once cholesterol is transported to the inner mitochondrial membrane it can be converted into pregnenolone by mitochondrial cytochrome P450 side-chain cleavage enzyme (p450scc; CYP11A1).[2] Further steps in steroidogenesis are facilitated by several steroidogenic enzymes within the mitochondria and the endoplasmatic reticulum. Most of these steroidogenic enzymes are cytochrome P450 enzymes (CYPs) or hydroxysteroid dehydrogenases (HSDs).[2] The first steps in both adrenal and gonadal steroid synthesis are driven by the same enzymatic steps (common pathway). The enzymes involved in the common pathway are StAR, CYP11A1, CYP17 and 3BHSD2. A defect in one of these enzymes can lead to both impairment of gonadal and adrenal steroid synthesis. The last steps in the steroid synthesis are driven by characteristic enzymes within the specific zonas of the adrenal or within the gonads.

The quantitative regulation of steroidogenesis occurs at the first step, the conversion of cholesterol to pregnenolone by CYP11A1. ACTH stimulates a rapid increase in adrenal steroidogenesis, by stimulating cholesterol delivery to the mitochondria where CYP11A1 is located, and a longer-term chronic cyclic AMP/protein kinase A based response that affects both gene transcription and translation of a variety of steroidogenic enzymes. However, the qualitative regulation of adrenal

steroidogenesis, determining the class of steroid produced, is principally determined by CYP17A1. The presence or absence of 17alpha-hydroxylase and 17,20 lyase activity determines which class of steroids will be produced. The discrimination between 17alpha-hydroxylase and 17,20 lyase activities is regulated by two posttranslational events, the serine phosphorylation of CYP17 and the allosteric action of cytochrome b5, both of which act to optimize the interaction of CYP17 with its obligatory electron donor, P450 oxidoreductase.[6] Adrenal steroidogenesis, the involved enzymes and produced (precursor) steroids are schematically shown in figure 1.

In healthy individuals adrenal steroidogenesis leads to the stepwise conversion of cholesterol into aldosterone, cortisol or adrenal androgens. Levels of precursor steroids are generally low. However, in patients with impaired enzymatic activity of one of the enzymes involved in adrenal steroidogenesis, elevated levels of steroid precursors before the enzymatic block are found. For example in 21OHD patients, levels of progesterone, 17OHP, androstenedione and 21-deoxycortisol are increased. In 21OHD CYP11B1 facilitates the conversion of 17OHP into 21-deoxycortisol (as indicated in figure 1).

Steroid hormone receptors

Adrenal steroid hormones exhibit their action via binding to a steroid receptor. Steroid hormone receptors are intracellular transcription factors that can be activated, by the specific and high affinity binding of ligand (steroid hormone) to exert positive or negative effects on the expression of target genes. The steroid hormone receptor family consists of nuclear receptors sharing a common structure.[7] The glucocorticoid receptor (GR; *NR3C1*), mineralocorticoid receptor (MR; *NR3C2*), progesterone receptor (PR; *NR3C3*) and androgen receptor (AR; *NR3C4*) are members of the nuclear receptor family, together composing subfamily C3. The GR, MR, PR and AR share structural similarities. All four receptors contain three functional domains: the N-terminal transactivation domain followed by the DNA-binding domain and the C-terminal ligand-binding domain.[8] A hinge region links both binding domains. Compared with the GR, the sequence identities of the N-terminal domains of the MR, PR and AR are 38, 24, and 16%, of the DNA binding domains 94, 91 and 79%, and of the ligand binding domains 57, 54 and 51%, respectively.[9,10] One of the common features of the ligand-binding domains of the GR, MR, PR and AR is their structural composition and organization. However, several structural features ensure ligand selectivity. For example, a unique hydrogen bond network between the receptor and the bound ligament establishes specific recognition between the ligand and receptor.[9] The shape of the steroid and the topology inside the binding pocket enhance selectivity. Furthermore, the relative position of the binding pocket within the receptor ligand binding domain plays a role in ligand selectivity.[9]

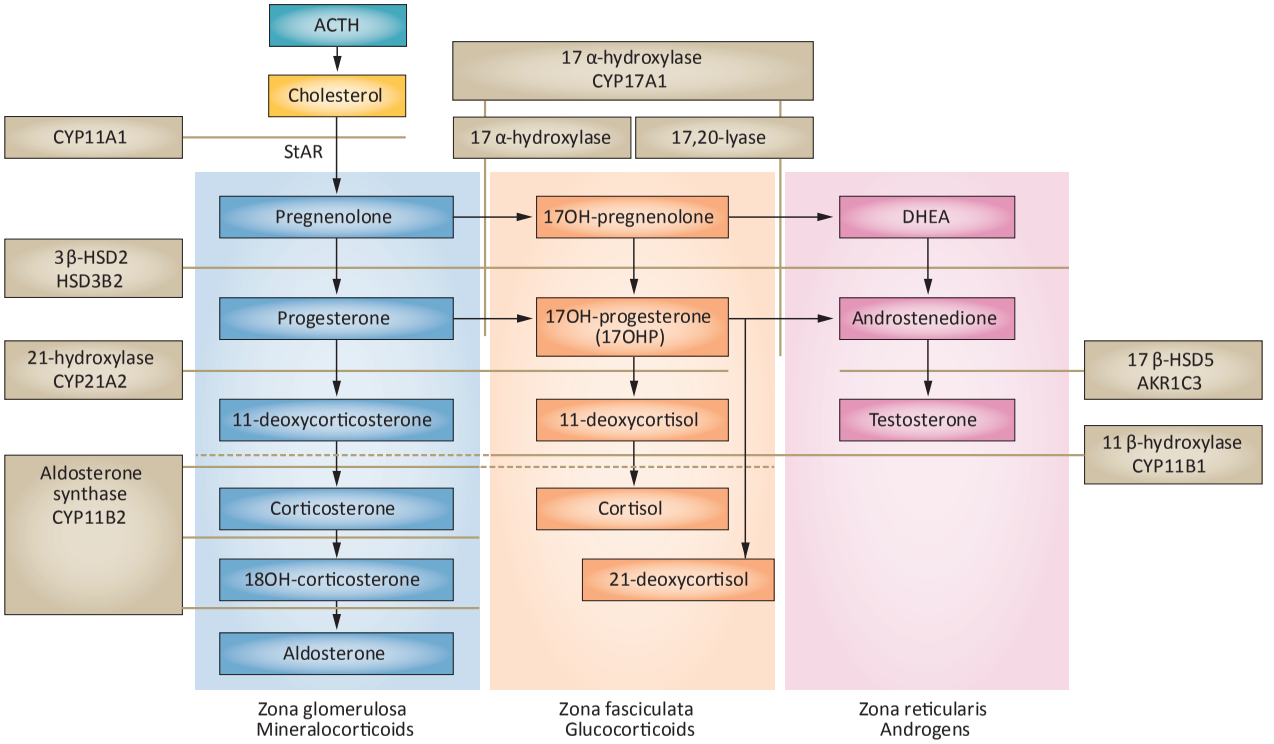


Figure 1 A schematic overview of adrenal steroidogenesis *(adapted from Han et al.[33] with permission).*

The endogenous ligand of the GR is the major glucocorticoid cortisol, but also exogenous glucocorticoids like dexamethasone, prednisolone and hydrocortisone can act as agonists of the GR. In high concentrations also aldosterone may act as a ligand for the GR.[10] The most important mineralocorticoid is aldosterone exhibiting its action by binding to the MR. Besides its affinity for aldosterone the MR has an equivalently high affinity for cortisol, corticosterone and progesterone.[10,11] The endogenous ligands for both the GR and MR are C21 steroids, based on a 21-carbon skeleton. In contrast, the C19 steroids, based on a 19-carbon skeleton, dihydrotestosterone, testosterone and androstenedione are the physiological ligands of the AR. Not all ligands exhibit agonistic effects. Some ligands have antagonistic properties, like progesterone on the MR. The exact mechanism how ligands fulfill their antagonistic action is not fully elucidated, one may hypothesize that it may cause inhibition of all steps involved in binding, translocalization and transactivation of the steroid receptors. As steroid precursors show structural similarity to aldosterone or cortisol, they may also act as a ligand for the steroid hormone receptors. Especially in patients with elevated levels of steroid hormone precursors, like in CAH, the effects of these steroid hormones as potential ligand of the GR, MR and AR are of great interest. The structural similarity of these steroid hormone precursors is illustrated in figure 2.

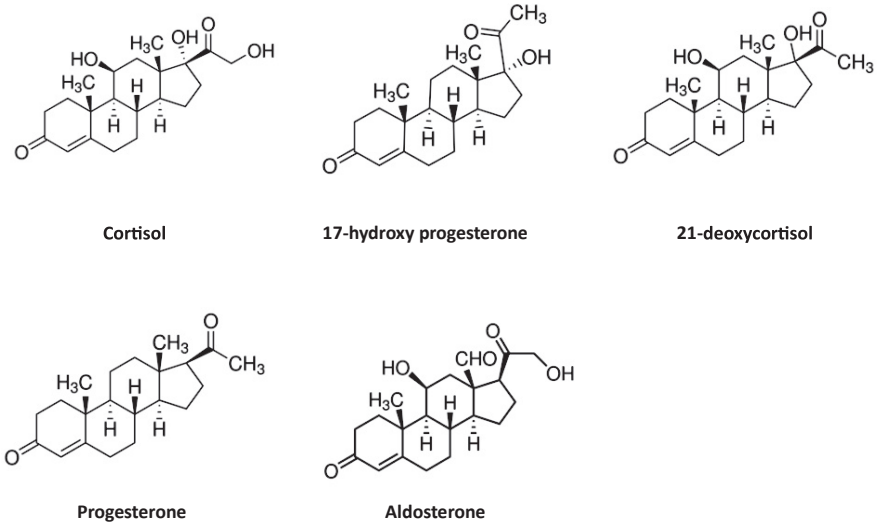


Figure 2 The chemical structures of cortisol, 17OHP, 21-deoxycortisol, progesterone and aldosterone illustrating their structural similarity.

At equilibrium the GR and MR are localized both in the cytoplasm and the nucleus, the AR is predominantly localized in the cytoplasm without the presence of a ligand.[12] Hormone induced nuclear translocation is described for both the GR, MR and AR.[12-15] Within the nucleus the ligand-activated receptors regulate transcription via three major pathways. Transactivation requires receptor dimerization and binding to *cis*-activating palindromic response elements located in the promotor region of target genes.[16,17] A second way of gene regulation is the binding to negative response elements causing transrepression.[18] The third mechanism of action is transrepression via protein-protein interactions by inactivation of transcription factors such as activator protein-1, nuclear factor-kappa B and NF-AT (e.g. playing a role in the anti-inflammatory action of glucocorticoids).[19-21] Furthermore, extragenomic effects have been described for both glucocorticoids and mineralocorticoids.[22-24] The presence of glucocorticoid receptor agonists is known to result in down-regulation of the glucocorticoid receptor,[25] suggesting that the presence of elevated concentrations of steroids and its precursors may also lead to steroid hormone resistance. A schematic and simplified overview of steroid hormone action is shown in figure 3.

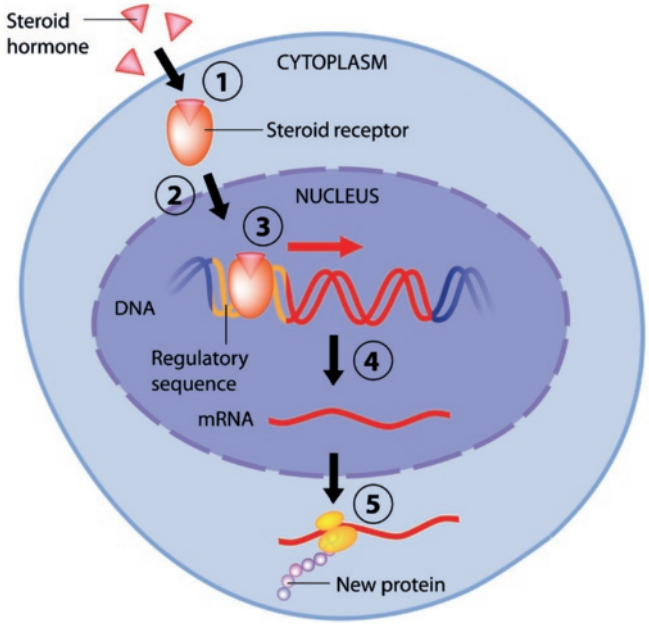


Figure 3 A schematic overview of steroid hormone action including binding to the steroid hormone receptor, translocation of the receptor/steroid hormone complex to the nucleus leading to gene transcription. *Image credit to Alila Medical Media (www.alilamedicalmedia.com).*

Clinical observations in CAH suggesting a role of adrenal steroid precursors in steroid metabolism

The structural similarity between the steroid hormone receptors as well as the steroid hormones and their precursors suggest that elevated levels of steroid hormone precursors may also lead to binding to steroid hormone receptors, and consequently activation or inhibition of glucocorticoid, mineralocorticoid and androgen receptor mediated action. Furthermore, several clinical observations suggest that adrenal steroid precursors in CAH patients may have some compensatory mineralocorticoid feature and play a role in glucocorticoid and/or mineralocorticoid metabolism:

- Untreated newborn patients with the SW CAH phenotype usually present with salt wasting crisis after the first week of life. However, most of them do not present with typical symptoms of infantile glucocorticoid deficiency such as hypoglycaemia or conjugated hyperbilirubinemia.[26,27]
- In countries where no neonatal screening for CAH is implemented males with the SV CAH phenotype often present with signs of androgen excess in childhood, without a history of Addisonian crises during illness or surgery prior to diagnosis.[28]
- Adult CAH patients who are lost to follow up and are not adherent to glucocorticoid therapy often do not develop adrenal crises.[29]
- Reduced fludrocortisone requirement after lowering precursor concentrations can be clinically observed even in the absence of an increased hydrocortisone dose. Therefore, this effect is very likely due to direct competition of steroid hormone precursors with fludrocortisone for binding to the mineralocorticoid receptor.[30]

All above described observations may be caused by potent effects of high circulating amounts of adrenal steroid precursors, in untreated or suboptimally treated patients, on the glucocorticoid and/or mineralocorticoid receptor. In untreated and poorly controlled CAH patients levels of steroid hormone precursors, including progesterone, 17OHP, 21-deoxycortisol and androstenedione, are elevated. These precursors are known to have structural similarities to cortisol,[31] and therefore may exhibit glucocorticoid properties. The exact role of the steroid precursors in the steroid metabolism in CAH patients still has to be elucidated. Previous studies showed that progesterone and 17OHP may contribute to mineralocorticoid deficiency in classic CAH patients, due to antagonistic properties on the human mineralocorticoid receptor.[30] Other studies suggested that at least some of these steroid precursors have glucocorticoid or mineralocorticoid activity interfering with the glucocorticoid and/or mineralocorticoid receptor.[32] In this thesis we will discuss the role of steroid precursors in both mineralocorticoid and glucocorticoid metabolism in more detail.

CHAPTER 1.4

Outline of the thesis

After a brief introduction on CAH (**Chapter 1.1**), a review of the literature on the cardiovascular and metabolic risk profile in both pediatric and adult CAH patients (**Chapter 1.2a and 1.2b**), and an introduction on the role of steroids and steroid precursors in CAH (**Chapter 1.3**) is given, this thesis continues with describing the results of original studies on these subjects. The most important questions we wanted to answer in this thesis were:

1. Is there an unfavorable cardiovascular and metabolic risk profile in adult and pediatric CAH patients?
2. Which factors contribute to an unfavorable cardiovascular and metabolic risk profile in adult and pediatric CAH patients?
3. What is the role of steroid precursors on the steroid metabolism in CAH patients *in vitro*?

In the first part of the thesis, we describe the results of a cross-sectional study evaluating the cardiovascular risk profile in adult CAH patients (**Chapter 2.1**) and the results of a placebo controlled trial on the use of pioglitazone to improve insulin sensitivity in adult CAH patients (**Chapter 2.2**).

In the second part of the thesis we focus on the cardiovascular and metabolic risk profile of pediatric CAH patients. **Chapter 3.1** describes the results of a retrospective study evaluating blood pressure levels during the first year of life in pediatric CAH patients. **Chapter 3.2** shows cross-sectional data on several cardiovascular and metabolic risk factors in pediatric CAH patients aged 8 to 16 years. Furthermore, we have evaluated cardiac function in the same group of pediatric CAH patients by electrocardiogram, conventional echocardiography, tissue Doppler imaging and 2D myocardial strain (rate) imaging (**Chapter 3.3**).

Data on the role of steroids and steroid precursors in CAH are presented in the third part of the thesis. **Chapter 4.1** describes the influence of steroids and steroid precursors whose concentrations are increased in CAH on the *in vitro* transactivation and translocation of the human mineralocorticoid receptor. In **Chapter 4.2** we present data on the role of steroids and steroid precursors accumulating in CAH on the transactivation of the human glucocorticoid receptor.

We conclude this thesis by reflecting on the main findings of this thesis, discussing the broader perspective of our results in relation to current literature, reflect upon the limitations of our studies and discuss the clinical implications of our work and the directions for future research in **Chapter 5**.

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CHAPTER 2

CARDIOVASCULAR AND METABOLIC RISK PROFILE IN ADULT PATIENTS WITH CONGENITAL ADRENAL HYPERPLASIA

CHAPTER 2.1

Adult patients with congenital adrenal hyperplasia have elevated blood pressure but otherwise a normal cardiovascular risk profile

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Abstract

Objective: Treatment with glucocorticoids and mineralocorticoids has changed congenital adrenal hyperplasia (CAH) from a fatal to a chronic lifelong disease. Long-term treatment, in particular the chronic (over-) treatment with glucocorticoids, may have an adverse effect on the cardiovascular risk profile in adult CAH patients. The objective of this study was to evaluate the cardiovascular risk profile of adult CAH patients.

Design: Case-control study.

Patients and Measurements: In this case-control study the cardiovascular risk profile of 27 adult CAH patients and 27 controls, matched for age, sex and body mass index was evaluated by measuring ambulatory 24-hour blood pressure, insulin sensitivity (HOMA-IR), lipid profiles, albuminuria and circulating cardiovascular risk markers (PAI-1, tPA, uPA, tPA/PAI-1 complex, hsCRP, adiponectin, IL-6, IL-18 and leptin).

Results: 24-Hour systolic (126.3 mmHg±15.5 *vs* 124.8 mmHg±15.1 in controls, $P = 0.019$) and diastolic (76.4 mmHg±12.7 *vs* 73.5 mmHg±12.4 in controls, $P < 0.001$) blood pressure was significantly elevated in CAH patients compared to the control population. CAH patients had higher HDL cholesterol levels ($P < 0.01$), lower hsCRP levels ($P = 0.03$) and there was a trend toward elevated adiponectin levels compared to controls. Other cardiovascular risk factors were similar in both groups.

Conclusion: Adult CAH patients have higher ambulatory blood pressure compared to healthy matched controls. Other cardiovascular risk markers did not differ, while HDL-cholesterol, hsCRP and adiponectin levels tended to be more favorable.

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder of adrenal steroidogenesis. In 95% of cases it is caused by 21-hydroxylase deficiency.[1] Deficiency of 21-hydroxylase results in impaired adrenal synthesis of cortisol and often also of aldosterone leading to increased secretion of ACTH by the pituitary gland, adrenal hyperplasia, and excessive production of adrenal androgens. Current treatment of CAH consists of administration of glucocorticoids and, if necessary, of mineralocorticoids to prevent adrenal crises and to suppress the abnormal secretion of adrenal androgens. Patients with CAH are at risk of developing signs and symptoms of Cushing's syndrome, as the therapeutic range of treatment with glucocorticoids is narrow and slightly supraphysiological doses of glucocorticoids are needed.[1,2] Cushing's syndrome is associated with insulin resistance and cardiovascular morbidity.[3] Therefore, patients with CAH may develop an adverse cardiovascular risk profile, which is of increasing clinical importance as nowadays nearly all CAH patients reach adulthood.

A recent Swedish population study described an increased risk ratio for all-cause mortality of 2.19 (confidence interval 1.91–2.51) for men and 2.86 (confidence interval 2.54–3.20) for women treated with glucocorticoids in Addison's disease.[4] The excess mortality in both males and females was mainly attributable to cardiovascular disease (risk ratio (RR) for cardiovascular death in men 1.97 (confidence interval 1.61–2.39); RR in women 2.31 (confidence interval 1.94–2.74)). The increased cardiovascular mortality in Addison's patients is most likely caused by excess glucocorticoid exposure. CAH patients are commonly treated with significantly higher doses of glucocorticoids than Addison's patients, already starting directly after birth. Therefore, evaluating the cardiovascular risk profile in CAH patients is of importance, with the oldest CAH patients now being in their sixties.

So far, few studies have focused on cardiovascular risk in CAH patients. An elevated body mass, an increased fat mass and insulin resistance have been described in adult and pediatric CAH patients.[5] Blood pressure has been studied mainly in pediatric and young adult CAH patients and showed a tendency towards high blood pressure in some, but not all, studies.[5] Lipid profiles in adult CAH patients did not show unfavorable changes.[5]

We hypothesized that adult CAH patients are at risk to develop an unfavorable cardiovascular risk profile due to lifelong treatment with glucocorticoids. As described above, results of prior studies concerning the cardiovascular risk profile in CAH patients are inconclusive. Furthermore, most studies were performed in small patient groups, or mainly in pediatric patients or without a proper control group. As adult CAH patients tend to become obese, especially proper matching for body mass is warranted. More recent markers for cardiovascular risk, like plasminogen activator inhibitor type 1 (PAI-1), tissue-type plasminogen activator (tPA), urokinase-type

plasminogen activator (uPA), tPA/PAI-1 complex and high sensitive CRP (hsCRP), have not been studied before in CAH patients. Therefore, the aim of this study was to evaluate insulin sensitivity, blood pressure, albuminuria, lipid profile, and other circulating cardiovascular risk markers in adult CAH patients and to compare to the cardiovascular risk profile of carefully matched control subjects.

Materials and Methods

Patients

Adult patients with proven congenital adrenal hyperplasia were included in this study. Inclusion criteria for patients were biochemically and genetically proven CAH and stable glucocorticoid and mineralocorticoid therapy for 3 months. For each individual patient a healthy, age-, sex- and body mass index (BMI)-matched control was recruited by advertisements. Inclusion criteria for control subjects were an unremarkable medical history and at present no evidence of disease, no medication (except for oral contraceptives) and Caucasian race. Exclusion criteria for patients and control subjects were age <18 years and inability to give informed consent. All patients were fully informed about the aim and design of the study and all the methods involved. They consented with the study protocol according to the recommendations of the medical ethical committee of the Radboud University Nijmegen Medical Centre.

Methods

Each participant visited the hospital on two consecutive days. On the first day, participants visited the hospital in the morning after abstinence of caffeine containing substances and an overnight (10-hour) fast. All participants received a physical examination including anthropometric measurements. Blood was drawn to assess fasting glucose, insulin, HDL cholesterol, LDL cholesterol, total cholesterol, triglycerides, and several circulating cardiovascular risk markers, like PAI-1, tPA, uPA, tPA/PAI-1 complex, hsCRP, adiponectin, IL-6, IL-18 and leptin. Plasma concentrations of total adiponectin, IL-6 and IL-18, hsCRP and leptin were determined using ELISAs (R&D Systems, Minneapolis, MN). ELISAs, developed by our department, were used for assessment of components of the plasminogen activation system (uPA, tPA and PAI-1) and its complexes (tPA:PAI-1).[6,7] PAI-1/tPA ratios were calculated. Insulin resistance (IR) was estimated using the homeostasis model assessment (HOMA) method [IR = insulin (µmol/ml)×glucose (mmol/l)/22.5].[8] Office blood pressure was measured using a Dinamap Vital Signs Monitor. Blood pressure was measured twice supine and once in upright position. Mean supine office blood pressure was calculated. Subsequently, ambulatory blood pressure was monitored for 24 hours (SpaceLabs model 90207). On the second day, participants returned to the hospital for disconnection of the ambulatory blood pressure monitoring device.

Statistics

For calculations and statistical analyses, the SPSS personal computer software package was used and $P<0.05$ was considered statistically significant. Abnormally distributed data were log-transformed. Differences between CAH patients and controls were statistically tested using unpaired Student's *t*-test or the Mann-Whitney U-test, as appropriate. Results are expressed as mean ± SEM, unless otherwise indicated.

Results

A total of 27 patients were included in this study. 20 patients had the salt-wasting type of CAH, 6 patients were simple virilizers and 1 patient had a non-classic type of CAH. The control group was well matched for age, sex and BMI. Key parameters of the selected population were similar to the whole population of adult CAH patients treated in our medical center. At the time of the investigation twenty-three patients were treated with hydrocortisone as the only glucocorticoid. One patient was treated with dexamethasone only, one patient was treated with cortisone acetate only and two patients received a combination of dexamethasone and hydrocortisone. Glucocorticoid doses were converted into hydrocortisone equivalents using anti-inflammatory equivalents (hydrocortisone 30 mg = cortisone acetate 37.5 mg = dexamethasone 0.75 mg). Hydrocortisone equivalents are also presented as mg/m^2. Five patients had plasma renin levels <5 mU/l, consistent with overtreatment. Characteristics of our study population, including current daily hydrocortisone and fludrocortisone dosage, are shown in table 1.

Blood pressure profiles

Office blood pressure measurements during hospital visit showed no significant differences in supine and upright systolic and diastolic blood pressure between CAH patients and controls. Supine and upright heart rates during hospital visit were not different in CAH patients compared to controls (table 1).

Mean 24-hour systolic (mean ± SD, 126.3±15.5 mmHg *vs* 124.8±15.1 mmHg, $P=0.019$) and diastolic (76.4±12.7 mmHg *vs* 73.5±12.4 mmHg, $P<0.001$) blood pressure was significantly elevated in CAH patients compared to the control population. Mean arterial pressure (MAP, 92.7±12.9 mmHg *vs* 90.0±12.3 mmHg in controls, $P<0.001$) and heart rate (77.1 bpm±17.9 *vs*74.2 bpm±15.5 in controls, $P<0.001$) also showed significantly higher levels in CAH patients than in controls. Measured blood pressure values and differences between day- and nighttime are shown in figure 1.

Table 1 Characteristics of the study population

	Patients (n=27)	Controls (n=27)
Gender (M/F)	12/15	12/15
Age (years)	30.2 ± 8.0	32.5 ± 11.7
Height (m)	1.67 ± 0.10	1.75 ± 0.09#
Weight (kg)	75.4 ± 13.1	83.3 ± 14.5#
BMI (kg/m²)	27.2 ± 4.6	27.3 ± 4.8
Waist circumference (cm)	86.9 ± 11.5	88.3 ± 12.0
Hip circumference (cm)	101.6 ± 9.7	103.9 ± 9.4
Waist/hip ratio	0.86 ± 0.08	0.85 ± 0.08
Office systolic blood pressure (mmHg)		
Supine	133 ± 12	133 ± 12
Upright	128 ± 13	131 ± 12
Office diastolic blood pressure (mmHg)		
Supine	83 ± 10	80 ± 10
Upright	91 ± 10	90 ± 10
Office heart rate (bpm)		
Supine	67 ± 13	66 ± 11
Upright	78 ± 14	79 ± 14
Daily hydrocortisone dosage (mg)*	23.4 ± 8.0	-
Daily hydrocortisone dosage (mg/m²)	12.6 ± 4.48	-
Daily fludrocortisone dosage (mg)**	0.11 ± 0.06	-
Plasma renin level (mE/l)	34.9 ± 69.9	-

Mean values ± 1 SD are given. M=male; F=female; bpm=beats per minute, # $P<0.05$, *Glucocorticoid doses were converted into hydrocortisone equivalents using anti-inflammatory equivalents **25 patients were treated with fludrocortisone

When analyzed separately for day and night, systolic blood pressure was not significantly elevated during the day (08.00 h to 22.00 h) nor during the night (22.00 h to 08.00 h), but diastolic blood pressure was elevated both during day- ($P<0.001$) and nighttime ($P=0.001$) in CAH patients compared to controls. CAH patients thus had a similar blood pressure dip during the night as compared to controls.

Urinary cardiovascular risk markers

Albumin excretion was comparable in CAH patients and controls (data not shown).

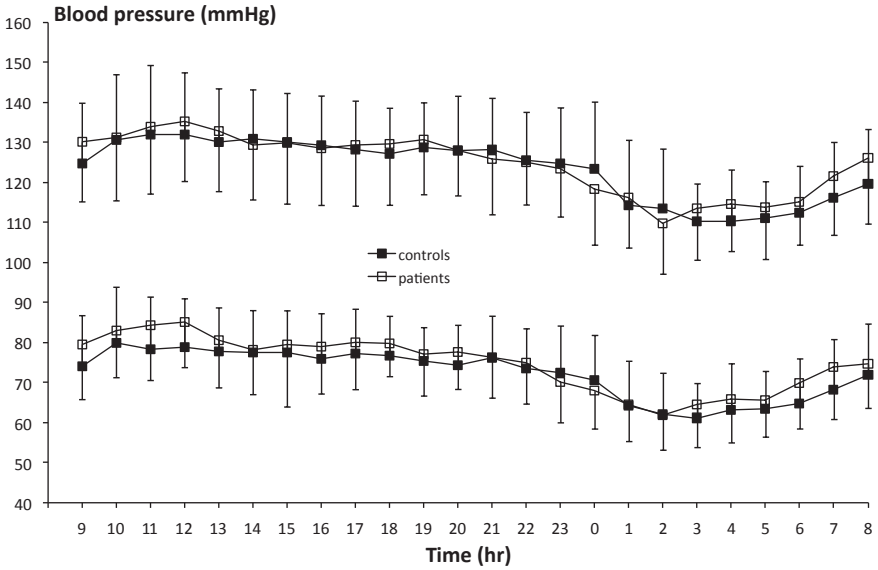

Figure 1 Mean 24-hour ambulatory blood pressure measurements (±1SD) in CAH patients (opens squares) and matched controls (closed squares).

Lipid profile

HDL cholesterol concentration was higher in CAH patients compared to controls. No significant differences in total cholesterol, LDL cholesterol and triglycerides between CAH patients and controls were observed (Table 2).

Table 2 Lipid profile in adult CAH patients and healthy, matched controls

	CAH patients (n=27)	Controls (n=27)	*P*-value
Total cholesterol (mmol/l)	4.6 ± 1.0	4.2 ± 1.0	0.22
Triglycerides (mmol/l)	1.0 ± 0.6	1.0 ± 0.6	0.93
HDL cholesterol (mmol/l)	1.4 ± 0.3	1.1 ± 0.2	< 0.01
LDL cholesterol (mmol/l)	2.8 ± 0.7	2.7 ± 0.9	0.87

Mean values ± 1 SD are given.

Insulin sensitivity

Fasting plasma glucose levels did not differ between CAH patients and controls (4.6±0.6 vs 4.8±0.6 mmol/L, *P* = not significant). Insulin levels also were comparable in CAH patients and controls (9.4±4.6 vs 10.1±4.8 pmol/L, *P* = not significant), as was calculated HOMA-IR (2.0±1.2 vs 2.2±1.0, *P* = not significant).

Circulating cardiovascular risk markers

A trend toward elevated adiponectin levels in CAH patients compared to controls was observed (Table 3). Significantly lower hsCRP levels were found in CAH patients compared to controls (*P* = 0.03). PAI-1, uPA, tPA, tPA/PA1-complex, PAI-1/tPA ratio, IL-6, IL-18 and leptin levels were not significantly different in CAH patients and controls (Table 3).

Table 3 Circulating cardiovascular risk markers in adult CAH patients and healthy, matched controls

	CAH patients (n=27)	Controls (n=27)	*P*-value
IL-18 (pg/mL)	57±24	53±26	0.56
IL-6 (pg/mL)	1.31±1.50	1.37±1.26	0.89
Adiponectin(ug/mL)	4.17±3.03	2.93±1.33	0.06
Leptin (ng/mL)	24±20 (n=26)	24±18	0.92
hsCRP (mg/L)*	1.06±1.49	1.79±1.83	0.03
uPA (ng/mL)	0.99±0.52	0.92±0.54	0.63
PAI-1 (ng/mL)	55±29	51±19	0.51
tPA (ng/mL)	4.86±2.13	6.01±3.74	0.17
tPA-PAI-1 complex (ng/mL)	6.95±3.67	9.11±5.52	0.10
PAI-1/tPA ratio	12.53±7.72	10.19±4.23	0.17

Mean values ± 1 SD are given.
*As hsCRP values were not distributed normally, we have calculated log(CRP) values

Clinical characteristics

Average BMI was high in CAH patients (27.2±4.6 kg/m^2). Waist and hip circumference and waist/hip ratio were not different in CAH patients compared to controls (data shown in table 1). It was not possible to evaluate BMI as an individual cardiovascular risk marker because controls were matched for BMI in our study.

Discussion

The present study shows that the cardiovascular risk profile of adult CAH patients is relatively unaffected compared to a carefully BMI, sex and age matched control group; only slightly elevated 24-hour blood pressure levels were found.

Our finding of an elevated 24-hour blood pressure profile in adult CAH patients was not reported in earlier studies in adult CAH patients.[9,10] However, studies in young adult and pediatric CAH patients did show a tendency towards hypertension.[5] Elevated blood pressure in CAH patients may be caused both by glucocorticoid and mineralocorticoid therapy, as glucocorticoid as well as mineralocorticoid excess is known to result in high blood pressure.[11,12] As 24-hour blood pressure is only slightly elevated it is hard to predict the clinical importance of this finding for the individual patient. Therefore, it is uncertain if the elevation in 24-hour blood pressure profile found in our population will lead to an increased cardiovascular mortality. The increase was found in ambulatory blood pressure, not in office blood pressure, suggesting that a given office blood pressure in a CAH patient may reflect slightly higher 24 h hypertensive burden and thus more risk compared to control subjects.

The finding of a tendency toward elevated adiponectin levels in adult CAH patients is in line with the study by Völkl *et al.* who reported significantly elevated adiponectin levels in 51 children and adolescents with CAH.[13] Adiponectin has insulin sensitizing and anti-inflammatory properties and high adiponectin levels are associated with lower risk of myocardial infarction and a reduced risk of type 2 diabetes.[14,15] Adiponectin levels are known to be decreased in obesity, insulin resistance and type 2 diabetes,[16] and administration of glucocorticoids and androgens have been shown to decrease adiponectin levels.[17,18] The higher adiponectin level found in CAH patients treated with glucocorticoids is thus somewhat surprising. With respect to their cardiovascular risk profile, elevated adiponectin levels might have a beneficial effect concerning the development of type 2 diabetes and myocardial infarction.

In contrast to earlier studies,[5] including one of our own,[19] our adult CAH patients were not insulin resistant when evaluated by the HOMA-IR method. The probable explanation for this finding is the careful matching in BMI. Alternatively, the HOMA method may not have been sensitive enough to detect differences in our population. The lipid profile of adult CAH-patients showed no unfavorable changes. This finding is in line with earlier studies evaluating lipid profiles.[5] In the present study we found significantly elevated HDL-cholesterol levels; Falhammer *et al.* showed a tendency towards higher HDL-cholesterol in female CAH patients 30 years of age or older.[9]

To our knowledge hsCRP levels have not been studied before in CAH patients. The trend towards lower hsCRP levels in CAH patients may be explained by the chronic treatment with glucocorticoids. It is well known that glucocorticoids have

anti-inflammatory effects, but data on the direct effect of glucocorticoid treatment on CRP levels are lacking.[20]

Most studies evaluating cardiovascular risk factors in CAH patients have focused on one or several risk factors. Recently, the CaHASE study evaluated the health status, including cardiovascular and metabolic risk, in a large British cohort of 199 adult CAH patients due to 21-hydroxylase deficiency.[21] Results were compared to Health Survey for England data. Similar to our study they showed elevated diastolic but not systolic blood pressure. Differences in blood pressure results between our study and the CaHASE study may be explained by the fact that in the CaHASE study no 24-hour ambulatory blood pressure measurement was performed and that the results were compared to reference values of the general population. Furthermore they report high frequencies of obesity (41%), hypercholesterolemia (46%), insulin resistance (29%) and osteopenia (40%) compared to reference values. As data in the CaHASE study are only compared to age- and sex-matched reference data it is hard to compare their results with those obtained in our study, using an age-, sex- and BMI-matched cohort, as increased BMI plays an important role in both cardiovascular and metabolic risk. Falhammar *et al.* recently evaluated cardiovascular and metabolic risk in adult male CAH patients.[22] Their findings are partly in agreement with ours: increased fat mass and similar lipid profiles, but no increased blood pressure, which may be due to the small (sub) sample sets.

Our study had limitations. We have used the HOMA-IR method to evaluate insulin sensitivity instead of the gold standard, the euglycemic clamp. Furthermore, the fact that we have chosen to use a BMI-matched control group, precludes us to evaluate the role of obesity as a cardiovascular risk factor in CAH patients. This may be relevant as the CAH group was on average overweight. Evaluation of the cardiovascular risk profile depends on the evaluated markers. In this study we did not evaluate endothelial dysfunction, endothelial vasodilative capacity and the endothelial response to glucocorticoid and mineralocorticoids. As we did not control for the effects of environmental and physiological factors like stress and health behavior we could not evaluate the role of these factors on blood pressure levels and other cardiovascular risk factors.

In summary, this study shows that adult CAH patients have an elevated ambulatory blood pressure, but otherwise a normal cardiovascular risk profile compared to healthy BMI, age and sex matched controls. One may speculate that elevated adiponectin and HDL-cholesterol and decreased hsCRP levels may even have some protective effect on the development of cardiovascular disease in these patients.

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CHAPTER 2.2

Pioglitazone improves insulin resistance and decreases blood pressure in adult patients with congenital adrenal hyperplasia

Kroese JM, Mooij CF, van der Graaf M, Hermus AR, Tack CJ.
European Journal of Endocrinology. 2009; 161(6):887-894

Abstract

Context: Patients with congenital adrenal hyperplasia (CAH) are chronically treated with supraphysiological doses of glucocorticoids, which are known to induce insulin resistance. Thiazolidinediones might reverse this effect and improve insulin sensitivity.

Objectives: To assess insulin sensitivity in CAH patients and the effect of pioglitazone treatment on insulin sensitivity in CAH patients. Secondary objectives were the effects of treatment with pioglitazone on blood pressure, body fat distribution, lipid, and steroid profiles.

Design: Randomized placebo controlled crossover trial.

Participants: Twelve CAH patients and 12 body mass and age-matched control subjects.

Intervention: Sixteen-week treatment with pioglitazone (45 mg/day) or placebo.

Main outcome measure: Insulin sensitivity measured by euglycemic clamp and oral glucose tolerance test. Further measures were 24-h blood pressure profiles, body fat distribution measured by magnetic resonance imaging, dual energy x-ray absorptiometry (DEXA) and bioimpedance procedures, liver fat by magnetic resonance spectroscopy, lipid, and steroid profiles.

Results: CAH patients were insulin resistant compared with healthy controls. Treatment with pioglitazone significantly improved insulin sensitivity in CAH patients (glucose infusion rate (GIR) from 28.5±11.6 to 38.9±11.0 µmol/kg per min, P=0.000, GIR in controls 46.2±23.4 µmol/kg per min, P<0.05 versus CAH). Treatment with pioglitazone decreased blood pressure (systolic: 124.0±13.6 vs 127.0±14.9 mmHg, P<0.001, diastolic: 72.8±11.5 vs 77.4±12.6 mmHg, P<0.001). No changes in body fat distribution, lipid, and steroid profiles were observed.

Conclusions: CAH patients are insulin resistant compared with matched control subjects. Treatment with pioglitazone improves insulin sensitivity and decreases blood pressure in CAH patients.

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive condition, which is in more than 95% of the cases caused by a mutation in the gene for 21-hydroxylase.[1] This defect results in increased secretion of ACTH, adrenal hyperplasia and increased production of androgens, but glucocorticoid deficiency. Current treatment comprises glucocorticoid (and, if necessary, mineralocorticoid) administration to prevent adrenal crises and suppress the abnormal secretion of androgens. However, because glucocorticoids are given in slightly supraphysiological doses and the therapeutic range for glucocorticoids is narrow, patients are at risk of developing iatrogenic Cushing's syndrome.[1]

Glucocorticoids induce insulin resistance in humans,[2] and may precipitate diabetes in susceptible subjects. Chronic exogenous or endogenous oversupply with glucocorticoids is associated with changes in fat distribution, typically toward more central/visceral fat. Whether the observed insulin resistance is related to these changes in body fat distribution is not known.

The use of supraphysiological doses of glucocorticoids and androgen excess in CAH patients are associated with the development of an unfavorable cardiovascular risk profile.[3] Data on the prevalence of cardiovascular morbidity or life expectancy in CAH patients are not yet available. In this respect, it is of interest that a recent population-based study in Sweden has showed that patients with Addison's disease have a clearly increased mortality rate. The excess mortality in both males and females was for a major part caused by cardiovascular disease. The authors of this study suggested that the increased cardiovascular mortality in Addison's patients is caused by excess glucocorticoid exposure.[4]

Of the few studies that have explored insulin sensitivity in CAH, most have concluded that CAH patients are indeed characterized by insulin resistance.[5-11] Many different methods have been used to assess insulin sensitivity, but studies using the euglycemic hyperinsulinemic clamp method, the gold standard to assess insulin sensitivity, are lacking. It has also been reported that CAH patients are at risk of developing other components of the metabolic syndrome.[3,12,13]

CAH patients are characterized by insulin resistance and have an unfavorable cardiovascular risk profile, so it might be a rational option to improve their insulin sensitivity with insulin sensitizers. Improving their cardiovascular risk profile might also improve their life expectancy. Thiazolidinediones (TZDs) improve insulin sensitivity in several conditions associated with insulin resistance. The improvement of insulin sensitivity is associated with beneficial changes in several parameters, like lipids, blood pressure, and vascular function, and changes in inflammatory markers.[14,15] Therefore, TZDs might be a rational therapeutic option in glucocorticoid-induced insulin resistance, as has been suggested by earlier studies.[16,17] Such improvement in

insulin sensitivity might have a twofold benefit: first, glucose tolerance in patients with limited β-cell function will improve and hence the chance to develop overt type 2 diabetes will decrease. Secondly, cardiovascular risk factors associated with insulin resistance will improve.[18]

In the present study, we tested the hypotheses that CAH patients who are chronically treated with glucocorticoids are insulin resistant and that treatment with pioglitazone improves insulin sensitivity in this group. Insulin sensitivity was assessed using the euglycemic clamp procedure. We also measured the effect of pioglitazone treatment on body fat distribution, including liver fat content, blood pressure, and lipid profile.

Materials and methods

Patients

Adult subjects with biochemically and genetically proven CAH on a stable corticosteroid replacement dose for 3 months were included in the study. Characteristics of the study population are shown in Table 1. Gender, phenotype, results of mutation analysis, medication, and mean salivary androstenedione levels of the study population are shown in Table 2. Exclusion criteria were as follows: age <18 years, inability to give informed consent, significant cardiovascular disease (defined as myocardial infarction or stroke 6 months preceding the study), significant renal disease (defined as a glomerular filtration rate (GFR) <30 ml/min), significant liver disease (defined as alanine aminotransferase and aspartate aminotransferase levels of more than three times the upper limit of normal), and mental disease. All patients were fully informed about the aim and design of the study and all the methods involved. They consented with the study protocol which was approved by the institutional review board of the Radboud University Nijmegen Medical Centre.

Insulin sensitivity in CAH patients treated with glucocorticoids was compared to insulin sensitivity measured in a group of body mass index (BMI) and age-matched normal subjects. These subjects were selected in a case–control design from a larger cohort earlier described.[19] Characteristics of the control group are shown in Table 1.

Methods

At screening, all patients underwent a full physical examination and electrocardiography. Blood was drawn for determination of liver function and renal function. To estimate GFR, the MDRD–GFR equation was used.[20]

After a 4-week run-in phase, patients were randomized to treatment with either placebo for 16 weeks, followed by pioglitazone (45 mg/day) for 16 weeks, or treatment with pioglitazone for 16 weeks, followed by placebo for 16 weeks in a randomized

Table 1 Characteristics of patients and controls

	CAH patients	Control subjects
Gender (M/F)	5/7	5/7
Age (yrs)	35.7 ± 8.9	37.6 ± 6.5
Height (m)	1.64 ± 0.11	1.73 ± 0.09
Weight (kg)	72.6 ± 15.1	79.5 ± 14.3
BMI (kg/m^2)	26.9 ± 4.7	26.6 ± 5.1
Waist (cm)	87.1 ± 10.7	
Hip (cm)	101.3 ± 10.5	
Waist/hip	0.86 ± 0.09	
Systolic blood pressure (mmHg)		
Supine	134 ± 14	124 ± 11
Upright	125 ± 12	
Diastolic blood pressure (mmHg)		
Supine	83 ± 8	78 ± 12
Upright	87 ± 11	
Heart rate (bpm)		
Supine	68 ± 6	70 ± 10
Upright	77 ± 8	

M=male, F=female, bpm=beats per minute

crossover design. Patients visited the clinic every 2 months for medication checks, adverse events, and measurement of weight, blood pressure, and edema formation. Safety measures included assessment of weight, blood pressure, and edema. At the end of the respective study periods, fasting glucose, lipid, and hormone levels (insulin, ACTH, cortisol, aldosterone, renin, 17-OH progesterone and androstenedione in serum, and 17-OH progesterone and androstenedione in saliva in the morning, afternoon, and evening) were assessed and insulin sensitivity was measured using a euglycemic hyperinsulinemic (steady-state plasma insulin level ~600 pmol/l) clamp. Glucose tolerance was measured using an oral 75-g glucose load. Blood glucose and insulin samples were collected at 30, 60, 90, and 120 min after the test load. Glucose tolerance was evaluated using the criteria of WHO.[21] Body fat distribution was assessed by two different methods, magnetic resonance imaging (MRI) and DEXA scanning. Total body DEXA scanning was performed using a Hologic QDR 4500 densitometer (Hologic, Bedford, MA, USA) to determine total bone mineral content (BMC, g), total areal bone mineral density (g/cm^2), fat mass, and lean mass. Twenty-four-hour ambulatory blood pressure measurement was performed using the

Table 2 Age, gender, phenotype, mutation analysis, medication and mean salivary androstenedione levels in 12 included congenital adrenal hyperplasia (CAH) patients

P	Age (years)	Gender	Phenotype[a]	Allele 1[b]	Allele 2[b]	BMI (kg/m²)
1	28	F	non-classic	c.841G>T (p.Val281Leu)	c.841G>T (p.Val281Leu) c.920-921insT(c.923dup) c.952C>T (p.Gln318X) c.1066C>T(p.Arg356Trp)	19.2
2	37	F	SW	del/conv	del/conv	37.6
3	33	M	SW	del/conv	c.290-13A/C>G	29.9
4	52	M	SV	c.515T>A (p.Ile172Asn)	del/conv	29.6
5	45	M	SV	c.290-13A/C>G	del/conv	26.2
6	28	F	SW	del/conv	c.IVS2-13A/C>G	25.7
7	27	F	SW	c.290-13A/C>G	c.329-336del (p.Gly110fs)	23.4
8	42	F	SW	c.290-13A/C>G	c.952C>T (p.Gln318X)	29.5
9	28	F	SW	c.1066C>T (p.Arg356Trp)	c.1066C>T (p.Arg356Trp)	24.5
10	28	M	SW	del/conv	del/conv	23.6
11	48	M	SV	del/conv	c.515T>A (p.Ile172Asn)	24.4
12	32	F	SW	c.707T>A (p.Ile236Asn), c.710T>A (p.Val237Glu), c.716T>A (p.Met239Lys)	del/conv	29.5

P = patient number; F = female; M = male; del = deletion; conv = conversion; HC = hydrocortisone; DXM = dexamethasone
[a]SW = classic salt wasting CAH; SV = classic simple virilising CAH
[b]Nucleotides are numbered according to the HGVS guidelines using Genbank entry NM_000500.5 as reference sequence, where the A of the first ATG is 1. The variable Leucine stretch in exon 1 was counted as 4, not 5 Leucines to comply with the widely accepted numbering used in the literature.
[c]Mineralocorticoid medication (9-α-fluorohydrocortisone acetate) was taken in one to two doses.
[d]Salivary levels of androstenedione are mean levels from six samples (see *Materials and Methods)*

ambulatory SpaceLabs model 90207 blood pressure monitoring system. Mean salivary androstenedione levels were calculated from six samples: three samples after placebo treatment and three samples after pioglitazone treatment (same time points as after placebo treatment). Undertreatment was defined as the presence of a mean level of salivary androstenedione above the upper reference morning (0800 h) level, i.e. more than 0.63 nmol/l in males and 1.1 nmol/l in females. Overtreatment

Daily glucocorticoid therapy	Daily mineralocorticoid therapy[c]	Mean salivary androstenedione levels (nmol/l)[d]
HC: 20 – 5 mg (8 a.m. – 11 p.m.)	-	0.07
HC: 25 – 10 mg (9 a.m. – 5 p.m.)	0.1 mg	0.08
HC: 15 – 5 – 7.5 mg (9.00 a.m. – 0.30 p.m. – 11.30 p.m.)	0.2 mg	0.51
HC: 17.5 – 10 mg (7.30 a.m. – 6.30 p.m.)	-	0.83
HC: 10 – 10 mg (8 a.m. – 11 p.m.)	0.0625 mg	0.14
HC: 6 – 4 – 4 mg (08 a.m. – 14 a.m. – 23 p.m.)	0.0625 mg	0.02
HC: 6 – 6 – 6 mg (6.30 a.m. – 4 p.m. – 10 p.m.)	0.1 mg	0.18
HC: 17.5 – 15 mg / HC: 15 – 15 mg (every other day, 6.30 a.m. – 5.30 p.m.)	0.150 mg	0.17
HC: 10 – 10 mg (8.30 a.m. – 10.30 p.m.)	0.0625 mg – 0.125 mg (every other day)	0.03
HC: 10 – 15 mg (6 a.m. – 9 p.m.), DXM: 0.25 mg (ante noctem)	0.4 mg	0.08
HC: 15 – 5 mg (8.30 a.m. – 11.30 p.m.)	-	0.50
DXM: 0.1 – 0.1 mg (7.30 a.m. – 11 p.m)	0.3 mg	0.44

was defined as the presence of a mean level of salivary androstenedione below the lower reference morning (0800 h) level, i.e. lower than 0.14 nmol/l in males and 0.16 nmol/l in females.

In control subjects insulin sensitivity was measured using the same euglycemic hyperinsulinemic clamp procedure as in CAH patients.

Procedures

Euglycemic clamp

Experiments were performed in the morning after an overnight (10 h) fast. Two i.v. cannula were inserted. One positioned retrogradely into a dorsal vein of the hand that was placed in a Plexiglas box, ventilated with heated air, for sampling of arterialized venous blood.[22] The second cannula was inserted in an antecubital vein of the contralateral arm for infusion of insulin and glucose. Insulin (Actrapid, NovoNordisk, Bagsvaerd, Denmark; diluted in NaCl of 0.9% to a concentration of 1 U/ml, with the addition of 2 ml whole blood per 50 ml) was infused at a rate of 60 mU/min per m^2 body surface area (360 pmol/min per m^2). Arterialized venous plasma glucose was measured in duplicate at 5-min intervals by the glucose oxidation method (Beckman Glucose Analyser II, Beckman, Fullerton, CA, USA). Plasma glucose was clamped at the fasting level by a variable infusion of glucose 20% solution. Plasma insulin was measured in all samples with an in-house RIA at the start of the clamp procedure and after 90 and 120 min.[23] Whole body glucose disposal was determined by glucose infusion rate (GIR) as: (mean glucose infusion 90–120 min (mg/min)/weight (kg))×(1000/180 g/mol) µmol/min per kg.

Magnetic resonance imaging and spectroscopy

Liver fat content and abdominal fat distribution were determined by proton magnetic resonance spectroscopy (MRS) and imaging (MRI) on a clinical 3T whole body MR system (Siemens Magnetom Tim Trio, Erlangen, Germany). All measurements were carried out during breath-holding for 15 s. Liver fat content was determined by single-voxel proton MR spectra with STEAM localization (echo time 20 ms; repetition time 3 s). Hepatic fat percentage was calculated without correction for differences in relaxation times by dividing the lipid methylene signal intensity at 1.3 ppm by the sum of the methylene lipid and water signal intensities and multiplying the result by 100. Abdominal fat distribution was derived from 16 T1-weighted FLASH-2D axial MR images from a region extending from 4 cm above to 4 cm below the fourth and fifth lumbar interspaces. Subcutaneous and visceral fat volumes were determined based on signal intensity.

Statistical analysis

For calculation and statistical analyses, the SPSS personal computer software package was used (SPSS Inc., Chicago, IL, USA) and $P<0.05$ was considered statistically significant. Differences between pioglitazone and placebo were statistically tested using Student's *t*-test or the Wilcoxon–Mann–Whitney test as appropriate. According to power calculations, detecting a 20% change in insulin sensitivity with a power of 80% at a significance level of 0.05 would require 11 subjects completing the study.

Results

A total of 12 CAH patients completed the study, seven females and five males. All patients had a biochemically and genetically proven CAH and were treated with stable corticosteroid doses for at least 3 months. Eight patients had the classic salt wasting type of CAH, three patients had the classic simple virilizing type of CAH and one patient had a nonclassical type of CAH. Evaluation of mean salivary androstenedione levels showed that six patients were adequately treated, five patients were over-treated and one patient was undertreated (data shown in Table 2).

Insulin sensitivity

CAH patients were insulin resistant compared with the control group using the euglycemic clamp procedure (GIR 28.5±11.6 vs 46.2±23.4 µmol/kg per min, CAH patients versus controls respectively, *P*=0.04, Fig. 1). Similarly, the insulin sensitivity index in CAH patients was significantly lower compared with control subjects (0.35±0.16 vs 0.56±0.30 µmol/kg per min (mU/l), *P*=0.03).

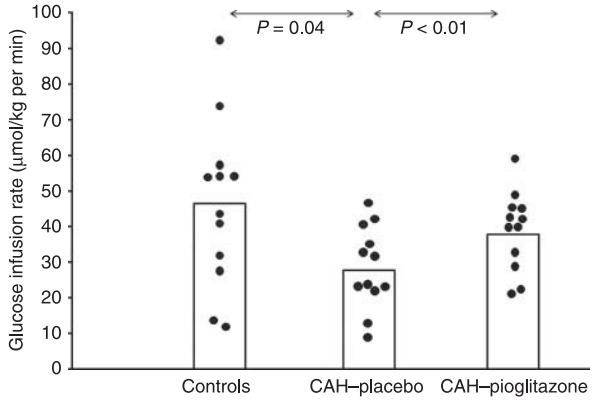

Figure 1 Glucose infusion rate (GIR, µmol/kg per min) in control subjects, CAH patients during placebo treatment and CAH patients during pioglitazone treatment, measured during a euglycemic clamp procedure. Mean values for each cluster are shown by the different boxes.

During treatment with pioglitazone, GIR significantly increased compared with placebo treatment (38.9±11.0 vs 28.5±11.6 µmol/kg per min, *P*=0.000, Fig. 1). Similarly, insulin sensitivity index improved during pioglitazone treatment compared with placebo (0.53±0.16 vs 0.35±0.16 µmol/kg per min (mU/l, *P*<0.001).[24] A non-significant decrease in homeostasis model assessment (HOMA)-estimated insulin

resistance (HOMA-IR) was observed after the use of pioglitazone (1.97±1.40 vs 1.80±0.99, *P*=0.34).[25] Plasma glucose and insulin levels during the clamps were similar during both treatments and in both groups.

Oral glucose tolerance test (OGTT) showed no significant differences in glucose levels after the use of pioglitazone compared to placebo. Insulin levels were lower, although nonsignificantly, after the use of pioglitazone. The $insulin_{AUC}$(mU/l per h) for the whole test period (0–120 min) was significantly lower after the use of pioglitazone compared with placebo (81±33 vs 111±69 mU/l per h, *P*<0.05), with no significant difference in $glucose_{AUC}$ (mmol/l per h) for the whole test period (13.8±2.5 vs 13.1±3.1 mlU/l per h, *P*=0.31).

Body fat distribution

The MRI–MRS data showed no statistically significant changes in subcutaneous fat, visceral fat, the visceral–subcutaneous fat ratio, and the percentage of liver fat after the use of pioglitazone compared to placebo (Table 3). Most patients had relatively low amounts of liver fat. The only patients with a high liver fat content during placebo (32.7%) showed a strong decrease in the percentage of liver fat after the use of pioglitazone (19.3%). Other patients did not show a decrease in the percentage of liver fat after the use of pioglitazone.

Table 3 Magnetic resonance imaging-magnetic resonance spectroscopy measurements of fat distribution, during placebo or pioglitazone in congenital adrenal hyperplasia patients

	Placebo	Pioglitazone	*P*-value
Subcutaneous fat (l)	1.98 ± 0.83	2.00 ± 0.75	0.76
Visceral fat (l)	0.58 ± 0.30	0.58 ± 0.25	0.93
Visceral:subcutaneous fat ratio	0.3 ± 0.2	0.3 ± 0.2	0.45
% liver fat	4.2 ± 9.0	3.6 ± 5.6	0.65

DEXA scanning showed no relevant changes in total body BMC, total lean mass, total fat mass, lean and fat mass in trunk, arms and legs, and the distribution of the fat mass after use of placebo or pioglitazone compared to baseline data.

Blood pressure profiles

Ambulatory 24-h blood pressure was significantly lower during pioglitazone compared with placebo treatment (systolic: 124.0±13.6 vs 127.0±14.9 mmHg, *P*<0.001, diastolic: 72.8±11.5 vs 77.4±12.6 mmHg, *P*<0.001, Fig. 2 and Table 4).

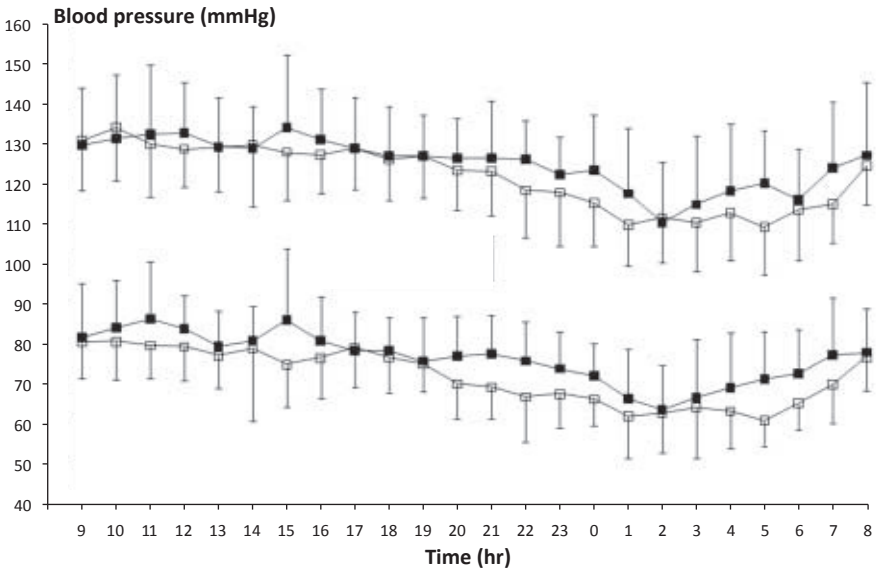

Figure 2 Twenty-four-hour ambulatory blood pressure during pioglitazone (open squares) or placebo treatment (closed squares) in CAH patients.

Clinical characteristics, lipid profile, and steroid profile

Clinical characteristics, like weight, BMI, waist, hip, and waist/hip ratio were not significantly different after the use of placebo or pioglitazone. No significant changes in lipid profile and levels of steroids, ACTH, and renin were observed after the use of pioglitazone compared to placebo.

Table 4 Twenty-four-hour ambulatory blood pressure during pioglitazone or placebo treatment in congenital adrenal hyperplasia patients

	Placebo	Pioglitazone	*P*-value
24-h SBP	127.0 ± 14.9	124.0 ± 13.6	0.000
24-h DBP	77.4 ± 12.6	72.8 ± 11.5	0.000
24-h HR	77.1 ± 13.9	75.1 ± 13.5	0.007
24-h MAP	93.4 ± 12.5	89.4 ± 11.1	0.000
Daytime SBP	129.9 ± 13.4	127.7 ± 12.1	0.017
Daytime DPB	79.9 ± 11.5	75.3 ± 11.0	0.000
Daytime HR	78.9 ± 12.9	78.1 ± 12.9	0.396
Daytime MAP	95.9 ± 11.3	91.9 ± 10.3	0.000
Nighttime SBP	116.5 ± 15.0	111.8 ± 11.9	0.010
Nighttime DBP	68.3 ± 12.6	62.6 ± 9.2	0.000
Nighttime HR	70.2 ± 13.3	66.6 ± 10.0	0.048
Nighttime MAP	84.3 ± 12.6	79.5 ± 9.2	0.000
Nocturnal drop in SBP (% fall)	10.7	12.2	0.341
Nocturnal drop in DBP (% fall)	14.5	16.5	0.390

SBP = systolic blood pressure, DBP = diastolic blood pressure, HR = heart rate, MAP = mean arterial pressure

Discussion

The main findings of the present study are that CAH patients who are chronically treated with glucocorticoids are insulin resistant as compared with body mass and age-matched normal subjects and that treatment with pioglitazone improves insulin sensitivity in this group of patients.

The finding that CAH is associated with insulin resistance is in line with earlier studies. These prior studies suggest that both overproduction of androgens and treatment with (supraphysiological doses of) glucocorticoids can induce insulin resistance.[5-11,26,27]

In our study, we document a significantly lower insulin sensitivity index and GIR in adult CAH patients who were chronically treated with glucocorticoids. The observed insulin resistance may well be the result of treatment with glucocorticoids.

Glucocorticoids are known to induce insulin resistance, although the mechanisms involved are incompletely understood.[28] Supraphysiological doses of glucocorticoids also increase lipolysis and plasma free fatty acids (FFA), which may lead to insulin resistance in muscles.[16,29]

Insulin resistance is known to be a risk factor for the development of cardiovascular disease and to precede the development of type 2 diabetes.[30,31] Therefore, CAH patients might be at risk to develop type 2 diabetes or cardiovascular disease later in life. Because CAH patients need to continue glucocorticoid treatment to suppress overproduction of androgens and as substitution of cortisol, we reasoned that an intervention with insulin-sensitizing drugs, like TZDs, might have a favorable effect on insulin sensitivity in CAH patients and as a result improve their cardiovascular risk profile.[15]

We observed a significant improvement of insulin sensitivity (measured both by clamp and by insulin response during OGTT) in CAH patients treated with pioglitazone, although not to the level of the healthy control group. Pioglitazone is known for its ability to improve insulin sensitivity in several conditions associated with insulin resistance. It has been hypothesized that TZDs have an insulin-sensitizing effect because of alterations in adipokine release, which modulates insulin sensitivity outside adipose tissue.[15] TZDs also promote the uptake and the storage of fatty acids in adipose tissue, which does lead to an increase in adipose tissue mass, but in this way TZDs spare other insulin-sensitive tissues as the liver and the skeletal muscle form the harmful metabolic effect of high concentrations of FFA.[15]

During pioglitazone treatment, ambulatory 24-h systolic and diastolic blood pressures decreased significantly. It has been noticed before that TZDs can improve blood pressure profiles, although the exact mechanism is not yet known. Insulin resistance is known to be a risk factor for the development of hypertension, so one may hypothesize that improvement of insulin sensitivity may also decrease blood pressure. Insulin resistance contributes to the development of hypertension through several mechanisms like angiotensin II and aldosterone actions, enhanced sympathetic nervous system activity, dyslipidemia, atherosclerosis, left ventricular hypertrophy and changes in renal function and structure, like glomerulosclerosis.[32] The anti-hypertensive effects of pioglitazone have been studied in Japanese male patients with type 2 diabetes.[33] This study showed a significant decrease in mean blood pressure (109±14 to 101±10 mmHg) after 3 months of treatment with pioglitazone (30 mg/day). Results of the present study are in line with these findings. Anti-hypertensive effects of pioglitazone have also been described in type 2 diabetics with difficult-to-control hypertension, nondipping diabetic patients, and in diet-induced obese rats.[34-36]

Although our study shows favorable effects of pioglitazone on insulin sensitivity and blood pressure in adult CAH patients, it has become uncertain whether pioglitazone is a valuable addition to current treatment strategies. Recent studies

have questioned the long-term safety of TZDs, especially with respect to long-term cardiovascular outcome. Frequently noticed side effects of TZDs include edema, weight gain, macular edema, and heart failure. Furthermore, TZDs tend to increase low-density lipoprotein cholesterol levels.[37] Rosiglitazone may even be associated with a higher risk of myocardial infarction and death due to cardiovascular causes,[38] but the RECORD study only confirmed the increased risk of heart failure.[39] The PROactive Study showed favorable effects of pioglitazone on reduction of all-cause mortality, nonfatal myocardial infarction, and stroke in type 2 diabetes patients.[40] Longer follow-up of treatment with TZDs is needed to evaluate their effects on congestive heart failure and cardiovascular death.[41] Treatment with TZDs also decreases bone formation and bone mass, possibly as a result of promoting adipogenesis above osteoblastogenesis.[42] This side effect seems particularly relevant in our group of patients, against the background of the already ongoing adverse effect of corticosteroids on the bone. Over this short treatment period, we did not observe any changes in BMC during treatment with pioglitazone. Nevertheless, with the currently available data on adverse effects of TZDs, the routine use of pioglitazone in CAH patients seems not to be indicated in a clinical setting.

Our study has limitations. Inherent to the short 16-week treatment period, we were not able to evaluate long-term effects of pioglitazone in our population. Furthermore, insulin sensitivity was used as a surrogate outcome, but no data on the prevalence of diabetes mellitus or cardiovascular disease are available. Furthermore, our study was probably underpowered to detect changes in fat distribution, fat mass, and liver fat.

In summary, this study shows that adult CAH patients treated with glucocorticoids are insulin resistant. Treatment with pioglitazone significantly improves insulin sensitivity and decreases blood pressure in CAH patients compared with the use of placebo. Despite these findings, there is currently not enough evidence to warrant the routine use of pioglitazone in this group of patients.

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CHAPTER 3

CARDIOVASCULAR AND METABOLIC RISK PROFILE AND CARDIAC FUNCTION IN PEDIATRIC PATIENTS WITH CONGENITAL ADRENAL HYPERPLASIA

CHAPTER 3.1

Blood pressure in the first year of life in children with congenital adrenal hyperplasia due to 21-hydroxylase deficiency: a pilot study

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Abstract

Aims: Evaluation of blood pressure in the first year of life in children with congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency.

Methods: Twenty-four children were included. Retrospective blood pressure values, fludrocortisone dosages, and serum renin, 17-hydroxyprogesterone (17-OHP) and androstenedione levels in the first year of life were evaluated. Blood pressure values were compared to reference values. Correlations between blood pressures and serum renin levels, and the dosage of fludrocortisone were calculated.

Results: Mean peak systolic blood pressure values were generally not elevated, most values were around the 50th percentile, except incidentally higher mean peak systolic blood pressure values most below the 95th percentile. No significant correlations between blood pressure and serum renin, androstenedione and 17-OHP levels and fludrocortisone dosage were found.

Conclusion: In this pilot study in CAH patients, blood pressure values do not seem to be elevated in the first year of life. Further investigations are necessary to evaluate blood pressure in the first year of life in CAH patients in more detail.

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder of adrenal steroidogenesis. In 95% of cases, it is caused by 21-hydroxylase deficiency.[1,2] A deficiency in 21-hydroxylase results in impaired adrenal synthesis of cortisol, and often aldosterone as well, leading to increased secretion of ACTH by the pituitary gland, and consequently adrenal hyperplasia and excessive production of adrenal androgens. In utero, the increased synthesis of adrenal androgens leads to virilization of the external female genitalia. Medical treatment consists of substitution of glucocorticoids and mineralocorticoids with resulting suppression of ACTH production and thereby adrenal androgen production. In most patients, supraphysiological dosages of glucocorticoids are necessary to suppress adrenal androgen production.

Nowadays, the classic type of CAH is generally diagnosed in the first weeks of life as a result of the neonatal screening program, and most children reach adulthood without serious complications. However, it has been suggested that treatment with supraphysiological dosages of glucocorticoids and mineralocorticoids may lead to hypertension, obesity and other cardiovascular risk factors.[3] Studies on blood pressure in children with CAH have been performed mostly in children older than 5 years of age.[4-8] In some of these studies, a tendency towards hypertension in pediatric CAH patients was shown. Studies on the incidence of hypertension in CAH patients in the first year of life are lacking.

The aim of this retrospective study was to evaluate the blood pressure in the first year of life in patients with the classical types of CAH (salt wasting, SW, and simple virilizing, SV) due to 21-hydroxylase deficiency. Furthermore, we evaluated the correlations between the blood pressure and serum renin levels, 17-hydroxyprogesterone (17-OHP) and androstenedione levels and dosage of fludrocortisone.

Patients and Methods

Patients

All patients who were born after the introduction of the neonatal screening on CAH in the Netherlands in 2000 and who were treated in the Radboud University Nijmegen Medical Centre because of a classic type of CAH (SW or SV) due to 21-hydroxylase deficiency were retrospectively studied. Diagnosis was confirmed by mutation analysis.[9] All patients were treated according to the local CAH treatment protocol: in the neonatal period medical treatment was started with hydrocortisone 3 mg per day in 3 doses (neonates 2–5 kg) and fludrocortisone 30 µg up to 62.5 µg twice a day. The dosage fludrocortisone was further increased when SW was suspected. The dosage of fludrocortisone and salt supplementation was further adapted to weekly measured

serum sodium and serum renin concentrations in the first weeks of life. The dosage of hydrocortisone was generally not changed until the age of 8–9 months. All patients received NaCl supplementation from the neonatal period up to 12 months of life (starting dosage NaCl 10%: 2 ml/kg/day).

Data Collection

Data on blood pressure measurements, serum renin, 17-OHP and androstenedione concentrations and fludrocortisone dosage during the first year of life were collected. Blood pressure measurements were performed using a Dinamap Pro 300 vital sign monitor (GE Healthcare, Finland) or incidentally by manual cuff blood pressure recording during clinical visits to the outpatient clinic. The blood pressure was measured by an experienced nurse with parents calming or feeding the child. When the child could not be calmed, we measured the blood pressure again at the end of the clinical visit when the infant was calmed or sleeping.

The mean arterial blood pressure (MAP) was calculated using the formula MAP = [(2 × diastolic pressure) + systolic pressure]/3.

Statistical Analysis

The collected data on blood pressures during the first year of life were compressed into 8 time periods (weeks 0–2, 3–8, 9–16, 17–24, 25–32, 33–40, 41–48 and 49–52) and presented as mean peak values.

SPSS (Windows, v. 16.0) was used for all statistical analyses. To compare peak systolic blood pressure values with reference values the one-sample t test was used. Blood pressure values were compared with reference values of 50th and 95th percentile systolic blood pressures as described by the Second Task Force on Blood Pressure Control in Children and by Flynn.[10,11] Correlation coefficients (c.c.) between blood pressure values (systolic, diastolic and MAP) on the one hand, and serum renin, 17-OHP and androstenedione concentrations and fludrocortisone dosage on the other hand were calculated using Spearman's rho. A value of $p < 0.05$ was considered significant (2-sided). Because of the rapidly changing reference values in the first 8 weeks of life, further statistical analyses were not performed in this period in our small population.

Results

Twenty-four patients were included in the study: 14 females and 10 males. All females presented with CAH at birth with ambiguous genitalia, except 1 patient (No. 22) who presented 17 days after birth with a positive neonatal screening. She had a mild enlargement of the clitoris that had not been noticed before. All males were diagnosed

Table 1 Gender, age at presentation, mutation analyses and type of CAH

Patient	Gender	Age at presentation (days)	DNA analysis allele 1	DNA analysis allele 2	CAH-type
1	F	0	del/conv	c.293-13A/C>G	SV/SW
2	F	0	del/conv	c.293-13A/C>G	SV/SW
3	F	0	c.518T>A (p.Ile173Asn)	c.1069C>T (p.Arg357Trp)	SV
4	F	0	del/conv	del/conv	SW
5	M	9	del/conv	c.293-13A/C>G	SV/SW
6	M	9	del/conv	c.293-13A/C>G	SV/SW
7	M	8	del/conv	c.293-13A/C>G	SV/SW
8	F	0	del/conv	c.293-13A/C>G	SV/SW
9	F	0	del/conv	c.1069C>T (p.Arg357Trp)	SW
10	M	8	c.293-13A/C>G	c.518T>A (p.Ile173Asn)	SV
11	M	6	del/conv	c.1451G>C (p.Arg484Pro)	SV/SW
12	M	9	c.1069C>T (p.Arg357Trp)	c.1069C>T (p.Arg357Trp)	SW
13	M	6	c.293-13A/C>G	c.332-339del8 (p.Gly111fs)	SV/SW
14	F	0	c.293-13A/C>G	c.293-13A/C>G	SV/SW
15	M	13	del/conv	c.293-13A/C>G	SV/SW
16	F	0	c.293-13A/C>G	c.293-13A/C>G	SV/SW
17	F	0	del/conv	c.1451G>C (p.Arg484Pro)	SV/SW
18	M	8	del/conv	c.955C>T (p.Gln319X)	SW
19	F	0	del/conv	c.332-339del8 (p.Gly111fs)	SW
20	M	7	c.332-339del8 (p.Gly111fs)	c.955C>T (p.Gln319X)	SW
21	F	0	c.293-13A/C>G	c.293-13A/C>G	SV/SW
22	F	17	c.518T>A (p.Ile173Asn)	c.518T>A (p.Ile173Asn) + c.710T>A (p.Ile237Asn) + c.713T>A (p.Val238Glu) + c.719T>A (p.Met240Lys) + c.844G>T (p.Val282Leu) + c.923dup (p.Leu308fs)	SV
23	F	0	c.293-13A/C>G	c.710T>A (p.Ile237Asn)+ c.713T>A (p.Val238Glu)+ c.719T>A (p.Met240Lys)	SV/SW
24	F	0	c.293-13A/C>G	c.332-339del8 (p.Gly111fs)	SW

DNA-mutation analyses performed as described by Higashi et al.[9]

with a positive neonatal screening and had no clinical symptoms. None of our patients developed hyponatremia. All patients started treatment with hydrocortisone and fludrocortisone within the first 2 weeks of life, with exception of patient No. 22 who was treated from day 18. The gender, age at presentation and mutation analysis of all patients are shown in table 1. Three patients had the SV genotype of CAH, 7 patients had the SW genotype and 14 patients had a genotype with a residual enzyme activity of 1–5%, usually described as SV-SW CAH.[2] Data on body weight, renin levels and dosage of fludrocortisone during the first year of life are shown in table 2.

A total of 189 blood pressure measurements in all 24 patients during the first year of life have been included in this study. Mean peak systolic blood pressure values for male and female CAH patients are shown in figure 1. Mean peak systolic blood pressure values were within the normal range, most measured around the 50th percentile, except mean peak systolic blood pressure values in females in weeks 9–16 ($p = 0.035$). Rapidly changing variable blood pressure values were measured in the first 8 weeks of life.

Table 2 Means (ranges) for weight, renin levels and daily dosage of fludrocortisone during the first year of life in 24 CAH patients

Time period (weeks)	Weight, kg	Renin level, mE/l	Daily dosage of fludrocortisone, µg
0-2	3.44 (2.73–4.48)	1,368 (37–16,136)	140 (62.5–200)
3-8	4.93 (3.19–5.35)	308 (3–1,808)	150 (62.5–187.5)
9-16	6.02 (4.36–7.08)	104 (3–562)	130 (62.5–200)
17-24	7.29 (5.10–8.60)	83 (3–426)	110 (50–187.5)
25-32	8.05 (5.59–9.40)	213 (3–1,113)	110 (50–150)
33-40	8.78 (6.30–11.0)	176 (3–585)	110 (50–150)
41-48	9.28 (6.70–11.1)	232 (3–988)	120 (60–200)
49-52	9.85 (7.70–12.1)	211 (3–1,379)	100 (60–150)

A positive correlation between mean peak systolic (c.c. 0.606, $p = 0.048$) and mean peak diastolic (c.c. 0.723, $p = 0.018$) blood pressures and serum renin concentrations in week 0–2 and a negative correlation between mean peak systolic blood pressure and serum renin concentrations in week 25–32 (–0.554, $p = 0.026$) were found. In all other time periods, there were no significant correlations between mean peak systolic or diastolic blood pressure values and renin levels (data not shown). No significant correlation was found between MAP, systolic or diastolic blood pressure

on the one hand and dosage fludrocortisone, serum 17-OHP and androstenedione concentrations on the other hand.

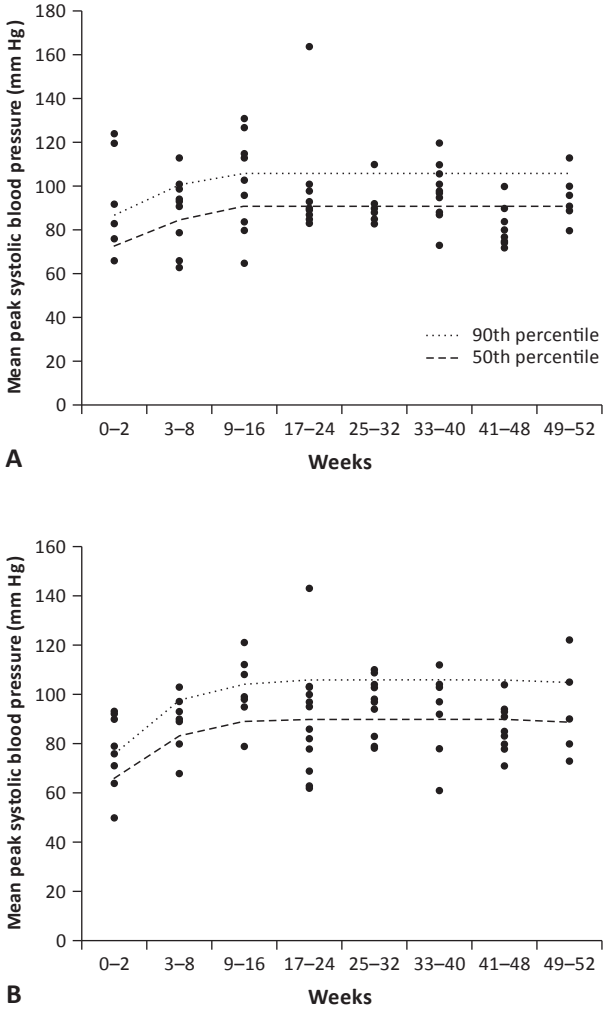

Figure 1 Mean peak systolic blood pressure values of male (**A**) and female (**B**) CAH patients in the first year of life.

Discussion

This report is the first to study blood pressure in the first year of life in children with CAH due to 21-hydroxylase deficiency. This pilot study shows that blood pressure values in the first year of life measured in our patient population are not significantly elevated compared with reference values.

Studies on the incidence of hypertension in older children with CAH showed variable outcomes. In one study, de Silva et al. showed that none of the CAH patients (n = 11, age 8.5–27.2 years, mean age 14.5 years) had a blood pressure above the 95th percentile, using a 24-hour ambulatory blood pressure measurement.[4] Roche et al. concluded that CAH patients (n = 38, age 6.1–18.2 years) had significantly elevated mean systolic blood pressures, and 58% of the investigated patients had systolic hypertension defined as systolic blood pressure measurements ≥95th percentile for age and sex.[5] Völkl et al. showed altered 24-hour blood pressure profiles with elevated systolic blood pressure values in CAH patients (n = 55, age 5.3–19.0 years).[6] Hoepffner et al. found average blood pressure values in the upper normal range in pediatric CAH patients (n = 23, age 6–17 years).[7] Only one study by Nebesio and Eugster focused also on blood pressure in children younger than 5 years of age.[8] That study showed higher blood pressure levels in children with CAH. Five out of 91 (5.5%) CAH patients had hypertension defined as blood pressure >95th percentile for age and gender (age 58 days to 12.6 years). The differences in outcome in these studies may be explained by the choice of method, age and type of CAH, differences in dosage of hydrocortisone and fludrocortisone, and other unknown factors that may influence the blood pressure in the study population.

Several risk factors may contribute to changes in blood pressure in children with CAH already in the first year of life. It is well known that mineralocorticoid excess leads to hypertension, e.g. in patients with primary aldosteronism or types of CAH with elevated levels of steroid metabolites with mineralocorticoid activity such as 11-hydroxylase deficiency.[12] Therefore, excessive treatment with mineralocorticoids might lead to elevated blood pressure. However, in our study we did not find any correlation between blood pressure on the one hand and type of CAH or fludrocortisone dosage on the other hand. These findings are in line with other studies on blood pressure values in pediatric CAH patients who showed no correlation between dosage of fludrocortisone and blood pressure levels either.[5-8] In our population, we measured incidentally elevated blood pressure in patients with decreasing renin levels. The absence of correlation between serum renin levels and blood pressure may be explained by the fact that we adapted the dosage of fludrocortisone very quickly when serum renin levels decreased or blood pressure increased. Therefore, normal blood pressure values as observed in our study may be the result of adaptation of the dosage of fludrocortisone to serum renin and blood pressure levels.

Treatment with supraphysiological doses of glucocorticoids may also play a role in the development of high blood pressure in CAH patients as glucocorticoid excess,[13] e.g. in patients with Cushing's syndrome, is known to result in high blood pressure as well.[14] Therefore, monitoring of the glucocorticoid treatment is essential not only to avoid undertreatment but also to detect overtreatment.

Our study has several limitations. A limited number of patients were included, complicating statistical analyses in subgroups of patients. Blood pressure measurements and laboratory examinations were not performed following a standardized study protocol. However, even considering suboptimal circumstances (e.g. stress during the measurements) that would probably lead to higher blood pressures, most blood pressure measurements were within normal range. A 24-hour blood pressure measurement giving more detailed information about diurnal changes was not performed, as ambulatory blood pressure measurement in children below 1 year is not possible. This retrospective study did not allow us to precisely study the correlation between blood pressure and mineralocorticoid or glucocorticoid dosage. Therefore, further prospective studies are needed to evaluate more accurately the incidence and consequences of hypertension in the first year of life.

In summary, in this retrospective pilot study, children with CAH did not show significantly elevated blood pressures in the first year of life. The correlation between blood pressure levels in the first year of life and the dosage of fludrocortisone and glucocorticoids should be carefully evaluated in prospective studies in order to detect and consequently prevent early cardiovascular risk factors in CAH children.

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CHAPTER 3.2

Cardiovascular and metabolic risk in pediatric patients with congenital adrenal hyperplasia due to 21 hydroxylase deficiency

Mooij CF, van Herwaarden AE, Sweep FCGJ, Roeleveld N, de Korte CL, Kapusta L, Claahsen-van der Grinten HL.
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Abstract

Background: The aim of the study was to evaluate the cardiovascular and metabolic risk profile in pediatric patients with congenital adrenal hyperplasia (CAH).

Methods: A cross-sectional study was performed in 27 CAH patients (8–16 years). Blood samples were taken to evaluate circulating cardiovascular risk (CVR) markers. Insulin resistance (IR) was evaluated by homeostatic model assessment (HOMA)-IR. Blood pressure (BP) was evaluated by office BP measurements and 24-h ambulatory BP measurements (24-h ABPM). Dual energy X-ray absorptiometry (DXA) scans were performed in patients >12 years.

Results: Body mass index (BMI) standard deviation score (SDS) was elevated (0.67), with seven patients being overweight and four obese. DXA scans showed percentage body fat SDS of 1.59. Office BP levels were higher than reference values. Twenty-four hour ABPM showed systolic hypertension (n=5), while 11 patients had a non-dipping BP profile. HOMA-IR was >75th percentile in 12 patients.

Conclusions: CAH patients develop an unfavorable CVR profile already in childhood with increased BMI, increased fat mass, elevated BP levels, a non-dipping BP profile and IR compared to population reference values.

Introduction

Studies in adult patients with congenital adrenal hyperplasia (CAH) showed that they seem to have a high risk of clustering of a number of cardiovascular risk factors: elevated blood pressure (BP), insulin resistance (IR), increased body mass index (BMI), increased fat mass [1-9] and an increased intima-media thickness (IMT).[10,11] Data on cardiovascular and metabolic risk factors in pediatric CAH patients are relatively scarce. Previous studies showed that pediatric CAH patients may already show signs of elevated BP, elevated BMI, increased fat mass, IR, hyperlipidemia and an increased IMT.[4,5,9,12-21] The unfavorable changes in the cardiovascular risk profile may be due to the effects of both treatment (with supraphysiological doses of glucocorticoids and in most cases mineralocorticoids) and high androgen levels. However, most of the studies on cardiovascular risks in pediatric CAH patients were performed in small patient groups or focused on one or several risk factors only.

The aim of this cross-sectional study was to investigate the cardiovascular and metabolic risk profile in pediatric CAH patients due to 21-hydroxylase deficiency by evaluating multiple cardiovascular risk factors.

Patients and methods

Patients

CAH patients with biochemically and genetically proven CAH due to 21-hydroxylase deficiency, aged 8–16 years were invited to this study. Exclusion criteria were known co-morbidities, such as cardiac disease, renal disease, or co-medication that interferes with BP. For patients aged 8–12 years, their parents consented with the study protocol, while for patients aged 12–16, both patients and parents consented according to the recommendations of the Local Medical-Ethical Committee.

Methods

Each participant visited the hospital on two consecutive days, combined with a regular visit to the outpatient clinic. Data on the current hydrocortisone and fludrocortisones dosages were collected from the medical record of the patient.

On the first day, participants visited the hospital at 8.30 am after an overnight fast. Before taking their morning medication, blood was drawn to assess the concentrations of androstenedione, 17-OHP, fasting glucose, HbA_{1c}, insulin, lipid profile (total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides), renin, and selected circulating cardiovascular risk (CVR) markers (leptin, adiponectin, hsCRP, tPA, PAI-1, and tPA-PAI-1 complex).

Glucose, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides were measured using an Architect c16000 (Abbott, Wiesbaden, Germany). Renin was measured by a radioimmunoassay (RIA) (Renin III, CisBio, Codolet, France) and hsCRP on a BN II nephelometer (Siemens, The Hague, The Netherlands). Total adiponectin was measured by a RIA (Millipore, St. Charles, MO, USA) with a LOQ of 0.5 mg/L, an intra-assay CV <5%, and an inter-assay CV <9% in the whole range. Leptin was measured using a RIA (Millipore, St Charles, MO, USA) with a lower limit of quantitation of 0.5 g/L. The inter-assay variation of this assay was <6% for the whole concentration range. Androstenedione and 17-OHP were measured by an in-house LCMS-MS method. The inter-assay variations and LOQs were 4.1% (at 3.6 nmol/L) and 0.05 nmol/L (10% CV), respectively, for androstenedione and 3.7% (at 3.0 nmol/L) and 0.10 nmol/L, respectively, for 17-OHP. Plasma concentrations of tPA, PAI-1 and tPA-PAI-1 complex were assayed with enzyme-linked immunosorbent assay procedures as described before.[22,23] In-house laboratory reference values for adults were available for PAI-1 (14.9±10.0 ng/mL) and tPA (2.93±1.16 ng/mL).

IR was estimated using the homeostasis model assessment (HOMA) method (IR=insulin [µmol/mL]×glucose [mmol/L]/22.5).[24] HOMA-IR data obtained were compared to the commonly used reference ranges for normal weight and obese Caucasians.[25] A HOMA-IR above the 75th percentile has been suggested to be associated with an unfavorable cardiometabolic risk.[25] Fasting total plasma cholesterol, LDL cholesterol and triglycerides <75th percentile for age and sex were considered normal, values in the 75th through 95th percentile range as borderline, and values >95th percentile as elevated.[26] For leptin and adiponectin, we compared our results with the concentrations in healthy controls, aged 6–16 years, using the same laboratory test by calculating standard deviation scores (SDS).[27]

Anthropometric measurements: All participants received a complete physical examination including anthropometric measurements and hip and waist circumferences. BMI and height SDS scores were calculated using Dutch national reference data based on the 5th national growth study by TNO Child Health, Leiden, The Netherlands (https://groeiweb.pgdata.nl/calculator.asp). Patients with BMI SDS >1 were classified as being overweight, while patients with BMI SDS >2 were classified as obese. Hip and waist circumferences and waist/hip ratio were measured and compared to reference values for Dutch children as well.[28]

Evaluation of BP: Office BP was measured using a Dinamap vital signs monitor (GE Healthcare, Hoevelaken, The Netherlands). BP was measured 3 times in an upright position and mean supine office BP was calculated. The systolic and diastolic office BPs were corrected for age, sex and height and expressed as SDS using published reference data.[29] Systolic (SBP) and/or diastolic BPs (DBP) >95th percentile for sex, age and height was classified as hypertensive. BP values in the 90th through 95th percentile range were classified as “pre-hypertensive”. Subsequently, ambulatory BP

was monitored for 24 h using a SpaceLabs 90217-A monitor (SpaceLabs Healthcare, Snoqualmie, WA, USA). BP was measured every 15 min between 08.00 am and 08.00 pm, and once every hour between 08.00 pm and 08.00 am. During the 24 h BP measurements, patients and their parents registered physical activities and time of going to sleep and waking up in a diary. Sex-specific SDSs for both age and height were calculated for the 24-h ABPM data using the published algorithm and reference values.[30] For all patients, the dip in "sleeping" BP compared to daytime BP was calculated for SBP, DBP and mean arterial pressure (MAP). A non-dipping BP profile was defined as a BP decrease of less than 10% during sleep.[31]

Evaluation of intima media thickness: Ultrasound evaluation of the IMT of the left and right carotid artery was performed on one of the 2 days the participant visited the hospital by a specially trained ultrasound technician. We used a Vivid E9 for the IMT measurements of the common carotid artery (CCA) with a Linear array transducer (GE 9LD, GE, Vingmed Ultrasound, Horten, Norway). Acquisitions were made in the longitudinal view through the center of the lumen. IMT measurements of the CCA, within 2 cm of the bifurcation, were performed using the IMT module in the EchoPAC workstation version 112 (GE Medical Systems, Horten, Norway). One researcher (CFM) was trained and performed the offline analysis of the images, being double checked by an experienced pediatric cardiologist (LK). The results were compared to carotid IMT (cIMT) measurements in healthy German children.[32]

Evaluation of body composition: A dual energy X-ray absorptiometry (DXA) scan was performed to evaluate body composition in patients older than 12 years on one of the 2 days. A DXA total-body scanner (Hologic Discovery A type, Hologic Inc, Bedford, MA, USA) was used to obtain regional and whole body composition measurements using a three-compartment model of body composition: lean tissue mass (LTM), fat tissue mass (FTM) and bone mineral content (BMC). LTM, FTM and BMC were determined using software algorithms based on regression equations. Percentage of body fat was calculated using the formula: 100·FTM/(FTM+LTM+BMC). The data obtained were compared to available reference values for both Canadian and Dutch children by calculating Z-scores. Reference values in healthy Canadian children were obtained by Hologic densitometers for BMC, LTM and FTM. Z-scores were calculated using the published formula and reference values.[33] Reference values in healthy Dutch children measured by Lunar were obtained for bone mineral density (BMD), BMC, LTM and % body fat.[34] As a different fan-beam instrument was used in the latter study,[34] we converted our data using the known conversion factors from Hologic to Lunar prodigy,[35] before calculating Z-scores for BMC, LTM, and percentage body fat. Because the data in healthy Dutch children were obtained using a different fan-beam instrument, we compared our data to both the Canadian and converted Dutch reference values.

Statistical analysis

All data were collected and stored in a secure database (Castor, www.castoredc.com), complying with the regulations of the Local Medical-Ethical Committee. For the statistical analyses, the SPSS personal computer software package version 22 (IBM SPSS Inc., Chicago, IL, USA) was used. Using descriptive statistics, we checked whether the study variables were normally distributed. If not, we presented median values plus ranges. Otherwise, we calculated mean absolute and SDS values with 95% confidence intervals (CI). If the 95% CI for an SDS value did not contain the zero value, we considered the values in our cohort to be different from the reference values used. Linear regression analyses were performed to evaluate the associations between different cardiovascular risk markers, treatment (daily dosages of hydrocortisone and fludrocortisones), and disease control (levels of 17-OHP, androstenedione, and renin). B-coefficients with 95% CIs above or below 0 were considered to indicate relevant associations.

Results

Patient characteristics

A total of 27 patients (17 boys/10 girls), aged 8.8–16.0 years (median 11.7 years) participated in this study. Twenty-four patients were classified as salt wasting CAH patients, two as simple virilizing CAH patients, and one as a non-classic CAH patient. A complete description of the anthropomorphic evaluation and the biochemical analysis is given in Table 1. The B-coefficients showing the associations between the different parameters are shown in Table 2. BMI SDS for the cohort was clearly higher compared to the reference population (BMI SDS 0.67; 95% CI: 0.16–1.18), with seven patients (25.9%; four boys, three girls) being overweight and four patients (14.8%; three boys; one girl) being obese.[28] BMI SDS was positively associated with the 17-OHP and androstenedione concentrations.

BP profiles

The office BP measurements showed a mean SBP SDS of 0.83 (95% CI: 0.49–1.18) and a mean DBP SDS of 0.56 (95% CI: 0.34–0.78). Five of the patients (18.5%) had systolic hypertension and four patients (14.8%) were pre-hypertensive. None of the patients showed diastolic hypertension, but three patients (11.1%) had a pre-hypertensive DBP. Office SBP SDS and DBP SDS were not associated with BMI SDS, therapy control, treatment, or HOMA-IR.

The 24-h ABPM values for SBP and DBP and MAP are shown in Table 3. Mean 24-h SBP and DBP SDSs in the cohort were within the normal range compared to both age- and height-specific reference values for each sex, although the 24-h SBP SDS and DBP

Table 1 Patient characteristics, current dosages of hydrocortisone and fludrocortisone, and results of biochemical analysis of fasting pre-morning medication blood concentrations of androstenedione, 17-OHP, renin, and several circulating cardiovascular risk markers in a cohort of 27 pediatric CAH patients aged 8 – 16 years

	Mean (95% CI)	Minimum value	Maximum value
Anthropometric measure			
Height (cm)	156.0 (150.5 – 161.4)	131.8	183.1
Height SDS	-0.04 (-0.45 – 0.36)	-1.83	1.60
Target height SDS	-0.01 (-0.31 – 0.30)	-2.06	1.48
Weight (kg)	48.9 (41.9 – 55.8)	26.2	100.2
BMI (kg/m^2)	19.5 (17.8 – 21.3)	14.7	33.3
BMI SDS	0.67 (0.16 – 1.18)	-1.14	3.72
Waist circumference (cm)	64.7 (59.8 – 69.5)	50.0	107.5
Hip circumference (cm)	73.4 (68.5 – 78.4)	57.0	106.0
Waist-hip ratio	0.88 (0.86 – 0.90)	0.82	1.01
Current treatment dosages			
Daily hydrocortisone dosage (mg/m^2)	12.2 (11.2 – 13.2)	8.5	17.4
Daily fludrocortisone dosage * (µg/m^2)	98.5 (75.8 – 121.1)	46	276
Hormonal evaluation			
Androstenedione (nmol/l)	4.0 (median)	0.05	32.10
17-OHP (nmol/l)	83.0 (median)	0.68	740.00
Renin (mU/l)	52.0 (median)	3.0	1900.0
Circulating cardiovascular risk markers			
Leptin (µg/l)	6.8 (median)	1.1	27.2
Leptin SDS	1.14 (0.45 – 1.88)	-0.83	4.33
Adiponectin (mg/l)	13.5 (11.2 – 15.8)	4.1	26.0
Adiponectin SDS	0.25 (-0.16 – 0.66)	-1.45	2.51
High sensitive CRP (mg/l)	1.13 (0.28 – 1.98)	0.16	8.06
tPA (ng/ml)	13.07 (9.36 – 16.79)	1.28	40.54
PAI-1 (ng/ml)	11.56 (9.61 – 13.52)	3.95	22.47
Complex tPA/PAI-1 (ng/ml)	34.03 (22.71 – 45.34)	4.02	129.36
PAI1/tPA ratio	1.34 (0.76 – 1.92)	0.48	8.07

* Only patients with salt wasting CAH (n=24) were treated with fludrocortisone.

Table 2 Associations among parameters in a cohort of 27 pediatric CAH patients.

Parameters	Daily dosage hydrocortisone	17-OHP
BMI SDS	0.6 (-0.2 – 1.4)	62.3 (2.5 -122.2)*
24h Diastolic BP SDS (height)	-0.28 (-1.52 – 0.97)	-77.6 (-170.3 – 15.1)
Sleeping diastolic BP SDS (age)	-0.67 (-1.93 – 0.59)	-71.2 (-167.5 – 25.1)
Sleeping diastolic BP SDS (height)	-0.62 (-1.73 – 0.49)	-66.7 (-151.4 – 18.0)
Daytime diastolic BP SDS (age)	-0.09 (-1.31 – 1.12)	-70.4 (-160.9 – 20.2)
Daytime diastolic BP SDS (height)	-0.06 (-1.25 – 1.13)	-73.0 (-161.2 – 15.2)
Percentage of the nocturnal dip in MAP	0.03 (-0.14 – 0.20)	-5.5 (-18.3 – 7.3)
Total cholesterol concentration	-0.74 (-2.04 – 0.57)	-41.3 (-144.8 – 62.2)
HOMA-IR	0.75 (0.11 – 1.39)*	28.4 (-25.7 – 82.6)
Leptin concentration (SDS)	0.26 (-0.32 – 0.87)	10.1 (-37.4 – 57.6)
Percentage bodyfat SDS (Dutch references)	1.7 (-0.4 – 3.8)	179.1 (5.3 – 353.0)*
Fat tissue mass SDS (Canadian references)	0.45 (-0.79 – 1.69)	97.3 (5.1 – 189.5)*
Percentage bodyfat on DXA scan	0.10 (-0.12 – 0.32)	7.3 (-12.3 – 26.9)
Bone mineral content SDS (Dutch references)	-0.42 (-2.30 – 1.45)	139.6 (-0.2 – 279.3)
Bone mineral content SDS (Canadian references)	-0.30 (-2.11 – 1.51)	134.3 (-0.01 – 268.5)
Lean tissue mass SDS (Dutch references)	-0.06 (-1.31 – 1.19)	87.6 (-6.2 – 181.4)
Lean tissue mass SDS (Canadian refences)	0.37 (-1.59 – 2.33)	134.2 (-16.0 – 284.3)

B-coefficients of linear regression analysis are presented with 95% CI. B-coefficients with 95% CI excluding the zero-value are marked with an asterisk (*).

SDS were higher than in the reference population. Four of the patients (14.8%) had systolic hypertension compared to both age- and height-specific references, one additional patient was hypertensive compared to height-specific references (total n=5; 18.5%). Three of these five patients were also classified as hypertensive based on the office BP measurement. In one patient (3.7%), 24-h diastolic hypertension was found compared to both age- and height-specific references. As shown in Table 3, the mean sleeping SBP and DBP SDSs and the MAP SDSs were increased by approximately 0.5 and 0.8 SDS, respectively, compared to the normal ranges for both age and height. The mean dip in BP during sleep in the cohort as a whole was just >10%. However, 13 patients (48.1%) had a dip <10% in SBP during sleep, and 11 patients (40.7%) had a dip <10% in MAP during sleep.

Androstenedione	Renin	HOMA-IR	BMI SDS
2.99 (0.22 – 5.77)*	-13.5 (-127.9 – 100.8)	0.59 (0.17 – 1.01)*	
-4.3 (-8.5 – -0.1)*	-49.2 (-220.4 – 122.1)	-0.61 (-1.29 – 0.08)	-0.50 (-1.09 – 0.08)
-4.5 (-8.8 – -0.2)*	-67.8 (-242.5 – 106.9)	-0.61 (-1.29 – 0.08)	-0.07 (-0.71 – 0.56)
-4.1 (-7.9 – -0.3)*	-50.0 (-205.2 – 105.1)	-0.23 (-0.97 – 0.51)	-0.10 (-0.66 – 0.47)
-3.7 (-7.9 – 0.5)	-69.0 (-233.7 – 95.7)	-0.7 (-1.3 – -0.05)*	-0.53 (-1.09 – 0.03)
-3.7 (-7.8 – 0.4)	-52.3 (-214.6 – 110.1)	-0.7 (-1.3 – -0.03)*	-0.53 (-1.07 – 0.02)
-0.14 (-0.75 – 0.48)	0.84 (-22.0 – 23.7)	-0.06 (-0.16 – 0.03)	-0.1(-0.17 – -0.03)*
-5.0 (-9.5 – -0.6)*	-100.3 (-280.0 – 79.4)	-0.06 (-0.84 – 0.72)	-0.16 (-0.82 – 0.51)
1.8 (-0.6 – 4.3)	112.1 (26.4 – 197.9)*		0.43 (0.12 – 0.73)*
-0.61 (-1.60 – 2.82)	57.3 (-23.1 – 137.7)	0.53 (0.26 – 0.81)*	0.50 (0.28 – 0.72)*
7.1 (-0.2 – 14.5)	156.3 (-255.1 – 567.8)	0.95 (-0.18 – 2.07)	1.03 (0.09 – 1.98)*
3.4 (-0.7 – 7.5)	3.0 (-223.8 – 229.8)	0.55 (-0.04 – 1.13)	0.81 (0.50 – 1.13)*
0.11 (-0.72 – 0.95)	9.4 (-30.8 – 49.7)	0.11 (0.02 – 0.21)*	0.11 (0.03 – 0.20)*
6.0 (0.4 – 11.7)*	-223.5 (-526.9 – 79.9)	0.14 (-0.87 – 1.16)	0.78 (0.01 – 1.55)*
6.0 (0.6 – 11.4)*	-162.1 (-468.4 – 144.2)	0.19 (-0.79 – 1.16)	0.81 (0.10 – 1.52)*
3.5 (-0.5 – 7.4)	-171.8 (-362.7 – 19.1)	0.04 (-0.64 – 0.71)	0.60 (0.13 – 1.06)*
5.4 (-0.9 – 11.7)	-220.5 (-540.3 – 99.3)	0.24 (-0.81 – 1.29)	1.04 (0.36 – 1.72)*

No associations were found between 24-h SBP or MAP and dosage of hydrocortisone or fludrocortisone, therapy control, HOMA-IR and body composition. In contrast, 24-h DBP SDS to height was negatively associated with androstenedione level (Table 2). DBP SDS during sleep was also negatively associated with androstenedione concentrations, while daytime diastolic BP SDS was negatively associated with HOMA-IR. The percentage of the dip in MAP during sleep was negatively associated with BMI SDS. The dip in BP was not associated with dosage of hydrocortisone, fludrocortisone, HOMA-IR, or therapy control.

Biochemical evaluation

The lipid profiles of our 27 CAH patients are shown in Table 4. These were normal in the vast majority of patients, with 63%–78% of our population having triglyceride, total cholesterol, and/or LDL cholesterol levels below the 50th percentile. Total

Table 3 The 24 hour blood pressure (BP) profiles of 27 pediatric CAH patients compared to sex- and age- and sex- and height-specific reference data for healthy children

	24-h BP (mean + 95% CI)	Daytime BP (mean + 95%CI)	Sleeping BP (mean + 95% CI)	% dip in sleeping BP (mean + 95% CI)
Systolic BP				10.7 (8.2 – 13.1)
Measured value (mmHg)	116 (112 – 120)	119 (115 – 123)	106 (102 – 110)	
SDS (age)	0.39 (-0.07 – 0.85)	0.17 (-0.31 – 0.65)	0.48 (0.00 – 0.96)	
SDS (height)	0.47 (-0.37 – 0.97)	0.23 (-0.28 – 0.74)	0.51 (0.04 – 0.98)	
Diastolic BP				15.5 (12.6 – 18.4)
Measured value (mmHg)	68 (66 – 70)	70 (68 – 72)	59 (57 – 62)	
SDS (age)	0.18 (-0.18 – 0.55)	-0.31 (-0.66 – 0.04)	0.53 (0.20 – 0.86)	
SDS (height)	0.14 (-0.20 – 0.47)	-0.29 (-0.64 – 0.07)	0.58 (0.21 – 0.95)	
Mean arterial pressure (MAP)				10.8 (8.2 – 13.3)
Measured value (mmHg)	84 (82 – 86)	86 (83 – 88)	76 (74 – 79)	
SDS (age)	0.35 (0.01 – 0.70)	-0.05 (-0.39 – 0.28)	0.80 (0.42 – 1.18)	
SDS (height)	0.37 (0.01 – 0.72)	0.00 (-0.37 – 0.36)	0.81 (0.41 – 1.20)	

Sex-specific SDSs, compared to both age and height, for the 24-h ABPM data were calculated using the algorithm and reference values published by Wühl *et al.*[30]

cholesterol levels were negatively associated with androstenedione levels (Table 2). All other cholesterol and triglyceride levels were not associated with therapy control, dosage of hydrocortisone, BMI SDS, or HOMA-IR.

Fasting glucose concentrations (mean 4.49 mmol/L; 95% CI: 4.35–4.62) and HbA_{1c} concentrations (mean 36.0 mmol/mol; 95% CI: 35.2–36.8) were normal in all 27 patients. The range in fasting insulin concentrations was wide (4.50–28.4 mU/L; median 11.0 mU/L). The mean HOMA-IR level in the cohort was 2.64 (95% CI: 2.05–3.24). We compared the HOMA-IR of our patients with BMI SDS <1 to reference values of non-overweight children and the HOMA-IR of patients with BMI SDS >1 to reference values for children with BMI SDS >1. Twelve patients (44.4%; seven boys, five girls) had a HOMA-IR above the 75th percentile, and eight patients (29.6%; four boys, four girls) had a HOMA-IR above the 90th percentile for sex and age. HOMA-IR levels were positively associated with the daily dosage of hydrocortisone, BMI SDS and renin concentration (Table 2).

The mean concentrations of CVR markers in our CAH patients are shown in Table 2. Adiponectin levels were on average similar to the selected reference values. Only one patient had an adiponectin SDS >2. Adiponectin levels were not associated with BMI SDS, hydrocortisone dosage or HOMA-IR.

The median leptin level in our cohort (6.8 µg/L; SDS 0.30) was similar to the average of 5.29 µg/L in healthy Polish children,[27] while the mean leptin SDS in our cohort was 1.14 (95% CI: 0.45–1.88). In 9 CAH patients (33.3%; four of these patients were classified as obese and three as overweight), the leptin SDS was >2. Leptin levels were positively associated with BMI SDS, HOMA-IR values, (for B-coefficients see Table 2) percentage of body fat SDS on the DXA scan (B-coefficient 3.8; 95% CI: 2.5–5.2), and FTM on the DXA scan (B-coefficient 0.52; 95% CI: 0.13–0.90). Leptin levels were not associated with daily hydrocortisone dose.

The high sensitivity CRP levels were normal in all patients according to the reference values of our laboratory. Compared to our laboratory reference values for adults, mean PAI-1 values in our cohort were comparable (−0.33 SDS; 95% CI: −0.53 to −0.14), but mean tPA values were increased (8.74 SDS; 95% CI: 5.54–11.95).

Intima media thickness

The cIMT was normal in our cohort (n=24) with a mean cIMT of 0.50±0.03 mm. Compared to reference values in healthy children, only one patient had a cIMT >75th percentile for age and sex.

Body composition

Data on body composition obtained by DXA scans are shown in Table 5. Compared to both Canadian and Dutch reference children, the CAH patients in our cohort had more body fat. No associations were found between fat mass or fat percentage and

Table 4 Lipid profiles of 27 pediatric CAH patients.

	Measured value (Mean and 95%CI)	< 50th percentile	50th-75th percentile	75-90th percentile	90th-95th percentile	> 95th percentile
Triglycerides	0.70 (0.58 – 0.82) mmol/l	63.0% (n=17)	11.1% (n=3)	18.5% (n=5)	3.7% (n=1)	3.7% (n=1)
Total cholesterol	3.80 (3.48 – 4.12) mmol/l	70.4% (n=19)	14.8% (n=4)	11.1% (n=3)	0%	3.7% (n=1)
LDL cholesterol	2.06 (1.79 – 2.32) mmol/l	77.8% (n=21)	14.8% (n=4)	3.7% (n=1)	0%	3.7% (n=1)
		< 5th percentile	5th-10th percentile	10-25th percentile	25th-50th percentile	> 50th percentile
HDL cholesterol	1.43 (1.23 – 1.56) mmol/l	3.7% (n=1)	7.4% (n=2)	7.4% (n=2)	29.6% (n=8)	51.9% (n=14)
Non-HDL cholesterol*	2.37 (2.09 – 2.64) mmol/l					

Measured values were compared to reference values published byDaniels et al.[26]
* For non-HDL cholesterol reference values were not available.

hydrocortisone dosage. Percentage body fat SDS (compared to Dutch references) was positively associated with BMI SDS, 17-OHP and androstenedione concentrations (Table 2). Positive associations were seen between FTM SDS (compared to Canadian references) and BMI SDS and 17-OHP concentrations and tentative associations between FTM SDS and androstenedione concentrations and HOMA-IR. The percentage body fat in our patients was positively associated with BMI SDS and HOMA-IR. LTM was positively associated with BMI SDS compared to both Dutch and Canadian references. No associations were observed between data on body composition and dosage of hydrocortisone per m^2 (BSA). The SDSs for BMC were positively associated with BMI SDS and with the androstenedione concentrations.

Table 5 Body composition of pediatric CAH patients evaluated by DXA scan compared to healthy Dutch and Canadian children

	CAH patients (n = 13) (Mean ± 95% CI)	SDS compared to healthy Dutch children* (Mean + 95% CI)	SDS compared to healthy Canadian children (Mean + 95% CI)
Lean tissue mass	41582.1 (36472.7 – 46691.5) g	-1.21 (-2.13 – -0.28)	-0.31 (-0.90 – 0.27)
Fat tissue mass	18762.4 (12806.7 – 24718.1) g		0.94 (0.04 – 1.85)
Fat percentage	29.0 (23.9 – 34.0) %	1.59 (1.10 – 2.07) **	
Bone mineral content	2048.11 (1843.83 – 2252.38) g	-0.06 (-0.67 – 0.54)	0.60 (-0.03 – 1.24)
BMC Z-score	0.63 (0.22 – 1.04)		
Bone mineral density	1.05 (1.01 – 1.09) g/cm^2		

* After correction of data for the type of fanbeam used according to published conversion ratio's.[35]
** After converting to natural logarithm of fat percentage as used by van der Sluis et al.[34]

Discussion

This study showed that, compared to population reference values, a number of unfavorable changes in the cardiovascular risk profile of our CAH patients already occur in childhood, i.e. overweight and obesity, an increased fat mass, elevated BP levels, a non-dipping BP profile, and increased IR. The lipid profile and cIMT were within the normal range. Only elevated HOMA-IR values were positively associated with the daily dosage of hydrocortisone per m^2. The prevalence of overweight and obesity in our studied pediatric CAH patients is higher (25.9% overweight; 14.8% obese) than in the healthy pediatric population suggesting that additional CAH-related factors may play a role.

First of all, treatment with glucocorticoids, both in physiological and supraphysiological doses, has been associated with (central) obesity in adult CAH patients in several studies.[4,15,36] However, we did not find an association between BMI SDS and current hydrocortisone dose. Secondly, obesity is associated with high serum leptin concentrations, suggesting leptin resistance.[37] Leptin acts via a specific receptor in the brain, the long form of the leptin receptor (OB-Rb) in the hypothalamic regions and the arcuate nucleus to regulate energy balance and body weight. Glucocorticoids and insulin are known to increase leptin secretion and catecholamines suppress leptin secretion. Our pediatric CAH patients had increased leptin levels, associated with BMI SDS, HOMA-IR and fat mass. This finding is in line with previous studies evaluating leptin levels in CAH patients.[4,9,12] Finally, an increased prevalence of obesity in adult CAH patients may be due to hyperandrogenism[38] which is known to cause IR, and consequently may lead to obesity and centralization of body fat. We found positive associations between BMI SDS and the 17-OHP and androstenedione concentrations. It may be expected that undertreated patients, with higher 17-OHP and androstenedione levels, will be treated with higher dosages of hydrocortisone to suppress androgens with consequently increased BMI due to high glucocorticoid dosages. In contrast, Reinehr et al. showed that otherwise healthy prepubertal obese children had significantly increased levels of androstenedione and other androgens.[39] Weight loss significantly lowered androstenedione concentrations suggesting that elevated precursors rather are the consequence than the cause of obesity.[39] Several hypotheses for the increased glucocorticoid and androgen levels in healthy obese prepubertal children were proposed, including an increased activation of the hypothalamic pituitary adrenal axis and an adrenal fasciculata-reticularis cell refractoriness to the inhibitory effect of leptin on ACTH-stimulated glucocorticoid production. The increased leptin concentrations in our cohort fit within this hypothesis. In CAH patients, a vicious circle may exist: alimentary obesity due to unhealthy lifestyle causes an increased ACTH stimulated secretion of androgens due to the above proposed mechanisms. In CAH patients, this may be more pronounced due to the enzymatic defect. A consequent increase in glucocorticoids to supraphysiological dosages further induces obesity.

The finding of increased fat mass, found by DXA scan, in our pediatric CAH patients is in line with previous studies using different methods (skin-fold thickness, bioelectrical impedance analysis and DXA scan) to evaluate body composition in both pediatric and young adult CAH patients.[9,16,40-42] In contrast to our findings, Falhammar et al.[6,43] found normal fat mass with the DXA scan in young adult CAH patients compared to controls. They explained the normal fat mass in young adults as a consequence of lowering glucocorticoid dosage over the years. Despite relatively low hydrocortisone dosage, we still found an increased fat mass in our pediatric population. The increased fat mass contributes to the increased BMI SDS in our

population, as BMI SDS scores were associated with the fat percentage SDS and FTM SDS.

Increased BP levels and a non-dipping BP profile

The prevalence of hypertension in pediatric CAH has been described in up to 58% of patients. [4,5,20,21,44-48] In our study, the prevalence of systolic hypertension was lower (18.5% in both office BP and 24-h ABPM). In previous studies, BP levels were associated with BMI, dose of glucocorticoids, dose of fludrocortisone, or suppressed plasma renin activity.[4,20,21,44,45] Both our study and the study by Subbarayan et al.[5] did not find these associations. However, regular control of renin levels to prevent mineralocorticoid overtreatment, as a cause of elevated BP, remains important in clinical care.

Data on the nocturnal dip in BP in pediatric CAH patients are scarce and the results vary.[44,46,49] A non-dipping SBP profile was described previously in 84% of the CAH patients (n=32).[44] Another study showed that the nocturnal dip in BP was maintained in all studied CAH patients (n=20), despite a higher BMI SDS (1.05) and higher daily hydrocortisone dose compared to our study.[46] The percentage dip in sleeping BP in our cohort was associated with BMI SDS. It is well known that obesity is associated with a non-dipping BP profile, an independent cardiovascular risk factor.[31] Further research in larger cohorts is needed to establish the presence of a non-dipping BP profile in pediatric CAH, and its association with BMI. All our patients were treated with hydrocortisone in a reverse circadian rhythm, that may affect sleeping BP and cause the absence of the nocturnal dip.[50] In the future, treatment with slow release hydrocortisone, mimicking a physiological cortisol secretion in CAH patients,[51] may have a favorable outcome on 24-h BP profiles in CAH patients.

Insulin resistance

IR in our cohort was associated with the daily hydrocortisone dose.[52] Lowering the hydrocortisone dose, if possible, may lead to reduction of IR as suggested by Subbarayan et al.[5] In contrast to previous studies,[1] we did not find an association between hyperandrogenism and IR.

The main limitation of our study is that we could not include a control group with healthy matched controls, due to regulations of the medical ethical committee in our center. Therefore, we carefully selected the reference groups used in this study, to use reference groups that are as similar as possible to the Dutch population. We were unable to calculate the cumulative steroid dosages, which may elucidate associations between long-term treatment and unfavorable changes in the cardiovascular risk profile. Furthermore, the size of our study population was too small to perform subgroup analyses. A future long running study evaluating the cardiovascular risk profile over many years from childhood into adulthood could give additional insights in the long-term effect of the current treatment regimen and unfavorable cardiovascular

risk factors in pediatric patients on the cardiovascular morbidity in adulthood, as current cross-sectional studies in adult CAH patients reflect the outcomes of the treatment regimens of several decades ago.

In conclusion, our study shows that CAH patients already develop an unfavorable cardiovascular and metabolic risk profile in childhood compared to population reference values. Increased BMI seems to play an essential role in the increased cardiovascular risk. Lifestyle interventions to lower BMI may lead to better hormonal control and a reduced risk of cardiovascular morbidity in adult life. Further studies are necessary to evaluate the role of lifestyle interventions on the cardiovascular risk profile of pediatric CAH patients.

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CHAPTER 3.3

Cardiac function in pediatric patients with congenital adrenal hyperplasia due to 21 hydroxylase deficiency

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Summary

Background: Hyperandrogenism and exogenous glucocorticoid excess may cause unfavorable changes in the cardiovascular risk profile of patients with congenital adrenal hyperplasia (CAH).

Objective: To evaluate the cardiac function in pediatric patients with CAH.

Patients and methods: Twenty-seven pediatric patients with CAH, aged 8-16 years, were evaluated by physical examination, electrocardiogram (ECG), conventional echocardiography, tissue Doppler imaging and two-dimensional (2D) myocardial strain (rate) imaging. Results were compared to 27 age- and gender- matched healthy controls.

Results: No signs of left ventricular hypertrophy or dilatation were detected on echocardiography. ECG revealed a high prevalence (25.9%) of incomplete right bundle branch block. Left ventricular posterior wall thickness in diastole (LVPWd) was significantly lower in patients with CAH compared to controls (5.55 vs 6.53 mm; P = .009). The LVPWd Z-score was significantly lower in patients with CAH yet within the normal range (–1.12 vs −0.35; P = .002). Isovolumetric relaxation time was significantly lower in patients with CAH (49 vs 62 ms; P = .003). Global longitudinal, radial and circumferential strain was not significantly different compared to controls. Global radial strain rate was significantly higher compared to healthy controls (2.58 vs 2.06 1/s; P = .046). Global longitudinal strain was negatively correlated with 24-hour blood pressure parameters.

Conclusion: Cardiac evaluation of pediatric patients with CAH showed no signs of left ventricular hypertrophy or ventricular dilatation. LVPWd was lower in patients with CAH than in controls but within the normal range. A shorter isovolumetric relaxation time in patients with CAH may be a sign of mild left ventricular diastolic dysfunction.

Introduction

Congenital adrenal hyperplasia (CAH) due to 21 hydroxylase deficiency (21OHD) is a disorder of adrenal steroidogenesis. 21OHD is characterized by androgen excess and cortisol deficiency, and depending on the residual enzyme activity also aldosterone deficiency.[1] Treatment consists of glucocorticoids and, if necessary, mineralocorticoids to prevent adrenal crises and to suppress abnormal secretion of adrenal androgens. In most cases, supraphysiological doses of glucocorticoids are needed to suppress androgen levels.

Both androgen excess and treatment with supraphysiological doses of glucocorticoids are associated with an unfavorable cardiovascular and metabolic risk profile in patients with CAH.[2,3] Several studies performed in adult and pediatric patients with CAH showed that they indeed cluster a number of cardiovascular risk factors: obesity, increased fat mass, insulin resistance and high blood pressure.[2,4,5] These unfavorable changes in the cardiovascular risk profile may cause cardiovascular morbidity later in life. This hypothesis was confirmed by a population-based national cohort study in Sweden.[6]

Few studies have previously evaluated the cardiac function in patients with CAH. One study evaluated the cardiac function in untreated newborn patients with CAH (n = 9), reporting cardiac dysfunction that had been reversed with glucocorticoid treatment.[7] Seven of the nine patients had an abnormal fractional shortening and rate-corrected velocity of circumferential fiber shortening at baseline that was improved in six of the patients after glucocorticoid treatment. The other previous studies on cardiac function in patients with CAH evaluated cardiac function in pediatric and adolescent patients, mean age ranges of 6.3-13.6 years, using conventional echocardiography and, in most studies, tissue Doppler imaging.[8-12] None of the studies used the newer echocardiographic technique, for example myocardial deformation, for the detection of early signs of myocardial damage. Left ventricular diastolic dysfunction was described in Italian adolescent male patients with CAH (n = 10)[8] and in pediatric patients with CAH from Cameroon (n = 19),[9] Egypt (n = 32)[10] and Turkey (n = 25).[11] The finding of impaired left ventricular diastolic function was based on the presence of a lower ratio of Doppler-derived E/A wave, prolonged mitral deceleration time and isovolumetric deceleration times in studied patients with CAH compared to controls. Left ventricular diastolic dysfunction was associated with testosterone levels and the late age of diagnosis in three of the studies;[9-11] in one study, no correlation with androgen levels was found.[11] Left ventricular hypertrophy, indicated by an increased left ventricular mass index, was described by two studies.[9,10] In both these studies, levels of left ventricular hypertrophy correlated with testosterone levels.[9,10] In addition, a study in Italian pediatric patients with CAH (n = 20) showed an association between left ventricular

mass and testosterone concentrations; however none of the studied patients had myocardial hypertrophy.[12] Marra et al compared the cardiac parameters in patients with CAH with a group of patients receiving a similar dose of glucocorticoids for juvenile idiopathic arthritis.[8] In none of the above studies, a correlation between the glucocorticoid dose and cardiac function was reported, which may be surprising as supraphysiological doses of glucocorticoids may also impair cardiac function.[13]

In this study, we systematically evaluated the cardiac function, and its correlations with the blood pressure, glucocorticoid and fludrocortisone doses and the concentrations of 17-OHP and androstenedione, in pediatric patients with CAH due to 21OHD by electrocardiography (ECG), by conventional echocardiography, by tissue Doppler imaging, and by 2D myocardial strain (rate) imaging. Echocardiographic data, including tissue Doppler and 2D myocardial strain, were compared to healthy gender- and age- matched controls.

Patients and methods

Patients

Patients with CAH, with biochemically and genetically proven 21OHD, aged 8-16 years and treated at a single tertiary hospital were included in this study. Exclusion criteria were known comorbidities as congenital cardiac disease, renal disease and comedication that might interfere with blood pressure measurements. As part of the routine clinical care, all patients were followed every 3 months at our outpatients clinic. All patients and their parents were fully informed about the aim and design of the study and the methods involved. Informed consent with the study protocol was obtained in all cases according to the recommendations of the local medical ethical committee. Full data on the cardiovascular and metabolic risk profile in the same group of patients with CAH were recently published by our group.[14] As a control group, we included 27 healthy age-and gender- matched children who were routinely referred for echocardiographic evaluation of an asymptomatic, innocent heart murmur or for screening purposes. In the controls, medical history, electrocardiogram and echocardiogram were not indicative of cardiac disease and no medication was used.

Data regarding the current hydrocortisone and fludrocortisone dosage of the patients with CAH were collected from their medical records. Serum levels of 17-OH-progesterone (17OHP), androstenedione and renin were measured in blood withdrawn after an overnight fast and before the patients took their morning medication as a part of our recent study evaluating the cardiovascular risk profile in pediatric 21OHD patients.[14] These data were used to evaluate possible correlations between these serum levels and the cardiac function. Data on 24-hour ambulatory blood pressure measurement (24-hour ABPM) were collected in the same study and

used to evaluate possible correlations between 24-hour ABPM values and cardiac function.[14]

Methods

Each patient visited the hospital on two consecutive days; cardiac assessment was carried out on one of these days by an ultrasound technician and supervised offline by one experienced pediatric cardiologist (LK). A 12-lead ECG was performed in rest in supine position. All patients and controls underwent a transthoracic 2D echocardiogram in supine and lateral position, in rest, according to the recommendations of the American Society of Echocardiography.[15] Images were obtained with a 3.0 MHz or a 5.0 MHz phased-array transducer, depending on the patient's age and weight, using a Vivid 7 echocardiographic scanner (GE, Vingmed Ultrasound, Horton, Norway).

Quantification of cardiac chamber size, ventricular mass and systolic and diastolic left ventricular function was measured in accordance with the recommendations for chamber quantification by the American Society of Echocardiography's Guidelines and Standard Committee and the Chamber Quantification Writing Group.[15]

Left ventricular systolic function was determined using fractional shortening (FS), ejection fraction (EF), end-systolic wall stress (ESWS) and rate-corrected velocity of circumferential fiber shortening (VCFc). FS was calculated by the following formula: ((LVIDd-LVIDs)/LVIDd)/100. Fractional shortening above 27% and EF 54% and higher were considered normal. The modified formula of Rowland and Gutgesell was used for calculating left ventricular end-systolic wall stress (ESWS).[16] Velocity of circumferential fiber shortening (VCFc) was calculated with the formula from Colan et al.[17]

An M-mode echocardiogram was performed in the parasternal long- and short-axis views to measure the internal dimensions of the left ventricle at end-diastole (LVIDd) and end-systole (LVIDs), the posterior and septal wall thickness at end-diastole (LVPWd, IVSd) and the left ventricular mass (LVM).

LVM was calculated using the following formula: $LVM = 0.8\{1.04[(\{LVIDd + IVSd + LVPWd]^3 - LVIDd^3)]\} + 0.6$;[18] and was afterwards indexed by the body mass surface area (BSA). Z-scores for LVIDd, LVIDs, IVSd and LVPWd were calculated using an online calculator (parameterz.blogspot.com).

Left ventricular diastolic function was evaluated using early (E) and late (A) diastolic transmitral peak flow velocity (E/A ratio), the systolic to diastolic pulmonary vein peak flow velocity (PV S/D ratio), early diastolic transmitral peak flow velocity (E) to early diastolic myocardial Doppler velocity (e') ratio (E/e' ratio) and isovolumetric relaxation time (IVRT). The E/A ratio and S/D ratio were obtained by pulse Doppler at the mitral valve inflow and the pulmonary vein inflow, respectively.

The myocardial strain, a measure for the deformation of the myocardium throughout the cardiac cycle, was determined using 2D speckle-tracking strain analysis in accordance with a previously published protocol.[19] Offline analysis was

performed using software for echocardiographic quantification (EchoPAC 6.1.0; GE Medical Systems, Horten, Norway). Timing of aortic valve closure (AVC) and mitral valve opening (MVO) was used to indicate end of systole and start of diastole, respectively. Myocardial segments were named and localized according to the statement of the Cardiac Imaging Committee of the Council on Clinical Cardiology of the American Heart Association.[20] Manual tracking of the endomyocardial borders was performed at the end-systolic frame. An automatic generation of the second epicardial tracing was created by the software, which also automatically divided the image into six equal segments. Quality of the tracking was verified for each segment and adjusted if needed. Three consecutive cardiac cycles were analyzed separately. Strain and strain rate curves were then exported for postprocessing in a custom-made tool in which the curves can be aligned, detrended and averaged for final global strain (rate) values estimates according to the consensus paper of Voigt et al[21] and to create a fully automated workflow to avoid errors by user interaction.

Strain values are dimensionless and are expressed in percentage. Strain rate is the temporal derivative of strain and is expressed as 1/s. The average values of peak systolic longitudinal, radial and circumferential strain, and strain rate of the three imported curves were calculated without correction for the length of the cardiac cycle as it is known that the systolic phase is relatively constant if heart rate changes <10%.[22] Global longitudinal myocardial strain (GLS) and strain rate (GLSR) were calculated by averaging the six segments of the 4-chamber long-axis view. Global radial and circumferential myocardial strain (GRS, GCS) and strain rate (GRSR, GCSR) were calculated by averaging four up to six present segments of the mid-cavity short-axis view.

For the matched controls, conventional echocardiography and myocardial strain data were obtained following the same protocol as described above.

Statistical analysis

All data were collected and stored in a secure database (Castor, www.castoredc.com), complying with the regulations of the local medical ethical committee. For the statistical analyses, the SPSS personal computer software package version 22 (IBM SPSS Inc., Chicago, IL, USA) was used. Student's *t* test (independent samples) was used to compare the cardiac function parameters between patients with CAH and the gender- and age- matched controls assuming a normal distribution of the data. Pearson and Spearman correlation tests were performed to evaluate the strength of the association between different cardiac function parameters, treatment (current daily dosages of hydrocortisone and fludrocortisone per m^2 BSA), levels of 17OHP, androstenedione and renin and 24-hour ambulatory blood pressure measurement SD scores in patients with CAH. A *P*-value of <.05 was considered statistically significant. Z-scores between −2 and 2 were interpreted as being normal.

Results

Twenty-seven patients with CAH (17 boys/10 girls; 24 patients were classified as salt wasting, two as simple virilizing and one as nonclassic), aged 8.8-16.0 years (median 11.7 years), participated in this study. Twenty-seven age- and gender-matched healthy controls were included. Patient characteristics, including daily dosages of hydrocortisone, fludrocortisone and data on the biochemical evaluation of 17OHP progesterone, androstenedione and renin levels, are shown in Table 1.

Table 1 Characteristics of 27 studied patients with CAH and gender and age matched controls

	Patients with CAH (Mean ± SD)	Controls (Mean ± SD)	*P*-value
Gender	17 males / 10 females	17 males / 10 females	
Age (years)	12.15 ± 2.29	12.25 ± 2.45	0.536
Anthropometric measure			
Height (cm)	156.0 ± 13.9	157.6 ± 15.6	0.425
Weight (kg)	48.9 ± 17.6	45.6 ± 15.3	0.315
BMI (kg/m^2)	19.5 ± 4.4	17.8 ± 2.6	0.066
BMI SDS	0.67 ± 1.29	0.04 ± 1.00	0.054
Current treatment dosages			
Daily hydrocortisone dosage (mg/m^2)	12.2 ± 2.6	-	
Daily fludrocortisone dosage * (µg/m^2)	98.5 ± 53.6	-	
Hormonal evaluation			
Androstenedione (nmol/l)	4.0 (median; range 0.05 - 32.10)	-	
17OHP (nmol/l)	83.0 (median; range 0.68 - 740.00)	-	
Renin (mU/l)	52.0 (median; range 3.0 - 1900.0)	-	

* Only patients with salt wasting CAH (n=24) were treated with fludrocortisone.

Cardiac function evaluated by electrocardiogram

ECGs showed a high prevalence of 25.9% (n = 7) of an incomplete right bundle branch block (IRBBB). A sinus rhythm was seen in 25 of the patients, with one patient having atrial rhythm and one patient with sino atrial rhythm. Conduction durations (PR

interval, QRS interval and QTc interval) were normal in all patients with CAH. No ST segment abnormalities were found. Based on ECG, none of the patients met the criteria for left ventricular (LV) or right ventricular (RV) hypertrophy.

Cardiac function evaluated by conventional echocardiography and tissue Doppler imaging

The echocardiographic parameters evaluated by conventional echocardiography for both the patients with CAH and the controls are shown in Table 2. One patient had increased LVIDd (Z-score 3.07), with a normal LVIDs (Z-score 1.32), resulting in re-evaluation by the pediatric cardiologist within 1 year. All other echocardiographic parameters were within the normal range in all studied patients with CAH.

Table 2 Cardiac function evaluated by conventional echocardiography in 27 pediatric patients with CAH and 27 age and gender matched controls

Echocardiographic parameter	Patients with CAH (mean ± SD)	Controls (mean ± SD)	*P*-value
FS (%)	37 ± 4	36 ± 4 (n=24)	0.638
EF (%)	66 ± 6	67 ± 5	0.701
LVIDd (mm)	46.4 ± 4.5	45.2 ± 4.9	0.226
LVIDd (Z)	0.95 ± 0.98	0.63 ± 0.75	0.197
LVIDs (mm)	29.3 ± 3.8	28.5 ± 3.9	0.323
LVIDs (Z)	0.57 ± 0.97	0.31 ± 0.95	0.330
IVSd (mm)	5.50 ± 0.87	5.70 ± 1.17	0.359
IVSd (Z)	-1.42 ± 0.76	-1.15 ± 1.01	0.200
LVPWd (mm)	5.55 ± 0.82	6.53 ± 1.70	**0.009**
LVPWd (Z)	-1.12 ± 0.59	-0.35 ± 1.05	**0.002**
LVM (g)	77 ± 24	75 ± 34	0.691
LVMI (g/m^2)	53 ± 10	52 ± 14	0.615
IVRT (ms)	49 ± 18	62 ± 11	**0.003**
MV E/A ratio	2.05 ± 0.63 (n=26)	2.36 ± 1.06 (n=26)	0.328

Compared to controls, a significantly lower LVPWd was found (5.55 ± 0.82 vs 6.53 ± 1.70 mm, *P* = .009) in patients with CAH. However, the average LVPWd Z-score of -1.12 in patients with CAH was within the normal range. A shorter IVRT was found in patients with CAH compared to controls (49 ± 18 vs 62 ± 11 ms, *P* = .003). All other evaluated echocardiographic parameters were similar for patients with CAH and controls.

No correlations were found between left ventricular parameters and BMI standard deviation score (SDS), hydrocortisone dose, androstenedione and 17OHP levels, respectively. LVPWd Z-score (not in mm) was weakly correlated with the daily dose of fludrocortisone (*r* = .386; *P* = .042). All other evaluated echocardiographic parameters did not correlate with the daily dose of fludrocortisone. The MV E/A ratio was significantly but weakly correlated with the systolic blood pressure during sleep SDS for age (correlation coefficient (*r* = .416; *P* = .035), the systolic blood pressure during sleep SDS for height (*r* = .423; *P* = .031), the diastolic blood pressure during sleep SDS for age (*r* = .393; *P* = .047) and the diastolic blood pressure during sleep SDS for height (*r* = .389; *P* = .049). LVIDs (in mm, not Z-score) was negatively correlated with the 24-hour diastolic blood pressure SDS for height (*r* = −.386; *P* = .047), the diastolic blood pressure in sleep SDS for age (*r* = −.432; *P* = .024) and the diastolic blood pressure in sleep SDS for height (*r* = −.441; *P* = .021). All other evaluated echocardiographic parameters did not correlate with 24-hour ambulatory blood pressure measurement parameters in our patients with CAH.

Myocardial deformation evaluated by 2D speckle tracking-derived strain imaging

2D speckle tracking-derived strain (rate) parameters of patients with CAH and controls are shown in Table 3. No relevant signs of myocardial deformation abnormalities were seen in our patients with CAH compared to controls. No correlations were found between 2D strain (-rate) parameters and BMI SDS, hydrocortisone dose, androstenedione and 17OHP levels, respectively. Global circumferential strain was weakly correlated with the daily dose of fludrocortisone (*r* = .539; *P* = .008). All other 2D speckle tracking-derived myocardial deformation (strain and strain rate) parameters were not correlated with the daily dose of fludrocortisone. Global longitudinal strain was negatively correlated with systolic 24-hour blood pressure SDS for height (*r* = −.400, *P* = .039), systolic daytime blood pressure SDS for height (*r* = −.412, *P* = .033), diastolic 24-hour blood pressure SDS for both age and height (*r* = −.428, *P* = .033; *r* = −.438, *P* = .022), diastolic daytime blood pressure SDS for both age and height (*r* = −.425, *P* = .027; *r* = −.426, *P* = .027). Time to peak GCS was correlated with systolic sleeping blood pressure SDS for age (*r* = .419; *P* = .047). All other 2D speckle tracking-derived myocardial deformation (strain and strain rate) parameters were not correlated with 24-hour ambulatory blood pressure measurement parameters in the studied patients with CAH.

Table 3 Myocardial deformation evaluated by 2D speckle tracking in 27 pediatric patients with CAH and 27 age and gender matched controls

Strain (rate) parameters	Patients with CAH (mean ± SD)	Controls (mean ± SD)	*P*-value
Global longitudinal strain (GLS) (%)	-20.4 ± 2.0	-19.5 ± 2.1	0.093
Global longitudinal strain rate (1/s)	-1.24 ± 0.15	-1.21 ± 0.16	0.520
Global radial strain (GRS) (%)	53 ± 14 (n=23)	46 ± 10 (n=20)	0.253
Global radial strain rate (1/s)	2.58 ± 0.79 (n=22)	2.06 ± 0.29 (n=20)	0.046
Global circumferential strain (GCS) (%)	-17.5 ± 4.2 (n=23)	-19.1 ± 3.3 (n=22)	0.350
Glocal circumferential strain rate (1/s)	-1.55 ± 0.26 (n=22)	-1.43 ± 0.25 (n=20)	0.264
Time to Peak GLS (%)	35 ± 2	35 ± 3	0.755
Time to Peak GRS (%)	33 ± 4 (n=23)	33 ± 4 (n=20)	0.631
Time to Peak GCS (%)	34 ± 4 (n=23)	32 ± 3 (n=22)	0.305

Discussion

This study was the first to evaluate cardiac function in a group of pediatric patients with CAH by both electrocardiogram, conventional echocardiography, tissue Doppler imaging and 2D myocardial strain (rate) imaging. Compared to healthy controls, the conventional echocardiographic parameters showed a significantly thinner LVPWd and significantly shorter IVRT. Electrocardiogram showed a high prevalence of 25.9% of an ICRBBB in patients with CAH.

Left ventricular characteristics in patients with CAH

The LVPWd (both in mm and as Z-score) was significantly lower in the patients with CAH when compared to the controls. As the mean Z-score of the LVPWd of our patients was −1.12, we believe it should be considered as normal without clinical relevance. Our finding was in contrast to the study by Metwalley et al, who reported a significantly higher left ventricular posterior wall thickness (without the international Z-scores) and LVM index indicating LV hypertrophy in a cohort of 32 Egyptian pediatric patients with CAH as compared to their controls.[10] The increased LVM index was positively correlated with testosterone levels,[10] supporting the hypothesis that increased levels of androgens may have an unfavorable effect on the development of ventricular hypertrophy in patients with CAH. The diagnosis of CAH was made at an older age in the Egyptian study compared to our patients. The longer exposure to high androgen levels in the Egyptian study might explain the difference on the left ventricular structure between our studies. Early diagnosis, as a result of the newborn

screening for CAH in the Netherlands with consequent initiation of treatment within the first weeks of life, may reduce the level and duration of androgen excess and perhaps help to prevent LV hypertrophy.

In several reports on cardiac function in pediatric patients with CAH, unfavorable changes in left ventricular function and left ventricular mass were correlated to testosterone levels partly also due to late age of diagnosis with longer exposure to elevated androgen levels.[8-10,12] We did not find an increased left ventricular mass (index) in our well-controlled group of patients with CAH in which treatment was started immediately after diagnosis based on positive newborn screening for 21OHD or directly after birth in female patients with ambiguous genitalia. Furthermore, we did not find any correlation between androgen levels and the other studied cardiac parameters. Preventing long-term androgen excess by early diagnosis and consequently early start of treatment may therefore be beneficial in reducing the incidence of left ventricular hypertrophy in patients with CAH. The role of neonatal screening in early diagnosis of 21OHD (male) patients is essential and may not only contribute to the prevention of Addisonian crisis but may also lead to reduction in morbidity, such as impaired cardiac function, later in life. During treatment with glucocorticoids, one should prevent overtreatment with glucocorticoids as cortisol excess also is associated with left ventricular hypertrophy.[23,24] Besides an increased left ventricular mass, also negative effects on the left ventricular systolic and diastolic function in patients with increased cortisol levels have been described.[23,25-29] It has been suggested that cortisol also acts directly on the myocard, as glucocorticoid receptors have been reported in the human heart.[30] An association between the degree of glucocorticoid excess (hypercortisolism) and cardiac changes has been reported, supporting the hypothesis of glucocorticoid excess-related cardiac alterations.[23,25,31]

Impaired left ventricular diastolic function in patients with CAH

A shorter IVRT was found in our patients with CAH compared to controls. A shortened IVRT may be the result of increased left atrial pressure.[32] Both a prolonged and shortened IVRT may be a sign of impaired left ventricular diastolic function. Mild left ventricular dysfunction has been described previously,[8-11] with both Metwalley et al and Marra et al reporting a prolonged IVRT. Marra *et al* [8] found a significant negative correlation between serum testosterone concentrations and IVRT with a prolonged IVRT being reported mainly in men, where Metwalley *et al* suggest that both high and low levels of androgens and/or high blood pressure may play a role in the etiology of left ventricular dysfunction.[10] IVRT in our patients was not correlated with androgen levels or 24-hour ABPM parameters. Although we did not find a correlation between blood pressure and the shortened IVRT in our patients, the increased blood pressure levels in our studied patients[14] may explain an increased atrial pressure resulting in a shortened IVRT as a sign of mild diastolic dysfunction.

Elevated blood pressure levels and cardiac function in patients with CAH

Twenty-four-hour ABPM evaluation showed a prevalence of systolic hypertension of 18.5%, with an absence of the nocturnal dip in blood pressure in 40.7% of the patients.[14] On the long-term, elevated blood pressure levels are associated with cardiovascular events and impaired cardiac function. Although this study did not show clear unfavorable effects of blood pressure on cardiac function, one may hypothesize that ongoing elevated blood pressure levels may cause impaired cardiac function later in life. In this context the weak, but significant, negative correlation between 24-hour blood pressure levels and the global longitudinal strain in our patients is interesting. Previous studies in adult hypertensive patients with preserved left ventricular ejection fraction showed that global longitudinal strain is significantly decreased in hypertensive adults compared to healthy controls.[33,34] Global longitudinal strain is known to be a more sensitive predictor of left ventricular dysfunction than left ventricular ejection fraction and may identify subclinical left ventricular dysfunction at an earlier stage.[35,36] Although at the time of our study, the global longitudinal strain was normal compared to controls, we suggest to perform a longitudinal follow-up study of this strain parameter to identify subclinical left ventricular dysfunction in adult patients with CAH. In addition, this study may further evaluate the correlation between the doses of fludrocortisone on the global longitudinal strain, as we found a weak, but significant, correlation between them.

IRBBB in patients with CAH

The prevalence of IRBBB in the studied patients with CAH is much higher than previously reported in cohorts of healthy children and adolescents (0.32%-7.0%).[37-39] An IRBBB in children is considered as a benign conduction disturbance of unknown etiology, and this conduction pattern usually normalizes in adulthood. There are no previous data on the association between elevated androgen levels and/or glucocorticoid treatment and IRBBB. It is not clear whether this ECG pattern will be clinically relevant in future. However, this particular finding had no clinical implications for our pediatric patients with CAH.

The main limitation of this study was the relatively small sample size of patients. Furthermore, the development of left ventricular dysfunction and other impairments in cardiac function over time was not evaluated. As patients with CAH are exposed to a number of cardiovascular risk factors, such as elevated blood pressure, one may hypothesize that later in life, the effects on the cardiac function may be more prevalent. Further (longitudinal) studies are needed to evaluate the cardiac function in adult patients with CAH with long exposure to elevated androgen levels and/or exogenous glucocorticoid excess.

In conclusion, we thoroughly evaluated the cardiac function in pediatric patients with CAH by multiple techniques showing normal left ventricular mass and systolic

function, with a shortened IVRT that may be a sign of mild left ventricular diastolic dysfunction. Based on the current study, there is no indication to routinely evaluate the cardiac function pediatric patients with CAH. Further studies are needed to evaluate the cardiac function in well-controlled patients with CAH in adolescence and adulthood.

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CHAPTER 4

STEROID METABOLISM IN CONGENITAL ADRENAL HYPERPLASIA

CHAPTER 4.1

Influence of 17-hydroxyprogesterone, progesterone and sex steroids on mineralocorticoid receptor transactivation in congenital adrenal hyperplasia

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Abstract

Background: Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency leads to accumulation of steroid precursors and adrenal androgens. These steroids may have a biological effect on the steroid receptor with clinical consequences on diagnostics and treatment in CAH patients. Therefore, we analyzed the effect of accumulated steroids [17-hydroxyprogesterone (17OHP), progesterone, androstenedione and testosterone] on aldosterone-mediated transactivation of the human mineralocorticoid receptor (hMR).

Methods: A transactivation assay using transiently transfected COS-7 cells was employed. Cells were co-transfected with hMR-cDNA, MMTV-luciferase and renilla-luciferase expression vectors. Transfected cells were incubated with six different steroid concentrations in addition to aldosterone (10^{-10}M). Luciferase and renilla activities were measured to quantify hMR transactivation.

Results: Linear regression analysis showed statistically significant linear inhibition of transactivation of the hMR by 10^{-10}M aldosterone in the presence of increasing 17OHP [$F(1,5) = 11.34$, $p = 0.019$] and progesterone [$F(1,5) = 11.08$, $p = 0.021$] concentrations. In contrast, neither androstenedione nor testosterone affected hMR transactivation by aldosterone at a concentration of 10^{-10}M.

Conclusion: Our study shows for the first time that neither androstenedione nor testosterone has a biological effect on aldosterone-mediated transactivation of the hMR. 17OHP and progesterone have an anti-mineralocorticoid effect in vitro that may clinically lead to an increased requirement of mineralocorticoids in poorly controlled CAH patients.

Introduction

Congenital adrenal hyperplasia (CAH) is a group of disorders affecting adrenal steroidogenesis. The incidence of classic CAH varies between 1 in 10,000 and 1 in 15,000 live births in most Caucasian populations.[1] In about 95% of the cases, CAH is caused by 21-hydroxylase deficiency,[2] resulting in impaired adrenal synthesis of cortisol. Cortisol deficiency triggers a counter-regulatory increase in pituitary ACTH secretion leading to accumulation of adrenal steroid precursors before the deficient enzymatic step and increased adrenal androgen production. 21-hydroxylase converts 17-hydroxyprogesterone (17OHP) to 11-deoxycortisol, the penultimate step in cortisol synthesis. Hence, 17OHP accumulates and is used as a marker for 21-hydroxylase deficiency.

Classic CAH is commonly subdivided in the salt wasting and simple virilizing forms depending on the residual enzymatic activity. Salt wasting patients have no residual 21-hydroxylase activity leading to severe salt loss, typically after the first week of life, and prenatal virilization of the female external genitalia. Patients with the simple virilizing form of CAH have a residual enzyme activity of 1-2% and usually have sufficient aldosterone production to prevent severe salt loss, whereas glucocorticoid synthesis is severely impaired. In both salt wasting and simple virilizing forms, elevated adrenal androgens cause prenatal virilization of the female external genitalia and postnatal androgen excess in both sexes.[2,3] Current treatment of CAH consists of lifelong glucocorticoid and, if necessary, mineralocorticoid treatment.[4] Treatment with glucocorticoids restores feedback within the hypothalamus-pituitary-adrenal axis, consequently achieving downregulation of adrenal androgen production. In many patients, however, supraphysiological doses of glucocorticoids are needed to normalize androgen levels.

Untreated and poorly controlled CAH patients are characterized by elevated levels of steroid hormone precursors, including progesterone and 17OHP, and androgens such as androstenedione and testosterone.[3,5-8] It has been shown that progesterone and 17OHP have antagonistic properties on the human mineralocorticoid receptor (hMR), and therefore may contribute to mineralocorticoid deficiency in classic CAH patients.[9] The aim of our study was to evaluate the effects of 17OHP, progesterone, androstenedione and testosterone on aldosterone-mediated transactivation and translocalization of the hMR. Furthermore, we studied the effect of the frequent mineralocorticoid receptor p.Ile180Val single nucleotide polymorphism (SNP) on transactivation of the hMR.

Material and Methods

Construction of Plasmids

The hMR cDNA was PCR amplified from the previously used pcDNA3.1-*NR3C2* construct[10] using specific primers with *Hind*III and *Eco*RV restriction sites for directional cloning into pcDNA6/V5-His-B vector (Invitrogen Corp., Carlsbad, Calif., USA). The p.Ile180Val SNP was recreated in the pcDNA6-hMR construct by site-directed mutagenesis using the QuikChange XL Site-Directed Mutagenesis Kit according to the manufacturer's protocol (Stratagene, Amsterdam, The Netherlands). The correct insertion of the hMR construct and the p.Ile180Val SNP as well as the integrity of the cDNA was checked by direct DNA sequencing. For intracellular localization assays Green Fluorescent Protein (GFP), an autofluorescent genetic reporter, was cloned into pcDNA6. The hMR cDNA and the hMR p.Ile180Val (hMR-I180V) construct were cloned into the pcDNA6-GFP vector using the same restriction enzymes as described above.

In vitro Transactivation Assays

Transactivation of hMR and hMR-I180V by different concentrations of aldosterone was investigated using a MMTV-luciferase assay. Approximately 2.5×10^4 COS-7 cells were grown in 500 ml of Dulbecco's minimal essential medium (DMEM) high glucose (4.5 g/l) with L-glutamine (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH) and penicillin/streptomycin (PAA Laboratories GmbH) in 24-well plates, and transiently transfected 24 h after seeding using FuGene® HD transfection reagent (Roche Applied Sciences, Burgess Hill, UK). Cells were transfected with 300 ng of pcDNA6-V5/HisB-hMR or pcDNA6-V5/HisB-hMR variant (p.Ile180Val) in the presence of 300 ng of a mouse mammary tumor virus (MMTV)-luciferase reporter construct (MMTV-luc) driving the firefly luciferase gene. Co-transfection with 50 ng of pRL-TK (Promega, Madison, Wisc., USA), a renilla luciferase vector, was performed to normalize data for transfection efficiency. In each set of experiments, 3 wells with COS-7 cells were co-transfected with 300 ng of pcDNA-hMR and 300 ng of pGL3-Basic (Promega) for data normalization and interassay comparison purposes as pGL3-Basic contains a coding region for firefly luciferase for monitoring transcriptional activity in transfected cells. Two days after transfection, cells were treated with aldosterone (Sigma Aldrich, Gillingham, UK) for 24 h in different concentrations (final concentrations made up in total of 500 µl full DMEM media: 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12} and 10^{-14}M), or in a 10^{-10}M concentration in addition to different concentrations of 17OHP (range 5-1,000 nM), progesterone (2.5-100 nM), androstenedione (1-250 nM) or testosterone (0.5-60 nM) (Sigma Aldrich). Concentrations of 17OHP, progesterone, androstenedione and testosterone used in the assays were based on biochemical findings in CAH patients.[5,6,7,8]

To evaluate the transactivational potential of 17OHP, progesterone, androstenedione and testosterone on the hMR in the absence of aldosterone, transfected cells were also incubated in 500 µl of full DMEM supplemented with different concentrations of these steroids.

Cells were lysed in 100 µl of passive lysis buffer (Promega). Consequently, 30 µl of cell lysate was used for the measurement of firefly and renilla luciferase activity, with a luminometer (Berthold, Bad Wildbad, Germany), using the Dual-Luciferase® Reporter Assay System (Promega) according to manufacturer's standard protocol. The hMR transactivation was calculated by the ratio of the steroid-dependent (firefly) luciferase and the steroid independent renilla (luciferase). Luciferase/renilla ratios were normalized for luciferase activity driven by pGL3-Basic. Data were normalized for the transactivation by a 10^{-10}M aldosterone concentration and are presented as fold transactivation compared to the transactivation by 10^{-10}M aldosterone (transactivation by 10^{-10}M aldosterone was set as 1.0-fold transactivation). All assays were performed in triplicate (n = 9). Statistical analysis was performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, Calif., USA). Results were analyzed by both linear regression analyses and ANOVA with Bonferroni adjustment for multiple comparisons (all possible comparisons were analyzed). Differences between the hMR wild type and the p.Ile180Val construct were analyzed using a t test. $p < 0.05$ was considered significant.

Intracellular Localization

The transactivational potential of the hMR-GFP construct was evaluated to ensure comparable transactivational potential to the hMR construct in the presence of 10^{-10}M concentrations of aldosterone. The hMR-GFP construct was used for an intracellular localization assay. Approximately 2×10^5 COS-7 cells were grown on glass coverslips in 6-well plates containing 2 ml of DMEM high glucose (4.5 g/l) with L-glutamine (PAA Laboratories GmbH) supplemented with charcoal stripped fetal bovine serum (Sigma Aldrich) and penicillin/streptomycin (PAA Laboratories GmbH). Twenty-four hours after seeding, the cells were transiently transfected using FuGene® HD transfection reagent (Roche Applied Sciences) with 2 µg of hMR-GFP or 2 µg of hMR-I180V-GFP. Forty-eight hours after transfection, cells were treated for 120 min with a combination of 10^{-10}M aldosterone and different concentrations of other steroids (17OHP, progesterone, androstenedione and testosterone) to study the effect of these steroids on the intracellular localization of the receptor. Cells were washed three times in 1× phosphate buffered saline (PBS) and fixed in 1 ml 100% methanol at -20°C for 15 min. Fixed cells were further washed three more times in 1× PBS and mounted on Vectorshield with 4', 6-diamidino-2-phenylindole (DAPI; exclusively nuclear staining). Results were obtained from three independent transfection experiments in which 150 transfected cells were classified in 4 categories:

(1) nuclear, (2) mainly nuclear, (3) equal nuclear and cytoplasmic, and (4) mainly cytoplasmic. Representative images were taken using confocal microscopy (Nikon Instruments Inc., Melville, N.Y., USA). To evaluate if treatment causes a difference in the number of cells counted as nuclear, mainly nuclear, equal nuclear or mainly cytoplasmic, respectively, one-way ANOVA was performed. Statistical analysis was performed using GraphPad Prism software version 5.0.

Results

Transactivation of the Mineralocorticoid Receptor by Aldosterone

Increasing concentrations of aldosterone caused an increase in potent transactivation of both the hMR and hMR-I180V. The dose-dependent effects on the transactivation are shown in a dose-response curve (fig. 1). An estimated concentration for 50% transactivation (EC-50) of the hMR of around 10^{-10}M aldosterone was calculated for both the wild type (2.4×10^{-11}M) and the p.Ile180Val SNP (1.2×10^{-11}M).

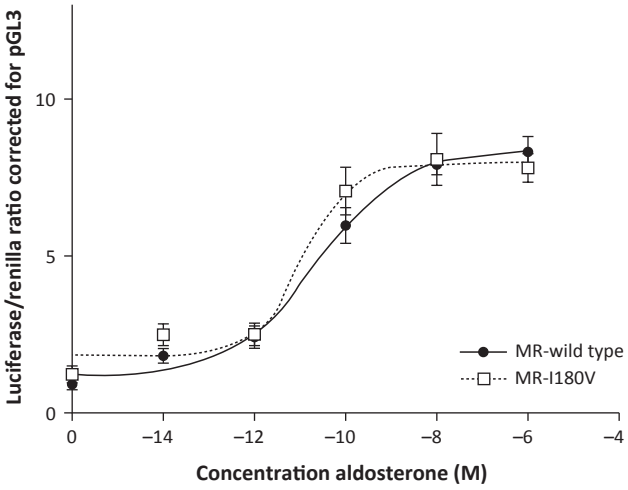

Figure 1 Dose-response curves showing the transactivation of the hMR (wild type) and the hMR-I180V SNP by different concentrations of aldosterone using a luciferase assay. The results are expressed as the ratio of (firefly) luciferase and renilla (luciferase) activity. Data are means ± SEM for each concentration (n = 9). MR = Mineralocorticoid receptor.

Effect of 17OHP, Progesterone, Androstenedione and Testosterone on hMR Transactivation

Increasing concentrations of 17OHP and progesterone inhibited aldosterone-mediated transactivation of the hMR in a dose-dependent fashion (fig. 2). Linear regression analyses showed a linear inhibition of transactivation of the hMR by 10^{-10}M aldosterone in the presence of increasing concentrations of 17OHP [$F(1,5) = 11.34$, $p = 0.019$] and progesterone [$F(1,5) = 11.08$, $p = 0.021$]. Variable concentrations of 17OHP [$F(6,48) = 111.9$, $p < 0.0001$] and progesterone [$F(6,48) = 62.11$, $p < 0.0001$] have a significant effect on transactivation of the hMR by aldosterone in the presence of 10^{-10}M aldosterone, as shown by ANOVA (suppl. tables 1, 2).

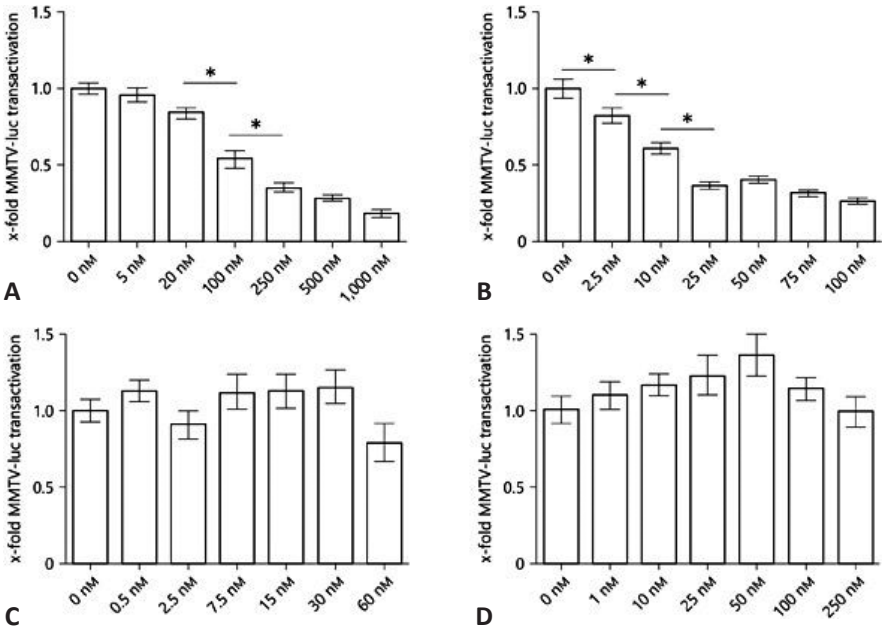

Figure 2 The effect of different concentrations of 17OHP (**A**), progesterone (**B**), testosterone (**C**) and androstenedione (**D**) on the transactivation of hMR by 10^{-10}M aldosterone concentration. The transactivation activity of 10^{-10}M aldosterone was set as 1.0. Results are expressed as x-fold transactivation of MMTV (firefly) luciferase (MMTV-luc). Data are means ± SEM for each concentration (n = 9). Significant differences in transactivation between two concentrations closest to each other are indicated by an asterisk ($p < 0.05$).

In contrast, treatment with increasing concentrations of androstenedione and testosterone did not have any measureable effect on hMR transactivation (fig. 2). No linear effect of increasing concentrations of androstenedione [F(1,5) = 0.709, p = 0.438] or testosterone [F(1,5) = 1.57, p = 0.265]on transactivation of the hMR by aldosterone was found. In addition, ANOVA showed that different concentrations of androstenedione or testosterone did not affect transactivation of the hMR by aldosterone (online suppl. tables 3, 4).

The effect of three different concentrations of 17OHP on the aldosterone-mediated transactivation of the hMR was also evaluated in the p.Ile180Val SNP construct (fig. 3). The inhibitory effect of 17OHP on hMR-I180V was found to be similar to its effect on the wild-type hMR (p > 0.05).

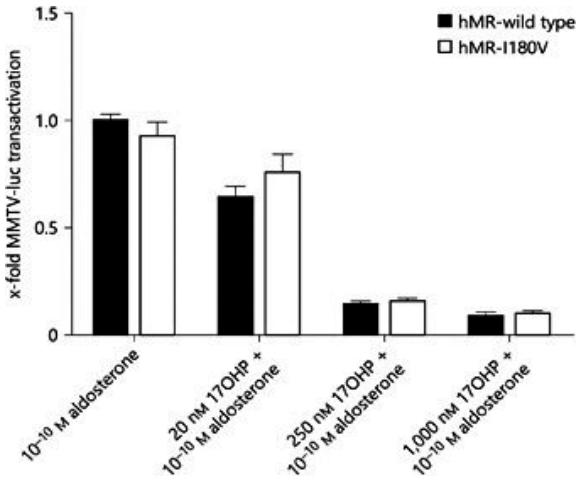

Figure 3 The effect of different concentrations of 17OHP on the transactivation of hMR by 10^{-10}M aldosterone concentration compared to the effect of different concentrations of 17OHP on the transactivation of the hMR-I180V SNP. The transactivation activity of 10^{-10}M aldosterone on the hMR (wild type) was set as 1.0. Results are expressed as x-fold transactivation of MMTV (firefly) luciferase (MMTV-luc). Data are means ± SEM for each concentration (n = 9)

Intracellular Localization of the hMR

The transactivation potential of both the hMR-GFP and the hMR construct were compared to assess whether the GFP had altered transactivational properties of the construct prior to performing an intracellular localization assay. The hMR-GFP construct was shown to have equal transactivational properties as the hMR construct (suppl. fig. 1).

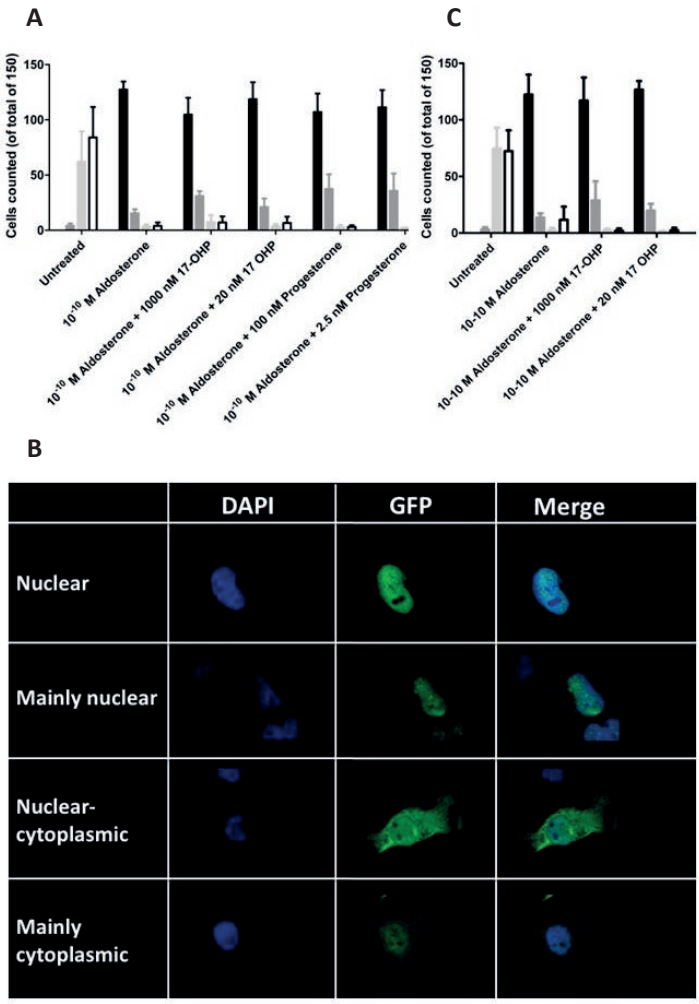

Figure 4 **A** Cellular localization of the hMR without the presence of aldosterone and in the presence of aldosterone with or without different concentrations of 17OHP and progesterone. Cells were localized using confocal microscopy as (1) nuclear (black bars), (2) mainly nuclear (dark grey bars), (3) equal nuclear-cytoplasmic (light grey bars) and (4) mainly cytoplasmic (white bars). **B** Images showing the four possible cellular localizations of the hMR: (1) nuclear, (2) mainly nuclear, (3) equal nuclear and cytoplasmic, (4) mainly cytoplasmic. Images were taken with a confocal microscope. Different images were taken showing DAPI staining, GFP and a merged image. **C** Cellular localization of the hMR-I180V without the presence of steroids and in the presence of aldosterone with or without different concentrations of 17OHP. Cells were localized using confocal microscopy as (1) nuclear (black bars), (2) mainly nuclear (dark grey bars), (3) equal nuclear - cytoplasmic (light grey bars) and (4) mainly cytoplasmic (white bars).

In untreated cells, the hMR was localized only in the cytoplasm or equally distributed in nucleus and cytoplasm (fig. 4a). Treatment with aldosterone for 120 min resulted in a clear translocation of the hMR with a predominantly nuclear localization.

17OHP and progesterone did not influence the translocation of the hMR to the nucleus in the presence of aldosterone (fig. 4b). Treatment with 17OHP, progesterone, androstenedione or testosterone did not result in significant differences in the intracellular localization of the hMR.

In the presence of aldosterone, the hMR-I180V-GFP was also mainly localized in the nucleus. 17OHP did not inhibit the translocation of the hMR-I180V-GFP to the nucleus in the presence of aldosterone (fig. 4c).

Discussion

We studied the effects of different adrenal steroid hormone precursors and androgens on the transactivational potential and localization of the hMR. Our study shows for the first time that excess concentrations of androstenedione and testosterone do not have a biological effect on the aldosterone-mediated transactivation of the hMR in vitro. Furthermore, 17OHP and progesterone have a strong anti-mineralocorticoid effect in vitro, which confirms previous findings.[9] This study highlights the anti-mineralocorticoid effect of elevated 17OHP concentrations as found in poorly controlled CAH patients.

These findings may have important implications for the clinical care provision. Based on our results, it can be suggested that elevated 17OHP and progesterone concentrations are likely to have an adverse effect on the mineralocorticoid effect in untreated and poorly treated CAH. This may potentially lead to increased requirement of mineralocorticoids and suboptimal control. In contrast, elevated androgens did not influence the mineralocorticoid transactivation in vitro. We therefore hypothesize that elevated androgens per se do not have a clinical relevant effect on mineralocorticoid treatment in the clinical care of CAH.

The current treatment strategy is based on normalizing adrenal androgens to prevent adverse effects of hyperandrogenism. Slightly elevated 17OHP concentrations are generally accepted because of the possible side effects of high dosages of glucocorticoids needed to achieve physiological 17OHP concentrations. Based on our results it can be suggested that lowering of highly elevated 17OHP concentrations may also have an additional positive effect on the dosage of mineralocorticoid treatment and consequently decrease the potential risk of adverse effects of mineralocorticoid treatment such as hypertension. Unfortunately, supraphysiological doses of glucocorticoids are generally necessary to lower 17OHP levels that may lead to adverse effects and long-term complications. Therefore, the treatment goal in CAH patients is normalization of adrenal androgens with slightly elevated 17OHP

levels.[4] Elevated renin levels may indicate the need for higher mineralocorticoid doses. However, based on our data, elevated renin concentrations may also reflect the anti-mineralocorticoid effect of elevated 17OHP concentrations. A fine balance between the use of supraphysiological dosages of glucocorticoids, mineralocorticoid treatment and normalizing 17OHP levels has to be achieved to prevent long-term complications of overtreatment with glucocorticoids on one hand and overtreatment with mineralocorticoids on the other hand.

The antagonistic properties of progesterone on the human, rat and sheep mineralocorticoid receptor have been previously described.[9,11-15] A 50% inhibition of the maximum transactivation of the mineralocorticoid receptor is caused by progesterone concentrations between 2 and 11 nM.[9,16-18] The inhibitory effect of progesterone described in our study is in line with those described in the studies mentioned above. Minor differences between the results of those studies may be explained by different cells and different luciferase constructs used.

The effect of slightly elevated 17OHP concentrations on the hMR have been studied previously.[9] The previously reported concentration of 135 nM, causing a 50% inhibition of transactivation of the hMR by a 10^{-9}M aldosterone, is in line with the antagonistic effect of 17OHP on aldosterone-mediated transactivation described in our study. In our study we evaluated the effect of even higher 17OHP concentrations, as found in untreated or poorly controlled CAH patients.

In contrast to the effect on transactivation, the translocation to the nucleus seems not to be affected by 17OHP or progesterone. The physiological human ligand of the hMR is aldosterone. After binding to aldosterone, the hMR undergoes a conformational change and partial dissociation of the ligand binding complex occurs, leading to translocation of the hMR to the nucleus. Within the nucleus, the activated receptors regulate transcription by different pathways including transactivation of target genes.[19-23] Intracellular localization studies on the hMR have shown that in the absence of steroids the hMR is localized in the cytoplasm and in the nucleus, aldosterone causes a rapid nuclear accumulation of the hMR.[19,24-27] Binding of aldosterone to the hMR causes dissociation of several associated proteins from the receptor, followed by dimerization and finally nuclear translocation of the activated receptor. The translocation assay performed in this study showed a similar subcellular localization with a predominant localization of the hMR in the cytoplasm in the absence of steroids. Treatment of the COS-7 cells expressing the hMR-GFP construct with aldosterone causes a quick translocation of the hMR to the nucleus of the cells. However, different concentrations of 17OHP and progesterone in addition to an aldosterone concentration of 10^{-10}M do not have an impact on the translocation of the hMR to the nucleus. This finding is in contrast to the described effects of hMR antagonists, such as spironolactone and eplerenone, which inhibit the translocation of the hMR to the nucleus.[19]

The mechanism of the inhibition of aldosterone-mediated transactivation of the hMR by progesterone and 17OHP remains unclear. It has been shown that 17OHP has a relatively high binding affinity for the hMR.[9] Therefore, competitive binding of the hMR between 17OHP and aldosterone, such as in patients with poorly controlled CAH, is very likely. We showed that 17OHP does not inhibit the translocation of the hMR to the nucleus. Therefore, we hypothesize that the anti-mineralocorticoid effect of 17OHP on the hMR is not due to an effect on the translocation of the hMR, but might be caused by effects on the transcription after translocation to the nucleus. It has been suggested by Hellal-Levy et al.[20] that binding of an antagonist to the hMR leads to an inactive conformation of the hMR. Due to instability, this complex of the mineralocorticoid receptor and its antagonist will not be converted into a transcriptionally active conformation.[20] This hypothesis may explain the antagonistic properties of 17OHP and progesterone on the hMR.

The mineralocorticoid receptor p.Ile180Val SNP (rs5522) is one of the most frequent SNPs in the hMR with a frequency of 10.2% of the G allele in a European population (HapMap project, www.hapmap.org). The mineralocorticoid receptor p.Ile180Val SNP has been associated with an increased hypertension risk.[28] As CAH patients have a tendency to develop elevated blood pressure,[29,30] the role of this SNP in CAH patients might be important with respect to their cardiovascular risk profile. We showed that the hMR p.Ile180Val SNP does not affect transactivation of the hMR by aldosterone. These findings are in line with the results by DeRijk et al.[31] In addition, 17OHP has the same antagonistic effect on the hMR-I180V SNP as on the wild-type hMR. Thus, the results of this study do not explain the increased hypertension risk in p.Ile180Val.

In conclusion, our study shows for the first time that neither androstenedione nor testosterone have a significant biological effect on the aldosterone-mediated transactivation of the hMR. In contrast, increased 17OHP and progesterone concentrations have an anti-mineralocorticoid effect due to an inhibition of aldosterone-mediated transactivation of the hMR. However, unlike hMR blockers, neither 17OHP nor progesterone inhibits the translocation of the hMR to the nucleus. Further studies are needed to explain the mechanism of this inhibition of transactivation by 17OHP.

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Supplementary table 1 Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of 17OHP on the aldosterone mediated transactivation of the hMR

Comparison	Mean difference.	t	P < 0.05?
1000 nmol/l vs 500 nmol/l	-0,09818	2,176	No
1000 nmol/l vs 250 nmol/l	-0,1691	3,748	Yes
1000 nmol/l vs 100 nmol/l	-0,3538	7,841	Yes
1000 nmol/l vs 20 nmol/l	-0,6562	14,54	Yes
1000 nmol/l vs 5 nmol/l	-0,7723	17,12	Yes
1000 nmol/l vs 0 nmol/l	-0,8160	18,09	Yes
500 nmol/l vs 250 nmol/l	-0,07093	1,572	No
500 nmol/l vs 100 nmol/l	-0,2556	5,664	Yes
500 nmol/l vs 20 nmol/l	-0,5581	12,37	Yes
500 nmol/l vs 5 nmol/l	-0,6741	14,94	Yes
500 nmol/l vs 0 nmol/l	-0,7179	15,91	Yes
250 nmol/l vs 100 nmol/l	-0,1846	4,092	Yes
250 nmol/l vs 20 nmol/l	-0,4871	10,80	Yes
250 nmol/l vs 5 nmol/l	-0,6032	13,37	Yes
250 nmol/l vs 0 nmol/l	-0,6469	14,34	Yes
100 nmol/l vs 20 nmol/l	-0,3025	6,704	Yes
100 nmol/l vs 5 nmol/l	-0,4186	9,277	Yes
100 nmol/l vs 0 nmol/l	-0,4623	10,25	Yes
20 nmol/l vs 5 nmol/l	-0,1161	2,573	No
20 nmol/l vs 0 nmol/l	-0,1598	3,542	Yes
5 nmol/l vs 0 nmol/l	-0,04373	0,9692	No

Supplementary table 2 Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of progesterone on the aldosterone mediated transactivation of the hMR

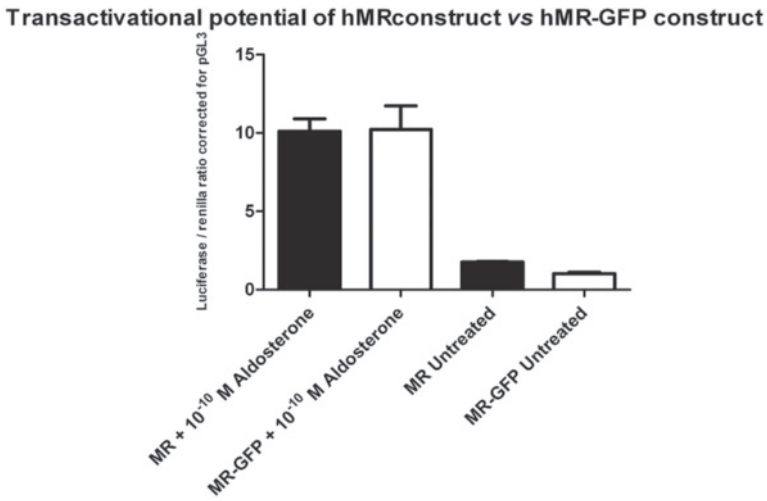
Comparison	Mean difference	t	P < 0.05?
100 nmol/l vs 75 nmol/l	-0,04790	0,9424	No
100 nmol/l vs 50 nmol/l	-0,1382	2,719	No
100 nmol/l vs 25 nmol/l	-0,09753	1,919	No
100 nmol/l vs 10 nmol/l	-0,3455	6,797	Yes
100 nmol/l vs 2.5 nmol/l	-0,5654	11,12	Yes
100 nmol/l vs 0 nmol/l	-0,7384	14,53	Yes
75 nmol/l vs 50 nmol/l	-0,09030	1,776	No
75 nmol/l vs 25 nmol/l	-0,04963	0,9763	No
75 nmol/l vs 10 nmol/l	-0,2976	5,855	Yes
75 nmol/l vs 2.5 nmol/l	-0,5175	10,18	Yes
75 nmol/l vs 0 nmol/l	-0,6905	13,58	Yes
50 nmol/l vs 25 nmol/l	0,04067	0,8001	No
50 nmol/l vs 10 nmol/l	-0,2073	4,078	Yes
50 nmol/l vs 2.5 nmol/l	-0,4272	8,405	Yes
50 nmol/l vs 0 nmol/l	-0,6002	11,81	Yes
25 nmol/l vs 10 nmol/l	-0,2480	4,878	Yes
25 nmol/l vs 2.5 nmol/l	-0,4679	9,205	Yes
25 nmol/l vs 0 nmol/l	-0,6408	12,61	Yes
10 nmol/l vs 2.5 nmol/l	-0,2199	4,327	Yes
10 nmol/l vs 0 nmol/l	-0,3929	7,729	Yes
2.5 nmol/l vs 0 nmol/l	-0,1729	3,402	Yes

Supplementary table 3 Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of testosterone on the aldosterone mediated transactivation of the hMR

Comparison	Mean difference	t	P < 0.05?
60 nmol/l vs 30 nmol/l	-0,3613	2,501	No
60 nmol/l vs 15 nmol/l	-0,3361	2,326	No
60 nmol/l vs 7.5 nmol/l	-0,3316	2,295	No
60 nmol/l vs 2.5 nmol/l	-0,1137	0,7870	No
60 nmol/l vs 0.5 nmol/l	-0,3324	2,301	No
60 nmol/l vs 0 nmol/l	-0,2067	1,431	No
30 nmol/l vs 15 nmol/l	0,02527	0,1749	No
30 nmol/l vs 7.5 nmol/l	0,02973	0,2058	No
30 nmol/l vs 2.5 nmol/l	0,2476	1,714	No
30 nmol/l vs 0.5 nmol/l	0,02890	0,2001	No
30 nmol/l vs 0 nmol/l	0,1546	1,070	No
15 nmol/l vs 7.5 nmol/l	0,004459	0,03086	No
15 nmol/l vs 2.5 nmol/l	0,2224	1,539	No
15 nmol/l vs 0.5 nmol/l	0,003627	0,02511	No
15 nmol/l vs 0 nmol/l	0,1294	0,8955	No
7.5 nmol/l vs 2.5 nmol/l	0,2179	1,508	No
7.5 nmol/l vs 0.5 nmol/l	-0,0008315	0,005755	No
7.5 nmol/l vs 0 nmol/l	0,1249	0,8646	No
2.5 nmol/l vs 0.5 nmol/l	-0,2187	1,514	No
2.5 nmol/l vs 0 nmol/l	-0,09299	0,6436	No
0.5 nmol/l vs 0 nmol/l	0,1257	0,8704	No

Supplementary table 4 Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of androstenedione on the aldosterone mediated transactivation of the hMR

Comparison	Mean difference	t	P < 0.05?
250 nmol/l vs 100 nmol/l	-0,1522	1,264	No
250 nmol/l vs 50 nmol/l	-0,3702	3,073	No
250 nmol/l vs 25 nmol/l	-0,2441	2,027	No
250 nmol/l vs 10 nmol/l	-0,1788	1,484	No
250 nmol/l vs 1 nmol/l	-0,1100	0,9129	No
250 nmol/l vs 0 nmol/l	-0,01177	0,09770	No
100 nmol/l vs 50 nmol/l	-0,2180	1,810	No
100 nmol/l vs 25 nmol/l	-0,09192	0,7631	No
100 nmol/l vs 10 nmol/l	-0,02654	0,2204	No
100 nmol/l vs 1 nmol/l	0,04224	0,3507	No
100 nmol/l vs 0 nmol/l	0,1404	1,166	No
50 nmol/l vs 25 nmol/l	0,1261	1,047	No
50 nmol/l vs 10 nmol/l	0,1915	1,589	No
50 nmol/l vs 1 nmol/l	0,2602	2,160	No
50 nmol/l vs 0 nmol/l	0,3584	2,976	No
25 nmol/l vs 10 nmol/l	0,06538	0,5427	No
25 nmol/l vs 1 nmol/l	0,1342	1,114	No
25 nmol/l vs 0 nmol/l	0,2324	1,929	No
10 nmol/l vs 1 nmol/l	0,06878	0,5710	No
10 nmol/l vs 0 nmol/l	0,1670	1,386	No
1 nmol/l vs 0 nmol/l	0,09820	0,8152	No



Supplementary figure 1 Transactivational potential of the hMR construct versus the hMR-GFP construct evaluated by a luciferase assay. The results are expressed as the ratio of (firefly) luciferase to renilla (luciferase) activity corrected for pGL3 (transfection efficiency). Data are means ± S.E.M. (n=9).

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CHAPTER 4.2

Adrenal steroid metabolites accumulating in congenital adrenal hyperplasia lead to transactivation of the glucocorticoid receptor

Pijnenburg-Kleizen KJ, Engels M, Mooij CF, Griffin A, Krone N, Span PN, van Herwaarden AE, Sweep FCGJ, Claahsen-van der Grinten HL.
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Summary

Patients with congenital adrenal hyperplasia (CAH) are often clinically less severely affected by cortisol deficiency than anticipated from their enzymatic defect. We hypothesize that adrenal steroid hormone precursors that accumulate in untreated or poorly controlled CAH have glucocorticoid activity and partially compensate for cortisol deficiency. We studied the in vitro effects of 17-hydroxyprogesterone (17OHP), progesterone (P), 21-deoxycortisol (21DF), and androstenedione (Δ4) on the human glucocorticoid receptor (hGR). Competitive binding assays were performed in HeLa cells. Nuclear translocation of the hGR was studied by transfection of COS-7 cells with a GFP-tagged hGR and fluorescence microscopy. Transactivation assays were performed in COS-7 cells and in HEK 293 cells after cotransfection with hGR and luciferase reporter vectors using a dual luciferase assay. 17OHP, P, and 21DF are able to bind to the hGR with binding affinities of 24–43% compared with cortisol. Δ4 has a low binding affinity. Incubation with 21DF led to complete nuclear translocation of the hGR, whereas treatment with 17OHP or P resulted in partial nuclear translocation. 21DF transactivated the hGR with an EC50 approximately 6 times the EC50 of cortisol. 17OHP and P transactivated the hGR with EC50s of more than 100 times the EC50 of cortisol. No hGR transactivation was detected after incubation with Δ4. 21DF, 17OHP, and P are able to bind, translocate, and transactivate the hGR in vitro and thus may have glucocorticoid activity. 21DF might have a clinically relevant agonistic effect on the hGR and could potentially partially compensate the cortisol deficiency in CAH patients.

Congenital adrenal hyperplasia (CAH) is an autosomal-recessive disorder caused by a deficiency of one of the enzymes involved in adrenal steroid synthesis. Ninety-five percent of cases are caused by 21-hydroxylase deficiency. This results in impaired synthesis of cortisol. In 75% of cases patients also suffer from clinically relevant aldosterone deficiency.[1] Due to the reduced negative feedback to the hypothalamus and pituitary gland, cortisol deficiency leads to increased pituitary secretion of ACTH. Consequently, the steroid hormone precursors prior to the enzymatic block accumulate and are shunted into the androgen-synthesis pathway. The presence of increased concentrations of adrenal steroid hormone precursors is a hallmark feature of patients with CAH and these precursors can be used as diagnostic markers.[2] CAH caused by 21-hydroxylase deficiency represents a spectrum of disease depending on the severity of the enzymatic defect. The most severe, classic CAH, is subdivided in a salt-wasting form (SW-CAH) and a simple-virilizing form (SV-CAH) without aldosterone deficiency. Nonclassic CAH (NCAH) is less severe with generally only mild symptoms of hyperandrogenism.[1] The treatment of classic CAH consists of lifelong replacement of glucocorticoids and if necessary also of mineralocorticoids. The goal of treatment is to prevent adrenal and salt-wasting crises and to suppress abnormal secretion of adrenal androgens by suppression of the pituitary gland. Treatment of NCAH is only indicated when there are severe symptoms of androgen excess and/or glucocorticoid deficiency.[2]

Patients with CAH are clinically often less severely affected by cortisol deficiency than anticipated from their enzymatic defect. For example, patients with SW-CAH have a severe cortisol and aldosterone deficiency due to a residual enzymatic activity of less than 1%.[1] They develop salt-wasting crises neonatally but only a minority present with hypoglycemia or conjugated jaundice, a classic symptom of infantile glucocorticoid deficiency.[3] Patients with SV-CAH have a residual enzymatic activity of 1–2% and a suboptimal increase of cortisol after stimulation with ACTH. In areas where neonatal screening is not implemented, male patients with SV-CAH often present with signs of androgen excess in (early) childhood, without a history of Addisonian crises during illness or surgery prior to their diagnosis.[1] Furthermore, many adult patients with classic CAH who are lost to endocrine followup and are not adherent to treatment with glucocorticoids do not develop adrenal crises for long periods of time.[4] Finally, between 30 and 40% of patients with NCAH diagnosed in adolescence or adulthood show a suboptimal cortisol response to synacthen stimulation, suggesting a potential risk of adrenal insufficiency during illness or surgery.[5-7] However, these patients usually do not report a history of signs or symptoms consistent with adrenal insufficiency during surgery or illness.[5,7]

These observations could hypothetically be explained by the presence of adrenal steroid hormone precursors that accumulate in patients with untreated and poorly controlled CAH. These precursors, such as 17-hydroxyprogesterone (17OHP),

progesterone (P), 21-deoxycortisol (21DF), and androstenedione (Δ4),[2] have structural similarities to cortisol.[8] We hypothesize that they may have clinically significant glucocorticoid activity and partially substitute for cortisol. To analyze their glucocorticoid properties, we studied their effects on binding, nuclear translocation, and transactivation of the human glucocorticoid receptor (hGR).

Materials and Methods

In vitro receptor binding assays

Competitive binding assays for the hGR were performed as described previously.[9] HeLa cells were cultured in DMEM High Glucose (4.5 g/L) with L-Glutamine supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all PAA Laboratories, GmbH). Whole cells ($0.2–1.0 \times 10^6$) were incubated in serum and phenol-red-free RPMI medium (final volume, 150 µL) for 1.5–2 hours at 37°C in a series of 0.5 mL microcentrifuge tubes containing 30nM ^{3}H-cortisol (PerkinElmer, Inc.) and an increasing amount of unlabeled competitor: cortisol, 17OHP, 21DF, Δ4 (all Steraloids) or P (Sigma-Aldrich). Nonspecific binding was assessed by means of 500-fold excess of dexamethasone (Steraloids). Radioactivity was counted in a β counter. Specific binding was expressed as the percentage of specific binding over binding of radioligand only, corrected for nonspecific binding.

In vitro nuclear translocation assays

For the intracellular localization assays, hGR DNA (pRShGRα) was PCR amplified using primers with BamHI and EcoRV restriction sites (Sigma-Aldrich). It was cloned into a pcDNA6-V5/HisB-EGFP vector (Invitrogen Corp.). The correct insertion of the hGR construct as well as the integrity of the cDNA was checked by direct DNA sequencing. The assays were carried out in COS-7 cells, which is a cell line that does not contain endogenous glucocorticoid receptor. The COS-7 cells were cultured in DMEM High Glucose (4.5 g/L) with L-Glutamine supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. COS-7 cells ($n = 1.5 \times 10^5$) were grown in six-well plates on glass coverslips. After 24 hours the cells were transiently transfected with 2 µg of GFP-hGR using the TransIT-LT 1 DNA transfection reagent (Mirus Bio). Fourty-eight hours after transfection the cells were treated for 60 minutes with one of the steroids (cortisol, 17OHP, P, 21DF, Δ4) in a concentration of 10^{-6}M. Afterward the cells were fixated on the coverslips in 100% methanol at –20°C for 15 minutes and mounted with Vectashield with DAPI (Vector Laboratories) on microscope slides. The cells were studied under the Zeiss Apotome Fluorescence microscope with Zeiss Axiovision imaging software (version 4.7.2) at a magnification of 200× for the localization of the hGR. Representative images were taken. The experiment was performed in duplicate.

In vitro transactivation assays

For the transactivation assays, the hGR DNA was cloned into a pcDNA6-V5/HisB vector (Invitrogen Corp.) using the same restriction enzymes as described above. The luciferase reporter vectors used for the transactivation assays were MMTV-luc and pRL-TK (Promega). MMTV-luc is a firefly luciferase reporter construct. Transcription is controlled by glucocorticoid response elements immediately upstream from the luciferase sequence. pRL-TK contains a renilla luciferase. It serves as an internal standard to normalize firefly luciferase light emission measurements with regard to transfection efficiency and the number of cells in each well.

The COS-7 cells were cultured in DMEM High Glucose (4.5 g/l) with L-Glutamine supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and seeded in 24-well plates in a density of 2×10^4 cells per well. Twenty-four hours after seeding, the COS-7 cells were transiently cotransfected using the TransIT-LT 1 DNA transfection reagent with 0.2 µg pcDNA6-V5/HisB-hGR, 0.3 µg MMTV-luc, and 0.01 µg pRL-TK per well. Two days after transfection the cells were treated with one of the steroids (cortisol, 17OHP, P, 21DF, Δ4). Steroid solutions in ethanol were made in concentrations of 200× the final desired concentration (10^{-9} to 10^{-4}M) and diluted 1:200 with DMEM prior to adding them to the transfected cells. Twenty-four hours after adding the steroid, firefly and renilla luciferase activity were measured using the Dual-Luciferase Reporter Assay System (Promega) on a Fluoroskan FL luminometer (Thermo Scientific). Firefly luciferase/renilla luciferase ratios were calculated to normalize for transfection efficiency. Each experiment was performed in triplicate.
To ensure that the COS-7 cell line did not contain relevant amounts of endogenous steroid receptors, the transactivation activities of the different steroids were also measured in COS-7 cells that had not been transfected with steroid receptor expression vectors. Likewise, the system was tested for the presence of endogenous steroids by measuring the transactivation activity after transfection with the hGR vectors but without addition of steroids. In neither approach relevant transactivation was measured.

The transactivation assay was repeated in HEK293 cells using the methods described above. These experiments were performed in duplicate.

Statistical analysis

Analyses were performed using GraphPad Prism software version 5.0 for Windows (GraphPad Software). Steroid concentrations were expressed on a log scale and dose-response curves were calculated using nonlinear regression. For the receptor binding assays, the concentration of unlabeled steroid that reduces binding of the radioligand by half (IC_{50}) was determined. The relative binding affinity of the study steroids compared with cortisol was calculated by $IC_{50:cortisol}/IC_{50:test\ steroid} \times 100\%$. For the transactivation assays, the estimated concentration for 50% transactivation

(EC_{50}) was determined. For calculation of the relative functional sensitivity of the hGR to the different steroids, the transactivation potential of cortisol was set at 100%. The sensitivity of the hGR to the other test compounds was calculated as $EC_{50:cortisol}$/ $EC_{50:test\ steroid}$ × 100%.

Results

Receptor-binding assay

The receptor-binding curves for the studied steroids are shown in Figure 1. The IC_{50} and the relative binding affinity of cortisol, 17OHP, P, 21DF, and Δ4 were calculated (Table 1). The results demonstrate that 17OHP, P, and 21DF bind to the hGR with relative binding affinities of 27, 43, and 24%, respectively. The binding affinity for Δ4 is dramatically less than that of cortisol, 0.4%.

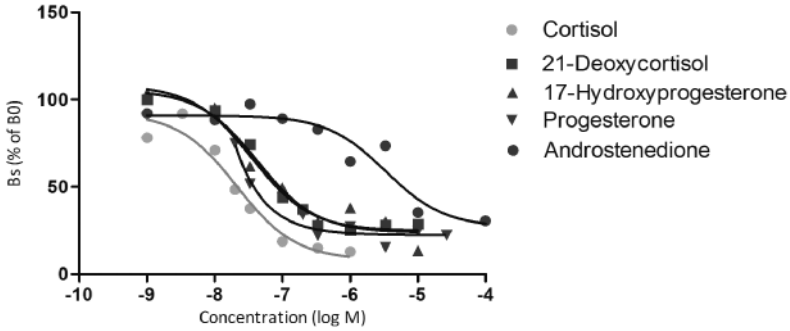


Figure 1 Binding assay. Competition of various steroids for binding of 3H cortisol to the hGR in HeLa cells. Binding data are expressed as the percentage of specific binding (Bs) remaining after adding increasing amounts of competitor.

Nuclear translocation assay

Incubation with cortisol in a concentration of 10^{-6}M resulted in complete nuclear translocation of the hGR within 60 minutes (Figure 2B). Adding 17OHP, P, and 21DF to the transfected cells in a concentration of 10^{-6}M resulted in respectively almost complete, partial and complete transport to the nucleus (Figure 2, C–E). After treatment of the cells with Δ4, the location of the hGR was still predominantly cytoplasmic (Figure 2F).

Table 1 Binding and transactivation capacity of cortisol, 17OHP, P, 21DF, and Δ4 to the hGR

	Binding assay		Transactivation assay in COS-7 cells		Transactivation assay in HEK293 cells	
Adrenal steroid metabolite	**IC_{50} (95% CI)**	**Relative binding affinity**	**EC_{50} (95% CI)**	**Relative functional sensitivity**	**EC_{50} (95% CI)**	**Relative functional sensitivity**
Cortisol	2.2×10^{-8} ($1.3 \times 10^{-8} – 3.8 \times 10^{-8}$)	100 %	1.7×10^{-8} ($1.0 \times 10^{-8} – 2.8 \times 10^{-8}$)	100 %	3.5×10^{-9} ($1.8 \times 10^{-9} – 6.9 \times 10^{-9}$)	100 %
17OHP	8.2×10^{-8} ($3.8 \times 10^{-8} – 1.6 \times 10^{-7}$)	27 %	2.2×10^{-6} ($1.5 \times 10^{-6} – 3.2 \times 10^{-6}$)	0.8 %	1.2×10^{-6} ($7.6 \times 10^{-7} – 1.9 \times 10^{-6}$)	0.3 %
P	5.1×10^{-8} ($3.1 \times 10^{-8} – 7.8 \times 10^{-8}$)	43 %	3.0×10^{-6} ($1.5 \times 10^{-6} – 5.9 \times 10^{-6}$)	0.6 %	2.3×10^{-6} ($1.2 \times 10^{-6} – 4.8 \times 10^{-6}$)	0.2 %
21DF	9.1×10^{-8} ($6.2 \times 10^{-8} – 1.3 \times 10^{-7}$)	24 %	1.0×10^{-7} ($5.2 \times 10^{-8} – 2.0 \times 10^{-7}$)	17 %	4.1×10^{-8} ($2.1 \times 10^{-8} – 8.2 \times 10^{-8}$)	8.5 %
Δ4	5.9×10^{-6} ($2.0 \times 10^{-6} – 2.2 \times 10^{-5}$)	0.4 %	-	-	-	-

IC_{50}: estimated concentration that reduces binding of the radioligand by 50%, in mol/liter.
Relative binding affinity: the binding affinity of cortisol is set at 100%. The binding of the other steroids to the hGR is calculated as $IC_{50\ cortisol}$ / $IC_{50\ test\ steroid}$ x 100%.
EC_{50}: estimated concentration for 50% transactivation, in mol/liter.
Relative functional sensitivity: the transactivation potential of cortisol is set at 100%. The sensitivity of the hGR to the other test compounds is calculated as $EC_{50\ cortisol}$ / $EC_{50\ test\ steroid}$ x 100%.

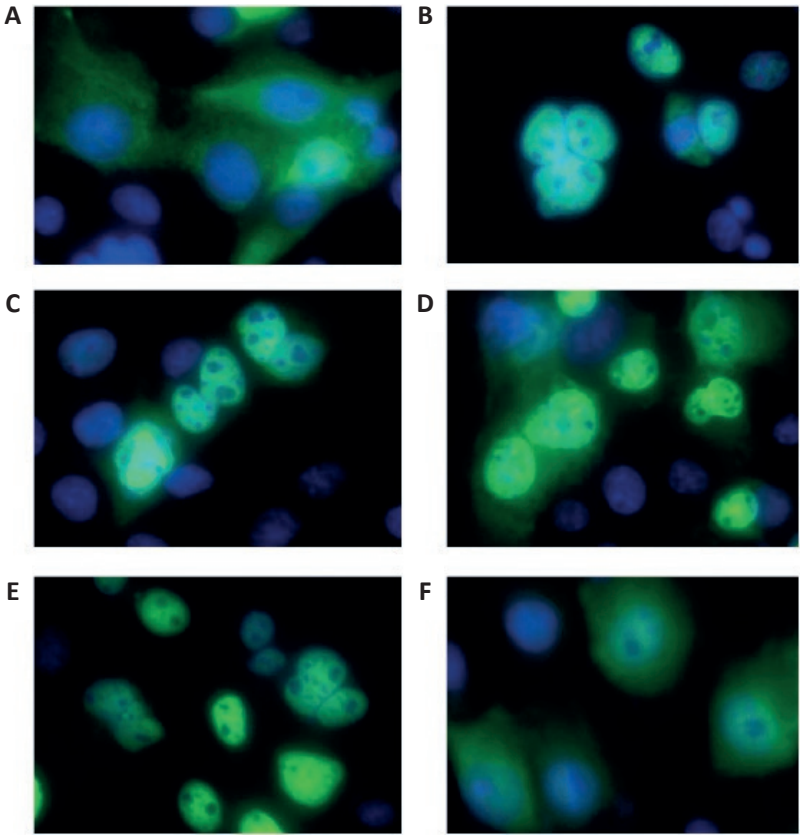


Figure 2 Localization of the hGR in COS-7 cells transfected with the hGR, without steroids (**A**) and after incubation with various steroids in a concefitration of 10^{-6}M (**B–F**). The nucleus is stained blue and the hGR is tagged with a green fluorescent protein. **B**, cortisol. **C**, 17-OHP. **D**, progesterone. **E**, 21DF. **F**, Δ4.

hGR transactivation assay in COS-7 cells

Cortisol activated the hGR with an EC_{50} of 1.7×10^{-8}M (Figure 3, A–D; Table 1). Exposure of the hGR to increasing concentrations of 17OHP, P, and 21DF resulted in increasing hGR transactivation, up to a maximum. A dose-response curve was fitted (Figure 3, A–C) and the EC_{50} values for these steroids were calculated. The EC_{50} value was 2.2×10^{-6}M for 17OHP, 3.0×10^{-6}M for P, and 1.0×10^{-7}M for 21DF (Table 1). Δ4 did not transactivate the hGR at the concentrations tested (Figure 3D).

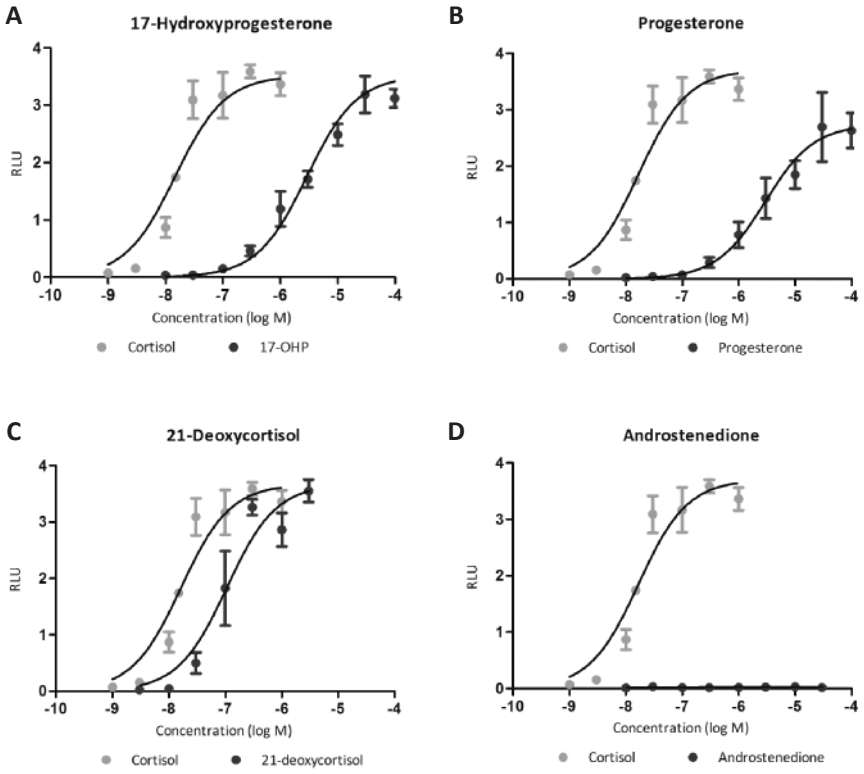
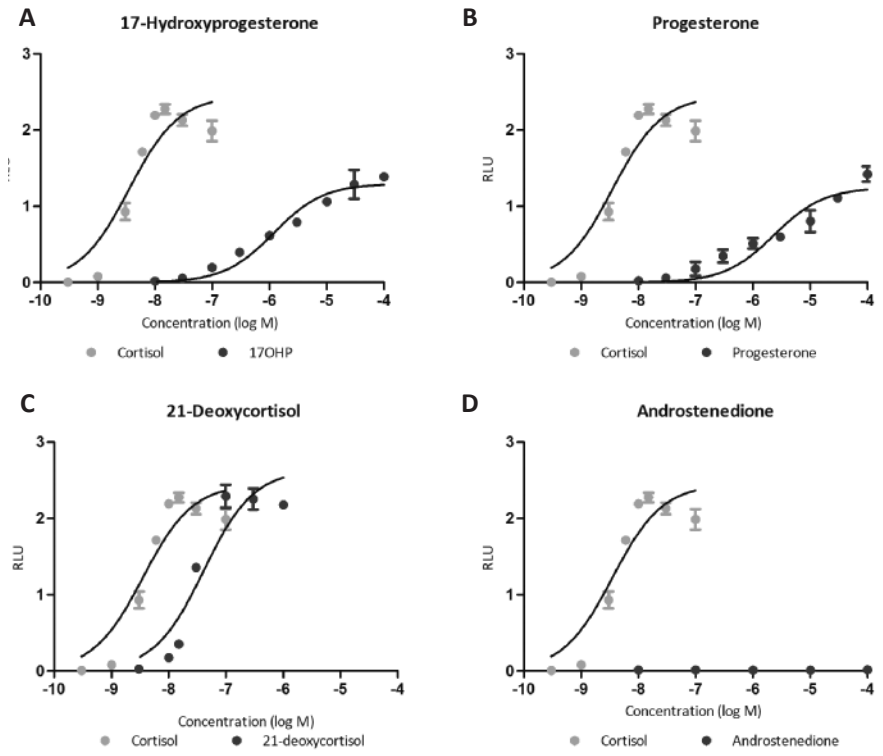


Figure 3 Transactivation of the hGR by 17OHP (**A**), P (**B**), 21DF (**C**), and Δ4 (**D**), in comparison with the transactivation of the hGR by cortisol.

hGR transactivation assay in HEK293 cells

The results for the transactivation assay in HEK293 cells are shown in Supplemental Figure 1 and Table 1. In these experiments, the EC_{50} for cortisol was 3.5×10^{-9}M. 17OHP, P, and 21DF activated the hGR with EC_{50} values of 1.2×10^{-6}M, 2.3×10^{-6}M, and 4.1×10^{-8}M, respectively.



Supplemental figure 1 Transactivation of the human glucocorticoid receptor (hGR) in HEK293 cells by 17-hydroxyprogesterone, 21-deoxycortisol and androstenedione, in comparison with the transactivation of the hGR by cortisol.

Discussion

We describe here the binding of 17OHP, P, 21DF, and Δ4 to the hGR and their effects on the nuclear translocation and transactivation of the hGR, in comparison with the effects of cortisol. We found that 21DF and to a lesser extent 17OHP and P can transactivate the hGR. This is consistent with the receptor-binding and nuclear translocation of the hGR we found after incubation with these steroids. It has previously been described that 17OHP and P bind to the hGR,[10-12] but to the best of our knowledge this has not been shown for 21DF previously. These agonistic properties on the hGR might explain the clinical observation that CAH patients are often less severely affected by cortisol deficiency than anticipated from their enzymatic defect.

Steroid hormone cross reactivity to receptors is a well-known phenomenon. For example, cortisol is known to have an agonistic effect on the mineralocorticoid receptor.[13] In addition, in a previous study of our group we have demonstrated that 17OHP and P can influence the transactivation of the human mineralocorticoid receptor: both have antimineralocorticoid effects in vitro.[14] The occurrence of cross reactivity of these steroids at both the hGR and the human mineralocorticoid receptor can be explained by the high degree of homology at the DNA-binding domains of these receptors.[15]

The EC_{50} of cortisol in our model is comparable to the EC_{50} previously described,[16] demonstrating the reliability of our in vitro model. Under the experimental conditions 21DF, 17OHP, and P have the capacity to transactivate the hGR. With the concentrations used in our experiments we were able to reconstruct complete dose response curves, up to the point where maximum transactivation was reached. In the two cell lines studied, a comparable profile was found: 21DF has the greatest transactivational capacity, 17OHP and P are also able to transactivate the hGR but only at much higher concentrations. Δ4 does not transactivate the hGR at the concentrations tested. Given that there was no transactivation detectable for Δ4 even in concentrations at least 100 times higher than the concentrations found in untreated CAH, we do not expect that relevant transactivation will occur with higher concentrations.[17] The fact that Δ4 has no agonistic effect on the hGR might be explained by the greater structural dissimilarity between Δ4 and cortisol compared with that of the other test compounds and cortisol.[8]

As illustrated by the EC_{50} values more than 100 times higher than the EC_{50} of cortisol, 17OHP and P are less potent agonists of the hGR than cortisol. In healthy volunteers the 17OHP concentration is substantially lower than that of cortisol: reference values for serum 17OHP are 2.0–10.8 nmol/L for males and 0.45–12.7 nmol/L for females, 0900-h reference values for serum cortisol are 190–550 nmol/L. In this situation cross-reactivity on the hGR will be negligible. However, in patients with classic CAH 17OHP concentrations can increase to very high concentrations of more than 1500 nmol/l.[18,19] Based on the dose-response curve we constructed, these concentrations might be high enough to result in relevant hGR transactivation.

Interestingly, the EC_{50} of 21DF is much closer to the EC_{50} of cortisol (approximately 6-fold in the transactivation assays in COS-7 cells, approximately 12-fold in the HEK293 cells). The serum concentrations of 21DF in untreated or poorly controlled patients with classic CAH can exceed 400 nmol/l.[18,19] Based on our results we hypothesize that these concentrations may lead to a clinically relevant transactivation of the hGR. Less transactivation can be expected in NCAH patients, given that in these patients the 21DF concentrations are lower and reach up to approximately 40 nmol/L.[6] We suggest that high serum 21DF concentrations in untreated or poorly controlled CAH patients may partially compensate for their cortisol deficiency.

In contrast, overtreatment with complete suppression of adrenal precursors including 17OHP and 21DF might lead to an increased risk of adrenal crises and hypoglycemia. Therefore, adequate stress dosing is crucial once glucocorticoid treatment is initiated in patients with CAH.

Considering that we have studied several elements of the hGR transactivation cascade with consistent results, we are confident that our results represent actual glucocorticoid properties of 21DF, 17OHP, and P. However, given that this is an in vitro model, our findings must be confirmed in additional studies. We have studied the transactivating properties of several steroids, and not the other mechanisms by which the glucocorticoid receptor exerts its actions: transrepression and nongenomic effects.[13,20,21] It might also be relevant to study other steroid precursors. Despite these limitations, we consider our results promising and potentially relevant for a significant group of patients with CAH.

In conclusion, 21DF and to a lesser extent 17OHP and P are able to transactivate the hGR in vitro and thus may have glucocorticoid activity. 21DF, which can be strongly elevated in patients with untreated or poorly controlled CAH, has the strongest agonistic effect on the hGR and may partially compensate for the cortisol deficiency in patients with CAH.

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CHAPTER 5

GENERAL DISCUSSION

Since the introduction of glucocorticoid and mineralocorticoid replacement therapy in the 1950s classic congenital adrenal hyperplasia (CAH) is no longer a potentially lethal condition. As a result of successful steroid hormone replacement regimens and careful follow up, CAH changed into a well treatable chronic condition. Consequently, long term health consequences, co-morbidities and quality of life became more important. Both CAH related factors, e.g. elevated androgen levels and chronic exposure to elevated steroid hormone precursor levels, and side effects of long term treatment with glucocorticoids and/or mineralocorticoids play a role in the current and future health status and quality of life of CAH patients. CAH is a unique type of adrenal insufficiency as it results in ACTH stimulated increased activity of the adrenal gland leading to the production of large amounts of steroid hormone precursors. As cardiovascular disease is a potential cause of morbidity in CAH patients, it is important to gain more insight in the effect of steroid hormone precursors as they may also contribute to an increased risk profile in CAH patients. Therefore, within this thesis we especially focused on cardiovascular and metabolic risk in CAH and the role of steroid hormones and its precursors on glucocorticoid and mineralocorticoid metabolism.

Cardiovascular and metabolic risk factors in CAH

Over the last years many studies, including studies by our group (chapters 2.1, 2.2, 3.1, 3.2, 3.3), have evaluated the cardiovascular and metabolic risk profile in both pediatric and adult CAH patients. The results of studies by other groups on this topic are summarized in chapter 1.2: unfavorable changes in the cardiovascular and metabolic profile of CAH patients including obesity, increased fat mass, elevated blood pressure and insulin resistance have been described. All studies differ regarding study population (different age groups, different countries), treatment regimens and control groups or reference data. Nevertheless, the evidence for unfavorable changes in the cardiovascular and metabolic risk profile in CAH patients is solid. Multiple risk factors contributing to the development of this unfavorable cardiovascular and metabolic risk profile have been suggested, and some of them were confirmed by the results of our studies: obesity, the use of supraphysiological doses of glucocorticoids and hyperandrogenism. One of the pitfalls of studying the cardiovascular risk profile in adult CAH patients is that the results may reflect the effects of the treatment regimen of decades ago. Therefore, our finding of unfavorable changes in the cardiovascular and metabolic risk profile in pediatric CAH patients including increased BMI, increased fat mass, elevated blood pressure levels, a non-dipping blood pressure profile and insulin resistance, is important, as it emphasizes that unfavorable changes in the cardiovascular risk profile of CAH patients can already occur during childhood. The importance of the various individual cardiovascular risk factors in the unfavorable

cardiovascular risk profile in CAH patients is still unclear. Based on our results at least three key factors can be identified playing an important role in the long term health consequences in CAH patients: obesity, the treatment with glucocorticoids and hyperandrogenism.

The role of obesity in the cardiovascular risk profile in CAH

One of the potential factors in the development of an unfavorable cardiovascular risk profile in CAH patients is obesity. Most studies report an increased BMI in CAH patients or an increased prevalence of obesity. Many of the studies by others describe an association between the dosage of glucocorticoids and the BMI. In contrast to other reports, we did not find an association between the glucocorticoid dose and BMI in pediatric CAH patients (chapter 3.2). Therefore, additional factors, as for example an unhealthy lifestyle, may explain the increased BMI in our patient group. It is well known that obesity plays an important role in the development of insulin resistance and dyslipidemia. Furthermore, positive associations between BMI and blood pressure levels have been described in CAH patients. Therefore, CAH patients with an increased BMI are at risk of BMI associated negative effects on their cardiovascular risk profile. The negative effects on the cardiovascular risk profile may even be more pronounced than in obese individuals without CAH, as other factors, like glucocorticoid treatment, may have an additional negative effect on the cardiovascular risk profile.

In chapter 3.2 we report a positive association between BMI SDS levels in pediatric CAH patients and 17-OH progesterone (17OHP) and androstenedione levels. In CAH patients elevated concentrations of androgens and steroid precursors before the enzymatic block are caused by an increased ACTH stimulated activity of the adrenal gland due to the lack of negative feedback on the pituitary gland by cortisol. In obese, but otherwise healthy individuals, an increased activation of the hypothalamic pituitary adrenal axis and adrenal fasciculata-reticularis cell refractoriness to the inhibitory effect of leptin on ACTH-stimulated glucocorticoid production have been described.[1] In CAH patients, elevated 17OHP and androstenedione levels may therefore be the result of obesity due to increased stimulation of the hypothalamic pituitary axis in obesity, causing elevated levels of steroid precursors due to the enzymatic deficiency in CAH, and may not indicate undertreatment in CAH patients. In addition, the elevated leptin levels in our studied patients may indicate resistance to the inhibitory effect of leptin on ACTH-stimulated adrenal steroidogenesis. Our clinical observation that normalization of 17OHP and androstenedione levels in overweight or obese CAH patients is more difficult to achieve fits within this hypothesis.

Based on the above mentioned findings we suggest that achieving a normal BMI (between -1 and +1 SDS for age and gender) may contribute to lowering androgen and 17OHP levels without the need of increasing the dosage of glucocorticoids; this may consequently contribute to a reduced cardiovascular and metabolic risk later in life. Lifestyle interventions, including dietary interventions, to optimize BMI may therefore be an important tool in the treatment of CAH patients, especially in those with an increased BMI. Preferably, aiming for a normal BMI starts as early in life as possible. A future study evaluating the effect of BMI reduction by life style interventions on glucocorticoid requirements, cardiovascular and metabolic risk parameters and androgen and 17OHP levels in CAH patients is necessary to evaluate our hypotheses.

The role of glucocorticoid treatment in the cardiovascular risk profile in CAH

Glucocorticoid exposure in CAH patients is associated with an unfavorable cardiovascular and metabolic risk profile. Over the past decades the mean daily dose of glucocorticoids used in the treatment of CAH patients was reduced, due to better treatment monitoring, to prevent overtreatment. However, supraphysiological doses of glucocorticoids are still required to reset the negative feedback loop on the pituitary gland and consequently normalize androgen levels in CAH patients. Not only the use of supraphysiological doses, but also the inability to mimic the physiological secretion pattern of cortisol, with a thrice daily treatment regimen, may negatively affect the cardiovascular and metabolic risk profile. The peak concentrations of glucocorticoids may be higher in a thrice daily treatment regimen compared to peak levels in healthy subjects. Furthermore, the use of a reversed circadian rhythm regimen with the highest dose of hydrocortisone before the night, is associated with an impaired nocturnal dip in blood pressure levels (chapter 3.2).

It can be hypothesized that a more physiological glucocorticoid replacement strategy leads to a reduction of unfavorable changes in the cardiovascular risk profile. Currently several new treatment modalities mimicking physiological glucocorticoid exposure are being evaluated, including continuous hydrocortisone infusion, once-daily modified-release hydrocortisone and delayed and sustained absorption hydrocortisone formulations.[2,3] However, further studies have to clarify if the use will lead to a significant reduction of the total daily glucocorticoid dosage and if the cardiovascular risk profile may improve over time as the long term effects on the cardiovascular and metabolic risk profile have not yet been studied. Additionally, new treatment modalities focusing on blocking the ACTH production, like Corticotropin-Releasing Factor Receptor-1 antagonists, are currently being studied.[2,4] Due to a complete block of ACTH production patients will undergo a chemical adrenalectomy, with consequently the need of only physiological glucocorticoid and mineralocorticoid replacement.

The role of hyperandrogenism in the cardiovascular risk profile in CAH

The association between elevated androgens and impaired insulin sensitivity has been reported in several studies.[5,6] Elevated androgen levels may also have a direct negative effect on the cardiac function, as discussed in chapter 3.4. As the effects of hyperandrogenism on left ventricular function and left ventricular mass are already detected at a young age, these patients may have an increased risk of cardiovascular morbidity and mortality later in life, especially when these effects on the heart are not reversible after normalizing androgen levels. We hypothesize that early diagnosis of CAH, and consequently reduced exposure to elevated androgen levels due to frequent monitoring and follow up, may explain the normal left ventricular mass in our studied pediatric CAH patients. The potentially negative effects of hyperandrogenism on the heart emphasizes the need of early diagnosis, and consequently early start of treatment, in CAH patients. In the Netherlands, early diagnosis of CAH is facilitated by the newborn screening program. However, in many countries, e.g. the United Kingdom, there is no newborn screening program for CAH leading to later diagnosis and delayed start of treatment. Future studies evaluating the cardiac function in untreated or lately diagnosed adult simple virilizing or non-classic CAH patients may further elucidate the role of long term androgen exposure on the heart.

The role of steroid precursors in CAH

One of the hallmark features of 21-hydroxylase deficiency is the presence of elevated levels of steroid hormone precursors before the enzymatic block due to ACTH stimulated increased adrenal activity. The clinical role of these steroid precursors on glucocorticoid and mineralocorticoid action is not yet elucidated. As we know that both glucocorticoid and mineralocorticoid excess are associated with unfavorable changes in the cardiovascular risk profile, we hypothesize that glucocorticoid or mineralocorticoid properties of elevated concentrations of these steroid precursors may also affect the cardiovascular risk profile of CAH patients. Besides elevated levels of adrenal steroid precursors like 17OHP, recent studies showed that less frequently measured adrenal 11-oxygenated 19-carbon steroid concentrations are elevated in 21OHD patients.[7,8] The role of these lesser known steroid precursors in the steroid metabolism in CAH patients have to be further elucidated.

In chapter 4 of this thesis we report the results of our research on the effect of steroid precursors on the mineralocorticoid and glucocorticoid receptor *in vitro*. Several steroid precursors, like 21-deoxycortisol and to a lesser degree, 17OHP and progesterone, are able to bind, translocate and transactivate the glucocorticoid receptor and therefore can exhibit glucocorticoid action. This may explain some of the clinical observations indicating residual glucocorticoid potential in CAH patients

mentioned in chapter 1.3 (the absence of hypoglycemia in neonates with classic CAH; the absence of Addisonian crises in adult patients who are lost to follow up or children with a delayed diagnosis in countries without neonatal screening). The glucocorticoid activity of the steroid precursors may have several clinical implications. Treatment with glucocorticoids lowers the concentrations of steroid precursors by restoring feedback on the pituitary leading to a decrease in ACTH levels and therefore may reduce endogenous glucocorticoid activity. As a result, patients may have an increased risk of developing an Addisonian crisis, especially in situations with increased stress if the combined exogenous and endogenous glucocorticoid activity is insufficient. In untreated CAH patients the adrenocortical function evaluated by a cosyntropin stimulation test is impaired with an inadequate release of cortisol after stimulation. However, based on the results in chapter 4.2 it may be more appropriate to evaluate the cumulative glucocorticoid potential after stimulation based on the concentrations of cortisol and steroid hormone precursors with glucocorticoid activity. Future research should be performed to gain insight in the cumulative glucocorticoid activity of present steroid hormone precursors in CAH patients. We suggest that both animal models and a glucocorticoid activity index based on *in vivo* concentrations of steroids and their precursors may help in elucidating glucocorticoid activity in CAH patients. Hypothetically, patients with sufficient endogenous cumulative glucocorticoid activity may not require glucocorticoid replacement therapy at all, or only in situations with a severely increased stress level, and treatment of CAH in those patients can focus on blocking androgen secretion and/or action to normalize androgen levels. So far, several antiandrogen treatment modalities have been described as alternative treatment options to reduce androgen levels in CAH. The described treatment options include adrenal androgen synthesis inhibitors (e.g. ketoconazole), androgen receptor antagonists (e.g. flutamide) and 5 alpha reductase inhibitors (e.g. finasteride). Normalizing androgen levels with an alternative treatment modality in patients with sufficient glucocorticoid activity may not have an effect on the production of glucocorticoid precursors and therefore may reduce the need of supraphysiological glucocorticoid dosages with beneficial effects on the cardiovascular risk profile. It can be hypothesized that some of the patients with a mild non-classic phenotype do not need treatment and follow-up by a physician if the cumulative glucocorticoid activity is sufficient. This might potentially lead to a reduced use of medical care and consequently financial costs in non-classic CAH patients.

The elevated concentrations of steroid hormones and steroid hormone precursors in CAH also have impact on the mineralocorticoid metabolism. The results presented in chapter 4.1 show that 17OHP and progesterone *in vitro* have antagonistic properties on the mineralocorticoid receptor. This may lead to a higher requirement of mineralocorticoid replacement therapy in poorly controlled CAH patients.

The fundamental studies described in chapter 4 both evaluated the *in vitro* effect of a single steroid hormone precursor on the glucocorticoid or mineralocorticoid receptor. However, in CAH patients generally multiple steroid hormones and steroid precursors are present at the same time in different concentrations. Our studies have not evaluated the competitive effect of these steroid precursors in the presence of other steroids (except for evaluating the anti-mineralocorticoid properties on the mineralocorticoid receptor in the co-presence of aldosterone). An important goal of future research on this topic is to evaluate the glucocorticoid and mineralocorticoid effects of these steroid hormone precursors in a more physiological situation.

Clinical recommendations and suggestions for future studies based on this thesis

Based on our studies and the studies by others several unfavorable changes in the cardiovascular risk profile of both pediatric and adult CAH patients are present. As mentioned previously, these unfavorable changes include increased BMI, increased body fat, increased blood pressure levels and insulin resistance. In our opinion the current knowledge on the cardiovascular risk profile of CAH patients warrants regular evaluation of some cardiovascular risk factors in the clinical care of both pediatric and adult CAH patients. We recommend regular evaluation of at least blood pressure levels and BMI at each follow-up appointment in pediatric and adult CAH patients, as it may lead to earlier detection of changes in these two major cardiovascular risk markers. As 24 hour ambulatory blood pressure measurement is more accurate in evaluating blood pressure levels compared to office blood pressure measurements performing a 24 hour blood pressure evaluation once every 2 - 3 years from the age of 12 or when office blood pressure levels are elevated can be considered. In obese CAH patients regular evaluation of blood pressure, insulin resistance and dyslipidemia is necessary and more important compared to normal weight CAH patients, as an increased BMI may play a role in the development of further unfavorable changes in the cardiovascular risk profile. Therefore we suggest to individualize evaluation of cardiovascular and metabolic risk markers in CAH patients with an increased BMI (more than + 1 SDS for age and gender). Based on the current knowledge and possible consequences of unfavorable changes in the lipid profile and insulin sensitivity we would not recommend standard evaluation of the lipid profile and insulin resistance by the HOMA-IR method in pediatric CAH patients. Future studies should evaluate the effects of dyslipidemia and insulin resistance and potential treatment consequences before regular evaluation should be incorporated into standard care.

Besides improving the screening of cardiovascular risk factors in CAH patients, also changes in the current treatment protocol are warranted to improve long term

health in CAH patients. First of all, it is of great importance to use the lowest possible glucocorticoid dose to reduce adverse effects of exposure to supraphysiological doses of glucocorticoids. Regular monitoring of salivary androstenedione can help to find the lowest acceptable dosage. As previously mentioned, new glucocorticoid treatment modalities may have beneficial effect on the cardiovascular risk profile in CAH patients. We furthermore propose that aiming for a normal BMI (between -1 and +1 SDS for age and gender) should be an independent treatment goal in CAH patients to improve long term health, because of the crucial role of an increased BMI in the unfavorable cardiovascular and metabolic risk profile. As discussed previously, elevated 17OHP levels may also be associated with increased BMI. Therefore, we hypothesize that life style interventions to lower BMI and consequently 17OHP levels may lead to a reduced replacement dosage of mineralocorticoids due to the anti-mineralocorticoid potential of 17OHP. Future studies evaluating the effect of life style interventions on the cardiovascular and metabolic risk profile in CAH patients, should also evaluate the effect of a reduced BMI on 17OHP concentrations and the mineralocorticoid requirement after lowering 17OHP concentrations.

If an unfavorable cardiovascular risk profile persists despite the proposed interventions other treatment options should be considered to reduce cardiovascular morbidity and mortality. Improving insulin sensitivity and lowering blood pressure levels for example may reduce cardiovascular morbidity and mortality in CAH patients later in life. As a proof of principle we have shown that adding pioglitazone, a drug known to improve insulin sensitivity, to the treatment of adult CAH patients indeed leads to improved insulin sensitivity and also decreases blood pressure. (Chapter 2.2) As the long term safety of the use of pioglitazone was questioned we do not suggest to add pioglitazone to the treatment regimen of CAH patients with impaired insulin sensitivity. Future research should evaluate the safety and efficacy of adding insulin sensitizing and/or blood pressure lowering drugs to the treatment of CAH patients with unfavorable changes in their cardiovascular risk profile.

The role of steroid precursors, and especially their combined glucocorticoid potential, should be one of the main points of interest for future studies within the field of CAH. Based on the results in chapter 4.1 and 4.2 future research should focus on quantifying the mineralocorticoid and especially glucocorticoid potential of steroid precursors *in vivo* and on developing a tool to calculate the combined glucocorticoid activity in patients with an enzymatic defect in adrenal steroidogenesis. The results of these studies may have important clinical implications, as discussed previously, as they may change the treatment regimens of CAH patients with sufficient combined glucocorticoid activity.

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CHAPTER 6

SUMMARY

Congenital adrenal hyperplasia (CAH) is a disorder of adrenal steroidogenesis due to a defect in one of the enzymes involved in steroid synthesis. In the majority of cases CAH is caused by 21-hydroxylase deficiency (21OHD). In this thesis we discuss CAH due to 21OHD. 21OHD causes impaired glucocorticoid synthesis, in some cases also impaired mineralocorticoid synthesis, accumulation of the precursors before the enzymatic block and excess of adrenal androgen production. Treatment of the most severe forms of CAH consists of lifelong replacement of glucocorticoids and, in cases with insufficient aldosterone production, also mineralocorticoids. As a result of the treatment with glucocorticoids and mineralocorticoids, nearly all patients with CAH in Western countries currently reach adulthood without severe complications. Therefore, long term health consequences of CAH and its treatment become more important. Knowledge on the role of steroid precursors in CAH on glucocorticoid and mineralocorticoid metabolism and its long term consequences is lacking. In this thesis we studied the cardiovascular and metabolic risk profile of both pediatric and adult CAH patients. In addition we described the role of steroid precursors on the steroid metabolism in CAH patients *in vitro*.

Chapter 1 contains a general introduction on CAH (**Chapter 1.1**), a review of the literature on the cardiovascular and metabolic risk profile in both pediatric and adult CAH patients (**Chapter 1.2a and Chapter 1.2b)** and an introduction on the role of steroids and steroid precursors in CAH (**Chapter 1.3**).

In **chapter 2** studies evaluating the cardiovascular and metabolic risk profile of adult CAH patients are presented. **Chapter 2.1** describes a case-control study evaluating the cardiovascular risk profile of adult CAH patients. The cardiovascular risk profile of 27 adult CAH patients and 27 controls, matched for age, sex and body mass index (BMI) was evaluated by measuring ambulatory 24-hour blood pressure levels, insulin sensitivity (by HOMA-IR), lipid profiles, albuminuria and several circulating cardiovascular risk markers (PAI-1, tPA, uPA, tPA/PAI-1 complex, hsCRP, adiponectin, IL-6, IL-18 and leptin). Based on 24-hour blood pressure measurement both systolic (126.3 mmHg ± 15.5 versus 124.8 mmHg ± 15.1 in controls, $P = 0.019$) and diastolic (76.4 mmHg ± 12.7 versus 73.5 mmHg ± 12.4 in controls, $P < 0.001$) blood pressure was significantly elevated in CAH patients compared to controls. Furthermore, this study showed that CAH patients had higher HDL cholesterol levels ($P < 0.01$), lower hsCRP levels ($P = 0.03$) and there was a trend toward elevated adiponectin levels compared to controls. The other evaluated cardiovascular risk factors were similar in both groups. Based on the results of this study we concluded that adult CAH patients have higher 24-hour systolic and diastolic blood pressure levels compared to healthy matched controls. Other cardiovascular risk markers did not differ from controls, while HDL-cholesterol, hsCRP and adiponectin levels tended to be more favorable in the studied adult CAH patients.

In **chapter 2.2** the results of a randomized placebo controlled crossover trial evaluating the effect of pioglitazone on insulin sensitivity in adult CAH patients are presented. As it is known that glucocorticoid treatment in CAH may induce insulin resistance, we hypothesized that thiazolidinediones, like pioglitazone, might reverse this effect and improve insulin sensitivity. Twelve adult CAH patients and twelve BMI and age-matched control subjects were included in this study. All studied patients were treated with pioglitazone (45 mg/day) or placebo during sixteen weeks in this crossover trial. Insulin sensitivity was measured using an euglycemic clamp and oral glucose tolerance test. Furthermore, the effects of treatment with pioglitazone on blood pressure, body fat distribution, lipid, and steroid levels were evaluated. The studied CAH patients were more insulin resistant compared with healthy controls. Sixteen weeks of treatment with pioglitazone significantly improved insulin sensitivity in CAH patients (glucose infusion rate (GIR) from 28.5 ± 11.6 to 38.9 ± 11.0 micromol/kg per min, P=0.000, GIR in controls 46.2 ± 23.4 micromol/kg per min, P<0.05 versus CAH). An additional favorable effect of treatment with pioglitazone was lowered 24-hour blood pressure levels (systolic: 124.0 ± 13.6 vs 127.0 ± 14.9 mmHg, P<0.001, diastolic: 72.8 ± 11.5 vs 77.4 ± 12.6 mmHg, P<0.001). No changes in body fat distribution, lipid, and steroid profiles were observed after treatment. In conclusion, adult CAH patients were insulin resistant compared with matched control subjects. Treatment with pioglitazone improved insulin sensitivity and decreased blood pressure in CAH patients. Although this study showed favorable effects of pioglitazone treatment on the cardiovascular risk profile in adult CAH patients we would not recommend its standard use in clinical care due to the results of other studies questioning the long term safety of pioglitazone.

Chapter 3 focused on the cardiovascular and metabolic risk profile and cardiac function in pediatric CAH patients. **Chapter 3.1** describes the results of a pilot study evaluating blood pressure levels within the first year of life in CAH patients. Twenty-four children were included. Retrospective blood pressure values, fludrocortisone dosages, and serum renin, 17-hydroxyprogesterone (17OHP) and androstenedione levels in the first year of life were evaluated. Blood pressure values were compared to reference values. Mean peak systolic blood pressure values were generally normal, around the 50th percentile, except incidentally higher mean peak systolic office blood pressure values. In this study we did not find correlations between blood pressure on one side and serum renin, androstenedione and 17OHP levels and fludrocortisone dosage on the other side. In conclusion, blood pressure values do not seem to be elevated in the first year of life. However, further investigations are necessary to evaluate blood pressure in the first year of life in CAH patients in more detail.

In **chapter 3.2** we analyzed the cardiovascular and metabolic risk profile of 27 pediatric CAH patients aged 8 to 16 years in a cross-sectional study. Blood samples were taken to evaluate circulating cardiovascular risk markers. Insulin resistance was

evaluated by HOMA-IR. Blood pressure was evaluated by office measurements and 24-hour ambulatory blood pressure measurements (24-h ABPM). Carotid intima media thickness (cIMT) was evaluated as surrogate marker of atherosclerosis. Dual energy X-ray absorptiometry (DXA) scans were performed in patients >12 years to evaluate body composition. The results of this study were compared to carefully selected reference values. The results of this study make it clear that unfavorable changes in the cardiovascular risk profile in CAH patients may occur already in childhood. BMI standard deviation score (SDS) was elevated (0.67) in the studied CAH patients, with seven patients being overweight (25.9 %) and four obese (14.8 %). DXA scans showed unfavorable changes in body composition with a percentage body fat SDS of 1.59. Office blood pressure levels were higher than reference values. Twenty-four hour ABPM showed systolic hypertension in 18.5 % of the studied CAH patients, while 11 patients (40.7 %) had a non-dipping blood pressure profile. The prevalence of insulin resistance, defined as a HOMA-IR >75th percentile for gender and age, was high (44.4 %). The lipid profile and cIMT were within the normal range in the studied patients. Only elevated HOMA-IR values were positively associated with the daily dosage of hydrocortisone per m^2. Based on this study increased BMI seems to play an essential role in the increased cardiovascular risk. In summary, the data presented in this chapter show that the unfavorable changes in the cardiovascular risk profile of pediatric CAH patients include an increased BMI, increased fat mass, elevated blood pressure levels, a non-dipping blood pressure profile and insulin resistance compared to population reference values.

Chapter 3.3 describes the cardiac function of the same 27 pediatric CAH patients. Only a few studies previously evaluated the cardiac function in CAH patients. The cardiac function was evaluated by electrocardiogram (ECG), conventional echocardiography, tissue Doppler imaging and two-dimensional (2D) myocardial strain (rate) imaging. Results were compared to 27 age- and gender-matched healthy controls. No signs of left ventricular hypertrophy or dilatation were detected on echocardiography. ECG revealed a high prevalence (25.9%) of incomplete right bundle branch block. Conventional echocardiography revealed that left ventricular posterior wall thickness in diastole (LVPWd) was significantly lower in patients with CAH compared to controls (5.55 vs 6.53 mm; *P*=0.009). The LVPWd Z-score was significantly lower in patients with CAH yet within the normal range (-1.12 vs -0.35; *P*=0.002). Isovolumetric relaxation time was significantly lower in patients with CAH (49 vs 62 ms; *P*=0.003). Global longitudinal, radial and circumferential strain was not significantly different compared to controls. Global radial strain rate was significantly higher compared to healthy controls (2.58 vs 2.06 1/s; *P*=0.046). In conclusion, the cardiac evaluation of pediatric patients with CAH showed no signs of left ventricular hypertrophy or ventricular dilatation. LVPWd was lower in patients with CAH than in controls but within the normal range. A shorter isovolumetric relaxation time in patients with CAH

may be a sign of mild left ventricular diastolic dysfunction. Further studies are needed to evaluate the cardiac function in adolescence and adulthood in well-controlled CAH patients. Compared to the results of other studies the cardiac function in our studied CAH patients seemed to be better, which may be explained by a longer duration of androgen excess in those studies due to a later diagnosis and start of treatment. Therefore, we believe that early diagnosis and start of treatment in CAH patients is essential to lower androgen levels with consequently less negative effects on cardiac function.

Chapter 4 aimed to evaluate the role of steroid precursors accumulating in CAH on glucocorticoid and mineralocorticoid metabolism *in vitro*. We hypothesized that the elevated concentrations of steroid precursors present in CAH patients may have a biological effect on the steroid receptors with clinical consequences on diagnostics and treatment in CAH patients. In **chapter 4.1** the results of our study evaluating the effects of 17OHP, progesterone, androstenedione and testosterone on aldosterone-mediated translocation and transactivation of the human mineralocorticoid receptor (hMR) are presented. A transactivation assay using transiently transfected COS7 cells was employed. Cells were co-transfected with hMR-cDNA, MMTV-luciferase and renilla-luciferase expression vectors. Transfected cells were incubated with six different steroid concentrations in addition to aldosterone (10^{-10}M). Luciferase and renilla activities were measured to quantify hMR transactivation. A hMR-green fluorescent protein (GFP) construct was used for an intracellular localization assay evaluating translocation. Linear regression analysis showed statistically significant linear inhibition of transactivation of the hMR by 10^{-10}M aldosterone in the presence of increasing 17OHP [$F(1,5) = 11.34$, $P=0.019$] and progesterone [$F(1,5) = 11.08$, $P=0.021$] concentrations. In contrast, neither androstenedione nor testosterone affected hMR transactivation by aldosterone at a concentration of 10^{-10}M. **Chapter 4.1** shows that 17OHP and progesterone have anti-mineralocorticoid effects in vitro that may clinically lead to an increased requirement of mineralocorticoids in poorly controlled CAH patients.

Chapter 4.2 focused on the effects of 17OHP, progesterone, 21-deoxycortisol (21DF), and androstenedione on the human glucocorticoid receptor (hGR). Competitive binding assays were performed in HeLa cells. Nuclear translocation of the hGR was studied by transfection of COS7 cells with a GFP-tagged hGR and fluorescence microscopy. Transactivation assays were performed in both COS7 cells and in HEK293 cells after cotransfection with hGR and luciferase reporter vectors using a dual luciferase assay. This study showed that 17OHP, progesterone, and 21DF are able to bind to the hGR with binding affinities of 24-43% compared with cortisol. Androstenedione has a low binding affinity. Incubation with 21DF led to complete nuclear translocation of the hGR, whereas treatment with 17OHP or progesterone resulted in partial nuclear translocation. 21DF transactivated the hGR with an EC50

approximately 6 times the EC50 of cortisol. 17OHP and progesterone transactivated the hGR with EC50s of more than 100 times the EC50 of cortisol. No hGR transactivation was detected after incubation with androstenedione. 21DF, 17OHP, and progesterone are able to bind, translocate, and transactivate the hGR in vitro and thus may have glucocorticoid activity. 21DF might have a clinically relevant agonistic effect on the hGR and could potentially partially compensate the cortisol deficiency in CAH patients.

A discussion of the studies of this thesis, clinical recommendations and future research perspectives can be found in **chapter 5**. Based on the studies presented in this thesis and studies by others we conclude that several unfavorable changes in the cardiovascular risk profile of both pediatric and adult CAH patients may be present. Unfavorable changes in the cardiovascular risk profile of CAH patients include increased BMI, increased body fat, increased blood pressure levels and insulin resistance. We recommend regular evaluation of at least blood pressure levels and BMI at each follow-up appointment, as it may lead to earlier detection of changes in these two major cardiovascular risk markers. Obesity may play an important role in the development of further unfavorable changes in the cardiovascular risk profile. Therefore, we suggest to individualize the follow up of obese CAH patients. Furthermore, we have shown that adrenal steroid precursors present in CAH patients have effects on both glucocorticoid and mineralocorticoid action *in vitro*. Especially, the glucocorticoid activity of 21DF may have a clinically relevant role in CAH patients, partially compensating for the cortisol deficiency. Future research should focus on quantifying the mineralocorticoid and glucocorticoid potential of steroid precursors *in vivo* and on developing a tool to calculate the combined glucocorticoid activity in patients with an enzymatic defect in adrenal steroidogenesis.

CHAPTER 7

SUMMARY IN DUTCH

Nederlandse samenvatting

Congenitale bijnierhyperplasie, in Nederland ook bekend als het adrenogenitaal syndroom (AGS), is een aandoening waarbij er sprake is van een deficiëntie van één van de enzymen die betrokken zijn bij de synthese van hormonen in de bijnier. In de meerderheid van de gevallen wordt AGS veroorzaakt door 21-hydroxylase deficiëntie (21OHD). In dit proefschrift bestuderen we AGS ten gevolge van 21OHD. 21OHD leidt tot een verminderde synthese van cortisol (een glucocorticoïd) en meestal ook tot een verminderde synthese van aldosteron (een mineralocorticoïd). Door een onvoldoende negatieve terugkoppeling op de ACTH secretie door de hypofyse ontstaat er overmatige stimulatie van de bijnier leidend tot bijnierhyperplasie (vandaar de Engelse benaming 'congenital adrenal hyperplasia' (CAH)). Door de overmatige stimulatie van de bijnier treedt er een accumulatie van voorloperhormonen (precursors) voor het enzymatische defect op en is er sprake van een sterk verhoogde productie van bijnierandrogenen. De behandeling van de ernstige vormen van AGS bestaat uit levenslange substitutie met glucocorticoïden, en indien er tevens sprake is van onvoldoende aldosteron productie, ook mineralocorticoïden. Vaak zijn suprafysiologische doseringen glucocorticoïden noodzakelijk om de androgeen productie in de bijnier te normaliseren. Succesvolle behandeling met glucocorticoïden en mineralocorticoïden heeft er toe geleid dat bijna alle patiënten in Westerse landen de volwassen leeftijd bereiken zonder ernstige complicaties. Daardoor worden lange termijn gevolgen van AGS steeds belangrijker. De kennis over de rol van hormoonprecursors bij AGS op het glucocorticoïde en mineralocorticoïde metabolisme en de lange termijn effecten van deze precursors is nog beperkt. In dit proefschrift hebben wij het cardiovasculaire en metabole risicoprofiel van zowel pediatrische als volwassen AGS patiënten bestudeerd. Daarnaast bestudeerden wij de rol van hormoonprecursors op het hormoonmetabolisme bij AGS patiënten *in vitro.*

Hoofdstuk 1 omvat een algemene introductie over AGS (**Hoofdstuk 1.1**), een overzicht van de literatuur over het cardiovasculaire en metabole risicoprofiel van zowel pediatrische als volwassen AGS patiënten (**Hoofdstuk 1.2a en Hoofdstuk 1.2b**) en een introductie op de rol die hormonen en hormoonprecursors spelen bij AGS (**Hoofdstuk 1.3**).

In **hoofdstuk 2** worden de resultaten van twee studies gepresenteerd waarin het cardiovasculaire en metabole risicoprofiel van volwassen AGS patiënten is bestudeerd. **Hoofstuk 2.1** beschrijft een case-control studie waarin het cardiovasculaire risicoprofiel van volwassen AGS patiënten is onderzocht. Het cardiovasculaire risicoprofiel van 27 volwassen AGS patiënten en 27 op leeftijd, geslacht en body mass index (BMI) gematchte controles werd in kaart gebracht door evaluatie van een 24-uurs ambulante bloeddrukmeting, het bepalen van de insuline gevoeligheid (via de HOMA-IR methode), het vetspectrum, albuminurie en het meten van verschillende circulerende cardiovasculaire risico markers (PAI-1, tPA, uPA, tPA/PAI-1 complex, hsCRP, adiponectine, IL-6, IL-18 en leptine). De 24-uurs bloeddruk metingen toonden aan dat zowel de

systolische (126.3 mmHg ± 15.5 versus 124.8 mmHg ± 15.1 bij controles, *P* = 0.019) als diastolische (76.4 mmHg ± 12.7 versus 73.5 mmHg ± 12.4 bij controles, *P*<0.001) bloeddruk significant verhoogd was bij AGS patiënten vergeleken met de controles. Verder bleken AGS patiënten een hoger HDL cholesterol (*P*<0.01) en lagere hsCRP concentratie (*P*=0.03) te hebben, daarnaast was er sprake van een trend wijzend op verhoogde adiponectine concentraties ten opzichte van controles. De andere in kaart gebrachte cardiovasculaire risicofactoren waren vergelijkbaar in beide groepen. Op basis van de resultaten van deze studie concludeerden wij dat volwassen AGS patiënten een verhoogde 24-uurs systolische en diastolische bloeddruk hebben vergeleken met gezonde gematchte controles. De andere cardiovasculaire risicomarkers verschilden niet van de controlegroep, terwijl HDL-cholesterol, hsCRP en adiponectine concentraties juist gunstig leken te zijn in de bestudeerde AGS patiënten.

Hoofdstuk 2.2 beschrijft de resultaten van een gerandomiseerde placebo gecontroleerde cross-over trial die het effect van pioglitazone op de insuline gevoeligheid bij volwassen AGS patiënten heeft onderzocht. Het is bekend dat behandeling met glucocorticoïden kan leiden tot insuline resistentie bij AGS patiënten. Onze hypothese was dat thiazolidinedionen, zoals pioglitazone, dit effect konden omdraaien en de insuline sensitiviteit zouden verbeteren. Twaalf volwassen AGS patiënten en twaalf BMI en leeftijd gematchte controles werden geïncludeerd in deze studie. Alle geïncludeerde patiënten werden behandeld met pioglitazone (45 mg/dag) of een placebo gedurende 16 weken. Insuline gevoeligheid werd gemeten door een euglycemische clamp en een orale glucose tolerantie test. Daarnaast werden de effecten van de behandeling met pioglitazone op de bloeddruk, lichaamsvetverdeling, het lipidenprofiel en steroïd concentraties geëvalueerd. De onderzochte AGS patiënten waren meer insulineresistent vergeleken met gezonde controles. Zestien weken behandeling met pioglitazone leidde tot een significante verbetering van de insuline gevoeligheid bij de AGS patiënten (*glucose infusion rate* (GIR) van 28.5 ± 11.6 naar 38.9 ± 11.0 micromol/kg per min, *P*=0.000, GIR in controles 46.2 ± 23.4 micromol/kg per min, *P*<0.05 versus CAH). Een bijkomend gunstig effect van de behandeling met pioglitazone was een verlaging van het 24-uurs bloeddruk profiel (systolisch: 124.0 ± 13.6 vs 127.0 ± 14.9 mmHg, *P*<0.001, diastolisch: 72.8 ± 11.5 vs 77.4 ± 12.6 mmHg, *P*<0.001). Er werden na de behandeling geen veranderingen ten aanzien van de lichaamsvetverdeling, lipidenprofiel en steroïd concentraties gevonden. Concluderend waren volwassen AGS patiënten insulineresistent vergeleken met gematchte controles. Behandeling met pioglitazone verbeterde de insuline gevoeligheid en leidde tot een verlaging van de bloeddruk bij AGS patiënten. Ondanks de gunstige effecten van pioglitazone op het cardiovasculaire risicoprofiel bij volwassen AGS patiënten, is er geen plaats voor het gebruik van pioglitazone in de standaard behandeling van AGS patiënten gezien de resultaten van recentere andere studies die de lange termijn veiligheid van pioglitazone in twijfel trekken.

Hoofdstuk 3 richt zich op het cardiovasculaire en metabole risicoprofiel en de cardiale functie bij pediatrische AGS patiënten. In **hoofdstuk 3.1** beschrijven wij de resultaten van een pilot studie waarin de bloeddruk waarden in het eerste levensjaar van AGS patiënten zijn geëvalueerd. Vierentwintig kinderen werden geïncludeerd. Retrospectieve analyse van bloeddrukmetingen, doseringen van fludrocortison en serum concentraties van renine, 17-hydroxyprogesteron (17OHP) en androsteendion vond plaats. De bloeddruk waarden werden vergeleken met referentiewaarden. De gemiddelde piek systolische bloeddruk waarden waren over het algemeen normaal, rond de 50^{e} percentiel, met uitzondering van incidenteel hogere gemiddelde piek systolische bloeddrukwaarden. In deze studie vonden wij geen correlaties tussen enerzijds de bloeddruk en anderzijds de dosering fludrocortison en de 17OHP, androsteendion en renine concentraties. Concluderend toont deze studie dat de bloeddruk niet verhoogd lijkt te zijn bij AGS patiënten in het 1^{e} levensjaar. Verder onderzoek is gewenst om de bloeddruk in het 1^{e} levensjaar bij kinderen met AGS beter te evalueren.

In **hoofdstuk 3.2** hebben we het cardiovasculaire en metabole risicoprofiel van 27 pediatrische AGS patiënten tussen de 8 en 16 jaar geanalyseerd in een cross-sectionele studie. Bloed werd afgenomen om circulerende cardiovasculaire risico markers te bepalen. Insulineresistentie werd bepaald middels HOMA-IR. De bloeddruk werd geëvalueerd door gestandaardiseerde bloeddrukmetingen in de spreekkamer en ambulante 24-uurs bloeddrukmetingen (24-u ABPM). De intima media dikte (IMT) van de arteria carotis werd bepaald als een surrogaat marker van atherosclerose. Een DEXA (Dual Energy X-Ray Absorptiometrie) scan werd verricht bij patiënten ouder dan 12 jaar om de lichaamssamenstelling in kaart te brengen. De data verkregen in deze studie werden vergeleken met zorgvuldig geselecteerde referentiewaarden. De resultaten beschreven in dit hoofdstuk maken duidelijk dat ongunstige veranderingen in het cardiovasculaire risicoprofiel bij AGS patiënten al op de kinderleeftijd op kunnen treden. De BMI standaard deviatie score (SDS) was verhoogd (0.67) in de bestudeerde pediatrische AGS patiënten. Zeven patiënten hadden overgewicht (25.9%) en vier patiënten waren obees (14.8%). DEXA scans toonden ongunstige veranderingen in de lichaamssamenstelling met een percentage lichaamsvet SDS van 1.59. Spreekkamer bloeddruk waarden waren hoger dan normaalwaarden. Vierentwintig uur ABPM toonde systolische hypertensie bij 18.5% van de bestudeerde AGS patiënten, terwijl 11 patiënten (40.7%) een non-dipping bloeddruk profiel hadden. De prevalentie van insulineresistentie, gedefinieerd als een HOMA-IR > 75^{e} percentiel voor geslacht en leeftijd, was hoog (44.4%). Het lipiden profiel en de IMT van de arteria carotis waren normaal in de geïncludeerde patiënten. Enkel de verhoogde HOMA-IR waarden waren positief geassocieerd met de dagelijkse dosering hydrocortison per m^{2}. Het verhoogde BMI lijkt een essentiële rol te spelen in het verhoogde cardiovasculaire risico in de AGS patiënten in deze studie. Samenvattend laten de data gepresenteerd in dit hoofdstuk zien dat ongunstige veranderingen in het cardiovasculaire risicoprofiel

van kinderen met AGS een verhoogd BMI, verhoogde vetmassa, verhoogde bloeddruk waarden, een non-dipping bloeddruk profiel en insuline resistentie omvatten.

Hoofdstuk 3.3 beschrijft de cardiale functie van dezelfde 27 kinderen met AGS. Slechts enkele studies hebben eerder de cardiale functie bij AGS patiënten in kaart gebracht. De cardiale functie werd geëvalueerd door een electrocardiogram (ECG), conventionele echocardiografie, tissue Doppler imaging en twee-dimensionale (2D) myocardiale strain (rate) imaging. De verkregen resultaten werden vergeleken met 27 op leeftijd en geslacht gematchte gezonde controles. Er werden geen tekenen van linker ventrikel hypertrofie of dilatatie waargenomen bij analyse middels ECG en/of echocardiografie. De ECG's toonden een hoge prevalentie (25.9%) van een incompleet rechter bundeltak blok. Conventionele echocardiografie toonde dat de dikte van de achterwand van de linker ventrikel in diastole (LVPWd) significant minder was in AGS patiënten vergeleken met controles (5.55 vs 6.53 mm; *P*=0.009). De LVPWd Z-score was ook significant lager in AGS patiënten vergeleken met controles, maar binnen de normale range (-1.12 vs -0.35; *P*=0.002). De isovolumetrische relaxatietijd was significant korter bij AGS patiënten (49 vs 62 ms; *P*=0.003). De globale longitudinale, radiale en circumferentiële strain verschilde niet significant tussen AGS patiënten en controles. De globale radiale strain rate was significant hoger vergeleken met gezonde controles (2.58 vs 2.06 1/s; *P*=0.046).

Concluderend toont de cardiale evaluatie van kinderen met AGS aan dat er geen tekenen van linker ventrikelhypertrofie of ventriculaire dilatatie zijn. De LVPWd was lager in AGS patiënten dan in controles, maar binnen de normale range. Een kortere isovolumetrische relaxatietijd bij AGS patiënten kan een teken zijn van milde diastolische dysfunctie van de linker ventrikel. Toekomstige studies zijn wenselijk om de cardiale functie bij goed gecontroleerde adolescente en volwassen AGS patiënten in kaart te brengen. Vergeleken met de resultaten van andere studies naar de cardiale functie bij AGS patiënten lijkt de cardiale functie in onze studie beter. Dit verschil kan mogelijk verklaard worden door een langere, chronische blootstelling aan verhoogde androgeen waarden in de patiënten in de andere studies aangezien de diagnose bij hen later werd gesteld en behandeling daardoor ook later werd opgestart. Wij zijn van mening dat vroege diagnose en behandeling van AGS patiënten essentieel is om de androgeen concentraties te verlagen, dit zou mogelijk kunnen leiden tot minder negatieve effecten op de cardiale functie.

In **hoofdstuk 4** hebben wij het effect van hormoonprecursors, die overmatig aanwezig zijn bij 21OHD, op het glucocorticoïde en mineralocorticoïde metabolisme *in vitro* geëvalueerd. Onze hypothese was dat de verhoogde concentraties van deze hormoonprecursors een biologisch effect kunnen hebben op de steroïd receptoren met klinische consequenties voor de diagnostiek en behandeling van AGS patiënten. In **hoofdstuk 4.1** beschrijven wij de resultaten van de studie die het effect van 17OHP, progesteron, androsteendion en testosteron op de aldosteron gemedieerde translocatie

en transactivatie van de humane mineralocorticoïde receptor (hMR) onderzocht. Het onderzoek werd uitgevoerd met een transactivatie assay met getransfecteerde COS7 cellen. De cellen werden geco-transfecteerd met hMR-cDNA, MMTV-luciferase en renilla-luciferase expressie vectoren. De getransfecteerde cellen werden geïncubeerd met zes verschillende concentraties steroïden in de aanwezigheid van aldosteron (10^{-10}M). De luciferase en renilla activiteit werd gemeten om de hMR transactivatie te kwantificeren. Een hMR-groen fluorescerend proteïne (GFP) construct werd gebruikt voor een intracellulaire lokalisatie assay om de translocatie in kaart te brengen. Lineaire regressie analyse toonde een statistisch significante lineaire inhibitie van transactivatie van de hMR door 10^{-10}M aldosteron in de aanwezigheid van oplopende concentraties 17OHP [$F(1,5) = 11.34$, $P=0.019$] en progesteron [$F(1,5)= 11.08$, $P=0.021$]. Daar tegenover stond dat zowel androsteendion als testosteron geen effect had op de hMR transactivatie door aldosteron in een concentratie van 10^{-10}M. **Hoofdstuk 4.1** toont aan dat 17OHP en progesteron een anti-mineralocorticoïde effect hebben *in vitro* dat klinisch zou kunnen leiden tot een verhoogde behoefte aan mineralocorticoïden bij matig en slecht ingestelde AGS patiënten.

Hoofdstuk 4.2 richt zich op de effecten van 17OHP, progesteron, 21-deoxycortisol (21DF) en androsteendion op de humane glucocorticoïde receptor (hGR). Competitieve bindingsassays werden uitgevoerd in HeLa cellen. Nucleaire translocatie van de hGR werd bestudeerd middels fluorescentie microscopie na het transfecteren van COS7 cellen met een hGR-GFP construct. Transactivatie assays werden uitgevoerd in zowel COS7 cellen als HEK293 cellen na co-transfectie met hGR en luciferase expressie vectoren gebruikmakend van een duaal luciferase assay. Deze studie toonde aan dat 17OHP, progesteron en 21DF in staat zijn om te binden aan de hGR met een bindings-affiniteit van 24-43% vergeleken met cortisol. Androsteendion had een lage bindings-affiniteit. Incubatie met 21DF leidde tot complete translocatie van de hGR naar de nucleus, waar incubatie met 17OHP of progesteron resulteerde in gedeeltelijke translocatie naar de nucleus. 21DF resulteerde in transactivatie van de hGR met een EC50 van ongeveer 6 keer de EC50 van cortisol. 17OHP en progesteron resulteerden in transactivatie van de hGR met EC50s van meer dan 100 keer de EC50 van cortisol. Incubatie met androsteendion leidde niet tot transactivatie van de hGR. 21DF, 17OHP en progesteron zijn dus in staat om te binden aan en te zorgen voor translocatie en transactivatie van de hGR *in vitro*, waardoor zij glucocorticoïde activiteit zouden kunnen hebben. 21DF zou een klinisch relevant agonistisch effect op de hGR kunnen hebben en mogelijk deels kunnen compenseren voor de cortisol deficiëntie bij AGS patiënten.

Een discussie van de studies in dit proefschrift, de klinische aanbevelingen en suggesties voor toekomstig onderzoek kunnen worden gelezen in **hoofdstuk 5**. Op basis van de studies in dit proefschrift en de studies verricht door anderen concluderen wij dat verschillende ongunstige veranderingen in het cardiovasculaire risicoprofiel

van zowel kinderen als volwassenen met AGS aanwezig kunnen zijn. Ongunstige veranderingen in het cardiovasculaire risicoprofiel van AGS patiënten omvatten een verhoogd BMI, toegenomen percentage lichaamsvet, verhoogde bloeddruk en insulineresistentie. Wij adviseren regelmatige evaluatie van minimaal de bloeddruk en de BMI tijdens iedere controle afspraak, aangezien dit kan leiden tot vervroegde detectie van veranderingen op het vlak van deze twee belangrijke cardiovasculaire risicomarkers. Obesitas speelt mogelijk een belangrijke rol in het ontwikkelen van verdere ongunstige veranderingen in het cardiovasculaire risicoprofiel. Daarom adviseren wij om de follow up van AGS patiënten met obesitas per individu vorm te geven afhankelijk van het persoonlijke cardiovasculaire risicoprofiel. Verder hebben wij aangetoond dat bijnier hormoonprecursors die in verhoogde concentraties aanwezig zijn bij 21OHD *in vitro* zowel een effect kunnen hebben op de glucocorticoïde als de mineralocorticoïde receptor. Vooral de glucocorticoïde activiteit van 21DF zou een klinisch relevante rol kunnen spelen bij AGS patiënten, waarbij 21DF gedeeltelijk de cortisol deficiëntie compenseert. Toekomstig onderzoek zou zich moeten richten op het kwantificeren van het mineralocorticoïde en glucocorticoïde effect van hormoonprecursors *in vivo* en op het ontwikkelen van een model om de gecombineerde glucocorticoïde activiteit te berekenen bij patiënten met een enzymatisch defect in de bijnier steroidogenese.

APPENDICES

LIST OF PUBLICATIONS
DANKWOORD
CURRICULUM VITAE

List of publications

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Succes is een combinatie van geluk en kwaliteiten. Dat is ondertussen mijn levensmotto geworden. Daarom wil ik graag een anekdote met jullie delen die illustreert dat je soms geluk nodig hebt om te komen waar je nu bent. Tijdens het promotiefeest van Hedi Claahsen konden de gasten een persoonlijke wens aan Hedi in een heliumballon stoppen. Indien de ballon uiteindelijk gevonden werd, kon de vinder de boodschap aan Hedi opsturen. Al twee dagen later ontving Hedi per post uit Noord Duitsland de wens die Livia Kapusta in de ballon had opgelaten: samen een artikel schrijven. Omdat het niet bij een wens moest blijven werden de plannen concreet gemaakt. Hedi had al direct een onderwerp en Livia kende een student die het onderzoek moest gaan uitvoeren. En die student, dat was ik. Zo spraken wij eind 2007 met elkaar over het uitvoeren van een pilotstudie naar de bloeddruk in het eerste levensjaar bij kinderen met AGS. Dat onderzoek, hoofdstuk 3.1 van dit proefschrift, is achteraf gezien het begin geweest van mijn promotie onderzoek. En de rest is geschiedenis, en heeft u kunnen lezen in de voorgaande hoofdstukken. Maar wat als de wind anders had gestaan en die ballon nooit gevonden was, of de vinder de gevonden boodschap niet aan Hedi had opgestuurd… *Succes is een combinatie van geluk en kwaliteiten*.

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Curriculum vitae

Christiaan Mooij werd op 14 augustus 1986 geboren in het Radboud ziekenhuis in Nijmegen. Samen met zijn jongere broer Johannes groeide hij op in Berg en Dal. In 2002 behaalde hij zijn diploma aan het Stedelijk Gymnasium in Nijmegen. Aansluitend startte hij met zijn studie Geneeskunde aan de Radboud Universiteit. Al tijdens zijn studie raakte Christiaan geïnteresseerd in de kindergeneeskunde en begon hij met het doen van wetenschappelijk onderzoek binnen de afdeling Kindercardiologie onder supervisie van Prof. L. Kapusta. In aansluiting hierop verrichtte hij in het kader van zijn wetenschappelijke stage onderzoek op de afdeling Kindercardiologie van het Boston Children's Hospital, Harvard Medical School in Boston, USA.

Tijdens de coschappen maakte Christiaan een eerste start met het onderzoek dat uiteindelijk zou uitgroeien tot het promotietraject dat beschreven is in dit proefschrift. Na afronding van zijn artsexamen in december 2008 startte hij als arts-onderzoeker binnen de afdelingen Kinderendocrinologie en Endocrinologie van het Radboudumc met het onderzoek naar het cardiovasculaire risicoprofiel en steroidmetabolisme bij AGS patiënten. Dit onder supervisie van Dr. H.L. Claahsen-van der Grinten en Prof. A.R.M.M. Hermus. Met de opzet voor het verdere onderzoek wist Christiaan een ZonMw AGIKO-beurs binnen te halen. Vanaf dat moment heeft hij zijn promotie onderzoek verder gecombineerd met zijn specialisatie tot kinderarts. In de periode 2010-2011 heeft hij één jaar als research fellow gewerkt aan de University of Birmingham in het Verenigd Koninkrijk.

In 2011 startte Christiaan officieel met zijn specialisatie tot kinderarts. Zijn opleiding vond voor het grootste gedeelte plaats in het Amalia Kinderziekenhuis van het Radboudumc (opleider: J.M.T. Draaisma). Tevens werkte hij als kinderarts in opleiding in het Canisius-Wilhelmina Ziekenhuis in Nijmegen (opleider: B. Semmekrot) en rondde hij met succes het OOR-ON profiel “Medisch Leiderschap” af met verschillende taken binnen de (Junior Afdeling van de) Nederlandse Vereniging voor Kindergeneeskunde. In april 2017 voltooide hij in het Emma Kinderziekenhuis van het Academisch Medisch Centrum in Amsterdam zijn opleiding tot kinderarts (opleider: D.K. Bosman).

De laatste fase van zijn opleiding tot kinderarts heeft Christiaan besteed aan de kinderendocrinologie. Vanaf augustus 2016 startte hij al met een vervolgspecialisatie tot kinderendocrinoloog in het Emma Kinderziekenhuis (AMC) en het VUmc te Amsterdam (opleider: Prof. A.S.P. van Trotsenburg), die hij in februari 2019 verwacht af te ronden.