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CONGENITAL ADRENAL HYPERPLASIA: PRE- AND POSTNATAL TREATMENT EFFECTS ON COGNITION, BEHAVIOR AND BRAIN RESTING-STATE FUNCTIONAL CONNECTIVITY

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Congenital Adrenal Hyperplasia: Pre-and postnatal treatment effects on cognition, behavior and brain resting-state functional connectivity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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"and thence we came forth to see again the stars"
Dante Aligheri

To my parents

POPULAR SCIENCE SUMMARY OF THE THESIS

Congenital adrenal hyperplasia (CAH) is a rare and inherited disorder. “Congenital” means present from birth; “adrenal” refers to the part of the body affected, the adrenal glands, the two organs sitting on the top of each kidney; and “hyperplasia” describes the change that happens to adrenal glands. These are three complex terms to indicate a rare inherited genetic disorder present from birth. The adrenal glands make hormones essential to regulating important functions in our body. In patients with CAH the adrenal glands will produce more male-like sex hormones called androgens. This overproduction starts already during fetal life. At birth, babies with CAH present some physical changes. The adrenal glands are larger than normal and girls with CAH may present with more masculine external genitalia. Thus, the sex assignment at birth can be challenging to determine in some cases.

However, CAH is treatable. Patients with CAH are treated with glucocorticoids (GCs) to reduce the overproduction of adrenal androgens and replace the hormones they cannot make. Moreover, because this disorder is inherited, to prevent virilization of the genitals, a synthetic hormone, dexamethasone (DEX), may be used during fetal life by treating the pregnant woman carrying a female fetus with CAH.

As is well known, all treatments may have negative side effects. Harmful side effects on cognition and behavior have been reported in patients treated for long periods with GCs and in healthy individuals treated with those hormones for a short time. Therefore, the treatment with GCs may negatively affect patients with CAH. In this thesis we looked at the effect of postnatal GC treatment on cognition and behavior in a large group of children and adolescents (7-17 years) with CAH. We also investigated the effect of DEX treatment in a small group of patients with CAH that received prenatal treatment. All patients were identified through the Swedish neonatal screening program and immediately treated postnatally. For the first time, the effect of pre-and postnatal GC treatment on resting-state brain functions was investigated. Specifically, we examined the impact of prenatal treatment with DEX in a group of adolescents and adults not having CAH and being treated during the first trimester of fetal life. We also assessed the effect of postnatal treatment with GCs in adolescents and adults with CAH.

Our results on cognitive performance (general intelligence, working memory, long-term memory) and behavioral outcomes are encouraging. Our sample of children and adolescents with CAH performed equally well as population controls. A possible explanation might be that all our patients identified through the screening were treated immediately after birth. Our small cohort of patients with CAH prenatally treated with DEX performed worse than the control group in one test assessing verbal intelligence. The parents of these children reported more social problems in their children. However, larger studies are needed to confirm our results based on a small cohort. When we analyzed the magnetic resonance imaging data, we detected more functional activity during rest in one brain region (the precuneus) in our adolescents and adult patients. This activation may reflect a reorganization of the brain due to the disease, treatment, or both. Looking at the resting-state functional connectivity in individuals prenatally treated with DEX during the first trimester of fetal life, we did not identify any differences between DEX-exposed individuals and controls. In conclusion, this thesis extends the knowledge in the literature on GC effects on functional connectivity in the brain and cognitive and behavioral outcomes in patients with CAH.

ABSTRACT

Congenital adrenal hyperplasia (CAH), an autosomal recessive disorder affecting adrenal steroid synthesis, is linked to impaired adrenal synthesis of cortisol and aldosterone. The increased production of androgen precursors in the adrenal cortex during fetal life leads to the virilization of external genitalia in girls with CAH already in utero. Prenatal virilization can be reduced or alleviated entirely by dexamethasone (DEX) treatment in affected girls, but with potentially adverse effects on growth parameters, cognition, behavior, brain structure and brain networks and metabolism. The effects of synthetic glucocorticoids (GCs) on fetal development are time- and dose-dependent, with different outcomes in early vs. late gestational treatment. Patients with CAH are also treated postnatally with life-long GC replacement therapy to mimic the physiological levels following the circadian rhythm. Because of the inherent difficulties in mimicking the natural rhythm of cortisol secretion, there is a risk of under- or over-treatment during the individual's life span, with potentially adverse effects on brain function and structure, metabolism and quality of life.

In this thesis the long-term effects of pre-and postnatal DEX treatment were investigated in a cohort of 206 individuals: patients with CAH not prenatally treated with DEX (n=71), a small cohort of patients with CAH prenatally treated with DEX (n=13), individuals at risk of CAH who were prenatally treated during the first trimester of fetal life (n=18) and population controls (n=104). Patients with CAH were diagnosed through the national neonatal screening program. We hypothesized that GC treatment could impact cognitive performance, behavior and resting-state functional connectivity of the brain. Compared to controls, we did not identify significant differences in cognitive performance in children and adolescents aged 7-17 years (mean age 11.5 years) with CAH compared with the population controls. However, patients with the salt-wasting (SW) genotype performed worse on a subtest assessing visuospatial working memory compared to patients with simple-virilizing (SV) CAH. The scores on this subtest were in the average range for the general population. In summary, early diagnosis may optimize treatment and benefit cognitive development. The small cohort of CAH cases prenatally treated with DEX (6 females, 5 males) showed poorer performance in a subtest assessing verbal intelligence than patients not treated with DEX. The behavioral outcomes evaluated using parental and self-rated questionnaires reflected a good overall adjustment in children and adolescents with CAH compared to controls. The parental questionnaires suggested more social problems in CAH patients. Moreover, the parents of children with CAH prenatally treated with DEX (8 girls, 5 boys) scored their children as having more social problems (males) and more withdrawn/depressed problems (girls). Additional studies in larger cohorts are needed to draw more definitive conclusions. To our knowledge there are no studies on resting-state functional connectivity in patients with CAH or persons at risk of CAH prenatally treated with DEX. When we performed whole-brain analyses to investigate the functional connectivity of the brain during rest in 31 patients with CAH (18 females), aged 16-33 years (mean age 23.7 years), we found increased functional connectivity in the precuneus in patients with CAH compared to controls. Post hoc analyses within the precuneus revealed that patients with SV CAH had stronger connectivity. The

altered functional connectivity may reflect a reorganization of the brain in patients with CAH. Looking at the resting-state functional connectivity in 18 participants (8 females), aged 16-33 years (mean age 20.8 years) exposed to DEX treatment during the first trimester of fetal life because of the risk of having CAH, we used two approaches: an exploratory whole-brain analysis and a seed-based analysis. We chose three brain regions (amygdala, hippocampus, superior frontal gyrus) for the seed-based analysis based on our previous findings and literature evidence. We did not find any differences in resting-state functional connectivity between DEX-exposed individuals and controls.

In conclusion, this thesis extends the existing literature on GC effects on functional connectivity in the brain and cognitive and behavioral outcomes in patients with CAH.

LIST OF SCIENTIFIC PAPERS

I. **Valeria Messina**, Leif Karlsson, Tatja Hirvikoski, Anna Nordenström, Svetlana Lajic
Cognitive function of children and adolescents with congenital adrenal hyperplasia: importance of early diagnosis
Journal of Clinical Endocrinology and Metabolism 2020;105(3):e683-691

II. **Valeria Messina**, Tatja Hirvikoski, Leif Karlsson, Sophia Vissani, Lena Wallensteen, Rita Ortolano, Antonio Balsamo, Anna Nordenström, Svetlana Lajic
Good overall behavioural adjustment in children and adolescents with classic congenital adrenal hyperplasia
Endocrine 2020;68(2):427-437

III. **Valeria Messina**, Annelies van't Westeinde, Nelly Padilla, Svetlana Lajic
Changes in resting-state functional connectivity in patients with congenital adrenal hyperplasia (submitted)

IV. **Valeria Messina**, Annelies van't Westeinde, Nelly Padilla, Svetlana Lajic
First trimester dexamethasone treatment is not associated with alteration in resting-state functional connectivity at adolescent or adult age (submitted)

Scientific publications not included in the thesis

Lena Wallensteen, Leif Karlsson, **Valeria Messina,** Anton Gezelius, Malin Thomsen Sandberg, Anna Nordenström, Tatja Hirvikoski, Svetlana Lajic.
Evaluation of behavioral problems after prenatal dexamethasone treatment in Swedish children and adolescents at risk of congenital adrenal hyperplasia
Hormones and Behavior 2018;98:219-224

Annelies Van't Westeinde, Marius Zimmermann, **Valeria Messina,** Leif Karlsson, Nelly Padilla, Svetlana Lajic
First Trimester DEX Treatment is not Associated with altered brain activity during working memory performance in adults
Journal of Clinical Endocrinology and Metabolism 2020;105(11):e4074-e4082.

Lena Wallensteen, Leif Karlsson, **Valeria Messina,** Anna Nordenström, Svetlana Lajic
Perturbed Beta-Cell Function and Lipid Profile After Early Prenatal Dexamethasone Exposure in Individuals without CAH
Journal of Clinical Endocrinology and Metabolism 2020;105(7):e2439-e2448

CONTENTS

LIST OF ABBREVIATIONS

21-OH	21-hydroxylase
ACTH	Adrenocorticotropic hormone
CAH	Congenital adrenal hyperplasia
CNS	Central nervous system
CRH	Corticosteroid releasing hormone
DEX	Dexamethasone
EAS	Emotionality-activity-sociability
GCs	Glucocorticoids
GR	Glucocorticoid receptors
GW	Gestational week
HC	Hydrocortisone
HPA	Hypothalamic-pituitary-adrenal
ICA	Independent component analysis
IQ	Intelligence quotient
MC	Mineralocorticoid
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
fMRI	Functional magnetic resonance imaging
MTL	Long-term memory
NEPSY	Developmental neuropsychological assessment
PFC	Prefrontal cortex
QoL	Quality of life
ROI	Region of interest
RSNs	Resting-state networks
SASC-R	Social anxiety scale for children-revised
SPAI-C-P	Social phobia and anxiety inventory for children-parental
SPSS	Statistical package for social science
SV	Simple virilizing
SW	Salt-wasting
VCI	Verbal comprehension index

WISC	Wechsler intelligence scale for children
WAIS	Wechsler intelligence scale for adult

1 INTRODUCTION

The first “case report” of congenital adrenal hyperplasia (CAH) appeared in 1865, describing a woman with a phallos 6 cm long with hypospadias, no testes, internal female genitalia and enlarged adrenal glands. This person died at 40 years of age after episodes of vomiting and diarrhea [1]. After almost 100 years, it was discovered that the death was caused by impaired cortisol production. Finally, in 1950 the first patient with CAH was treated with synthetic glucocorticoids (GCs) [2].

CAH is a rare inherited disorder, affecting approximately 1:10 000 - 1:15 000 newborn babies (in Sweden 1:9800) [3]. It is characterized by decreased production of cortisol and aldosterone. The consequent increment in the production of androgen precursors leads to the virilization of external genitalia in the CAH-affected girls already in utero [4-6].

Management of the disease requires lifelong replacement therapy with GC to replace cortisol and reduce adrenal androgen excess. In the more severe cases mineralocorticoid (MC) is needed to replace aldosterone and prevent adrenal and salt-wasting (SW) crises. However, mimicking the physiological circadian and ultradian secretion rhythms is challenging and suppressing high androgen levels requires high doses of GCs, especially during puberty [7].

Thus, in the context of CAH it is likely that patients experience suboptimal levels of GCs, with an ensuing disrupted circadian and ultradian rhythm throughout development. Together with early exposure to high androgen levels, this may alter the development of neuro-circuitry that plays a vital role in cognitive functions (e.g., pre-frontal cortex) and emotion regulation (amygdala, hippocampus), potentially leading to negative long-term outcomes related to cognitive processes, mood and altered structure and function of the brain.

2 BACKGROUND

2.1 CONGENITAL ADRENAL HYPERPLASIA

CAH comprises a group of recessively inherited disorders affecting the steroid synthesis in the adrenal cortex [8-10]. The most common form is due to mutations in the 21-hydroxylase (21OH) enzyme involved in producing cortisol and aldosterone [11]. If not stated otherwise, this thesis will refer to 21-hydroxylase deficiency (21OHD).

Cortisol and all other steroid hormones are synthesized from cholesterol in the adrenal cortex, which is composed of three layers: zona glomerulosa, fasciculata and zona reticularis. The zona glomerulosa is involved in aldosterone production, which is essential for regulating sodium and potassium. The zona fasciculata, the intermediate zone of the adrenal cortex produces cortisol. Androgens are synthesized in the zona reticularis (Figure 1).

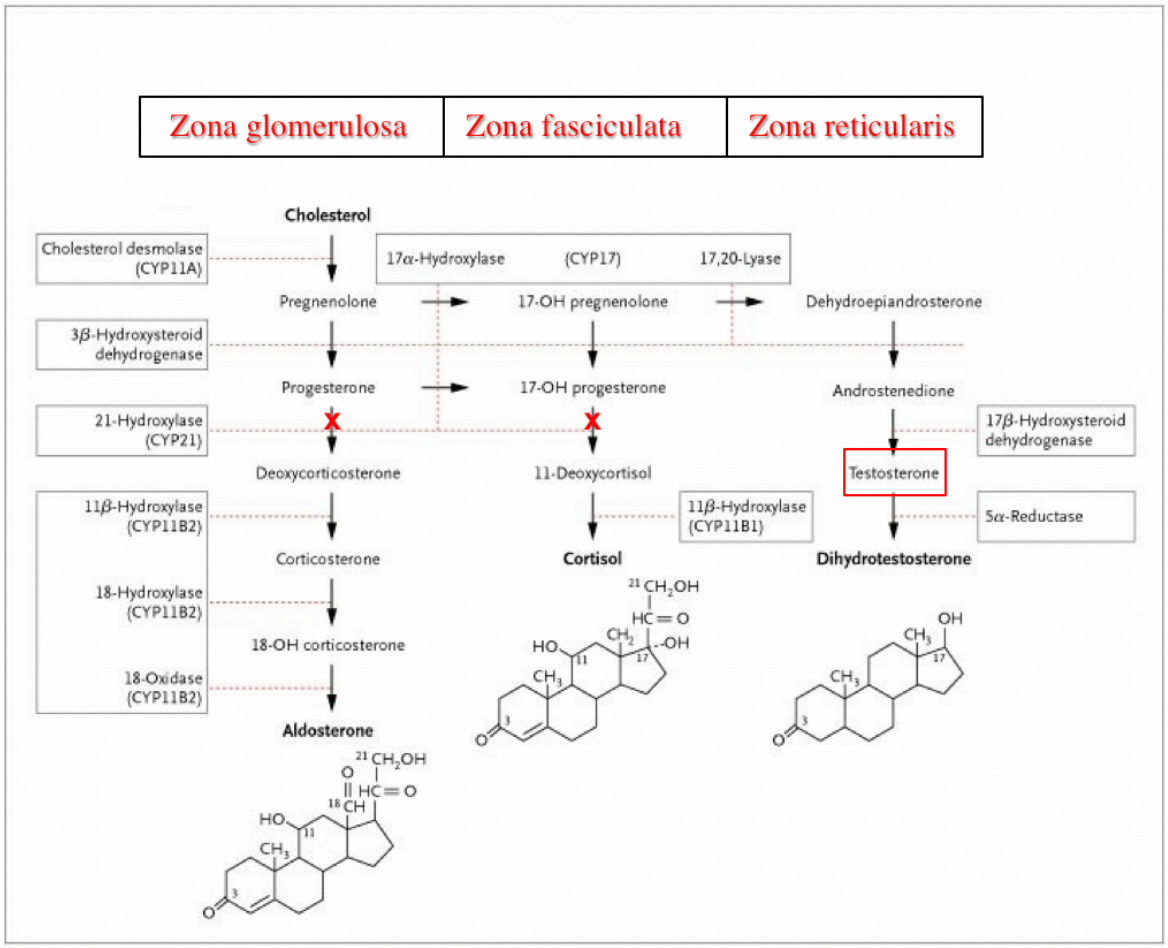

Figure 1. Pathways of Steroidogenesis

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In CAH, a disrupted cortisol synthesis, leads to increased secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland with a consequent overproduction of 17-hydroxy progesterone (17-OHP) and adrenal androgens (Figure 1). This occurs due to lack of negative feedback on both corticotropin releasing factor (CRF) and ACTH itself. Thus, patients with CAH have cortisol deficiency and androgen overproduction, in addition to aldosterone deficiency in the most severe cases [12].

The clinical classification of CAH is based on the severity of the disease. It is characterized by a good genotype and phenotype correlation with the CYP21A2 genotype and the 21OHD. The mildest mutated allele determines the severity of the disorder. There is a spectrum of CAH, from mild disease (non-classic, NC CAH) to the most severe form known as SW CAH, marked by almost complete loss of enzyme function. The SW form occurs with an incidence of 1:10 000-23 000 in the general population. The condition is depicted by salt loss, cortisol and mineralocorticoid deficiency and androgen overproduction due to no or very low levels of 21-hydroxylase activity (<1-2%) [11-13]. These patients are usually diagnosed during the neonatal period because of lethargy, vomiting, weight loss, hyponatremia, and hyperkalemia due to aldosterone deficiency. This severe condition may be fatal if not treated in time due to the SW crisis. The milder, simple virilizing form (SV CAH, ~1-10% enzyme activity) comprises cortisol deficiency and androgen overproduction [14, 15]. Patients with the SV form are less at risk of fatal SW crisis owing to a slightly higher production of aldosterone [16]. However, during febrile episodes and gastroenteritis, children with SV CAH have a 10% risk of developing a SW crisis [17].
The virilization of the external genitalia in girls with SV and SW CAH (classic CAH) is caused by an accumulation of androgen precursors (dehydroepiandrosterone DHEA, DHEA sulfate, androstenedione and 21-deoxycortisol) that occurs already in utero, sometimes to the extent that they are assigned to the wrong gender in the neonatal period [12, 18, 19].

The NC CAH form is characterized by 50–80% loss of enzymatic (21-hydroxylase) function, and consequently, it is undetected at birth due to the absence of symptoms. It is diagnosed during childhood due to early pubarche and accelerated growth or hirsutism and fertility problems in women [20].

Ten common mutations in *CYP21A2* (Pro30Leu, I2 splice, del 8 bp E3, Ile172Asn, Cluster E6, Val281Leu, Gln318Stop, Arg356Trp, Pro453Ser) have been identified in more than 95% of patients with CAH. However, more than 200 rare mutations have been described [21].

2.1.1 Neonatal screening program

A prompt diagnosis through the neonatal screening program for CAH and subsequent treatment administered immediately after birth with GC (hydrocortisone) can be life-saving, especially in the most severe forms (SW) [6].

Pang developed a technique for neonatal screening for CAH for the first time in 1977, using elevated blood levels of 17OHP as the biomarker for CAH [22]. Since then, it has been used worldwide in more than 50 countries, including Sweden, which introduced screening for CAH in 1986 [3, 23, 24]. The test involves the measurement of 17OHP on filter paper cards [23]. In Sweden, the recall level of plasma 17OHP is currently 60 nmol/L for full-term infants (in or after gestational week 37) [4, 25]. However, the risk of false-positive results is higher in preterm or sick newborns due to their higher levels of 17OHP compared with full-term or healthy babies [26]. The neonatal screening program in Sweden has stressed the importance of screening in predicting disease severity through the analyses of 17OHP complemented by an early genetic confirmation with CYP21A2 genotyping [27].

Although a clear benefit has been shown for neonatal screening of CAH for mortality [5, 26], it is not implemented yet in all European countries, mainly because of the high false-positive rate in preterm and sick newborns. The combined gestational age and birth weight adjusted cut-off levels in the past years have reduced false-positive cases [3, 28]. It is worth noting that neonatal screening also plays an important role in detecting patients with a milder form of CAH, enabling an early initiation of treatment that improves growth in children and adolescents with CAH [26].

2.1.2 Diagnosis

In the absence of a neonatal screening program affected girls are more easily diagnosed due to ambiguous genitalia. In boys, however, the condition may be undetected, with a high risk of adrenal crises after birth and resultant death [23, 26]. Before introducing neonatal screening, a female preponderance in patients with CAH had been identified, suggesting higher mortality in boys caused by a lack of identification of symptoms and consequent adrenal crises [29].

Detecting increased potassium, failure to thrive in the neonatal period, and sodium loss may help diagnose boys with CAH [30]. The biochemical marker used for diagnosing 21 hydroxylase deficiency is 17OHP and a random sample with a level above 240 nmol/L is diagnostic of classic CAH at any age [9]. The 17OHP value to exclude CAH at adult age is < 6.0 nmol/L, whereas in children it is < 2.5 nmol/L [9] and a Synachten stimulated 17OHP level > 30 nmol/L should lead to further confirmation of the diagnosis with genotyping.

Undiagnosed patients with SV CAH are commonly identified during childhood as a result of symptoms of excess androgen production such as acne, pubic hair and growth acceleration [31].

Patients with NC CAH are instead diagnosed later in life, from childhood to adulthood, on account of hyperandrogenic signs. Usually, children may present with accelerated growth and women mainly present with increased facial or body hair, irregular menses or infertility [32].

2.2 MANAGEMENT OF CAH

2.2.1 Postnatal treatment in children and adults

Consensus for the management of CAH has been established in childhood but not in adult patients [12, 33].

The treatment aims to restore the lack of endogenous cortisol and aldosterone and suppress high androgen levels. However, at the same time, care must be taken to avoid potential side effects of over-treatment, such as short stature and adverse effects on metabolism [6]. Treatment is individualized depending on the patient's age, sex and disease severity [33].

GC treatment in children with CAH aims to optimize growth and pubertal development [34]. Usually, children with CAH are treated with hydrocortisone (HC) because it has less harmful effects on growth and weight compared to prednisolone or dexamethasone (DEX) [35]. The total HC dose can vary between 10 and 15 mg/m2 body size divided into three or four doses per day. The highest dose is taken at 08.00 or earlier, depending on the child's age and the last dose at about 19:00-20:00 [13]. Children are treated with higher doses of HC during puberty because of alterations in cortisol pharmacokinetics [7].

In adults the treatment regimen is based on the sex of the patient, as well as the degree of virilization in women. The therapy focuses on fertility and preventing negative metabolic effects and osteoporosis in women. For instance, young women seeking fertility may be treated for a short period with a high dose of a long-acting GC. Treatment for a middle-aged man targets risk reduction of osteoporosis and cardio-metabolic disorders [36]. Adults with CAH are treated with HC or prednisolone, while DEX should be avoided.

In case of aldosterone deficiency, replacement with mineralocorticoid is achieved with fludrocortisone. Usually, it is administered immediately after the neonatal screening to avoid hyponatremia. Owing to its biological half-life, it is typically administered once a day, with doses ranging from 50 to 200 □g [12].

New knowledge about the genetics and pathophysiological processes related to CAH has helped improve the management of existing therapies. In addition, novel modified-release GC therapies aim to replicate the natural secretion rhythm more closely and improve the metabolic outcome and quality of life (QoL) in patients with CAH [33].

2.2.2 Challenges in the treatment of CAH

The oral therapy with the conventional immediate release hydrocortisone, administered 3-4 times/day, should mimic the circadian rhythm. Although effective in replacing cortisol, there is evident difficulty in mimicking the normal physiological state. Hence, patients are at risk of under-replacement, characterized by increased androgen levels, hypotension, abdominal pain, or over-replacement with impaired growth in children and adolescents, Cushingoid symptoms with hypertension, impaired glucose tolerance and mood disorders [37].

Adolescence is challenging, with a high risk of suboptimal treatment due to hormonal changes. Non-compliance with the therapy during adolescence may contribute to adverse health outcomes and impaired QoL in adulthood [38, 39]. Hence, monitoring is vital to target an appropriate treatment at different ages.

Moreover, during the transition to adult care, once final height is reached, a change to longer-acting GCs may be recommended to improve adherence to the therapy as well adrenal control [37]

To avoid salt-losing crises during periods of fever or illness, it is also important to inform families on how to act and inject Solu-Cortef® (HC) intramuscularly in case of emergency.

2.2.3 Surgical correction

Since the 1950s, reconstructive surgery has been practiced in females with CAH with severely ambiguous genitalia to normalize the appearance of the genitalia and limit the risk of stigmatization [12]. However, the procedure has been much debated during the past decade [40]. Clitoral surgery was performed in the past at an early age, with or without vaginoplasty. In recent years retrospective studies have shown unsatisfactory cosmetic and functional surgical outcomes and surgical interventions have become more restrictive, suggesting the importance of involving the patients in the decision process [41-43]. Genital surgery can lead to psychological stress for the patients and the family, and optimal management of each case with the help of a multidisciplinary team is required. The team includes not only pediatricians, endocrinologists and expert surgeons but also psychologists because integrating emotional and psychological support plays a vital role during the care process. Moreover, in surgery they may also experience complications, including urinary incontinence, clitoral pain, and sexual difficulties [44], emphasizing the importance of psychological support.

However, without surgical intervention, self and social stigma may emerge by virtue of genital appearance, leading to withdrawal from social interaction. Indeed, self-stigma caused by seeing oneself as different from peers has been reported from childhood to adulthood in patients with CAH [45, 46].

2.3 PRENATAL TREATMENT WITH DEX

Since the mid-1980s, pregnant women at risk of having a fetus affected by CAH have been treated worldwide with the GC DEX to suppress fetal androgen production and minimize prenatal virilization of a female fetus. DEX treatment must be initiated before gestational week 7 to be effective because virilization of external genitalia takes place from the 6th to the 8th week of gestation. As soon as the genotyping of the fetus is known, through chorionic villous sampling in gestational weeks 11-12, the treatment is discontinued for unaffected girls and all boys. Only girls with CAH are treated until term [47, 48]. Consequently, about 7 of 8

fetuses (unaffected girls and boys) are treated without any personal benefit and are exposed to high doses of synthetic GC during early gestation (dose of 20 μg/kg/d given to the mother). For this reason, many ethical concerns have been raised by clinicians. Recently, prenatal determination of fetal sex (approximately GW 6) has become possible using cell-free fetal DNA from maternal blood [49, 50]. It can be used to avoid unnecessary DEX treatment in boys. However, girls at risk of CAH are still treated with this strategy until the diagnosis can be established, irrespective of whether they have CAH.

Because the safety of DEX treatment has been questioned, an international consensus statement suggests that prenatal therapy should be pursued only within a clinical study and with the informed consent of parents [51].
However, recent data from a questionnaire assessing the current medical practice in 36 medical centers in 14 European countries raised new concerns due to the high variability in DEX treatment in different countries [52].

Moreover, systematic evidence related to the safety of the treatment for the fetus and the mother is still scarce. In Sweden, prenatal DEX treatment has been used since 1985 and as a clinical trial since 1999 (PREDEX, PI, S Lajic) [53]. The PREDEX study evaluated the long-term effects of prenatal DEX treatment on maternal health during treatment, fetal and postnatal growth and metabolism, cognition and behavior [54-61]. Recently, we also evaluated the effects on brain structure and function [62, 63].

Based on the harmful effects assessed in the studies, prenatal treatment with DEX is currently not offered in Sweden until more data on treatment safety or earlier prenatal diagnosis is available.

2.4 GLUCOCORTICOIDS

GCs (cortisol in humans) play an important role in regulating different and essential basal body processes/functions. These include homeostasis, by inducing physiological and behavioral adaptation [64]; metabolism, by stimulating glycogen formation in the liver and mobilizing energy [65]; immune system, by inhibiting the production of cytokines and eicosanoids (e.g., prostaglandins and thromboxanes) [66].

2.4.1 Synthesis and release: HPA axis and the circadian rhythm

One of the central systems involved in cortisol secretion is the hypothalamic-pituitary-adrenocortical (HPA) axis (Figure 2).

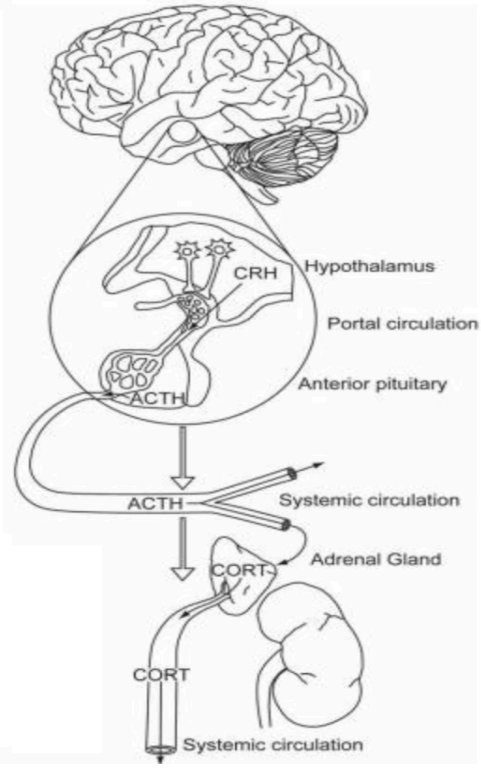

Figure 2. HPA axis

Reprinted from Herman JP, et al. Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response, Comprehensive Physiology, 2016, 15;6(2):603-21. Copyright 2016 American Physiological Society.

The HPA axis develops during fetal life (between GW 8 and 12)[67]. It has been shown that environmental perturbation during fetal life or exposure to a high level of GC may affect the normal development of the HPA axis, with detrimental effects for the fetus later in life because of abnormal HPA functioning.

Normally, a physiologic signal (low cortisol level) induces the release of corticotrophin hormone (CRH) and arginine vasopressin (AVP) by cells in the paraventricular nucleus of the hypothalamus (PVN), which reach the anterior pituitary gland and stimulate the release of adrenocorticotropic hormone (ACTH) into the circulation. Finally, in response to ACTH, cortisol is produced by conversion of cholesterol through a series of enzymatic reactions. [68]. To stabilize GC secretion and repair/restore homeostasis a negative feedback mechanism is activated in the HPA axis. This feed-forward feedback in the adrenal pituitary constitutes the ultradian rhythm, the expression of the pulsatile release of cortisol and ACTH [69]. The ultradian rhythm promotes the normal gene expression of GC target genes [70, 71]. The daily rhythm of HPA activity, regulating the cortisol secretion, is instead regulated by an internal pacemaker, the suprachiasmatic nucleus [72].

The morning cortisol peak usually occurs at 0800 h before starting daily activities. Concentrations start decreasing during the day, with the lowest levels in the mid-afternoon and midnight and then up again between 0200 h and 0400 h, usually after nocturnal sleep

until awakening [73] (Figure 3). It has been suggested that the nocturnal rise depends on the higher brain energetic demand at the end of the night [74, 75].

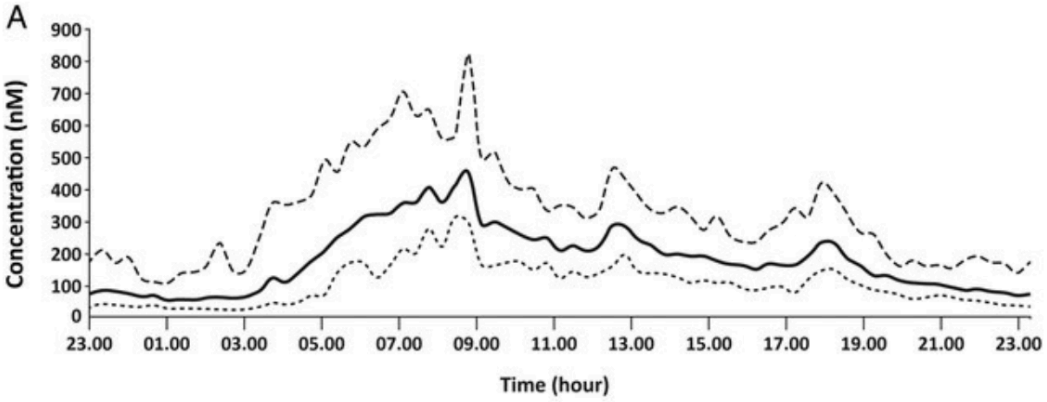

Figure 3. Cortisol concentrations in healthy volunteers (mean, 10th and 90th centile).

Figure readapted from Porter, J., et al. Is physiological glucocorticoid replacement important in children? (2017), Archives of Diseases in Childhood 102(2): 199-205. Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license.

2.4.2 Stress response

GCs are released in a circadian cycle from the adrenal gland and by catecholamine signaling in response to physiologic or psychological signals and stress [13]. To react to stressors ranging from mild (i.e. social interactions) to severe (i.e. dangerous situations), predictable or unpredictable, the brain activates a coordinated response from the organism, which is mediated by a complex regulatory system [76].

A stress response involves two steps, where the brain and the body act in synchronicity. In the first step, the brain interprets a physical or psychological signal as a threat. Depending on stressor typology, physical or psychological, different brain networks are activated in response to stress. The brainstem and hypothalamus are usually involved in regulating physical stressors [77, 78], whereas limbic structures, comprising the prefrontal cortex (PFC), amygdala and hippocampus, among others, take part in regulating psychological stressors [79]. Once the stress has been identified, the sympathetic-adrenal medulla (SAM) and HPA axes will be activated. The SAM will secrete epinephrine and norepinephrine to prepare the body for a fight or flight response. The HPA axis will release cortisol into the bloodstream. The psychological stressor will also elicit a cognitive response involving different brain areas important for memory and attention [80]. The cognitive response is regulated by three brain areas: PFC, amygdala and hippocampus. The PFC, innervated by GABAergic neurons, responds to environmental changes facilitating behavior plasticity. Although its involvement

is complex as different anatomical subdivisions play different roles in the stress response, the prelimbic cortex and the infralimbic cortex coordinate a top-down control [81]. The amygdala, involved in emotional processing, particularly the basolateral nucleus (BLA), is essential for memory consolidation of adverse events and fear-related responses and coordinates a bottom down control [82, 83]. The hippocampus, which has direct projections with the amygdala and PFC, responds to physical and psychological stressors. In particular, its paraventricular nucleus (PVN) triggers the activation of the HPA and SAM axes [79]. Moreover, the hippocampus mediates the negative feedback of the HPA axis [76].

2.5 EFFECT OF PRENATAL TREATMENT WITH GC

2.5.1 Glucocorticoids and brain

Cortisol plays a vital role during the stages of pregnancy. During sensitive periods of prenatal life, the fetus is exposed to different levels of maternal cortisol promoting organogenesis, neuronal maturation, and finally, fetal maturation at the end of the pregnancy when the level of cortisol is higher to prepare the fetus for birth [84]. A balanced cortisol level during pregnancy is vital for normal fetal development, and it is regulated by the placental 11β-hydroxysteroid dehydrogenase type 2 enzyme (11β-HSD2) [85]. In the case of synthetic GC administration the range of placental inactivation for the supraphysiologic level of GCs varies according to the type of GC. DEX is not metabolized by placental 11β-HSD2. Hence it easily crosses the placenta [86]. This means that the fetus will be exposed to a GC dose that is 60 times higher than the endogenous cortisol levels [87]. Such a high dose may affect fetal programming, altering the brain development trajectory and the brain's functional organization, with consequent long-term adverse health outcomes on cognition and behavior [88, 89].

2.5.2 Effect of prenatal exposure to GCs on brain development

GCs are essential for normal brain development, but exposure to high levels of GCs may have damaging long-term consequences. Fetal life, especially early gestation, is a sensitive period during which the brain may be vulnerable to GC perturbation. The prenatal period is characterized by important neurologic advances. Between GW 8 to 16, the neurogenesis begins. Neurons migrate until they reach the final destination, with ensuing arborization and proliferation [90, 91]. They will stop proliferating at about GW 20 to 24 and start migrating to the cortical plate. However, new organizational changes in the cerebral cortex will continue until term. Different neuronal stages can be observed in the third trimester of pregnancy, including differentiation, dendritic arborization, proliferation and myelination [92].

From GW 29 to 41, the cortical grey matter volume will constantly increase, with an approximately 50% increase in the cortical volume between GW 34 and 40 [93]. The hippocampus and amygdala, important regions for the mediation of the stress response and

cognitive processes, develop rapidly from GW 6 to 8 until term. There is evidence that GRs are present in the hippocampus already by GW 24, but knowledge regarding the time course is limited [94]. Interestingly, it has also been shown that both structural networks and functional networks are present already during fetal life [95, 96]. Structural networks, in particular, have an adult-like topological organization (85%), suggesting the presence of a small-world organization already during fetal life [97]. In contrast, functional networks are present in an immature state, with the sensorimotor areas being the most active [98].

Exposure to high GC levels has been linked to altered neurogenesis, dendritic atrophy and synaptic loss in brain regions expressing a high density of GR [99, 100]. Given that the amygdala, hippocampus and structural networks start developing during the first trimester of pregnancy, it is conceivable to expect an alteration later in life.

The majority of human and animal studies focusing either on the effect of maternal stress during prenatal life or on the impact of prenatal GC exposure on functional and structural connectivity have reported contradictory results. Discrepancies are related to differences in dose and time of medication, stage of development, cohort size and age. Moreover, many studies have investigated GC effects on neurodevelopment, mainly during the second and third trimesters in infants and children. Thus, when comparing these studies with CAH patients prenatally treated during the first trimester, it is crucial to consider this difference in time exposure.

Hippocampal damage, reduced hippocampal volume and decreased neuronal proliferation have been reported in primates treated with GC during the second and third trimesters [101, 102], with more severe changes correlated with higher doses [103].

Similar results have been reported in human studies. A study by Davis et al. evaluating brain development and affective problems in 54 full-term children (6-10 years) prenatally treated with two doses of betamethasone during the third trimester reported a thinner rostral anterior cingulate cortex in the treated children compared to controls [104]. This region is involved in affective disorders. Thus, the authors suggest that children may be at risk of developing mental problems later in life.

In another longitudinal study Buss et al. investigated the effect of early maternal prenatal stress and pregnancy anxiety on brain gray matter in offspring (children aged 6-9 years). The authors identified changes in brain morphology. More specifically, altered grey matter volume was reported in different brain regions (e.g., the prefrontal cortex, the premotor cortex, the medial temporal lobe, the lateral temporal cortex), suggesting a risk of developing neurodevelopmental and psychiatric disorders, as well as cognitive and intellectual impairment later in life [105].

A recent prospective longitudinal study evaluating the association between prenatal maternal cortisol concentrations during the third trimester and neurodevelopment in children reported

an association between high maternal cortisol levels and cortical thickness in frontal brain regions [106].

However, only a few studies have investigated the association between prenatal exposure to GCs and brain structure and function changes. Furthermore, it is difficult to conclude, especially in patients prenatally treated during the first trimester, due to time exposure differences.

2.5.3 Effect of prenatal exposure to GCs on cognition

Most results from studies investigating the effects of prenatal treatment on cognitive outcomes found a correlation between GC dose and timing of the treatment. The cognitive functions in the majority of the studies were evaluated either by a trained psychologist or assessed indirectly by parental questionnaires.

A negative effect on cognitive performance has been reported in pre-school children, adolescents and adults exposed to two or more doses of betamethasone during late gestation. Pre-school children performed worse on tests assessing general intelligence, whereas adolescents and adults performed worse on tests assessing executive functions, particularly attention and processing speed [107, 108].

However, not all studies have reported negative effects. There is growing evidence that moderate exposure to stress during late pregnancy may increase cognitive maturation [109] and enhance cognitive performance in children [106].

2.5.4 Effect of prenatal exposure to GCs on behavior

Prenatal GC treatment has been linked to the risk of developing mood and psychiatric problems [110, 111]. The majority of the studies, however, have reported outcomes related to the effects at an early age. Thus it is difficult comparing the results with our cohort.

A recent Finnish study investigating the effect of GC in children of mothers treated during late pregnancy because they were at risk of pre-term delivery showed that the treatment was significantly associated with mental and behavioral disorders in 11-year-old children [112].

Evidence of the long-term effect of prenatal treatment with cortisol has also been reported in the context of preterm birth treatment with DEX on adolescent (14-17 years) brain development. Patients had reduced brain volume and behavioral problems upon entering adolescence [113].

Many studies have also assessed the effects of exposure to maternal psychosocial distress during pregnancy, as well as the impact caused by maternal exposure to stressful life events (i.e. a natural disaster). Different measures have been used to assess maternal stress (self-

reported questionnaires vs. cortisol levels measured in saliva). Studies investigating the effects of exposure to maternal cortisol from early to late gestation showed that higher maternal cortisol levels in earlier gestation were associated with more affective problems in girls (age 6.5 years), with a larger amygdala predicting affective issues [114]. Moreover, the higher cortisol levels also predicted anxiety problems in children (age 6-10 years)[115]. Laplante et al. showed that maternal exposure to a natural disaster predicted difficulties in temperament in the offspring (aged 6 months)[116].

However, it is worth noting that all the findings from previous studies are based on different nature of exposure (maternal stress vs. synthetic GC) and different methodologies (questionnaires vs. cortisol levels). Thus, considering many uncontrolled factors (e.g., shared genetics) that remain to be elucidated, the results should be interpreted with caution when considering the effects of prenatal DEX exposure in CAH.

2.5.5 Effect of prenatal DEX exposure in CAH

Study findings related to the effect of prenatal exposure to DEX in individuals at risk of having CAH treated during the first trimester are conflicting. The differences are usually correlated to the sample size, the methodology used to assess the outcomes, and the individuals' age. Usually, the cohorts are quite small due to the rarity of the disease, and there is a variation between those at risk of CAH treated for a short period and female patients with CAH prenatally treated until term. Moreover, behavioral outcomes are often detected through self-report or parental questionnaires, with or without the assessment of a trained psychologist. These differences make it difficult to compare results across studies.

Two studies have not shown alteration in cognitive performance in children prenatally treated with DEX [117, 118], but the two studies differ regarding the age of the cohorts and methodologies. In contrast, an observational follow-up that initially included 140 children (5-12 years of age) reported slower mental processing in girls with CAH exposed to DEX during their fetal life [119].

In our previous research we showed evidence of impaired verbal working memory in first trimester DEX-treated (non-CAH) participants in a cohort of children and adolescents (7-17 years) [60], with effects more pronounced in girls [56]. However, in our recent study at adult age we reported a significant improvement in verbal working memory, and we did not observe any differences between our DEX-treated group and controls [57].

For behavioral outcomes, parents in our Swedish cohort did not report behavioral problems, although they rated their children as more sociable compared with the population controls [53]. However, DEX-treated children reported more social anxiety in self-assessed questionnaires [60]. Moreover, findings from the same cohort depicted a more neutral behavior in males prenatally treated with DEX, suggesting a potential treatment effect on gender role behavior in males [120].

In the first pilot study on prenatal treatment with DEX from Trautman et al. in a cohort of infant and preschool children parents reported more shyness and less sociability in their preschool children (5.5 years) and more internalizing problems (e.g., depression, anxiety, social anxiety, somatic complaints) in their toddlers at 2-3 years [118].

Recently, our group started investigating the effects of prenatal exposure on functional brain connectivity. In our first study, looking at the brain activation during a working memory task in an adult cohort (mean age 25.6), we did not find any alteration in brain activity during the working memory performance. However, impaired cognitive performance was reported during adolescence [63]. Looking at the brain structure in the same adult cohort, we identified structural brain alterations, such as an enlarged amygdala and increased volume in the superior frontal gyrus, suggesting that prenatal treatment with DEX may have an organizational effect on brain development [62].

These findings may suggest a potential negative effect of prenatal treatment with DEX on patients treated during the first trimester.

2.6 EFFECT OF POSTNATAL TREATMENT WITH GC

2.6.1 Glucocorticoids and brain functional connectivity

In this thesis we investigate the effects of chronic exposure to GCs. However, it is worth noting that acute exposure to GCs may promote behavioral resilience and adaptation to stress, likely preserving dendritic spine density in the hippocampus and amygdala through a non-genomic mechanism of action [121, 122]

Several studies have indicated that prolonged exposure to high GC doses may cause impairment in brain function and brain structure [123-126]. Before going into detail, it is essential to highlight the brain's functional organization in healthy people.

It has been suggested that the brain is organized in networks. In particular, three large scale cognitive networks have received attention: the default mode network (DMN), involved in self-referential processing, the executive network (CEN), involved primarily in working memory and executive functioning, and the salience network (SN) that plays a key function when identifying internal and external stimuli to guide behavior [127]. Dysfunctional brain architecture in one or more of these networks may be related to psychopathology, such as depression, anxiety and dementia.

The DMN, active during the resting state, comprises different networks specialized in a specific processing domain [128, 129]. The DMN includes large parts of the prefrontal cortex, posteromedial and inferior parietal cortex and parts of the temporal cortex that are functionally correlated [130]. The DMN is active during the resting state but is deactivated during a cognitive task requiring external attention.

The CEN, including the dorsolateral prefrontal cortex and the posterior parietal cortex, conversely to the DMN, is activated during a cognitive task requiring attention. Particularly noteworthy is that these two regions are anticorrelated networks, suggesting that they are involved in different cognitive tasks [131].

The SN, which comprises the anterior insula and the dorsal anterior cingulate cortex, is responsible for switching between the DMN and the central executive network, as demonstrated by the triple network model [132].

Considering that these networks underlie regions such as the hippocampus, amygdala (SN) and prefrontal cortex (CEN and DMN), with a high presence of GC receptors, we could expect that a disrupted cortisol imbalance may affect the networks.

2.6.2 Effect of postnatal treatment on functional connectivity

One study conducted at rest in patients with Cushing's syndrome, another disorder of cortisol imbalance, suggests that long-term cortisol excess results in increased connectivity within the default-mode network (DMN), particularly in the medial temporal lobe and prefrontal cortex [125]. The precuneus, reduced in volume in our study in CAH patients [124], is functionally connected to the CEN and the default mode networks. Thus, the altered structure resulting from prolonged cortisol imbalance might lead to altered functional connectivity of both the CEN and default mode networks.

Cortisol operates through stimulation of the GC and mineralocorticoid receptors, which are distributed throughout the brain and may affect excitatory glutamate signaling, especially in the frontal cortex, in addition to regulating glucose metabolism [133]. Thus, long-term disturbances in cortisol may impact widespread brain networks but are expected to mainly affect hubs that contain a high density of cortisol receptors, such as the prefrontal cortex and the hippocampus [133], or hubs that have a high energy demand, such as the precuneus [134].

Interestingly, recently it has also been suggested that cortisol affects brain functioning differently in women and men. Indeed, a reduced functional connectivity of the amygdala with different cortical structures has been reported in women, while a positive correlation has been observed in men [135]. However, it has been shown that the brain networks have the capacity to compensate in case of genetic or environmental risk [136]. Indeed, studies in children with diabetes have demonstrated compensatory hyper-connectivity [137].

Moreover, alterations in the default mode network have been associated with mood disorder. In particular, decreased functional connectivity between the posterior cingulate cortex (PCC) and the precuneus has been associated with an increased rumination, causing persistent negative thinking in depressed patients [138, 139].

2.6.3 Effect of postnatal treatment on cognition

GCs play an essential role in memory consolidation and retention [140] and exert their functions by binding the GR and MR receptors, involved in memory consolidation and encoding [141, 142].

However, an excess of GCs may have a negative effect on the process involved in memory formation [143]. Many studies have been done on Cushing´s syndrome, a disease characterized by prolonged exposure to high cortisol levels, showing deficits in cognitive domains, particularly learning and memory [144, 145].

Deficits in verbal and visual memory [146], processing speed and attention [147], as well as general intellectual ability [148] have also been reported in patients with Cushing´s disease. The deficits persist after short [149] or long remission [150, 151].

In line with those findings a longitudinal study investigated the long-term effects of hypercortisolism on cognition in 18 patients, tested at three time points after treatment (12, 24 and 36 months), revealed deficits in general intelligence, executive functions, attention and non-verbal memory, suggesting a long-term impact of high GC exposure. Of note, patients with Cushing´s syndrome present structural and functional brain alterations, such as changes in white and grey matter [152] and reduced hippocampal volume [153], which correlate with impaired cognitive function [154]. A negative impact of GC treatment on learning and memory has been shown in children and adults with asthma. The impairment correlates with the GC dose [155, 156], suggesting that the effect of GCs is dose-dependent. A decline in declarative memory has also been identified in one study in 30 healthy adults tested after 3 days of exposure to two prednisone doses [156].

2.6.4 Effect of postnatal treatment on behavior

Most reviews on the effects of GC treatment on behavior are based on individual cases or case-series reports and present a high risk for neuropsychiatric conditions (e.g., suicidal behavior and impact on mood) [156, 157]. A risk for mood problems seems to increase with age, which is correlated with the magnitude of the initial dose. Moreover, long-acting GCs, such as betamethasone or DEX, significantly increased the risk for mood disorders (e.g., depression and anxiety) [157]. However, the exact mechanism involved in the development of mood disorder is still unclear [158]. High cortisol levels have been observed to inhibit the brain-derived neurotrophic factor (BDNF), which promotes neurogenesis and synaptic plasticity in brain regions such as the hippocampus and prefrontal cortex [159]. A low level of BDNF may contribute to depression and anxiety problems.

Studies on Cushing´s syndrome, characterized by prolonged exposure to excess glucocorticoid levels, have shown increased mood-related problems, which correlate with the degree and time of GC exposure [160]. A reduction of cortisone levels has been associated with an improvement in symptoms of depression and anxiety in this patient group [161].

Moreover, disturbances in the circadian rhythm of cortisol secretion have also been linked to problems with mood (e.g., to increased self-reported negative affect) [162], and circadian disruption has been linked to mania or depression in bipolar disorder [163], suggesting the effects of the circadian rhythm on mood and psychiatric symptomatology.

2.6.5 Effect of postnatal treatment in CAH

Studies on cognition in patients with CAH have reported conflicting outcomes in three domains: general intelligence, executive functioning and learning and memory. Moreover, most of the studies have focused on the spatial ability domain in females to investigate possible masculinizing effects on spatial ability due to prenatal exposure to high androgen levels [164-166] rather than on the effects of the postnatal GC treatment. However, recently, a meta-analysis concluded that prenatal exposure to androgens does not support the spatial differences between the sexes [167].

Sex, age, disease severity, SW crises, treatment strategy and availability of neonatal screening programs in the investigated cohort have been reported as factors mediating cognitive functioning and therefore may be a plausible explanation for the conflicting outcomes.

The majority of evidence on cognitive abilities comes from studies in adults.

Johanssen et al. investigated the cognitive functions of 35 women with CAH (17-51 years) compared to a control group from the general population matched for educational level. Using a short form of the WAIS test, they reported lower full-scale IQ and Performance scores in women with CAH, especially in SW cases. Moreover, hyponatremic crises contributed to lower scores in Full-Scale IQ and verbal IQ in women with CAH [168]. These results are in line with a previous study performed by Helladay et al. on 22 women (17-34 years), showing lower general IQ in the CAH group compared to the control group [169].

Notably, in the studies mentioned above, patients with CAH were not screened at birth, which implies that patients might have more possibilities to have been exposed to early hyponatremia or hypoglycemia.

Evidence of normal or even higher intelligence in adult CAH cohorts has also been noted. An American group, comparing 24 women with SW CAH (21-71years) with 10 unaffected siblings and 8 controls with polycystic ovary syndrome, did not report differences in general intellectual ability in women with CAH. However, due to the small sample size, lack of control for parental education or socioeconomic status, as well as the nature of the control group, it is difficult to draw meaningful conclusions [170].

A recent study investigated the cognitive functions of 30 children with CAH (6-16 years) compared to 20 age- and sex-matched healthy controls also reported lower scores in the Full-Scale IQ [171].

A follow-up study performed by Berenbaum et al. on 104 patients with CAH (62 females, 42 males) and 88 unaffected relatives (31 females, 57 males) tested on four separate occasions from childhood to young adulthood reported normal intelligence in patients with CAH compared to controls. Drawing a firm conclusion in this study is also problematic because of the different cohorts assessed each time (only a small proportion was tested four times), the nature of the control group, which consisted of unaffected siblings and the lack of statistical control for parental education or socioeconomic status. On the other hand, some studies have reported impaired working memory in children and adults with CAH [172], as well as a deficit in an overlapping construct of short-term memory, resulting in impaired spatial perception and quantitative performance [173], suggesting that impairment in short-term memory may alter higher cognitive functions.

Our recent study, including adolescents and adults with CAH, also reported negative effects on cognition [174]. In particular, patients with CAH performed poorly in verbal and visual-spatial working memory subtests compared to controls. It is known that excess GC may have a negative effect on the process involved in memory formation [143].

Studies on behavioral effects in children and adolescents with CAH are scarce. Most of the studies regarding behavioral outcomes have focused on girls with CAH and investigating the influence of early androgen exposure on sex-specific issues based on the hypothesized link between androgen effects and behavioral development.

Some studies have reported good overall psychological adjustment in girls and boys with CAH [175-178]. In contrast, other studies have shown that boys with CAH have more behavioral problems compared to controls, especially in the internalizing scale [179]. Other studies have reported more aggression in girls with CAH compared to healthy females and more interest in masculine sports and physical games [180, 181]. A Swedish study on psychological and psychosexual outcomes in girls and women with CAH showed more gender-atypical behavior that correlates with the genotype [182]. Moreover, women with CAH showed an interest in more male-typical occupations, motor vehicles and non-heterosexual orientation.

Findings on QoL in males are conflicting, likely due to differences in methodologies, samples, and the complexity of assessing such a multidimensional concept as QoL, which includes different aspects. Some studies have reported impaired QoL in adult males with CAH [183] with impaired sexual wellbeing [184] and a high probability of sick leave and disability pension [185]. In contrast, other studies did not identify differences in QoL but reported a less active sexual life [186].

These studies suggest that long-term treatment with GC and the effect of the disease per se may increase the risk of worsening cognitive abilities in patients with CAH, leading possibly to impaired QoL. However, the neonatal screening program's efficacy in identifying patients with the most severe form of CAH seems essential in preventing adrenal crisis that may contribute to unfavorable health outcomes later in life.

3 RESEARCH AIMS

This thesis aims to increase the knowledge of long-term outcomes related to cognitive abilities, behavior and functional connectivity in the brain in patients with CAH, pre- and postnatally treated with synthetic GCs. We also assessed the effect of first-trimester prenatal treatment with DEX on functional brain connectivity in individuals without CAH. We hypothesized that both pre-and postnatal treatment with GCs may alter the developmental trajectories of the brain with effects on brain functions.

3.1 RESEARCH QUESTIONS

1) Do pre- and postnatal treatments in patients with CAH affect cognition? Is there a correlation between the severity of the disease and cognitive outcomes? (Study I)

2) Do pre- and postnatal treatments in patients with CAH affect the behavioral outcome? (Study II)

3) Is the whole-brain functional connectivity at rest altered in adolescents and adults with CAH? (Study III)

4) Does prenatal treatment with DEX during the first trimester affect whole-brain functional connectivity at rest in adolescents and adults without CAH? (Study IV)

4 MATERIALS AND METHODS

4.1 STUDY POPULATION

This thesis is part of the PREDEX study, a larger study investigating the effects of prenatal treatment with DEX in individual with or at risk of CAH. The entire cohort comprises in total 265 subjects, see figure 3. Between the 1984 and 2010, 77 mothers at risk of having a child with CAH were treated with DEX (max dose: 20µ/kg per day). Due to 4 miscarriages, in total 73 mother were treated with DEX (4 mothers were treated twice) [56]. The final cohort, included in the long-term follow-up study comprised 60 DEX treated children. Of those 60, 45 did not have CAH and therefore were treated only during the first trimester. The remaining 15 children had CAH and the 8 girls were treated until term while 7 boys with CAH were treated during the first trimester. Moreover, 78 patients with CAH, who did not receive the prenatal treatment were also included in the more extensive study to evaluate the long-term effects of the GC replacement therapy and the disease *per se*. These 78 CAH patients without prenatal treatment were also used as a control group for the CAH-DEX group.

A detailed description of the number of participants included in the different 4 studies of the present thesis is listed in figure 5.

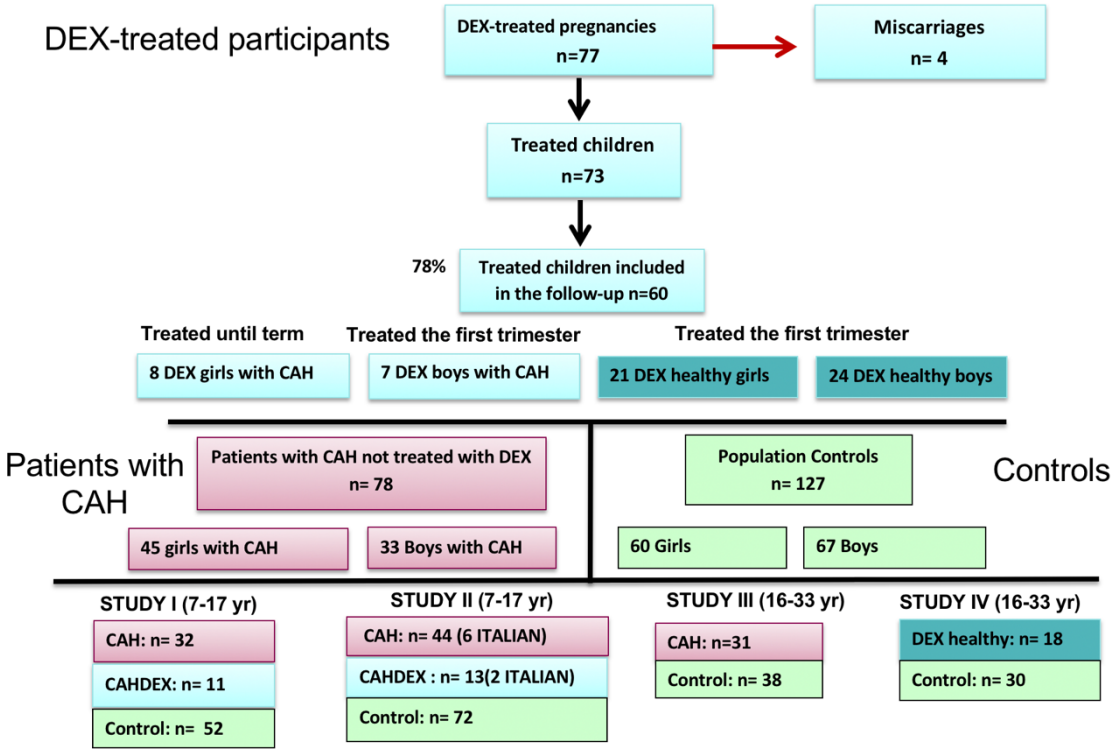

Figure 5. *Flowchart of the PREDEX cohort, with DEX participants, patients with CAH and controls*

The control group (n=127) was recruited from the Swedish national population registry and matched for sex and age with the CAH and DEX-treated participants. For sake of simplicity all the control group was randomly selected from the Stockholm County.

The participants initially received an invitation letter, comprising two different letters, one for the parents and on for the child. All participants gave their informed consent to participate in the study.

The entire protocol comprises different investigations, divided into different sessions. Initially medical parameters and blood sampling were collected. Cognitive data was collected successively using a neuropsychological test battery, including parental and self-reporting questionnaires. Trained psychologists assessed all the participants and all the psychological tests and questionnaires were administered in a fixed order in the presence of the trained psychologists. Thus, participants could ask for clarification if needed. Finally, brain imaging was assessed for participants ≥ 16 years of age.

In addition, the following parameters were collected: socio-economic status (estimated as level of education for the parent and adult participants), general well-being ,measured using a 10 point-visual analogue scale (1 was the lowest score and 10 the highest score), alcohol and drug consumption and smoking behavior for participants ≥16 years.

The study was approved by the Regional Ethics Committee in Stockholm (Dnr 99-153 and 2011/1764-32). All the studies were performed at the Karolinska University Hospital.

4.2 OVERVIEW OF STUDY I-IV

4.2.1 Study I

"Cognitive function of children and adolescents with congenital adrenal hyperplasia: importance of early diagnosis"

Study population

The cohort of this study comprised 43 children and adolescents with CAH (age range 7-17 years; 23 girls, including one sibling pair). Of these 43 children/adolescents, 31 were not prenatally treated with DEX (17 females, 15 males). Eleven (24%) (5 boys, 6 girls) had been prenatally treated with DEX.
Phenotype among patients with CAH: SW: n=30; SV: n=12; NC n=1.
Genotype: Null genotype n=12 (no 21-hydroxylase activity; Non-null n=30 (some residual 21-hydroxylase activity). The genotype was not known in one patient. The control group comprised 52 individuals (age range 7-17 years; 27 girls, including 3 siblings of 3 patients with CAH who opted to participate in the study). All patients with CAH, except one (not born

in Sweden), were identified through the Swedish national neonatal screening program. The study participation rate for the CAH cohort was 81.0% and for the population controls 55.0%.

Assessment of cognition: outcome measures

General intellectual ability was assessed using two subtests from the Wechsler Intelligence Scale for Children-III (WISC-III): the Block Design subtest and the Vocabulary subtest, estimating respectively non-verbal intelligence and verbal intelligence [187].
The Digit Span subtest from the WISC-III was selected to assess executive functions (i.e. verbal working memory) [187]. The Span Board subtest from the WMS-III was used to investigate visual-spatial working memory [188]. The participants were administered the Coding subtest of the WISC-III to measure speed in information processing [187]. The Stroop Interference Test was selected to evaluate the ability to inhibit an overlearned response [189].
To assess learning and long-term memory was administered the list learning subtest of the NEPSY (A Developmental Neuropsychological Assessment) [190].

Statistics

Before analysis the raw scores of the Wechsler scale subtests were transformed into scaled scores based on age-specific Swedish norms [188] as well as NEPSY, according to the American manual [189]. The raw scores of the Span Board subtest as well as the results of the Stroop Interference test were transformed into T-scores, using the norm tables respectively from the Wechsler Nonverbal Scale of Ability (WNV) [191] and the American norms [189].

Two-way analyses of variance (ANOVAs) with sex (male vs. female) and group (CAH vs. control) as the independent variables were used to compare CAH patients not prenatally treated with DEX to general population controls. Interactions ($p<0.05$) between sex and group were followed by separate post hoc comparisons between patients and controls of the same sex. Mann-Whitney U tests were used to compare cognitive performance in children with CAH prenatally with DEX with those with CAH not prenatally DEX-treated. In all analyses we also controlled for parental education. One-way ANOVAs compared test performance between children/adolescents with CAH (not prenatally treated) with different phenotypes (SW, SV) and genotype groups. Pearson's bivariate correlation was used to investigate the impact of the GC replacement dose (mg/m2/day) to test cognitive performance. Effect sizes were expressed as Cohen's d and classified as large when $d\geq0.80$, medium when $d\geq0.50$ and small when $d\geq0.20$ [192]. A two-tailed alpha level of $p<0.05$ was used to compare groups. We did not correct for multiple comparisons to avoid missing clinically relevant effects. All analyses were planned *a priori*. SPSS version 23 (IBM, Armonk, NY, USA) was used for statistical analysis.

4.2.2 Study II

"Good overall behavioral adjustment in children and adolescents with classic congenital adrenal hyperplasia

Study population

The CAH cohort consisted of 44 children and adolescents with CAH not prenatally treated with DEX (CAH: 22 boys) and 13 children/adolescents with CAH prenatally treated with DEX (CAH-DEX: 5 boys). The control group included 72 individuals (34 boys; 8 were healthy siblings of 5 patients with CAH included in the study). All participants were between 7 and 17 years old.

Assessment of behavior: outcome measures

Parent-completed questionnaires

Three parental ratings were used to assess behavioral problems, social phobia and temperament: The Child Behavior Checklist for ages 4-18 years (CBCL/4-18), the Social Phobia and Anxiety Inventory for Children – Parent Report (SPAI-C-P) and the Emotionality-Activity-Sociability-Shyness Temperament Survey for children (EAS).

The CBCL/4-18 is a 113-item standardized parental-child instrument for psychiatric screening providing both broad- and narrowband scales. It was used to quantify internalizing and externalizing problems and specific subscales such as depressive, social and attention problems and delinquent and aggressive behaviors [193]. The CBCL total competence score, which measures adaptive functioning, is a sum of scores from three subscales: 1) Activities scale – the number, amount and quality of the child´s participation in different leisure activities and everyday tasks; 2) Social scale – participation in social activities, number of close friends and weekly contact with them; and 3) School scale – (e.g., academic performance and need for a special class or support) [193].

The SPAI-C-P is a 26-item parent-report measure covering cognitive, physiological and behavioral symptoms of social phobia according to the DSM-IV classification system [194, 195]. The SPAI-C-P includes subscales for 'Public Performance` (PP), 'Assertiveness/General Conversation' (AGC) and 'Traditional Social Encounters' (TSE). Temperament was quantified using the parent-reported EAS with subscales describing four dimensions of temperament: Sociability, Activity, Shyness and Emotionality [196]

Child-completed questionnaires

The Social Anxiety Scale for Children-Revised (SASC-R), a self-reported scale, was used to assess children's self-perception of social anxiety and avoidance [197]. The scale comprises three subscales: Fear of Negative Evaluation (SASC-FNE) - fear of negative evaluation from

peers; Social Avoidance and Distress in new social situations or with unfamiliar peers (SAD-New); and Social Avoidance and Distress in general situations (SAD-General). The Scholastic Competence subscale from the Self-Perception Profile for Children was used to estimated their scholastic ability [198]. The questionnaire includes school-related items reflecting the children's perception of their competence at school.

Statistics

Multiple linear regression analyses were used to analyze diagnostic status (CAH, CAH-DEX, control), sex, age, parental education and city of origin on behavioral outcomes (dependent variables). The alpha level was set at $p<0.05$ (two-tailed). Interactions ($p<0.10$) between sex and group (CAH, control) were followed up by separate post hoc analyses between patients and controls of the same sex. Effect sizes were calculated using Cohen's d based on group differences adjusted for parental education and country of origin in the linear model. In the model a positive effect size represents higher scores in the CAH cohort and negative values represent higher scores in the controls. Effect sizes were categorized as large ($d\geq0.80$), moderate ($d\geq0.50$) and small ($d\geq 0.20$). We did not control for multiple comparisons to avoid missing small, though potentially clinically relevant, effects. Two multiple regression analyses were performed to determine the effect of genotype (null vs. non-null) and phenotype (SW vs. SV) on social problems within the CAH group. Sex, age, and GC dose at testing were included in both regression analyses to assess which factors contributed to an increased risk of social problems. Finally, a multiple linear regression analysis was performed to compare behavioral outcomes in children/adolescents with CAH who were prenatally treated with DEX with those with CAH not exposed to DEX in utero. SPSS version 24 (IBM, Armonk, NY, USA) was used for statistical analysis.

4.2.3 Study III

"Changes in resting-state functional connectivity in patients with congenital adrenal hyperplasia"

Study population

Forty-two patients with CAH, not prenatally treated with DEX, underwent brain functional magnetic resonance imaging (fMRI). After participation, patients with a reported history of neuropsychological problems and/or current medication for a psychiatric disorder or central stimulant treatment were not included in the analyses (one CAH patient, five controls). Moreover, eight participants (three CAH patients, five controls) were excluded from the analyses because of excessive motion artifacts during the scanning sessions. One participant was excluded because of enlarged ventricles and another because of signal loss in the frontal cortex related to metallic braces. Thus, the final sample consisted of 31 patients with CAH (18 females) and 38 controls (24 females). Two patients in the CAH group had NC CAH, 13 had simple-virilizing (SV) CAH and 16 had SW CAH. In the CAH group there were two

pairs of siblings. In the control group two participants were siblings to two individuals in the CAH group. The age range for all participants was from 16-33 years.

Demographic and clinical characteristics, assessment of executive functions

Participants also completed a battery of neuropsychological tests described elsewhere [174]. The present study focused on executive functions, in particular on tests of visuo-spatial working memory (Span Board Forward and Backward) from the Wechsler Memory Scale [188]; verbal working memory (Digit Span) from WAIS-IV [247] and inhibition/selective attention as assessed by the Stroop task [189].

Non-imaging statistics

All the variables were tested for normality and homogeneity before each analysis. Analyses were performed using SPSS version 23 (IBM, Armonk, NY, USA). For the demographic and clinical data, one-way ANOVAs were conducted to compare CAH and controls on age, height, weight, BMI, education and self-reported mental wellbeing (using a 10-point visual analogue scale) at the time of scanning. A p-value threshold of 0.05 was used for significance determination. A non-parametric test (Mann-Whitney) was performed to compare CAH and controls for drug and alcohol use and smoking. ANOVAs were conducted to analyze between group differences in performance on tests assessing cognitive functions. Further, based on the observed group differences, the association between phenotype and visuo-spatial working memory performance (Span Board Forward) was tested in the whole cohort and the CAH group separately, testing the linear relationships.

Magnetic Resonance Imaging data acquisition

All data were acquired with a 3T MR scanner (Discovery MR750, General Electric, Milwaukee, WI, USA) using an 8-channel head coil. This study is based on the resting-state fMRI, included in a set of structural and functional acquisitions, following a specific protocol described previously [124]. Images were acquired with a planar echo imaging sequence (TR 2000 ms; TA echo time 30 ms; voxel size 3.0 x 3.0 x 3.0 mm3; 41 slices; thickness; 3.0 mm; flip angle; 70°). The acquisition time for the resting-state fMRI was 8 minutes (min) (70 min for the entire protocol). Participants were instructed to keep their eyes closed for the whole sequence during the resting-state scan.

Resting-state fMRI data analysis

Data pre-processing

Resting-state fMRI was pre-processed using FMRIB's Software Libraries version 5.0.11 (FMRIB Laboratory, University of Oxford, England, UK) [199]. Briefly, the pre-processing included co-registration of each participant's structural image with the functional image and head motion correction using MCFLIRT [200], slice timing correction and brain extraction using a brain extraction tool (BET) [201], and finally, spatial smoothing with a Gaussian kernel of 5 mm full width. Each participant's functional images were registered to the participant's structural images using the FMRIB's Linear Image Registration Tool (FLIRT) [200, 202] and to the standard space (MNI152) images using non-linear registration with a warp resolution of 10 mm. After data pre-processing, the "aggressive" option of the independent component analysis (ICA)-based automatic removal of motion artifacts (ICA-AROMA) was used to identify and remove motion artifacts from the time series [203, 204]. ICA-AROMA is a robust strategy not requiring a study-specific training dataset. Motion-related components are automatically detected and removed from the initial data set through an ordinary least squares regression [203].

Independent component analysis

The cleaned individual resting-state data was then fed into the spatial ICA to extract resting-state networks. ICA was performed using MELODIC v 3.15 software (FSL, Oxford, UK) [205].

First, ICA was performed separately for each of the two group conditions (patients with CAH and controls) to map the resting-state networks (RSNs). The number of independent components was set to 60. RSNs were then classified based on their spatial similarity to the functional networks described in healthy people [206] by visually inspecting the aggregate spatial maps, time courses and the power spectrum. Next, the entire group of the pre-processed data, consisting of 69 participants, was concatenated and entered into an ICA group to identify common functional connectivity patterns for the whole cohort.

Dual regression analysis

Dual regression was used to regress the obtained group ICA components back into the individual participant's space for all 69 participants [205]. Next, group comparisons were performed using the FSL randomize tool [207] with 5000 permutations to identify differences in resting-state connectivity between CAH and controls after controlling for age and sex. Significant clusters were identified with threshold-free cluster enhancement (TFCE) with a significance threshold of $p<0.05$ [207].

Association between functional connectivity and executive functions

The association between functional connectivity and executive functions (verbal- and visuospatial WM, inhibition) in patients with CAH in the whole brain was also tested. To

investigate the association, FSL's randomized tool was used [207] in patients with CAH while controlling for age and sex. The inputs for the within-network analyses were the participant-specific time series from the dual regression analysis.

Association between functional connectivity in the precuneus and *CYP21A2* phenotype and medication dose

For the region in which a significant group difference in functional connectivity was found, we performed additional exploratory analyses to assess the relationship between functional connectivity of that region and medication dose (hydrocortisone equivalence in mg/m2 body surface at the time of scanning) in the CAH patient group and with disease severity as to phenotype in the entire cohort. Healthy controls were categorized as healthy phenotypes. First, using the cluster tool to define our cluster index and fslmaths to extract our mask [199] (FMRIB's Software Libraries version 5.0.11), a mask was created for our regions of interest (ROIs) based on the significant outcome from the randomized analyses. Next, the mean time series for each participant was extracted from this ROI using fslmeants [199] so that an estimate of the mean functional connectivity of the precuneus was obtained per participant. Linear regression was then performed to test the association between functional connectivity and medication dose in the CAH patient group and functional connectivity and phenotype in the entire cohort. The phenotype was grouped based on severity: SW, SV and NC CAH. Patients with NC CAH were excluded from the association analysis because of the small sample size (n=2). The control group was also included as a "healthy" phenotype.

Association between functional connectivity of the precuneus and visuospatial working memory

We also tested the relationship between functional connectivity in our ROI and performance on a test assessing visual-spatial working memory in patients and controls separately, testing the linear relationships.

4.2.4 Study IV

"First-trimester DEX treatment is not associated with alteration in resting-state connectivity at adolescent or adult age"

Study population

This study included 18 (8 female) DEX-treated participants (mean age = 20.4 years; age range, 16.3 to 26.4 years) and 30 (24 female) controls (mean age = 20.3 years; age range,

16.7 to 26.4 years). Exclusion criteria and outcomes measures are described in previous studies [57, 63, 174]

Assessment of cognition and behavior: outcome measures

A well-being self-report estimation based on a 10-point visual analogue scale was obtained for each participant after the scanning. In addition, a screening questionnaire regarding lifestyle and health-related problems was filled by the participants. All participants were healthy based on a self-reported lifestyle and health-related problems questionnaire.

Neuropsychological tests included verbal and nonverbal intelligence (Wechsler Adult Intelligence Scale (WAIS)-IV Vocabulary and WAIS-IV Matrices [247], executive functions and working memory performance (WAIS-IV Digit Span [247] and Span Board Test [248]), learning and memory (Wechsler Memory Scale [WMS]-III List Learning Test [248]), processing speed and interference (WMS-III Coding [248] and the Stroop Task [189]). Self-rated questionnaires were used for assessment of executive functioning (Barkley Deficit in Executive Functioning Scale–Short Form [249]) and depressive and anxiety symptoms (Hospital Anxiety and Depression Scale [250-251] and Liebowitz Social Anxiety Scale–Self-Report [252]).

Magnetic Resonance Imaging data acquisition

The study procedure has been described previously [57, 174, 208]. Briefly, the participants completed neuropsychological tests and, on a separate day (mean difference 263 days, range 0-800 days), a 70-min magnetic resonance imaging (MRI) brain scan on a 3T MR scanner (Discovery MR750, General Electric) with an 8-channel head coil. Resting-state fMRI was used in the present study. The acquisition time for the resting-state fMRI was 8 min. During the resting-state functional MRI, participants were instructed to keep their eyes closed for the whole sequence.

Resting-state fMRI data analysis

Data pre-processing

Resting-state fMRI data were pre-processed using FMRIB's Software Libraries version 5.0.11 (FMRIB Laboratory, University of Oxford, England, UK) [199]. Briefly, pre-processing included co-registration of each participant's structural image with the functional image and head motion correction using MCFLIRT [200], slice timing correction and brain extraction using a BET [201], and finally, spatial smoothing with a Gaussian kernel of 5 mm full width.

Each participant's functional images were registered to the participant's structural images using the FMRIBs Linear Image Registration Tool (FLIRT) [200, 202] and to the standard space (MNI152) images using non-linear registration with a warp resolution of 10 mm.

After data pre-processing, the "aggressive" option of the ICA-based AROMA was used to identify and remove motion artifacts from the time series [203, 204].

Independent component analysis

The cleaned individual resting-state data was fed into the spatial ICA to extract resting-state networks. ICA was performed using MELODIC v 3.15 software (FSL, Oxford, UK) [205]. First, ICA was performed separately for each of the two groups (participants prenatally treated and controls) to map the RSNs. The number of independent components was set to 60. RSNs were then classified based on their spatial similarity to the functional networks described in healthy people [206] by visually inspecting the aggregate spatial maps, time courses and the power spectrum. Next, the entire group of the pre-processed data, consisting of 56 participants, was concatenated and entered into an ICA group to identify common functional connectivity patterns for the whole cohort.

Dual regression analysis

Dual regression was used to regress the obtained group ICA components back into the individual participant's space for all 56 participants [205]. Next, group comparisons were performed using the FSL randomize tool [207] with 5000 permutations to identify differences in resting-state connectivity between DEX-treated participants and controls after controlling for age and sex. Significant clusters were identified with TFCE with a significance threshold of $p<0.05$ [207].

Seed-based connectivity analyses

For seed-based connectivity analysis, we chose brain regions based on our structural brain study findings [62] and previous evidence on the vulnerability to high GC dosages. Specifically, three regions have been identified using the Harvard-Oxford cortical Atlas (FSL)(https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/ Atlases): the amygdala, hippocampus and superior frontal gyrus. Right and left masks were then created for each region. Finally, the time series of these three regions were modeled with GLM analysis using the FMRI Expert Analysis Tool [209, 210] to estimate the ROI connectivity maps for each study participant. For the group-level comparisons, age and sex were used as covariates. Group-level maps were clustered using standard values of $Z=2.3$ and FWE $p<0.05$.

5 RESULTS

5.1 STUDY I

CAH vs. Control

We did not identify deficit in general intellectual ability, executive function or learning and memory between patients with CAH and controls. No significant interactions between sex and diagnostic status were identified. In addition, were not observed any significant correlations between current HC dose and cognitive function. We did not find significant differences between patients with SW vs. SV phenotype or null vs. non-null genotype in any measure of cognitive ability. However, significantly lower test scores (though in the normal range) were observed in patients with SW CAH compared with SV CAH in the Span Board forward subtest (p=0.039). In separate follow-up analyses results were non-significant (p=0.650).

CAH vs. CAHDEX

Girls with CAH, prenatally treated with DEX scored lower on the Vocabulary subtest of the WISC-III (p=0.037), although in the normal range. No differences were identified between boys prenatally treated with DEX during the first trimester of fetal life, and CAH.

5.2 STUDY II

CAH vs. Control

No differences between CAH and control participants were identified in the three parent-completed questionnaires or the child-completed questionnaires. However, in the CBCL parents reported more social problems in CAH children (p=0.042). A significant interaction between CAH and sex was observed in social problems (p=0.032). Post hoc analyses revealed that parents scored their daughters with CAH as having significantly more problems than control girls (p=0.043). In contrast, boys with CAH did not differ from control boys (p=0.067).

CAH vs. CAHDEX

In the CBCL the parents of children with CAH prenatally treated with DEX scored their children as having more withdrawn/depressed problems (p=0.014), and a significant interaction between DEX and sex (p=0.014) was observed. However, follow up analyses did not reach statistical significance in girls (p=0.266) or boys (p=0.210). A significant interaction was observed between DEX and sex (p=0.015) in the Social problems subscale of the CBCL. In particular, more social problems in CAH DEX-treated boys were detected in follow up analyses compared with CAH not prenatally treated with DEX (p=0.003).

5.3 STUDY III

Demographic and clinical characteristics, assessment of executive functions and association between disease severity (phenotype) and visuo-spatial working memory

Patients with CAH were older (approximately 3 years) (p=0.001) and shorter (p= 0.002) than controls. Moreover, they had a slightly higher body mass index (BMI) (p = 0.044) than the control group.

For the neuropsychological assessment, patients with CAH performed significantly worse than controls on the Span-board forward test (assessing visuo-spatial WM) (p=0.001). Males with CAH performed worse than control males on the Stroop Interference Test (p=0.044). We did not identify any other differences between groups.

A linear association was confirmed in the whole cohort between phenotype and visuo-spatial working memory performance (Span board, forward (B= -1.152; p=0.009)). No differences between SW and SV CAH groups were showed from post-hoc analyses. Both phenotype groups (SV CAH (B= -1.94 p>0.001) and SW CAH (B=-2.27, p>0.001)) performed worse on the visuo-spatial working memory test compared to controls.

Group-independent component analysis

Twenty-three of 60 components for the patients with CAH and 29 of 60 for the controls were considered to reflect the RSNs previously described in the literature for healthy individuals [206, 211]. All other plausible components were noise-related artifacts, such as false activations in ventricles, cerebral spinal fluid, head motion, blood vessels or components that did not match any networks. We identified 12 networks based on their spatial configuration [206, 211]. The obtained RSNs included the visual (pole and medial), motor leg-hand and motor-face regions, cerebellum, auditory/language, dorsal attention, salience, default mode (medial PFC and PCC), frontoparietal (executive) and basal-ganglia networks.

Dual regression analysis

The CAH group showed a pattern of increased connectivity in the left precuneus cortex compared to the control group (voxels=53; x= -50; y= -14; z=8; p=0.008) (Figure 3).

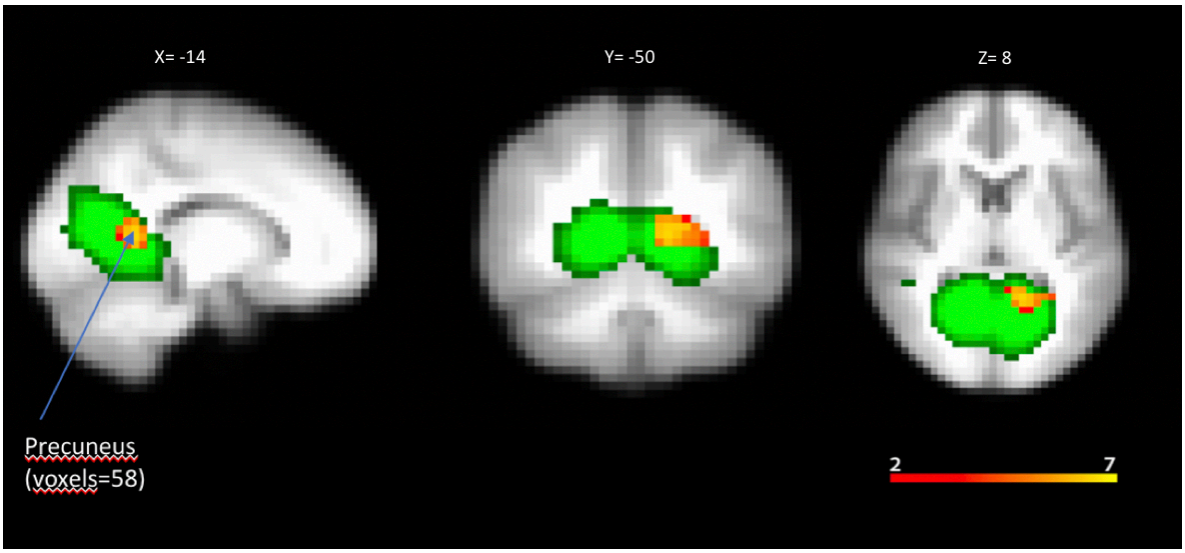

Figure 3. *Within network connectivity: independent component analysis. Functional network with increased functional connectivity in patients with CAH.*

The DMN extract using group ICA is overlaid on the average MNI-152 brain template in green color. Within DMN, significant group differences were observed for a cluster encompassing the precuneus region (peak MNI coordinates: X=-14, Y=-50, Z=8; P= 0.008).

Association between resting-state functional connectivity and executive functions

No association was identified between whole-brain functional connectivity and executive functions (verbal and visuospatial WM, inhibition) in patients with CAH.

Association between resting-state functional connectivity of the precuneus and *CYP21A2* phenotype and medication dose

A linear association between phenotype and functional connectivity (B= 5.65, p=0.009) was observed. In post hoc analyses no differences were found between SW and SV. However, only SV had significantly stronger connectivity compared to controls (B= 17.65 p>0.001), whereas SW did not (B= 4.91, p= 0.10) (Fig.4)

There was no correlation between total hydrocortisone replacement and functional connectivity in the precuneus.

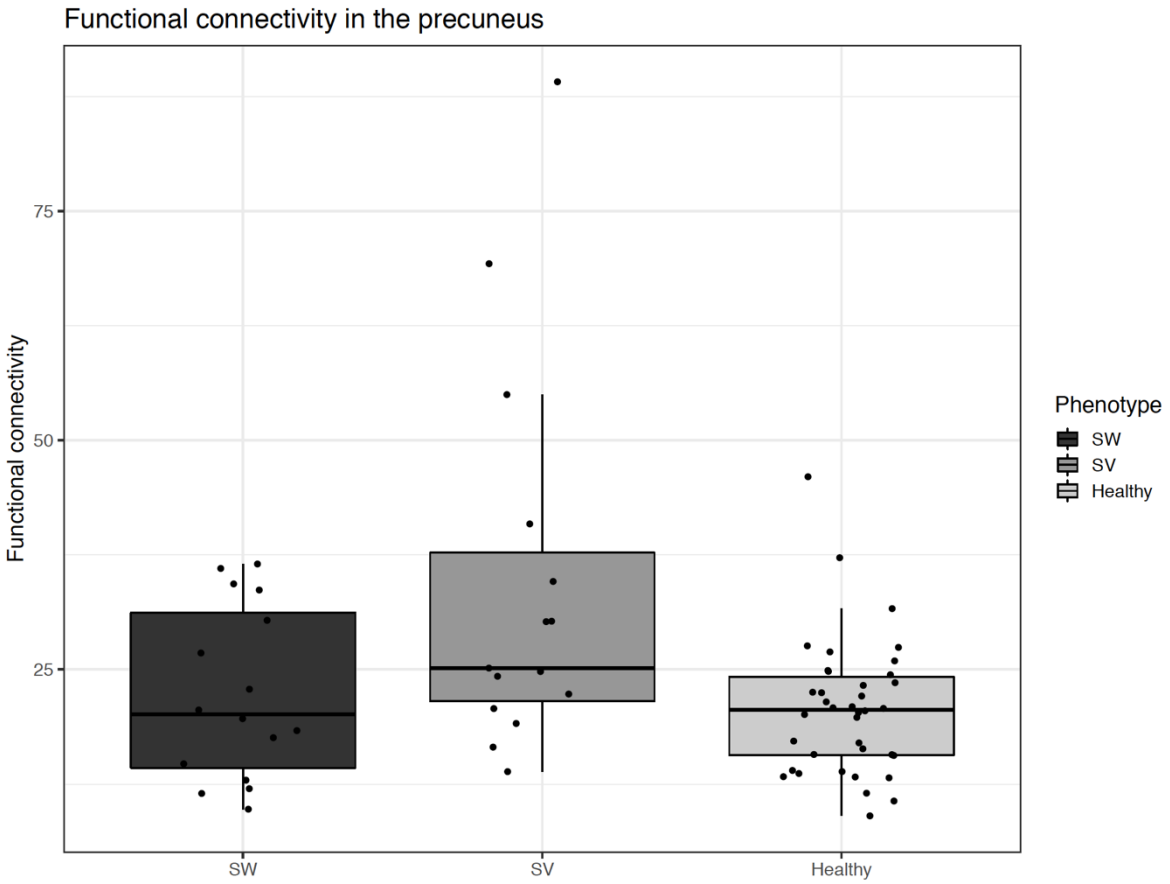

Figure 4. *Association between phenotype and functional connectivity of the precuneus*

SW(salt-wasting) n= 16
SV (simple virilizing) n= 13
Healthy (controls) n= 38

Figure 4 illustrates the association between functional connectivity and phenotype in the ROI (precuneus) for the whole cohort (CAH and control group). A significant linear association was observed between phenotype and functional connectivity. Only SV had higher functional connectivity compared to controls.

Association between functional connectivity of the precuneus and visuospatial working memory

No significant linear relationship was identified between functional connectivity in the precuneus and performance on the forward visuo-spatial working memory task in the CAH or whole cohort.

5.4 STUDY IV

Demographic characteristics and assessment of cognition and behavior

No group differences in age, height, weight, BMI and overall well-being were identified between DEX-treated participants and control participants at the time of scanning. For the neuropsychological assessment, we did not identify any differences between groups, neither in cognition nor in behavior.

Group-independent component analysis

Twenty-seven of 60 components for the participants prenatally treated with DEX and 32 of 60 for the controls reflected the RSNs previously described in the literature for healthy individuals [206, 211]. Other components were considered noise-related artifacts components that did not correspond any networks. Twelve networks were identified based on their spatial configuration [206, 211], including visual (pole and medial), motor leg-hand and motor-face regions, cerebellum, auditory/language, dorsal attention, salience, default mode (medial prefrontal cortex (PFC) and PCC), frontoparietal (executive) and basal-ganglia networks (Figure 1)

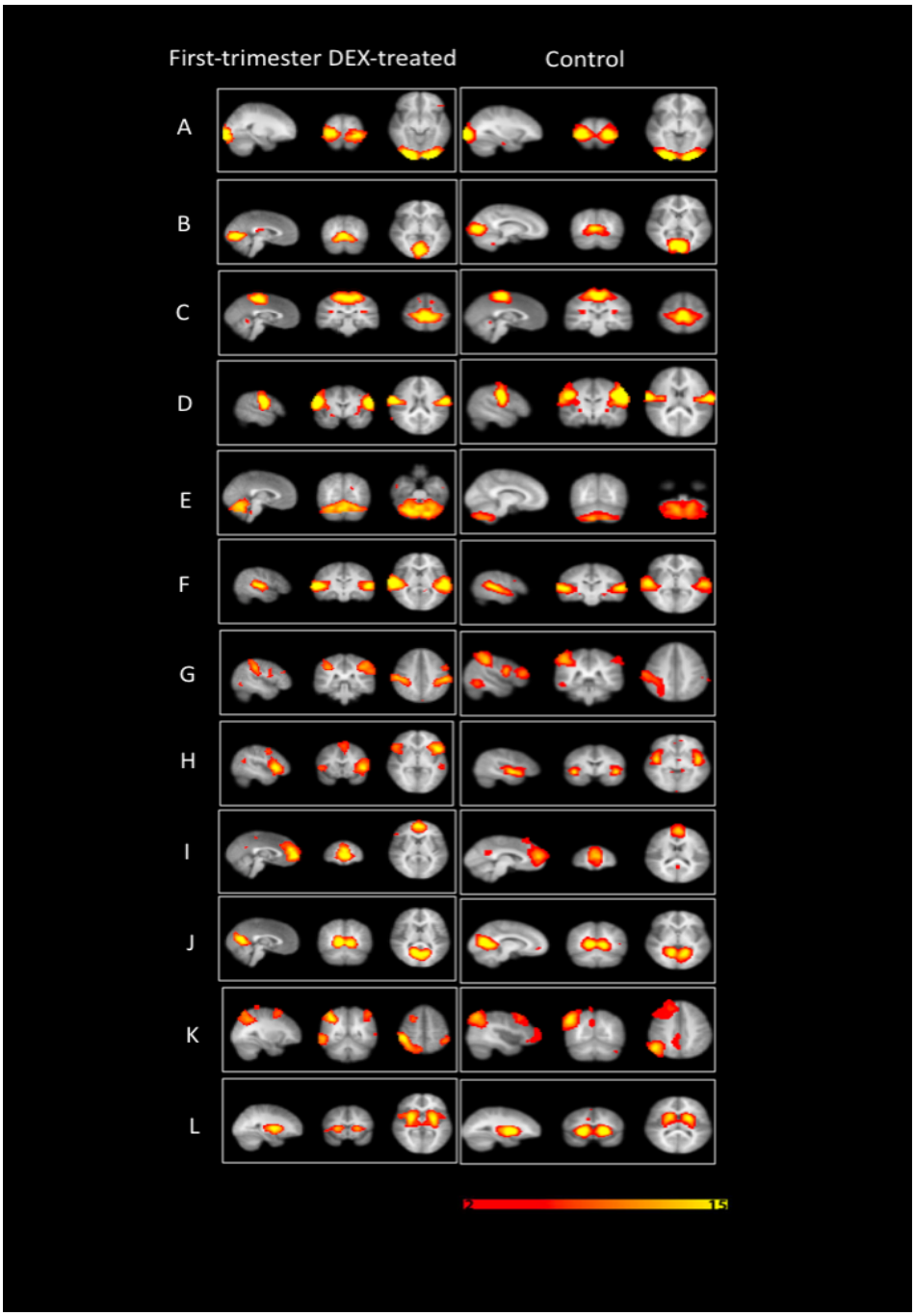

Figure 1. *Resting-state functional networks. (A) Visual (pole), (B) visual (medial), (C) motor leg-hand, (D) motor face regions (E) cerebellum, (F) auditory/language, (G) dorsal attention, (H) Salience network, (I) Default Mode Network (mPFC), (J) Default Mode Network (PCC), (K) frontoparietal (executive network), (L) (basal-ganglia).*

Dual regression analysis

No differences in resting-state functional connectivity were observed in any of the networks between the DEX-treated participants and the controls.

Seed-based connectivity analyses

No differences were identified in resting-state functional connectivity in any of the chosen

seed regions (amygdala, superior frontal gyrus, hippocampus) in DEX-treated participants vs. controls.

6 DISCUSSION

6.1 COGNITION AND BEHAVIOR

Effects of postnatal treatment with GCs

Studies on cognitive function in persons with CAH have shown inconsistent results, which may reflect differences in age, sex, cohort size, number of salt-losing crises, type of treatment or differences in methodology. Moreover, most findings refer to studies in adults.

In Study I we observed that our cohort of children and adolescents performed equally well to controls in tests assessing cognitive functions. Previous studies in children with CAH have shown impairment in working memory [172], leading to negative performance in spatial and quantitative tasks [173]. The authors suggest that sub-optimal treatment with GCs likely explains cognitive impairments. Hence, early adrenal crises and hormone imbalance cannot be dismissed [172, 173]. Some of the studies reporting adverse outcomes in children and adults with CAH are from countries that, at that time, did not have neonatal screening [168, 169, 172, 173]. Thus, it is conceivable that the study participants might have experienced early episodes of hyponatremia or hypoglycemia [168, 169]. All the participants in our study have been detected through neonatal screening, suggesting the efficacy and importance of the screening, not only in saving lives [26] but also in preventing early crises that may impact cognitive functioning later in life [25].

Notably, when we compared patients with different phenotypes (SW vs. SV), despite both performing in the average range for the population, children and adolescents with SW CAH scored poorer in the test measuring visuospatial working memory (Span Board forward). However, we could not investigate a possible interaction with sex due to the small sample size of females with the SV form. In this case results from previous studies are also inconsistent. However, patients with severe phenotypes are more vulnerable to salt-losing crises [168] and may be treated with a higher GC dose, which may have deleterious consequences in brain areas involved in memory functions. It is known that GCs, acting through GR and MR located in brain areas that play an important role in higher cognitive functions and emotion regulation, are essential for optimal cognitive performance [212-214]. However, while an adequate cortisol concentration is beneficial for cognitive functioning, elevated concentrations may have harmful effects [215, 216]. Negative effects have been reported in healthy individuals treated with GCs [212, 217] and in patients exposed to high cortisol levels, such as Cushing´s syndrome or depressive and post-traumatic stress disorders [147, 148, 218]. In our study children with SW CAH performed poorer on the subtest assessing visuospatial-working memory (Span Board forward). Working-memory tasks such as Span Board or Digit Span rely on the interaction of different brain networks [219], including the prefrontal cortex [220, 221]. Hence, prolonged exposure to high cortisol levels may affect brain regions involved in short-term and/or working memory [217, 222-225].

We could not identify any association between GC dose at testing and cognitive performance. However, GC dose at testing may not be the best predictor, given that the GC dosage changes over time, especially during puberty. Thus, future studies using the cumulative dose are warranted. In a recent study from our group investigating brain structure in adolescents and adults with CAH we reported alterations in the prefrontal, parietal and superior occipital cortex, regions that are part of the working memory network [208]. Impairment in working memory performance, specifically verbal and visual working memory, processing speed and inhibition reported in our study on adult CAH may stem from alterations in brain structure [174], suggesting that the cumulative effects of GC may contribute to reduced cognitive performance.

Other factors may also have a negative impact on executive function. For instance, parents reported that children have more fatigue, negatively affecting their QoL [226]. Fatigue has also been reported in a Brazilian self-assessment study by children and adolescents with CAH, treated with prednisolone [227]. In boys with CAH an increased prevalence of attention-deficit/hyperactivity disorder and anxiety disorders have also been described [228]. We did not assess fatigue in our cohort, but future studies in CAH may consider investigating the influence of this parameter on cognitive function.

All CAH participants in Study I were treated with HC. No studies have studied the long-term effect of different treatment strategies. Thus, we cannot conclude that the choice of HC might have contributed to the more positive cognitive outcomes. Therefore, future longitudinal studies are also needed to investigate the effects of different treatments on patients with CAH. However, our results suggest that good health care management during childhood and adolescence, adherence to therapy and early diagnosis through a screening program are essential for optimal cognitive functioning. Indeed, recently, a study in adolescents and adults (aged 15-72 years) recruited from the Karolinska University Hospital, investigating the adherence to therapy and their QoL reported high adherence to therapy, regardless of the treatment (HC or prednisolone). A better adherence to therapy was associated with higher QoL in younger CAH patients [229].

Because of our positive findings in cognition, when we assessed behavioral outcomes in our cohort, we expected a reasonable adjustment in behavioral outcomes. The initial studies on behaviors in children and adolescents with CAH have evaluated the impact of high androgen levels on behaviors in girls and women with CAH, underlining the organizational effects of prenatal exposure on human brain development in modeling gender-related behavior [181].

Studies in girls with CAH have reported their tendency to masculinization. Girls with CAH seem to prefer male-typical activities [230, 231] and, as reported by their mothers, may exhibit a more aggressive temperament (externalizing problems) [232]. However, in general most studies have shown that in females with CAH, the most critical factor affecting psychosocial outcomes and, in turn also, QoL is related to the type of GC treatment used [233], surgical outcomes [42, 234] and experience of stigma and anticipation in the context of their sexual life [46].

Results from Study II on behavioral outcomes showed a good overall adjustment in our cohort of children and adolescents with CAH. A plausible explanation for this is strict adherence to treatment, likely due to proper parental and health care management and good psychological support. However, from the parent-completed CBCL questionnaires, we observed more internalizing problems (social problems) in children and adolescents with CAH compared to population controls.

Studies investigating social problems in patients with CAH are rare, but the literature suggests that social issues are common in children with chronic illness [235].
CAH is a chronic condition that impairs QoL [233], and it is quite demanding for patients and families because of its complexity. Positive parenting is crucial for the psychological development of the child. Parenting a child with chronic illness may cause high stress due to the many responsibilities and demanding tasks, promoting parental overprotection [236]. Furthermore, other factors (such as body image and self-stigma) may increase internalizing problems.

Effects of prenatal treatment with DEX

In Study I we also reported findings related to the cognitive performance in a small subgroup of children and adolescents that received DEX treatment during the gestational period (boys only the first trimester and girls until term) compared to a group of patients with CAH not prenatally treated with DEX. Although the results may suggest that girls treated with DEX performed poorly on a subtest assessing verbal intelligence, despite being in the normal range for the population, we cannot draw a definitive conclusion due to the lack of statistical power to detect differences between the two cohorts. Thus, more longitudinal studies in larger cohorts are needed to confirm or contradict these results. However, our findings align with a previous study indicating slower mental processing in a cohort of CAH-DEX girls compared to controls [119]. Our results still raise concerns about the safety of DEX treatment, suggesting the need for further studies on this topic.

Study II investigated behavioral outcomes by comparing children and adolescents with CAH who were prenatally treated with DEX with children and adolescents with CAH not prenatally treated with DEX. Parents of DEX-treated children and adolescents reported more withdrawn-depressed problems as well as more social problems in DEX-treated boys. In our previous studies on the topic parents did not report more behavioral problems compared to the population controls, but DEX-treated children did report more social anxiety [60, 237].

However, we cannot draw any conclusions because of the small sample size and lack of statistical power. Future longitudinal studies in larger cohorts are required to better evaluate the consequences of prenatal treatment for CAH.

6.2 RESTING-STATE FUNCTIONAL CONNECTIVITY IN CAH

Study III is the first to investigate the functional brain connectivity in patients with CAH at rest. When we performed the whole-brain analyses, we observed increased functional connectivity in patients with CAH in the left precuneus cortex.
The precuneus, characterized by high energetic demand, is part of the default mode network and represents an important hub in the working-memory network. [238]. During a working memory task, the precuneus is involved in shifting attention when needed; conversely, during rest, it is engaged in self-referential thinking or consolidation of episodic memory (memories of everyday events) [239]. Thus, the precuneus is involved in two of the three networks of the triple-network model, consisting of the default mode network, central executive network and salience network. [131]. Altered functional connectivity in one network may cause disengagement with the other networks, causing cognitive or affective problems.
Patients with CAH performed worse on a test assessing visuospatial working memory (Span Board forward), and males with CAH performed worse on the Stroop Interference test. Although our participants were detected through neonatal screening, we cannot exclude that salt-losing or hypoglycemic crises later in life that may have affected cognition. However, when we looked at the association between genotype and whole-brain functional connectivity, we did not identify any correlation between the functional connectivity and executive function outcomes in patients with CAH. Studies in larger groups are needed to investigate these relationships further.

One explanation of the altered functional connectivity might be a compensatory mechanism that helps the person to maintain good performance. We recently found increased activity in a more dorsal part of the precuneus in males with CAH during working memory performance (Van't Westeinde, submitted). We also performed additional exploratory analyses to assess the relationship between functional connectivity in the precuneus, where we found a significant group difference in functional connectivity and medication dose (hydrocortisone equivalence in mg/m2 body surface at the time of scanning) in the CAH patient group as well as an association with disease severity as to phenotype in the entire cohort. The total hydrocortisone replacement dose at testing did not correlate with functional connectivity in the precuneus, suggesting that an optimal treatment strategy may optimize brain functioning. We found, however, that patients with the SV phenotype showed higher functional connectivity in the precuneus, but due to the small cohort, we cannot perform additional statistical analyses to assess the impact of genotype on working memory performance.

We also suggested that, due to the known relationship between structural and functional connectivity [240], our finding on higher functional connectivity may be connected to the underlying changes in brain structure that we observed in our recent study in the precuneus, parietal and superior occipital cortex [208]. Hence, the higher activity at rest in the precuneus in patients with CAH might be interpreted as a compensation mechanism for reduced volume. The precuneus is characterized by a high metabolic rate and might be more vulnerable to hormonal imbalances [239]. Studies in patients with Cushing's disease in

remission, a condition characterized by cortisol excess, have shown elevated functional connectivity within the DMN [125, 241]. Similar results have also been detected in resting-state studies of patients with stress-related disorders [242]. Both disorders are characterized by dysregulated HPA axis functions, which might lead to hypercortisolism. Cortisol imbalances through altered glucose metabolism may affect functional neuronal activity. However, our study has some limitations that need to be addressed. Our CAH group was almost 3 years older than the control group, with a risk that we may underestimate the CAH findings. Moreover, we could not perform subanalyses to assess the impact of sex and genotype in functional connectivity. Larger studies are needed to investigate the impact of those important factors, as well as the influence of accumulated GC dose over time.

6.3 RESTING-STATE FUNCTIONAL CONNECTIVITY IN PERSONS TREATED WITH DEX DURING THE FIRST TRIMESTER OF FETAL LIFE

In this last study we presented novel findings on resting-state functional connectivity in adolescent and young adult participants who were at risk of having CAH and therefore had been treated with the synthetic GC DEX during the first trimester of prenatal life. We looked at whole-brain connectivity and chose three brain regions (amygdala, hippocampus, superior frontal gyrus) based on our previous findings and literature evidence. We did not observe any differences in resting-state functional connectivity in DEX-treated patients in the whole brain or between three a priori specified ROIs and the rest of the brain. In addition, there were no differences in cognitive performance or symptoms of depression or anxiety compared to population controls.

Studies evaluating the long-term effect of treatment during the first trimester on brain, cognition and behavior are scarce. Our previous studies investigated the effects of prenatal treatment during the first trimester in children, adolescents and adults.
Children and adolescents (7-17 years) performed worse in verbal working memory tasks, with a sex effect seen in girls [56]. However, we noted a catch-up in working memory performance at adult age, with CAH DEX individuals performing equally well as controls [174]. Notably, when we evaluated the brain's structure in the adult cohort, our findings suggested a prenatal programming effect of high GC levels during the first trimester on the structure of the brain [62]. We detected an enlarged amygdala, increased volume in the prefrontal gyrus and alteration in the white-matter structure. Thus, despite good cognitive performance, we expected to find compensatory functional connectivity alterations in the DEX-treated group due to alterations in brain structure. However, we did not see these anticipated changes in the whole brain or the regions where we found a structural difference. Moreover, our studies did not identify any group differences in mood symptoms in the adult cohort [57, 62].

We suggested that the structural alterations that we observed may reflect an adaptive mechanism of the brain to normalized brain function and functional connectivity patterns. In fact, we did not identify differences in brain activity in the same cohort during verbal and visuospatial working memory tasks [63].

Structural networks develop earlier than functional networks, and already at birth, they present an adult-like topological organization [96]. In contrast, functional networks exhibit more dramatic changes during development [243], suggesting that GC excess during the first trimester may primarily affect brain structure, whereas GC exposure during the second and third trimester of pregnancy may result in changes in both structural [104, 114] and functional connectivity [244], affecting cognition and behavior [107, 108, 114]. However, we cannot exclude that we could not identify any difference because the methodology is not sensitive enough to detect differences in brain organization and functions. Future studies using different methodologies (e.g., graph analysis) are needed to gain a better assessment of segregation and integration [245, 246]. Individuals prenatally treated with DEX are exposed to high GC dosages that may alter the brain developmental trajectory. Thus, future studies are warranted to assess these aspects of brain organization in DEX-treated individuals.

Although the current results are reassuring, more studied in larger cohort and with different methodologies are needed.

6.4 ETHICAL CONSIDERATIONS

There are many ethical considerations connected with the current research project. **The first ethical dilemma concerns the prenatal treatment itself.** Since the mid-1980s, prenatal treatment with DEX has been administered in Sweden and in many medical centers worldwide to reduce virilization in girls with CAH. DEX is a synthetic hormone that crosses the placenta reaching the fetus, and the treatment must be initiated early in pregnancy to be effective. One can reasonably ask whether *it is ethical that it is ethical that 7 of 8 fetuses are exposed to high doses of GCs during the first trimester without any benefit ?*

According to the Declaration of Helsinki, the physician's duty is to promote and safeguard the health, wellbeing and rights of patients (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/). The treatment is indeed effective in reducing virilization and the need for genital reconstructive surgery in girls with CAH. However, healthy girls, boys and pregnant mothers will be exposed to high doses of GCs without benefit and in the context of inadequate data regarding the long-term risks of this treatment.

Evidence suggests that exposure to GCs already in utero may predispose to disease later in adulthood (Barker hypothesis).

When clinicians started using DEX treatment in Sweden in 1985 and in other countries, there was insufficient knowledge regarding the efficacy and safety of this treatment. After the first results from the retrospective studies in Sweden, the clinicians in charge of this therapy decided to put the treatment on hold, waiting for results from larger cohorts and studies in other countries. Thus, the primary goal of our work is to accumulate more knowledge on plausible side effects of prenatal DEX treatment in the context of CAH to determine whether the treatment must be stopped or if it may be used as part of the clinical care of patients with CAH.

The second ethical dilemma concerns the methods used to collect all the data: psychological assessment and MRI scans.

When we test children and adolescents, we get informed consent from the parents. But *is it ethical to take permission from the parents? Do children and adolescents understand the purpose of the research and the potential benefits? Is it ethical to test children and adolescents for long periods, which may cause stress and fatigue?*

We put all our effort into trying to choose a good test battery with fewer negative consequences on children and adolescents. When we started testing children and adolescents to estimate full-scale IQ, we used the short version of the WISC-III, but the battery required too much time to complete and the children often became fatigued. Therefore, we decided to use only subtests with a stronger correlation with the four index scores.

Moreover, is it ethical to test healthy children and adults to identify any potential adverse effects of the treatment that was not their choice? If any adverse effects are found, how will that affect the relationship with the mother?

Furthermore, we use the MRI scan to investigate the neuro-structural and functional correlates of cognitive deficit. Is it ethical to ask them to lie down for more than one hour inside the scanner, completely immobile? Is that stressful? We gave them detailed information related to the entire procedure, explaining the possibility of discontinuing participation at any time.

There are also ethical considerations that we need to take into account regarding the retrospective study design. For our first study, clinicians started collecting all the information by calling all the mothers that started the treatment in the mid-1980s. The therapy was defined as “safe” and effective at that time. Many contacted families declared that they did not inform their children about the prenatal treatment. This request may be too invasive, especially when it was clear to them that the therapy's safety was ambiguous and required clarification. Several families did not want to take part in the follow-up. However, it is also an ethical dilemma to leave the families in this greater uncertainty if the studies and follow-ups are not performed. The follow-up studies aim to identify even slight differences such as minor neurocognitive deficits in their children already at an early age to introduce early help and improve academic performance.

A written informed consent was given by the participants before inclusion and the study was approved by the Regional Ethics Committee in Stockholm (Dnr 99-153 and 2011/1764-32)

7 CONCLUSIONS AND FUTURE PERSPECTIVES

Our studies on cognitive functions and behavioral outcomes suggest good overall cognitive and behavioral adjustment in children and adolescents with CAH who did not receive prenatal treatment with DEX. The CAH cohort performed equally well as the population controls matched for sex and age for specific cognitive functions (e.g., general intellectual ability, executive functions, long-term memory). However, when we compared CAH subjects based on disease severity (SW vs. SV), we observed a poorer performance in children/adolescents with SW CAH in spatial working memory (Span Board forward), despite being in the normal range for the population. When we assessed the behavioral and temperamental outcomes using parent- and child-completed questionnaires, we did not find differences between the CAH and control group, except in the subscale of the CBCL assessing social problems. Compared with the control group, parents reported more social problems in children/adolescents with CAH.

Optimal treatment with hydrocortisone, good adherence to therapy during childhood and parent education on how to intervene in case of salt crises during stress periods might be essential in promoting good overall cognitive and behavioral outcomes. However, adrenal crises during adult age, likely due to non-compliance with therapy and lack of education on how to act in case of adrenal crises, may contribute to adverse outcomes.

When we looked at the whole-brain functional connectivity during rest in a cohort of adolescents and adults with CAH, we observed an alteration in functional connectivity in the precuneus, an important hub in the working-memory network. We suggested that this alteration reflects a functional reorganization in response to the CAH disease. In addition, the observed changes in functional connectivity might be dependent on phenotype.

Finally, study IV assessed brain functional connectivity at rest, both in the whole brain and in three ROIs (amygdala, hippocampus, superior frontal gyrus), in patients at risk of CAH treated with DEX during the first trimester of fetal life. We reported promising results as we did not observe any differences in functional connectivity during rest in the whole brain or seed-based connectivity analyses at adolescent or young adult age. However, future studies in larger samples and more sensitive methodologies are needed to confirm (or contradict) these findings and further determine treatment safety.

Further studies are recommended to address the impact of the accumulated GC load, multiple adrenal crises and different treatments on cognition and behavior. Moreover, more longitudinal studies with a larger cohort are necessary to investigate the effect of prenatal treatment in patients treated during the first trimester and the entire gestational period.

In conclusion, it is crucial to evaluate the postnatal GC treatment and use the last evidence to improve clinical management of the disorder, offering prompt interventions to ensure a better QoL and development in children with CAH and those who have been treated prenatally with DEX.

Chronic diseases require daily attention and health management that may limit physical and social activities. Furthermore, learning how to cope with a chronic condition is a stressful experience not only for the child but also for the parents and the whole family.

Thus, emotional and psychological support is vital to promote effective coping and adaptation to the child's chronic illness and achieve a better QoL in persons with CAH.

7.1 CLINICAL IMPLICATIONS

Our findings suggest that optimal disease control plays an essential role in preventing impaired health outcomes. Moreover, evidence from unfavorable outcomes in adults highlights the importance of a better-structured transition from child to adult care.

Monitoring the adherence to therapy, especially in adult age, is important for a better QoL in patients with CAH. A recent study suggested that a multidisciplinary team (physicians, endocrinologists, gynecologists and psychologists) may improve therapy adherence, preventing morbidity and mortality, especially due to adrenal crises [229].

The opportunity to discuss the patients experience related to his/her medical condition, treatment issues, and possible treatment options should be part of the routine care. Moreover, a systematic follow-up, comprising a psychological assessment to detect small behavioral changes on time should be part of the management to contribute to tailor a specific intervention. Recently, a qualitative interview with parents of children with CAH reported the need for parents to know how to help their children become independent [245], suggesting the importance of empowering young people to transmit all their knowledge and skills to become independent and manage their health care. Thus, adolescent transition programs, including training and education related to different aspects of their condition and the impact on their everyday life and social relations, need to be implemented to promote better QoL.

The effects of prenatal treatment on CAH results are still conflicting, mainly due to the small sample size. However, it is important to consider the adverse effects on individuals without CAH exposed to the treatment without benefit. Thus, for countries still offering the treatment, it is vital to follow the current practice recommendations, i.e. the treatment must be administrated within a clinical frame of reference. The clinicians have a professional obligation to provide all the information related to potential side effects to the mothers before obtaining informed consent. Recently, some concerns have been raised because of the discrepancy in treatment use in different countries in Europe, underling the importance of standard guidelines [52].

Systematic follow-up for patients already treated should be introduced in the care management to address possible adverse effects in girls with CAH treated until term and in

mothers and fetuses treated during the first trimester. Earlier prenatal diagnosis is needed to avoid treatment of those cases that do not benefit from the prenatal treatment.

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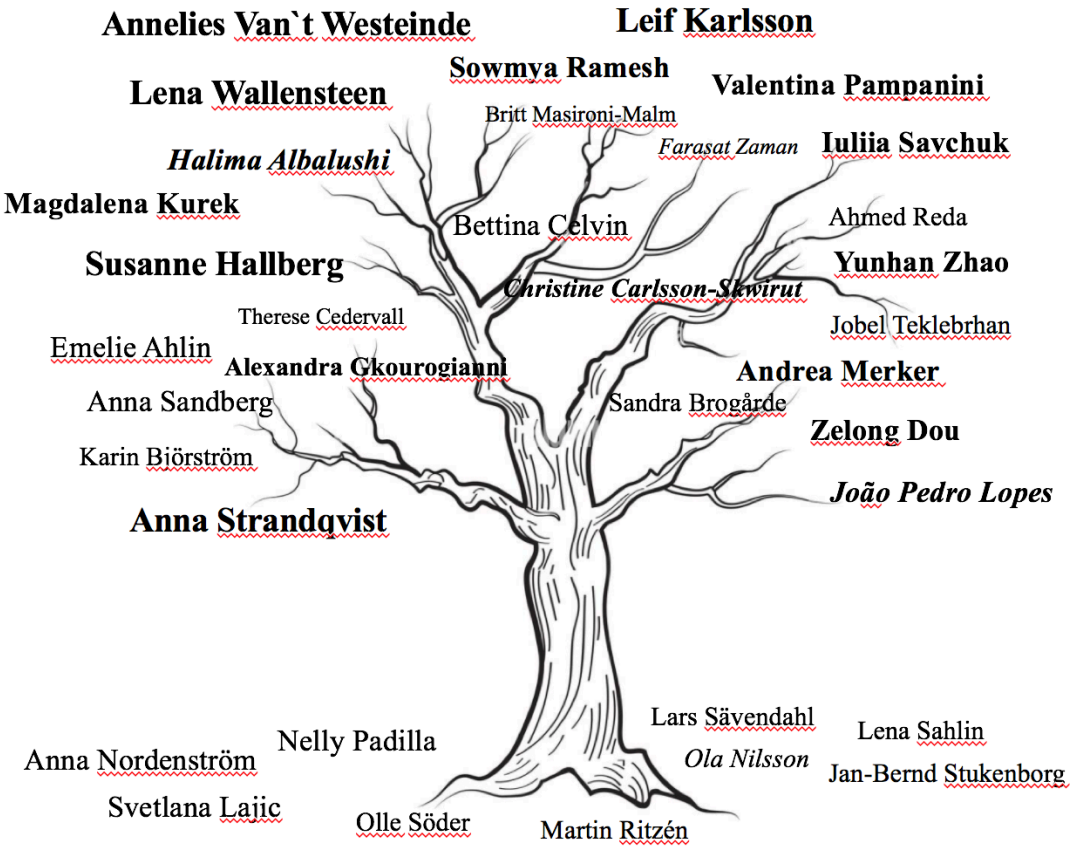

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