

FROM THE DEPARTMENT OF WOMEN'S
AND CHILDREN'S HEALTH
Karolinska Institutet, Stockholm, Sweden

**METABOLIC AND COGNITIVE EFFECTS AFTER EARLY
PRENATAL DEXAMETHASONE TREATMENT**

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Cover: My daughter Julia at GW 13

Metabolic and cognitive effects after early prenatal dexamethasone treatment

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*To my family, for your never ending
support and encouragement.*

POPULAR SCIENCE SUMMARY OF THE THESIS

Exposure to stress hormones (glucocorticoids, GC) during fetal life is known to affect the child in many ways, including negative effects on cognition, brain morphology, behavior and metabolism. Our research confirms these findings. We have shown that first trimester GC treatment in pregnant women at risk of having a child with a disease called congenital adrenal hyperplasia (CAH) adversely affects cognition and metabolism in treated children. Moreover, our results suggest that girls seem more vulnerable than boys to GC exposure, as girls performed worse in verbal and non-verbal IQ tests and verbal working memory, but no effect was found in boys. In addition, girls, but not boys, had affected glucose and insulin metabolism, with a lower HOMA- β index, an estimation of pancreas ability to secrete insulin, an indication of reduced insulin sensitivity. Finally, older teenagers and young adults of both sexes had higher blood lipids than a group of matched (age, sex) controls.

CAH is a disease caused by a defect in the adrenal steroid synthesis, leading to a shortage of cortisol and androgen excess. This disease is potentially life-threatening after birth if not treated with cortisone. However, girls are affected already during fetal life with virilization of the external genitalia because of the high androgen level, sometimes to the extent that sex-assignment is difficult to determine at birth. If the mother is treated with GC during pregnancy, androgen levels will be low and virilization reduced. However, because the genital development occurs in early gestation, GC treatment must begin as soon as possible, preferably in gestation week 6-8 to be effective. At that time in pregnancy, it is not yet possible to determine whether the fetus has the disease or not. Because CAH has a recessive inheritance (parents with a mutation have a 25% risk of having a child with the disease) and only the girls with CAH benefit from the treatment, 7/8 children (statistically) will be treated unnecessarily. Thus, adverse side effects without any benefit are not acceptable.

This thesis investigates potential long-term effects in children without CAH who were exposed to GC during the first trimester (as it was not known if they were affected with CAH or not). Forty-two treated children and young adults (age range, 4-26 years) and 75 age- and sex-matched controls from the general population were included in the studies. The results show that GC exposure during fetal life has adverse effects on metabolism and cognition, with girls overall being more vulnerable than boys. Thus, prenatal GC treatment in pregnancies at risk of CAH is questionable given that most treated cases do not benefit from the treatment.

ABSTRACT

Congenital adrenal hyperplasia (CAH), due to 21-hydroxylase deficiency (21OHD), is a disease with an inborn error of the adrenal steroid synthesis. This enzyme deficiency leads to cortisol shortage and androgen excess. If left untreated, CAH is potentially life-threatening especially in the neonatal period, but girls are also affected in the prenatal period with virilization caused by the surplus of adrenal androgen. Prenatal dexamethasone (DEX) treatment will minimize the androgen levels and reduce virilization. However, because the development of genitalia occurs in early gestation, the treatment must start in gestational week (GW) 6-8 to be efficient. Because of the recessive mode of inheritance and because genotyping of the fetus is not possible until GW 12, statistically, 7 of 8 fetuses will be treated unnecessarily during the first trimester of fetal life. This quandary emphasizes the importance of investigating the potential risks of DEX treatment. Glucocorticoid (GC) exposure during fetal development is known to negatively affect the child (e.g. cognition, behavior and metabolism and altered brain morphology).

This thesis is part of a long-term study of children without CAH who were prenatally treated with DEX because of the potential risk of having CAH. Specifically, the thesis investigates the effects of DEX treatment on cognition (study I), behavior (study II), metabolism (study III) and blood pressure (study IV). Forty-two DEX-treated children and young adults without CAH (age range, 4-26 years) and 75 controls from the general population matched for age and sex were included in the studies. We identified a negative effect on cognition in DEX-treated girls but not in boys. Girls did worse on test assessing verbal IQ, non-verbal IQ and verbal working memory. There were no differences in behavioral problems, evaluated by parents and self-rated questionnaires in treated versus non-treated children. We found a lower HOMA- β in girls, but not in boys (another sex-dimorphic effect), suggesting a lower beta-cell function due to prenatal DEX exposure. In the younger age group (<16 years), fasting glucose levels were higher in the treated group in both sexes. In the older age group (\geq 16 years), total cholesterol and LDL cholesterol levels were higher in the exposed group in both sexes. When we assessed 24-hour ambulatory blood pressure, the only significant finding was higher pulse pressure in the younger age group during nighttime measurements.

In conclusion, early prenatal DEX treatment seems to have a sex-dimorphic effect on cognition and glucose metabolism. It also affects blood lipids in both sexes. Owing to these findings, and other negative findings previously shown in this cohort, the safety of prenatal DEX treatment is questionable. New, and an earlier prenatal diagnosis is needed to avoid treating healthy fetuses and males with CAH.

LIST OF SCIENTIFIC PAPERS

- I. **Lena Wallensteen** , Marius Zimmermann, Malin Thomsen Sandberg , Anton Gezelius, Anna Nordenstrom, Tatja Hirvikoski, Svetlana Lajic
Sex-dimorphic effects of prenatal treatment with dexamethasone.
Journal of Clinical Endocrinology and Metabolism, 101(10): 3838-3846, 2016
- II. **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, 98: 219–224, 2018
- III. **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, 105(7): e2439–e2448, 2020
- IV. Leif Karlsson, **Lena Wallensteen**, Anna Nordenström, Rafael Krmar, Svetlana Lajic
Effects on blood pressure after early prenatal dexamethasone exposure in individuals without CAH
Manuscript

Related publications not included in the thesis:

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, 68(2):427-437, 2020

CONTENTS

1	Introduction	1
2	Background.....	2
2.1	Congenital Adrenal Hyperplasia (CAH)	2
2.2	Virilization.....	4
2.3	Prenatal treatment of CAH.....	5
2.4	Glucocorticoids.....	7
2.4.1	Regulation and synthesis of cortisol	7
2.4.2	Cortisol and human physiology	9
2.4.3	Cortisol and fetal development	11
2.5	Cognition	13
2.5.1	Psychometric intelligence	13
2.5.2	Memory	13
2.5.3	Executive function (EF).....	14
2.5.4	Cognition and glucocorticoids.....	14
2.5.5	Cognition and prenatal glucocorticoid exposure.....	15
2.6	Behavior.....	18
2.6.1	Behavior and glucocorticoids	19
2.6.2	Behavior and prenatal glucocorticoid exposure	19
2.7	Metabolism and glucocorticoids	21
2.7.1	HPA axis and prenatal glucocorticoid exposure	21
2.7.2	Metabolism and prenatal glucocorticoid exposure	22
2.7.3	Blood pressure and prenatal glucocorticoid exposure	23
3	Hypothesis and aims.....	25
4	Methods	26
4.1	Study population.....	26
4.2	Cognition - Study I.....	28
4.3	Behavior - Study II.....	29
4.4	Metabolism - Study III	30
4.5	Blood pressure – Study IV	30
5	Statistics.....	32
5.1	Study-specific statistics	32
6	Results.....	34
6.1	Cognition - Study I.....	34
6.2	Behavior - Study II.....	35
6.3	Metabolism - Study III	36
6.4	Blood pressure - Study IV	37
7	Discussion.....	38
7.1	Cognition	39
7.2	Behavior.....	41
7.3	Metabolism	42
7.4	Blood pressure	44

8	Ethical considerations	46
9	Conclusion and points of perspective	48
10	Acknowledgements	49
11	References	51

LIST OF ABBREVIATIONS

11 β HSD	11 beta Hydroxysteroid Dehydrogenase
17-OHP	17 Hydroxyprogesterone
21-OH	21 Hydroxylase
ABPM	Ambulatory Blood Pressure Measurement
ACC	Anterior Cingulate Cortex
ACTH	Adrenocorticotrophic Hormone
ADH	Antidiuretic Hormone
ADHD	Attention Deficit Hyperactivity Disorder
ANOVA	Analysis of Variance
BP	Blood Pressure
CAH	Congenital Adrenal Hyperplasia
CBG	Corticosteroid Binding Globulin
CNS	Central Nervous System
CRH	Corticosteroid Releasing Hormone
CVS	Chorion Villi Sampling
CVD	Cardio Vascular Disease
CVH	Cerebral Ventricular Hemorrhage
CYP-21	21-Hydroxylase Cytochrome P450 gene
DBP	Diastolic Blood Pressure
DEX	Dexamethasone
DNA	Deoxyribonucleic Acid
EAS	Emotionality-Activity-Sociability-Shyness Temperament
EF	Executive Function
FDI	Freedom from Distractibility Index
GC	Glucocorticoid
sGC	Synthetic Glucocorticoid

GFR	Glomerular Filtration Rate
GH	Growth Hormone
GR	Glucocorticoid Receptor
GW	Gestation Week
HDL	High-Density Lipoprotein
HOMA	Homeostatic Model Assessment
HPA	Hypothalamic Pituitary Adrenal
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IQ	Intelligence Quotient
LDL	Low Density Lipoprotein
LMS	Lambda-Mu-Sigma
MC	Mineralocorticoid
MR	Mineralocorticoid Receptor
MRI	Magnetic Resonance Imaging
fMRI	Functional Magnetic Resonance Imaging
MTL	Medial Temporal Lobe
NEC	Necrotizing Enterocolitis
NEPSY	Developmental Neuropsychological Assessment
OGTT	Oral Glucose Tolerance Test
PFC	Prefrontal Cortex
POI	Perceptual Organization Index
PP	Pulse Pressure
PSI	Processing Speed Index
mRNA	Messenger Ribonucleic Acid
SASC-R	Social Anxiety Scale for Children Revised
SBP	Systolic Blood Pressure

SDS	Standard Deviation Score
SPAI-C-P	Social Phobia and Anxiety Inventory for Children-Parental Rating
SPSS	Statistical Package for the Social Sciences
SRY	Sex-determining Region Y
SV	Simple Virilizing
SW	Salt Wasting
VCI	Verbal Comprehension Index
WISC	Wechsler Intelligence Scale for Children
WM	Working Memory

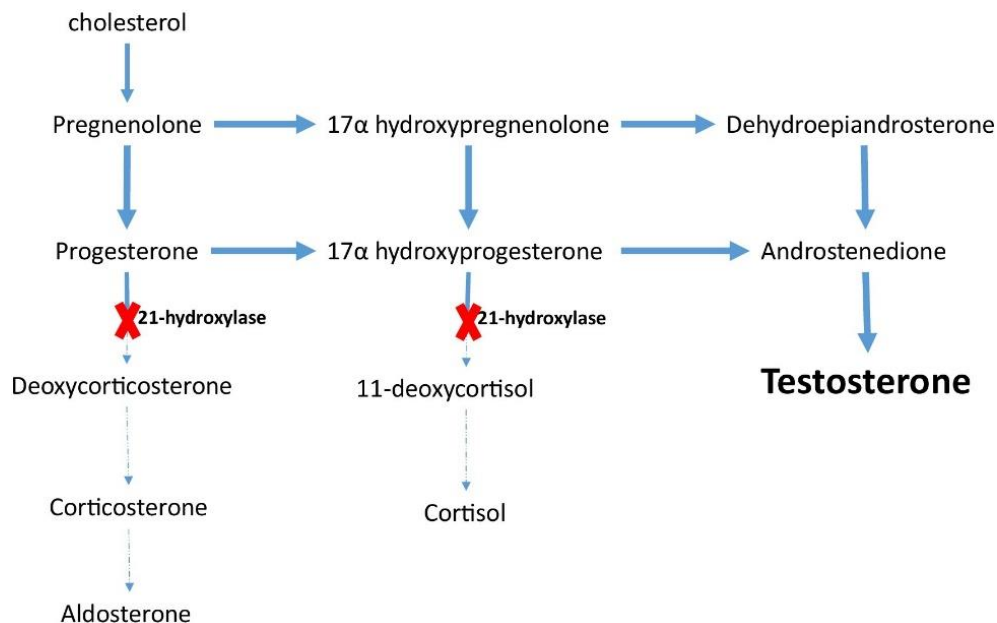
1 INTRODUCTION

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is one of the most common causes of ambiguous genitalia in females. Most individuals with CAH have mutations in the gene coding for this enzyme leading to a reduced synthesis of glucocorticoid (GC) and mineralocorticoid (MC) in the adrenal cortex [1]. Accumulation of steroid precursors will give an increased androgen synthesis in the adrenals already during early fetal development. Consequently, the external genitalia of the female fetus will become virilized, sometimes to the extent that sex assignment is difficult to determine at birth [2]. Prenatal dexamethasone (DEX) treatment aims to reduce the androgen overload and ameliorate the virilization in girls with CAH. To be effective, the treatment has to start before genotyping of the fetus is possible and the majority of the fetuses will thus be treated unnecessarily during embryogenesis [3]. Possible side effects of the treatment are of major concern because children not needing treatment should not be exposed to unnecessary risks [4]. This thesis assesses the potential long-term side effects of prenatal DEX treatment in the context of CAH, focusing on the children and adults not having CAH but exposed to DEX during the first trimester of fetal life.

2 BACKGROUND

2.1 CONGENITAL ADRENAL HYPERPLASIA (CAH)

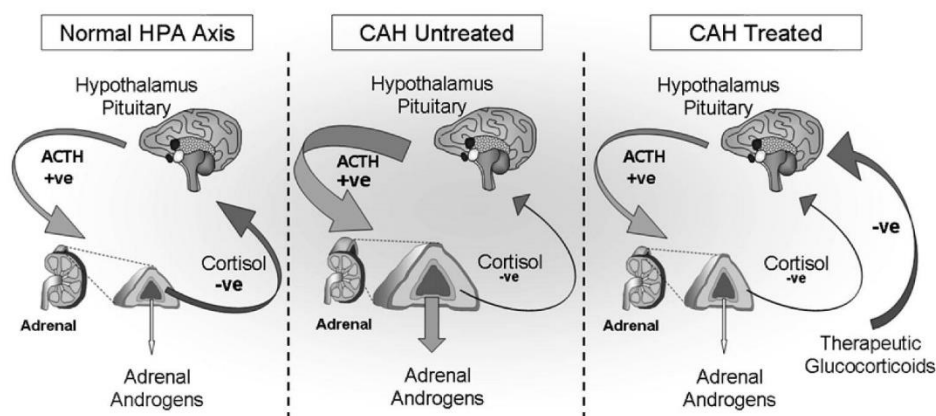
CAH is caused by an enzyme deficiency in the steroid synthesizing pathway in the adrenal cortex (Figure 1). Over 95% of patients with CAH have a deficient 21-hydroxylase enzyme which leads to a shortage of cortisol and aldosterone, accumulating 17-hydroxyprogesterone (17-OHP) and an increased androgen synthesis [1].



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Figure 1. Deficiency of 21-OH leads to accumulation of precursors and an increase in testosterone production.

Cortisol is synthesized in the adrenal cortex and released to the circulation in response to adrenocorticotrophic hormone (ACTH) synthesized in the pituitary. ACTH synthesis is in turn stimulated by the hypothalamic release of the corticotropin-releasing hormone (CRH) and the antidiuretic hormone (ADH). There is also a negative feedback mechanism due to increased circulating cortisol, which inhibits pituitary and hypothalamic hormonal synthesis. These hormones and their interactions are called the hypothalamus-pituitary-adrenal (HPA) axis [5].



Reprint from Hardy et al 2010 with permission from Elsevier[6]

Figure 2. Changes in the adrenal steroid synthesis; in CAH and its response to glucocorticoid treatment.

Because the adrenal cortex cannot synthesize enough cortisol in individuals with CAH, the pituitary will increase ACTH release to stimulate steroid synthesis. This situation leads to an accumulation of steroid precursors and an overproduction of androgens instead of an increased cortisol synthesis. High androgen levels during fetal development affect the external genitalia and virilize the female fetus [7] (Figure 2).

The characteristic of 21OH deficiency depends on the severity of the mutation and reduction of enzyme synthesis [8, 9]. The phenotype ranges from mild in non-classic (late-onset) CAH, to the more severe phenotype in the classic form, with or without salt-wasting (SW) [1]. SW CAH is life-threatening in the neonatal period, during starvation, infection, or both. The shortage of MC (Aldosterone) leads to a SW crisis risk due to the individual's inability to control water and electrolyte balance, resulting in hyponatremia. Cortisol deficiency may lead to severe hypoglycemia. Because the newborn infant usually has a starving period during the first days of life, before the mothers' breast milk production increases, the risk of adrenal crisis is most flagrant. Neonatal screening programs have been introduced to analyze the level of 17-OHP in the newborn child (the major precursor that accumulates in the steroid synthesis pathway), and the goal is to prevent a life threatening SW crisis [10-12]. The incidence of 21OHD in Sweden is 1/10 000 children, where about 50% of these children have no symptoms before diagnosis because of the neonatal screening [13].

Children with **classic CAH** are usually diagnosed short after birth either by ambiguous genitalia in girls, via the neonatal screening program for CAH or by clinical signs of adrenal insufficiency [12]. Females, with classic CAH, are virilized to different degree, sometimes

to the extent that sex may be hard to establish at birth. The genitalia of boys with CAH are not affected but they may have hyperpigmentation of the scrotum. Classic CAH is divided into two sub-types, depending on the ability to synthesize aldosterone: Salt wasting CAH (SW CAH) and Simple virilizing CAH (SV CAH) [1]. In SW CAH, the child has insufficient cortisol and aldosterone production with a considerable risk of SW crisis. In SV CAH, the child has a less deficient aldosterone synthesis but a severe cortisol deficiency and an excess of androgens, enough to virilize female fetuses [14].

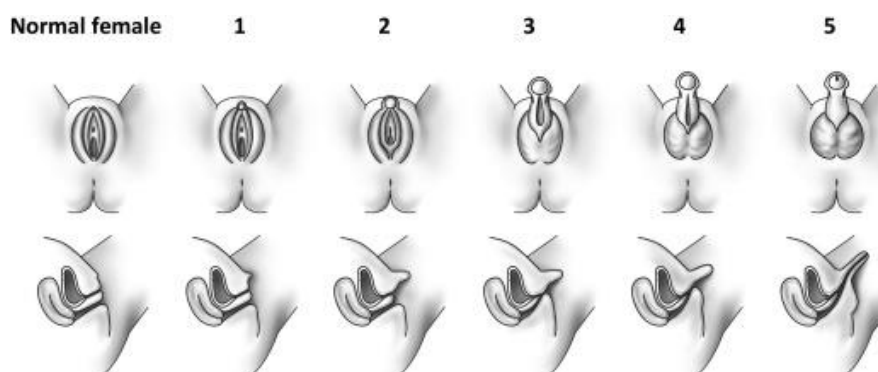
Individuals with **non-classic CAH** (late-onset CAH) have milder disease, although the pituitary still produces an excess of ACTH to meet the demand of cortisol, with an androgen overload. Symptoms of androgen excess is usually presented later in life since the levels are not high enough to cause prenatal virilization in females. Hence, these children may be missed in the screening process due to lower 17-OHP levels, compared to levels obtained in classic CAH [15]. Therefore the diagnosis may be delayed until childhood or even until adult age when the clinical signs of hyperandrogenism become more evident. Children may show signs of precocious puberty and in women it may cause hirsutism (i.e. excessive growth of facial or body hair). Males may escape diagnosis completely. The symptoms of androgen excess are reduced by GC treatment, but there is no absolute cortisol or aldosterone deficiency [15].

Treatment of CAH starts as soon as the child has been diagnosed and aims to replace the shortage of cortisol and aldosterone. However, for various reasons, treatment is hard to calibrate perfectly. Individuals may be either undertreated, or more commonly, over-treated because of the fear of adrenal crisis [14]. The difficulty in calibrating the cortisol treatment may lead to long-term effects due to the under- or over replacement of GC [16].

2.2 VIRILIZATION

Classic CAH, affects the genital development during gestational week (GW) 8-12 [17, 18]. The severity of genital virilization in girls correlates somewhat to the severity of the genotype, although there are also other influencing factors since girls with the same mutation may have a different degree of virilization [19, 20]. The virilization is classified according to the Prader stages (on a scale from 1-5), with stage 5 indicating the most severe virilization [21]. The internal genitalia, i.e. the uterus and the ovaries, are though unaffected. In Prader 1, only the clitoris is enlarged but the labia, urethra and vaginal orifices are unaffected. In Prader 5, the urethral orifice is located almost at the tip of the enlarged clitoris and the vaginal and

urethral orifices are fused to a common urogenital sinus. The labia are also fused into a scrotum-like pouch [22] (Figure 3).



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Figure 3. Prader scale of female external genitalia.

Depending on the severity of the virilization reconstructive surgery may be needed. Surgery may be indicated to separate the vaginal and urethral orifices, but it can also be suggested to reduce the clitoris' size. Surgery on the clitoris may lead to reduced innervation and sensation, which is why decision regarding surgery preferably is postponed until the girl is old enough to decide on her own. Moreover, if genitoplasty is performed in infancy, there is often a need for further reconstructive surgery at puberty [24].

2.3 PRENATAL TREATMENT OF CAH

Prenatal DEX treatment of CAH has been available since the middle of the 1980s. It is based on the hypothesis that virilization can be reduced if the mother's DEX treatment achieves adequate fetal adrenal suppression. However, to be effective, the treatment has to start before GW 7, because the development of the genitalia occurs during GW 8-12 [3, 17, 25, 26]. The necessity to start the treatment early in pregnancy becomes a dilemma since genotyping is not yet possible at that time in gestation. In addition, because CAH has a recessive mode of inheritance, the statistical probability that the fetus is a girl with CAH is only one out of eight. Accordingly, most fetuses will be exposed to DEX treatment unnecessarily until GW 12, when genotyping on DNA from a chorionic villus sampling (CVS) is possible. This means that healthy children and boys with CAH do not have any known benefit that could balance the treatment's potential risks.

Today, early fetal sex-typing (SRY detection), using cell-free fetal DNA from maternal blood, can avoid boy's prenatal treatment, if done after 4.5 weeks of gestation [27]. However, the unnecessary treatment of healthy girls cannot be avoided, and a majority (three out of four), will still be exposed unnecessarily. In this context, a careful risk assessment of the therapy is of great importance, especially given that the plausible long-term effects of the DEX treatment are poorly understood. Because there were uncertainties about the safety of this treatment, an international meeting in Gloucester, Massachusetts in 2002, was held. A consensus was reached that prenatal DEX treatment of CAH should only be offered as part of a clinical trial, with written consent from the mother only after information about the potential risks [11].

Long-term effects of synthetic GC (sGC) in growing children and adults have been studied for many years. Thus, there is good knowledge of the benefits of treatment regarding various diseases and side effects. Using this knowledge, we can decide whether a treatment should be initiated or not. The same risk-benefit analysis is legitimated when exposing a fetus to medical treatment. However, to make accurate decisions, more information is needed about the treatment's long-term effects.

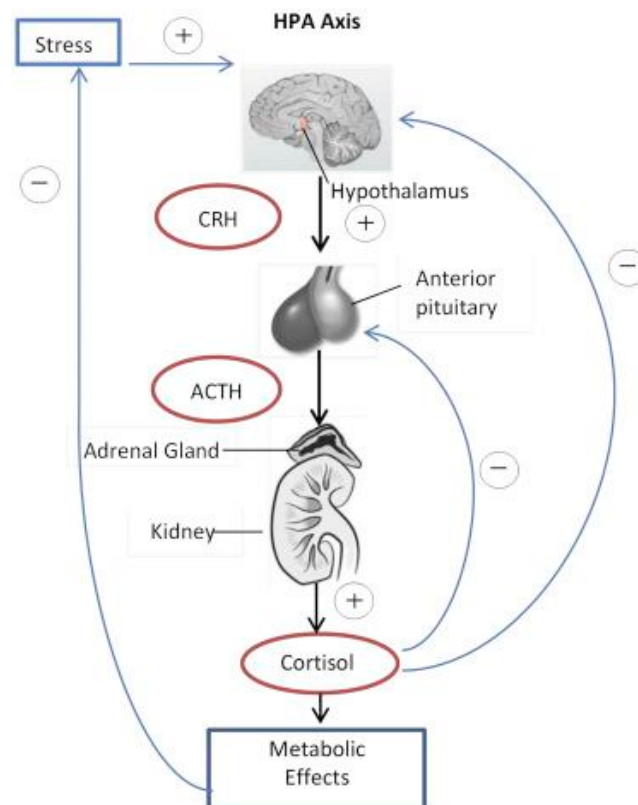
In Sweden, prenatal treatment of CAH has been used since 1984, and since 1999 only within the frame of a clinical follow-up study (the PREDEX-study) [28]. Since then, our research group has systematically evaluated the long-term effects of DEX-treated individuals until adult age. Our research team studies the impact of DEX on maternal health during treatment, fetal and postnatal growth, metabolic health, programming effects on whole-genome methylation and cognitive development including behavioral aspects [4, 26, 28-35]. In addition, we also investigate the possible effects on brain structure and function and the correlation with cognitive function, using functional magnetic resonance imaging (fMRI) [35, 36].

The thesis aims to assess possible long-term effects of early prenatal DEX treatment in the context of CAH. The focus has been on evaluating the impact of DEX therapy on cognition, behavior and metabolic health in healthy/non-CAH individuals exposed to DEX during the first trimester of fetal life.

2.4 GLUCOCORTICOIDS

2.4.1 Regulation and synthesis of cortisol

The HPA axis (Figure 4) regulates the synthesis of cortisol, ACTH and CRH and controls the body's basal cortisol level as well as stress-induced levels. The amount of circulating cortisol is therefore, dependent on this system and its degree of sensitivity and activity, and consequently impacts almost all bodily tissues at basal state and during increased physical or emotional stress [5].



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Figure 4. The HPA-axis

Cortisol cannot be stored in the adrenal gland making the cortisol level dependent on rapid synthesis. However, the quantity and activity of cortisol are also regulated by other mechanisms. Some 95% of the total cortisol amount secreted into the circulation are bound to either GC-binding globulin (CBG) or albumin, which inactivates it. Only about 5% of the total amount of cortisol is unbound to protein and thus metabolically active. The CBG level is therefore also a regulator of the amount of active cortisol. In situations of increased stress, in addition to increased synthesis, the CBG will down-regulate its synthesis to increase the amount of active cortisol [5, 38-40]. Moreover, the 11β -hydroxysteroid dehydrogenase (11β -HSD) enzymes regulate the activity of cortisol. 11β -HSD2 inactivates

cortisol to cortisone and 11 β -HSD1 activates cortisone to cortisol [41]. These enzymes are an effective way of regulating cortisol activity. Depending on the nature of the organ, different tissues have these enzymes activated to different degrees. In the placenta, 11 β -HSD2 is highly expressed to reduce the amount of maternal cortisol reaching the fetus [41].

Cortisol exerts its effects by binding to the GC receptor (GR) and the MC receptor (MR). The GR can only be activated by GC, but the MR may be activated by both MR and cortisol. MR has a tenfold higher affinity for cortisol than GR and is presumed to be the primary receptor activated in basal state. In times of increased stress, the GR is of primary importance to initiate the synthesis of proteins needed in the specific tissues. The negative feedback loop is mostly dependent on GR, especially in states of high stress levels. The diverse properties of the MR and GR make the balance between them in different tissues important in the homeostasis of the HPA axis [38, 42]. The GR and MR are proteins located in the cell's cytosol and when bound to GC or MC they form dimers that enter the nucleus. In the nucleus the dimers either repress or enhance transcription of specific genes through binding to the GC response elements in the genome. There are also MR and GR receptors in the cell membrane that are thought to have non-genomic effects [41].

Activation of the MR and GR has different effects in the cell. MR activation primarily leads to increased expression of proteins associated with regulating ion and water transport in different cell types, whereas activation of GR has various effects in different tissues due to its multiple isoforms. At translation of the GR protein, different mechanisms alter the final protein characteristics. For instance, there are multiple initiation sites on the gene, alternative splicing and post-translational modifications leading to different length and nucleotide sequences of the RNA, all of which affects the final protein [41, 43].

GR and MR are widely distributed in the human body including the central nervous system (CNS). In the CNS, MR is mostly confined to the structures associated with the limbic system. In contrast GR is abundant in the CNS, but has the highest density in the hypothalamus, hippocampus, amygdala and prefrontal cortex [44]. The receptors also have different effects depending on their location in the CNS. In the hippocampus, which is dense in both receptors, MR is hypothesized to be associated with regulating behavior while the GR is facilitating the storage of information [45].

The different isoforms of the GR, the varying MR/GR ratio, the 11 β -HSD activity and the amount of CBG are all part of regulating cortisol's diverse effects in different tissues [45]. In the kidneys, for example, the MR expression is high. On the other hand, the 11 β -HSD2 is

also more active than 11β -HSD1, leading to a lower level of active cortisol to prevent a cortisol-induced MR effect on salt and water homeostasis [46].

Because GR is the primary receptor in times of increased cortisol level and the primary target for sGC, the distribution and function of GR are of most importance in increased times of stress and the major contributor to side effects of sGC [47]. sGCs differ in their affinity to the MR and GR compared to endogenous cortisol. sGCs have a higher affinity to GR and less to MR compared to cortisol and are also more efficient activators of the GR receptor. In addition, sGCs are less affected by 11β -HSD enzymes and thus less inactivated. Moreover, they don't bind to carrier proteins to the same extent as cortisol. These properties of sGCs are part of the physiology behind the efficacy but also the adverse effects of sGCs when used in the treatment of different medical conditions [41].

2.4.2 Cortisol and human physiology

Cortisol has numerous effects on almost all organ systems. The effects usually differ in the organs due to earlier mentioned modulatory effects of the GR. The HPA axis's homeostasis is the key to normal function in the basal state and situations of increased stress. Below is a summary of the most prominent effects of cortisol in the human body

Glucose and lipid metabolism

Cortisol affects the liver, pancreas, adipose tissue and muscle cells to produce precursors to facilitate glucose production. A large contributor to glucose production is the liver. Cortisol activates gluconeogenesis and suppresses glycogen synthesis to generate energy to muscles and the CNS. The substrate for glucose production is primarily glycogen but fatty acids and amino acids also contribute [48]. In fat tissue, cortisol promotes lipolysis to supply the liver with free fatty acids as a substrate in glucose production. In muscle cells and other organs with high protein content (e.g., skin, bone and connective tissue) cortisol increases protein degradation to provide the liver with amino acids for the same reason. In addition, cortisol affects the pancreas by reducing insulin secretion and increasing the glucagon level, which facilitates lipolysis but also stimulates gluconeogenesis in the liver [49, 50]. This mechanism is essential in times of starvation. However, an unhealthy excess of cortisol leads to drainage of the body's storage of proteins (in muscle, bone and other connective tissues) and increased lipolysis that results in an increase of glucose and lipids in the circulation [5].

Cardiovascular system

Blood pressure (BP) and water balance are also affected by cortisol. Cortisol affects the arterioles by increasing their sensitivity to neuromodulators (e.g. catecholamines), which results in vasoconstriction and increased blood pressure. Further, it suppresses the effects of other elements (such as prostaglandins and histamines) leading to diminished vasodilation. In addition, cortisol has a positive effect on myocardial contraction which also contributes to the increased blood pressure [51]. Cortisol can also impact the kidneys by activating the MR. MR-activation increases the glomerular filtration rate (GFR), enhances excretion of potassium and retention of sodium and by that, increases water retention. Cortisol deficiency leads to hypotension and vice versa. However, cortisol-induced hypertension is thought to primarily result from an increased responsiveness and constriction of the arteries; the contribution of the kidneys may be of lesser importance [51, 52].

Immune system

Cortisol affects the immune system and inflammatory responses in an inhibitory fashion. Cortisol decreases the synthesis of a number of mediators of inflammation, including prostaglandins, thromboxanes and leukotrienes. Cortisol also stabilizes the lysosomes and reduces the local proteolytic enzyme release and thereby swelling [5]. Furthermore, cortisol decreases the leukocyte's recruitment to a specific inflammatory site by inhibiting the production of peptides that attracts leukocytes in the capillaries and suppresses the differentiation and maturation of monocytes and lymphocytes. These anti-inflammatory properties of GC are effective in treatment of inflammatory diseases (e.g., rheumatoid arthritis) and inflammation in general [53].

Bone and calcium metabolism

Bone maturation and growth, as well as mineral density are affected by cortisol. The osteoblasts are affected by a reduction in their activity, increased apoptosis and a decrease in osteoblastogenesis. The osteoclasts are instead activated with an upgraded activity and survival. These combined effects lead to bone resorption and an increase of circulating amino acids, which can be used for gluconeogenesis. Cortisol also reduces the absorption of calcium in the intestine and the kidneys, leading to a secondary increase in parathyroid hormone and an enhanced osteoclast activity [54]. Thus, an increase in endogenous cortisol or treatment with sGC, may lead to osteoporosis.

An excess of GC affects childhood growth negatively due to adverse effects on osteoblast and growth hormone (GH) activity [6, 54]. At physiological level, cortisol is essential for

normal GH secretion but an excess of GC can impact the GH-axis negatively by inhibiting secretion of GH releasing hormone and increasing release of the inhibitory peptide Somatostatin. In addition, GC can affect translation of genes involved in the GH axis (e.g. the GH receptor and Insulin-like growth factor-I) [55].

Central Nervous system (CNS)

Cortisol is not only essential for neuronal growth and survival but also for differentiation. An excess (or a lack of) cortisol influences cognition, behavior and mood [44]. Cognition, behavior and emotions involve the limbic system, the parietal cortex and the prefrontal cortex. These areas are abundant in GR and MR [56]. In animal studies, MR activation seems to enhance neurogenesis and proliferation, whereas activating GR inhibits neurogenesis [57]. This shows the importance of the balance between the GR and MR activation and which receptor is dominant in the different brain areas to develop optimal cognitive and psychological development and general wellbeing [58]. This may be an explanation for the negative effects of long-term stress and long-term sGC treatment has on the CNS, since high levels of cortisol and sGC primarily activate the GR [47]. The cortisol levels, regulated by the HPA axis, also control the circadian rhythm, which is important for normal cognitive functions, behavior and mood, and altered circadian rhythm has a negative impact on both [59, 60]. Cortisol also impact neurotransmitters (e.g., serotonin, dopamine and glutamate), with effects on cognitive and behavioral CNS functions. The association between GC and depression is well studied but the underlying mechanisms are not clear [61]. Patients with severe depression and anxiety usually have high cortisol levels and they often exhibit cognitive impairments. The same effects have been found in patients with Cushing's syndrome (a cortisol-producing tumor) with a decrease in cognitive function and development of depression [62, 63].

2.4.3 Cortisol and fetal development

Cortisol as well as other adrenal hormones has vital effects on the human body throughout life, but they are also important during fetal development. Cortisol play an important role in fetal maturation by the end of the pregnancy but are also crucial during organogenesis [64]. For example, cortisol is important for neuronal maturation, remodeling and survival and altered GC levels during development may result in both structural and functional changes in the brain, as well as in many other fetal organs [47, 56, 65, 66]. At the end of pregnancy, cortisol levels increase to mature the organs, especially the lungs, by increasing the surfactant production in the alveolar cells, which helps the alveoli keep open after birth.

Mothers at risk of premature delivery receive sGC to reduce neonatal morbidity and respiratory distress syndrome in the child [67, 68].

Fetal development of the adrenal glands

The fetal adrenal glands develop from GW 4-8 and are large compared to other fetal organs throughout pregnancy. The fetal adrenal growth is dependent on ACTH production and other growth factors [48]. Fetal production of adrenal hormones starts about GW 7 and fetal cortisol synthesis is thought to be of most importance during GW 8-12, whereafter the fetal production decreases again. After GW 14, cortisol's fetal production is low until the last trimester when it increases again to prepare the child for delivery [69]. Based on animal studies, it is probable that MR and GR's ratio also varies throughout gestation. Studies on human fetal brains have also found that the amount of GR, MR, cortisol and ACTH differ depending on the time of gestation [39, 47, 70, 71].

Fetal development of the brain

By GW 8, the rudimentary structures of the human brain are developed. The critical period for brain development of the neocortex is from GW 8 until about midgestation. Most of the neurons are formed and have migrated during that period and starts to interact with each other by GW 15 [72, 73]. This indicates that the brain may be particularly vulnerable during this period of fetal development as to possible alterations in GC level.

The role of placenta during fetal development

The placenta protects the fetus from the mother's increasing cortisol levels during pregnancy due to the high expression of 11β -HSD2 in the placenta. The cortisol level in the fetal circulation is about 13 times lower than in the maternal circulation [74]. The levels of 11β -HSD2 in the placenta increase as the pregnancy proceeds but drops significantly in late gestation, leading to increased fetal cortisol levels that prepare the child for postnatal life. However, in studies assessing the fetal effects of maternal stress, the fetus seems to still be affected by higher levels of cortisol despite the placental 11β -HSD2 enzyme [75]. The mechanism for the increase of cortisol in the fetal circulation is not fully understood. However, one hypothesis asserts that maternal cortisol reaches a level that exceeds the capacity of the 11β -HSD2. An alternative hypothesis is that high cortisol levels downregulate the presence of 11β -HSD2, possibly by DNA methylation [74, 76, 77].

The effect on the fetus from sGC will differ depending on the type of sGC administered to the mother. Some sGCs (e.g., DEX and betamethasone) are minimally inactivated by the placenta and will reach the fetus unimpeded, while the placental enzyme largely inactivates hydrocortisone and prednisolone [41]

2.5 COGNITION

Cognition, behavioral problems and mood have been studied in pre- and postnatal sGC exposure. Not only excess of GC but also deficiency of cortisol has been found to negatively affect cognition, mood and behavior [63, 78].

2.5.1 Psychometric intelligence

The definition of psychometric intelligence and the best way to assess it has been debated and have changed over time. However, it is usually referred to as organized in a hierarchical structure, with the general intelligence factor (g) on top, with underlying cognitive abilities underneath [79-81]. Depending on the theory, the abilities vary in content and number. The most common theory on which most intelligence tests are based is the Cattell-Horn-Carroll theory, which has a three-level hierarchy based on a factor analysis of different standardized tests. A commonly used test in children is the Wechsler Intelligence Scale for Children (WISC), which also addresses the properties of IQ (intelligence quotient) similarly providing full-scale IQ and intelligence indices (standardized population mean=100, SD=5), as well as subtests (standardized population mean=10, SD=3) in a hierarchical structure [82, 83].

2.5.2 Memory

The memory process can be divided into different phases; encoding, consolidation and storage and retrieval. Separate but partly overlapping brain networks are thought to be involved in different parts of the memory process [44]. Encoding is the process by which the brain perceives the information and processes it into a memory. Different types of sensory input are processed in the encoding phase, usually defined as visual, acoustic or semantic input. The medial temporal lobe (MTL) has a crucial part in the memory process, both during encoding and consolidation. The MTL structure includes the amygdala, the hippocampus and the area surrounding the hippocampus [44, 58, 84]. In the consolidation phase, the memories become more stable, making it possible to store them for longer periods and retrieve them later. The prefrontal cortex (PFC) seems to have an essential part in long-term memory storage and retrieval [44, 63, 85, 86]. The memory process is dependent on the neurons' ability to change, i.e. their plasticity. Memory is the result of changes in the synapses;

neurotransmitter release, dendritic growth, formation of new synapses and up- or down-regulation of receptors [44, 87].

2.5.3 Executive function (EF)

Executive functions (EFs) are a set of higher-order cognitive processes that regulate and control lower-order cognition, mood and behavior. EFs are strongly associated with general intelligence (g) and related to success in school, working life and social and psychological development and wellbeing [88-90]. EF can be defined as composed of different basic cognitive processes that together regulate goal-directed behavior [88]. The three core EF's are listed below.

Inhibition and interference control: The control of attention to a specific task and suppression of attention to other stimuli, i.e. the ability to resist disturbances and stay focused. It also includes the ability to inhibit impulses and thus, controls our behavior, thoughts and emotions [88].

Working memory (WM): The ability to remember information and mentally process it (as opposed to short-term memory which does not include the aspect of mental processing) [90]. WM is often divided into verbal and nonverbal (visual-spatial) WM.

Cognitive flexibility: the ability to shift attention or perspectives to new information or between different tasks.

EF's are associated with the PFC, which matures late and is not fully developed until well past adolescence [88]. Correspondingly, the EF has been demonstrated to develop rapidly in school-age children and peak between 20 and 30 years of age. [91].

2.5.4 Cognition and glucocorticoids

Optimal levels of cortisol play an important role in the memory process [38]. Non-optimal levels of cortisol, on the other hand, impair this process [63]. The effects of cortisol can be visualized, as an inverted U-shaped curve where too low and too high GC levels are negative to the memory process. The length of exposure is also an important factor. Shorter periods of high cortisol levels may facilitate memory, whereas long-term exposure is harmful [63]. The adverse effects on memory are not only be dependent on the duration of the exposure, but it has also been shown to be dose-dependent [92]. Cognitive impairments seem to be reversible but some studies have shown that they may become permanent [62]. Chronic overexposure to GC (endogenic and exogenous) increases the risk of long-term deficits [58, 62, 63, 93]. In a long-term follow-up study of Cushing patients, cognitive impairments found at diagnosis

(attention, non-verbal WM, and verbal WM) still existed several years post-surgery [62]. Several studies on healthy individuals treated with sGC (for other reasons than cortisol deficiency) have shown negative side effects on cognitive function, especially memory. In addition, sGC treatment has also been found to induce structural changes such as reduced volume of the hippocampus and anterior cingulate cortex (ACC), which are part of the limbic system, affecting both emotions and cognition [58, 93, 94].

2.5.5 Cognition and prenatal glucocorticoid exposure

Numerous human and animal studies have assessed third trimester GC exposure. It is difficult to say if these results apply to our cohort of early gestation GC treatment. Most likely, there is time-specific differences and the fetus' vulnerability probably differs in early compared to late prenatal development [95].

Studies of mid- to late gestational GC exposure have shown structural effects on the brain in both animals and humans, especially in the areas associated with memory and learning [47]. In primates, prenatal sGC treatment has been associated with reduced hippocampus volume, reduced number of neurons, and a reduced number of GRs [96-98]. Most animal studies have assessed the offspring at birth and have not evaluated them in childhood or as adults.

However, a study exposing primates to both late and early prenatal sGC and assessing them at two time points, at birth and 2 years of age, found a reduced neuronal proliferation rate in the hippocampus at birth, regardless of the timing of GC exposure. However, at the age of 2 years, the effect could no longer be found. The follow-up study at 2 years of age, however, only included males and a possible effects in females might have been missed [99, 100]. It might also have been an effect of the CNS's known plasticity and that structural changes may be reduced or diminished with time [101]. Human studies examining the effects of late prenatal GC treatment (due to risk of premature delivery) have shown similar results. A brain MRI study of children 6-10-years of age, born at term, but exposed to sGC prenatally, found a significantly thinner cingulate cortex and prefrontal cortex. The largest effect was seen in the rostral ACC (rACC), with a 30% cortical reduction in thickness compared to controls [102]. The ACC is part of the limbic cortex and the rACC is involved in emotion processing and learning [86].

Studies on cognitive effects after late prenatal GC treatment have shown varying results depending on the number of sGC doses administered. Most of the studies evaluating one- or two-dose regimens found no association with major cognitive impairments, regardless of their age at assessment or the study design [103-106]. In many of these studies, a trained

psychologist evaluated the children, but some also used parent-completed questionnaires. One study even showed better results in children exposed to two doses of prenatal GC compared to non-treated controls [107]. In contrast, studies evaluating the effect of multiple sGC doses in late gestation have shown a negative impact on full-scale intelligence in preschool children and lower performance in tests assessing attention and speed in adults [108, 109]. High endogenous cortisol levels in mid-to-late gestation, due to maternal stress, have also been associated with lower full-scale IQ, verbal IQ and non-verbal IQ (WISC). Further, parental ratings concerning the child's development, have shown negative effects on cognitive development and higher ratings on attention problems [110-114].

Little is known about the possible changes in the CNS structure caused by early gestational DEX treatment in individuals at risk of CAH. To our knowledge, our research group is the only one that has published results from a study investigating sGC-exposed healthy individuals (non-CAH) and brain structure. A fMRI was performed in prenatally short-term DEX-treated healthy young adults and healthy population controls. DEX treatment was associated with a bilateral enlargement of the amygdala, larger left superior frontal gyrus and a widespread increased diffusivity of white matter compared to the healthy non-treated controls. There was no relationship between altered brain structure and cognition [35]. Maternal stress in early pregnancy has also been shown to have similar results, but only in girls. Children assessed with MRI at 7 years of age were found to have a larger right amygdala volume associated with high maternal cortisol levels in early gestation. No association was found in boys [115].

Many studies investigating long-term effects of early DEX treatment in children at risk of CAH have focused on the cognitive outcome owing to the numerous animal studies showing changes in brain structure, behavior and memory [47]. The methods used by these studies to assess cognition varied. Some studies used direct assessment by a psychologist in addition to parent-rated questionnaires and others have only used parental questionnaires rating the child's cognitive development, scholastic performance, or both. Because CAH is a rare disease and prenatal treatment has only been in use since the 1980s, it is challenging to design studies with sufficient statistical power. Besides, the prenatally treated individuals consist of several subgroups, i.e. long-term treated girls with CAH, short-term treated boys with CAH, and short-term treated healthy individuals of both sexes. Accordingly, in the stratified analyses, sample sizes, and consequently, statistical power decrease (especially in analyses of possible sex-dimorphic effects).

Studies investigating cognitive functions, based on parent-rated developmental questionnaires, have not shown any negative effects of DEX treatment on cognitive development, cognition or scholastic performance [116, 117]. The first study, published in 1995, assessed 40 children (age range: 6 months - 5.5 years) 26 DEX-treated (3 with CAH and 23 non-CAH) and 14 untreated (3 with CAH and 11 non-CAH) [117]. The other study, published in 2004, included 487 children (age 1 month - 12 years), 174 DEX-treated (48 with CAH and 125 non-CAH) and 313 untreated (195 with CAH and 118 non-CAH). This study had a large cohort but all children above 6 years of age (44 DEX-treated and 162 untreated) were rated only by three questions in the Child Behavior Check List's (CBCL) school competence section [116].

Two research groups in addition to ours have evaluated cognition with direct assessment by psychologists. The American research group (2012) found a possible (near significant [$p=0.066$]) DEX effect in healthy short-term DEX-treated children, with lower performance on the sequential processing test, which assesses short-term memory [118]. None of the other cognitive tests showed any differences (the Kaufman Assessment Battery for Children and the Wide Range Assessment of Memory and Learning). This study assessed 140 children (aged 5–12 years), 67 DEX-treated (16 with CAH and 51 non-CAH) and 73 untreated (37 with CAH and 47 non-CAH) [118].

The Polish group (2014) used almost the same psychometric test battery as we did, but restricted the cohort to girls. Thirty-three girls aged 6-23 years participated in the study. Of these 33 girls, 17 were DEX-treated (9 with CAH and 8 without CAH) and 16 untreated (all with CAH). They did not have a group of non-treated girls without CAH to compare the DEX results of the girls not affected by CAH. Nevertheless, they found that DEX-treated girls with CAH performed better in subtests used to estimate both verbal IQ ($p=0.03$) and non-verbal IQ ($p=0.04$) compared to non-treated girls with CAH [119]. Furthermore, the short-term DEX-treated healthy girls performed worse than the DEX-treated girls with CAH and at the same level as non-treated girls with CAH. These findings suggest a possible negative effect in the healthy DEX-treated girls as individuals with CAH are known to perform worse on WM tasks compared to non-CAH controls [120-122]. However, it's probably presumptuous to draw any conclusions about the DEX-treated healthy girls as the study did not have a non-treated control group. Moreover, the American research group did not find positive cognitive effects of DEX in long-term-treated girls with CAH; rather, the girls with CAH performed overall worse if they had been long-term-treated with DEX. However, these findings did not reach statistical significance [118].

In our original cohort of DEX treated individuals, published 2007, we found that healthy DEX-treated children performed poorer than non-treated controls in the WISC subtest Digit span assessing verbal WM (n=26 DEX treated)[29]. The difference between these two groups was found to have a large effect size (Cohen's $d=0.95$). The children also rated their scholastic performance lower than controls and the test performance in Digit span significantly correlated with their self-rating in scholastic performance. No differences between groups were found in full scale IQ, learning and long-term memory, nor did parent-rated questionnaires show any differences in scholastic performance [29]. When evaluating them again as adults, these between-group differences could not be replicated (n=23 DEX treated) [123].

2.6 BEHAVIOR

The limbic system, including the limbic cortex, amygdala, hypothalamus and hippocampus, are important structures in processing emotional stimuli and are known to affect our mood and behavior [124, 125]. Structural changes have been found within the limbic system with respect to different behavioral problems, especially aggression [126-128]. The areas associated with the limbic system are abundant in GC receptors and are highly dependent on appropriate levels of cortisol for normal neuronal development and plasticity [63].

Behavioral problems in childhood can be characterized as either internalizing or externalizing problems. Internalizing problems refer to inward-focused behavior (e.g., anxiety and depression) while externalizing problems refer to outward-focused behavior, also referred to as "acting out" (e.g., hyperactivity, aggression and disruptive behavior) [129]. Behavioral problems in childhood are often assessed using questionnaires, filled in by the parents or by the child. There is relatively good agreement between the parents and the child's assessment of the child's problems in younger children. However, as the child grows older, the parent-child agreement decreases, especially in items measuring withdrawal and attention problems [130]. One common parent-rated questionnaire is the CBCL, which addresses both internalizing problems (e.g., anxiety, withdrawal and somatic complaints) and externalizing problems (e.g., rule-breaking and aggression) [131]. The different questionnaires often include subscales measuring different behavioral constructs that may or may not be associated. In the Emotionality-Activity-Sociability-Shyness Temperament Survey for children (EAS), shyness (tendency to feel awkward or worried during social encounters, especially with unfamiliar people) and sociability (tendency to seek out others' company, e.g., through different social activities) are two separate constructs but can be intercorrelated [132].

2.6.1 Behavior and glucocorticoids

Emotional and psychiatric side effects of GC treatment in humans are thoroughly studied and sGC treatment is associated with a potential risk of developing depression and psychosis [63, 133, 134]. Children treated for nephrotic syndrome with high doses of sGC showed depression, anxiety and aggression that increased over time and was positively associated with the dosage [134]. However, studies focusing on endogenous cortisol levels and behavioral problems show inconsistent and ambiguous results. Some studies have found that high cortisol levels are associated with internalizing problems, whereas others have found that high cortisol levels are associated with externalizing problems [127]. Different types of behavioral problem may also co-exist, causing difficulty when analyzing possible associations.

Behavioral problems and other psychiatric diagnoses have also been associated with structural findings in the CNS [124, 127]. Studies in children with externalizing problems, such as aggression and conduct problems, have found altered volumes in the prefrontal region, amygdala and hippocampus [126, 128]. However, if these findings are related to changes in the HPA axis is not confirmed

2.6.2 Behavior and prenatal glucocorticoid exposure

Studies on late gestation GC exposure have shown behavioral effects in animal offspring (e.g., reduced locomotor activity in guinea pigs, interpreted as a sign of anxiety, and abnormal behavior in primates, expressed as increased clinging and reduced social interaction) [135, 136]. Human studies on late gestation sGC treatment due to a risk of preterm birth have shown diverse results depending on the number of sGC doses administered, similar to studies addressing the cognitive effects. One or two doses have not shown any significant effects on behavior or mental health [103-105]. However, results indicate negative effects on behavior after multiple sGC doses. Children assessed at 3 years of age by parental questionnaires and again at 6 years of age by parental and teachers questionnaires showed more aggressive/destructive behavior and more hyperactivity/distractibility compared to controls at both ages [137]. When studying sGC-treated children's behavior and mental health at 12 years of age by teachers' questionnaires (the Rutter score) the prenatally DEX-treated children were found to have an increase in total behavioral problems and an increase in the sub-score for inattention. However, when assessing the same individuals again at 16 years of age by parental questionnaires and self-reports regarding attention-deficit hyperactivity disorder (ADHD) symptoms, the results did

not persist [138]. Another similar study, investigating the long-term effects of multiple doses of prenatal sGC on attention and hyperactivity, found poorer achievement in psychologist-assessed measures of attention and speed. However, when the individuals rated themselves for the same problems, no differences could be found [109]. These results imply that there might be a difference in the parental and self-reported questionnaires compared to the teacher's evaluation, direct assessments, or interviews by a psychologist [130].

Maternal stress in mid- to late gestation has also shown to have negative behavioral effects in infants [139-142]. Based on maternal questionnaires (the infant behavior questionnaire, IBQ) the children have been described as having more "difficult behavior" (sub score: Distress to limitation) and more "negative reactivity" (sub score: Fear). Furthermore, evaluation in 4-year-old children, based on parental ratings, found that the children scored higher in total emotional/behavioral problems than the controls. They also found that boys, but not girls, were rated as having more hyperactivity/inattention problems [141]. In addition, several studies have reported an association between maternal stress during pregnancy and later autism spectrum disorder, as well as ADHD in the children [143-145]. However, in these studies, the child's genetics were not analyzed and hence the possible effect of shared genetics was not determined.

The behavioral effects caused by early gestation GC exposure are not as comprehensively studied as late gestation exposure. However, primates exposed to DEX in early or late gestational showed that early, but not late, exposure was associated with reduced social behavior and increased food-seeking behavior [146]. Human studies on early gestation GC exposure have primarily evaluated increased cortisol exposure due to maternal stress. Maternal stress level is often measured by self-ratings of perceived stress, but some studies also measure the cortisol level in serum or saliva additional to the maternal ratings. An increase in maternal cortisol levels in early gestation (measured in saliva) has been found to be associated with both a larger right amygdala volume and more affective problems in girls but not in boys, which would indicate a sex-dimorphic effect [115]. Natural disasters, maternal illness, or both in early gestation have also been shown to be associated with "poorer temperament" in infants (higher scores in the dimensions: fussy/difficult, unadaptable, and dull) [147].

The possible behavioral effects of prenatal DEX treatment in children at risk of having CAH have previously been evaluated and the published results are all based on parental or self-assessed questionnaires. Young children (n=26 DEX, mean age: 2.5±1.3 years),

prenatally treated with DEX, were reported by their mothers to be less sociable, more emotional and shyer compared to how the mothers of the non-treated controls rated their children. Moreover, they were found to have more internalizing problems and higher scores in total behavioral problems [117]. However, because this study had a mixture of healthy and CAH children in both groups and the assessment was made at a noticeably young age, the results are somewhat difficult to evaluate. In our first Swedish cohort of DEX-treated children (n=26, mean age 11±2.3), the behavioral problems were rated by the parents and the children themselves. The DEX-treated healthy children rated themselves as having more social anxiety compared to non-treated healthy children. However, the parents did not report increased social anxiety in their children. Moreover, the parents found their children more sociable than the parents' ratings of the children in the non-treated control group [29]. As previously discussed, the concordance between parental and self-assessment for evaluating behavior is not always high. Thus, many of the results from studies that evaluate temperament or behavior only by parental questionnaires are difficult to interpret [130]. In our study, assessing behavior in DEX-treated children with CAH (n=11, mean age 11.7 years), the parents rated the DEX-treated children as more withdrawn/depressed and the boys (but not girls) as having more social problems. None of these problems was confirmed in their self-ratings [148].

2.7 METABOLISM AND GLUCOCORTICIDS

As mentioned previously, cortisol has multiple metabolic effects, including altered glucose metabolism and cardiovascular changes [50-52]. Cortisol level, both at basal state and increased stress, is regulated by the HPA axis and changes in the homeostasis of the HPA axis may lead to different metabolic effects depending on how the HPA axis is affected. The sensitivity of the HPA axis may be altered at different levels (the hypothalamus, pituitary or adrenal cortex) leading to harmful cortisol responses with implications for various organ systems [127]. Besides affected synthesis of cortisol, changes in the GR quantity, MR/GR ratio and enzyme activity may also alter the effects of cortisol. Additionally, other parts of the brain are involved in regulating the HPA axis (e.g., the amygdala, hippocampus, prefrontal cortex) and structural or functional effects in these areas may also impact metabolism [149].

2.7.1 HPA axis and prenatal glucocorticoid exposure

Numerous animal studies have shown that prenatal exposure to sGC alters the HPA axis, although with somewhat variable results. In some studies, the prenatal sGC treated offspring have shown a reduced HPA-activity while others instead found an increased activity [150, 151]. Several studies in rodents have also found sex-dimorphic effects, with different results

in males compared to females [135, 152-154]. In addition, the physiological changes found in guinea pig offspring have been found to transfer into the third generation, which suggests an epigenetic effect. DNA methylation has been suggested to be one of the epigenetic mechanisms [155, 156]. Following prenatally sGC-treated sheep from infancy up to adulthood, showed that the HPA axis activity changed as the sheep matured and the adrenals became increasingly ACTH-resistant with age [157-159]. Contrary to the findings in sheep, primates exposed to prenatal sGC resulted in a sensitized HPA axis [98, 146, 160].

Mid- to late gestation GC treatment in humans born *preterm* has resulted in a lower stress-induced cortisol level in infants, assessed at 4-6 weeks of age. This finding indicates a postnatal suppressive effect on the HPA axis, which is consistent with animal studies on sheep [159, 161-163]. However, when testing children born *full-term* but prenatally sGC-treated in mid-to-late gestation, the results showed an increase in cortisol levels after induced stress (heel-stick), indicating a sensitized HPA axis similar to the primate studies described above [160, 164]. The same research group also studied the effects on the HPA axis in regard to maternal stress in the second and third trimester and found corresponding results as in sGC-treated the full-term babies. The postnatal cortisol levels were higher and more prolonged after inducing stress (heel-stick) in the gestational stress-exposed group [164]. Other studies assessing maternal stress have shown similar results with an increased basal cortisol level [165]. When assessing the prenatal sGC effects on the HPA axis in older children, the results differ as well. At 6-11 years of age, one study found the HPA axis to be sensitized, with higher cortisol levels than in the control group after exposing them to psychosocial stress (a public speaking task) [166]. On the other hand, another study found the opposite results in children after late gestation sGC treatment. Lower cortisol and ACTH levels were found after a similar stress test at 8-9-years of age [167]. Still, other research groups have not found any differences at all [168]. In summary, the results of late gestation GC treatment on the HPA axis are not fully clarified, although many studies found altered homeostasis in one way or another.

To our knowledge, studies on HPA axis response in individuals who were DEX-treated in early gestation have not yet been studied.

2.7.2 Metabolism and prenatal glucocorticoid exposure

Animal studies have shown that primates treated with DEX in late gestation had offspring with higher insulin levels at 8 months of age [160]. Another study looking at early prenatal DEX treatment in sheep found no differences in insulin, glucose or amino acid metabolism at

5 years of age. However, they found that insulin inhibition of lipolysis was increased in the DEX-exposed group. The authors argue that decreased lipolysis may contribute to the development of obesity later in life [169].

Human studies have found inconsistent results. Adults exposed to late gestation sGC treatment showed a lower insulin response to an oral glucose tolerance test (OGTT), indicating altered insulin sensitivity [168]. There are also findings of reduced beta-cell function, measured as HOMA- β after late sGC treatment. However, other studies could not identify any metabolic effects [170-172].

In the cohort of early prenatally DEX-treated individuals at risk of CAH, only one study was published besides the one included in this thesis. A French research group found that DEX-treated individuals (non-CAH) exhibited a higher glucose level at 30 minutes during a glucose stimulation test (OGTT) and lower insulin levels after glucose and arginine stimulation, suggesting a reduced beta-cell function [173].

2.7.3 Blood pressure and prenatal glucocorticoid exposure

Prenatal GC exposure and long-term effects on BP can result from altered activity in HPA axis, but it can also be a direct effect on different organs. In the kidneys, prenatal sGC may affect the nephrogenesis with a reduced number of nephrons. In animal studies, there are convincing associations with reduced nephron number and hypertension [174-176].

Furthermore, sGC can increase the reactivity of the arteries smooth muscle cells, leading to increased arterial resistance and BP [177, 178]. In sheep, early (but not late) prenatal sGC exposure resulted in increased BP but no effects were found on the HPA axis or vascular reactivity [179]. When analyzing the number of glomeruli and the GFR, they found that DEX-treated offspring had lower number of glomeruli and higher filtration rate, which they argued might be causing the hypertension found in the same animals [180]. On the other hand, another research group found that a short DEX exposure in early gestation was associated with increased responsiveness in coronary arteries in 1-week-old and 4-month-old sheep. However, they found no difference in BP but argued that the responsiveness might represent an early stage in hypertension development [181, 182].

In primates, the results differ as well. One study found that neither early nor late gestation DEX treatment resulted in altered BP, glomerular number or kidney size [183]. Another study showed that primates treated with DEX in late gestation had offspring with higher BP at 12-14 months of age compared to non-treated controls [160].

Several human studies on late gestation GC exposure (in pregnancies at risk of preterm labor) and the possible effect on postnatal BP have presented somewhat contradicting results. A few follow-up studies show no effect on BP or pulse pressure in children or young adults [168, 170, 171, 184-187]. However, some results have indicated higher BP already in adolescence [188]. Additionally, two studies have shown possible effects on aortic stiffness. Both of these studies were follow-up studies during adolescence or young adulthood with no negative findings on BP after sGC exposure, although the increased aortic stiffness might be a risk factor in developing high BP at an older age [170, 172]. Maternal stress has also been shown to affect BP already at 5-6 years of age [189].

3 HYPOTHESIS AND AIMS

The overall aim of the thesis was to identify whether first-trimester DEX treatment has any negative consequences on health in children at risk of CAH. The overall hypothesis was that prenatal DEX treatment has negative long-term effects on somatic health, cognition and behavior.

The specific aims of the studies outlined herein are:

Study I

Identify possible long-term cognitive effects due to early prenatal DEX treatment in healthy children at risk of CAH.

Study II

Identify long-term behavioral effects due to early prenatal DEX treatment in healthy children at risk of CAH.

Study III

Study the effects of first-trimester DEX treatment on glucose metabolism, lipid profiles and renal function in children and adults exposed to DEX during the first trimester of fetal life.

Study IV

Study the effects of first-trimester DEX treatment on BP in children and adults exposed to DEX during the first trimester of fetal life.

4 METHODS

4.1 STUDY POPULATION

This thesis is part of a larger study, the PREDEX study on long-term effects of prenatal DEX treatment in individuals with and without CAH. The study's objective was to include the entire Swedish cohort of prenatally DEX-treated individuals at risk of CAH. Because there is only one laboratory in Sweden that analyzes all CVS and the possible CYP 21 mutations (Karolinska University Hospital, Department of Clinical Genetics), the identification of all DEX-treated mothers was possible.

In total, 77 mothers were treated from 1986-2010. In 1999, all mothers who met the treatment criteria were only treated as part of the PREDEX study, i.e. a long-term prospective, non-randomized clinical study. In 2010, our group decided to halt the inclusion due to our initial reports on plausible negative effects on cognition in treated cases [4, 29] (Figure 5).

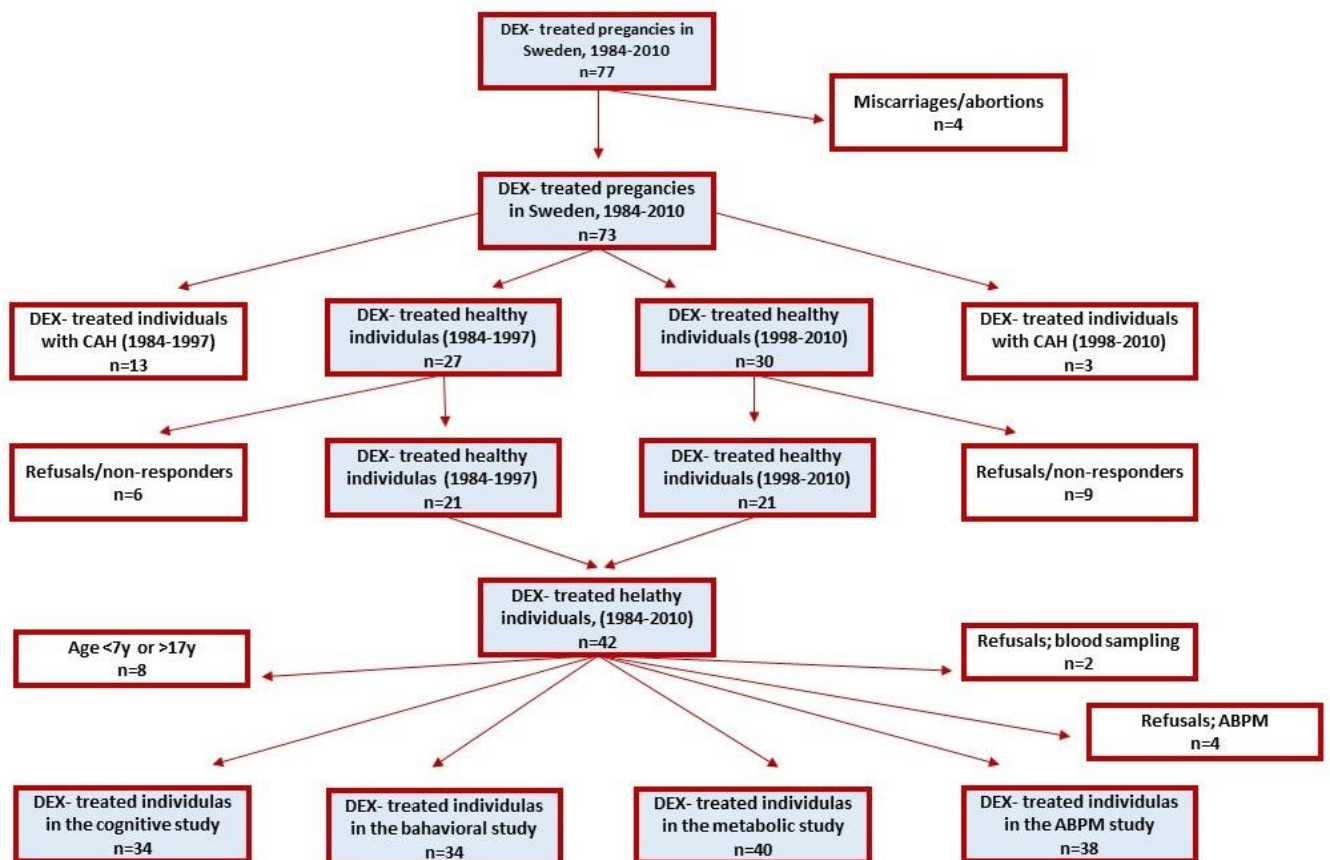


Figure 5. Flowchart of the DEX-treated individuals in this thesis.

Four of the pregnancies resulted in miscarriages or termination, which resulted in 73 born children [31]. Six of the 73 treated mothers (7 pregnancies) did not respond to our follow-up request within the PREDEX study (8). 57 (78%) of the 73 DEX-treated children did not have CAH and this sub-cohort is the focus of the present thesis. Seven families declined to participate in the follow-up or could not be reached for follow-up (8 pregnancies) and one child died in an accident at a young age. In the cohort of individuals born before 1989, 10 declined participation for cognitive testing at the time of the first assessment (2002-2004) but agreed to participate when they were asked again to participate in the second follow-up (2011-2016), where 4/10 families responded positively [29].

In total, 42 children and young adults without CAH and who were exposed to DEX during the first trimester of fetal life (age range, 4-26 years) were included in the present thesis. For a detailed description of the number of participants per study, see figure 5.

Prenatal treatment protocol for CAH: Women who previously had given birth to a child with classic CAH were offered treatment with DEX starting about GW 7 in the event of a subsequent pregnancy. A dose of 20 $\mu\text{g}/\text{kg}$ and day (of the maternal pre-pregnancy weight), divided into 3 doses per day (with a maximum of 1.5 mg/day) was prescribed. Healthy/non-CAH fetuses and CAH boys were treated during the first trimester, as the treatment was terminated after the genotyping results were obtained. Girls with CAH were treated until term. Genotyping was performed on DNA obtained from a chorionic villous sampling approximately GW 12 [190] (Figure 6).

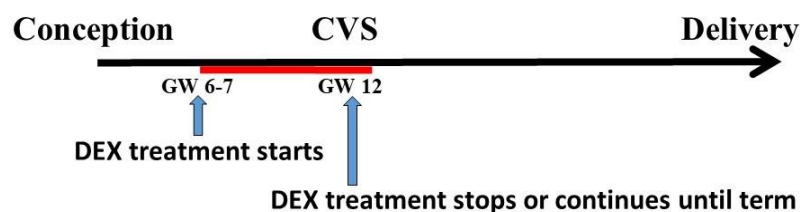


Figure 6. Flowchart of the prenatal DEX treatment protocol.

The control group in the studies consisted mostly of individuals from the general population randomly selected from the Swedish civil registry and was matched for age and sex with the DEX-treated cases. A subgroup of the controls comprised healthy siblings of the DEX-treated children. For practical reasons, all population controls were selected from the Stockholm County area except for the healthy siblings. Invitation letters were sent to 394 individuals in the Stockholm County area: approximately 100 controls accepted to participate and 62 actively declined participation. The rest either did not respond to the invitation or were not

possible to contact by telephone. Of the positive responders, not all participated in all the assessments and thus the number of controls differs between Study I-IV.

All study participants underwent several investigations within the frame of the PREDEX study: medical examination, blood sampling, cognitive testing, including parental and self-reporting questionnaires and 24-hour ambulatory BP monitoring (ABPM). Participants from age 16 years also underwent fMRI of the brain and a test assessing emotional regulation. The two latter investigations are not part of the present thesis. The majority of the participants underwent all tests, but a few of the youngest choose not to participate in the blood sampling and the ABPM tests [34, 35, 148, 191].

4.2 COGNITION - STUDY I

Cognition was assessed using standardized tests administered by a trained psychologist as well as by parent-rated and self-rated questionnaires. Thirty-four children between 7 and 17 years of age at the time of testing were included in the cognition study and 66 controls matched for age and sex (mean age: 10.5 ± 2.6 years).

Outcome measures

One subtest in each of the four indices of the Wechsler Intelligence Scales for Children (WISC-III) was used. Vocabulary from the Verbal Comprehension Index (VCI), as an estimation of verbal psychometric intelligence, Block Design from the Perceptual Organization Index (POI), as an estimation of non-verbal psychometric intelligence, Digit Span from the Freedom from the Distractibility Index (FDI) to assess verbal WM and attention and Coding from the Processing Speed Index (PSI) to assess processing speed [192].

To assess learning, long-term memory and interference, two subtests from A Developmental Neuropsychological Assessment (NEPSY) were used: Wordlist and Memory for faces [193].

To further assess the child's verbal processing speed and ability to inhibit an overlearned response, the Stroop color-word test was used [194].

Visual-spatial WM was tested using the Spatial Span Board from the Wechsler Nonverbal Scale of Ability [195].

Handedness was measured by the Manual Preference Test from a previous version of the NEPSY [196].

To evaluate the children scholastic performance the parents as well as the children filled out the questionnaires. For the parental evaluation we used the school competence section in the

Child Behavior Check List (CBCL) [197] and for the children the Scholastic Competence subscale from the Self-Perception Profile for Children were used [198].

4.3 BEHAVIOR - STUDY II

Behavioral problems and possible psychopathology were assessed using questionnaires: Two parent-rated and two self-rated scales for the children. One of the parent-rated questionnaires evaluated the child's temperament. 34 DEX-treated children were in aged 7 - 17 years at the time of testing and were thus included in the behavioral study together with 67 controls matched for age and sex (mean age: 10.5±2.6 years)

Outcome measures

Parent-rated questionnaires:

The CBCL is a psychiatric screening instrument for children consisting of 113 questions about the child's behavior. It's divided into eight syndrome scales: Aggression, Anxiety/Depression, Attention problems, Rule-breaking behavior, Somatic complaints, Social problems, Thought problems and Withdrawal/Depression. The syndrome scales can be aggregated into two broadband scales: Internalizing problems and Externalizing problems. Moreover, a Total problem score can be calculated that includes all items. In addition, there is a section assessing the child's adaptive behavior, including Activities, Social relations and Scholastic competence. These combined results yield a total competence score [199].

The Social Phobia and Anxiety Inventory for Children - Parent Report (SPAI-C-P) is a 26-item parent-rated questionnaire covering cognitive, physiological and behavioral symptoms of social phobia in children according to the DSM-IV. The questionnaire measures the child's anxiety level and is divided into three subscales: Public Performance, Assertiveness/General Conversation and Traditional Social Encounters [200, 201].

The Emotionality-Activity-Sociability-Shyness Temperament Survey for children (EAS) estimates the child's temperament. The survey is divided into four subscales: Sociability, Activity, Shyness and Emotionality [132].

Self-rated questionnaires:

The Social Anxiety Scale for Children-Revised (SASC-R) consists of three subscales: Fear of Negative Evaluation, Social Avoidance and Distress in new social situations and General Social Avoidance and Distress [202].

4.4 METABOLISM - STUDY III

All participants were instructed to fast from midnight the night before the blood sampling procedure. They were also instructed to bring a portion of the first-morning urine in a test tube provided the day before. All participants were offered topical anesthesia to reduce pain and discomfort due to blood sampling. All blood samples were analyzed at an accredited laboratory: Department of Clinical Chemistry, Karolinska University Hospital, Sweden. Forty DEX-treated children and 75 controls, matched for age and sex, were included (mean age: 16.3 ± 6.2 years).

Outcome measures

Height and weight were measured during the medical examination. Blood sampling was performed in the morning to evaluate the following metabolic markers:

Renal function: P-cystatin C, cystatin C GFR, P-sodium, P-potassium and U-albumin/creatinine. The GFR was calculated using the Schwartz bedside (Creatinine) and CAPA (cystatin C) formulas [203-205].

Blood count: B-Hemoglobin, B-Erythrocytes, B-Leukocytes and B-Thrombocytes.

Glucose metabolism: fP-glucose, fP-insulin, fS-C-peptide and B-HbA1. Beta-cell function and insulin resistance were estimated using the homeostatic model assessment, HOMA- β and HOMA-IR [206, 207].

Blood lipids: fS-total cholesterol, fS-HDL (high-density lipoprotein), fS-LDL (low-density lipoprotein) and fS-TG (triglycerides).

4.5 BLOOD PRESSURE – STUDY IV

BP was recorded on the non-dominant arm using an appropriately sized arm cuff and measured every 30 minutes during waking hours and 60 minutes during sleep. All participants were instructed to notify what time they went to bed and what time they woke up the next day. In all, 38 DEX-treated children and 70 controls matched for age and sex were included. The mean age of DEX-treated children and controls was 15.6 ± 6.4 years and 16.9 ± 5.5 year, respectively.

Outcome measures

ABPM was conducted with the oscillometric monitor model 90207 (Space Labs, Redmond, WA) which has been validated in children and adolescents [208, 209]. The ABPM results included 24-h, daytime and nighttime mean systolic and diastolic BP, mean arterial pressure (MAP) and mean heart rate. Nighttime blood pressure dipping was defined as the difference

between the mean systolic and diastolic daytime BP and mean systolic and diastolic nighttime BP expressed as a percentage of the daytime mean. Pulse pressure was defined as the difference between mean systolic pressure and mean diastolic pressure. The individuals' ABPM values were compared to published pediatric normative data for ABPM [210]. Ambulatory hypertension was defined as mean daytime or nighttime systolic or diastolic BP value(s) >95th percentile [210].

5 STATISTICS

For all analyses (Papers I-IV), variables were tested for normality and homogeneity of variance prior to data analyses. If the assumption of normality were not met, the variables were analyzed using nonparametric methods. Group differences were analyzed using DEX (treated, untreated) and sex (male, female) as the independent variables and age as a covariate, if applicable, in conjunction with other appropriate covariates when necessary. In general, all tests were two-tailed and p-values of < 0.05 were considered statistically significant. Interactions between DEX and sex were followed up with sex-specific tests. We did not control for multiple comparisons to avoid missing small but potentially clinically relevant effects.

All statistical analyzes were performed in SPSS 23 (IBM, Armonk, NY, USA), except for study IV, where the analyses were performed in R, version 4.0.2 [211, 212].

5.1 STUDY-SPECIFIC STATISTICS

In study I, raw scores from the Wechsler scales were transformed to scaled scores using age-specific Swedish norm tables before analyses [213]. For NEPSY and the Stroop color-word test, raw scores were transformed according to the American manuals [193, 194]. Because the raw scores were transformed into age-specific standardized scores, we did not include age at testing as a covariate in the analyses. General two-way ANOVAs with factors DEX (treated, untreated) and sex (male, female) as independent variables were used to calculate group differences. Effect sizes were calculated using Cohen's d , with positive results representing higher test scores in healthy controls and negative results representing higher test scores in the DEX-treated group. Effects were categorized as large when $d \geq 0.80$, moderate when $d \geq 0.50$ and small when $d \geq 0.20$.

In study II, linear regression analysis was performed to analyze group differences in behavioral outcomes (rating scales) using DEX, sex and parental education as predictors. Parental education was included as an estimate of the families' socioeconomic backgrounds. Effect sizes were calculated using Cohen's d , with positive results representing higher test scores in DEX-treated children and negative results representing higher test scores in the healthy control group. Effects were categorized as large when $d \geq 0.80$, moderate when $d \geq 0.50$ and small when $d \geq 0.20$.

In study III, linear regression analysis was performed to estimate group differences for metabolic outcomes using DEX, sex and age as predictors. We further divided the cohort into two age groups (one group <16 years and one group ≥ 16 years) to estimate the effects of DEX in pre- and post-pubertal children.

In study IV, raw ABPM data were normalized to standard deviations scores (SDS) using the LMS (Lambda-Mu-Sigma) method. The LMS is a linear transformation that calculates the SDS of our individuals' ABPM values compared to mean ambulatory BP in pediatric normative data relative to height and sex previously published by Wuhl et al [210]. By applying this method, the data analysis for both sexes and all heights could be combined. Linear regression analysis was performed to estimate group differences using DEX, sex and age as predictors. We also divided the cohort into pre- and post-pubertal children to investigate the outcome similar to the metabolic outcomes.

6 RESULTS

6.1 COGNITION - STUDY I

In study I, we examined possible cognitive effects after first-trimester DEX treatment in children without CAH. We found a sex-dimorphic effect on several cognitive measures. The DEX-treated girls scored significantly lower on three of four sub-tests from the WISC-III with large size effects: Block Design, Vocabulary and Digit Span (Figure 7).

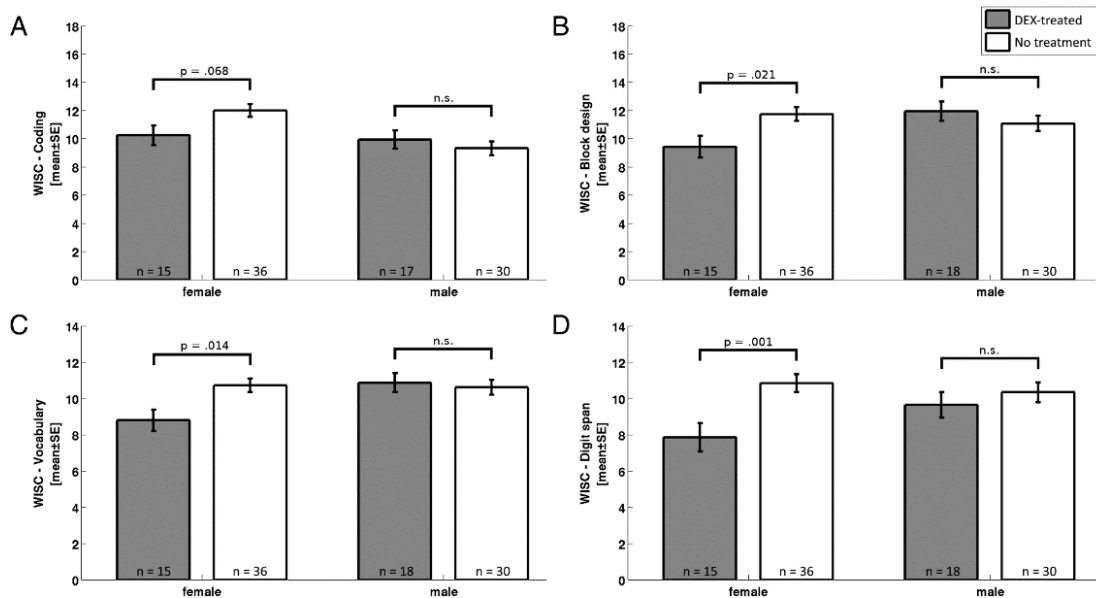


Figure 7. Group averages for WISC-III. (A) Coding ($p=0.068$, $d=0.66$), (B) Block design ($p=0.021$, $d=0.81$), (C) Vocabulary ($p=0.014$, $d=0.84$), and (D) Digit span ($p=0.001$, $d=1.00$).

In the Span Board-backwards test, the DEX treated girls scored significantly lower than the control girls with a moderate size effect ($p=0.034$, $d=0.79$). No effect could be seen in the Span Board-forward test (Figure 8).

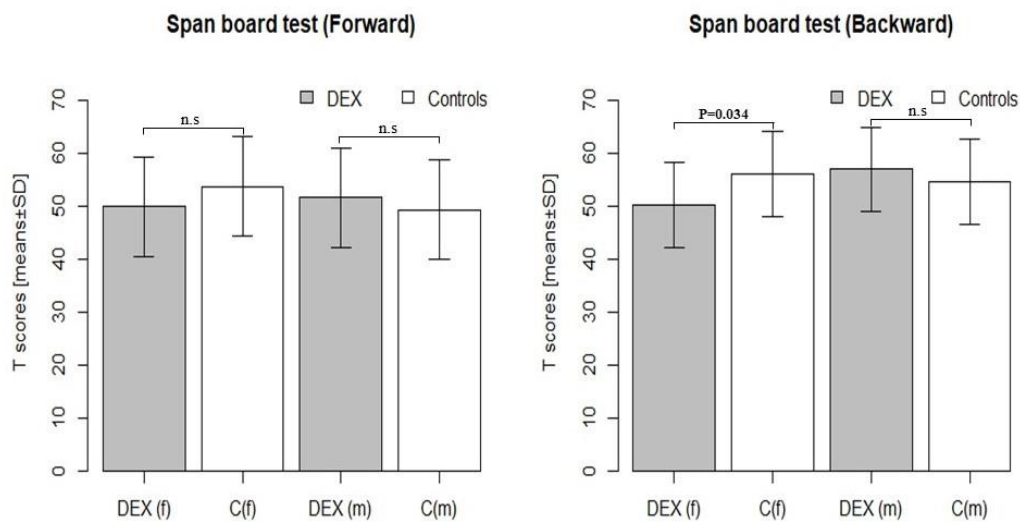


Figure 8. Group averages for span board forward (A) and backward (B). DEX-treated girls scored lower than female controls on span board backwards ($p=0.034$, $d=0.72$).

In boys, there were no significant findings in any of the cognitive tests and no differences between groups were identified in girls or boys for handedness, NEPSY or the Stroop color-word test.

Results from the CBCL questionnaires did not show any differences in scholastic competence when rated by the parents. However, when analyzing the results of their self-perceived scholastic ability, the DEX-treated children rated their ability lower than controls ($p=0.001$). In girls the effects size was large ($d=1.28$) and in boys moderate ($d=0.51$).

6.2 BEHAVIOR - STUDY II

In study II, we evaluated possible behavioral effects of DEX treatment in healthy children (non-CAH). We did not find any significant differences compared to the control group.

CBCL - There were no differences between the groups on the scales measuring adaptive functioning (CBCL total competences score), in the two broad-band scales: Internalizing problems and Externalizing problems or any of the eight specific syndrome scales. However, DEX treated girls were scored by their parents to have more attention problems and social problems, although the difference was not significant (Table 1).

CBCL	DEX (f)	C(f)	β (CI)	d	p
Attention problems	4.1 (3.8)	1.7 (2.2)	1.2 (-0.5, 2.8)	0.53	0.154
Social problems	2.3 (3.0)	0.8 (1.4)	0.7 (-0.5, 1.9)	0.45	0.225

Table 1 Results from CBCL, showing group means and standard deviations for girls. Regression coefficients (β), with 95% confidence intervals (CI lower, CI upper) effect sizes (Cohen's d , adjusted for parental education) and p -values for the main effects of DEX.

SPAI-C-P - Parental estimations of their children's social anxiety level, divided into the three subscales (Assertiveness/General Conversation, Traditional Social Encounters and Public Performance), did not show any significant differences (all $ps>0.05$).

EAS - Parental estimation of children's temperament using the EAS subscales shyness, emotionality, sociability and activity did not reveal any significant group differences (all $ps>0.05$). However, we found a near-significant result showing that parents rated their DEX-treated girls as being less shy compared to the parental ratings of the controls ($p=0.068$, $d=-0.68$).

SASC-R - We found that girls scored higher on all three self-reported anxiety subscales (Fear of Negative Evaluation, Social Avoidance and Distress- New and Social Avoidance and Distress - General), although the differences were non-significant (all $p>0.05$) (Table 2).

SASC-R	DEX (f)	C(f)	β (CI)	d	p
Fear of Negative Evaluation (FNE)	18.0 (7.2)	14.6 (5.3)	4.0 (-0.8, 8.8)	0.68	0.103
Social Avoidance and Distress (SAD)- New	15.3 (4.7)	13.3 (4.0)	1.0 (-2.4, 4.4)	0.25	0.543
Social Avoidance and Distress (SAD)- General	8.0 (3.2)	6.7 (2.5)	0.1 (-2.0, 2.2)	0.06	0.14
Total	41.3 (13.5)	34.6 (9.6)	5.1 (-3.6, 13.9)	0.48	0.243

Table 2 Results from SASC-R, showing group means and standard deviations for girls. Regression coefficients (β), with 95% confidence intervals (CI lower, CI upper) effect sizes (Cohen's d, adjusted for parental education) and p-values for the main effects of DEX.

6.3 METABOLISM - STUDY III

In study III we investigated possible metabolic effects after first-trimester DEX treatment in children without CAH.

Renal function: No significant DEX effects were found in the whole cohort for renal function. However, after dividing the group by age (<16 years and ≥ 16 years), we found a significantly higher P-potassium level in the DEX-treated younger children ($p=0.025$).

Blood count: No differences were found in blood count when analyzing the whole group, but after separating them into two age groups (<16 years and ≥ 16 years), the younger DEX-treated children had a slightly higher leukocyte count ($p=0.003$).

Glucose metabolism: We observed a significant DEX effect on HOMA- β with lower beta-cell function in the whole group of DEX-treated children ($p=0.049$). After post hoc analyzes, the lower HOMA- β values persisted in DEX-treated girls ($p=0.024$) but not in boys ($p=0.550$). No significant differences were found when analyzing fP-glucose, fP-insulin and B-HbA1. When separating the individuals into two age groups (<16 years and ≥ 16 years), we found a significantly higher fP-glucose in the younger DEX-treated children ($p=0.001$) and a lower HOMA- β value ($p=0.048$). As in the whole cohort results, the post hoc analysis revealed that the difference in HOMA- β persisted in girls ($p=0.05$) but not in boys ($p=0.540$). For fP-glucose, there was no interaction with DEX and sex. In the older age group (≥ 16 years), no significant DEX effects on glucose metabolism were observed.

Blood lipids: There were no significant effects on blood lipid profiles when the entire DEX-treated cohort was compared to the controls. However, when the age groups were analyzed separately, a significant effect of DEX was identified in the older age group (≥ 16 years) for cholesterol ($p=0.003$) and LDL cholesterol ($p=0.009$). No interactions were found between DEX and sex. In the younger age group (< 16 years), there were no significant findings.

6.4 BLOOD PRESSURE - STUDY IV

In study IV we examined the possible effects on BP after prenatal DEX treatment in healthy children. When analyzing the cohort across all ages, we did not identify any differences in 24-h BP. When dividing the cohort into two age groups (< 16 years and ≥ 16 years), the younger DEX-treated children had significantly higher mean nighttime PP ($\beta=11.6$, $t(34)=2.1$, $p=0.039$). No significant differences were observed in the older age group.

7 DISCUSSION

Prenatal DEX treatment in the first trimester, result in negative effects on cognition and metabolism. Furthermore, DEX treatment seems to affect girls in a higher degree than boys. Our results are partly confirmed by other studies on DEX treatment in the context of CAH [118, 173]. However, since studies on first-trimester DEX treatment in CAH risk pregnancies are relatively few, we are required to compare our results with studies assessing other types of GC exposure and at different periods of fetal life.

Animal studies may be applicable, but there are probably many differences between humans and animals because different species have different maturity levels at birth, different gestational length, different cognitive development, etc. [214]. Results from studies on maternal stress may, to some extent, be relevant for our results. However, the stress level is often difficult to validate. Also, the cortisol level measured in maternal plasma and amniotic fluid is not always associated with the mothers' estimated stress level [139]. Maternal stress also induces a rise in endogenous cortisol in contrast to DEX exposure. The different GCs have different GR affinity and different potency for activating the GR. In addition, they differ in their sensitivity to the 11β -HSD2 enzyme [41]. Effects due to mid to late gestation sGC treatment in pregnancies at risk for prematurity may be comparable to our results. However, there are probably differences because sGC exposure affects the fetus during different stages of development. Organogenesis occurs in early gestation, but the organs' maturation usually occurs in later pregnancy [64, 65]. The fetal HPA axis also evolves during gestation and the levels of these hormones changes as the pregnancy progresses [71].

These facts make our field of research and the results obtained in this thesis novel. Our affected cognitive functions and metabolism findings are in line with animal and human studies of maternal stress and late gestation sGC treatment [47, 65, 66, 76, 144, 151, 214-216]. However, there are not many convincing results from other research groups investigating cognition after DEX treatment in non-CAH individuals, perhaps due to the assessment methods, the lack of a control group and small group size [217]. However, a French research group found similar results to ours on glucose metabolism and further studies are hopefully in progress from other groups [173]. Our studies found no evidence of negative DEX effects on behavior, which is consistent with other groups studying the prenatal treatment of CAH, but in contrast to the studies on late gestation sGC exposure and maternal stress [138, 144, 151, 217, 218]. However, the majority of the individuals exposed to late prenatal exposure are also born preterm. In addition, they often need neonatal medical care for a prolonged period,

probably leading to anxiety and stress in the mothers, which might also influence the outcome [219, 220].

7.1 COGNITION

In study I, DEX-treated girls performed worse than controls in three out of four of the WISC-III subtests, and in the fourth subtest there was a trend in the same direction. Further, the DEX-treated girls also performed worse in the backward part of the Spatial Span board test, assessing visual-spatial WM. In WISC-III there are four index scores (VCI, PSI, POI and FDI), but it also renders an estimate for verbal IQ and non-verbal IQ. Our interpretation is that DEX exposure during early gestation affects verbal WM, visual-spatial WM, verbal IQ and non-verbal IQ in girls but not in boys [31]. Accordingly, the finding of a lower result on the digit span test (assessing verbal WM) in DEX-treated healthy girls in our first study published 2007 should be regarded as confirmed [29]. The differences showed medium to large effect-sizes in all the significant test results ($d=0.72-1.00$). Moreover, DEX-treated girls rated their scholastic ability lower than controls with a difference over 1 SDS ($p=0.001$, $d=1.28$), consistent to the results of our earlier study [29].

There are few other studies published on early DEX treatment in pregnancies at risk for CAH and its effects on children's cognitive outcome, to compare our results with. The studies differ in the set-up in regard to cohort, methods and outcomes. There are primarily three research groups assessing cognition concerning first-trimester prenatal DEX treatment: Our Swedish research group in Stockholm, the American research group in New York and the Polish group in Warsaw [217]. Two American papers have been published (1995 and 2004) assessing cognition by parental questionnaires on cognitive development. Neither of these studies found negative effects of DEX treatment. However, both of these studies had a mixture of children with CAH and children without CAH in the DEX-treated and non-treated groups. Further, they had no control group of healthy non-DEX exposed children to compare the results of the DEX-treated individuals without CAH [116, 117]. There are two studies published with a similar setup as ours, with direct assessment by a psychologist but with some differences in methods and cohort. The American group found that healthy short-term DEX-treated children (boys and girls combined) performed worse on short-term memory, however the result was only near significant ($p=0.066$) [118]. The Polish group found a significant difference between girls with CAH, depending on whether they had been exposed to prenatal DEX treatment. The DEX-treated girls with CAH performed better than the non-DEX-treated girls with CAH and the healthy girls performed worse than the DEX-treated girls with CAH and at the same level as non-treated girls with CAH [119]. They

suggested a potentially negative effect in the healthy DEX-exposed girls because individuals with CAH are known to perform worse in tests assessing WM compared to healthy controls [120-122]. However, they did not have a healthy, non-treated control group, making the results in the DEX-treated healthy girls challenging to interpret.

Maternal stress in early pregnancy has also shown to have a sex-dimorphic effect in inhibitory control (an aspect of executive functions) and structural changes in the amygdala [115, 221]. Children tested at 6-9 years of age assessed by direct psychometric testing and MRI of the brain, found lower performance in girls in tests assessing inhibitory control, but no effect could be seen in boys [221]. The study also showed a reduced performance in girls and boys on the sequential memory task, assessing visuospatial WM. These children were also examined with an MRI of the brain, which showed an association between early gestational maternal stress and a larger amygdala volume on the right side in girls but not in boys [115]. Moreover, they found that mid-gestational stress was associated with a reduction in gray matter volume in several areas associated with memory and EF (e.g., the prefrontal and temporal cortex) in both sexes [222]. These findings of early prenatal excess of cortisol are consistent with our findings of reduced executive function in girls and structural changes in the amygdala [29, 31, 35]. WM is primarily associated with the prefrontal cortex, the temporal lobe and the parietal lobe. Not surprisingly, lesions in these areas result in impaired cognitive abilities [223, 224]. Both animal and human studies show structural changes in these areas after prenatal GC exposure. Reduction of the hippocampal volume, a reduced number of neurons in the hippocampus, decreased size and activity level in the ACC and reduced volume of the amygdala have been reported [35, 78, 96, 98, 102, 115, 128].

The sex-dimorphic effect and the reason for this finding is not apparent; however, one can speculate that it may be because men and women activate different areas during WM tasks, suggesting that they use different strategies to solve the same problem [225]. Women often activate the limbic system and the prefrontal cortex more extensively than men during WM, whereas men use the parietal and temporal areas more [225, 226]. There are also sex differences in total brain size, volumes of gray matter vs. white matter, size differences in brain regions associated with cognition and differences in what hemisphere is primarily used during WM tasks [225-229]. Because we assess children and teenagers, CNS maturation during adolescence is another factor to consider. During adolescence, the brain changes both structurally and functionally. Some parts (e.g., the frontal and parietal cortices) are not fully mature until well past adolescence [230]. Maturation in adolescence seems to occur at

different rates for males and females with respect to total brain volume and gray matter volume, with girls peaking approximately 2 years earlier than boys [230-232]. In some regions (such as the prefrontal cortex), the difference in time of maturation is even larger [227]. Our study did not divide the children into different age groups, nor did we associate the results to their pubertal stage, which might have given us more information about the data from a developmental perspective. However, when we assessed the cognitive abilities in the DEX-treated healthy adults in our cohort, we did not find any differences in WM or intelligence regardless of sex [123]. The lack of negative outcome in the older individuals might be a result of compensatory mechanisms in girls, that obscure a true difference, but it might also be caused by the plasticity of the brain and the neurons ability to grow and reorganize [233]. However, since we also found structural changes in the brain in these individuals, the theory of complete neuronal recovery seem less convincing.

7.2 BEHAVIOR

In study II, we found no significant association with DEX treatment and negative behavior or temperament based on parental and self-reported questionnaires. Thus, our findings do not support our hypothesis that early fetal DEX treatment affects behavior or temperament in childhood or adolescence. In our first group of prenatally DEX-treated children, we found that the children rated themselves as having more social anxiety [29]. In the extended study presented in this thesis, we found that girls (but not boys) scored higher on all subscales of SASC-R assessing social anxiety, although no significant differences could be found. Further we found a difference in the CBCL syndrome scales for attention problems and social problems with higher parental ratings in DEX-treated girls. However, this difference did not either reach significance. Indications of increased problems with attention and anxiety have been found in follow-up studies investigating effects of prenatal maternal stress and in studies assessing children exposed to sGC in mid- to late gestation [115, 137, 139, 141].

In the American study from 1995, the DEX-treated children were rated by their parents as less sociable, more emotional and shy than the non-treated controls. They also had more internalizing problems and total behavioral problems [117]. However, this study had a mixture of healthy and CAH children (boys and girls) and the parental evaluations were made at an early age (mean=2.5±1.3 years, range: 6 months - 5.5 years). Their later extended study (mean=5.55±3.46 years, range: infancy - 12 years) found no differences in behavioral outcomes [116]. Similar results were found in the Polish cohort, with no effects on behavior due to prenatal DEX treatment [119].

Our interpretation is that there are no major behavioral effects of DEX treatment when using an indirect assessment method (parental questionnaires) [234]. However, despite the lack of significant results, we found differences between girls and boys when assessing anxiety. DEX-treated girls scored themselves as having more social anxiety in coherence with the sex-dimorphic results on cognition and metabolism. Girls seem to be more affected by prenatal GC exposure than males.

7.3 METABOLISM

In study III, the results indicate a negative effect on glucose metabolism due to prenatal DEX treatment. We speculate that this finding may have considerable impact on health as the individual grows older. If the reduced HOMA- β in females suggests that beta-cell activity is affected already in childhood, the risk of impaired glucose tolerance (IGT), impaired fasting glucose levels (IFG) and eventual type 2 diabetes will be considerable. Individuals with type 2 diabetes have a significantly greater risk of developing cardiovascular disease (CVD) than non-diabetic individuals [235, 236]. Notably, IFG alone is not a risk factor for developing CVD; instead, it seems as though IGT is a prominent risk factor [237].

HOMA- β , is an estimation of beta-cell function, is based on fasting levels of glucose and insulin. The method assumes that insulin secretion and is directly dependent on the coexisting plasma-glucose level. However, that may not always be true. If an individual's insulin sensitivity is extremely high, such as in athletes, the insulin level will be lower at the same glucose level, leading to a lower HOMA- β , even though there is a perfectly normal beta cell function. Thus, it is reasonable to propose that HOMA- β is a measure of insulin secretion rather than a measure of beta-cell function [238, 239]. However, our results showed neither a difference in insulin levels nor HOMA-IR. Consequently, we assume that the difference in HOMA- β indicates reduced beta-cell activity not caused by higher insulin sensitivity in the DEX-treated group.

Our findings are also coherent with a recently published French study in which healthy DEX-treated individuals were found to have a probable reduced beta-cell activity [173]. The insulin secretion was tested in multiple ways, not only by calculating HOMA- β values. They examined fasting blood samples, performed an oral OGTT and performed a euglycemic, hyperinsulinemic clamp. Further, they tested the insulin secretory response to i.v. glucose infusion and after arginine stimulation. The results showed a significantly lower insulin response in the DEX-treated individuals, which they argue is due to a probable decrease in beta-cell mass or function. They also estimated that the change in response in insulin

secretion might alter glucose tolerance with time [173]. These results strengthen our hypothesis that early gestation DEX treatment has a negative effect on beta-cell activity.

Our results are also in line with the many animal and human studies assessing long-term effects on glucose metabolism after prenatal GC exposure. In sheep, findings have revealed altered glucose homeostasis in adult offspring. The insulin levels were higher in adult female offspring after an intravenous glucose tolerance test suggesting increased insulin resistance [240, 241]. No effect could be found in male offspring, suggesting a possible sex-dimorphic effect. In humans, young adults exposed to GC treatment during late gestation because of the risk of being born preterm displayed a significantly lower HOMA- β compared to untreated controls [172]. Human studies assessing prenatal exposure to maternal stress have also shown similar results with impaired glucose tolerance in the exposed, especially if the stress exposure was early to mid-gestation [242, 243].

We also found higher total cholesterol and LDL cholesterol levels in the older age group of DEX-treated individuals, but no differences were found in the younger age group. The difference in the results between age groups may be caused by changes in the lipid-profile during childhood and puberty, with a natural decrease in serum total cholesterol, HDL and LDL cholesterol levels [244, 245]. Thus, the DEX-effect seen in older teenagers might be caused by an absence of the natural decline in blood lipid levels during puberty, which might explain the lack of results seen in the younger children as the decline has not yet begun. However, our attempt to divide the group according to age to detect possible pre- and post-pubertal effects, as earlier argued, might not have been sufficient in this regard. There was probably a mix of pre-pubertal and pubertal children in the younger age group, which might have obscured potential differences [246]. High cholesterol level, especially LDL, is a well-known risk factor for coronary heart disease (CHD). If the individuals also have reduced insulin sensitivity, the risk of CHD will increase even further [245].

The finding of a higher leukocyte count in the DEX-treated young cohort is difficult to interpret. Nevertheless, it could be speculated that the higher count is due to a programming effect on the immune system. We have previously found altered DNA methylation in peripheral T cells, although the effect on DNA methylation was mainly associated with immune functioning and inflammation, not specifically to leukocyte count [34]. GC treatment is also known to induce apoptosis in some cell types of the immune system (e.g. eosinophils and T cells) but in other cell types it may also inhibit apoptosis and induce survival, e.g. neutrophils [247].

Younger children have a wider reference interval in leukocyte count than older children and adults, which might explain why we see differences in this age group and not the older one. In that case, the result should be considered a false-positive [248]. Naturally, it may also be due to a random higher number of low-grade infections in the DEX-treated group. Our interpretation is that further studies are needed before any conclusion is drawn.

The finding of higher potassium levels in the DEX-treated group might be caused by an effect on glomeruli function or by altered aldosterone synthesis or function. Effects on the number of nephrons and their function have been shown in studies exposing rodents to prenatal GC [180, 249]. However, there are also studies in primates that contradict these findings [183]. Particularly noteworthy is that epigenetic changes associated with DEX were also found in our cohort (e.g., the CYP11B2-gene), which codes for aldosterone synthase, an enzyme involved in aldosterone synthesis [34].

7.4 BLOOD PRESSURE

Study IV, which assessed possible effects on the cardiovascular system, failed to show convincing results. The only significant finding regarding BP was a higher pulse pressure (PP) at nighttime in the younger cohort. PP is calculated as the difference between systolic BP (SBP) and diastolic BP (DBP). Increased PP is caused by an increased systolic blood pressure but an unaffected diastolic blood pressure and is known to be a risk factor in the development of heart disease in adults [250, 251]. The elasticity and compliance of the large arteries and flow pulsatility are the major factors in the composition of PP. Aortic elasticity declines with age, except in younger children in which there is no correlation between aortic stiffness and age until approximately 8 years [252].

Studies investigating the incidence of hypertension in school aged children have found that there is a higher incidence if elevated SBP than elevated DBP. Furthermore, elevated SBP, PP and heart rate have been shown to be associated with childhood obesity [253]. The suggested mechanism of higher SBP in children, is thought to be due to hyperactivity in the sympathetic nervous system rather than early stages of arteriosclerosis. Studies have shown that obese individuals have higher sympathetic activity even at normal BP [254]. We did not find any differences in BMI or heart rate between the groups in our study, but we have found effects on glucose- and lipid metabolism. Since there are correlations between insulin secretion and the sympathetic nervous system, a possible increase in sympathetic activity (that we have not investigated) might be one reason for the found reduced insulin secretion in these individuals [255].

Studies on *late* gestation GC exposure (in pregnancies at risk of preterm labor) have shown effects on aortic stiffness during adolescence or young adulthood but no findings on BP [170, 172]. Possibly is the increased aortic stiffness found in these studies also due to an increase in sympathetic activity and a risk factor in developing high BP at an older age.

8 ETHICAL CONSIDERATIONS

This research field and its endpoints are part of an ethical dilemma. Is it ethical to treat 7 of 8 children (or if early sex assessment is done, 3 of 4 children) to relieve suffering of one? Pediatric endocrinologists have discussed this question for many years and there remain diverse opinions on the matter. In many countries, prenatal DEX treatment is still available, however, in Sweden we have decided not to use this treatment because of the negative effects on cognition and the overall uncertainties of the treatment. It is vital to accumulate as much knowledge as possible regarding possible side effects of prenatal DEX treatment in otherwise healthy individuals to make a rational decision on its future use in the clinical care of families with a genetic risk of CAH. Our research is part of that process.

The discussion among health care professionals throughout the world concerns whether it is ethical to treat children that do not benefit from the treatment if the negative side effects are minor and what defines a “minor” side effect. Is an effect on cognition of importance if cognitive functions are still within the normal range? In many countries, the physicians believe that the benefit for the virilized girl outweighs the side effects that have been identified in treated cases. However, in Sweden, it was decided to halt further treatment until more knowledge was gathered about the long-term consequences. After several studies showing negative results in healthy individuals, our opinion persists in that the results are not “minor” and that the treatment is not justifiable.

Another ethical consideration is the actual process of enrolling and assessing the individuals in the study. Is it ethical to test these healthy (non-CAH) children and young adults to see determine whether they had suffered negative side effects of a treatment their mother decided to accept before they were born? One can speculate that this may interfere with the relationship between the mother and child. Some of the treated individuals did not even know that they were exposed to GC during gestation, which may also be an issue of conflict. Because of these ethical issues, we decided not to approach the adult participants directly but instead contacted the mothers to discuss the potential participation of their child. Thus, the child was contacted only after the mother had sanctioned it.

Another question to address is whether neuropsychological testing is always in the best interest of the individual being tested. All the children and their parents have been presented with the results after testing. If a child is found to have some problems with i.e. concentration, there are different reactions to that knowledge. Some families may be grateful and using the new information, they will be able to address the school to get a full evaluation and, in the

end, suitable help. Another family may feel differently by that result, especially if they had not seen any previous difficulties.

As for blood tests and ABPM in children, there is always a discussion about how much discomfort we can expose a child to and especially when the result is part of a study and there is no direct benefit for the child. Blood sampling may be scary for many children and sometimes it may hurt. Blood pressure measurements may also be perceived as a discomfort to many children. We have not tried to persuade the children nor the parent to perform the blood tests or ABPM if they were hesitant or scared. We have also used anesthetic plaster to numb the skin and always had an experienced pediatric nurse perform the drawing of blood. They were also told that they could stop the ABPM at any time if they wanted.

All participants and their parents received written and verbal information before signing a written consent. Approval from the Stockholm Research Ethics Committee was obtained before start of the study. All data-carrying personal information was coded to ensure anonymity.

9 CONCLUSION AND POINTS OF PERSPECTIVE

The results from the studies presented in this thesis, supports the hypothesis that prenatal DEX treatment is not to be considered safe. Additional findings reported from our research group support this statement. We have published several papers demonstrating adverse effects on cognitive functions in DEX-treated children [29, 31, 123]. Furthermore, we have found alterations in brain structure and differential DNA methylation associated with this treatment [34, 35]. In contrast to our findings, a recent review of 18 papers assessing prenatally DEX-treated individuals found no association with DEX treatment and cognition or behavior [217]. However, the review also observed that the papers differed in aspects previously mentioned, i.e. inclusion of a healthy control group and assessment methods. The paper also displays a table summarizing the quality criteria of the different studies, using the Newcastle-Ottawa assessment scale. The only research group that scored 9/9 was ours and the major difference was found in the criteria “Comparability”. This criteria comprise the inclusion of matched control groups and controls for additional important factors (e.g. parental educational level, parental socioeconomic status, geographic areas) [217].

Within the frame of the PREDEX study, we will continue investigating the possible effects of DEX treatment on the HPA axis using an ACTH stimulation test (Synacthen-test) and the effects on bone mineralization by density scans (DEXA scan). We also plan to do further assessments of the glucose-insulin homeostasis, such as an OGTT and ongoing studies on brain structure and brain function will hopefully further elucidate the neural correlations underlying the observed cognitive deficits.

The fact that we see cognitive effects in children but not adults makes it even more important to perform even longer follow-up, when all the Swedish DEX-treated children have become adults. The adult DEX cohort will then be much larger than today and we may then get more convincing results. The same argument is also eligible for the findings on metabolic effects of DEX treatment where the age groups differ in their results, making longer follow needed.

Looking ahead, it is necessary to increase our knowledge of how prenatal DEX treatment affects non-CAH individuals, in order to get an international consensus whether the treatment should be offered or not. Not all countries agree with the Swedish standpoint in the matter. New, and even earlier, prenatal diagnosis is needed to avoid treating the fetuses that do not benefit from DEX therapy. However, the possible negative effects on girls with CAH must also be addressed. Thus, the worldwide medical society should use this therapy with caution.

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