LONG-TERM STEROID HORMONE CONCENTRATIONS IN HEALTHY CHILDREN, IN OBESITY, AND IN DISEASE

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Lange termijn steroïde hormoon concentraties in gezonde kinderen, in obesitas, en in ziekte

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CHAPTER 1

GENERAL INTRODUCTION

Hypothalamus – Pituitary – Adrenal axis

Cortisol production by the adrenals is primarily an effect of activity of the hypothalamus – pituitary – adrenal (HPA) axis. The median paraventricular nucleus (PVN) in the hypothalamus contains neurons producing corticotrophin-releasing hormone (CRH), which is released in to the hypophyseal portal system. CRH activates corticotroph cells in the anterior pituitary, together termed the corticotroph, by binding the G protein-coupled CRH-receptor 1.[1,2] Upon CRH stimulation, the corticotroph cells both secrete adrenocorticotropic hormone (ACTH) and increase its synthesis by cleaving pro-opiomelanocortin (POMC). The secreted ACTH subsequently binds to the melanocortin-2 receptor on the cell-membrane of cells in the zona fasciculata of the adrenal cortex.[1,3] ACTH stimulation of the zona fasciculata both maintains the endocrine tissue in this part of the adrenal cortex, and stimulates the secretion of cortisol into the bloodstream.[4] Increased serum cortisol concentrations in turn decrease CRH and ACTH secretion, forming a negative feedback loop (figure 1.1).[1] Cortisol exhibits negative feedback at the level of the pituitary, hypothalamus and higher brain areas affecting the HPA-axis activity. Inhibition of ACTH production by cortisol happens within seconds to minutes and is dose-dependent, and lasts as long as serum cortisol concentrations are rising. Approximately 2 to 4 hours later, ACTH secretion shows a delayed inhibition lasting several hours up to a day. Ultimately, an increase in cortisol concentrations can reduce basal ACTH production and secretion.[5]

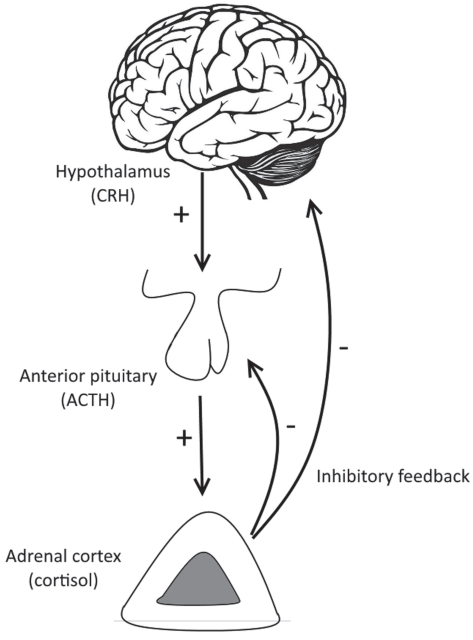

Figure 1. Hypothalamus – Pituitary – Adrenal axis, a negative feedback loop.

Cholesterol is the main precursor for steroid synthesis. Cholesterol is taken up from the circulation in the form of low- density lipoprotein (LDL) by endocytosis. LDL is hydrolyzed to cholesterol, and converted to, amongst other steroids, cortisol through an enzymatic cascade in the mitochondria and endoplasmic reticulum, as shown in figure 1.2.[6] Total cortisol synthesis is on average 10-20 mg/day in adults, and approximately 10 mg/day in children.[6,7] Around 95% of circulating serum cortisol is protein-bound, primarily to cortisol-binding globulin (CBG), but also to albumin. Cortisol measured in serum is the cumulative of free and bound cortisol. Some factors are known to influence CBG concentrations, such as estrogens, liver cirrhosis, and hyperthyroidism, these factors also change the serum cortisol concentration. However, the free circulating cortisol concentration, which is the metabolically active fraction, remains unchanged.[6] Free cortisol is lipophilic, and readily diffuses across the plasma membrane. Within the cytoplasm, cortisol binds to the glucocorticoid receptor (GR). Upon binding its ligand, the GR dissociates from a protein complex in the cytoplasm and is translocated into the cell nucleus, where it exerts its effect either by directly binding the glucocorticoid response elements on the DNA, or through forming complexes with transcription factors. As a result, transcription of several hundred to thousand genes are either up- or down-regulated.[6,8] The GR is expressed in nearly every human peripheral tissue. The affinity of cortisol for the mineral corticoid receptor (MR) is even greater than for the GR. MR expression is limited to several tissues, in particular the kidneys, saliva glands and the colon. To prevent binding of cortisol to

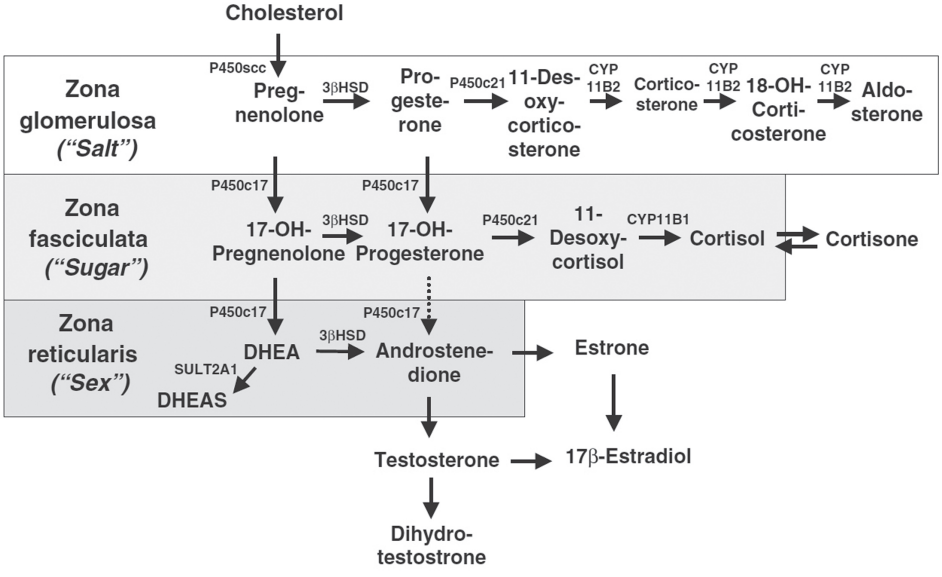

Figure 2. Adrenal steroid synthesis cascade.
Adapted from Arlt and Stewart, 2005.[6]

the MR in these cells, cortisol is converted to inactive cortisone by 11 β-hydroxysteroid dehydrogenase type 2 (11βHSD2).[6] The MR does play a role in GC signaling in the brain and is implicated in the negative feedback loop at the level of the hypothalamus and anterior pituitary.[9]

The extensive mechanism regulating HPA-axis activity is subject to a variety of factors. Through synapses between the suprachiasmatic nucleus and the PVN, the HPA axis is controlled by the circadian clock. Additionally, the central clock system, located to the SCN, influences the ACTH sensitivity of the zona fasciculata. This results in the highest cortisol peak early in the morning, prior to awakening, and lowest concentrations in late evening and night (Figure 1.3), with additional smaller peaks due to daily events such as eating.[1,10] Glucocorticoids are essential for normal life, but even more so in adverse conditions. In 1950, Selye already recognized the adaptive reaction of HPA-axis activation in the case of stress, resulting in a sharp increase in the production and secretion of glucocorticoids, to prepare the body for stressful circumstances.[11] Increase of the HPA-axis activity occurs in seconds after the onset of a stressor, which is followed by an increase in cortisol in several minutes. When the stressor becomes chronic, so does the increased HPA-activity, which is further supported by a sensitization for glucocorticoids.[12] Both psychological and physiological stress, either exogenous or endogenous, all cause an increase in HPA-axis activity[13-15], but the increase in activity is stressor-specific, which is mainly regulated by neurons in the hypothalamic PVN.[15] Stress responses may however be out of balance with the stressor, which could in certain instances result in stress-related disease.[16]

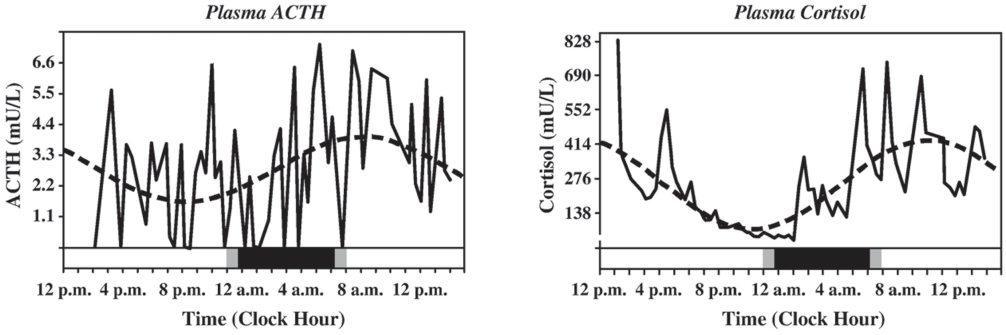

Figure 3. Twenty-four-hour plasma ACTH and cortisol curve.
Adapted from Haus et al, 2007.[17]

Measuring Cortisol

A variety of methods for quantifying steroids have been developed, of which two are mainly applied in practice. One of these methods, immunoassay, is based on the specificity of antibodies against cortisol. Several variations of immunoassays

have been developed, using different antibody sources which may be enzyme, ligand or radiolabel- linked, or make use of competitive binding of enzyme-linked cortisol versus endogenous cortisol in the sample. Immunoassays provide several benefits, particularly the relative low costs, easy applicability and no need for highly specialized personnel. This makes immunoassays a popular and widely-used method. Nevertheless, immunoassays are also subject to several limitations, especially the limited sensitivity and potential cross-reactivity with other components. Another more recently developed method for steroid quantification is the combination of liquid chromatography with mass spectrometry (Liquid Chromatography tandem Mass Spectrometry, LC-MS/MS). With this method, steroids are separated, mainly based on polarity, after which the molecules are ionized and separated based on mass over charge ratio. LC-MS/MS allows for a superior sensitivity compared to immunoassays, requiring lower sample volumes, and does not suffer from cross reactivity. These benefits make LC-MS/MS to be the advocated method for steroid quantification.[18] On the other hand, the specialized equipment is costly in acquisition and maintenance, and requires highly trained staff. Another advantage for LC-MS/MS is that it provides the option of quantifying multiple components simultaneously, which may help in reducing the relative costs per sample/measurement.

Cortisol is routinely measured in plasma, urine and saliva. As cortisol concentrations vary strongly during the day, the time point of measurement should be taken into account. In addition, acute stress may lead to physiologically elevated cortisol concentrations, which is especially relevant in plasma measurement which requires a venipuncture. Saliva cortisol may be falsely elevated when small amounts of blood are present in the saliva, because cortisol concentrations are much higher in blood compared to saliva, so even minute blood traces may contribute significantly. In urinalysis, urine is collected during a 24h period, giving information on the average cortisol concentrations during those 24 hours. Urine collection is however highly dependent on patient compliance, as urine may in some circumstances proof challenging. This also has implications for the reliability of the measurement. In plasma, total cortisol concentrations are measured, whereas in saliva and urine the cortisol concentrations reflect the free and unconjugated fraction, respectively. The total cortisol concentration is dependent on CBG concentrations, which can vary due to e.g oral anticonception, pregnancy, and insulin, changing the total but not the free plasma cortisol concentrations. These matrices are suited for assessing cortisol reactivity, but may be less fitting for long-term cortisol measurement. Recent studies have applied human scalp hair as a matrix for cortisol measurement. Scalp hair grows steadily at 1 cm per month, which provides the possibility to measure average cortisol concentrations of a selected period of weeks to multiple months, and scalp hair cortisol concentrations are not influenced by acute stress. In chapter 3 we describe a LC-MS/MS based method for cortisol and steroid hormone profiling in human scalp hair. This method was further evaluated and reference ranges are described, as presented in chapter 4.

Cortisol effects

Glucocorticoids (GCs) affect myriad physiological processes in the human body.[6] Cortisol effects are mainly mediated through the glucocorticoid receptor, which is expressed in virtually all cells, and regulates transcription of up to 20% of the human genome.[8] The cortisol-GR complex interacts with numerous cofactors, conferring tissue-specific cortisol effects.[19] One of the most well-known glucocorticoid effects is its anti-inflammatory effect. To this, glucocorticoids have to thank their popularity as therapeutic drug in inflammatory and auto-immune disease.[20] In fact, cortisol has a wide-spread effect on the immune-system, amongst others reducing the lymphocyte and eosinophil counts in blood by redistribution and lymphocyte apoptosis, and suppression of pro-inflammatory cytokine expression.[21] Glucocorticoids also influence the glucose metabolism, which becomes apparent by the rise in cortisol concentration due to hypoglycemia. Cortisol increases blood glucose levels by stimulation hepatic gluconeogenesis and reducing insulin sensitivity in peripheral tissues.[22] Moreover, GCs are important regulators of lipid metabolism and adipose tissue, although the exact mechanisms and effects in physiological conditions are not yet fully understood. In hypercortisolemic conditions, adipogenesis occurs with a characteristic distribution toward increased abdominal adipose tissue, which is metabolically adverse. Triglyceride concentrations in blood increase and triglyceride accumulation in the liver is promoted by GCs.[23,24] Glucocorticoids are further implicated in stress-related behavior, influence other endocrine systems, have effects on the cardiovascular system, affects skin, muscle and bone morphology, growth, and development.[6] In children, an important complication of hypercortisolism in addition to the previous symptoms mentioned is growth retardation, causing a reduced adult height.[25] Glucocorticoids are essential for normal life, but may have detrimental effects in supra-physiological concentrations, as becomes apparent in Cushing's syndrome, which is discussed in the following paragraph.

HPA – axis abnormalities

Malfunctions in the HPA- axis may either lead to hyper- or hypocortisolism, which, taken together with the secondary effects and associated complications, have major health consequences and adverse effect on the quality of life, and may even be life-threatening.

Cushing's syndrome and disease

Chronic hypercortisolism, resulting in Cushing's syndrome (CS), can lead to a broad spectrum of symptoms. However, the clinical presentation may also be subtle and changing, which is easily overlooked. Indeed, the average time from disease onset to diagnosis is 3 to 5 years. CS is thought to be a rare disorder, with an incidence of approximately 2-3 per million persons per year. However, as the diagnosis is frequently missed and the prevalence is higher in selected patient groups with e.g. obesity or diabetes, CS may be more common than studies report.[26] Patients with

adrenal incidentaloma's and subclinical hypercortisolism have improved glucose metabolism, lower blood pressure, and reduced body weight after adrenal surgery, which underscores the importance of also identifying subtle cases of CS.[27,28] Approximately 70% of endogenous CS cases are ACTH-dependent, either produced by a pituitary adenoma (Cushing's disease (CD)) or by an ectopic tumor. In 30%, cortisol is produced autonomously in the adrenal, either by a adrenal adenoma or carcinoma, or by micro- or macronodular adrenal hyperplasia.[29] The clinical manifestations of CS are diverse, and may comprise of adipose tissue accumulation in the face, neck, and trunk (abdomen), weight gain, thinning skin with striae, proximal muscular weakness and atrophy, hypertension, glucose intolerance, fatigue, depressive symptoms, acne, hirsutism, hypertension, dyslipidemia, oligo-/amenorrhea, osteoporosis, and in children growth retardation. Treatment is focused on the underlying etiology, but even after biochemical remission, the long-term mortality rate remains increased.[30]

Hypocortisolism

Hypocortisolism, or adrenal insufficiency (AI), may find its origin on all levels of the HPA-axis. A deficiency in cortisol production due to disease within the adrenal cortex, termed primary AI, is most often caused by autoimmune adrenalitis (Addison's disease), which may be part of an autoimmune polyglandular syndrome, responsible for approximately 80% of primary AI cases. A genetic mutation resulting in cortisol synthesis enzymes deficiency is another cause, which causes a syndrome called congenital adrenal hyperplasia (CAH). No treatment existed until 1949, when cortisone treatment became available, which turned a lethal disease into a chronic one as life-long GC replacement therapy is needed.[31] Another 10% of primary AI cases is caused by tuberculosis. Symptoms are mostly nonspecific, such as weakness, fatigue, anorexia, and weight loss. Adrenal insufficiency may however result in a life-threatening adrenal crisis, with severe hyponatremia and hypovolemic shock, often induced by physiological stress or illness. Characteristic for primary AI is hyperpigmentation of the skin, specifically on skin areas exposed to pressure, e.g elbows, palmar creases and the lips. This is caused by stimulation of the melanocortin-1 receptor by high concentrations of ACTH.[32] In secondary AI, the pituitary function is affected, causing ACTH deficiency. Causes are multiple, e.g. prolonged use of exogenous GC's, neoplasia, head injury, radiotherapy, pituitary surgery, genetic, and autoimmune, and the HPA-axis is often not the only endocrine axis affected. Symptoms and treatment are comparable to primary AI, with exception of the ACTH induced hyperpigmentation and normal aldosterone levels and hence sodium and water homeostasis.[33] The etiology of tertiary AI lies at the hypothalamus, and is most commonly induced by long-term glucocorticoid treatment.[32] Hydrocortisone (synthetic glucocorticoid identical to cortisol) thrice daily is the advised GC replacement therapy regimen in AI, with a dosing tapered to the individual.[31] However, it remains difficult to mimic the endogenous cortisol secretion, which is reflected by the increased mortality ratio, mainly due to cardiovascular and

infectious disease.[34,35] The long-term cortisol concentrations in children with adrenal hyperplasia are compared with concentrations in healthy children in chapter 7.

Congenital adrenal hyperplasia

A special case of primary AI is congenital adrenal hyperplasia (CAH). CAH is caused by a genetic defect in one of the enzymes of the steroid synthesis pathway, leading to a decreased cortisol synthesis capacity, with subsequent increase in CRH and ACTH, causing adrenal hyperplasia. In 95% of the cases, the genetic defect lies in the gene for 21-hydroxylase (Figure 1.2, P450c21) and inherits as a autosomal recessive disorder.[36] In case of 21-hydroxylase deficiency, the androgen precursor 17α-hydroxyprogesterone (17OHP) is no longer converted into 11-deoxycortisol, which results in an overflow of androgen precursors which are secreted and converted into active androgens (e.g. testosterone) in peripheral tissues. This androgen excess causes prenatal virilization in girls, leading to ambiguous genitalia at birth, and increased growth rate with premature closure of the epiphyseal plates. The severity of the phenotype is mainly affected by the remaining enzymatic functionality; less than 20% causes classical CAH, with severe cortisol deficiency and androgen excess. When enzyme function is less than 2%, aldosterone synthesis is affected as well, resulting in salt wasting classical CAH, comprising approximately 75% of classical CAH patients. From approximately 20 to 50% enzyme function, cortisol production is normal but androgen excess causes premature pubarche, acne, hirsutism and oligo- or amenorrhea, and is often not diagnosed until childhood or adolescence. This form is called non-classic CAH. Nowadays, CAH is part of postnatal screening, but before this was introduced classic CAH was often lethal in the first weeks of life.[36,37] In classical CAH, hydrocortisone treatment is not only focused on GC replacement, but also on reducing androgen excess by decreasing ACTH production. This calls for supra-physiological GC dosing, with adverse side-effects of its own, forcing the clinician to balance between GC overdosing with subsequent Cushing syndrome on one hand, and androgen excess with virilization, reduced fertility, or premature epiphyseal fusion on the other.[38,39] In chapter 8, measurement of long-term cortisol and androgen precursor hormones are evaluated as monitoring tool in treatment follow-up in children with CAH.

Cortisol sensitivity

Hypercortisolism without Cushingoid features may indicate a reduced cortisol sensitivity, as is the case in generalized glucocorticoid resistance. This is a condition which is usually the result of mutations in the glucocorticoid receptor gene, causing a defective cortisol signaling cascade. In these patients, CRH and ACTH are elevated, increasing cortisol production by the adrenals, because the GR is also important in the negative feedback of cortisol at the hypothalamus and pituitary. As the peripheral tissue is resistant against the higher cortisol levels, no Cushingoid symptoms develop. However, the increased adrenal stimulation may also increase adrenal androgen

synthesis, with negative effects such as acne, hirsutism, masculinization, precocious puberty and fertility problems. Additionally, the increased cortisol concentrations may induce increased mineralocorticoid receptor stimulation, causing hypokalemia and hypertension, in case the 11β-hydroxysteroid dehydrogenase type 2 capacity is overcome in kidney cells.[40-42] Glucocorticoid resistance syndromes are rare. However, the high variability in both therapeutic and side-effects of glucocorticoid treatment suggests that a more subtle variation in glucocorticoid sensitivity is rather common. This variation in glucocorticoid sensitivity is, at least in part, attributable to the GR gene. The GR is encoded by the *NR3C1* gene, spanning approximately 150kb, including 9 exons. This gene gives rise to a number of different translational isoforms and alternative splice variants of the GR. Alternative splicing at the 3'-end results in use of alternative exons 9, giving rise to GR-α and GR-β. Whereas GR-α is a ligand-dependent transcription factor, affecting transcription both directly and in concert with cofactors upon cortisol binding, GR-β does not bind ligand and is transcriptionally inactive. GR-β is thought to function as a dominant negative inhibitor of GR-α.[43] A single nucleotide polymorphism (SNP, rs6198, GR-9β, minor allele frequency 0.09) located in the 3' untranslated region of GR-β increases GR-β mRNA stability, and subsequent increased expression of GR-β. This has been associated with a relative GC resistance, causing a pro-inflammatory phenotype with increased risk of amongst others rheumatoid arthritis and systemic lupus erythematosus, and has later been associated with increased risk of cardiovascular risk.[44-46] Another SNP, with a minor allele frequency of 0.013, ER22/23EK (rs6189 and rs6190), results in a relative increased expression of the translational isoform GRα-A compared to GRα-B. As GRα-B is a more active in GRE transactivation, the increased GRα-A levels result in a relative GC resistance, with a phenotype different from the GC resistance due to GR-9β. ER22/23EK has been associated with a beneficial metabolic profile.[47-49] Although the exact molecular mechanism has not been clarified yet, the SNP N363S (rs56149945) has been shown to increase the GR transactivating capacity and results in increased glucocorticoid sensitivity with adverse metabolic consequences.[46,50,51] *Bcl*I, a SNP with a minor allele frequency of 0.28, is also associated with glucocorticoid hypersensitivity, through an unknown molecular mechanism. *Bcl*I is associated with a decreased risk for autoimmunity but, like N363S, with adverse metabolic parameters.[43,46] The associations of SNP's in the GR gene with obesity and body fat distribution in children have been assessed in chapter 6.

Cortisol effects on a population level

In Cushing's syndrome, with severe hypercortisolism, patients develop an adverse metabolic profile with obesity, hyperglycemia, dyslipidemia and hypertension. These symptoms together form the Metabolic syndrome. These symptoms also constitute (part of) the side-effects of glucocorticoid treatment. Also in subtler conditions, e.g. in subclinical hypercortisolism and slightly increased cortisol sensitivity, these adverse metabolic effects are observed.[27,28,43]

Obesity and the metabolic syndrome are major health concerns, affecting 1 in every 5 adults in the western world. Risks of subsequent disease are increased, most notably for cardiovascular disease. Childhood obesity prevalence has tripled in the past decades. As a result, 17% of all US children are currently obese. Worldwide, approximately 40 million children under the age of 5 years are obese, both in the western world and developing countries. Childhood obesity is an important risk factor for premature onset of the metabolic syndrome and cardiovascular disease in later life.[52,53]

The observation that glucocorticoids can induce obesity and subsequent metabolic complications, already in relatively subtle situations such as subclinical hypercortisolism and increased cortisol sensitivity, raises the question whether intra-individual variations may be implicated in the onset of obesity in the general population. This hypothesis has received extensive attention in studies with contradicting results, eventually leading to a study and review by Abraham *et al*.[54] in 2013, concluding that no strong associations between cortisol and obesity could be established. These studies based their research on measuring cortisol in saliva, plasma and/or urine. Although these matrices are suitable for cortisol measurement, they represent cortisol concentrations of only a pinpoint in time, with the exception of urine which is usually measured over a 24-hour span. As cortisol varies strongly due to circadian rhythm, acute stress and pulsatile excretion, these matrices may not be the most suited for establishing long-term cortisol effects. Recently, methods to measure long-term cortisol exposure have been developed, which may yield new insights into the relation between cortisol and metabolic adversities.[55,56] In chapters 5 and 6, the relation between long-term glucocorticoids and childhood obesity are assessed.

Thesis outline and aims

This thesis can be subdivided in two parts. The first part focusses on validating a novel method for measuring long-term cortisol concentrations in scalp hair, which has been developed in adults, for use in pediatric clinical practice and research. Additionally, we focused on improving this method by developing a LC-MS/MS based method for scalp hair measurement and validating this method in children as well.

In the second part of this thesis, both the immune-assay based and LC-MS/MS based methods are applied to challenges in clinical research and practice. The associations between glucocorticoids and metabolic adversities appear convincing in the case of Cushing's disease, but remain to be proven in more subtle forms of hypercortisolism. Recent studies provide strong support for a positive association between long-term cortisol measured in scalp hair and cardiovascular disease and the metabolic syndrome in adults.[57,58] We assessed the relation between long-term cortisol and obesity in children. Additionally, we evaluated measurement of long-term cortisol concentrations and adrenal precursor hormones as a monitoring tool in the follow-up of hydrocortisone treatment in children with adrenal insufficiency and congenital adrenal hyperplasia.

In chapter 2 we aimed at validating the immunoassay based scalp hair cortisol measurement in children aged 4–14 years by assessing the effects of factors potentially influencing hair cortisol concentrations, such as age, gender, puberty, use of hair products, washing frequency and distance to scalp variation. Additionally, we aimed to establish reference ranges for use in clinical practice. In chapter 3, the aim was to establish a LC-MS/MS based method for long-term glucocorticoid and steroid profiling in scalp hair. The aim of chapter 4 was to establish reference ranges of long-term glucocorticoid and steroid hormones in healthy children aged 4-18 years, and assess the effects of potentially confounding factors as described in the aim of chapter 2.

Chapter 5 is a pilot study in which we aimed to assess the association of long-term cortisol with childhood obesity. In this chapter 6, the aim was to analyze the associations of long-term glucocorticoid concentrations with childhood obesity and body fat distribution. In this study, we additionally aimed to evaluate the effects of SNPs in the GR gene on childhood obesity.

To assess on how good hydrocortisone treatment in children with adrenal insufficiency resembles physiological cortisol synthesis in the long term, we compared long-term cortisol concentrations in children with adrenal insufficiency with concentrations measured in healthy age and gender matched controls in chapter 7. In chapter 8 we aimed to evaluate the measurement of long-term cortisol and androgen precursor hormones in scalp hair as monitoring tool in the treatment follow-up of children with congenital adrenal hyperplasia.

Chapter 9 contains the general discussion of the studies performed, along with the implications of the results and suggestions for further research.

REFERENCES

1. Jacobson L. Hypothalamic-pituitary-adrenocortical axis regulation. *Endocrinol Metab Clin North Am* 2005; **34**(2): 271-92, vii.
2. Sam S, Frohman LA. Normal physiology of hypothalamic pituitary regulation. *Endocrinol Metab Clin North Am* 2008; **37**(1): 1-22, vii.
3. Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. *Science* 1992; **257**(5074): 1248-51.
4. Thomas M, Keramidas M, Monchaux E, Feige JJ. Dual hormonal regulation of endocrine tissue mass and vasculature by adrenocorticotropin in the adrenal cortex. *Endocrinology* 2004; **145**(9): 4320-9.
5. Keller-Wood ME, Dallman MF. Corticosteroid inhibition of ACTH secretion. *Endocr Rev* 1984; **5**(1): 1-24.
6. Arlt W, Stewart PM. Adrenal corticosteroid biosynthesis, metabolism, and action. *Endocrinol Metab Clin North Am* 2005; **34**(2): 293-313, viii.
7. Linder BL, Esteban NV, Yergey AL, Winterer JC, Loriaux DL, Cassorla F. Cortisol production rate in childhood and adolescence. *J Pediatr* 1990; **117**(6): 892-6.
8. Oakley RH, Cidlowski JA. The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. *J Allergy Clin Immunol* 2013; **132**(5): 1033-44.
9. Reul JM, Gesing A, Droste S, et al. The brain mineralocorticoid receptor: greedy for ligand, mysterious in function. *Eur J Pharmacol* 2000; **405**(1-3): 235-49.
10. Nicolaides NC, Charmandari E, Chrousos GP, Kino T. Circadian endocrine rhythms: the hypothalamic-pituitary-adrenal axis and its actions. *Ann N Y Acad Sci* 2014; **1318**: 71-80.
11. Selye H. Stress and the general adaptation syndrome. *Br Med J* 1950; **1**(4667): 1383-92.
12. Dallman MF, Pecoraro NC, La Fleur SE, et al. Glucocorticoids, chronic stress, and obesity. *Prog Brain Res* 2006; **153**: 75-105.
13. Boonen E, Van den Berghe G. Understanding the HPA response to critical illness: novel insights with clinical implications. *Intensive Care Med* 2015; **41**(1): 131-3.
14. Kyrou I, Tsigos C. Stress hormones: physiological stress and regulation of metabolism. *Curr Opin Pharmacol* 2009; **9**(6): 787-93.
15. Pacak K, Palkovits M. Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. *Endocr Rev* 2001; **22**(4): 502-48.
16. de Kloet ER, Joels M, Holsboer F. Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 2005; **6**(6): 463-75.
17. Haus E. Chronobiology in the endocrine system. *Adv Drug Deliv Rev* 2007; **59**(9-10): 985-1014.
18. Handelsman DJ, Wartofsky L. Requirement for mass spectrometry sex steroid assays in the Journal of Clinical Endocrinology and Metabolism. *J Clin Endocrinol Metab* 2013; **98**(10): 3971-3.
19. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* 1999; **20**(3): 321-44.
20. Kavanaugh A, Wells AF. Benefits and risks of low-dose glucocorticoid treatment in the patient with rheumatoid arthritis. *Rheumatology (Oxford)* 2014; **53**(10): 1742-51.
21. McKay LI, Cidlowski JA. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr Rev* 1999; **20**(4): 435-59.
22. Mueller KM, Themanns M, Friedbichler K, et al. Hepatic growth hormone and glucocorticoid receptor signaling in body growth, steatosis and metabolic liver cancer development. *Mol Cell Endocrinol* 2012; **361**(1-2): 1-11.
23. Lee MJ, Pramyothin P, Karastergiou K, Fried SK. Deconstructing the roles of glucocorticoids in adipose tissue biology and the development of central obesity. *Biochim Biophys Acta* 2014; **1842**(3): 473-81.
24. Rose AJ, Herzig S. Metabolic control through glucocorticoid hormones: an update. *Mol Cell Endocrinol* 2013; **380**(1-2): 65-78.
25. Hoppmann J, Wagner IV, Junghans G, et al. How early can one diagnose Cushing's disease? An early diagnosis in a case of prepubertal Cushing's disease. *Journal of pediatric endocrinology & metabolism : JPEM* 2014; **27**(11-12): 1043-7.
26. Findling JW, Raff H. Screening and diagnosis of Cushing's syndrome. *Endocrinol Metab Clin North Am* 2005; **34**(2): 385-402, ix-x.
27. Terzolo M, Pia A, Ali A, et al. Adrenal incidentaloma: a new cause of the metabolic syndrome? *J Clin Endocrinol Metab* 2002; **87**(3): 998-1003.
28. Reincke M, Nieke J, Krestin GP, Saeger W, Allolio B, Winkelmann W. Preclinical Cushing's syndrome in adrenal "incidentalomas": comparison with adrenal Cushing's syndrome. *J Clin Endocrinol Metab* 1992; **75**(3): 826-32.
29. Arnaldi G, Mancini T, Tirabassi G, Trementino L, Boscaro M. Advances in the epidemiology,

pathogenesis, and management of Cushing's syndrome complications. *J Endocrinol Invest* 2012; **35**(4): 434-48.

30. van Haalen FM, Broersen LH, Jorgensen JO, Pereira AM, Dekkers OM. Mortality remains increased in Cushing's disease despite biochemical remission: a systematic review and meta-analysis. *Eur J Endocrinol* 2015; **172**(4): R143-R9.
31. Grossman A, Johannsson G, Quinkler M, Zelissen P. Perspectives on the management of adrenal insufficiency: clinical insights from across Europe. *Eur J Endocrinol* 2013; **169**(6): R165-75.
32. Charmandari E, Nicolaides NC, Chrousos GP. Adrenal insufficiency. *Lancet* 2014; **383**(9935): 2152-67.
33. Toogood AA, Stewart PM. Hypopituitarism: clinical features, diagnosis, and management. *Endocrinol Metab Clin North Am* 2008; **37**(1): 235-61, x.
34. Bensing S, Brandt L, Tabaroj F, et al. Increased death risk and altered cancer incidence pattern in patients with isolated or combined autoimmune primary adrenocortical insufficiency. *Clin Endocrinol (Oxf)* 2008; **69**(5): 697-704.
35. Giordano R, Marzotti S, Balbo M, et al. Metabolic and cardiovascular profile in patients with Addison's disease under conventional glucocorticoid replacement. *J Endocrinol Invest* 2009; **32**(11): 917-23.
36. Claahsen-van der Grinten HL, Stikkelbroeck NM, Otten BJ, Hermus AR. Congenital adrenal hyperplasia--pharmacologic interventions from the prenatal phase to adulthood. *Pharmacol Ther* 2011; **132**(1): 1-14.
37. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Endocr Rev* 2000; **21**(3): 245-91.
38. Speiser PW, Azziz R, Baskin LS, et al. A Summary of the Endocrine Society Clinical Practice Guidelines on Congenital Adrenal Hyperplasia due to Steroid 21-Hydroxylase Deficiency. *Int J Pediatr Endocrinol* 2010; **2010**: 494173.
39. Stikkelbroeck NM, Otten BJ, Pasic A, et al. High prevalence of testicular adrenal rest tumors, impaired spermatogenesis, and Leydig cell failure in adolescent and adult males with congenital adrenal hyperplasia. *J Clin Endocrinol Metab* 2001; **86**(12): 5721-8.
40. Kino T, De Martino MU, Charmandari E, Mirani M, Chrousos GP. Tissue glucocorticoid resistance/hypersensitivity syndromes. *J Steroid Biochem Mol Biol* 2003; **85**(2-5): 457-67.
41. Malchoff CD, Malchoff DM. Glucocorticoid resistance and hypersensitivity. *Endocrinol Metab Clin North Am* 2005; **34**(2): 315-26, viii.
42. van Rossum EF, Lamberts SW. Glucocorticoid resistance syndrome: A diagnostic and therapeutic approach. *Best Pract Res Clin Endocrinol Metab* 2006; **20**(4): 611-26.
43. Gross KL, Cidlowski JA. Tissue-specific glucocorticoid action: a family affair. *Trends Endocrinol Metab* 2008; **19**(9): 331-9.
44. Derijk RH, Schaaf MJ, Turner G, et al. A human glucocorticoid receptor gene variant that increases the stability of the glucocorticoid receptor beta-isoform mRNA is associated with rheumatoid arthritis. *J Rheumatol* 2001; **28**(11): 2383-8.
45. van den Akker EL, Russcher H, van Rossum EF, et al. Glucocorticoid receptor polymorphism affects transrepression but not transactivation. *J Clin Endocrinol Metab* 2006; **91**(7): 2800-3.
46. Koper JW, van Rossum EF, van den Akker EL. Glucocorticoid receptor polymorphisms and haplotypes and their expression in health and disease. *Steroids* 2014; **92**: 62-73.
47. van Rossum EF, Koper JW, Huizenga NA, et al. A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids in vivo, is associated with low insulin and cholesterol levels. *Diabetes* 2002; **51**(10): 3128-34.
48. van Rossum EF, Voorhoeve PG, te Velde SJ, et al. The ER22/23EK polymorphism in the glucocorticoid receptor gene is associated with a beneficial body composition and muscle strength in young adults. *J Clin Endocrinol Metab* 2004; **89**(8): 4004-9.
49. Russcher H, van Rossum EF, de Jong FH, Brinkmann AO, Lamberts SW, Koper JW. Increased expression of the glucocorticoid receptor-A translational isoform as a result of the ER22/23EK polymorphism. *Mol Endocrinol* 2005; **19**(7): 1687-96.
50. Manenschijn L, van den Akker EL, Lamberts SW, van Rossum EF. Clinical features associated with glucocorticoid receptor polymorphisms. An overview. *Ann N Y Acad Sci* 2009; **1179**: 179-98.
51. Russcher H, Smit P, van den Akker EL, et al. Two polymorphisms in the glucocorticoid receptor gene directly affect glucocorticoid-regulated gene expression. *J Clin Endocrinol Metab* 2005; **90**(10): 5804-10.
52. Daniels SR, Arnett DK, Eckel RH, et al. Overweight in children and adolescents:

pathophysiology, consequences, prevention, and treatment. *Circulation* 2005; **111**(15): 1999-2012.

53. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of obesity and trends in body mass index among US children and adolescents, 1999-2010. *JAMA* 2012; **307**(5): 483-90.
54. Abraham SB, Rubino D, Sinaii N, Ramsey S, Nieman LK. Cortisol, obesity, and the metabolic syndrome: a cross-sectional study of obese subjects and review of the literature. *Obesity (Silver Spring)* 2013; **21**(1): E105-17.
55. Manenschijn L, Koper JW, Lamberts SW, van Rossum EF. Evaluation of a method to measure long term cortisol levels. *Steroids* 2011; **76**(10-11): 1032-6.
56. Sauve B, Koren G, Walsh G, Tokmakejian S, Van Uum SH. Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clin Invest Med* 2007; **30**(5): E183-91.
57. Manenschijn L, Schaap L, van Schoor NM, et al. High long-term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease. *J Clin Endocrinol Metab* 2013; **98**(5): 2078-83.
58. Stalder T, Kirschbaum C, Alexander N, et al. Cortisol in hair and the metabolic syndrome. *J Clin Endocrinol Metab* 2013; **98**(6): 2573-80.

PART I

LONG-TERM STEROID HORMONE CONCENTRATIONS IN HEALTHY CHILDREN

METHODS AND REFERENCE RANGES

CHAPTER 2

VALIDATION AND REFERENCE RANGES OF HAIR CORTISOL MEASUREMENT IN HEALTHY CHILDREN

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ABSTRACT

Background

Cortisol is produced in a circadian rhythm controlled by the hypothalamus-pituitary-adrenal axis, making it cumbersome to measure long-term cortisol exposure. Hair has proven to be a reliable matrix for long-term cortisol measurement in adults and can be used as diagnostic tool for (cyclic) Cushing's syndrome. The diagnostic applicability in children has not been studied, nor have the effects of development and hair care been evaluated in children. We aimed to establish reference ranges of hair cortisol concentrations (HCC) in healthy children and to evaluate the effects of age, gender, puberty and characteristics of hair care.

Methods

In 128 healthy children aged 4-14yr, HCC were measured in a small 3cm hair-lock from the back of the head.

Results

HCC increased with age (p=0.04) up to age 10yr, with a mean of 5.0, 5.8, 6.8 and 8.5 pg/mg at age 4-5, 6-7, 8-9 and 10-14 years respectively. Children age 4-7yr had significantly lower HCC compared to healthy adults (p=0.007). We did not find any influence on hair cortisol of gender, puberty or hair care characteristics.

Conclusion

Hair cortisol concentrations can be reliably measured in childhood and reference ranges increase with age. HCC in children is not dependent on hair care or hair characteristics.

INTRODUCTION

The glucocorticoid stress hormone cortisol is the end product of the hypothalamus-pituitary-adrenal (HPA) axis. Both a deficiency and an excess of cortisol have detrimental effects, resulting in both physical and psychiatric symptoms as manifested in Cushing's syndrome and Addison's disease. Cortisol is measurable in several matrices, each with its own merits and drawbacks. Cortisol measurements in serum and saliva provide a snap-shot indication of cortisol concentrations but are subject to the circadian rhythm, pulsatile excretion and acute stress, thereby impeding interpretation [1]. Urinary free cortisol is less subject to these drawbacks and provides a cortisol concentration spanning 24 hours. However, 24h urine collection is often cumbersome and unreliable. Moreover, most of the deleterious effects of hyper- and hypocortisolism are mediated by long-term exposure. A more suitable matrix for long-term cortisol measurement is scalp hair. Hair is readily accessible, sampling is non-invasive, samples can easily be stored at room temperature and cortisol can be measured using an relatively simple method. Measurement of hair cortisol has been shown to be a valuable diagnostic tool in situations of extreme hypercortisolism such as seen in Cushing's syndrome [2], but also hair cortisol concentrations (HCC) within the physiological range have been associated with the metabolic syndrome [3], obesity [4] and cardiovascular disease [5]. Recently, HCC are being explored as a stress biomarker in children [6-8].

Hair cortisol measurements have been validated in adults [2,9] and so far most studies measuring hair cortisol were in adults. A prerequisite to study hair cortisol in children is the availability of reference values, according to age, gender and pubertal stages. These have hitherto not been published.

In this study we aimed at measuring HCC in healthy children aged 4-14 years to establish reference ranges. Furthermore, we evaluated factors potentially influencing HCC such as age, gender, puberty, use of hair products, washing frequency and distance to scalp variation.

METHODS

Healthy children were enrolled during visitation of primary schools and from the siblings of children attending the pediatric outpatient clinic. Children were excluded if they were using glucocorticoids, both systemic and topical, or medication known to alter glucocorticoid metabolism. In total, HCC were measured in 128 children aged 4 through 14yr. Only two children were non-Caucasoid by appearance. Parents were requested to fill out a questionnaire about general health, the use of medication and the hair care characteristics of their child. Hair care characteristics, which were queried are hair washing frequency (< 1x/week, 1-2x/week, 3-4x/week, >4x/week), the use of hair products on the day of sampling (yes/no and type: none, mousse, gel, wax, spray, other), frequent sweating at the scalp (yes/no) and hair treatment in the

last six months (bleaching, dyeing, perming). Hair color was noted during preparation for analysis. In all children hair samples were obtained and anthropometrics were measured by one trained staff-member as follows: Standing height was measured in centimeters with the precision of 1 millimeter with a wall mounted stadiometer. Body weight was measured in kilograms with the precision of 100 gr with electric scales. Waist circumference (WC) and hip circumference (HC) were assessed in centimeters with the precision of 1 mm, taken on bare skin with both feet standing on the floor. The WC was measured mid-way between the lowest rib and the upper side of the pelvis. The HC was measured at the height of the widest range of the buttocks and the upper side of the os pubis. WC and HC measurements were taken twice and the mean was used as outcome. In children aged 7 and above Tanner puberty staging was assessed by a trained research nurse. Puberty was defined as Tanner stage M>1 in girls and testis volume ≥ 4cc in boys. Approval was given by the local Medical Ethics Committee, written informed consent was obtained from parents and informed assent from participants.

Hair collection

Of each participant, a lock of hair were cut from the posterior vertex as close to the scalp as possible using small surgical scissors. This area has been shown to have the lowest inter-lock variation [10]. Hair locks were taped to a paper form with scalp end marking and stored at room temperature until analysis.

Hair analysis

Hair samples were prepared and analyzed as described previously [9]. Briefly, the proximal three cm hair segment was weighed (samples approximately between 25 and 40mg) and minced using small surgical scissors. The hair was extracted in 1mL methanol for 16h at 52°C with gentle shaking. The extract was transferred to a glass tube and the methanol was evaporated at 37°C under a constant stream of nitrogen and reconstituted in 250µL phosphate buffered saline (PBS) pH8.0. After vortexing, the samples were analyzed using a commercially available cortisol ELISA kit (DRG Instruments, GmbH, Marburg, Germany) following the manufacturer's directions. Cross reactivity as reported by the manufacturer are as follows: Corticosterone 29.0%, Cortisone 3.0%, 11-Deoxycortiol <1.0%, 17-OH Progesterone <0.5%, Testosterone <0.10% and Estradiol <0.10%.

Assay variation

The intra-assay and inter-assay variation were analyzed on internal quality controls used for routine saliva cortisol measurement, measured in duplicate on five consecutive assays. The intra-assay coefficients of variance (CV) were 3.1% at 4.4ng/mL, 2.3% at 21.3 and 2.6% at 35.0 ng/mL. The inter-assay CV's were 7.0%, 2.3% and 8.2% respectively.

Variation within lock between proximal and distal segments (Distance to scalp variation)

To asses intra-individual variation over time, we measured HCC in 15 participants in the distal 3cm segment adjacent to the proximal 3cm (from 3cm to 6cm distal from the scalp) of hair within a single hair lock. These participants were randomly selected with the requirement that hair length was at least 6cm. The Spearman correlation between the two measurements is reported.

Statistical analysis

Statistical analyses were performed using SPSS 20.0 (IBM Corp., Armonk NY, USA). HCC were log10-transformed to achieve normality. Anthropometrics standard deviation (SD) scores were calculated based on the 1997 Dutch nationwide growth study [11] using Growth Analyser RCT (Growth Analyser B.V., Rotterdam, the Netherlands). Waist circumference to height ratio was calculated by dividing waist circumference (cm) by height (cm). Relations between hair cortisol and age and anthropometrics were analyzed by linear regression. Age-specific reference ranges are based on linear regression 95% prediction intervals. Differences in HCC between age groups were tested using Students *t*-test. Proximal and distal hair samples were compared using Spearman's correlation and differences were analyzed using Wilcoxon signed ranks test. Effects of hair care and hair characteristics on cortisol concentrations were assessed using one-way Analysis of Variance (ANOVA).

RESULTS

General characteristics of participants are shown in Table 1. Hair cortisol concentrations increased significantly with age (log-transformed β=0.031, R=0.18, p=0.04, Figure 1), which is emphasized by the significantly lower HCC in children aged 4-7 years compared to 141 healthy adults as previously measured [9] (Mean and 95% CI: 4.6 [3.3, 6.1] vs. 7.5 [6.1, 9.1] pg/mg, p=0.008) and compared to children aged 8-14yr (Mean and 95% CI: 4.6 [3.3, 6.2] vs. 9.0 [6.9, 11.6] pg/mg, p=0.001). Cortisol concentrations were not different between children aged 8-14 and adults (p=0.14). Neither puberty nor gender influenced HCC (p= 0.88 and p=0.78 respectively). Waist circumference

Table 1. General characteristics and reference ranges per age category

Variable	N=128
Age, mean and [range], yr.	8.4, [4.25-14.13]
Female, N and %	65, 50.8%
BMI SD score, mean and SD	-0.25, 0.99
Waist circumference SD score, mean and SD	0.72, 0.83
Puberty, N and %	26, 20.3%

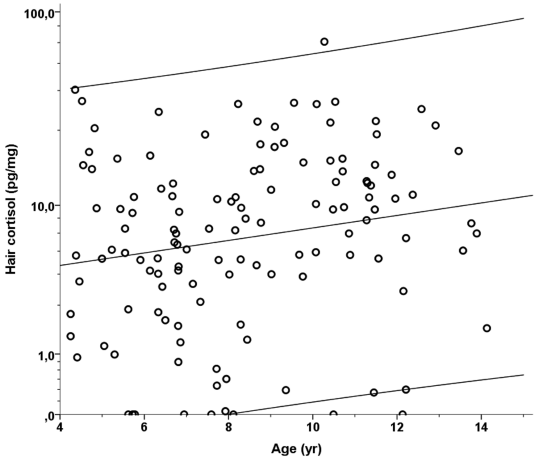

Figure 1. Hair cortisol distribution over age. Each dot represents a single individual. Lines show linear regression and limits of 95% prediction interval.

showed a significant association with HCC (log-transformed β=0.13, r=0.19, *p*=0.04, adjusted for age. Figure 2), the association between waist to height ratio did not reach statistical significance (log-transformed β=0.16, r=0.19, *p*=0.07, adjusted for age). Hair cortisol was not correlated with BMI (*p*=0.50, adjusted for age).

Age-specific reference ranges

Age-specific hair cortisol reference ranges based on linear regression 95% prediction intervals are listed in Table 2. HCC in children above age 10 were not significantly different from adults and were hence pooled in one category.

Hair care

Hair care characteristics in terms of washing frequency, hair product use or type of hair product did not influence hair cortisol content (*p*=0.33, *p*=0.73 and *p*=0.22, respectively), nor was hair cortisol content affected by hair color or frequent sweating at the scalp (*p*=0.72 and *p*=0.25, respectively). Adjustment for age did not change these results. None of the children participating in the current study dyed, bleached or permed their hair.

Distance to scalp variation

Intra-individual differences in hair cortisol measurements between the proximal 3cm hair segment and a distal 3cm hair segment (3-6 cm distal from scalp) measured in 15 children (male N=8) showed a significant correlation (Spearman *r*=0.76 *p*=0.001). No significant difference in HCC between proximal and distal segments was observed (*p*=0.43).

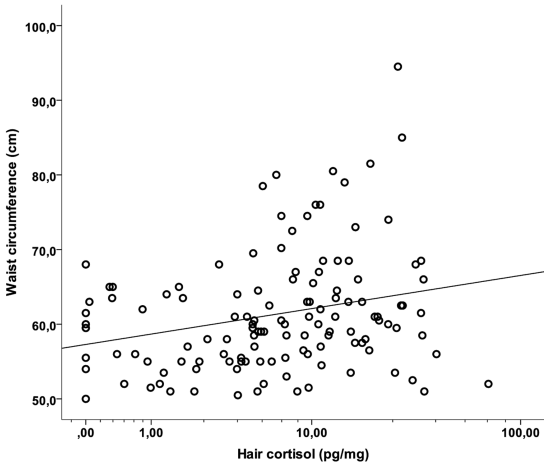

Figure 2. Waist circumference plotted against hair cortisol concentration. Line shows unadjusted correlation. Linear regression adjusted for age: log transformed β=0.13, p=0.04.

Table 2. Hair cortisol concentration reference ranges per age category

Variable	Hair cortisol concentration (pg/mg)
Age 4-5, n=27	5.0 [0 – 43.4]
Age 6-7, n=34	5.8 [0 – 49.1]
Age 8-9, n=27	6.7 [0.1 – 56.0]
Age 10-14, n=40	8.5 [0.3 – 69.5]

Reference ranges are described by linear regression mean and 95% prediction interval limits per age category

DISCUSSION

Hair cortisol concentrations in healthy children were readily measurable, and increased significantly with age. In other matrices, e.g. serum or saliva, the relation between age and cortisol concentrations has yielded conflicting results. Knutsson *et al.* found no effects of age on serum cortisol in a study in 235 healthy children [12]. However, two larger studies on serum cortisol in 762 and 1482 healthy children aged 0-18, respectively, reported high cortisol variance in neonates and infants, after which cortisol rapidly decreased to subsequently increase steadily with age up to adulthood [13,14]. More specifically, the median serum cortisol concentration in the study by Elmlinger *et al.* was 218 nmol/L in girls and 253 nmol/L in boys aged 4-6 years and rose steadily up to 466 nmol/L (girls) and 440nmol/L (boys) in 14 year olds. These study results support our findings in hair cortisol. Very few studies report hair cortisol measurement in children. Dettenborn *et al.* reported a decrease in hair cortisol over age in 28 children 1 to 9 years of age [15]. This seems contradictory to our findings, however the numbers per age category in that

study are low and the sample mainly composed of children under 4 years of age (mean age 3.6 years) including only 8 children aged above 5, whereas we studied children 4-14 years old (mean age 8.4 years). The differences may be explained in the relative overrepresentation of very young children, as serum cortisol show high and variable concentrations during infancy[13]. The high variability in HCC at young age has also been demonstrated by Karlén *et al,* measuring hair cortisol in children of 1, 3, 5 and 8 years of age [16]. HCC showed a large variation in the very young, which decreased with increasing age. However, no clear difference was visible between the age of 5 and 8 years. HCC may be further elevated during the first years of life when hair morphology and growth is subject to major changes [17]. After the age of 2, hair has been observed to increase in cross-sectional surface area and in the percentage of medullated hair with age up to puberty [18]. The influence of these morphological changes on hair cortisol content is unknown. Theoretically, linear hair growth rate may also influence HCC. Research on hair growth rate in healthy children is scarce. Growth rates of 0,3-0,4 mm per day have been reported [17]. One study suggests a slightly higher hair growth rate in childhood in boys age 3-9yr. [17,19]. It seems unlikely that the hair growth rate contributes significantly to hair cortisol content, as hair grows slowly, there is ample time for circulating cortisol to achieve an equilibrium at the hair follicle and to completely diffuse into the forming hair shaft, even in the case of a slight increase in growth rate. However, differences in growth rate may have consequences if consecutive segments of hair are used to construct a timeline of cortisol exposure. For such applications, it would be useful to have more data about the hair growth rate in children.

To validate hair cortisol measurement in children, factors that could influence cortisol concentrations were studied. HCC were not affected by hair color, hair washing frequency or use of hair products. The effect of topical use of glucocorticoid treatment, e.g. hydrocortisone cream, was not assessed, as this was a criterion for exclusion. Despite recent evidence showed extensive sweating at the scalp may increase HCC [20], no effect of frequent sweating at the scalp as reported through a questionnaire was found in the present study. However, noting extensive sweating at time of sampling as suggested by the authors was not performed. Hair segments up to six cm from the scalp were measured in the current study. No significant difference in HCC were found between the proximal (cm 0 - 3) and distal (cm 4 – 6) hair segments, and distal and proximal hair segments showed a high correlation, supporting the previous evidence of stable incorporation of cortisol in hair. With the current method HCC can be reliably measured at least up to six cm. As none of the children participating in the current study had undergone any hair treatment, e.g. dyeing, straightening or perming, the effects of these treatments could not be assessed. The same applies to ethnicity, as almost all participants were Caucasian. These findings are in line with the previously described findings in adults [5].

Stressing the value of hair cortisol as a reflection of long-term systemic cortisol exposure, hair cortisol was significantly correlated with waist circumference in children, since visceral fat redistribution is one of the hallmarks of cortisol tissue

effects. Also in adults positive correlations between waist and hair cortisol levels have been demonstrated [3,9]. In addition, recently strong evidence was presented that HCC in adults are related to metabolic syndrome and cardiovascular disease [3,5].

Several limitations of the current study need to be mentioned. First, the population is primarily Caucasian, limiting the applicability of the reference ranges to other ethnicities. However, hair color has been observed not to influence HCC [15], so there are no clear indications that HCC is different in other ethnicities. Additionally, the current reference ranges do not cover all pediatric age categories. The findings described here and in other studies [13] underline the need for reference ranges measured in all ages, rather than extrapolating results to other ages. Hair cortisol measurement in children under the age of 4 years deserve extra attention, as hair characteristics in this age group change dramatically [17-19]. Although Figure 1 suggests HCC increases up to approximately the age of 10, no distinct cut-off age at which the increase in HCC levelled off could be determined in the current study using a linear regression model. HCC measurement using different methods for extraction or different ELISA kits render different results [10,15], and while immunoassays will remain in use for HCC measurement, especially in settings where equipment and money are scarce, methods with a higher sensitivity such as gas- and liquid chromatography mass spectrometry, will eventually be widely employed. The comparison of results between varying methods is an important focus for future research [21].

A strength of this study is the sample size, exceeding 20 children per 2-year age group with even distribution in gender. The additional validation of these data and this technique is supported by the information on pubertal staging and on potential confounders. With these reference data we have a foundation for further research on the value of using HCC in pediatric clinical practices.

CONCLUSION

Hair cortisol concentrations in childhood can be reliably measured. HCC reference ranges increase with age between 4 and 9 years of age until concentrations comparable to adults are reached in puberty. This study emphasizes the need for age-specific reference ranges. Hair cortisol measurement has the potential of becoming a valuable tool in diagnosing hypothalamus-pituitary-adrenal diseases in children in the future. Further research is needed to clarify its value in pediatric clinical practice and research.

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REFERENCES

1. Carroll TB, Aron DC, Findling JW, Tyrrell B. Glucocorticoids and Adrenal Androgens. In: Gardner DG, Shoback D, eds. Greenspan's Basic & Clinical Endocrinology: McGraw-Hill Companies; 2011.
2. Manenschijn L, Koper JW, van den Akker EL, et al. A novel tool in the diagnosis and follow-up of (cyclic) Cushing's syndrome: measurement of long-term cortisol in scalp hair. *J Clin Endocrinol Metab* 2012; **97**(10): E1836-43.
3. Stalder T, Kirschbaum C, Alexander N, et al. Cortisol in hair and the metabolic syndrome. *J Clin Endocrinol Metab* 2013; **98**(6): 2573-80.
4. Veldhorst MAB, Noppe G, Jongejan MHTM, et al. Increased Scalp Hair Cortisol Concentrations in Obese Children. *J Clin Endocr Metab* 2014; **99**(1): 285-90.
5. Manenschijn L, Schaap L, van Schoor NM, et al. High long-term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease. *J Clin Endocrinol Metab* 2013; **98**(5): 2078-83.
6. Groeneveld MG, Vermeer HJ, Linting M, Noppe G, van Rossum EF, van IJzendoorn MH. Children's hair cortisol as a biomarker of stress at school entry. *Stress* 2013; **16**(6): 711-5.
7. Hostinar CE, Gunnar MR. Future directions in the study of social relationships as regulators of the HPA axis across development. *J Clin Child Adolesc Psychol* 2013; **42**(4): 564-75.
8. Vanaelst B, Huybrechts I, Bammann K, et al. Intercorrelations between serum, salivary, and hair cortisol and child-reported estimates of stress in elementary school girls. *Psychophysiology* 2012; **49**(8): 1072-81.
9. Manenschijn L, Koper JW, Lamberts SW, van Rossum EF. Evaluation of a method to measure long term cortisol levels. *Steroids* 2011; **76**(10-11): 1032-6.
10. Sauve B, Koren G, Walsh G, Tokmakejian S, Van Uum SH. Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clin Invest Med* 2007; **30**(5): E183-91.
11. Fredriks AM, van Buuren S, Burgmeijer RJ, et al. Continuing positive secular growth change in The Netherlands 1955-1997. *Pediatr Res* 2000; **47**(3): 316-23.
12. Knutsson U, Dahlgren J, Marcus C, et al. Circadian cortisol rhythms in healthy boys and girls: relationship with age, growth, body composition, and pubertal development. *J Clin Endocrinol Metab* 1997; **82**(2): 536-40.
13. Bailey D, Colantonio D, Kyriakopoulou L, et al. Marked biological variance in endocrine and biochemical markers in childhood: establishment of pediatric reference intervals using healthy community children from the CALIPER cohort. *Clin Chem* 2013; **59**(9): 1393-405.
14. Elmlinger MW, Kuhnel W, Ranke MB. Reference ranges for serum concentrations of lutropin (LH), follitropin (FSH), estradiol (E2), prolactin, progesterone, sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEAS), cortisol and ferritin in neonates, children and young adults. *Clin Chem Lab Med* 2002; **40**(11): 1151-60.
15. Dettenborn L, Tietze A, Kirschbaum C, Stalder T. The assessment of cortisol in human hair: associations with sociodemographic variables and potential confounders. *Stress* 2012; **15**(6): 578-88.
16. Karlen J, Frostell A, Theodorsson E, Faresjo T, Ludvigsson J. Maternal Influence on Child HPA Axis: A Prospective Study of Cortisol Levels in Hair. *Pediatrics* 2013; **132**(5): e1333-40.
17. Barth JH. Normal hair growth in children. *Pediatr Dermatol* 1987; **4**(3): 173-84.
18. Duggins OH, Trotter M. Age changes in head hair from birth to maturity. II. Medullation in hair of children. *Am J Phys Anthropol* 1950; **8**(3): 399-415.
19. Duggins OH, Trotter M. Changes in morphology of hair during childhood. *Ann N Y Acad Sci* 1951; **53**(3): 569-75.
20. Russell E, Koren G, Rieder M, Van Uum SH. The detection of cortisol in human sweat: implications for measurement of cortisol in hair. *Ther Drug Monit* 2014; **36**(1): 30-4.
21. Albar WF, Russell EW, Koren G, Rieder MJ, Van Uum SH. Human hair cortisol analysis: Comparison of the internationally-reported ELISA methods. *Clin Invest Med* 2013; **36**(6): E312.

CHAPTER 3

LC-MS/MS BASED METHOD FOR LONG-TERM STEROID PROFILING IN HUMAN SCALP HAIR

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Clinical Endocrinology, 2015;83(2):162-166

ABSTRACT

Background

Liquid chromatography – tandem mass spectrometry (LC-MS/MS) is the method of choice for quantification of steroids. Human scalp hair provides the possibility to measure long term retrospective steroid concentrations, which is especially useful for steroids with large time-dependent fluctuations in concentration, such as the glucocorticoid cortisol.

Aim

We set-up and validated a LC-MS/MS based method for long-term steroid profiling, quantifying cortisol, cortisone, testosterone, androstenedione, dehydroepiandrosterone sulfate (DHEAS), and 17-α-hydroxyprogesterone (17OHP).

Method

Hair locks were cut from the posterior vertex of healthy male and female volunteers and washed in isopropanol. Steroids were extracted using methanol, extract was cleaned up by solid phase extraction and measured on a Waters XEVO-TQ-S LC-MS/MS. Lower limit of quantification, precision, matrix interference and intra-individual variation were determined.

Results

The functional sensitivity of our steroid analysis was < 1.3, < 9.3, 2.3, <1.3, <15.9, 1.87 pg/mg hair for cortisol, cortisone, testosterone, androstenedione, dehydroepiandrosterone sulfate (DHEAS), and 17OHP respectively. Measured over a nine-month period the inter-run CV were below 16% for all steroids. Intra-individual coefficients of variation were below 15% for all steroids, except 17OHP (19.7%).

Conclusion

The authors present a LC-MS/MS based method for long-term steroid profiling in human scalp hair, potentially providing novel insights by a multitude of clinical and research applications in the field of endocrinology.

INTRODUCTION

Steroid measurement in human scalp hair was originally set-up for use in determination of exogenously administrated steroids, e.g. performance enhancing drugs in sports and forensic medicine[1,2]. In the past decade, measurement of endogenously produced steroids, especially the glucocorticoid hormone cortisol, has drawn increasing attention. As human scalp hair at the posterior vertex grows 1 cm per month, cortisol in a 1 cm hair segment reflects the mean systemic cortisol exposure of one month. Thus, hair provides a long-term cortisol measurement, offering the ability to measure months to years in retrospect, and is thought to reflect the free cortisol fraction. Hair cortisol has shown to be a valuable biomarker in research on both mental [3,4] and physical health[5,6]. It has been posted as a risk factor for the metabolic syndrome[7] and cardiovascular disease[8]. Moreover, measurement of long-term cortisol exposure using hair has been shown to be clinically relevant in e.g. diagnosing (cyclic) Cushing's syndrome[9] and monitoring of hydrocortisone treatment[10,11].

Most studies reporting on cortisol concentrations in scalp hair are based on immunoassays, either in-house developed or commercially available saliva-cortisol assays [6,12-14]. Immunoassays are technically straightforward, explaining their popularity, despite drawbacks such as cross reactivity and the limitation to single component measurement per assay. Superior methods based on mass spectrometry have been available for decades, but were costly and unsuited for high-throughput routine laboratory measurements. In recent years, mass spectrometry has overcome these drawbacks and, with its superior specificity and sensitivity and its multi-analyte capabilities, has become the preferred method for steroid analysis in high-quality clinical research[15].

Taking advantage of the multi-analyte analysis, we established a LC-MS/MS assay for profiling glucocorticoids and sex steroids in human scalp hair. Here, we describe the method and present the validation of this novel technique providing long-term steroid profiles through a non-invasive procedure. To date, only one report on the use of an LC-MS/MS based method for scalp hair steroid profiling has been published. [16] The current method adds to this by providing results on 17α-hydroxyprogesterone (17OHP) and dehydroepiandrosterone sulfate (DHEAS) thereby expanding potential applicability of scalp hair analyses for example in therapy monitoring of patients with congenital adrenal hyperplasia.

MATERIALS AND METHODS

Chemicals

Steroids were purchased from Sigma-aldrich (Zwijndrecht, the Netherlands). Deuterated cortison-d8 was purchased from Buchem B.V. (Apeldoorn, The Netherlands). Androstendione-d5, cortisol-d3, 17α-hydroxyprogesterone-d8, testosterone-d5, and dehydroepiandrosterone sulfate-d6 were purchased in a dried mixture from Perkin Elmer (Turku, Finland). LC-MS grade isopropanol and LC-MS grade methanol

(MeOH) were purchased from Biosolve B.V. (Valkenswaard, The Netherlands). Stock solutions of cortison-d8 in methanol was prepared by weighing, as were stock solutions of each unlabeled steroid. Working solutions were prepared by diluting stock solutions in MeOH to a concentration between 1 and 1000 nmol/L. Of each working standard (S1-S10), 100 µl was mixed with 100 µl of diluted internal standard working solution and dried under a gentle stream of N_2 at 50°C. The dry residue was reconstituted in 100 µl of initial mobile phase. Two standard curves of ten calibration standards were prepared on each day of analysis.

Controls

Two internal human control materials were established by pooling extract from a large supply of scalp hair, which was evaporated to dryness under a flow of N_2, and reconstituted in methanol at a concentration of 200 mg/mL. This pool was divided in 100 µL aliquots and stored at -20°C in glass vials. On each day of analysis the two internal controls were measured twice.

Specimens

Hair sampling

Hair locks were obtained from healthy adult male and female volunteers, not using any medication. Approximately 100-200 hairs were cut from the posterior vertex, close to the scalp, using small surgical scissors, marking the scalp-end. The proximal 3 cm of hair, reflecting the most recent three months, were cut in 1 cm segments, weighed and transferred to disposable glass tubes.

Sample preparation

Hair samples were washed by gently shaking in 2 mL LC-MS grade isopropanol at room temperature. They were allowed to dry for a minimum of 2 days. Ten to 30 mg of whole, nonpulverised hair was weighed out and transferred into a glass tube, capped with glass marbles. Subsequently, 1.4 mL MeOH and 100 µl internal standards were added and the hair was incubated for 18 h at 25°C for extraction. Samples were centrifuged at 4302x g (4500 rpm), 4°C for 5 minutes and 1 ml of the clear supernatant was transferred into a new glass tube. The MeOH was evaporated at 50°C under a constant stream of N_2 until the samples were completely dried. The dry residu was resuspended using 1 ml 2% MeOH and vortexed for 1 min. Hair extracts were subsequently cleaned by solid phase extraction using Oasis HLB 30µm 96-wells extraction plates on a 96-wells positive pressure manifold (Waters Corporation, Milford, MA, USA). Wells were conditioned with 1 mL of MeOH followed by 1 mL Milli-Q. Subsequently, samples were loaded with the resuspended extract (1 mL), washed with 1 mL 30% LC-grade MeOH in Milli-Q, and eluted twice with 300 uL MeOH. Samples were evaporated to dryness at 50°C under a constant flow of N_2 and stored at 4°C until analysis. Before injection, the dry residu was resuspended in the initial mobile phase.

LC-MS/MS analysis

Conditions and procedures

All steroid hormones were measured simultaneously with a LC-MS/MS method. The chromatographic separation was performed on a Waters® Acquity™ UPLC HSS T3 1.8 µm column and in-line filter frit 0.2 µm with a H_2O /MeOH gradient (Waters Corporation, Milford, MA, USA). The Waters XEVO-TQ-S system (Waters Corporation, Milford, MA, USA) was equipped with an ESI source operating in the electrospray positive mode except for DHEAS (negative ESI). The HPLC flow rate was set to 250 µl/min with mobile phase consisting of solution A, H_2O with 1.25 mM ammonium acetate, and solution B, LC-grade MeOH with 1.25 mM ammonium acetate, using the following gradient elution: Initial – 1.5 min 70% A, 30% B; 8 min 35% A, 65% B; 8.1 min 100% B; 8.1-9.5 min 100% B; 9.55 70% A, 30% B; 9.55-10 70% A, 30% B. A volume of 40 µl sample solution was injected. The ion transitions are given in Table 1. Peak integration and calculations of concentrations against the standard curve were performed using Waters Masslynx vs. 4.1 (Waters Corporation, Milford, MA, USA). Steroid peak integrations were reviewed and manually integrated by two independent persons when automated peak integration feature incorrectly of partially integrated peaks.

Table 1. Mass spectrometer parameter settings and steroid ion-transitions

Steroid	Internal standard	Precursor ion	1st – 2nd transition	Time	ESI	Collision V
Cortisol	Cortisol-d3	363.168	121.108 - 97.100	3.84	+	24 – 24
Cortisone	Cortisone-d8	361.200	163.100 - 105.100	3.60	+	24 – 36
Testosterone	Testosterone-d5	289.232	97.097 - 109.074	6.19	+	20 – 24
17α-OHP	17α –OHP-d8	331.168	97.097 - 109.073	6.58	+	22 – 26
Androstenedione	Adion-d5	287.168	97.097 - 109.970	5.93	+	20 – 22
DHEAS	DHEAS-d6	367.104	80.007 – 96.99*	3.43	-	66 – 32

ESI, Electrospray Injection charge; V, Voltage; CV, Coefficient of variation in %; OHP, Hydroxyprogesterone;

Lower limit of quantification

The functional sensitivity (lower limit of quantification, LLoQ) is the lowest analyte concentration that can be reproducibly measured with an intermediate precision CV of ≤ 20 %. The LLoQ was determined in hair extracts of male and female adults, by serial dilution of hair extract, starting with 40 mg/mL to 0.3125 mg/mL. The LLoQ was calculated in pg/mg hair.

Precision

Precision was determined using human scalp hair extraction pools as internal controls. We measured six steroids in duplicate over a period of 9 months.

Intra-individual variation

Intra-individual variation due to repeated sampling and measurements was determined by analyzing 5 separate hair samples per individual in parallel, obtained from 5 volunteers (4 male, 1 female). All samples were obtained from the posterior vertex at the same moment.

To assess the variety of steroid concentrations along the consecutive hair segments, we constructed time-lines of hair samples of three consecutive segments of 1 cm followed by two segments of 3 cm, totaling to 9 cm, in 6 volunteers (3 male, 3 female). Hence, subjects for this analysis were selected on a hair length of at least 9 cm.

Matrix interference

To evaluate the effect of hair matrix on LC-MS/MS response we added different concentration of non-deuterated steroids to different weights of hair (2.5-5-10-20 mg). Concentration ranges of non-deuterated cortisol, cortisone, 17OHP, androstenedione, DHEAS and testosterone added to the hair extracts were 31-509 pg, 24-396 pg, 3.6-62 pg, 23-388 pg, 524-8456 pg and 8.4-136 pg. The mean recoveries were calculated as follows: (spiked sample – blanc sample) / measured spiking concentration * 100%.

RESULTS

Precision

Over a nine-month period, coefficients of variation for cortisol, cortisone, 17OHP, androstenedione, DHEAS and testosterone in internal hair pools were 14.8, 15.3, 15.0, 14.9, 13.5, 16.0 %, respectively.

Extracting 10 mg hair from 2 individuals gave an intra-day CV for cortisol, cortisone, 17OHP, androstenedione, DHEAS and testosterone of 1.9% (range 0.9-2.9%), 4.7% (range 4.5-4.8%) , 7.7% (range 6.2-9.1%), 2.5% (range 2.0-3.0%), 6.3% (range 5.7-6.9%) and 3.1% (range 2.3-3.9%), respectively.

Using human hair pools the LLoQ of Cortisol, Cortisone, 17-OHP, Androstenedione, DHEAS and Testosterone was < 1.3, < 9.3, 1.87, <1.3, <15.9 and 2.3 pg/mg hair respectively for a 10mg hair sample.

Intra-individual variation

In five volunteers, five hair samples per individual were obtained which were separately extracted and measured, to determine the total variation of repeated sampling and measurement. The mean CV was 14.0% for cortisol, 14.8% for cortisone, 19.7% for 17-OHP, 8.5% for androstenedione, 10.0% for DHEAS, and 14.8% for testosterone. The intra-individual variation was higher compared to the intra-day variation of the described method.

Matrix interferences

No distinct trend in the effect of different hair sample weights used for extraction procedure (range 2.5-20 mg) on the recovery of the six studied steroids was observed.

We found a mean recovery of the non-deuterated steroid added to the hair extract of 110% for cortisol, 111% for cortisone, 126% for androstenedione, 113% for testosterone, 102% for 17OHP and 108% for DHEAS. With the present LC-MS/MS procedure we are able to measure steroids in hair sample extracts derived from 2.5 up to 20 mg.

Wash-out

Figure 1 shows mean steroid concentrations in 6 individuals (3 male) along successive hair segments. Steroid concentrations decrease gradually from proximal to more distal hair segments. Only cortisone showed a significant decrease in the most proximal 3 cm hair (p=0.01).

DISCUSSION

We present a LC-MS/MS based method for long-term steroid profiling in human scalp hair, potentially providing novel insights in a variety of clinical challenges in the field of endocrinology. Using this method, we were able to quantitatively measure cortisol, cortisone, 17OHP, androstenedione, testosterone and DHEAS in human scalp hair samples in concentrations well above the limit of quantification. This method resembles the method described by Gao *et al.*[16], but lacks the need for an online SPE system and adds the quantification of the clinically relevant adrenal steroids DHEAS and 17OHP. Due to the high sensitivity provided by state-of-the-art mass spectrometers, only small hair samples of volumes are needed. With the current method, we prefer 10 mg as the optimal amount of hair, although lower weights up to 2.5 mg also gave good results. Limiting the maximum amount of hair is advised, as extraction of larger hair samples may introduce matrix interference, limiting the reliability of the measurement. The minimum amount of 2,5 mg hair is the lowest amount that has been reported until now. In previous studies measuring glucocorticoids in scalp hair by LC-MS/MS reported using much higher weights, ranging from 50[17] to 150 mg[18], whereas Gao *et al.*[16] used 10mg hair locks. The low amount needed for this assay is of added value, as the need for high volumes of hair is an important barrier for the use of hair steroid profiling in patient care or in large epidemiological studies.

In a study on hair cortisol using fluorescence detection (HPLC-FLU), Gao *et al.*[19] showed a decrease in hair cortisol contents in distal segments along the hair shaft. This was previously reported by Kirschbaum *et al.*[20] using an immunoassay. In our enzyme linked immunoassay (ELISA) method, we did not observe this decrease.[14] However, using the current LC-MS/MS method, we observed a gradual decrease in steroids along the length of the hair shaft, most pronounced in the more distal segments. Distal hair samples may have suffered more insults such as UV-damage and repeated water and soap exposure, making these parts more susceptible to leak contents during e.g. a washing step. In the previously described immunoassay method, hair was not washed prior to analysis. [14] The current method does include a washing step using iso-propanol,

we speculate this might contribute to the observed decrease of steroid content along the hair shaft. This finding should be taken into account in the assessment of time-lines over longer periods.

To date, a variety of methods on measurements of steroids in hair, mostly cortisol, have been published. Most methods are based on immunoassays while LC-MS/MS methods on endogenous hair steroids are rising. These methods vary in the extraction protocol and the assay used. Efforts for international validation and external quality assurance schemes are ongoing and the first results show high correlation between the varying methods[21]. In our studies on validating hair cortisol measurement using an ELISA in both adults[14] and children[22], no contributions of hair treatment (e.g. washing frequency, hair product use, hair dyeing etc.) to steroid concentrations have been found. This remains to be confirmed for the LC-MS/MS based method. Additionally, reference ranges and the effects of age and gender need to be established.

In conclusion, we describe a sensitive and robust LC-MS/MS method for the quantification of long-term adrenal and sex steroid concentrations retrospectively in human scalp hair. Measurement of cortisol in hair is a promising tool in the diagnosis of (cyclic) Cushing's syndrome[9], for treatment monitoring in e.g. adrenal insufficiency[11], as a risk factor for cardiometabolic diseases[7,8], and is valuable for numerous other applications[5,23]. The high sensitivity of the LC-MS/MS based measurement and the addition of other adrenal and sex steroids broadens the scope of clinical use of hair analysis to a variety of endocrine diseases e.g. Addison's disease, congenital adrenal hyperplasia, female hyperandrogenism and male hypogonadism.

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REFERENCES

1. Boumba VA, Ziavrou KS, Vougiouklakis T. Hair as a biological indicator of drug use, drug abuse or chronic exposure to environmental toxicants. *Int J Toxicol* 2006; **25**(3): 143-63.
2. Deshmukh N, Hussain I, Barker J, Petroczi A, Naughton DP. Analysis of anabolic steroids in human hair using LC-MS/MS. *Steroids* 2010; **75**(10): 710-4.
3. Staufenbiel SM, Penninx BW, Spijker AT, Elzinga BM, van Rossum EF. Hair cortisol, stress exposure, and mental health in humans: a systematic review. *Psychoneuroendocrinology* 2013; **38**(8): 1220-35.
4. Steudte S, Kirschbaum C, Gao W, et al. Hair cortisol as a biomarker of traumatization in healthy individuals and posttraumatic stress disorder patients. *Biol Psychiatry* 2013; **74**(9): 639-46.
5. Wester VL, Lamberts SW, van Rossum EF. Advances in the assessment of cortisol exposure and sensitivity. *Curr Opin Endocrinol Diabetes Obes* 2014; **21**(4): 306-11.
6. Sauve B, Koren G, Walsh G, Tokmakejian S, Van Uum SH. Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clin Invest Med* 2007; **30**(5): E183-91.
7. Stalder T, Kirschbaum C, Alexander N, et al. Cortisol in hair and the metabolic syndrome. *J Clin Endocrinol Metab* 2013; **98**(6): 2573-80.
8. Manenschijn L, Schaap L, van Schoor NM, et al. High long-term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease. *J Clin Endocrinol Metab* 2013; **98**(5): 2078-83.
9. Manenschijn L, Koper JW, van den Akker EL, et al. A novel tool in the diagnosis and follow-up of (cyclic) Cushing's syndrome: measurement of long-term cortisol in scalp hair. *J Clin Endocrinol Metab* 2012; **97**(10): E1836-43.
10. Gow R, Koren G, Rieder M, Van Uum S. Hair cortisol content in patients with adrenal insufficiency on hydrocortisone replacement therapy. *Clin Endocrinol (Oxf)* 2011; **74**(6): 687-93.
11. Noppe G, van Rossum EFC, Vliegenthart J, Koper JW, van den Akker ELT. Elevated hair cortisol concentrations in children with adrenal insufficiency on hydrocortisone replacement therapy. *Clin Endocrinol* 2014; **81**(6): 820-5.
12. Dettenborn L, Tietze A, Kirschbaum C, Stalder T. The assessment of cortisol in human hair: associations with sociodemographic variables and potential confounders. *Stress* 2012; **15**(6): 578-88.
13. Karlen J, Ludvigsson J, Frostell A, Theodorsson E, Faresjo T. Cortisol in hair measured in young adults - a biomarker of major life stressors? *BMC Clin Pathol* 2011; **11**: 12.
14. Manenschijn L, Koper JW, Lamberts SW, van Rossum EF. Evaluation of a method to measure long term cortisol levels. *Steroids* 2011; **76**(10-11): 1032-6.
15. Handelsman DJ, Wartofsky L. Requirement for mass spectrometry sex steroid assays in the Journal of Clinical Endocrinology and Metabolism. *J Clin Endocrinol Metab* 2013; **98**(10): 3971-3.
16. Gao W, Stalder T, Foley P, Rauh M, Deng H, Kirschbaum C. Quantitative analysis of steroid hormones in human hair using a column-switching LC-APCI-MS/MS assay. *J Chromatogr B Analyt Technol Biomed Life Sci* 2013; **928**: 1-8.
17. Vanaelst B, Michels N, De Vriendt T, et al. Cortisone in hair of elementary school girls and its relationship with childhood stress. *European journal of pediatrics* 2013; **172**(6): 843-6.
18. Kamps AW, Molenmaker M, Kemperman R, van der Veen BS, Bocca G, Veeger NJ. Children with asthma have significantly lower long-term cortisol levels in their scalp hair than healthy children. *Acta paediatrica* 2014; **103**(9): 957-61.
19. Gao W, Xie Q, Jin J, et al. HPLC-FLU detection of cortisol distribution in human hair. *Clin Biochem* 2010; **43**(7-8): 677-82.
20. Kirschbaum C, Tietze A, Skoluda N, Dettenborn L. Hair as a retrospective calendar of cortisol production-Increased cortisol incorporation into hair in the third trimester of pregnancy. *Psychoneuroendocrinology* 2009; **34**(1): 32-7.
21. Russell E, Kirschbaum C, Laudenslager ML, et al. Toward Standardization of Hair Cortisol Measurement; Results of the First International Inter-laboratory Round Robin. *Ther Drug Monit* 2014.
22. Noppe G, Van Rossum EF, Koper JW, et al. Validation and Reference Ranges of Hair Cortisol Measurement in Healthy Children. *Horm Res Paediatr* 2014: 97-102.
23. Gow R, Thomson S, Rieder M, Van Uum S, Koren G. An assessment of cortisol analysis in hair and its clinical applications. *Forensic science international* 2010; **196**(1-3): 32-7.

CHAPTER 4

LONG-TERM STEROID HORMONE PROFILING IN SCALP HAIR OF HEALTHY CHILDREN

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Submitted

ABSTRACT

Background

Human scalp hair is a novel matrix for determining long-term steroid concentrations, with wide-spread applicability. Recently we published a Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) based method for long-term steroid hormone profiling in human scalp hair. The aim of this study is to establish age-adjusted reference ranges and evaluate the effects of potential confounders and environmental factors such as hair washing frequency, hair product use, and hair color. This knowledge is vital for application of this method in research and clinical practice.

Methods

Healthy children age 4-18 years were enrolled through elementary and secondary school visits. The proximal 3 cm hair was analyzed by LC-MS/MS, measuring cortisol, cortisone, androstenedione, 17α-hydroxyprogesterone (17OHP), testosterone and dehydroepiandrosterone sulfate (DHEAS). Height and weight were measured. Questionnaires on hair care characteristics were filled out by a subset of children.

Results

Age-adjusted reference ranges were established. Cortisol and cortisone show a slight increase over age. androstenedione, 17OHP, and testosterone show a marked increase over age, leveling off around the age of 14yr. The median testosterone concentrations are significantly higher in boys from the age above 12yr, compared to girls. Hair washing frequency and use of hair products did not affect hair steroid concentrations, whereas ethnicity and hair color were associated with a difference in hair steroid hormone concentrations.

Conclusion

This study provides age-adjusted reference ranges for clinical use. The non-invasive measurement of a complete long-term steroid hormone profile broadens the potential applications in both clinical practice and in research.

INTRODUCTION

Measurement of endogenously produced hormones is a cornerstone in the endocrine clinic. Routinely used matrices for hormone quantification are serum, urine, as well as saliva. A relatively novel matrix which is rapidly rising in popularity is human scalp hair.[1] Hair has several unique characteristics, such as providing a long-term retrospective reflection of cumulative hormone concentrations and non-invasive sampling. These features make the use of scalp hair attractive for both research and clinical practice. Studies measuring glucocorticoids in hair have linked increased long-term cortisol and cortisone to obesity[2-4], the metabolic syndrome [5] and cardiovascular disease.[6,7] Additionally, measurement of cortisol in hair may be of use in the diagnosis of Cushing's disease[8,9], as well as in evaluating cortisol levels and prevention of hydrocortisone overtreatment in adrenal insufficiency.[10-13] Recent studies have shown that a broad spectrum of steroids are measurable in hair matrix[14-16], widening the potential application of scalp hair as a matrix in endocrinology.

The spectrum of endogenous steroids in scalp hair measurable by LC-MS/MS thus far, consists of cortisol, cortisone, androstenedione, 17α-hydroxyprogesterone (17OHP), dehydroepiandrosterone, dehydroepiandrosterone sulfate (DHEAS), testosterone, epitestosterone, corticosterone and progesterone.[14-16] It has been shown that the distance of the hair segment from the scalp influences steroid concentrations, as concentrations decrease along the hair shaft.[15,16] Inter-lock variance as reported using LC-MS/MS is approximately 15%[16], which is comparable to previous immunoassay based studies.[17] The setup and validation of an LC-MS/MS based method requires costly investments in time and equipment, as well as the need for specialized personnel. Although the described methods have been validated extensively, basic prerequisite knowledge about effects of environmental factors linked to the hair matrix when using an LC-MS/MS based method, e.g. hair color, hair treatment, but also effects of age and gender, is lacking. Prior to application of hair analysis in research or clinical practice, the effects of these factors need to be evaluated and reference ranges in a healthy population should be established.

The aim of this study is to establish steroid profiles in healthy children aged 4-18 years, assessing the effects of hair matrix linked factors such as color, treatment and hair product use, and provide age adjusted reference ranges for cortisol, cortisone, testosterone, 17OHP, dehydroepiandrosterone sulfate (DHEAS), and androstenedione.

METHODS

Healthy participants were enrolled as described previously[18], through visitation of primary and secondary schools, and siblings of children attending the pediatric outpatient clinic. Criteria of exclusion were chronic disease, the systemic use of glucocorticoids (GC) or other medications affecting steroid metabolism. This study was approved by the medical ethics committee, written consent was obtained from parents and children from the age

of 12 years, and informed assent was given by children aged <12 years. Questionnaires were filled out in a subset of children by either parents/caretakers or children >12yr of age, covering hair care characteristics, general health and use of medication. Information was obtained about washing frequency (<1, 1-2, 3-4, or >4 times per week), use of hair product on the day of sampling (yes/no, type of product: none, mousse, gel, wax, spray, other), frequent sweating at the scalp (yes/no), hair treatment, e.g. dyeing, bleaching, or perming, in the past three months, topical use of GCs and current smoking status (yes/no). In children who did not fill out a questionnaire, hair treatment was set to 'no' for children under the age of 10 years and current smoking status was set to 'no' under the age of 12 years. Ethnicity was grouped as western and non-western, based on parental country of birth. Hair color was coded by two researchers independently and consensus was reached for samples with different coding.

Hair collection and preparation

Hair locks were cut from the posterior vertex, as close to the scalp as possible, using small surgical scissors. The posterior vertex has the lowest variation in repeated measurement[17]. The hair locks were taped to a paper form with scalp end marking and stored in envelopes at room temperature. The hair locks were cut in 1 cm segments, the proximal 3 cm of hair was used for a single analysis, reflecting the past 3 months. Subsequently, hair samples were transferred to glass tubes, weighed (mg), and washed for 2 minutes in Liquid Chromatography (LC) grade iso-propanol at room temperature, and left to dry for at least 48 hours.

Hair Analysis

Steroids were extracted in 1.5 mL LC-grade methanol for 18h at 25°C in the presence of deuterated steroids, the extract was cleaned using solid phase extraction and steroids were quantified following a LC-MS/MS based method described elsewhere[16] using a Waters Xevo TQ-S system (Waters Corporation, Milford, MA, USA). Steroid concentrations are reported in pg per mg hair. Steroid measurement was successful in 96.5%, 97.2%, 94.4%, 78.6%, 82.1% and 82.1% for cortisol, cortisone, 17-OHP, androstenedione, DHEAS and testosterone respectively.

Statistical analysis

Statistical analysis were performed in R version 3.1.1[19]. Age specific reference ranges of steroid concentrations was estimated by estimation of the 2.5th and 97.5th percentile curves by a semi-parametric model with Box-Cox transformation, available in the 'Generalized Additive Models for Location, Scale and Shape' package [20]. Model fit was compared using Akaike's 'an information criterion' combined with visual inspection to prevent over fitting. Steroid concentrations were transformed to age-adjusted z-scores for subsequent analyses. Associations of hair characteristics, topical GC use, smoking, gender and ethnicity with steroid concentrations was assessed using linear regression models, adjusted for gender and ethnicity.

RESULTS

Steroid profiles in a total of 288 children at the age of 4.3 – 18.8 years were measured to establish reference ranges. The general characteristics of participants are shown in Table 1. Questionnaires on hair care characteristics, topical GC use and smoking status was completed in a subset of 180 children.

Reference ranges

Reference ranges of steroids over age are listed in Table 2. The reference ranges are defined as the predicted 2.5th and 97.5th percentiles, shown in Figure 1. DHEAS and testosterone z-scores were significantly lower in girls compared to boys (β=-0.57, $p<0.001$ and β=-0.61, $p<0.001$ resp.), hence the predicted centiles for these steroids were

Table 1. General characteristics

Variable	N=288
Age, years	11.6 (4.1)
Sex, girls/boys	139/149 (48/52%)
Ethnicity, non-western/western	41/231 (15.1%)
Height, cm	156.3 [132.5, 169.0]
Weight, kg	44.0 [26.0, 58.1]
Body mass index	17.6 [15.3, 20.1]
Hair color	
Red	1 (0.4%)
Blond	42 (15.4%)
Dark blonde	79 (29.0%)
Brown	136 (50.0%)
Black	14 (5.1%)
Hair dyed yes/no	7/201 (3.4%)
Washing frequency	
<1x/wk	3 (1.7%)
1-2x/wk	44 (25.0%)
3-4x/wk	90 (51.1%)
>4x/wk	39 (22.2%)
Excessive sweating yes/no	20/148 (13.5%)
Use of hair product yes/no	71/109 (39.4%)
Mousse	3 (4.2%)
Hairspray	6 (8.5%)
Wax	17 (23.9%)
Gel	17 (23.9%)
Other	14 (19.7%)
Multiple	14 (19.7%)
Use of topical steroids yes/no	12/213 (5.3%)
Smoking yes/no	11/243 (4.5%)

Data described as mean (standard deviation), median [interquartile range] or number(percentage).

Table 2. Age adjusted reference ranges for steroid profiles in scalp hair

	Cortisol			Cortisone			Androstenedione			17OHP		
Age	**2,5**	**50,0**	**97,5**	**2,5**	**50,0**	**97,5**	**2,5**	**50,0**	**97,5**	**2,5**	**50,0**	**97,5**
4	0,11	1,01	15,6	2,2	7,6	52,0	0,08	0,19	0,78	0,04	0,18	0,64
5	0,16	1,31	17,0	2,4	7,9	48,8	0,07	0,18	0,74	0,06	0,21	0,62
6	0,20	1,52	16,8	2,6	8,1	45,5	0,07	0,18	0,75	0,08	0,24	0,61
7	0,25	1,66	15,8	2,8	8,4	43,0	0,09	0,21	0,91	0,11	0,27	0,64
8	0,29	1,78	14,8	3,1	8,9	42,0	0,11	0,29	1,23	0,13	0,31	0,72
9	0,35	1,93	14,1	3,4	9,6	41,7	0,15	0,37	1,61	0,14	0,35	0,82
10	0,39	1,97	12,7	3,7	10,0	40,7	0,18	0,46	2,06	0,15	0,38	0,92
11	0,41	1,91	11,0	4,0	10,4	39,3	0,23	0,59	2,67	0,16	0,41	1,00
12	0,46	1,98	10,2	4,4	11,2	39,5	0,30	0,77	3,53	0,18	0,47	1,10
13	0,58	2,27	10,6	5,1	12,4	41,2	0,38	0,99	4,63	0,22	0,55	1,30
14	0,73	2,66	11,4	5,8	13,6	42,8	0,47	1,23	5,86	0,25	0,66	1,60
15	0,87	2,96	11,6	6,3	14,5	43,2	0,55	1,47	7,10	0,28	0,77	1,92
16	0,94	3,01	10,9	6,6	14,6	41,3	0,63	1,68	8,28	0,29	0,85	2,22
17	0,97	2,91	9,7	6,5	14,1	37,9	0,70	1,87	9,40	0,28	0,91	2,59
18	0,96	2,73	8,5	6,3	13,2	34,0	0,76	2,05	10,5	0,26	0,96	3,04

Predicted 2.5th , 50th and 97.5th percentiles as reference range cutoffs for age, per year.

modelled separately for boys and girls. Cortisol and cortisone shows a slight increase over age with a reduction in variance in adolescents. Androstenedione, 17OHP and testosterone show a marked increase over age, leveling off at approximately the age of 14 years. The median testosterone concentrations are significantly higher in boys than in girls, especially in adolescent children. Due to a larger variance of testosterone in girls, the 97.5th percentile line is higher in girls than in boys. For DHEAS, concentrations increase slightly with age in boys, but not in girls.

Effects of determinants on steroid measurement

Table 3 shows the effects of participant and hair related characteristics on steroid z-scores. As only one child had red hair and two children washed their hair less than once a week, these results were not included in the analysis. All steroids are lower in children of western descent than in children of non-western descent, except for DHEAS which is lower in children of non-western ethnicity. Smoking and topical GC use was not associated with a difference in steroid concentrations. Hair color was associated with differences in steroid concentrations. Blond hair was associated with lower 17OHP and black hair was associated with higher DHEAS and testosterone. No influence of excessive sweating at the scalp and use of hair products was found. Also when analyzed per hair product type, no effects on steroids was found (ANCOVA adjusted for sex and ethnicity, all p-values > 0.3). Hair dye was associated with a lower cortisone and a higher androstenedione. A washing frequency of once to twice per

DHEAS Boys			DHEAS Girls			Testosterone boys			Testosterone Girls		
2,5	**50,0**	**97,5**	**2,5**	**50,0**	**97,5**	**2,5**	**50,0**	**97,5**	**2,5**	**50,0**	**97,5**
2,97	8,61	32,03	2,72	8,10	30,13	0,03	0,19	2,07	0,02	0,09	1,90
3,26	9,86	39,03	2,66	8,36	33,54	0,03	0,15	1,46	0,01	0,07	1,42
3,53	11,10	46,95	2,61	8,63	37,47	0,03	0,13	1,12	0,01	0,07	1,29
3,76	12,35	55,96	2,54	8,89	42,04	0,03	0,15	1,09	0,02	0,08	1,59
3,96	13,60	66,27	2,47	9,15	47,38	0,04	0,18	1,19	0,02	0,12	2,19
4,13	14,85	78,14	2,39	9,42	53,68	0,05	0,20	1,23	0,03	0,17	3,02
4,27	16,10	91,87	2,31	9,68	61,17	0,05	0,22	1,17	0,04	0,22	3,91
4,38	17,35	107,87	2,22	9,95	70,14	0,06	0,24	1,17	0,05	0,27	4,63
4,46	18,60	126,63	2,13	10,21	80,99	0,09	0,31	1,40	0,06	0,30	5,10
4,52	19,85	148,77	2,04	10,48	94,23	0,13	0,44	1,83	0,07	0,32	5,30
4,55	21,09	175,12	1,94	10,74	110,57	0,19	0,59	2,29	0,07	0,33	5,33
4,56	22,34	206,69	1,84	11,01	130,97	0,25	0,74	2,66	0,07	0,34	5,34
4,54	23,59	244,85	1,74	11,27	156,76	0,30	0,86	2,87	0,08	0,35	5,30
4,51	24,84	291,38	1,64	11,54	189,81	0,35	0,94	2,97	0,08	0,34	5,10
4,45	26,09	348,65	1,54	11,80	232,80	0,40	1,02	3,02	0,07	0,32	4,69

week was associated with lower 17OHP. No other associations were found between hair washing frequency and steroid concentrations.

DISCUSSION

This study is the first to provide reference ranges of LC-MS/MS measured steroid concentrations in human scalp hair in healthy children of 4 to 18 years old, for cortisol, cortisone, 17-OHP, androstenedione, testosterone and DHEAS. Measuring steroids in scalp hair provides long-term cumulative steroid concentrations over one to several months, unaffected by circadian rhythm or acute stress. This method can potentially be applied to a wide range of endocrinological issues such as diagnosis and follow-up of children with (cyclic) Cushing's syndrome or disease, adrenal insufficiency, and congenital adrenal hyperplasia. To date, studies on steroid measurement in human hair are scarce, especially in children. Vanaelst *et al.*[21] measured cortisone concentrations in hair of 223 elementary school girls, mean age 8.4 yr., associating cortisone levels with stress, life events and coping strategies. The median cortisone concentration of 8.6 pg/mg found by Vanaelst et al. corresponds to the results found in the current study (8.9 pg/mg at age 8yr.). Another group who measured cortisol in scalp hair from 10 healthy prepubertal children and 10 children with asthma, reported measuring a median of 4.3 pg/mg and 2.0 pg/mg in the two groups, respectively[22]. This is slightly higher than the median concentration of 1.8 pg cortisol per mg hair in 8-year old children in this study.

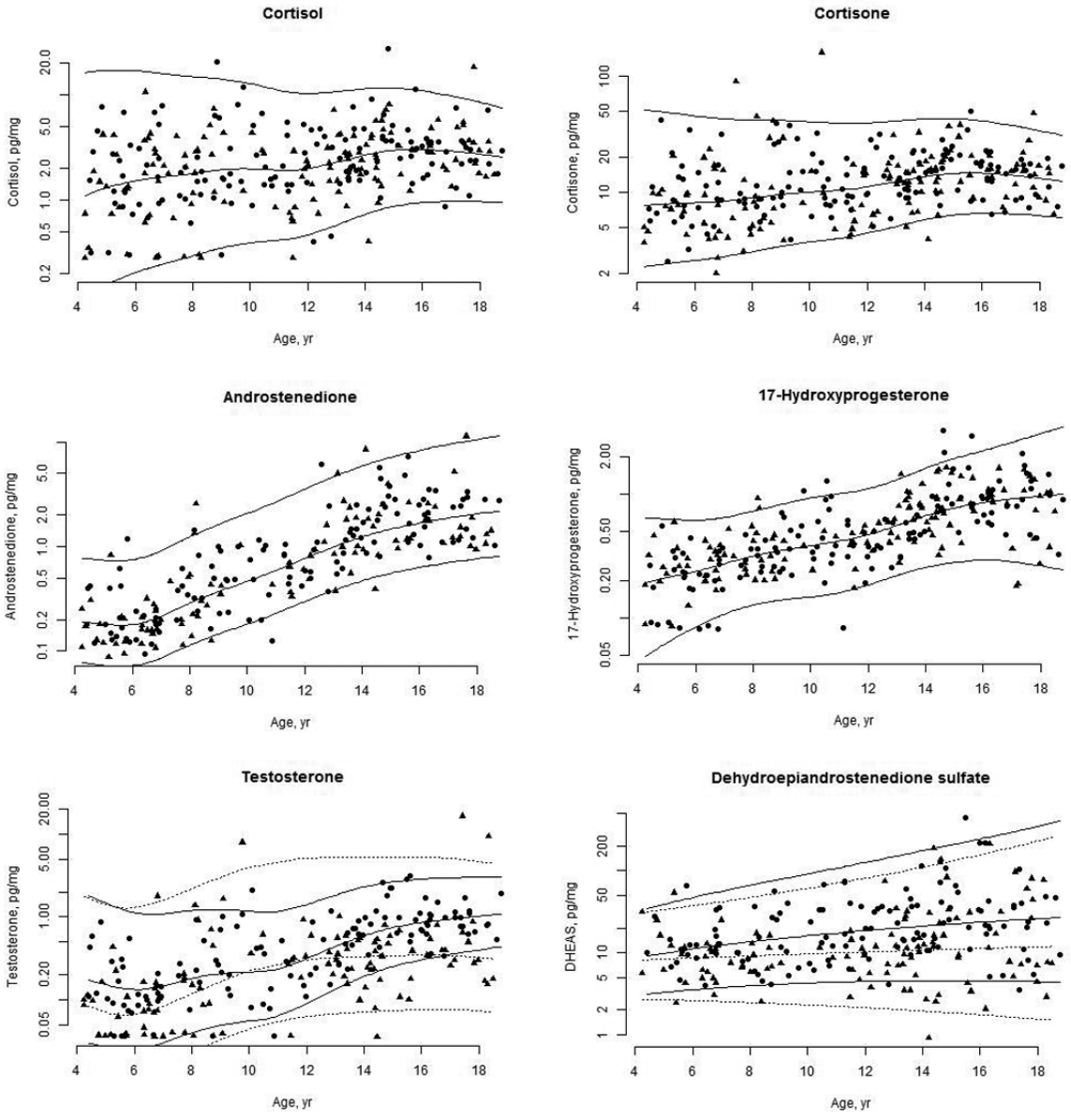

Figure 1. Reference ranges steroid concentrations over age. Steroid concentrations plotted against age, lines showing predicted 2.5th, 50th and 97.5th percentiles for cortisol, cortisone, androstenedione and 17-hydroxyprogesterone (17OHP). For DHEAS and testosterone the dotted lines represent ranges in girls and solid lines in boys. Circles represent boys, triangles girls.

The difference may derive from the small sample-size or differences in methods in the study by Kamps et al.[22] They reported processing 95-150 mg of hair, whereas we regard 40 mg as the absolute maximum amount of hair, and prefer to process 10-20 mg of hair, to avoid matrix interference.[16] Since 2012 three studies reported on steroids in scalp

Table 3. Associations with Hair steroid concentrations

	Cortisol			Cortisone			Androstenedione			17OHP			DHEAS			Testosterone		
	Beta	**SE**	**P**	**Beta**	**SE**	**P**	**Beta**	**SE**	**P**	**Beta**	**SE**	**P**	**Beta**	**SE**	**P**	**Beta**	**SE**	**P**
Sex:																		
Boys	Ref			Ref			Ref			Ref			Ref			Ref		
Girls	-0,21	0,12	0,09	-0,20	0,12	0,11	-0,20	0,13	0,13	0,09	0,13	0,46	0,00	0,13	0,99	-0,01	0,13	0,95
Ethnicity:																		
Western	Ref			Ref			Ref			Ref			Ref			Ref		
Nonwestern	**0,54**	**0,18**	**0,003**	**0,46**	**0,17**	**0,009**	**0,73**	**0,18**	**0,000**	**0,61**	**0,18**	**0,001**	**-0,41**	**0,19**	**0,03**	**0,61**	**0,18**	**0,001**
Top. GC use: yes	0,12	0,31	0,71	-0,11	0,29	0,71	0,41	0,29	0,16	-0.21	0,32	0,52	0,10	0,32	0,75	0,01	0,34	0,98
Smoking: yes	0,00	0,31	0,99	-0,24	0,30	0,43	-0,20	0,30	0,52	0,18	0,32	0,58	0,40	0,33	0,23	-0,12	0,33	0,72
Hair color:																		
Brown	Ref			Ref			Ref			Ref			Ref			Ref		
Blond	-0,05	0,18	0,78	-0,10	0,19	0,60	-0,32	0,20	0,12	**-0,41**	**0,19**	**0,04**	-0,11	0,21	0,61	0,11	0,22	0,62
Dark-blond	-0,05	0,15	0,72	0,10	0,15	0,51	-0,30	0,16	0,06	0,06	0,15	0,69	-0,09	0,16	0,57	0,04	0,17	0,82
Black	0,26	0,32	0,41	0,53	0,33	0,10	0,52	0,32	0,11	0,23	0,34	0,50	**0,72**	**0,33**	**0,03**	**0,97**	**0,34**	**0,005**
Hair treatment: yes	-0,56	0,41	0,18	**-1.02**	**0,40**	**0,01**	**0,86**	**0,43**	**0,05**	-0,10	0,43	0,82	0,18	0,43	0,68	-0,36	0,40	0,37
Washing frequency																		
1-2x/wk	0,26	0,19	0,18	-0,13	0,19	0,50	**-0,42**	**0,19**	**0,03**	0,12	0,21	0,55	-0,16	0,20	0,43	-0,15	0,19	0,44
3-4x/wk	Ref			Ref			Ref			Ref			Ref			Ref		
>4x/wk	-0,10	0,20	0,63	-0,17	0,20	0,39	-0,12	0,20	0,56	-0,07	0,21	0,74	-0,01	0,23	0,97	0,03	0,21	0,88
Excessive sweating: yes	0,40	0,24	0,10	0,28	0,23	0,22	-0,25	0,24	0,30	-0,25	0,25	0,33	0,08	0,28	0,79	0,17	0,26	0,50
Hair product use: yes	-0,13	0,16	0,42	-0,10	0,16	0,51	0,02	0,16	0,91	-0,10	0,17	0,55	-0,24	0,17	0,16	-0,17	0,16	0,30

Beta's, standard error (SE) and P-values (P) of linear regression model, all models adjusted for sex and ethnicity. Hair steroids are provided in z-scores adjusted for age

hair of adults. When compared to children at the age of 16 – 18 years participating in the current study, the concentrations reported by Gao *et al.*[15] for cortisol, cortisone, androstenedione and testosterone are all approximately twice the concentration measured in the current study. Cortisol and cortisone concentrations measured in two Chinese cohorts (mean age 41 and 38 years) by Chen *et al.*[23] were even higher (median concentrations cortisol 15.9 and 5.1pg/mg, cortisone 57.9 and 62.1 pg/mg) whereas DHEAS was lower (12.6 and 6.9 pg/mg). The differences in cortisol and DHEAS concentrations between the two cohorts are by itself already remarkable. Deshmukh *et al.*[14] reported testosterone concentrations in scalp hair ranging from 0.3 to 11.8 pg/mg. These differences may in part be explained by differences in the method, for example the use of other extraction solvent[14] or a longer extraction period, e.g. of five days versus 18 hours.[23]

Several studies have established steroid reference ranges in children, measured in serum. Bailey *et al.*[24] included 1482 children of 0-18yr and measured a range of hormones, amongst which cortisol. Mean cortisol concentrations increased steadily from the age of 4 into adolescence, which is in line with the increasing median concentrations found in the current study. Kulle *et al.*[25,26] reported age- and sex-specific reference data for serum concentrations of adrenal and sex steroids in children. Total serum cortisol and cortisone showed an increase with age after the age of 1 yr, as did 17OHP. Comparable to the current study, Kulle et al. found no differences in median testosterone concentrations between boys and girls up to the age of 12 years, after which concentrations increased particularly in boys[26]. Androstenedione showed an increase approximately initiating around age 9 and increased more significantly around age 12.[25] Although the current study embodies hormone concentrations measured in a different matrix, the associations with age do show resemblance to hormone studies performed in serum. In contrast, DHEAS, of which the concentrations in hair increase linearly with age, do not resemble the marked increase in concentrations around adrenarche in serum.[27,28] Although we have no explanation for this difference, one could speculate that the sulfate form of steroids might less readily diffuse into hair follicle cells, or that DHEAS undergoes desulphation upon uptake. Martel *et al.*[29] showed that several enzymes that are able to convert DHEAS into functional steroids are active in skin.

To our knowledge, this is the first study assessing the effects of potential confounders of the measurement of endogenous steroids in human scalp hair using LC-MS/MS. We found significant effects of ethnicity and hair color on steroid concentrations. Several studies have addressed the effects of potential confounders on cortisol concentrations measured in hair using immunoassays. Sauvé *et al.*[17] found no effects of natural hair color on the assay result. Dettenborn *et al.*[30] compared individuals with different hair colors, finding no significant differences in cortisol. Based on the hair colors reported, Caucasian participants were probably overrepresented, although no data on ethnicity is mentioned. The most striking differences in steroid concentrations associated with hair color in the current study are seen in testosterone and DHEAS, which are increased

in black hair. This suggests that differences in the makeup of hair represented by the color may contribute to the hair steroid content. Furthermore, residual confounding in ethnicity may play a role in these findings, as ethnicity was defined in a dichotomous variable (western vs. nonwestern), which probably underrepresents the differences between ethnicities in the nonwestern group. We found no major effects due to hair product use, excessive transpiration at the scalp or washing frequency, which is in line with the other studies which assessed these variables in hair cortisol measurement.[11,17,18,30] A hair washing frequency of 1-2 times per week was associated with lower concentration of androstenedione. Hypothesizing that a higher hair washing frequency may decrease hair steroid content, this finding is the opposite of expected, and may well be a type 1 error. It should be stressed that associations of steroid concentrations with topical glucocorticoid use, smoking and hair treatment are based on small numbers (N=12, 11 and 7, respectively). Steroid measurement in human scalp hair is a non-invasive method, providing insight in long-term steroid concentrations that, when measured in other matrices are subject to circadian fluctuations and pulsatile excretion. Strengths of this study are its heterogeneous population sample with a wide age range and multiple ethnicities. Ethnicity in the Netherlands is strongly associated with immigration, culture, different social economic status and education level.[31] It is therefore difficult to identify the causal factor in ethnicity affecting steroid levels. One limitation in the current study is that ethnicity is defined as a dichotomous variable. Further studies with larger numbers making sub-categorization may be needed. To establish reference ranges, using a non-parametric approach is advised requiring a minimum of 120 observations [32]. However, when partitioning for covariates such as age and gender, the sample size needed increases, especially for continuous variables such as age. As changes of steroid concentrations occur gradually over time, the concentrations measured for a certain age are assumed to contribute to the estimation of reference values of adjacent age categories. This can be applied by the estimation of smooth centile curves, which is a popular approach in defining reference ranges for variables in pediatric populations[20,33,34].

The non-invasiveness and easy storage of samples make this method exceptionally suitable for the pediatric clinical practice. Although clinical validation is warranted, long-term steroid profiling in scalp hair may provide new insights and novel clinical applications in a range of endocrinological diseases, e.g. diagnosis and treatment follow-up in Cushing's syndrome, adrenal insufficiency, congenital adrenal hyperplasia, male hyper- and hypogonadism, and female hyperandrogenism. This study provides the background knowledge needed for implementation of hair steroid profiling in clinical research.

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REFERENCES

1. Wester VL, Lamberts SW, van Rossum EF. Advances in the assessment of cortisol exposure and sensitivity. *Curr Opin Endocrinol Diabetes Obes* 2014; **21**(4): 306-11.
2. Chan J, Sauve B, Tokmakejian S, Koren G, Van Uum S. Measurement of cortisol and testosterone in hair of obese and non-obese human subjects. *Exp Clin Endocrinol Diabetes* 2014; **122**(6): 356-62.
3. Veldhorst MAB, Noppe G, Jongejan MHTM, et al. Increased Scalp Hair Cortisol Concentrations in Obese Children. *J Clin Endocr Metab* 2014; **99**(1): 285-90.
4. Wester VL, Staufenbiel SM, Veldhorst MAB, et al. Long-Term Cortisol Levels Measured in Scalp Hair of Obese Patients. *Obesity* 2014; **22**(9): 1956-8.
5. Stalder T, Kirschbaum C, Alexander N, et al. Cortisol in hair and the metabolic syndrome. *J Clin Endocrinol Metab* 2013; **98**(6): 2573-80.
6. Manenschijn L, Schaap L, van Schoor NM, et al. High long-term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease. *J Clin Endocrinol Metab* 2013; **98**(5): 2078-83.
7. Pereg D, Gow R, Mosseri M, et al. Hair cortisol and the risk for acute myocardial infarction in adult men. *Stress* 2011; **14**(1): 73-81.
8. Manenschijn L, Koper JW, van den Akker EL, et al. A novel tool in the diagnosis and follow-up of (cyclic) Cushing's syndrome: measurement of long-term cortisol in scalp hair. *J Clin Endocrinol Metab* 2012; **97**(10): E1836-43.
9. Thomson S, Koren G, Fraser LA, Rieder M, Friedman TC, Van Uum SH. Hair analysis provides a historical record of cortisol levels in Cushing's syndrome. *Exp Clin Endocrinol Diabetes* 2010; **118**(2): 133-8.
10. Gow R, Koren G, Rieder M, Van Uum S. Hair cortisol content in patients with adrenal insufficiency on hydrocortisone replacement therapy. *Clin Endocrinol (Oxf)* 2011; **74**(6): 687-93.
11. Manenschijn L, Koper JW, Lamberts SW, van Rossum EF. Evaluation of a method to measure long term cortisol levels. *Steroids* 2011; **76**(10-11): 1032-6.
12. Ibrahim C, Van Uum S. Hair analysis of cortisol levels in adrenal insufficiency. *CMAJ* 2014; **186**(16): 1244.
13. Noppe G, van Rossum EFC, Vliegenthart J, Koper JW, van den Akker ELT. Elevated hair cortisol concentrations in children with adrenal insufficiency on hydrocortisone replacement therapy. *Clin Endocrinol* 2014; **81**(6): 820-5.
14. Deshmukh NI, Barker J, Petroczi A, Naughton DP. Detection of testosterone and epitestosterone in human hair using liquid chromatography-tandem mass spectrometry. *Journal of pharmaceutical and biomedical analysis* 2012; **67-68**: 154-8.
15. Gao W, Stalder T, Foley P, Rauh M, Deng H, Kirschbaum C. Quantitative analysis of steroid hormones in human hair using a column-switching LC-APCI-MS/MS assay. *J Chromatogr B Analyt Technol Biomed Life Sci* 2013; **928**: 1-8.
16. Noppe G, de Rijke YB, Dorst K, van den Akker EL, van Rossum EF. LC-MS/MS-based method for long-term steroid profiling in human scalp hair. *Clin Endocrinol (Oxf)* 2015; **83**(2): 162-6.
17. Sauve B, Koren G, Walsh G, Tokmakejian S, Van Uum SH. Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clin Invest Med* 2007; **30**(5): E183-91.
18. Noppe G, Van Rossum EFC, Koper JW, et al. Validation and Reference Ranges of Hair Cortisol Measurement in Healthy Children. *Horm Res Paediat* 2014; **82**(2): 97-102.
19. R Core Team, R Foundation for Statistical Computing. R: A language and environment for statistical computing. *http://www.R-project.org* 2013.
20. Rigby RA, Stasinopoulos DM. Generalized additive models for location, scale and shape. *J Roy Stat Soc C-App* 2005; **54**: 507-44.
21. Vanaelst B, Huybrechts I, Bammann K, et al. Intercorrelations between serum, salivary, and hair cortisol and child-reported estimates of stress in elementary school girls. *Psychophysiology* 2012; **49**(8): 1072-81.
22. Kamps AW, Molenmaker M, Kemperman R, van der Veen BS, Bocca G, Veeger NJ. Children with asthma have significantly lower long-term cortisol levels in their scalp hair than healthy children. *Acta paediatrica* 2014; **103**(9): 957-61.
23. Chen Z, Li J, Zhang J, et al. Simultaneous determination of hair cortisol, cortisone and DHEAS with liquid chromatography-electrospray ionization-tandem mass spectrometry in negative mode. *J Chromatogr B Analyt Technol Biomed Life Sci* 2013; **929**: 187-94.
24. Bailey D, Colantonio D, Kyriakopoulou L, et al. Marked biological variance in endocrine and biochemical markers in childhood: establishment of pediatric reference intervals using healthy community children from the CALIPER cohort. *Clin Chem* 2013; **59**(9): 1393-405.
25. Kulle AE, Riepe FG, Melchior D, Hiort O, Holterhus PM. A novel ultrapressure liquid

chromatography tandem mass spectrometry method for the simultaneous determination of androstenedione, testosterone, and dihydrotestosterone in pediatric blood samples: age- and sex-specific reference data. *J Clin Endocrinol Metab* 2010; **95**(5): 2399-409.

26. Kulle AE, Welzel M, Holterhus PM, Riepe FG. Implementation of a liquid chromatography tandem mass spectrometry assay for eight adrenal C-21 steroids and pediatric reference data. *Horm Res Paediatr* 2013; **79**(1): 22-31.
27. Guran T, Firat I, Yildiz F, Kaplan Bulut I, Dogru M, Bereket A. Reference values for serum dehydroepiandrosterone-sulphate in healthy children and adolescents with emphasis on the age of adrenarche and pubarche. *Clin Endocrinol (Oxf)* 2014.
28. Arlt W. Dehydroepiandrosterone and ageing. *Best Pract Res Clin Endocrinol Metab* 2004; **18**(3): 363-80.
29. Martel C, Melner MH, Gagne D, Simard J, Labrie F. Widespread tissue distribution of steroid sulfatase, 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD), 17 beta-HSD 5 alpha-reductase and aromatase activities in the rhesus monkey. *Mol Cell Endocrinol* 1994; **104**(1): 103-11.
30. Dettenborn L, Tietze A, Kirschbaum C, Stalder T. The assessment of cortisol in human hair: associations with sociodemographic variables and potential confounders. *Stress* 2012; **15**(6): 578-88.
31. Flink IJ, Beirens TM, Looman C, et al. Health-related quality of life of infants from ethnic minority groups: the Generation R Study. *Qual Life Res* 2013; **22**(3): 653-64.
32. Shaw JL, Binesh Marvasti T, Colantonio D, Adeli K. Pediatric reference intervals: challenges and recent initiatives. *Crit Rev Clin Lab Sci* 2013; **50**(2): 37-50.
33. Rodd C, Metzger DL, Sharma A, Canadian Pediatric Endocrine Group Working Committee for National Growth C. Extending World Health Organization weight-for-age reference curves to older children. *BMC Pediatr* 2014; **14**: 32.
34. Stanojevic S, Wade A, Stocks J, et al. Reference ranges for spirometry across all ages: a new approach. *Am J Respir Crit Care Med* 2008; **177**(3): 253-60.

PART II

LONG-TERM STEROID HORMONE CONCENTRATIONS IN OBESITY AND IN DISEASE

CHAPTER 5

INCREASED SCALP HAIR CORTISOL CONCENTRATIONS IN OBESE CHILDREN

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ABSTRACT

Context

Pathologically increased cortisol exposure induces obesity but it is not known whether relatively high cortisol within the physiological range is related to childhood obesity.

Objective

To compare hair cortisol concentrations between obese and normal weight children.

Design

An observational case-control study.

Participants

Twenty obese children (Body Mass Index-standard deviation score, BMI-SDS>2.3) and 20 age- and sex-matched normal weight children (BMI-SDS<1.1) aged 8-12 years were recruited.

Main outcome measures

Scalp hair samples from the posterior vertex were collected and hair cortisol concentrations were measured using ELISA. Body weight, height and waist circumference were measured. From the obese children additional data on blood pressure and blood lipid concentrations were collected.

Results

In both groups 5 boys and 15 girls were included: their mean age was 10.8±1.3 vs. 10.8±1.2 years (obese vs. normal weight, n.s.). Body weight, BMI, BMI-SDS and waist circumference were higher in the obese compared with the normal weight children (69.81±17.17 vs. 35.46±7.23 kg, 29.6±4.9 vs. 16.4±1.6 kg/m^2, 3.4±0.5 vs. -0.2±0.8 SDS, 94±13 vs. 62±6 cm, $p<0.001$ all). Hair cortisol concentration was higher in obese compared with normal weight children (median [IQR]: 25 [17, 32] vs. 17 [13, 21] pg/mg, $p<0.05$).

Conclusions

Hair cortisol concentrations, a measure for chronic cortisol exposure, were higher in obese compared with normal weight children. This suggests chronic activation of the hypothalamus-pituitary-adrenal axis in obese children and may provide a novel target for treatment of obesity in children.

INTRODUCTION

Childhood obesity is often accompanied by co-morbidities including hypertension, impaired glucose tolerance and dyslipidemia. The clustering of these cardiometabolic abnormalities is known as the metabolic syndrome[1,2]. The symptoms of the metabolic syndrome resemble those of Cushing's syndrome, a disease which is characterized by hypercortisolism. This raises the question whether chronically elevated cortisol concentrations in the general population play a role in the development of obesity and the metabolic syndrome.

Several studies have shown a positive association between cortisol levels and obesity[3,4], body fat distribution[5], insulin sensitivity[3,6], or the metabolic syndrome[7] in children or adolescents. Contrarily, other studies observed an inverse association between cortisol measures and adiposity[8,9]. Also in adults conflicting results have been published: some studies report a positive association between cortisol and obesity and the metabolic syndrome[10,11], whereas in a review it was recently concluded that there is no strong relationship between cortisol and obesity or the metabolic syndrome[12].

In most studies cortisol concentrations have been measured in saliva, urine or serum. However, due to the circadian rhythm, pulsatile secretion and the daily variation in the secretion of cortisol, none of these sampling matrices is a good measure of chronic cortisol concentrations. A more suitable matrix appears to be scalp hair. Unbound cortisol and other lipophilic substances are incorporated in scalp hair, which grows with an average rate of one centimeter per month in adults[13]. A hair sample of one centimeter therefore is thought to represent the individual mean exposure of free cortisol of one month. Recently, several studies have shown that hair cortisol is a reliable marker of long-term endogenous cortisol concentrations[14,15]. For instance, hair cortisol has been shown to be increased in patients with hypercortisolism and when analyzed retrospectively, hair cortisol concentrations corresponded with clinical course in patients treated for hyper- or hypocortisolism[16,17]. Also in populations with higher chronic stress elevated concentrations of hair cortisol have been shown compared with control groups[18-20].

In a cohort of adults, Stalder *et al.* showed a positive association between Body Mass Index (BMI) and hair cortisol concentration[21]. Moreover, in normal weight adults hair cortisol concentration was positively correlated with waist circumference and waist-hip ratio[16]. In two other studies in adults, subjects in the highest quartile of hair cortisol concentration had a 2.4 fold increased risk of metabolic syndrome[22] and a 2.7 fold increased risk of cardiovascular disease[23]. However, it is questionable whether cortisol concentrations are disturbed early in the clinical course of obesity, *i.e.* in pre-pubertal children.

In the present study we used scalp hair samples to study long-term cortisol concentrations in children. The objective was to study whether obese children have higher hair cortisol concentrations than children with a normal body weight.

MATERIALS AND METHODS

Subjects

In this cross-sectional case-control study, 20 obese children (Body Mass Index-standard deviation score, BMI-SDS > 2.3) and 20 normal weight children (BMI-SDS <1.1) aged between 8 and 12 years were included. Obese children were recruited at the obesity outpatient clinic of the department of Pediatrics of 2 hospitals in Rotterdam, the Netherlands: the Erasmus MC - Sophia Children's Hospital and the Sint Franciscus Gasthuis. All obese children aged between 8 and 12 years visiting these obesity outpatient clinics obtained oral and written information about the study. For each child who gave consent to participate in the study, an age- and sex-matched normal weight child was included. The age- and sex-matched normal weight children were recruited at primary schools or among healthy siblings of children visiting the general outpatient clinic of the department of Pediatrics of the Erasmus MC - Sophia Children's Hospital. Exclusion criteria were: chronic disease, use of any type of medication and short scalp hair (<2 cm). The study was conducted according to the Declaration of Helsinki and was approved by the Medical Ethics Committees of the Erasmus MC and the Sint Franciscus Gasthuis. Written informed consent was obtained from the parents or caretakers of all participants. Children aged 12 years also signed an informed consent form themselves.

Measurements

Anthropometric parameters

Body weight and height were measured and BMI was calculated. BMI-SDS was calculated based on data from the Dutch National Growth Study 2010 using Growth analyser software. Obesity was defined according to the International Task Force Obesity cut-off values for BMI. With respect to Dutch children overweight and obesity were defined as a BMI-SDS of more than 1.1 and 2.3, respectively[24]. Waist circumference was measured using a non-elastic flexible tape measure with the subject standing without clothes covering the waist area.

Other measurements

Ethnicity was represented in 2 categories: Caucasian (2 parents Caucasian) and non-Caucasian (1 or 2 parents non-Caucasian).

Within the scope of standard care at the obesity outpatient clinics, a blood sample was drawn and blood pressure and heart rate were measured and it was checked whether the child had the metabolic syndrome, based on the criteria of the International Diabetes Federation[25]. Blood pressure was expressed as SDS adjusted for height and gender[26]. The attending pediatrician decided for each individual patient which parameters were to be analyzed in the blood; in any case, concentrations of total, highdensity lipoprotein (HDL)- and low-density lipoprotein (LDL)- cholesterol as well as triglycerides were measured. Serum total cholesterol and triglycerides were determined enzymatically, and LDL-cholesterol and HDL-cholesterol were determined

using a homogeneous assay on a Hitachi 917 (Roche Diagnostics). In the normal weight children no additional measurements were performed.

Hair cortisol measurements

A lock of approximately 100 hairs was cut from the posterior vertex of the scalp, as close to the scalp as possible. The hair samples were taped to a piece of paper and the proximal side of the hairs was marked. The hair samples were stored at room temperature in an envelope until analysis. The total length of the hair was divided in segments of 1 cm and the part closest to the scalp was used for further analysis. A minimum of 10 mg of hair was weighed and put into a glass vial. The hair samples were cut into small pieces of 1-2 mm in length, and 1 ml of methanol was used to extract cortisol from the hair samples. The vial was sealed and incubated overnight for 16h at 52°C while gently shaking. After incubation, the methanol was transferred to another vial and evaporated under nitrogen. Subsequently, the samples were dissolved in 250 µl phosphate buffered saline (pH 8.0) and vortexed for 1 min. Before analysis, the samples were vortexed again for 30 s. A commercially available ELISA kit for salivary cortisol (DRG GmbH, Marburg, Germany) was used to measure cortisol concentrations. Cross reactivity of other steroids with the kit's antibodies was reported as follows: corticosterone (29.00%), cortisone (3.00%), 11-deoxycortisol (<1.00%), 17OH progesterone (<0.50%), other hormones (<0.10%). Intra-assay variation was below 5% and the interassay variation below 8% as stated by the manufacturer[16].

Statistical analysis

Statistical procedures were performed using SPSS version 20 (IBM SPSS Statistics, Chicago, IL, USA). Differences in age, body weight, BMI, BMI-SDS and waist circumference between obese children and normal weight children were analyzed with one-way ANOVA. Hair cortisol concentrations were not normally distributed and were logarithmically transformed and subsequently analyzed with one-way ANOVA. Correlations of hair cortisol with BMI-SDS and waist circumference were analyzed using Pearson's correlations. A p-value <0.05 was regarded as statistically significant.

RESULTS

Subject characteristics are presented in table 1. In both groups, 5 boys and 15 girls were included. The mean age of the obese and normal weight children was 10.8 ± 1.3 years and 10.8 ± 1.2 years, respectively (n.s.). In the group with obese children, 9 were Caucasian and 11 were non-Caucasian whereas in the group with normal weight children 15 were Caucasian and 5 were non-Caucasian. The body weight, BMI, BMI-SDS and waist circumference were significantly higher in the obese children compared with the normal weight children (p<0.001 all).

None of the normal weight children was familiar with chronic diseases, including diabetes mellitus type 2. None of the obese children had diabetes mellitus type

Table 1. Subject characteristics of the obese children and the normal weight children

	Obese (n=20)	Normal weight (n=20)
Age (yr)	10.8 ± 1.3	10.8 ± 1.2
Sex (m/f)	5/15	5/15
Ethnicity (Caucasian/non-Caucasian)	9/11	15/5
Body weight (kg)	69.81 ± 17.17***	35.46 ± 7.23
BMI (kg/m^2)	29.6 ± 4.9***	16.4 ± 1.6
BMI-SDS	3.4 ± 0.5***	-0.2 ± 0.8
Waist circumference (cm)	94 ± 13***	62 ± 6

Data are presented as mean ± st. dev. unless otherwise indicated
BMI: Body Mass Index, BMI-SDS: Body Mass Index – standard deviation score
One-way ANOVA: *** p<0.001 between obese and normal weight children

2, but 3 subjects were diagnosed with the metabolic syndrome. The obese children had a systolic blood pressure of 116 ± 13 mmHg, a diastolic blood pressure of 62 ± 7 mmHg, a hearth rate of 86 ± 12 beats/minute, a total cholesterol concentration of 4.22 ± 0.84 mmol/l, a HDL-cholesterol concentration of 1.20 ± 0.32 mmol/l, a LDL-cholesterol concentration of 2.53 ± 0.74 mmol/l and a triglycerides concentration of 1.21 ± 0.66 mmol/l, respectively. The children had no other medical diagnoses.

The cortisol concentration in scalp hair was (median with [interquartile range]) 25 [17, 32] pg/mg in the obese children and 17 [13, 21] pg/mg in the normal weight children (p<0.05, figure 1).

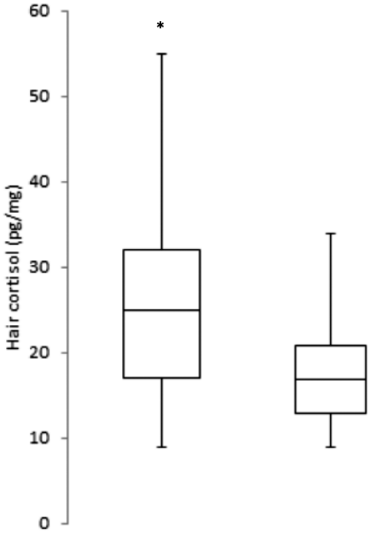

Figure 1. Scalp hair cortisol concentrations in obese children and normal weight children (median, Q1-Q3, min-max). * p<0.05 One-way ANOVA after logarithmically transformation.

There was a correlation between log hair cortisol concentration and BMI-SDS (r=0.407, p<0.01, figure 2A) and between log hair cortisol concentration and waist circumference (r=0.430, p<0.01, figure 2B).

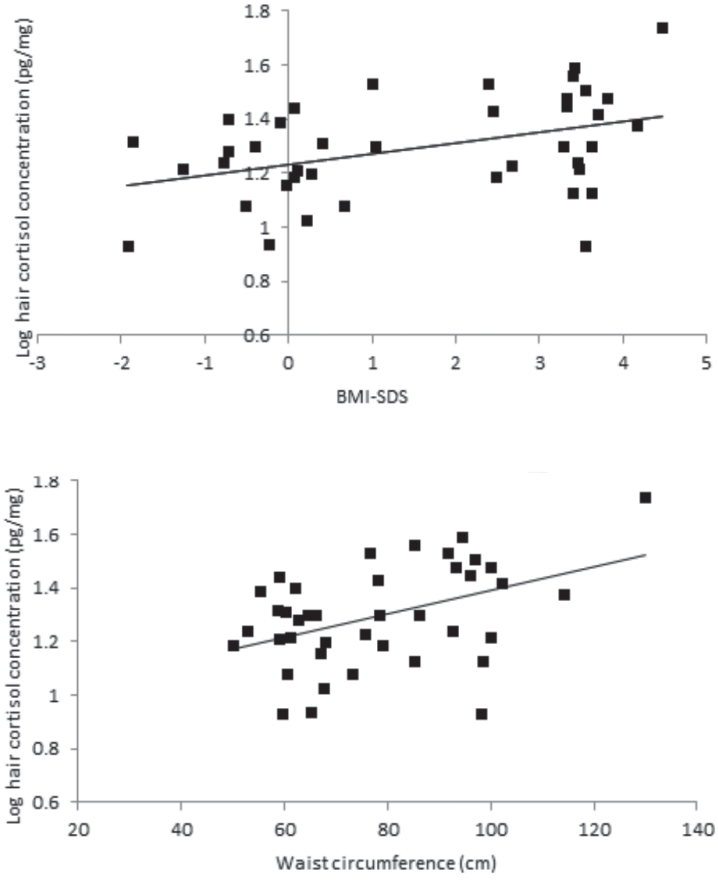

Figure 2. Correlation between BMI-SDS and hair cortisol concentration after logarithmically transformation (Pearson's correlation coefficient r=0.407, p<0.01, A) and correlation between waist circumference and hair cortisol concentration after logarithmically transformation (Pearson's correlation coefficient r=0.430, p<0.01, B) in children aged between 8 and 12 years.

There was one outlier with respect to hair cortisol concentration in the group with obese children; this was a morbidly obese subject with a BMI-SDS of 4.5 and a waist circumference of 130 cm (see figure 2). Repeating analyses without this subject resulted in similar results, *i.e.* a significant difference in hair cortisol concentration between obese and normal weight children (p<0.05) and a correlation between log hair cortisol concentration and BMI-SDS (p<0.05).

DISCUSSION

This case-control study shows that obese children have higher hair cortisol concentrations than children with a normal body weight. Hair cortisol concentrations were positively correlated with the BMI-SDS and with the waist circumference. These cortisol concentrations are a marker of long-term, *i.e.* 1 month, systemic cortisol exposure. This may indicate chronic activation of the hypothalamus-pituitary-adrenal (HPA) axis in obese children, however, more research is needed to unravel the role of cortisol in childhood obesity. To our knowledge our study is the first to show increased hair cortisol concentrations in obese children compared with normal weight children. In the adult population, a relationship between BMI and hair cortisol concentration already has been shown[21]. Moreover, an increased risk of metabolic syndrome or cardiovascular disease with increased hair cortisol concentrations has been observed[22,23]. Thus, the disturbed cortisol concentrations are present early in the course of obesity.

Cortisol is released after activation of the HPA-axis in response to a stressor, which may be of physical or psychological nature. Increased cortisol concentrations have several effects throughout the body; increased activity of the HPA-axis is therefore associated with a variety of conditions[27-30]. Several studies have shown a positive association between cortisol concentrations and obesity or the metabolic syndrome in adults[10,11] and in children or adolescents[3-7]. On the other hand, associations between perceived or experienced stress and obesity have been shown[29,30]. For instance, BMI-SDS was higher in those adolescents with moderate or higher perceived stress compared with those with low perceived stress[31]. In Hispanic girls aged 8-11 years with an increased cortisol awakening response, increased school-related stress was associated with increased visceral and subcutaneous adipose tissue[32]. It is not known whether chronically increased cortisol concentrations are cause or consequence of the development of obesity and metabolic syndrome. Due to the cross-sectional nature of the present study, again no conclusions about causality can be drawn. However, if - in parallel to Cushing's syndrome - increased cortisol exposure at the tissue level is the driving force behind this relation, reducing cortisol concentrations may provide a novel target for obesity treatment in children.

Several limitations and directions for future studies can be mentioned. In the present study, 75% of the participants were female. All obese subjects aged between 8 and 12 years visiting the obesity outpatient clinics of the 2 hospitals were approached to participate in the study. Because more girls than boys visited the pediatric obesity outpatient clinics, a phenomenon often seen in the obesity outpatient clinics in the Netherlands, more girls than boys were approached and subsequently included in the study. Whether there are differences in hair cortisol concentrations between boys and girls at this age needs further study. However, because we included age- and sex-matched normal weight subjects as a control group, possible gender differences in hair cortisol concentrations do not affect the results of the present study.

The distribution of the ethnicity of the children was slightly different between the two groups: there were more non-Caucasian children in the obese group than in the normal-weight group. The relationship between obesity and hair cortisol concentration may be mediated by ethnicity; after ANCOVA with ethnicity as covariate, there was a trend for a difference in hair cortisol concentration between obese and normal-weight children. It is not known yet whether there are differences in hair cortisol concentration between different ethnicities, and this needs further study. Another explanation may be that obesity occurs more frequently in non-Caucasian children[33] and that the group with obese children therefore contained more children with a non-Caucasian ethnicity compared with the group with normal-weight children. Future studies with larger sample sizes are needed to evaluate the effect of perceived or experienced stress, pubertal status, ethnicity, or socioeconomic status on hair cortisol and obesity in children.

In the present study the growth rate of human scalp hair of 1 centimeter per month in adults was also assumed for children aged between 8 and 12 years[13]. However, it is not completely clear whether the growth rate of scalp hair indeed is the same in adults and children. The linear growth of scalp hair in children is poorly studied and there may be differences in growth rate between different ages and between boys and girls[34]. If the assumed growth rate appears to be incorrect, the timeline of long-term hair cortisol assessment in children may be somewhat different from the 1 month as presented in the present study. However, if this is the case it would be a minor difference of at most a few days. Also in children scalp hair cortisol concentrations are a measure for long-term cortisol concentrations.

In conclusion, obese children have higher long-term cortisol concentrations than normal weight children. This may provide a novel target for future obesity treatment in children.

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REFERENCES

1. Weiss R, Dziura J, Burgert TS, et al. Obesity and the metabolic syndrome in children and adolescents. *N Engl J Med* 2004; **350**(23): 2362-74.
2. Lobstein T, Jackson-Leach R. Estimated burden of paediatric obesity and co-morbidities in Europe. Part 2. Numbers of children with indicators of obesity-related disease. *Int J Pediatr Obes* 2006; **1**(1): 33-41.
3. Reinehr T, Andler W. Cortisol and its relation to insulin resistance before and after weight loss in obese children. *Horm Res* 2004; **62**(3): 107-12.
4. Reinehr T, Kulle A, Wolters B, et al. Steroid hormone profiles in prepubertal obese children before and after weight loss. *J Clin Endocrinol Metab* 2013; **98**(6): E1022-30.
5. Barat P, Gayard-Cros M, Andrew R, et al. Truncal distribution of fat mass, metabolic profile and hypothalamic-pituitary adrenal axis activity in prepubertal obese children. *J Pediatr* 2007; **150**(5): 535-9, 9 e1.
6. Adam TC, Hasson RE, Ventura EE, et al. Cortisol is negatively associated with insulin sensitivity in overweight Latino youth. *J Clin Endocrinol Metab* 2010; **95**(10): 4729-35.
7. Weigensberg MJ, Toledo-Corral CM, Goran MI. Association between the metabolic syndrome and serum cortisol in overweight Latino youth. *J Clin Endocrinol Metab* 2008; **93**(4): 1372-8.
8. Chalew SA, Lozano RA, Armour KM, Zadik Z, Kowarski AA. Reduction of plasma cortisol levels in childhood obesity. *J Pediatr* 1991; **119**(5): 778-80.
9. Hillman JB, Dorn LD, Loucks TL, Berga SL. Obesity and the hypothalamic-pituitary-adrenal axis in adolescent girls. *Metabolism* 2012; **61**(3): 341-8.
10. Ward AM, Fall CH, Stein CE, et al. Cortisol and the metabolic syndrome in South Asians. *Clin Endocrinol (Oxf)* 2003; **58**(4): 500-5.
11. Marin P, Darin N, Amemiya T, Andersson B, Jern S, Bjorntorp P. Cortisol secretion in relation to body fat distribution in obese premenopausal women. *Metabolism* 1992; **41**(8): 882-6.
12. Abraham SB, Rubino D, Sinaii N, Ramsey S, Nieman LK. Cortisol, obesity, and the metabolic syndrome: a cross-sectional study of obese subjects and review of the literature. *Obesity (Silver Spring)* 2013; **21**(1): E105-17.
13. Kintz P, Villain M, Cirimele V. Hair analysis for drug detection. *Therapeutic drug monitoring* 2006; **28**(3): 442-6.
14. Stalder T, Kirschbaum C. Analysis of cortisol in hair--state of the art and future directions. *Brain, behavior, and immunity* 2012; **26**(7): 1019-29.
15. Russell E, Koren G, Rieder M, Van Uum S. Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. *Psychoneuroendocrinology* 2012; **37**(5): 589-601.
16. Manenschijn L, Koper JW, Lamberts SW, van Rossum EF. Evaluation of a method to measure long term cortisol levels. *Steroids* 2011; **76**(10-11): 1032-6.
17. Manenschijn L, Koper JW, van den Akker EL, et al. A novel tool in the diagnosis and follow-up of (cyclic) Cushing's syndrome: measurement of long-term cortisol in scalp hair. *J Clin Endocrinol Metab* 2012; **97**(10): E1836-43.
18. Yamada J, Stevens B, de Silva N, et al. Hair cortisol as a potential biologic marker of chronic stress in hospitalized neonates. *Neonatology* 2007; **92**(1): 42-9.
19. Van Uum SH, Sauve B, Fraser LA, Morley-Forster P, Paul TL, Koren G. Elevated content of cortisol in hair of patients with severe chronic pain: a novel biomarker for stress. *Stress* 2008; **11**(6): 483-8.
20. Skoluda N, Dettenborn L, Stalder T, Kirschbaum C. Elevated hair cortisol concentrations in endurance athletes. *Psychoneuroendocrinology* 2012; **37**(5): 611-7.
21. Stalder T, Steudte S, Alexander N, et al. Cortisol in hair, body mass index and stress-related measures. *Biological psychology* 2012; **90**(3): 218-23.
22. Stalder T, Kirschbaum C, Alexander N, et al. Cortisol in hair and the metabolic syndrome. *J Clin Endocrinol Metab* 2013; **98**(6): 2573-80.
23. Manenschijn L, Schaap L, van Schoor NM, et al. High long-term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease. *J Clin Endocrinol Metab* 2013; **98**(5): 2078-83.
24. Hirasing RA, Fredriks AM, van Buuren S, Verloove-Vanhorick SP, Wit JM. [Increased prevalence of overweight and obesity in Dutch children, and the detection of overweight and obesity using international criteria and new reference diagrams]. *Nederlands tijdschrift voor geneeskunde* 2001; **145**(27): 1303-8.
25. Zimmet P, George K, Alberti MM, et al. The metabolic syndrome in children and adolescents - an IDF consensus report. *Pediatric Diabetes* 2007; **8**(5): 299-306.
26. de Kort SW, Willemsen RH, van der Kaay DC, Hokken-Koelega AC. The effect of growth hormone treatment on metabolic and cardiovascular risk factors is similar in

preterm and term short, small for gestational age children. *Clin Endocrinol (Oxf)* 2009; **71**(1): 65-73.

27. Bose M, Olivan B, Laferrere B. Stress and obesity: the role of the hypothalamic-pituitary-adrenal axis in metabolic disease. *Current opinion in endocrinology, diabetes, and obesity* 2009; **16**(5): 340-6.
28. Chrousos GP. Stress and disorders of the stress system. *Nat Rev Endocrinol* 2009; **5**(7): 374-81.
29. Gundersen C, Mahatmya D, Garasky S, Lohman B. Linking psychosocial stressors and childhood obesity. *Obes Rev* 2011; **12**(5): e54-63.
30. Pervanidou P, Chrousos GP. Metabolic consequences of stress during childhood and adolescence. *Metabolism* 2012; **61**(5): 611-9.
31. van Jaarsveld CH, Fidler JA, Steptoe A, Boniface D, Wardle J. Perceived stress and weight gain in adolescence: a longitudinal analysis. *Obesity (Silver Spring)* 2009; **17**(12): 2155-61.
32. Donoho CJ, Weigensberg MJ, Emken BA, Hsu JW, Spruijt-Metz D. Stress and abdominal fat: preliminary evidence of moderation by the cortisol awakening response in Hispanic peripubertal girls. *Obesity (Silver Spring)* 2011; **19**(5): 946-52.
33. de Wilde JA, van Dommelen P, Middelkoop BJ, Verkerk PH. Trends in overweight and obesity prevalence in Dutch, Turkish, Moroccan and Surinamese South Asian children in the Netherlands. *Arch Dis Child* 2009; **94**(10): 795-800.
34. Barth JH. Normal hair growth in children. *Pediatr Dermatol* 1987; **4**(3): 173-84.

CHAPTER 6

LONG-TERM GLUCOCORTICOID CONCENTRATIONS AS A RISK FACTOR FOR CHILDHOOD OBESITY AND ADVERSE BODY FAT DISTRIBUTION

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ABSTRACT

Background

Childhood obesity is an important risk factor for premature development of the metabolic syndrome (MetS) at adulthood. There is need for understanding of the mechanisms underlying the MetS and obesity. Patients with Cushing's disease suffer from similar metabolic complications, leading to the hypothesis that inter-individual cortisol variation may contribute to the onset of obesity. Additionally, glucocorticoid receptor (GR) gene polymorphisms resulting in differential glucocorticoid (GC) sensitivity, have been associated with an adverse metabolic profile.

Aim

To study associations of GC levels in scalp hair, as a marker of long-term systemic GC concentrations, and genetically determined GC sensitivity with obesity and body-fat distribution in children.

Methods

We performed a cross-sectional study of cortisol and cortisone concentrations over a 3-month period, measured by LC-MS/MS in hair of 3019 6-year-old children participating in the Generation R study. Genotyping of GR-gene polymorphisms was performed.

Results

Of all children, 4.3% was obese and 13.4% overweight. Cortisol was significantly associated with risk of obesity (OR 9.4 (3.3 – 26.9)) and overweight (OR 1.4 (1.0 – 2.0)). Cortisone was associated with risk of obesity (OR 1.9 (1.0 – 3.5)). Cortisol and cortisone were significantly positively associated with BMI, fat mass (FM) index, and android/gynecoid FM ratio. GR polymorphisms were not associated with adiposity parameters.

Conclusion

Long-term cortisol concentrations are strongly associated with an increased risk of childhood obesity and adverse body-fat distribution. Future research may reveal whether these are causal relations and may be a target for therapy.

INTRODUCTION

Childhood overweight and obesity, an important risk factor for premature development of the metabolic syndrome and cardiovascular disease later in life,[1] has tripled in prevalence since the 1980s, currently affecting approximately 17% of all US children and adolescents, with an even higher prevalence in ethnic minorities.[2] The observation that hypercortisolism (e.g. Cushing's disease or use of prescribed glucocorticoid (GC) medication) results in the development of (central) obesity, lead to the hypothesis that inter-individual variation in activity of the hypothalamus-pituitary-adrenal (HPA) axis may be implicated in the onset of obesity in the general population.

Multiple studies have targeted the association between GC concentrations and obesity, yielding contrasting results on the association between cortisol concentrations and adiposity, either reporting a positive association[3-5] between cortisol and obesity, no association[6-9] and negative associations.[10-13] Most of these studies comprised relatively small sample sizes and measured cortisol in serum, saliva or urine. Although cortisol concentrations can reliably be measured in these matrices, they represent cortisol concentrations at a single point in time up to 24 hours when urinary analysis is performed. These measurements are highly influenced by the circadian rhythm, pulsatile excretion and acute stressors. However, obesity is a chronic phenomenon, hence glucocorticoid concentrations need to be chronically increased to contribute to the development of overweight and obesity. A relatively recent development in the measurement of glucocorticoids provides the option to measure long-term glucocorticoid exposure by analyzing cortisol and cortisone concentrations in scalp hair, which is thought to be less affected by acute changes in glucocorticoid synthesis.[14] As hair grows at approximately 1 cm per month, average glucocorticoid concentrations over multiple weeks to months can be measured.[15,16] The measurement of cortisone in saliva was shown to reflect serum free cortisol levels.[17] Stalder *et al.*[18] also measured cortisone in scalp hair and showed significant associations with the metabolic syndrome in adults. Since the availability of long-term cortisol measurement, several studies have reported positive associations between cortisol and adiposity parameters[16,19-23], the metabolic syndrome[18] and cardiovascular disease.[24] However, none of these studies investigated body composition in more detail. A different approach to assess the association between cortisol exposure and body composition is by using polymorphisms in the glucocorticoid receptor (GR) gene, known to affect cortisol sensitivity. For example, the combined single nucleotide polymorphisms (SNPs) ER22/23EK reduce GC sensitivity,[25] and have been associated with a beneficial body composition in young adults.[26] On the other hand, the SNPs N363S and *Bcl*I have been shown to increase GC sensitivity, and have been associated with an adverse body composition.[27-29] To date, studies focused on the association of long-term cortisol exposure with childhood obesity are scarce. Recently, our pilot study suggested that hair cortisol levels are higher in obese children compared to normal weight children.[22] The relationship with body

composition has not yet been studied. Hypothesizing that cortisol already has effects on body composition and fat distribution at young age, this study aimed to investigate the relationships of long-term glucocorticoid concentrations and polymorphisms in the GR gene with obesity and fat distribution in children of the general population.

METHODS

This study is embedded in the Generation R Study, a population based cohort study from fetal life onwards situated in Rotterdam, the Netherlands.[30] This study has been approved by the local medical ethics committee and informed consent has been obtained from the parents or caretakers. In total 6690 children visited the Generation R research center at age 6 yr. As the current study started at a later point in time, 3490 were asked to participate in hair sample studies, of which 3034 children (86.9%) were willing to participate. Participants were excluded in case of systemic glucocorticoid use (n=8). Genetic data were available in 4046 of the 6690 children visiting the Generation R research center (Flow chart in supplements, supplemental figure S1.). Maternal education status was used as a proxy for social economic status. Educational status information was collected through questionnaires at the child's age of six years, and subdivided in three categories; low (no or primary school only), mid (secondary school, college) and high (bachlor's degree or higher).

Obesity and fat distribution

At the age of 6 years, height and body weight were measured without shoes and heavy clothing. Height was measured using a stadiometer (Holtain Limited, Crosswell, Crymych, United Kingdom) with a precision of 0.1 cm. Weight was measured to the gram with an electronic scale (SECA 888, Almere, The Netherlands). Body mass index (kg/m^2) was calculated and cut-off values as developed by the International Obesity Task Force for overweight and obesity were used.[31] Fat distribution was assessed by dual-energy X-ray absorptiometry (DXA) (iDXA, General Electronics Lunar, 2008, Madison, WI, USA), equipped with enCORE software v12.6, using low radiation dose (standard 3 μGy). Fat mass index was calculated as total body fat mass divided by height squared (kg/m^2), the android-gynecoid fat mass ratio was calculated by dividing the fat mass in the android region by the fat mass in the gynecoid region.

Hair glucocorticoid measurement

Hair samples of approximately 100 strands were cut from the posterior vertex using small surgical scissors, as close to the scalp as possible. Hair locks were then taped to a piece of paper with the scalp end marked, and stored in an envelope at room temperature until further analyses. Parents were requested to fill out a questionnaire for their child on hair washing frequency, time since last wash, hair product use, and use of glucocorticoids. Cortisol and cortisone were measured as described previously,[32]

with the exception that hair samples were minced by hand using small surgical scissors, instead of using 1 cm segments. Briefly, the proximal 3 cm of hair samples were weighed using an electrical scale and minced. Hair samples were then washed in LC-grade isopropanol for 2 minutes at room temperature, and left to dry for at least 2 days. Deuterium labeled cortisol and cortisone were added prior to extraction. Extraction was performed using LC-grade methanol (MeOH), for 18 hours at 25°C in the presence of deuterated steroids. Subsequently, the extract was cleaned using solid phase extraction and steroids were quantified on a Xevo TQS LC-MS/MS (Waters Chromatography).

Glucocorticoid receptor genotyping

The glucocorticoid receptor single nucleotide polymorphisms *Bcl*I (rs41423247), GR-9β (rs6198), N363S (rs56149945, previously designated rs6195) and ER22/23EK (rs6189/rs6190) were genotyped in cord blood, as described previously.[33] Genotyping was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA, USA), and Abgene QPCR ROX mix (Abgene, Hamburg, Germany). The genotyping reaction was amplified using the GeneAmp PCR system 9600 at 95°C for 15 min, followed by 40 cycles at 94°C (15 sec.) and 60°C (1 min.). Fluorescence detection was performed by the 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), genotypes were determined using SDS software v2.3 (Applied Biosystems, Foster City, CA, USA). Genotyping was successful in 97-99% of the samples, with an error rate <1% for all polymorphisms. Haplotypes were inferred using PHASE software v2.1 (M. Stephens, Dept. of Statistics, University of Washington, Seattle, USA). The constructed haplotypes and their distribution are depicted in supplemental figure S2.

Statistical analysis

Statistical analyses were performed using R software v3.1.2.[34] Missing covariates (ethnicity 2.9%, topical glucocorticoid use 4.9%, hair washing frequency 4.0%, time since last hair wash 4.3%, and hair product use 4.4%) were imputed by multivariate imputation by chained equations using the R mice package.[35] Multiple logistic regression models were performed for the associations between long-term glucocorticoid concentrations and obesity and overweight. Associations between glucocorticoid concentrations and body mass index and body fat distribution was analyzed using multiple linear regression models. The associations between GR polymorphisms and body mass index and body fat distribution were analyzed using multiple linear regression models, and were performed in all children in which genetic data and data on body mass index and body fat distribution were available at the age of six years, N=4046 (See flow-chart, supplemental figure S1.). All models were adjusted for age, sex, ethnicity and topical glucocorticoid use, unless stated otherwise. Models for android/gynecoid fat mass ratio were additionally adjusted for height. Multiple

Linear regression models were also used for analyzing the associations between the GR SNPs and body mass index and body fat distribution. These models were adjusted for age, sex and ethnicity. To adjust for ethnicity in the genetic linear regression models, genome wide association study derived principal components identifying ethnicity were used.[36] Covariates were regarded confounders when they were either correlated with both independent and dependent variables, or when the effect size of the association between the independent and dependent variables changed by more than 10% upon inclusion of the covariate in the model. Variables that were assessed for confounding were hair washing frequency, time since last hair wash, hair product use, and maternal education. Dependent variables which are not normally distributed were log-transformed. Cortisol and cortisone concentrations were either divided in quintiles, or log-transformed after which outliers as defined using Tukey's definition of outliers (Q1-1.5*IQR and Q3+1.5*IQR) were excluded.

RESULTS

Obesity, overweight, body mass index and fat distribution

Characteristics of the 2953 children (supplemental figure S1) in whom long-term cortisol was measured are listed in Table 1. According to the World Obesity Federation criteria, 4.3% of the children were obese and 13.4% were overweight.

Hair cortisol levels were significantly associated with obesity (OR's 9.4 (3.3 – 26.9) for highest cortisol quintile), and also with overweight (OR's 1.39 (1.0 – 2.0) for highest cortisol quintile). Cortisone was associated with obesity (OR 1.9 (1.0 – 3.5) for highest cortisone quintile), but was not significantly associated with overweight (Figure 1, Table 2). Hair cortisol and cortisone concentrations were both significantly associated with BMI and fat mass index (Figure 2, Table 3). Cortisol and cortisone were also significantly associated with a relative increase in abdominal fat mass, reflected by the android/gynecoid fat mass ratio, which is a hallmark of glucocorticoid-induced tissue-effects. These results were not changed by additional adjustment for maternal education level. Interaction terms for gender, age and ethnicity were not statically significant (p's>0.10).

Glucocorticoid receptor polymorphisms

In 1753 children, data on both the GR gene SNPs and long-term glucocorticoid concentrations were available (Flow chart, supplemental figure S1). The SNPs were not associated with long-term glucocorticoid concentrations (supplemental table S2). The GR-gene polymorphisms were not associated with overweight/obesity, BMI or body fat distribution, as listed in supplemental table S1. Furthermore, we observed no significant interactions between long-term glucocorticoid concentrations and GR gene polymorphisms on overweight, obesity, BMI and body fat distribution (data not shown).

Table 1. Subject-characteristics

Participant Characteristics	N=2953
Age (years)	6.2 (0.6)
Sex	
Boys	1421 (48.1%)
Girls	1532 (51.9%)
Ethnicity	
European	1871 (63.4%)
Non-European	1082 (36.6%)
Maternal education (high)	1595 (54.0%)
Overweight	396 (13.4%)
Obesity	128 (4.3%)
Hair characteristics	
Washing frequency	
< once a week	553 (18.7%)
1-2 times a week	1430 (48.5%)
3-4 times a week	739 (25.0%)
>4 times a week	230 (7.8%)
Time since last hair wash	
<24h	1035 (35.1%)
24-48h	864 (29.3%)
>48h	1053 (35.6%)
Use of hair product (yes)	
None	2121 (71.8%)
Gel	364 (12.3%)
Other	231 (7.8%)
Wax	113 (3.8%)
Foam/mousse	86 (2.9%)
Use of glucocorticoids (yes)	233 (7.9%)
Inhalation	79 (2.8%)
Nasal spray	11 (0.4%)
Cutaneous	126 (4.5%)
Anthropometrics	
Height (m)	1.20 (0.10)
Weight (kg)	23.3 (4.5)
Body mass index(kg/m2)	16.2 (1.9)
Body fat distribution	
Fat mass index (kg fm/height^2)	3.69 [2.37 – 6.92]
Android/ gynecoid fm ratio	0.24 [0.15 – 0.42]
Hair GC concentrations	
Cortisol pg/mg	1.7 [0.4 – 38.3]
Cortisone pg/mg	7.7 [2.7 – 33.5]

Values are means (standard deviation), percentages or median [95% range]. Fm, fat mass; GC, glucocorticoid.

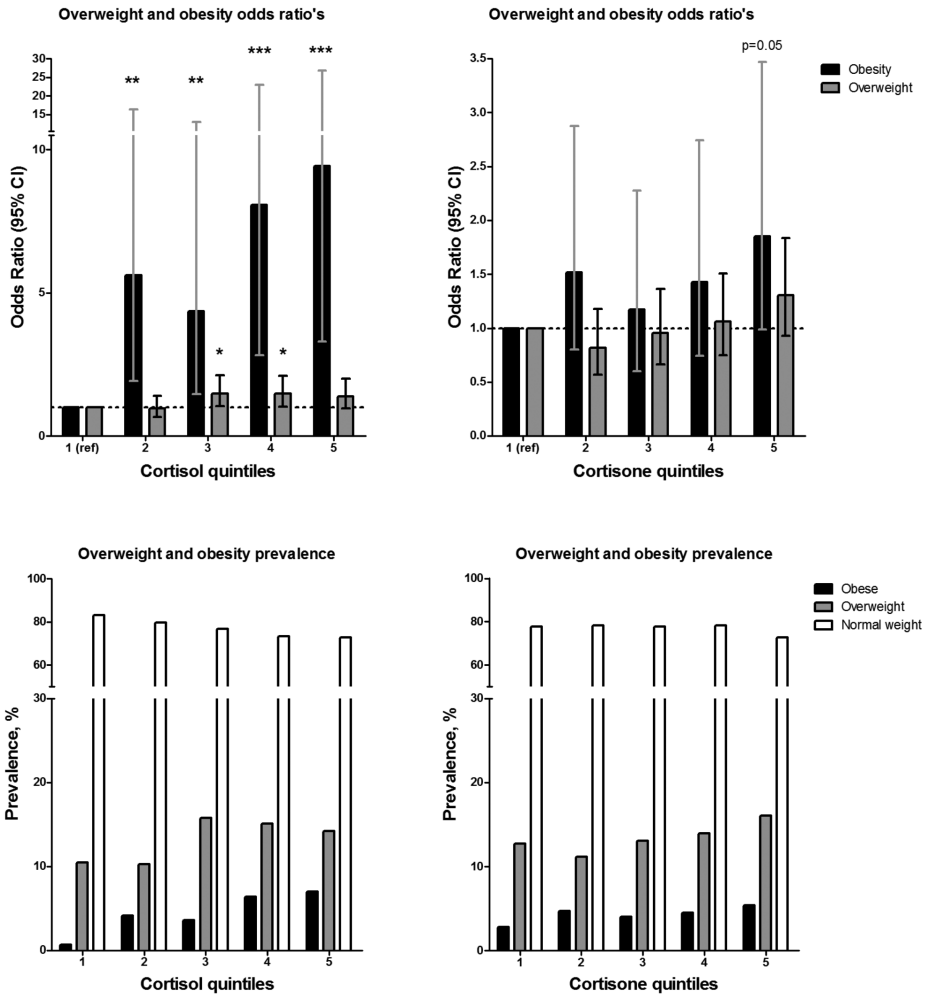

Figure 1. Association of long-term glucocorticoid concentrations with overweight and obesity. Logistic regression models adjusted for age, sex, ethnicity and topical glucocorticoid use. Obesity and overweight based on the World Obesity Foundation age and sex specific cut-offs. *p<0.05; **P<0.01; ***p<0.001.

DISCUSSION

This study shows surprisingly strong associations of long-term cortisol concentrations with obesity and adverse body fat distribution, already at the age of 6 years, which is independent of social economic status (as assessed by maternal education status). These results underline the previous associations between long-term cortisol measured in scalp hair and body composition reported in children,[21,22] and adults[16,18,37]. This is the first study in which obesity is associated with long-term cortisol concentrations

Table 2. Association of long-term glucocorticoid concentrations with overweight and obesity

	1	2	3	4	5
Cortisol quintiles					
Overweight	10,5%	10,3%	15,8%	15,1%	14,2%
Obese	0,69%	4,12%	3,60%	6,35%	7,02%
+	11,2%	14,4%	19,4%	21,4%	21,2%
Cortisone quintiles					
Overweight	12,7%	11,2%	13,1%	14,0%	16,0%
Obese	2,79%	4,71%	4,01%	4,54%	5,40%
+	15,5%	15,9%	17,1%	18,5%	21,4%

Percentage of children categorized as overweight or obese based on the World Obesity Foundation age and sex specific cut-offs.

Table 3. Associations between long-term glucocorticoid concentrations and body composition/fat distribution

	Cortisol	Cortisone
BMI	0.19 [0.12 – 0.26]	0.10 [0.03 – 0.17]
Fat mass index, SDS	0.05 [0.01 – 0.09]	0.06 [0.02 – 0.10]
Android/Gynecoid fm, SDS	0.04 [0.00 – 0.08]	0.06 [0.02 – 0.10]

Beta's en 95%CI based on linear regression models adjusted for age, sex, ethnicity and topical glucocorticoid use.

measured in hair by LC-MS/MS, which has superior sensitivity and specificity compared to immunoassays. Additionally, this study is the first of its magnitude including 2953 children whereas a previous studies, e.g. by Veldhorst et al.[22] included 20 obese and 20 normal weight children and should be regarded as a pilot-study. This study also adds the exploration of fat mass indices through measurements by DEXA scans, which aids in assessing fat distribution with high precision.

In literature, the majority of studies assessing the relation between HPA-axis activity and body composition, measured cortisol in serum, saliva and urine, and yielded conflicting results.[13] Based on these studies, Abraham *et al.*[13] concluded there was no strong relationship between systemic cortisol and obesity or the metabolic syndrome. However, cortisol concentrations measured in serum, saliva or urine represent concentrations of only a short time-period to a single point in time. Diurnal rhythm, pulsatile excretion and acute stress cause fluctuations in the concentration, causing these measurements to poorly reflect the long-term glucocorticoid exposure. However, the development of obesity and aspects of the metabolic syndrome require a prolonged exposure to increased glucocorticoid concentrations and other contributing factors. As scalp hair grows with approximately one centimeter per month, measuring cortisol and cortisone in the proximal three cm provides average concentrations spanning

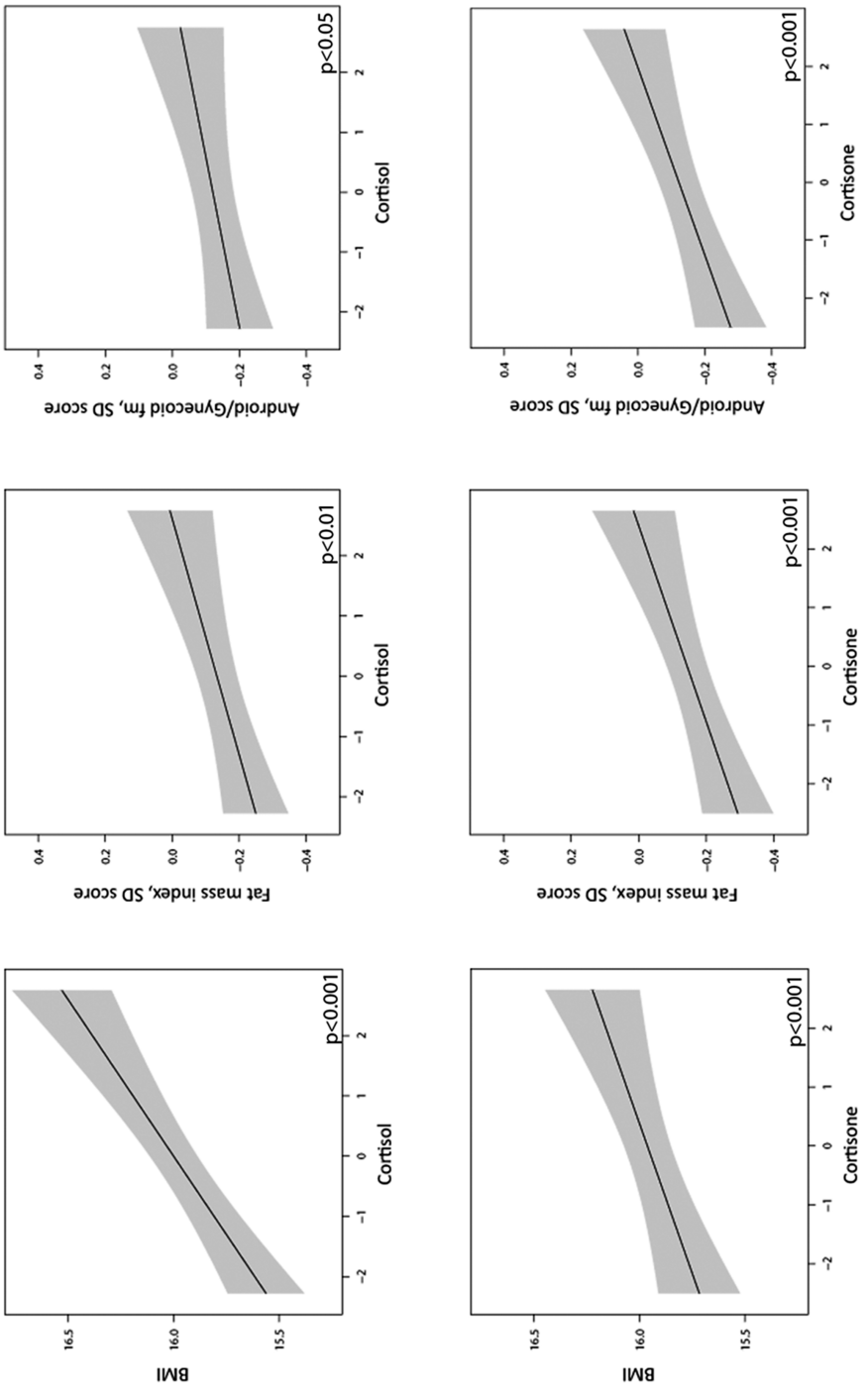

Figure 2. Associations between long-term glucocorticoid concentrations and body composition/fat distribution. Linear regression models adjusted for age, sex, ethnicity and topical glucocorticoid use.

three months, with a significantly reduced contribution of acute changes in HPA-axis activity. Using this technique, cortisol has repeatedly been associated with (abdominal) obesity,[22,37] , the metabolic syndrome,[18] and cardiovascular disease,[24] findings which are underscored by the current results of cortisol and obesity in children.

Increased glucocorticoid sensitivity due to single nucleotide polymorphisms in the glucocorticoid receptor gene has been shown to be associated with increased BMI and body fatness both in adults and elderly,[27,38] as well as in children.[39,40] In the current study, we analyzed the associations of SNPs in the GR gene, known to alter cortisol sensitivity, in over 4000 children at the age of 6 years. No associations were found with BMI or fat mass. It is unlikely that this would be a lack of power, as the sample sizes in the previous studies were less than one tenth of the current sample. Barat *et al.*[39] conducted a study in a group of prepubertal obese children aged 6 to 13 years (n=46), showing Bcl1 carriers to have a higher fat percentage and to be relatively cortisol resistant. Both the selection of obese children and the relative higher age may explain the contradiction with the current study. In the study by Voorhoeve *et al.*[40], two cohorts of apparently healthy adolescents in the age of with a mean age of 11.3 and 12.9 years with a generational difference of 20 years were included. Only in the younger (age 11.3yr) and later established cohort, and only in boys an association between Bcl1 G-allele carriage and increased body mass index and fat mass was established. One explanation for the contradicting results between these studies and the current findings may be that the associations between cortisol sensitivity and body fatness may be more pronounced in adolescence compared to children at 6 years of age. A different study, although very early in life, assessing the association between GR gene SNPs with growth characteristics in neonates, did not report a significant effect of differential GR gene SNPs.[33,41] Additionally, in the current study no associations with GR gene SNPs and long-term glucocorticoid concentrations were found. This is the first study to assess the association between scalp-hair glucocorticoids and GR polymorphisms. As the GR is involved in cortisol feedback, an altered GR glucocorticoid sensitivity would be expected to result in altered cortisol concentrations. This has previously been tested by Barat et al.[39] measuring a smaller reduction in cortisol concentrations after dexamethasone suppression tests in Bcl1 G-allele carriers. HPA-axis negative feedback is however not solely regulated by the glucocorticoid receptor. Indeed, the mineral corticoid receptor is expressed in the hypothalamus and is thought to play a role in basal cortisol concentrations, whereas the glucocorticoid receptor mainly mediates stress reactive feedback of the HPA axis.[42] HPA-axis activity in stress Long-term cortisol and cortisone concentrations measured in scalp-hair are speculated to better reflect the basal cortisol concentrations rather than the short-term stress-induced cortisol peaks. Additionally, an interaction between the MR and the GR in HPA-axis stress reactivity was shown in mice, with increased expression of the MR inhibiting excess HPA-axis stress reactivity in low GR circumstances.[43] It may be speculated that polymorphisms in the MR affects GR polymorphisms phenotypes. This would not be

detected using dexamethasone suppression tests as dexamethasone is known to have no mineralocorticoid effect.

Although we categorized overweight and obesity based on the commonly used age and sex-specific BMI cut-off values reported by the World Obesity Foundation,[31] the body fat distribution in school age children may not be adequately represented by the body mass index. Indeed, total body fat mass and abdominal fat mass were shown to be stronger associated with cardiovascular risk factor compared to, and independent from body mass index.[44] Hence, fat mass index and distribution were assessed by low-dose DEXA scans. The ratio between abdominal fat mass and fat in the hip region was used as a proxy for waist-hip circumference, to assess whether glucocorticoids were preferably associated with the metabolically adverse abdominal fat accumulation. Long-term glucocorticoid concentrations were positively associated with both fat mass index and the abdominal to hip fat mass ratio.

The main strengths of this study are the large number of participating children, the population-based design, and the prospective data collection in a specialized research center. A long-term measurement for glucocorticoids was used, which is less affected by acute factors and the circadian rhythmicity compared to other matrices. The method for glucocorticoid measurement was LC-MS/MS based. Compared to immune-assays, this provides improved sensitivity and lacks the cross-reactivity, and hence is the advocated method for steroid quantification.[45] One drawback of this study is that its design does not easily allow for establishing causality, due to potential residual confounding and reverse causality.

The fact that high glucocorticoid concentrations have adverse metabolic effects is best demonstrated in Cushing's syndrome, which may be induced by both endogenous and exogenous glucocorticoid excess. Obesity, hyperglycemia and dyslipidemia are hallmark symptoms of Cushing's syndrome. Obesity is induced by glucocorticoid excess through multiple mechanisms. For example, glucocorticoids tend to increase appetite, they stimulate adipogenesis through preadipocyte differentiation and hypertrophy particularly in central fat, and glucocorticoids negatively affects brown adipose tissue, which is thought to decrease energy consumption. These mechanisms and others are more extensively described in a comprehensive review by Fardet and Fève.[46] It seems plausible that less marked increases in glucocorticoid exposure has similar effects, albeit less florid. Additionally, multiple factors may be inducing a chronic cortisol excess, e.g. an altered HPA setting caused by an adverse environment intrauterine or in early life (the Barker hypothesis), chronic stress, relative sleep deprivation, chronic inflammation, and high glycemic index food consumption. These potential factors were not addressed in the current study and may be a focus for future research.

Concluding, there is a strong association between chronic glucocorticoid exposure and obesity in children, already at the age of 6 years. Future research may reveal whether this is a causal relationship and whether cortisol may be a future target for preventive and therapeutic strategies to combat obesity.

ACKNOWLEDGEMENTS

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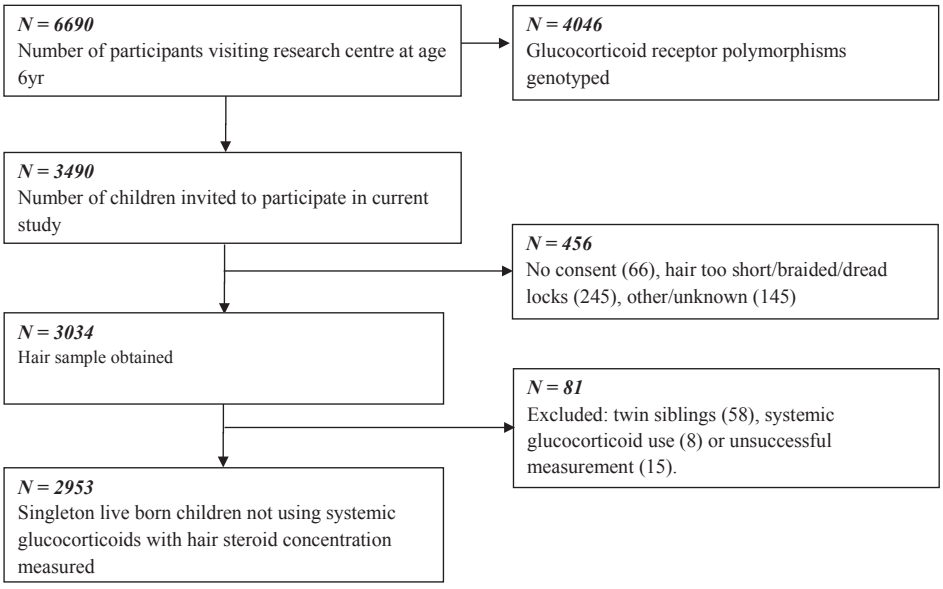
REFERENCES

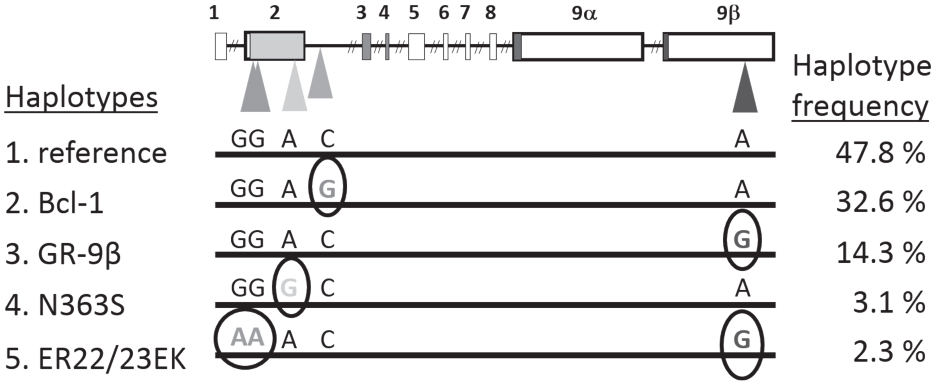
1. Daniels SR, Arnett DK, Eckel RH, et al. Overweight in children and adolescents: pathophysiology, consequences, prevention, and treatment. *Circulation* 2005; **111**(15): 1999-2012.
2. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of obesity and trends in body mass index among US children and adolescents, 1999-2010. *JAMA* 2012; **307**(5): 483-90.
3. Ljung T, Andersson B, Bengtsson BA, Bjorntorp P, Marin P. Inhibition of cortisol secretion by dexamethasone in relation to body fat distribution: a dose-response study. *Obes Res* 1996; **4**(3): 277-82.
4. Marin P, Darin N, Amemiya T, Andersson B, Jern S, Bjorntorp P. Cortisol secretion in relation to body fat distribution in obese premenopausal women. *Metabolism* 1992; **41**(8): 882-6.
5. Pasquali R, Cantobelli S, Casimirri F, et al. The hypothalamic-pituitary-adrenal axis in obese women with different patterns of body fat distribution. *J Clin Endocrinol Metab* 1993; **77**(2): 341-6.
6. Duclos M, Gatta B, Corcuff JB, Rashedi M, Pehourcq F, Roger P. Fat distribution in obese women is associated with subtle alterations of the hypothalamic-pituitary-adrenal axis activity and sensitivity to glucocorticoids. *Clin Endocrinol (Oxf)* 2001; **55**(4): 447-54.
7. Duclos M, Marquez Pereira P, Barat P, Gatta B, Roger P. Increased cortisol bioavailability, abdominal obesity, and the metabolic syndrome in obese women. *Obes Res* 2005; **13**(7): 1157-66.
8. Steptoe A, Kunz-Ebrecht SR, Brydon L, Wardle J. Central adiposity and cortisol responses to waking in middle-aged men and women. *Int J Obes Relat Metab Disord* 2004; **28**(9): 1168-73.
9. Stewart PM, Boulton A, Kumar S, Clark PM, Shackleton CH. Cortisol metabolism in human obesity: impaired cortisone-->cortisol conversion in subjects with central adiposity. *J Clin Endocrinol Metab* 1999; **84**(3): 1022-7.
10. Phillips DI, Barker DJ, Fall CH, et al. Elevated plasma cortisol concentrations: a link between low birth weight and the insulin resistance syndrome? *J Clin Endocrinol Metab* 1998; **83**(3): 757-60.
11. Reynolds RM, Labad J, Strachan MW, et al. Elevated fasting plasma cortisol is associated with ischemic heart disease and its risk factors in people with type 2 diabetes: the Edinburgh type 2 diabetes study. *J Clin Endocrinol Metab* 2010; **95**(4): 1602-8.
12. Stolk RP, Lamberts SW, de Jong FH, Pols HA, Grobbee DE. Gender differences in the associations between cortisol and insulin in healthy subjects. *J Endocrinol* 1996; **149**(2): 313-8.
13. Abraham SB, Rubino D, Sinaii N, Ramsey S, Nieman LK. Cortisol, obesity, and the metabolic syndrome: a cross-sectional study of obese subjects and review of the literature. *Obesity (Silver Spring)* 2013; **21**(1): E105-17.
14. Wester VL, Lamberts SW, van Rossum EF. Advances in the assessment of cortisol exposure and sensitivity. *Curr Opin Endocrinol Diabetes Obes* 2014; **21**(4): 306-11.
15. Sauve B, Koren G, Walsh G, Tokmakejian S, Van Uum SH. Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clin Invest Med* 2007; **30**(5): E183-91.
16. Manenschijn L, Koper JW, Lamberts SW, van Rossum EF. Evaluation of a method to measure long term cortisol levels. *Steroids* 2011; **76**(10-11): 1032-6.
17. Perogamvros I, Keevil BG, Ray DW, Trainer PJ. Salivary cortisone is a potential biomarker for serum free cortisol. *J Clin Endocrinol Metab* 2010; **95**(11): 4951-8.
18. Stalder T, Kirschbaum C, Alexander N, et al. Cortisol in hair and the metabolic syndrome. *J Clin Endocrinol Metab* 2013; **98**(6): 2573-80.
19. Manenschijn L, van Kruysbergen RG, de Jong FH, Koper JW, van Rossum EF. Shift work at young age is associated with elevated long-term cortisol levels and body mass index. *J Clin Endocrinol Metab* 2011; **96**(11): E1862-5.
20. Chan J, Sauve B, Tokmakejian S, Koren G, Van Uum S. Measurement of cortisol and testosterone in hair of obese and non-obese human subjects. *Exp Clin Endocrinol Diabetes* 2014; **122**(6): 356-62.
21. Noppe G, Van Rossum EFC, Koper JW, et al. Validation and Reference Ranges of Hair Cortisol Measurement in Healthy Children. *Horm Res Paediat* 2014; **82**(2): 97-102.
22. Veldhorst MAB, Noppe G, Jongejan MHTM, et al. Increased Scalp Hair Cortisol Concentrations in Obese Children. *J Clin Endocr Metab* 2014; **99**(1): 285-90.
23. Wester VL, Staufenbiel SM, Veldhorst MA, et al. Long-term cortisol levels measured in scalp hair of obese patients. *Obesity (Silver Spring)* 2014; **22**(9): 1956-8.
24. Manenschijn L, Schaap L, van Schoor NM, et al. High long-term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease. *J Clin Endocrinol Metab* 2013; **98**(5): 2078-83.

25. van Rossum EF, Koper JW, Huizenga NA, et al. A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids in vivo, is associated with low insulin and cholesterol levels. *Diabetes* 2002; **51**(10): 3128-34.
26. van Rossum EF, Voorhoeve PG, te Velde SJ, et al. The ER22/23EK polymorphism in the glucocorticoid receptor gene is associated with a beneficial body composition and muscle strength in young adults. *J Clin Endocrinol Metab* 2004; **89**(8): 4004-9.
27. Huizenga NA, Koper JW, De Lange P, et al. A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. *J Clin Endocrinol Metab* 1998; **83**(1): 144-51.
28. Dobson MG, Redfern CP, Unwin N, Weaver JU. The N363S polymorphism of the glucocorticoid receptor: potential contribution to central obesity in men and lack of association with other risk factors for coronary heart disease and diabetes mellitus. *J Clin Endocrinol Metab* 2001; **86**(5): 2270-4.
29. Roussel R, Reis AF, Dubois-Laforgue D, Bellanne-Chantelot C, Timsit J, Velho G. The N363S polymorphism in the glucocorticoid receptor gene is associated with overweight in subjects with type 2 diabetes mellitus. *Clin Endocrinol (Oxf)* 2003; **59**(2): 237-41.
30. Kruithof CJ, Kooijman MN, van Duijn CM, et al. The Generation R Study: Biobank update 2015. *European journal of epidemiology* 2014; **29**(12): 911-27.
31. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. *Bmj* 2000; **320**(7244): 1240-3.
32. Noppe G, de Rijke YB, Dorst K, van den Akker EL, van Rossum EF. LC-MS/MS-based method for long-term steroid profiling in human scalp hair. *Clin Endocrinol (Oxf)* 2015; **83**(2): 162-6.
33. Geelhoed MJ, Steegers EA, Koper JW, et al. Glucocorticoid receptor gene polymorphisms do not affect growth in fetal and early postnatal life. The Generation R Study. *BMC medical genetics* 2010; **11**: 39.
34. R Core Team, R Foundation for Statistical Computing. R: A language and environment for statistical computing. *http://wwwR-projectorg/* 2013.
35. Van Buuren S, Groothuis-Oudshoorn CGM. mice: Multivariate Imputation by Chained Equations in R. *Journal of Statistical Software* 2011; **45**(3): 1-67.
36. Medina-Gomez C, Felix JF, Estrada K, et al. Challenges in conducting genome-wide association studies in highly admixed multi-ethnic populations: the Generation R Study. *European journal of epidemiology* 2015; **30**(4): 317-30.
37. Wester VL, Staufenbiel SM, Veldhorst MAB, et al. Long-Term Cortisol Levels Measured in Scalp Hair of Obese Patients. *Obesity* 2014; **22**(9): 1956-8.
38. Geelen CC, van Greevenbroek MM, van Rossum EF, et al. BclI glucocorticoid receptor polymorphism is associated with greater body fatness: the Hoorn and CODAM studies. *J Clin Endocrinol Metab* 2013; **98**(3): E595-9.
39. Barat P, Corcuff JB, Tauber M, Moisan MP. Associations of glucocorticoid receptor and corticosteroid-binding globulin gene polymorphisms on fat mass and fat mass distribution in prepubertal obese children. *J Physiol Biochem* 2012; **68**(4): 645-50.
40. Voorhoeve PG, van den Akker EL, van Rossum EF, et al. Glucocorticoid receptor gene variant is associated with increased body fatness in youngsters. *Clin Endocrinol (Oxf)* 2009; **71**(4): 518-23.
41. Manenschijn L, van den Akker EL, Ester WA, et al. Glucocorticoid receptor gene haplotypes are not associated with birth anthropometry, blood pressure, glucose and insulin concentrations, and body composition in subjects born small for gestational age. *Eur J Endocrinol* 2010; **163**(6): 911-8.
42. Berardelli R, Karamouzis I, D'Angelo V, et al. Role of mineralocorticoid receptors on the hypothalamus-pituitary-adrenal axis in humans. *Endocrine* 2013; **43**(1): 51-8.
43. Harris AP, Holmes MC, de Kloet ER, Chapman KE, Seckl JR. Mineralocorticoid and glucocorticoid receptor balance in control of HPA axis and behaviour. *Psychoneuroendocrinology* 2013; **38**(5): 648-58.
44. Gishti O, Gaillard R, Durmus B, et al. Body mass index, total and abdominal fat distribution and cardiovascular risk factors in school-age children. *Pediatr Res* 2015.
45. Handelsman DJ, Wartofsky L. Requirement for mass spectrometry sex steroid assays in the Journal of Clinical Endocrinology and Metabolism. *J Clin Endocrinol Metab* 2013; **98**(10): 3971-3.
46. Fardet L, Feve B. Systemic glucocorticoid therapy: a review of its metabolic and cardiovascular adverse events. *Drugs* 2014; **74**(15): 1731-45.

SUPPLEMENTAL MATERIALS



Supplemental figure S1. Flow chart of participants in study.



Supplemental figure S2. Schematic representations of the glucocorticoid receptor gene, the variant haplotypes established in 4046 children, and their frequency.

Supplemental Table S1. Associations of glucocorticoid receptor gene polymorphisms with body composition and body fat distribution. N=4046

	ER22/23EK		GR-9β		N363S		*Bcl*I	
	OR (95% CI)	**P**	**OR (95% CI)**	**P**	**OR (95% CI)**	**P**	**OR (95% CI)**	**P**
≥Overweight	1.08 (0.51 – 1.35)	0.45	0.98 (0.82 – 1.18)	0.86	1.35 (0.93 – 1.98)	0.12	1.05 (0.91 – 1.20)	0.51
	Effect est. (95% CI)	P	Effect est. (95% CI)	P	Effect est. (95% CI)	P	Effect est. (95% CI)	P
BMI	-0.05 (-0.32 – 0.22)	0.73	0.06 (-0.06 – 0.18)	0.30	-0.04 (-0.28 – 0.20)	0.74	0.04 (-0.04 – 0.13)	0.13
FMI	-0.04 (-0.19 – 0.11)	0.62	0.02 (-0.04 – 0.09)	0.53	-0.02 (-0.11 – 0.16)	0.72	0.04 (-0.00 – 0.09)	0.09
A/G fm ratio	-0.14 (-0.30 – 0.01)	0.06	0.01 (-0.06 – 0.08)	0.76	0.00 (-0.13 – 0.13)	0.99	0.04 (-0.01 – 0.08)	0.08

Logistic and linear regression models adjusted for age, sex and ethnicity. ≥Overweight: overweight and obesity combined. OR: Odds ratio. Est: Estimate. FMI, Fat mass index; A/G fm ratio, Android / gynecoid fat mass ratio.

Supplemental Table S2. Associations of glucocorticoid receptor gene polymorphisms with long-term glucocorticoid concentrations. N=1753

	ER22/23EK		GR-9β		N363S		*Bcl*I	
	Effect est. (95% CI)	**P**	**Effect est. (95% CI)**	**P**	**Effect est. (95% CI)**	**P**	**Effect est. (95% CI)**	**P**
Cortisol	0.02 (-0.14 – 0.17)	0.84	0.04 (-0.05 – 0.12)	0.40	0.04 (-0.11 – 0.19)	0.61	0.06 (-0.02 – 0.13)	0.12
Cortisone	0.04 (-0.14 – 0.22)	0.66	0.01 (-0.07 – 0.09)	0.73	0.07 (-0.15 – 0.29)	0.54	0.05 (-0.03 – 0.12)	0.70

Linear regression models adjusted for age, sex, ethnicity (GWAS Principal components) and topical glucocorticoid use.

CHAPTER 7

ELEVATED HAIR CORTISOL CONCENTRATIONS IN CHILDREN WITH ADRENAL INSUFFICIENCY ON HYDROCORTISONE REPLACEMENT THERAPY

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Clinical Endocrinology, 2014; 81(6):820-825

ABSTRACT

Background

Glucocorticoid replacement therapy in patients with adrenal insufficiency needs to be tailored to the individual patient based on body composition and clinical signs and symptoms as no objective method for assessment of treatment adequacy is available. Current treatment regimens are often not satisfactory, which is shown by the adverse metabolic profile and doubled mortality rates in treated adrenal insufficiency patients. Measurement of cortisol concentrations in hair reflect the long-term systemic cortisol exposure and may be of use in refinement of hydrocortisone treatment.

Objective

We aimed to study whether long-term cortisol (hydrocortisone) levels, as measured in scalp hair, are similar in children with adrenal insufficiency and healthy children.

Material and methods

We set up a case control study, measuring anthropometric characteristics and hair cortisol concentrations (HCC) in 54 hydrocortisone substituted children with adrenal insufficiency (AI patients) in the age of 4-18 years and 54 healthy children matched for gender and age.

Results

Mean HCC were significantly higher in AI patients compared to healthy controls (mean 13.3 vs 8.2 pg/mg, p=0.02). AI patients also had a higher BMI (p<0.001) and waist circumference (WC) (p=0.02). HCC was significantly associated with BMI (p=0.002) and WC (p=0.002). HCC explained 13% of the difference in BMI and 29% of the difference in WC between AI patients and controls.

Conclusion

Hydrocortisone treated AI patients have increased HCC and adverse anthropometric characteristics compared to healthy controls. HCC measurement may be of value in identifying overtreatment and thereby improve hydrocortisone replacement therapy.

INTRODUCTION

Chronic adrenal insufficiency (AI) resulting in glucocorticoid deficiency may result from either adrenal (mostly autoimmune) destruction, termed Addison's disease or primary adrenal insufficiency (PAI), from the lack of adrenocorticotropic hormone (ACTH) due to a central defect (termed secondary adrenal insufficiency or hypopituitarism) or a genetic defect affecting steroid synthesis enzymes, as is the case in congenital adrenal hyperplasia (CAH)[1,2]. In childhood, the preferred glucocorticoid (GC) hormone replacement therapy is hydrocortisone (synthetic cortisol), administered orally thrice daily[3,4]. Replacement dose and timing should be titrated per individual, because pharmacokinetics and pharmacodynamics vary, as does the individual cortisol need[1,4]. Under dosing is associated with impaired health and may lead to potentially fatal adrenal crisis. But also chronic overdosing should be prevented, as this is associated with diminished glucose tolerance, obesity, dyslipidaemia and reduced bone mineral density[5,6]. Although cortisol is routinely measured in serum, saliva or urine, its use in therapy monitoring is limited since these parameters only reflect cortisol concentration at a single point in time. Measuring cortisol day-curves has been suggested as therapy monitoring tool. However, this requires hospital admission and is thus expensive, time consuming, and it is difficult to interpret and has been shown not to be superior to clinical assessment[4,7,8]. Hence, individual titration is based primarily on clinical parameters. Subtle over and under treatment are difficult to detect clinically on the short term as the clinical symptoms are non-specific and slow to develop[7], but may have profound health effects in the long run. Attaining the optimal dose is challenging, which is especially true in paediatric patients due to developmental changes in body composition and metabolism requiring continuous dose adjustments and is further complicated by difficulties concerning therapy adherence which are highly prevalent in adolescents[9]. A new tool for monitoring and improving glucocorticoid replacement therapy would be most welcome.

A recently developed method in which the long-term systemic exposure to cortisol can be analysed in human hair gives new perspective[10]. Scalp hair at the posterior vertex displays regular growth at an average rate of one cm per month[11,12], providing a retrospective calendar of mean cortisol exposure and hypothalamus pituitary adrenal (HPA) axis activity of recent months to years. Hair cortisol measurement can be applied as a diagnostic and follow-up tool for Cushing's syndrome and disease and even cyclic Cushing's[13]. Cortisol analysis in hair has been applied in research addressing, amongst others, stress[14-20] and glucocorticoid related endocrine diseases[21,22]. Moreover, increased hair cortisol within physiological ranges is associated with an increased risk of cardiovascular diseases and the metabolic syndrome[23,24]. Measuring cortisol concentrations in the hair of adrenal insufficiency patients may provide a new tool for monitoring hydrocortisone treatment, similar to measurement of the percentage of glycated haemoglobin (HbA1c) as a measure for long term glucose concentration in diabetes.

Our aims were to determine whether hair cortisol concentrations (HCC) can be measured in children with chronic adrenal insufficiency who are treated with hydrocortisone, and whether HCC in hydrocortisone treated children were comparable to healthy children.

MATERIALS & METHODS

Participant recruitment

Patients with adrenal insufficiency from the outpatient clinic of the department of paediatrics of Erasmus MC- Sophia children's hospital were asked for participation. Inclusion criteria were children aged 0-18yr, diagnosed with adrenal insufficiency due to Addison's disease, hypopituitarism (central or secondary adrenal insufficiency, CAI) or Congenital Adrenal Hyperplasia (CAH), who were treated with hydrocortisone for six months or longer following national guidelines for steroid dosing. Patients with confirmed therapy non-compliance were excluded. Patient recruitment was performed from May 2011 until August 2013. A total of 56 patients with adrenal insufficiency were recruited after informed consent was given. Hair samples were obtained and anthropometric characteristics, being weight (kg), height (m) and waist circumference (WC, cm), were measured. Patients were asked to fill out a questionnaire about hair-treatment and dosing regimens. Written informed consent was obtained from the parents/guardians and from patients 12 years and older. For comparison, 55 healthy children, matched for age and sex, were recruited by elementary schools visits and by asking siblings and friends of patients to participate. Exclusion criteria for healthy controls were hair <2cm, use of medication potentially influencing the HPA axis, chronic disease or significant illness in the past 3 months. This study was approved by the local ethical committee.

Hair sample collection and analysis

Hair samples of about 100 hairs were taken from the posterior vertex of the scalp, as close to the scalp as possible, using small surgical scissors. The samples were taped to a piece of paper, the scalp end marked, and stored in an envelope at room temperature until analysis. For analysis, the three most proximal centimetres were weighed and cut into fine pieces of 1-2 mm in length in glass vials. For extraction, 1 mL methanol was added and samples were incubated overnight for 16 hours at 52°C, while gently shaking. After incubation, the methanol was transferred into a disposable glass tube and evaporated at 37°C under a stream of nitrogen. The extract was then reconstituted in 250 microliter phosphate buffered saline (pH 8.0) and vortexed for 1 minute. Cortisol concentration was then measured using a commercially available immunoassay kit for salivary cortisol (DRG Instruments GmbH, Marburg, Germany), according to manufacturer's instruction manual. The inter-assay coefficients of variation (CV) measured on five consecutive assays were 7.0% at 4.4ng/mL, 2.3% at 21.3ng/mL and 8.2% at 35.0 ng/

mL. The intra-assay CV's were 3.1%, 2.3% and 2.6% respectively. Sauvé *et al.*[12] showed the total variation of repeated sampling, extraction and measurement to be 15.6%. Cross-reactivity reported by the manufacturer are as follows: Corticosterone 29.0%, Cortisone 3.0%, 11-Deoxycortiol <1.0%, 17-OH Progesterone <0.5%, other natural steroids all <0.10%.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 21 (IBM Corp. Armonk, New York, USA). The standard deviation scores (SDS) of height, waist circumference (WC) and body mass index (BMI) were calculated based on data from the fourth Dutch nationwide growth study 1997[25], using Growth Analyser (Growth Analyser BV, Rotterdam, The Netherlands). Patients and controls were compared using paired samples *t*-test. HCC was log-transformed to achieve normal distribution and is reported as the geometric mean. Differences in HCC and anthropometric characteristics between matched case and control pairs were calculated and correlations of difference in HCC (ΔHCC) with difference in anthropometrics (ΔWC SDS, ΔBMI SDS and ΔHeight SDS) were analysed using Pearson correlation. Patients with CAH were reciprocally compared based on the presence of salt-losing and subgroups of AI patients based on aetiology were compared to all controls using one-way analysis of variance (ANOVA), adjusted for age and sex. Hair cortisol and hydrocortisone dose associations with anthropometric characteristics in the total group of study participants were analysed using linear regression. In linear regression analyses of Hair cortisol concentrations in the total group, the presence of adrenal insufficiency was included to the model as independent variable to assess the additive effect of HCC to diagnosis. A p-value<0.05 was considered statistically significant.

RESULTS

In total 56 AI patients were included in the study. Two patients were excluded from analysis because of an admitted therapy nonadherence, resulting in a final sample of 54 patients. Ten patients had been diagnosed with Addison's disease and 31 patients with CAH (17 with salt losing form) of whom 30 patients had 21-hydroxylase deficiency, and one patient had 11-β-hydroxylase deficiency. Thirteen patients had been diagnosed with central adrenal insufficiency (CAI), of which five cases were caused by a pituitary tumour (four patients with craniopharyngioma, one with rhabdomyosarcoma). Nine patients with CAI had multiple pituitary deficiencies, all receiving thyroid hormone and growth hormone replacement, four patients were treated with oestrogens, one patient with testosterone and one patient with vasopressin. Characteristics of the 54 adrenal insufficiency patients and 55 healthy controls, matched for age and sex, are shown in Table 1. Characteristics of children with adrenal insufficiency grouped by aetiology are summarized in Table 2.

Table 1. Characteristics and hair cortisol concentrations

		Total Adrenal Insufficiency (n=54)	Healthy Controls (n=54)	
Age, years	mean (range)	11.6 (4.2-17.5)	11.4 (4.3-18.0)	
Male	n (%)	25 (46%)	26 (46%)	
Height for age (SDS)	mean (SD)	-0.61 (1.34)	0.27 (0.80)	*p<0.001*
Waist circumference (SDS)	mean (SD)	1.22 (0.90)	0.89 (0.90)	*p*=0.018
BMI (SDS)	mean (SD)	0.84 (1.25)	-0.04 (0.82)	*p<0.001*
Dosage HC (mg/m2/day)	mean (SD)	10.9 (2.7)		
Hair cortisol (pg/mg)	gm [95%CI]	13.3 [10.3 – 17.2]	8.2 [5.9 – 11.3]	*p*=0.022

P-values from paired samples T test. Gm: geometric mean

Table 2. Baseline characteristics of AI patients divided by aetiology

		Addison's disease (n=10)	Congenital Adrenal Hyperplasia (n=31)	Secondary adrenal insufficiency (n=13)
Age, years	mean (range)	13.7 (8.3 – 17.5)	10.8 (5.1 – 17.2)	12.0 (4.2-16.7)
Male	n (%)	5 (50%)	15 (48%)	5 (39%)
Height SDS	mean (SD)	-0.83 (0.99)	-0.39 (1.37)	-0.96 (1.48)
Waist circumference SDS	mean (SD)	1.74 (0.56)	1.15 (0.93)	0.98 (0.99)
BMI SDS	mean (SD)	0.17 (1.33)	0.88 (1.08)	1.28 (1.44)
Hair cortisol (pg/mg)	Gm [95%CI]	11.8 [5.9 – 22.8]	13.4 [9.7 – 18.3]	14.6 [7.1 – 29.1]
Dosage HC (mg/m2/day)	mean (SD)	9.8 (1.9)	11.9 (2.9)	9.4 (1.5)

SDS= Standard deviation score, Gm=geometric mean

AI patients were found to have a significantly lower height SDS ($p<0.001$) and a higher BMI SDS ($p<0.001$) and waist circumference (WC) SDS ($p=0.018$) compared to healthy controls. Hair cortisol levels in AI patients were significantly higher than in healthy controls (p=0.022, Fig. 1). HCC was higher in all aetiology subgroups compared to healthy controls, but this difference only reached statistical significance in subgroup of patients with salt losing CAH ($p=0.003$, adjusted for age and gender). ΔHCC correlated significantly with ΔWC SDS ($r=0.40$, $p=0.013$). The correlation with ΔBMI SDS did not reach statistical significance ($r=0.26$, $p=0.054$) and ΔHCC was not correlated with Δheight SDS ($p=0.80$). In AI patients, hair cortisol was not correlated with daily hydrocortisone dose in mg/m^2 ($p=0.41$). When cases and controls were grouped together, HCC showed a significant association with BMI SDS (log transformed β=0.74, $p=0.002$) and WC SDS (log transformed β=0.69, $p=0.002$), but not with height SDS ($p=0.98$). Adding HCC to the linear regression model decreased the partial effect size for AI, showing that 12.7% of the difference in BMI SDS and 29.2% of the difference in WC SDS between AI patients and controls was explained by HCC. Daily hydrocortisone dose was not associated with height or BMI SDS ($p=0.47$

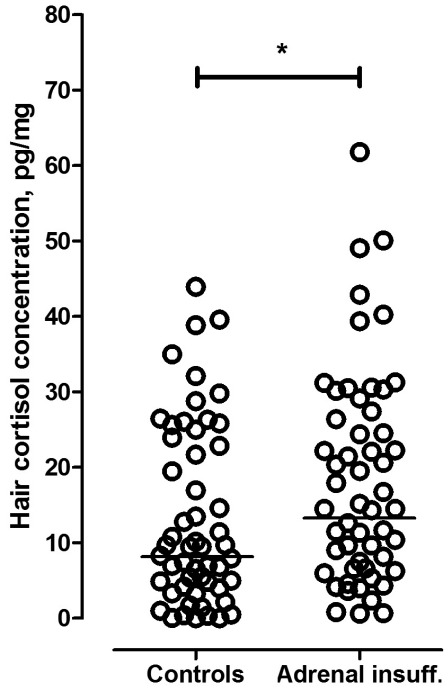

Figure 1. Hair cortisol concentrations (HCC, pg/mg hair) in healthy controls and children with adrenal insufficiency. Black lines show geometric mean. *p=0.022.

and p=0.25 respectively) but did show a significant correlation with WC SDS (r=0.32, p=0.05). Patients with salt losing form of CAH had a higher BMI SDS (mean [95%CI] 1.13 [0.64 – 1.62] vs. 0.49 [-0.15 – 1.12]), WC SDS (mean [95%CI] 1.38 [0.94 – 1.83] vs. 0.81 [0.27 – 1.35]) and HC dose (mean[95%CI] 12.6 [11.3 – 13.9] vs. 11.0 [9.3 – 12.6]) compared to non-salt losing patients, although these differences did not reach statistical significance when adjusted for age and sex (p=0.12, p=0.12 and p=0.13, respectively). HCC was significantly higher in patients with salt losing CAH compared to non-salt losing CAH patients (gm[95%CI] 18.2 [12.8 – 25.7] vs 8.1 [5.0 – 12.9], p=0.009, adjusted for age and sex).

DISCUSSION

To our knowledge, this is the first study reporting hair cortisol concentrations in children under GC replacement therapy. In patients with adrenal insufficiency at the age of 4-18 years receiving GC replacement therapy, hair cortisol concentrations were found to be significantly higher than in healthy controls. Additionally, waist circumference and BMI SDS were significantly increased in hydrocortisone treated patients, which can partially be explained by increased HCC. These finding probably

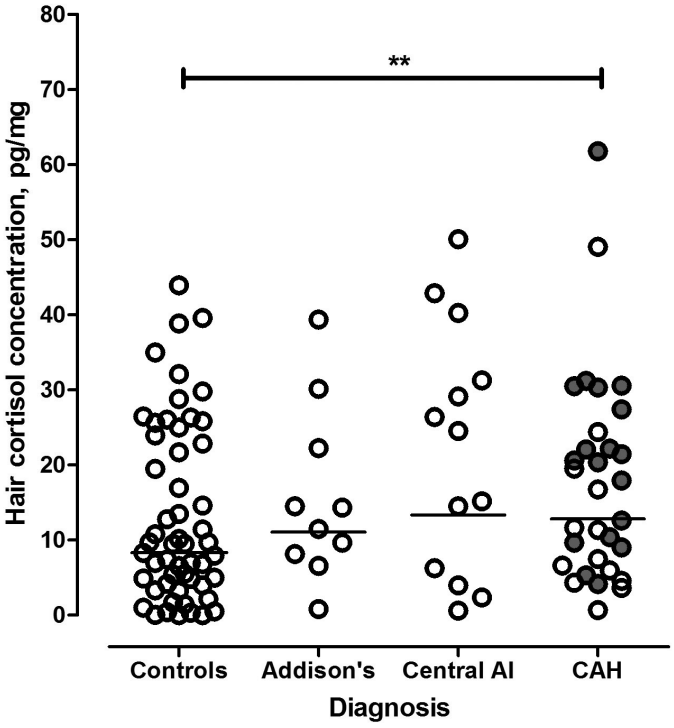

Figure 2. Hair cortisol concentrations (HCC, pg/mg hair) in healthy controls and children with adrenal insufficiency grouped per aetiology. Black lines show geometric mean. **p=0.012, adjusted for age and gender. Filled circles indicate patients with salt losing CAH.

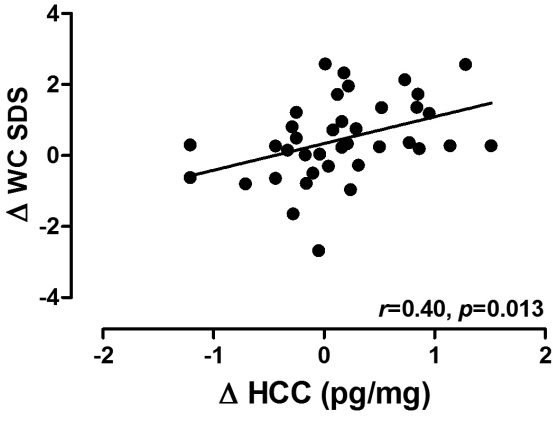

Figure 3. Correlation between paired delta in waist circumference (WC) SDS and paired delta in log-10 transformed hair cortisol concentration (HCC). Pearson's correlation r and p-value depicted in graph.

correlated with inadequate chronic serum cortisol levels, which was revealed by high HCC. This suggests that adrenal insufficiency patients are on average over-treated with hydrocortisone, resulting in the hallmark sequelae of hypercortisolism such as increased BMI and central adiposity. All patients in this study are treated according to national GC replacement guidelines, still effects of GC overtreatment were observed and long-term cortisol exposure, measured as hair cortisol, was significantly higher than in healthy controls. This is in line with previous studies showing increased serum cortisol concentrations in AI patients after GC ingestion, compared to healthy individuals[7,26]. HCC in AI patients are partially overlapping with concentrations in healthy controls, the extreme values in AI patients are the most informative results which may indicate glucocorticoid over replacement. Hydrocortisone overdosing is difficult to identify, as treatment adequacy in AI patients is primarily evaluated through the administered GC dose and clinical assessment of the symptoms of over- and under treatment[4,7]. Serum cortisol and urinary free cortisol (UFC) measurements are not useful in treatment adjustment[4,7,27]. Clinical symptoms indicating overtreatment are, amongst others, weight-gain, increased waist circumference, metabolic syndrome, sleep disturbances and skin changes such as acne. These are nonspecific symptoms which are often slow to develop[4,28]. Filipsson *et al.* demonstrated overdosing of GC, defined as an hydrocortisone equivalent dose of at least 20 mg per day, to be related to an adverse metabolic profile, including increased BMI and serum triglycerides and total cholesterol, in a large cohort of 2424 patients with hypopituitarism, with and without GC treatment[5]. These observations have been replicated in patients with Addison's disease[29]. Moreover, patients with Addison's disease treated with GCs were shown to have a two-fold mortality rate, mainly due to an increase in cardiovascular disease, infectious diseases and cancer and the authors speculated that this was, at least in part, attributable to GC overtreatment[30].

Medicinal therapy is influenced by absorption, distribution, metabolism and excretion. These factors vary between individuals, complicating prediction of the treatment effect in general. This is particularly true during childhood and adolescence, as absorption and excretion change over age, distribution is influenced by changes in body composition and metabolism is affected by changing hepatic enzyme activity and clearance[31]. This is even further complicated in cortisol pharmacokinetics, which undergo marked changes during puberty[32]. Hence, the lack of a correlation between the hydrocortisone dose and HCC was not surprising. This is also represented by the large range of salivary cortisol concentrations after GC ingestion[33]. In a study on HCC in patients with adrenal insufficiency, Gow *et al.*[21] did report a correlation between HCC and hydrocortisone dose. However, the patients studied by Gow *et al.* received a large range of hydrocortisone doses from 10 to 60 mg per day and were all adults. Hydrocortisone dose in these patients was not adjusted for BSA or body weight, which may further increase the difference of cortisol exposure between patients.

Objectively measuring long-term systemic cortisol exposure may improve current GC dosing regimens. Our results suggest measurement of hair cortisol concentration may be a novel method for identifying patients receiving supraphysiological hydrocortisone doses. Measurement of HCC has been shown to be of value in diagnosing and follow-up of Cushing's syndrome and cyclic Cushing's disease[13]. Moreover, elevated cortisol exposure within physiological ranges, as identified through HCC measurement, has been associated with increased risk for cardiovascular disease and the metabolic syndrome[23,24]. In adult AI patients on GC replacement therapy, Gow *et al.* reported increased HCC in patients compared to healthy controls similar to our results. No difference in HCC was found between 10 patients treated with cortisone acetate and patients treated with hydrocortisone, implicating this method can be applied in cortisone acetate treated patients as well.

One of the strengths of the current study is the sample size of hydrocortisone treated paediatric patients with adrenal insufficiency, with a large age range from 4 to 18 years, and the comparison with age and gender matched healthy controls. This study has a wide scope of adrenal insufficiency aetiologies. Moreover, as this study has an observational and cross-sectional design, no causal relation between hydrocortisone overdosing, HCC and anthropometric effect can be demonstrated. Longitudinal intervention studies focused on a more homogeneous patient population are required for this, and may be a subject for future research. The current study is limited to the analysis of anthropometric data as metabolic data on aspects such as glucose and lipid metabolism were not routinely available. Application of the described method to children under 4 years of age receiving GC treatment has not been studied. Previous studies have shown higher HCC and a larger range in healthy children at the age of 1 and 3 years[34]. For this age-group, a separate study should be conducted. Also for use of glucocorticoid treatment with other aims than replacement, e.g. anti-inflammatory treatment, additional studies are required. In another study on HCC in children performed by Karlén *et al.*[34] comparable ranges in HCC were found in children at the age of 8. Due to the non-physiologic administration of GC in adrenal insufficiency patients, HCC equal to those of healthy controls may prove unachievable, which is especially true for CAH patients, where GC treatment is not only focused on replacement, but also on decreasing ACTH secretion and resulting androgen excess. This does not decrease the validity of measuring cortisol in hair in GC treated patients, but it may require the establishment of reference ranges in well-controlled AI patients. One limitation of hair cortisol measurement is that it provides no direct information on systemic cortisol peak exposure directly after ingestion, as the maximal resolution of cortisol exposure is approximately one month. Additional measurement of for instance salivary cortisol day curves after GC ingestion may yield more information on short term peak cortisol exposure and nadir concentrations[33]. Furthermore, the potential influence of GC stress dosing on HCC has not been assessed in the current or previous studies, and warrants further research.

Measurement of cortisol concentrations in scalp hair provides clinicians with a long-term reflection of systemic cortisol exposure. The method is relatively simple, easily implemented and non-invasive, making it especially suitable in the paediatric clinical practice. Measurement of cortisol concentrations in scalp hair reflecting long-term systemic cortisol exposure at the tissue level may provide a new method for identifying hydrocortisone overtreatment in patients with adrenal insufficiency. This may improve current glucocorticoid replacement quality, limiting long-term negative health effects of previously unobserved overtreatment.

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REFERENCES

1. Quinkler M, Beuschlein F, Hahner S, Meyer G, Schofl C, Stalla GK. Adrenal Cortical Insufficiency-a Life Threatening Illness With Multiple Etiologies. *Dtsch Arztebl Int* 2013; **110**(51-52): 882-8.
2. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Endocr Rev* 2000; **21**(3): 245-91.
3. Bonfig W, Bechtold S, Schmidt H, Knorr D, Schwarz HP. Reduced final height outcome in congenital adrenal hyperplasia under prednisone treatment: deceleration of growth velocity during puberty. *J Clin Endocrinol Metab* 2007; **92**(5): 1635-9.
4. Husebye ES, Allolio B, Arlt W, et al. Consensus statement on the diagnosis, treatment and follow-up of patients with primary adrenal insufficiency. *J Intern Med* 2014; **275**(2): 104-15.
5. Filipsson H, Monson JP, Koltowska-Haggstrom M, Mattsson A, Johannsson G. The impact of glucocorticoid replacement regimens on metabolic outcome and comorbidity in hypopituitary patients. *J Clin Endocrinol Metab* 2006; **91**(10): 3954-61.
6. Jodar E, Valdepenas MP, Martinez G, Jara A, Hawkins F. Long-term follow-up of bone mineral density in Addison's disease. *Clin Endocrinol (Oxf)* 2003; **58**(5): 617-20.
7. Arlt W, Rosenthal C, Hahner S, Allolio B. Quality of glucocorticoid replacement in adrenal insufficiency: clinical assessment vs. timed serum cortisol measurements. *Clin Endocrinol (Oxf)* 2006; **64**(4): 384-9.
8. Quinkler M, Hahner S. What is the best long-term management strategy for patients with primary adrenal insufficiency? *Clin Endocrinol (Oxf)* 2012; **76**(1): 21-5.
9. Ingerski LM, Hente EA, Modi AC, Hommel KA. Electronic measurement of medication adherence in pediatric chronic illness: a review of measures. *J Pediatr* 2011; **159**(4): 528-34.
10. Manenschijn L, Koper JW, Lamberts SW, van Rossum EF. Evaluation of a method to measure long term cortisol levels. *Steroids* 2011; **76**(10-11): 1032-6.
11. Hayashi S, Miyamoto I, Takeda K. Measurement of human hair growth by optical microscopy and image analysis. *Br J Dermatol* 1991; **125**(2): 123-9.
12. Sauve B, Koren G, Walsh G, Tokmakejian S, Van Uum SH. Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clin Invest Med* 2007; **30**(5): E183-91.
13. Manenschijn L, Koper JW, van den Akker EL, et al. A novel tool in the diagnosis and follow-up of (cyclic) Cushing's syndrome: measurement of long-term cortisol in scalp hair. *J Clin Endocrinol Metab* 2012; **97**(10): E1836-43.
14. Davenport MD, Tiefenbacher S, Lutz CK, Novak MA, Meyer JS. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *Gen Comp Endocrinol* 2006; **147**(3): 255-61.
15. Dettenborn L, Tietze A, Bruckner F, Kirschbaum C. Higher cortisol content in hair among long-term unemployed individuals compared to controls. *Psychoneuroendocrinology* 2010; **35**(9): 1404-9.
16. Kalra S, Einarson A, Karaskov T, Van Uum S, Koren G. The relationship between stress and hair cortisol in healthy pregnant women. *Clin Invest Med* 2007; **30**(2): E103-7.
17. Kramer MS, Lydon J, Seguin L, et al. Stress pathways to spontaneous preterm birth: the role of stressors, psychological distress, and stress hormones. *Am J Epidemiol* 2009; **169**(11): 1319-26.
18. Manenschijn L, van Kruysbergen RG, de Jong FH, Koper JW, van Rossum EF. Shift work at young age is associated with elevated long-term cortisol levels and body mass index. *J Clin Endocrinol Metab* 2011; **96**(11): E1862-5.
19. Pereg D, Gow R, Mosseri M, et al. Hair cortisol and the risk for acute myocardial infarction in adult men. *Stress* 2011; **14**(1): 73-81.
20. Van Uum SH, Sauve B, Fraser LA, Morley-Forster P, Paul TL, Koren G. Elevated content of cortisol in hair of patients with severe chronic pain: a novel biomarker for stress. *Stress* 2008; **11**(6): 483-8.
21. Gow R, Koren G, Rieder M, Van Uum S. Hair cortisol content in patients with adrenal insufficiency on hydrocortisone replacement therapy. *Clin Endocrinol (Oxf)* 2011; **74**(6): 687-93.
22. Thomson S, Koren G, Fraser LA, Rieder M, Friedman TC, Van Uum SH. Hair analysis provides a historical record of cortisol levels in Cushing's syndrome. *Exp Clin Endocrinol Diabetes* 2010; **118**(2): 133-8.
23. Manenschijn L, Schaap L, van Schoor NM, et al. High long-term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease. *J Clin Endocrinol Metab* 2013; **98**(5): 2078-83.
24. Stalder T, Kirschbaum C, Alexander N, et al. Cortisol in hair and the metabolic syndrome. *J Clin Endocrinol Metab* 2013; **98**(6): 2573-80.

25. Fredriks AM, van Buuren S, Burgmeijer RJ, et al. Continuing positive secular growth change in The Netherlands 1955-1997. *Pediatr Res* 2000; **47**(3): 316-23.
26. Mah PM, Jenkins RC, Rostami-Hodjegan A, et al. Weight-related dosing, timing and monitoring hydrocortisone replacement therapy in patients with adrenal insufficiency. *Clin Endocrinol (Oxf)* 2004; **61**(3): 367-75.
27. Falorni A, Minarelli V, Morelli S. Therapy of adrenal insufficiency: an update. *Endocrine* 2013; **43**(3): 514-28.
28. Grossman A, Johannsson G, Quinkler M, Zelissen P. Therapy of endocrine disease: Perspectives on the management of adrenal insufficiency: clinical insights from across Europe. *Eur J Endocrinol* 2013; **169**(6): R165-75.
29. Giordano R, Marzotti S, Balbo M, et al. Metabolic and cardiovascular profile in patients with Addison's disease under conventional glucocorticoid replacement. *J Endocrinol Invest* 2009; **32**(11): 917-23.
30. Bensing S, Brandt L, Tabaroj F, et al. Increased death risk and altered cancer incidence pattern in patients with isolated or combined autoimmune primary adrenocortical insufficiency. *Clin Endocrinol (Oxf)* 2008; **69**(5): 697-704.
31. Funk RS, Brown JT, Abdel-Rahman SM. Pediatric pharmacokinetics: human development and drug disposition. *Pediatr Clin North Am* 2012; **59**(5): 1001-16.
32. Charmandari E, Hindmarsh PC, Johnston A, Brook CG. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency: alterations in cortisol pharmacokinetics at puberty. *J Clin Endocrinol Metab* 2001; **86**(6): 2701-8.
33. Smans L, Lentjes E, Hermus A, Zelissen P. Salivary cortisol day curves in assessing glucocorticoid replacement therapy in Addison's disease. *Hormones (Athens)* 2013; **12**(1): 93-100.
34. Karlen J, Frostell A, Theodorsson E, Faresjo T, Ludvigsson J. Maternal Influence on Child HPA Axis: A Prospective Study of Cortisol Levels in Hair. *Pediatrics* 2013; **132**(5): e1333-40.

CHAPTER 8

SCALP HAIR 17-HYDROXYPROGESTERONE AND ANDROSTENEDIONE AS A LONG-TERM THERAPY MONITORING TOOL IN CONGENITAL ADRENAL HYPERPLASIA

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ABSTRACT

Background

Glucocorticoid replacement therapy in Congenital Adrenal Hyperplasia (CAH) is challenging, especially in children, because both over- and under-dosing may have profound and long-lasting adverse effects. Clinical follow-up parameters are largely non-specific and slow to develop. Steroid concentrations in scalp hair may be a useful monitoring tool, as it provides information on both long-term steroid precursor and glucocorticoid exposure.

Aim

We aimed to evaluate scalp hair steroid precursor concentrations as a monitoring tool for treatment follow-up in children with CAH.

Methods

Scalp hair 17-hydroxyprogesterone (17-OHP) and androstenedione concentrations, measured by LC-MS/MS, of children with CAH (N=26) are correlated to concentrations in serum and saliva, and were compared to scalp hair concentrations in patient-controls with adrenal insufficiency (AI) (N=12) and healthy controls (N=293).

Results

Hair cortisol concentrations were higher in children with CAH, compared to both healthy controls ($p<0.001$) and patient-controls ($p=0.05$), and did not differ significantly between patient-controls with AI and healthy controls. Concentrations of androstenedione in scalp hair were strongly correlated with concentrations in serum ($\rho=0.72$, $p<0.001$) and saliva ($\rho=0.82$,$p=0.002$). This was also seen for 17-OHP in hair with serum ($\rho=0.94$, $p<0.001$) and saliva ($\rho=0.69$,$p=0.009$). Both hair 17-OHP and androstenedione were higher in CAH patients (mean concentration 17-OHP 2.9 pg/mg; androstenedione 1.3 pg/mg), when compared to healthy controls (17-OHP 0.44 pg/mg; androstenedione 0.65 pg/mg) and when compared to patients with AI (17-OHP 0.12 pg/mg; androstenedione 0.32 pg/mg).

Conclusion

This study shows that scalp hair 17-hydroxyprogesterone and androstenedione concentrations seem to be a promising parameter for treatment monitoring in patients with CAH.

INTRODUCTION

Congenital adrenal hyperplasia (CAH) can be caused by a number of autosomal recessive genetic defects in the cortisol synthesis cascade. In approximately 95% of cases, the defect is located in the steroid 21-hydroxylase (P450c21) gene, causing a steroid 21-hydroxylase deficiency leading to cortisol deficiency. The subsequent loss of negative feedback in the hypothalamus – pituitary – adrenal (HPA) axis results in an overproduction of adrenocorticotrophic hormone (ACTH), resulting in adrenal hyperplasia and steroid(-precursor) excess.[1] Several forms of CAH are identified, based on the clinical presentation. Less than 1-2% residual enzyme activity causes the classical salt-wasting form, in which also aldosterone is deficient, whereas below approximately 20% residual activity patients develop the 'simple virilizing' form without salt-wasting. Both forms are characterized by hyperandrogenism, causing premature skeletal maturation with epiphyseal closure, precocious puberty, and in girls virilization, often causing ambiguous genitalia at birth. Patients with nonclassical CAH, who usually have 20-50% enzyme activity left, mostly have sufficient cortisol synthesis capacity. Although about 40% of these patients have an suboptimal cortisol respone after synacthen, they suffer primarily from androgen excess and its consequences.[2] Hydrocortisone is the preferred glucocorticoid (GC) for replacement therapy in children.[3] Therapy prevents cortisol deficiency and thus adrenal crises, and subsequently reduces androgen excess by providing negative feedback at the hypothalamus and pituitary.[2] Attaining the optimal dosing scheme is challenging. Therapy generally results in supra-physiologic cortisol concentrations, which may partially explain the reduced adult height, increased body mass index, reduced fertility, impaired exercise performance, adverse metabolic profile, impaired quality of life, and an increased mortality risk present in CAH patients.[4-7]

The central theme in treatment follow-up in children is focused on preventing both GC and steroid hormone excess. Overtreatment with GC is primarily evaluated through clinical signs of Cushing's syndrome, e.g. growth retardation, increased weight and adverse body fat distribution. Steroid hormone excess is evaluated through clinical signs of increased growth velocity, advanced skeletal maturation, and steroid hormone specific symptoms such as hirsutism, acne and virilization.[8] As these symptoms are often slow to develop, patients may be over- or undertreated for a considerable time before these signs are noted. Current international guidelines advise assessment of therapy monitoring by measuring steroid hormone precursors in plasma or saliva in a consistent manner, aiming at mildly elevated steroid levels as normal levels of 17-OHP and the other steroids indicate overtreatment[8]. Several limitations apply to using hormone concentrations in these matrices; They are highly dependent on the time of measurement and intake of medication, provide information of short-term steroid hormone concentrations only, and may be falsely increased due to e.g. acute stress with ACTH increases or blood traces in saliva. A relatively newly used matrix is scalp hair, in which concentrations are not time-dependent, sampling is non-invasive,

and long-term steroid exposure can be quantified.[9-11] In a previous immunoassay based study, we reported scalp hair cortisol concentrations to be increased in CAH patients compared to healthy children.[12] The development of liquid chromatography tandem mass spectrometry (LC-MS/MS) based methods for scalp hair steroid profiling has provided considerable improvements, as this method has superior sensitivity and specificity compared to immunoassays, and provides the capability of measuring multiple components among which 17-OH-progesterone and androstenedione, in a single run.[13,14] Benefiting from these recent technical advances, adrenal precursor hormones essential for CAH follow-up can be quantified simultaneously with cortisol.

The aim of this study is to evaluate scalp hair as a matrix for long-term steroid precursor hormone profiling as a novel monitoring tool for treatment follow-up in pediatric congenital adrenal hyperplasia patients by assessing the correlations between concentrations of the adrenal steroid precursor hormones 17-hydroxyprogesterone and androstenedione in scalp hair with those in plasma and saliva. Additionally, we aimed to compare cortisol and steroid precursor hormones in scalp hair in patients with CAH to healthy controls and to patients with adrenal insufficiency (AI) (without steroid hormone excess) treated with hydrocortisone, as patient controls.

METHODS

Participants

Patients with congenital adrenal hyperplasia and/or adrenal insufficiency, attending the pediatric outpatient clinic of the Sophia children's hospital (Erasmus MC, Rotterdam, The Netherlands) were requested to participate. Healthy children were enrolled through primary and secondary school visits in two cities in the Netherlands, Rotterdam and Amersfoort. This study was approved by the local medical ethics committee. All parents and children above the age of 12 years provided informed consent, children under the age of 12 years provided informed assent. Inclusion criterion for all children was an age between 0 and 19 years. The inclusion criterion for patients and patient-controls was the use of glucocorticoid replacement therapy for adrenal insufficiency, exclusion criteria were use of other medications known to alter steroid or glucocorticoid metabolism, e.g. anti-epileptic drugs, or hair shorter than 2 cm. Controls were excluded when systemic glucocorticoid were used or when hair was shorter than 2 cm at the posterior vertex.

Data collection

Of all participants, anthropometrics were measured as described previously,[12] and information on hair characteristics and hair care were obtained, and hair was sampled. The standard deviation scores (SDS) of height, weight and body mass index (BMI) were calculated based on data from the Dutch nationwide growth study 1997, using Growth Analyser (Growth Analyser BV, Rotterdam, The Netherlands). As parental height was not available for healthy controls, target-height SDS was not measured. In all patients,

information on medication use was obtained. Serum hormone concentrations used in the current study were drawn on the day of hair sampling. Routinely measured saliva steroid hormone precursor concentrations measured in patients with CAH within three months from hair sampling were used in this study, as hair steroid concentrations reflect an average concentration of the past three months (see below). Saliva was collected through passive drooling on three consecutive days after awakening, prior to hydrocortisone intake. The average concentration was calculated and used for analyses. Serum samples were collected either before or after the outpatient clinic visit, after intake of medication according to the regular thrice daily hydrocortisone schedule, following the ESPE clinical practice guidelines.[15] Serum and saliva concentrations were measured in CAH patients only.

Measurements of cortisol and steroid precursor hormones

Scalp hair locks of approximately 100 hairs were cut from the posterior vertex, as close to the scalp as possible, using small surgical scissors. The proximal three cm of hair, reflecting the most recent three months, were used for analysis. The proximal three cm of hair was cut in segments of 1 cm in a single vial and measured in a single run. The samples were then weighed, washed in 2 mL LC-grade iso-propanol, and left to dry for at least two days. Subsequently, steroids were extracted in LC-grade methanol, the extract was cleaned using solid phase extraction, and steroids were quantified by liquid-chromatography tandem mass spectrometry (LC-MS/MS), as described elsewhere.[14] Steroid hormone concentrations were divided by the hair sample weight, and expressed as picogram per milligram hair (pg/mg). In subject 17-OH-progesterone and androstenedione were measured in serum samples with LC-MS/MS. Steroid hormones were measured simultaneously with a method using the CHS™ MSMS Steroids Kit (Perkin Elmer, Turku, Finland). The Steroids Kit uses a combined solvent extraction and protein precipitation method with acetonitrile containing the deuterated internal standards 2H5-androstenedione and 2H8-17α-hydroxyprogesterone. The internal standards undergo processing identical to the analytes. The chromatographic separation was performed on a Waters® Acquity™ UPLC HSS T3 1.8 µm column (diameter 1 mm, length 10 cm) and in-line filter frit 0.2 µm with acetonitrile/MeOH gradient. A Waters XEVO-TQ-S system (Waters, Milford, MA, USA) equipped with an ESI source operating in the electrospray positive mode. Multiple reaction monitoring was applied for the detection of the analytes using both quantifiers and qualifiers. The corresponding inter-assay coefficients of variation and lower limit of quantification (LLOQ) are the following: 17-OH-progesterone 6.1%; LLOQ 0.10 nmol/L and testosterone <5%; LLOQ: 0.07 nmol/L. Both 17α-OH-progesterone and androstenedione were measured in saliva by commercially available ELISA methods of DRG Instruments (GmbH, Marburg, Germany) (17OHP: SLV-3140 kit; Androstenedione: SLV-4780 kit). Both 17α-OH-progesterone and androstenedione were measured in saliva by commercially available ELISA methods of DRG Instruments (GmbH, Marburg, Germany) (17OHP: SLV-3140 kit; Androstenedione: SLV-4780 kit).

Statistical analysis

All analyses were performed using SPSS Statistics version 21.0.0.1 (IBM, Armonk, NY, USA). Adrenal (precursor) hormones do not follow a Gaussian distribution, therefore, log10-transformation was applied and the geometric mean was calculated to describe the data. General characteristics between patient and control groups were compared using one-way analysis of variance (ANOVA) or the Pearson Chi-Square test. Differences in adrenal hormone concentrations were assessed using the Mann-Whitney U test. To assess the effects of potential confounders, multiple linear regressions were performed on the log10-transformed concentrations, adjusted for age, sex, ethnicity and hair color. Correlations between steroid hormones in different matrices were assessed using the Spearman correlation coefficient. A p-value below 0.05 was considered to indicate statistically significant differences.

RESULTS

In total 27 children with congenital adrenal hyperplasia were included in this study, of which 26 children were diagnosed with 21-hydroxylase deficiency. One child had 11β-hydroxylase insufficiency and thus was excluded from the study. All children were treated with hydrocortisone thrice daily, except for one patient who was treated with prednisone, in whom no cortisol concentrations in saliva or hair were measured. Of the 26 patients with CAH, 16 (61.5%) had a salt-wasting form and were treated with fludrocortisone. Scalp hair analyses were additionally performed in 12 patient-controls with adrenal insufficiency without steroid hormone excess, all treated with hydrocortisone. Of these 12 patient-controls, two were diagnosed with primary adrenal insufficiency, and 10 with hypopituitarism, in which case they were treated for all deficient hormones adhering to the specific guidelines. Table 1 shows the basic characteristics of the patients with CAH and AI, and 293 healthy controls. BMI was significantly higher in both patient groups than in healthy controls. Children with AI were significantly shorter than children with CAH and healthy controls. Adhering to the international guidelines, children with CAH received higher hydrocortisone doses compared to children with AI (12.9 vs. 8.7 mg/m^2/24h). Hair cortisol concentrations were highest in children with CAH, compared to both healthy controls (p<0.001) and patients with AI (p=0.05). Cortisol concentrations in hair did not differ significantly between patients with AI and healthy controls (p=0.32).

Correlations between matrices

Serum and saliva hormone concentrations were not measured at every outpatient-clinic visit, hence these variables were available for only a subset of the CAH patients (N=19 for serum, N=13 for 17-OH-progesterone in saliva, N=11 for androstenedione in saliva). Figure 1 shows the correlations between steroid precursor hormones measured in scalp hair with concentrations measured in serum and saliva during

Table 1. General characteristics

	CAH N=26	AI N=12	Controls N=293	P-value
Sex, female	12 (46.2%)	5 (41,7%)	141 (48.1%)	0.89
Age, yr. (range)	10.6 (5 – 17)	10.0 (2 – 18)	11.5 (3 – 19)	0.23
BMI	21.6 (6.9)	19.8 (5.4)	17.9 (3.3)	
BMI – SD score	1.10 (1.23)	0.55 (1.65)	-0.11 (1.00)	<0.001
Height	145.0 (18.0)	132.9 (30.5)	150.7 (22.9)	
Height – SD score	-0.14 (1.28)	-1.34 (1.56)	0.15 (0.95)	<0.001
HC dose, mg/m^2/24h (range)	12.9 (8.8 – 16.7)	8.7 (7.2 – 10.3)	-	<0.001
Pubertal	13 (50.0%)	5 (41.7%)		0.63
Non-western ethnicity	6 (23.1%)	2 (18.2%)	42 (14.3%)	0.58
Hair color black	4 (15.4%)	1 (8.3%)	14 (5.1%)	0.13
Hair cortisol, pg/mg	8.5 (3.2)	4.2 (5.3)	2.2 (2.4)	<0.001

Data shown as (geometric) mean (SD) or (range). P-values derived through Pearson Chi-Square or one-way ANOVA. AI: Adrenal insufficiency; CAH: Congenital adrenal hyperplasia; BMI: Body Mass Index; SD: Standard deviation; HC: hydrocortisone.

routine follow-up in patients with CAH. Concentrations of androstenedione in scalp hair were strongly correlated with concentrations in serum (ρ=0.72, p<0.001) and saliva (ρ=0.82, p=0.002). Scalp hair 17-OH-progesterone was strongly correlated with concentrations of 17-OH-progesterone in serum (ρ=0.94, p<0.001) and saliva (ρ=0.69, p=0.009).

Scalp hair steroid precursor hormones between groups

Concentrations of scalp hair steroid precursor hormones measured in patients with AI or CAH and healthy children were compared, as shown in figure 2. Both 17-OHP and Adion were significantly higher in patients with CAH (mean (95%CI) 17-OHP 2.9 pg/mg (1.4 – 6.4); Adion 1.3 pg/mg (0.7 - 2.3)), when compared to healthy controls (mean (95%CI) 17-OHP 0.44 pg/mg (0.40 – 0.48); Adion 0.65 pg/mg (0.56 – 0.75)) and when compared to patients with AI due to Addison's disease or hypopituitarism (mean (95%CI) 17-OHP 0.12 pg/mg (0.11 – 0.13); Adion 0.32 pg/mg (0.19 - 0.54)). These differences remained statistically significant when adjusted for age, sex, ethnicity and black hair color. Both 17-OHP and androstenedione concentrations in scalp hair were also significantly lower in patient-controls when compared to healthy controls (p<0.001 and p=0.03 resp.).

Within the patients with CAH, 17-OH-progesterone and androstenedione concentrations in hair were not different between boys and girls (p=0.40 and p=0.93, respectively). In pubertal children with CAH, hair 17-OH-progesterone and androstenedione were significantly higher than in pre-pubertal children with CAH (mean (95%CI) 17-OHP 8.7 pg/mg (3.3 – 22.7) vs. 1.0 pg/mg (0.38 – 2.62); Adion 2.6 pg/mg (1.1 – 6.11) vs. 0.73 pg/mg (0.35 – 1.53)).

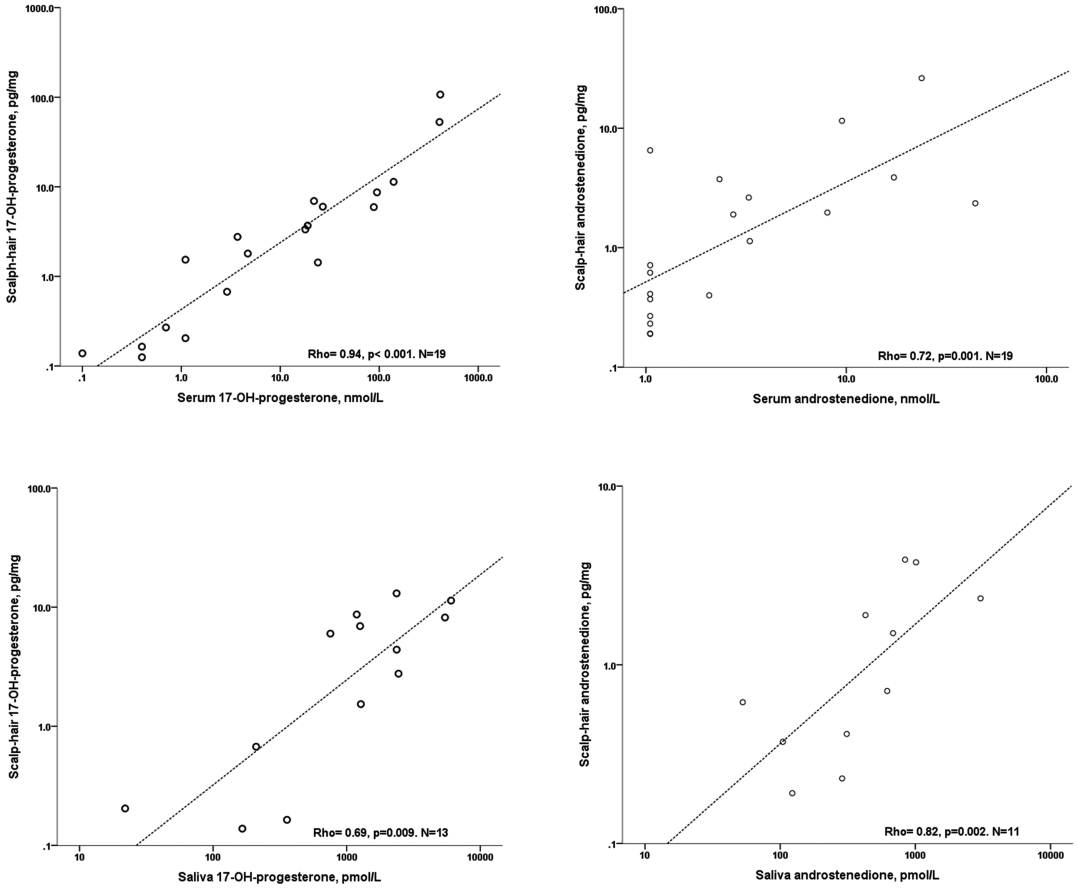

Figure 1. Correlations between 17-hydroxyprogesterone and androstenedione in scalp hair with serum and saliva in CAH patients. Correlations assessed using Spearman's correlation coefficient, Spearman's Rho and p-value are displayed in the figures.

DISCUSSION

In the current study, we show 17-hydroxyprogesterone and androstenedione measured in scalp hair of children with CAH to be strongly correlated with concentrations measured in serum and saliva. The latter two matrices are usually used for treatment follow-up. Scalp hair steroid precursor hormone concentrations were, as anticipated, significantly higher in children with CAH compared to both healthy controls and children with AI due to Addison's disease or hypopituitarism. This study is the first to apply steroid hormone measurements in scalp hair to patients with adrenal insufficiency, with or without steroid hormone excess. We and others have measured scalp hair cortisol concentrations in adrenal insufficiency patients on glucocorticoid replacement therapy, showing high concentrations in patients compared to healthy controls.[12,16,17]

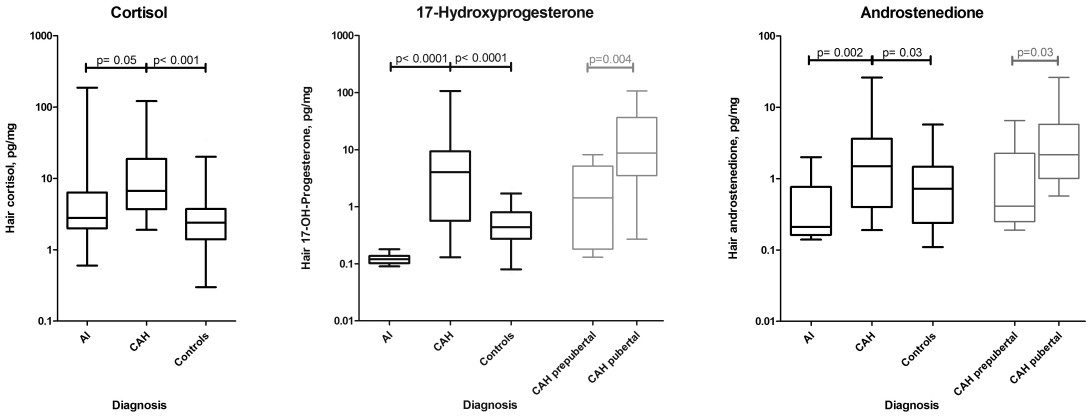

Figure 2. Cortisol,17-hydroxyprogesterone and androstenedione concentrations in scalp hair in CAH and (patient)control groups.Box-plots showing scalp hair steroid hormone concentrations in patients with adrenal insufficiency (AI), congenital adrenal hyperplasia (CAH) and healthy controls. Additionally, 17-OH-progesterone and androstenedione were compared in CAH patients before and after puberty. P-values calculated using the Mann-Whitney U test.

This is in line with the results of the current study, with cortisol concentrations being especially increased in patients with CAH, which is concordant with the higher dosing hydrocortisone in this group. The current method adds the value of simultaneously measuring multiple adrenal (precursor) hormones which are important monitoring parameters in congenital adrenal hyperplasia.[8]

Treatment monitoring in patients with CAH focuses on replacing cortisol and preventing steroid hormone excess on the one hand, and preventing cortisol overexposure with Cushingoid symptoms on the other hand. However, preventing steroid hormone excess requires supra-physiologic glucocorticoid doses. The clinician is therefore tasked with finding the optimal glucocorticoid dose, with reducing steroid hormone excess without inducing Cushing's syndrome. The continuous bodily development in pediatric patients, with changes in body size, body composition and metabolism, further complicates establishing and maintaining an optimal dose. The health status of adult patients with CAH shows that CAH treatment remains a challenge, with side-effects often presenting only on the long term with reduced height, cardio-metabolic complications, reduced fertility, and impaired subjective health status.[5]

The opportunity of measuring steroid hormones in scalp hair, provides the clinician insight in both the long-term cortisol exposure and steroid hormone exposure. As the hair sampling method used does not depend on patient compliance as for instance saliva and urine sampling do, scalp hair measurement seems to be a valuable therapy monitoring tool. From a practical viewpoint, a lock of 100 hairs is a small lock of approximately 0.5 cm across, the scalp-area can be sampled repetitively every

3-4 months, hair sampling is not labor-intensive and non-invasive and therefore also suited for the pediatric patient population.

This study is a cross-sectional study comparing steroid hormone concentrations in scalp hair with concentrations in other matrices. A strong point in the current study is that steroid hormone measurement in scalp hair was highly correlated to both serum and saliva concentrations. Additionally, both patient-controls with AI without steroid hormone excess and a large group of healthy controls were enrolled to compare with patients with CAH. However, also several limitations apply to the current study. The cross-sectional design of the study, although clearly showing a strong correlation between concentrations in multiple matrices, does not allow for proving that scalp hair steroid hormone profiling can be applied to adjust treatment regimens in patients. For this, longitudinal measurements would be needed, showing treatment adjustments to be reflected in scalp hair analysis. This may however prove challenging, as treatment adjustments are often subtle when disease control is good. Conducting research in healthy children, especially in their teens, is a challenge in itself, as participation is based on and informed consent from their caretakers but also on willingness and assent from the children. Puberty staging was included in the physical examination of the children, however due to puberty staging it proved difficult to acquire assent from healthy participating children. After eliminating this specific part of the examination, healthy children were willing to participate. The result is however that in the current study, Tanner stages could not be used as a covariate, which is a significant drawback. To compensate for this in part, we included a relatively large control-group. Another remark is that in this study the most proximal three cm hair was analyzed, reflecting concentrations over approximately three months, while concentrations in both serum and saliva only reflected the concentrations on a snap-moment within this time span.

Our study shows that 17-hydroxyprogesterone and androstenedione concentrations in scalp hair seem to be a promising parameters for treatment monitoring in patients with congenital adrenal hyperplasia. It provides insight into long-term cortisol and steroid hormone exposure, and may add to the evaluation of treatment regimens which are currently mainly evaluated on the basis of, often nonspecific, clinical evaluations of both cortisol and steroid hormone excess.

REFERENCES

1. Speiser PW, White PC. (2003) Congenital adrenal hyperplasia. *N Engl J Med*;349:776-88.
2. Claahsen-van der Grinten HL, Stikkelbroeck NM, Otten BJ, *et al.* (2011) Congenital adrenal hyperplasia--pharmacologic interventions from the prenatal phase to adulthood. *Pharmacol Ther*;132:1-14.
3. Debono M, Price JN, Ross RJ. (2009) Novel strategies for hydrocortisone replacement. *Best Pract Res Clin Endocrinol Metab*;23:221-32.
4. Marra AM, Improda N, Capalbo D, *et al.* (2015) Cardiovascular abnormalities and impaired exercise performance in adolescents with congenital adrenal hyperplasia. *J Clin Endocrinol Metab*;100:644-52.
5. Arlt W, Willis DS, Wild SH, *et al.* (2010) Health status of adults with congenital adrenal hyperplasia: a cohort study of 203 patients. *J Clin Endocrinol Metab*;95:5110-21.
6. Falhammar H, Frisen L, Norrby C, *et al.* (2014) Increased mortality in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J Clin Endocrinol Metab*;99:E2715-21.
7. Bouvattier C, Esterle L, Renoult-Pierre P, *et al.* (2015) Clinical outcome, hormonal status, gonadotrope axis and testicular function in 219 adult men born with classic 21-hydroxylase deficiency. A French national survey. *J Clin Endocrinol Metab*:jc20144124.
8. Speiser PW, Azziz R, Baskin LS, *et al.* (2010) A Summary of the Endocrine Society Clinical Practice Guidelines on Congenital Adrenal Hyperplasia due to Steroid 21-Hydroxylase Deficiency. *Int J Pediatr Endocrinol*;2010:494173.
9. Kirschbaum C, Tietze A, Skoluda N, *et al.* (2009) Hair as a retrospective calendar of cortisol production-Increased cortisol incorporation into hair in the third trimester of pregnancy. *Psychoneuroendocrinology*;34:32-7.
10. Manenschijn L, Koper JW, Lamberts SW, *et al.* (2011) Evaluation of a method to measure long term cortisol levels. *Steroids*;76:1032-6.
11. Sauve B, Koren G, Walsh G, *et al.* (2007) Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clin Invest Med*;30:E183-91.
12. Noppe G, van Rossum EFC, Vliegenthart J, *et al.* (2014) Elevated hair cortisol concentrations in children with adrenal insufficiency on hydrocortisone replacement therapy. *Clin Endocrinol*;81:820-5.
13. Gao W, Stalder T, Foley P, *et al.* (2013) Quantitative analysis of steroid hormones in human hair using a column-switching LC-APCI-MS/MS assay. *J Chromatogr B Analyt Technol Biomed Life Sci*;928:1-8.
14. Noppe G, de Rijke YB, Dorst K, *et al.* (2015) LC-MS/MS based method for long-term steroid profiling in human scalp hair. *Clin Endocrinol (Oxf)*:DOI: 10.1111/cen.12781.
15. Clayton PE, Miller WL, Oberfield SE, *et al.* (2002) Consensus statement on 21-hydroxylase deficiency from the European Society for Paediatric Endocrinology and the Lawson Wilkins Pediatric Endocrine Society. *Horm Res*;58:188-95.
16. Gow R, Koren G, Rieder M, *et al.* (2011) Hair cortisol content in patients with adrenal insufficiency on hydrocortisone replacement therapy. *Clin Endocrinol (Oxf)*;74:687-93.
17. Staufenbiel SM, Andela CD, Manenschijn L, *et al.* (2015) Increased Hair Cortisol Concentrations and BMI in Patients With Pituitary-Adrenal Disease on Hydrocortisone Replacement. *J Clin Endocrinol Metab*;100:2456-62.

CHAPTER 9

DISCUSSION

Hormone concentrations are measured in varying matrices

Measurement of hormone concentrations is the corner-stone of endocrine practice, with determining the concentrations at the target organs being the ultimate goal. This is however rarely feasible; thus hormone concentrations are measured in other matrices thought to reflect target organ levels. Due to particular hormone and matrix characteristics, e.g. molecular structure, lipophilicity, and protein binding, not all matrices are suitable for measurement of any hormone. Several commonly used matrices for hormone measurement are blood, saliva and urine. Human scalp hair analysis is a relatively novel method, applied for measurement of steroid hormones.[1-4]

Serum and plasma are probably the most routinely applied matrix for hormone measurement in clinical practice. Several advantages are its relatively easy availability through venipuncture, concentrations are often directly interpretable and is less dependent on factors such as local metabolism or renal function. However, several limitations do apply. For example, hormone concentrations may be dependent on binding protein concentrations.[5,6] Serum hormone measurement usually provides a concentration on a single point in time, which may be cumbersome to interpret for hormones with short half-times and large variances in synthesis, excretion and metabolism in time. Moreover, blood sampling can be stress-full for some people, which may influence stress-reactive hormone concentrations such as cortisol.

Saliva sampling may be a good alternative to serum samples. Sampling is non-invasive, and concentrations are highly correlated to serum sampling, reflecting the unbound, free hormone fraction[7]. Saliva sampling can be performed at home and samples can be send by post mail, which makes it an easy method for establishing concentration curves by sampling at several times during a day, without the need for hospital admission. Drawbacks are, like blood sampling, the short time-frame for which a single measurement interpretable and as such the potential influence of acute stress. Additionally, minute blood dilution due to for example eating or tooth brushing may result in falsely elevated concentrations, although Granger *et al.*[8] reported significant blood contamination to be rare. Lastly, sampling is dependent on patient compliance, which sometimes proves to be less than desired.[9]

Another routinely applied matrix, e.g. in diagnosis of Cushing's syndrome, pheochromocytoma and carcinoid, is urine.[10-12] 24-hour urine sampling provides a reliable reflection of hormone synthesis and excretion, and sampling is non-invasive, although often proves to be cumbersome. 24-hour urine collection is highly dependent on patient compliance, which is directly the largest drawback.[13,14] More-over, renal function and total urine volume, as well as short-term stress due to for example inter-current disease should be taken in to account. Inter-day variation in urine free cortisol concentrations may be large, and multiple 24-hour urine portions should be collected before CS can be excluded.[14]

Another technique that closely approaches free hormone concentrations at the target organ is based on micro-dialysis. Using an automated sampling system

of portable size, Bhake *et al.*[15] create high resolution 24-hour hormone curves while subjects reside in their familiar surroundings being able to partake in their everyday activities. The micro-dialysis system samples free hormone concentrations subcutaneously over adjustable time-periods, separated by air-bubbles between time-frames. This technique probably best reflects hormone tissue-concentrations through a relatively limited invasive method, but requires specialized devices and is limited to measurement during single days.

Human scalp hair as a source for steroid hormone measurement is relatively new. In 2000 Cirimele *et al.*[3] showed a range of glucocorticoids to be measurable in human scalp hair, both synthetic exogenous glucocorticoids such as prednisone and dexamethasone, but also endogenously synthesized cortisol and cortisone. In more recent years, human scalp hair has increasingly been applied for measurement of chronic glucocorticoid exposure in a broad variety of clinical research settings.[4,16,17] Several advantages apply to hormone measurement in scalp hair. It is non-invasive and does not rely on patient compliance, other than assent in donating a small lock of hair. The result reflects the retrospective free cortisol fraction and is therefore not influenced by binding protein concentrations, which can be elevated due to for example oral anticonception. A single hair lock can provide average hormone concentrations of recent months to even years, which renders the improved possibility of diagnosis of cyclic Cushing's syndrome.[18] Factors such as hair washing, hair product use or hair treatment seems to have none or a limited effect on the result.[19] Hair color does appear to effect the result, although the clinical relevance seems small to moderate (chapter 4). The minimal resolution of hair analysis is approximately half a month, hence short-duration nadir or peak concentrations may be diluted and therefore not measured. For this, urine, saliva of serum measurement would be more appropriate. Moreover, hair processing is relatively more labor-intensive for the laboratory employees compared to fluid matrices, as it requires steroid extraction prior to analysis. As hair follicles are part of the skin, one of the target organs of a multitude of hormones, scalp hair hormone concentrations may better reflect concentrations at the target organs compared to e.g. serum or urine concentrations.

Any matrix used for hormone concentrations measurement provides merely a reflection of hormone concentration at the hormone target organs. Additionally, it provides no information on tissue sensitivity, which is dependent on multiple factors such as genetic receptor polymorphisms and receptor expression, nor on hormone metabolism/degradation.[20] Hence interpretation of a single hormone concentration measured in either serum, saliva, urine or scalp hair, should be done with care and in light of the clinical condition of a patient.

Methodology

Steroid hormone quantification methods have long been based on immunosorbent assays (immunoassays). Generally, these assays are based on mice or rabbit derives

hormone specific monoclonal antibodies, coated on to a well. Samples containing the hormone of interest are added, which are bound by the antibodies, after which the binding saturation is quantified using differing techniques, e.g. through adding enzyme-linked antibodies, enzyme-linked hormones (competitive) or by adding radioactive labeled antibodies. Immunosorbent assays are widely applied in medical diagnostic testing, often using automatic sampling robotics. Also for scalp hair cortisol analysis, several immunosorbent assays have been developed and applied with promising results.[4,16,21,22] Immunosorbent assays do have their limits however. Especially in quantification of low concentrations of sex-steroid hormones, immunoassays proved insufficient,[23,24] leading to pleads for alternative methods of steroid quantification with higher accuracy, sensitivity and specificity. The combined technique of liquid chromatography and mass spectrometry, providing the aforementioned requirements, is becoming more readily available for clinical applications such as steroid hormone quantification. Hormones are separated in time first through liquid chromatography which is primarily based on polarity. Subsequently, hormones are selected based on molecular mass to charge ratio. The hormones fulfilling the preselected criteria are then fragmented through ion-collision, after which hormone specific fragments are selected once again (Tandem mass spectrometry) on mass-to-charge ratio and quantified using a mass spectrometer.

Although liquid chromatography tandem mass spectrometry (LC-MS/MS) is generally superior to immunoassays in terms of sensitivity and specificity, both techniques have advantages and disadvantages,[25-27] some of which have been listed in table 9.1.

Immunosorbent assays generally do not require specialized devices, but machinery which is present at every normally equipped medical diagnostics laboratory. The technique needs only basic staff-training and hence can be performed by most laboratory personnel. New assays can be readily implemented, are usually widely (commercially) available and need little to no lab-adjustments. This is in contrast to state-of-the-art LC-MS/MS, which requires specialized personnel and machinery which are costly both in acquisition and in maintenance. This hampers the wide availability of LC-MS/MS based assays, and hence hampers standardization of techniques across multiple centers.[25]

Sensitivity of immunosorbent assays are adequate for a range of serum hormone concentrations which are readily measurable. However, despite a range of assays that have been developed, the technique proves insufficient for reliable quantification of sex-steroid hormone, especially in the lower concentration ranges. As such, the Journal of Clinical Endocrinology and Metabolism announced in 2013 that LC-MS/MS was the preferred method for steroid hormone measurement, and declared to adopt this preference in their manuscript acceptation policy.[23,28]

Monoclonal antibodies are specific for a single epitope. Steroid hormones are structurally closely related (see Chapter 1, Figure 2) and thus show large resemblances, despite large variances in physiological effects. This causes antibodies used in

immunosorbent assays to potentially bind structurally similar steroid hormones, a phenomenon termed cross-reactivity. This may cause falsely elevated hormone concentrations and thus false-positive test results, as shown by Monaghan *et al.*[26] in patients treated with Metyrapone (a drug inhibiting 11-betahydroxylase, thereby preventing the conversion of 11-desoxycortisol to cortisol) measuring falsely elevated cortisol concentrations using an immunoassay, but not when samples were analyzed by LC-MS/MS. Newly developed immunoassays are tested for cross-reactivity against a selection of relevant hormones or hormone metabolites, which is expressed in a percentage reflecting contribution in an equal concentration. However, cross-reactivity for untested metabolites cannot be ruled out. Neither can relevant contribution of an increased concentration of unknown hormone(metabolites) be excluded in positive tests as these concentrations are generally not separately quantified. Due to the multiple separation/selection steps in LC-MS/MS, a high specificity can be assured. Additionally, multiple components can be quantified in a single run, including (inactive) hormone metabolites or synthetic alternatives (e.g. synthetic glucocorticoids such as prednisone or dexamethasone). Furthermore, interference due to unknown components causing false-positive test results can often be discerned during the result interpretation phase. Additionally, the option of measuring multiple components in a single run ultimately reduces costs per analyte, and is a strong advantage over immunoassays. However, not all cross-reactivity may be clinically irrelevant per se, potentially reducing clinical sensitivity in rare cases when using methods with higher specificity.

Table 1. ELISA vs. LC-MS/MS pros and cons

ELISA	LC-MS/MS
+ Low acquisition costs	- High acquisition costs
+ Basic lab technology	- Specialized machinery
+ basic trained lab-personnel	- Specially trained personnel required
- Generally inferior sensitivity	+ High sensitivity
- Cross reactivity	+ High specificity, noticeable interference
- Single component	+ Multiple components in single run

Basic determinants of scalp hair steroid hormones

In chapters two and four, we tested the effects of several potentially confounding factors on scalp-hair hormone measurement. Most determinants of scalp hair steroid hormone concentrations tested in these studies, such as hair washing frequency, hair product use, hair treatment and sweating at the scalp did not consistently or only marginally influence hormone concentrations measured. Darker hair color was associated with higher concentrations of DHEAS and testosterone. In different study-samples cortisol and cortisone concentrations were found to be higher in black hair.[29,30] Hence, hair color

should be taken into account in interpretation of scalp hair hormone concentrations. The exact mechanism has not yet been revealed, but hair melanin concentrations have been suggested to increase the scalp hair binding capacity. Rollins *et al.*[31] assessed codeine concentrations in individuals with hair colors ranging from red to black. They reported higher codeine concentrations in scalp hair with higher melanin concentrations, with up to 15-fold higher codeine concentrations in black hair.[31] These marked differences were not observed in hair cortisol and cortisone concentrations. The largest difference we observed in over 2800 children were mean HCC 1.4 pg/mg in blond children and 2.4 pg/mg in children with black hair. In an adult population, Staufenbiel *et al.*[30] reported a linear regression coefficient of 0.084 for black hair compared to blond hair. The differences in hair codeine concentrations due to hair color are vastly larger than HCC differences. As such, hair melanin concentrations may not be as relevant in hair steroid hormone incorporation. An alternative pathway may be that the higher melanin content of dark hair protects from cortisol decline by UV radiation. In a recent study, Wester *et al.*[32] demonstrated hair cortisol concentrations to be reduced after UV radiation and after exposure to natural sunlight. Hence sunlight exposure might be another variable to be taken into account when assessing hair steroid hormone concentrations. Several studies assessed an array of possible confounding determinants of hair steroid hormone concentrations[19,29,30]. Although several factors have been found to be associated with altered concentrations, the differences are rather limited, as may be their relevance. In large epidemiological studies, it is important to collect data of possible confounders, as these may significantly bias study results. In clinical application of scalp hair steroid hormone measurements, these factors are of less importance as the absolute hormone concentrations differences that discern health from disease are usually much larger than confounder effects. For example, Manenschijn *et al.*[18] described a 14-fold increase in mean HCC in Cushing's syndrome patients compared to healthy controls. Additionally, in chapter 8 we reported a six fold higher 17-OHP concentration in children with congenital adrenal hyperplasia compared to healthy children.[33]

Scalp hair hormone origin

The exact mechanism of cortisol incorporation in the hair shaft has not yet been elucidated. Several theories have been postulated, e.g. through passive or active diffusion from blood, the sebaceous gland or through sweat at the scalp. All mechanisms potentially contribute, although there seems to be a consensus that diffusion from blood and surrounding tissues probably has the largest contribution.

An alternative method of cortisol incorporation in hair is postulated based on research by Ito *et al.*[34] describing the presence of a complete HPA-axis homolog within the hair follicle. According to Ito *et al.* cortisol is produced locally in the hair follicle, thus cortisol measured in scalp hair may potentially originate from local synthesis. Human hair follicles were cultured in $1*10^{-7}$M CRH for a total of four days, after which cortisol and its metabolites were quantified using HPLC. It is unclear why the hair follicles were incubated for multiple days in the presence of CRH or ACTH, as the systemic

HPA-system is a swift-acting negative feedback loop, with cortisol release in a matter of minutes after HPA stimulation. Nevertheless, Ito *et al.* conclude that human hair follicles contain a complete HPA axis including steroid synthesis, secretion and negative feedback. In what proportion this contributes to systemic cortisol concentrations or the scalp hair cortisol content is unclear. The expression of many genes related to steroidogenesis and the HPA-axis in hair follicles has been described, but gene expression profiling in isolated hair follicles does not confirm this unequivocally.[35] A comprehensive study of gene expression in various human tissues, including skin, was published by Dezso *et al.*[36], showing that steroidogenic and HPA-axis related genes are indeed expressed in skin, however this expression reaches levels that are three to six orders of magnitude lower than those in the adrenal gland. We consider it likely that in hair follicles similar observations would be made. For this reason, we think that a serious role for peripheral (skin and hair follicles) HPA and steroidogenesis is doubtful. Additionally, in Addison's disease, cortisol synthesis is impaired due to adrenal destruction through an autoimmune process, in which auto-antibodies against 21-hydroxylase, an obligatory enzyme in cortisol synthesis, often are present and are thought to play a role in pathophysiology.[37] For hair follicles to synthesis cortisol, the enzyme 21-hydroxylase ought to be present. Nevertheless, alopecia due to autoimmune destruction of hair follicles is not associated with Addison's disease. Neither can cutaneous cortisol synthesis prevent an Addison's crisis, despite increased ACTH levels having a marked effect on skin melanocyte stimulation, resulting in the marked skin tanning. Patients suffering from adrenal insufficiency generally receive hormone replacement therapy. This is however inadequate to suppress pituitary ACTH synthesis, as is evident from androgen excess in patients with congenital adrenal hyperplasia.[38] One would expect markedly elevated scalp hair cortisol concentrations if high systemic ACTH concentrations would stimulate hair follicle cortisol synthesis. However, studies reporting on hair cortisol in patients with adrenal insufficiency report cortisol concentrations comparable to healthy controls, with slightly increased concentrations which are thought to be the result of hydrocortisone overdosing rather than local cortisol synthesis.[39,40] Concluding, the clinical relevance of the presence of an HPA-axis homolog within the hair-follicle remains to be established, but seems to be limited in the light of current knowledge.

Long-term glucocorticoids measured in scalp hair is associated with obesity

Chapters 5 and 6 describe a clear association between long-term glucocorticoid concentrations and body composition.[41] Hair cortisol concentrations in the highest quintile is associated with eight-fold increased risk of obesity in six-year old children. The studies described in chapters 5 and 6 are cross-sectional observational association studies. Hence, no inference on causality of increased endogenous glucocorticoid exposure with obesity can be drawn. The potential of glucocorticoids to induce obesity is however well known from glucocorticoid therapeutic side-effects, and Cushing's

syndrome, of which obesity is a hallmark symptom.[42] GCs typically increase abdominal fat mass causing centripetal obesity, which is in line with the association between long-term cortisol concentrations and abdominal fat mass described in chapter 6. The combination of this prerequisite knowledge with the findings of an association of increased long-term endogenous cortisol concentrations with obesity, renders intervention-studies to confirm the obesogenic properties of cortisol obsolete. Further research into the causality and directionality of the associations is however warranted.

One approach to further investigate the GC-obesity association's direction is through applying longitudinal research, measuring long-term cortisol concentrations and body-composition development over time. The Generation R study, a population-based cohort study from fetal life onwards,[43] would be an ideal platform to implement this study, as body-composition is measured throughout childhood, along with a score of potential determinants of mediators of cortisol concentrations and body composition. One of the important advantages of these observational studies in children is that the development of body composition can be tracked, as obesity often finds its origin in childhood, and contributing factors of the onset of obesity may be identified. Base-line cortisol concentrations have been established at the age of six,[29] additional measurements at later ages would expand analytical possibilities and clarify the direction and relevance of the described associations. Additionally, determinants of increased cortisol exposure may be identified.

Ultimate confirmation of the relation between cortisol and adverse body composition could be reached through intervention studies directed at reducing cortisol in obese individuals. Several interventions may apply, probably in combination with a patient oriented, individualized approach. Interventions may have a social focus, e.g. reducing environmental stressors, may focus at psychological or physical determinants such as cognitive behavior therapy, sleeping behavior, and eating behavior, or may directly influence cortisol exposure through e.g. pharmacological interventions targeted at the cortisol-cortisone shuttle enzymes. However, prior to initiation of intervention studies, additional observational research on determinants of increased endogenous cortisol exposure is important to properly focus future research on obesity reduction.

Clinical applications of hair steroid hormone analysis

Glucocorticoid and other steroid hormone analysis in scalp hair may benefit endocrine practice in diagnosis and treatment follow-up of several clinical issues. Scalp hairs properties of slow and steady growth with continuous logging of mean hormone concentrations at the tissue level offers a reliable long-term retrospective overview of hormone exposure.[20] As reported by Manenschijn *et al.* this offers the unique possibility to diagnose cyclic Cushing's syndrome months after the active symptomatic period, but may also aid in diagnosis of Cushing's syndrome or Cushing's disease.[16,18]

In Chapter 7 we have shown cortisol concentrations to be elevated in children with adrenal insufficiency compared to healthy controls, which may in part be attributed

to hydrocortisone over-exposure.[40] Additionally, in chapter 8, we describe scalp hair steroid precursor hormones 17-OH-progesterone and androstenedione to be markedly elevated in children with congenital adrenal hyperplasia.[33] These data suggest that scalp hair analysis may be of value in treatment follow-up of patients with congenital adrenal hyperplasia and perhaps adrenal insufficiency in general. Current treatment follow-up, based on clinical assessment of cortisol over- or under dosing markers and serum or saliva androgen precursor hormone concentrations.[44] Treatment adjustment based on these markers remains a clinical challenge, as clinical symptoms are often slow to develop. Serum and saliva concentrations reflect HPA-axis activity at a single point in time only and is dependent on acute stress, time of medication intake and time of day, hampering interpretation for medication adjustments. Scalp hair sampling is independent of timing on the day or with regard to medication intake, and enables measurement of a long term mean cortisol exposure and androgen steroid precursor synthesis (i.e. HPA-axis activity), providing the clinician with an additional aid in adjusting treatment regimens.

Although the increased scalp hair 17-OHP and androstenedione content in children with CAH and the strong correlation with serum a saliva concentrations are promising, the current study (Chapter 8) does not correlate hair androgen precursor content with clinical end-points.[33] This is however the primary endpoint in clinical practice. Potential focus for future studies could be the association of androgen precursor hormones in scalp hair and its correlation with clinical endpoints and disease control markers such as growth rate and body composition. However, longitudinal observational studies on the association of scalp hair steroids with clinical outcomes could be challenging as the common dose-adjustments in developing children are often small and happen in children with continuous growth and changes in body compensations. These dose changes are perhaps too small to detect significant associated changes in scalp hair hormone concentrations unless studies are performed for considerably long durations in large numbers of children, which would be costly and troubled by the relative rarity of CAH. Nevertheless, these studies would be optimal to ensure reliable implementation of scalp hair analysis on adrenal precursor hormones in clinical treatment monitoring.

Measurement of scalp hair steroid hormones is a non-invasive approach which is unique in providing a reflection of long-term steroid hormone concentrations. Although several determinants of scalp hair steroid concentrations should be taken into account, especially when performing large-scale epidemiological studies, the clinical relevance of these determinants seems limited. Measurement of scalp hair steroid hormones can be of value in multiple endocrine challenges, as appears to be in optimizing treatment of children with congenital adrenal hyperplasia. Also in clinical research scalp hair analysis proves to be a valuable technique, benefitting from the non-invasive sampling, easy storage and measurement of multiple steroid hormones in a single run. Additional research is warranted and bound to be performed as scalp hair analysis is applied more and more.

REFERENCES

1. Kintz P, Cirimele V, Jeanneau T, Ludes B. Identification of testosterone and testosterone esters in human hair. *J Anal Toxicol* 1999; **23**(5): 352-6.
2. Kintz P, Cirimele V, Ludes B. Physiological concentrations of DHEA in human hair. *J Anal Toxicol* 1999; **23**(6): 424-8.
3. Cirimele V, Kintz P, Dumestre V, Goulle JP, Ludes B. Identification of ten corticosteroids in human hair by liquid chromatography-ionspray mass spectrometry. *Forensic science international* 2000; **107**(1-3): 381-8.
4. Sauve B, Koren G, Walsh G, Tokmakejian S, Van Uum SH. Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clin Invest Med* 2007; **30**(5): E183-91.
5. Elmlinger MW, Kuhnel W, Ranke MB. Reference ranges for serum concentrations of lutropin (LH), follitropin (FSH), estradiol (E2), prolactin, progesterone, sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEAS), cortisol and ferritin in neonates, children and young adults. *Clin Chem Lab Med* 2002; **40**(11): 1151-60.
6. Qian X, Droste SK, Gutierrez-Mecinas M, et al. A rapid release of corticosteroid-binding globulin from the liver restrains the glucocorticoid hormone response to acute stress. *Endocrinology* 2011; **152**(10): 3738-48.
7. Mezzullo M, Fanelli F, Fazzini A, et al. Validation of an LC-MS/MS salivary assay for glucocorticoid status assessment: Evaluation of the diurnal fluctuation of cortisol and cortisone and of their association within and between serum and saliva. *J Steroid Biochem Mol Biol* 2016.
8. Granger DA, Cicchetti D, Rogosch FA, Hibel LC, Teisl M, Flores E. Blood contamination in children's saliva: prevalence, stability, and impact on the measurement of salivary cortisol, testosterone, and dehydroepiandrosterone. *Psychoneuroendocrinology* 2007; **32**(6): 724-33.
9. Ingerski LM, Hente EA, Modi AC, Hommel KA. Electronic measurement of medication adherence in pediatric chronic illness: a review of measures. *J Pediatr* 2011; **159**(4): 528-34.
10. Holleman F, Endert E, Prummel MF, van Vessem-Timmermans M, Wiersinga WM, Fliers E. Evaluation of endocrine tests. B: screening for hypercortisolism. *Neth J Med* 2005; **63**(9): 348-53.
11. Kim HJ, Lee JI, Cho YY, et al. Diagnostic accuracy of plasma free metanephrines in a seated position compared with 24-hour urinary metanephrines in the investigation of pheochromocytoma. *Endocr J* 2015; **62**(3): 243-50.
12. Nuttall KL, Pingree SS. The incidence of elevations in urine 5-hydroxyindoleacetic acid. *Ann Clin Lab Sci* 1998; **28**(3): 167-74.
13. Sninsky BC, Nakada SY, Penniston KL. Does socioeconomic status, age, or gender influence appointment attendance and completion of 24-hour urine collections? *Urology* 2015; **85**(3): 568-73.
14. Nieman LK, Biller BM, Findling JW, et al. The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* 2008; **93**(5): 1526-40.
15. Bhake RC, Leendertz JA, Linthorst AC, Lightman SL. Automated 24-hours sampling of subcutaneous tissue free cortisol in humans. *J Med Eng Technol* 2013; **37**(3): 180-4.
16. Manenschijn L, Koper JW, Lamberts SW, van Rossum EF. Evaluation of a method to measure long term cortisol levels. *Steroids* 2011; **76**(10-11): 1032-6.
17. Wester VL, van Rossum EF. Clinical applications of cortisol measurements in hair. *Eur J Endocrinol* 2015; **173**(4): M1-10.
18. Manenschijn L, Koper JW, van den Akker EL, et al. A novel tool in the diagnosis and follow-up of (cyclic) Cushing's syndrome: measurement of long-term cortisol in scalp hair. *J Clin Endocrinol Metab* 2012; **97**(10): E1836-43.
19. Noppe G, Van Rossum EFC, Koper JW, et al. Validation and Reference Ranges of Hair Cortisol Measurement in Healthy Children. *Horm Res Paediat* 2014; **82**(2): 97-102.
20. Wester VL, Lamberts SW, van Rossum EF. Advances in the assessment of cortisol exposure and sensitivity. *Curr Opin Endocrinol Diabetes Obes* 2014; **21**(4): 306-11.
21. Karlen J, Ludvigsson J, Frostell A, Theodorsson E, Faresjo T. Cortisol in hair measured in young adults - a biomarker of major life stressors? *BMC Clin Pathol* 2011; **11**: 12.
22. Kirschbaum C, Tietze A, Skoluda N, Dettenborn L. Hair as a retrospective calendar of cortisol production-Increased cortisol incorporation into hair in the third trimester of pregnancy. *Psychoneuroendocrinology* 2009; **34**(1): 32-7.
23. Rosner W, Vesper H, Endocrine S, et al. Toward excellence in testosterone testing: a consensus statement. *J Clin Endocrinol Metab* 2010; **95**(10): 4542-8.
24. Wartofsky L, Handelsman DJ. Standardization of hormonal assays for the 21st century. *J Clin Endocrinol Metab* 2010; **95**(12): 5141-3.
25. Ankarberg-Lindgren C, Norjavaara E. Sensitive RIA measures testosterone concentrations in

prepubertal and pubertal children comparable to tandem mass spectrometry. *Scand J Clin Lab Invest* 2015; **75**(4): 341-4.

26. Monaghan PJ, Owen LJ, Trainer PJ, Brabant G, Keevil BG, Darby D. Comparison of serum cortisol measurement by immunoassay and liquid chromatography-tandem mass spectrometry in patients receiving the 11beta-hydroxylase inhibitor metyrapone. *Ann Clin Biochem* 2011; **48**(Pt 5): 441-6.
27. Wang C, Shiraishi S, Leung A, et al. Validation of a testosterone and dihydrotestosterone liquid chromatography tandem mass spectrometry assay: Interference and comparison with established methods. *Steroids* 2008; **73**(13): 1345-52.
28. Handelsman DJ, Wartofsky L. Requirement for mass spectrometry sex steroid assays in the Journal of Clinical Endocrinology and Metabolism. *J Clin Endocrinol Metab* 2013; **98**(10): 3971-3.
29. Rippe RC, Noppe G, Windhorst DA, et al. Splitting hair for cortisol? Associations of socio-economic status, ethnicity, hair color, gender and other child characteristics with hair cortisol and cortisone. *Psychoneuroendocrinology* 2016; **66**: 56-64.
30. Staufenbiel SM, Penninx BW, de Rijke YB, van den Akker EL, van Rossum EF. Determinants of hair cortisol and hair cortisone concentrations in adults. *Psychoneuroendocrinology* 2015; **60**: 182-94.
31. Rollins DE, Wilkins DG, Krueger GG, et al. The effect of hair color on the incorporation of codeine into human hair. *J Anal Toxicol* 2003; **27**(8): 545-51.
32. Wester VL, van der Wulp NRP, Koper JW, de Rijke Y, Van Rossum EF. Hair cortisol and cortisone are decreased by natural sunlight. *Psychoneuroendocrinology.* 2016 Oct. **72**:94-6.
33. Noppe G, de Rijke YB, Koper JW, van Rossum EF, van den Akker EL. Scalp hair 17-hydroxyprogesterone and androstenedione as a long-term therapy monitoring tool in congenital adrenal hyperplasia. *Clin Endocrinol (Oxf)* 2016. doi: 10.1111/cen.13078. [Epub ahead of print]
34. Ito N, Ito T, Kromminga A, et al. Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. *FASEB J* 2005; **19**(10): 1332-4.
35. Kim SJ, Dix DJ, Thompson KE, et al. Gene expression in head hair follicles plucked from men and women. *Ann Clin Lab Sci* 2006; **36**(2): 115-26.
36. Dezso Z, Nikolsky Y, Sviridov E, et al. A comprehensive functional analysis of tissue specificity of human gene expression. *BMC Biol* 2008; **6**: 49.
37. Husebye ES, Allolio B, Arlt W, et al. Consensus statement on the diagnosis, treatment and follow-up of patients with primary adrenal insufficiency. *J Intern Med* 2014; **275**(2): 104-15.
38. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Endocr Rev* 2000; **21**(3): 245-91.
39. Gow R, Koren G, Rieder M, Van Uum S. Hair cortisol content in patients with adrenal insufficiency on hydrocortisone replacement therapy. *Clin Endocrinol (Oxf)* 2011; **74**(6): 687-93.
40. Noppe G, van Rossum EFC, Vliegenthart J, Koper JW, van den Akker ELT. Elevated hair cortisol concentrations in children with adrenal insufficiency on hydrocortisone replacement therapy. *Clin Endocrinol* 2014; **81**(6): 820-5.
41. Veldhorst MAB, Noppe G, Jongejan MHTM, et al. Increased Scalp Hair Cortisol Concentrations in Obese Children. *J Clin Endocr Metab* 2014; **99**(1): 285-90.
42. Pivonello R, De Martino MC, Iacuaniello D, et al. Metabolic Alterations and Cardiovascular Outcomes of Cortisol Excess. *Front Horm Res* 2016; **46**: 54-65.
43. Kruithof CJ, Kooijman MN, van Duijn CM, et al. The Generation R Study: Biobank update 2015. *European journal of epidemiology* 2014; **29**(12): 911-27.
44. Speiser PW, Azziz R, Baskin LS, et al. A Summary of the Endocrine Society Clinical Practice Guidelines on Congenital Adrenal Hyperplasia due to Steroid 21-Hydroxylase Deficiency. *Int J Pediatr Endocrinol* 2010; **2010**: 494173.

CHAPTER 10

SUMMARY

SAMENVATTING

SUMMARY

The adrenal glands produce a series of steroid hormones which are important in a large variety of physiological processes and altered hormone concentrations at the target-organ level often results in disease. Measurement of hormone concentrations at the target organs is however complicated, which is why concentrations are measured in surrogate matrices, e.g. serum, saliva and urine. A novel method applies scalp hair as a matrix for long-term glucocorticoid hormone measurement. In the first part of this thesis, reference ranges of steroid hormones in scalp hair of healthy children are established and determinants of scalp hair steroid hormone concentrations are assessed.

As introduced in **chapter 1**, the glucocorticoid hormone cortisol is produced by the adrenals in a circadian rhythm and production is elevated in stress. Cortisol synthesis is under control of the hypothalamus-pituitary-adrenal axis in a negative feedback loop. Increased exposure to glucocorticoids results in the Cushing's syndrome, which shows an overlap in symptoms with the metabolic syndrome. Alternatively, a deficiency in the ability to synthesis cortisol, or adrenal insufficiency, results in a syndrome which is potentially life-threatening without treatment. A subtype of adrenal insufficiency, caused by a genetic defect in one of the cortisol synthesis enzymes, causes congenital adrenal hyperplasia, a syndrome marked by adrenal insufficiency combined with androgen excess. The second part of this thesis is aimed as assessing the relation of endogenous long-term cortisol exposure to obesity in children. Additionally, the value of scalp hair steroid hormone concentrations in children with congenital adrenal hyperplasia is evaluated.

Part I

In **chapter 2**, we established reference ranges for scalp hair cortisol, measured with an enzyme linked immunosorbent assay, in healthy children aged 4 through 14 years old. Hair cortisol concentrations (HCC) increase slightly with age up to 10 years, after which concentrations reach a plateau. HCC were not associated with gender, puberty, nor with hair care characteristics such as hair washing frequency or hair product use.

In **chapter 3**, a liquid-chromatography tandem mass-spectrometry (LC-MS/MS) based method for simultaneous quantification of six steroid (precursor) hormones (cortisol, cortisone, 17-hydroxyprogesterone, androstenedione, dehydroepiandrosterone-sulphate, and testosterone) in scalp hair is presented.

Chapter 4 describes the reference ranges of scalp hair steroid (precursor) hormones in healthy children aged 4 through 18 years old, measured by LC-MS/MS. Hair washing frequency and hair product use did not affect hair steroid hormone concentrations, whereas non-western ethnicity was associated with an increase in hair steroid hormone content and black hair color was associated with an increased concentration of DHEAS and testosterone.

Part II

In **chapter 5**, we compared hair cortisol concentrations in obese children with age- and sex-matched healthy controls. Cortisol concentrations were significantly increased in obese children compared to normal weight children, suggesting increased chronic HPA-activity in obesity.

In **chapter 6**, we found hair cortisol concentrations in a population-based cohort of 6-year old children to be directly associated with increased body mass index and increased total and abdominal fat mass. Increased cortisol was strongly associated with an 8-fold increased risk of obesity. Genetic single nucleotide polymorphisms in the glucocorticoid receptor gene, affecting cortisol sensitivity, were not associated with cortisol concentrations nor with body composition.

In **chapter 7**, we compared hair cortisol concentrations measured by ELISA in children with adrenal insufficiency (AI) treated with hydrocortisone, to healthy controls. We found an increased mean cortisol concentration in AI patients compared with controls, and highest concentrations in the group of patients with congenital adrenal hyperplasia. This is in line with increased hydrocortisone dosing in patients with congenital adrenal hyperplasia to suppress androgen excess. Concluding, hair cortisol may be useful in identifying hydrocortisone overdosing in patients with adrenal insufficiency.

Chapter 8 describes cortisol, 17-hydroxyprogesterone and androstenedione concentrations measured with LC-MS/MS in scalp hair of children with congenital adrenal hyperplasia, adrenal insufficiency without androgen excess, and healthy children. As expected, 17-hydroxyprogesterone and androstenedione were markedly increased in children with congenital adrenal hyperplasia compared to the control groups. Additionally, androgen precursor hormone concentrations in scalp hair showed a good correlation with serum and saliva androgen precursor hormones. This indicates that scalp hair steroid profiling may prove a useful and non-invasive method for treatment monitoring in patients with congenital adrenal hyperplasia.

Chapter 9 contains a general discussion in which the research described in this thesis is reviewed in a broader perspective, and suggestions for future research are proposed.

SAMENVATTING

De bijnieren maken meerdere steroïde hormonen welke van belang zijn voor een breed scala aan fysiologische processen in het lichaam. Een veranderde hormoon concentratie op het niveau van het doelorgaan kan resulteren in ziekte. Het meten van de hormoon concentratie op het niveau van de doelorganen is gecompliceerd en vaak niet mogelijk. In plaats daarvan worden concentraties gemeten in andere matrices, zoals bloed, speeksel en urine. Een recente methode maakt gebruik van hoofdhaar voor de bepaling van lange termijn concentraties van glucocorticoïd hormonen (cortisol). Het eerste deel van dit proefschrift beschrijft de bepaling steroïde hormonen in het hoofdhaar van gezonde kinderen, het vaststellen van normaalwaarden en het evalueren van determinanten van hoofhaar hormoon concentraties.

De introductie, hoofdstuk 1, beschrijft dat het glucocorticoïd hormoon cortisol wordt gesynthetiseerd in de bijnieren in een circadiaans (dag-nacht) ritme, welke onder andere wordt beïnvloed door stress. Cortisol synthese staat onder controle van de hypothalamus-hypofyse-bijnier as (HPA-as) door middel van negatieve terugkoppeling. Verhoogde blootstelling aan cortisol of andere glucocorticoïd hormonen leidt tot het syndroom van Cushing, waarvan de symptomen overlappen met de symptomen van het metabool syndroom. Aan de andere hand leidt een tekort aan cortisol, bijnier insufficiëntie (AI), tot een syndroom welke mogelijk levensbedreigend is zonder adequate behandeling. Een subtype van AI wordt veroorzaakt door een genetische mutatie in een van de enzymen die nodig zijn voor het synthetiseren van cortisol, wat leidt tot het adrenogenitaal syndroom, een syndroom gekarakteriseerd door bijnier insufficiëntie en een overproductie van androgene steroïde hormonen. Het tweede deel van dit proefschrift is gericht op het analyseren van de relatie tussen endogene cortisol blootstelling en obesitas in kinderen. Tevens wordt het bepalen van hoofdhaar steroïde hormonen concentraties in kinderen met adrenogenitaal syndroom geëvalueerd.

Deel I

In **hoofdstuk 2** hebben we normaalwaarden bepaald van hoofdhaar cortisol concentraties (HCC), gemeten met een enzyme linked immunosorbent assay (ELISA), in gezonde kinderen tussen de 4 en 14 jaar oud. Cortisol concentraties nemen langzaam toe met de leeftijd tot 10 jaar, waarna concentraties een plateau bereiken. HCC was niet geassocieerd met geslacht, puberteit, noch met hoofdhaar verzorgingsaspecten zoals de frequentie van haar wassen of het gebruik van haarproducten.

Hoofdstuk 3 beschrijft een liquid chromatography tandem mass spectrometry (LC-MS/MS) methode voor het simultaan kwantificeren van zes steroïde hormonen (cortisol, cortisone, 17-hydroxyprogesterone, androstenedione, dehydroepiandrosterone-sulphate, and testosterone) in hoofdhaar.

In **hoofdstuk 4** beschrijven we de normaalwaarden voor hoofdhaar steroïde (precursor) hormonen gemeten in gezonde kinderen tussen de 4 en 18 jaar oud,

gemeten met LC-MS/MS. Hoofdhaar steroïde hormoon concentraties werden niet beïnvloed door de haarwasfrequentie of het gebruik van haarproducten. Niet-westerse afkomst was wel geassocieerd met een verhoogde concentratie van de meeste hormoon concentraties, en zwart haar was geassocieerd met een verhoogde concentratie DHEAS en testosteron.

Deel II

In **hoofdstuk 5** zijn de hoofdhaar cortisol concentraties van kinderen met obesitas vergeleken met de concentraties kinderen met een normaal gewicht. Dit suggereert een chronische verhoogde activiteit van de HPA-as in obesitas.

In **hoofdstuk 6** hebben we gevonden dat in een populatie gebaseerd cohort van 6-jaar oude kinderen HCC positief geassocieerd is met een hogere body mass index en een hoger totale en abdominale vetmassa. Hogere HCC was geassocieerd met een 8-voudige verhoging van het risico op obesitas. Genetische polymorfisme in het gen voor de glucocorticoïd receptor, welke de cortisol gevoeligheid beïnvloeden, waren niet geassocieerd met HCC noch met de lichaamssamenstelling bij kinderen op de leeftijd van 6 jaar.

In **hoofdstuk 7** is de HCC gemeten met ELISA vergeleken tussen kinderen met bijnierinsufficiëntie met hydrocortison behandeling en gezonde kinderen. De gemiddelde HCC in kinderen met bijnierinsufficiëntie was hoger vergeleken met gezonde controles, en de groep met adrenogenitaal syndroom had gemiddeld de hoogste HCC. Dit komt overeen met de hogere dosering hydrocortison gebruikt ter behandeling van kinderen met adrenogenitaal syndroom om de androgene steroïde hormoonproductie te onderdrukken. Concluderend is HCC wellicht waardevol in het opsporen van hydrocortison overdosering bij kinderen met bijnierinsufficiëntie.

Voor **hoofdstuk 8** hebben we cortisol, 17-hydroxyprogesteron (17-OHP) en androstenedione concentraties gemeten met LC-MS/MS in hoofdhaar van kinderen met adrenogenitaal syndroom, kinderen met bijnierinsufficiëntie zonder androgeen hormoon overproductie, en gezonde kinderen gemeten. In lijn met de verwachtingen waren 17-OHP en androstenedione sterk verhoogd in kinderen met adrenogenitaal syndroom vergeleken met de controle groepen. Daarnaast tonen de 17-OHP en androstenedione concentraties gemeten in hoofdhaar een goede correlatie met concentraties gemeten in serum en speeksel. Dit geeft aan dat het meten van steroïde hormoon-profielen in hoofdhaar waarschijnlijk een bruikbare methode is voor het vervolgen van de behandeling van kinderen met adrenogenitaal syndroom.

Hoofdstuk 9 betreft een algemene discussie waarin het onderzoek beschreven in dit proefschrift bediscussieerd wordt in een breder perspectief een waarin suggesties voor vervolgonderzoek worden gedaan.

APPENDIX

DANKWOORD

CURRICULUM VITAE

PORTFOLIO

PUBLICATION LIST

DANKWOORD

Iedereen die op welke manier dan ook heeft bijgedragen aan het tot stand komen van dit proefschrift wil ik hartelijk bedanken voor zijn of haar bijdrage. Natuurlijk kan ik er niet omheen enkele mensen bij naam te noemen. Aangezien het onmogelijk is hierin compleet te zijn, houd ik het kort.

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CURRICULUM VITAE

1985	geboren in Noordwijk, ZH
1997 - 2003	Atheneum, Northgo College, Noordwijk
2003 – 2012	Studie Geneeskunde, LUMC
2006 – 2007	Penningmeester bestuur Medische Faculteit der Leidse Studenten, LUMC
2007 – 2011	Pre-master Biomedical Sciences, LUMC
2008	Masterclass Epidemiology, dept. of Epidemiology, LUMC
2008 – 2012	Young Excellence Class o.l.v. Prof. Dr. R. Westendorp, Leyden Academy on Vitality and Ageing (LAVA)
2008 – 2012	Master Biomedical Sciences, Health profile, LUMC. *(cum laude)* Junior research project 1: Beta-galactosidase as senescence marker in human fibroblasts, LUMC, o.l.v. Prof. R. Westendorp en dr. A.B. Maier. Junior research project 2: Hair cortisol, a new monitoring tool for congenital adrenal hyperplasia? Erasmus MC, o.l.v. prof. dr. E.F. van Rossum en dr. E.L. van den Akker.
2012 – 2015	Promotie onderzoek 'The Role of the Stress Hormone Cortisol in the Development of Obesity and the Metabolic Syndrome in Children', laboratorium Interne Geneeskunde (sectie endocrinology) en The Generation R study group, o.l.v. prof.dr. E.F. van Rossum en dr. E.L. van den Akker.
2016 – heden	Opleiding tot internist, Erasmus MC Rotterdam en Reinier de Graaf Gasthuis Delft. Opleiders: dr. S.C.E. Klein Nagelvoort Schuit, dr. P.L.A. van Daele en dr. H. Boom.

PORTFOLIO

Name:	Gerard Noppe
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PhD period:	May 2012 – June 2015
Promotor:	Prof. Dr. E.F.C. van Rossum
Co-promotor:	dr. E.L.T. van den Akker

Courses:

2012	Science school 'European Society of Pediatric Endocrinology', Stress and disease.
2012	NIHES Biostatistical methods I. Basic Principles
2012	NIHES Biostatistical methods II. Classical regression models
2013	Molmed course Basic and Translational Endocrinology
2013	MRI and radiation safety course, dept. radiology, Erasmus MC.
2014	Molmed course introduction into R, statistical computing.

Teaching, education and other activities:

2012	Organization weekly seminar dept. of Epidemiology and Biostatistics
2013	Chair PhD-meeting, The Generation R study group.
2014 – 2015	Endocrinology practical lectures on adrenal and thyroid diseases.
2015	Journal club endocrinology, 1st year medical students.

Endocrinology course tutor first year medical students

Conference presentations:

2012	European Society of Pediatric Endocrinology Meeting, poster: **Hair cortisol reflects the supra-physiologic hydrocortisone exposure in children with congenital adrenal hyperplasia**. Leipzig, Germany. Most online visited poster.
2013	Science days department Internal Medicine, poster: **Hair cortisol reflects the supra-physiologic hydrocortisone exposure in children with congenital adrenal hyperplasia**. Antwerp, Belgium.
2013	Endocrine Society Meeting, poster: **Validation of hair cortisol as a measurement for long-term cortisol exposure in healthy children.** San Francisco, USA.
2014	Dutch Endocrine Meeting, oral: **A novel method for long-term steroid profiling in human scalp hair.** Noordwijkerhout, the Netherlands.
2014	Science days department Internal Medicine, Poster: **A novel method for long-term steroid profiling in human scalp hair.** Antwerp, Belgium.

2015 Science days department Internal Medicine, Poster: **Long-term glucocorticoid concentrations as a risk factor for childhood obesity and body fat distribution.** Noordwijkerhout, the Netherlands

2015 Dutch Endocrine Meeting, Oral: **Long-term glucocorticoid concentrations as a risk factor for childhood obesity and body fat distribution.** Noordwijkerhout, the Netherlands

2015 European Society of Endocrinology Meeting, Poster: **Long-term glucocorticoid concentrations as a risk factor for childhood obesity and body fat distribution.** Dublin, Ireland.

PUBLICATION LIST

G. Noppe, P. Dekker, C. de Koning-Treurniet, J. Blom, D. van Heemst, R.W. Dirks, H.J. Tanke, R.G. Westendorp, A.B. Maier. Rapid flow cytometric method for measuring senescence associated beta-galactosidase activity in human fibroblasts; Cytometry A. 2009 Nov;75(11):910-6.

A.Z. Juniarto, **G. Noppe**, N. Maharani, E.L. van den Akker, R. Susanto, S.M. Faradz, F.H. de Jong, S.L. Drop. Salivary 17-hydroxyprogesterone (17-OHP) and androstenedione in monitoring efficacy of treatment among Indonesian patients with Congenital Adrenal Hyperplasia. JAFES, 2012 Oct;27:203-8.

M.G. Groeneveld, H.J. Vermeer, M. Linting, **G. Noppe**, E.F. van Rossum, M.H. van IJzendoorn. Children's hair cortisol as a biomarker of stress at school entry. Stress,2013 Nov;16(6):711-5.

M.A. Veldhorst, **G. Noppe**, M.H. Jongejan, C.B. Kok, S. Mekic, J.W. Koper, E.F. van Rossum, E.L. van den Akker. Increased scalp hair cortisol concentrations in obese children. JCEM, 2014 Jan; 99(1): 285-90.

G. Noppe, E.F. van Rossum, J.W. Koper, L. Manenschijn, G.J. Bruining, Y.B. de Rijke, E.L. van den Akker. Validation and reference ranges of hair cortisol measurement in healthy children. Horm Res Paediatr, 2014 Aug; 82:97-102.

G. Noppe, E.F. van Rossum, J. Vliegenthart, J.W. Koper, E.L. van den Akker. Elevated hair cortisol concentrations in children with adrenal insufficiency on hydrocortisone replacement therapy. Clin Endo, 2014 Dec; 81(6):820-5.

G. Noppe, Y.B. de Rijke, K. Dorst, E.L. van den Akker, E.F. van Rossum. LC-MS/MS based method for long-term steroid profiling in human scalp hair. Clin Endo, 2015 Aug; 83(2):162-6.

J. Vliegenthart, **G. Noppe**, E.F. van Rossum, J.W. Koper, H. Raat, E.L. van den Akker, Socioeconomic status in children is associated with hair cortisol levels as a biological measure of chronic stress. PNEC, 2016 Mar; 65: 9-14.

R. Rippe, **G. Noppe**, D.A. Windhorst, H. Tiemeier, E.F. van Rossum, V.W. Jaddoe, F.C. Verhulst, M.J. Bakermans-Kranenburg, M.H. van IJzendoorn, E.L. van den Akker, Splitting hair for cortisol? Associations of socio-economic status, ethnicity, hair color, gender and other child characteristics with hair cortisol and cortisone. PNEC, 2016 Apr; 66: 56-64.

G. Noppe, Y.B. de Rijke, J.W. Koper, E.F. van Rossum, E.L. van den Akker, Scalp hair 17-hydroxyprogesterone and androstenedione as a long-term therapy monitoring tool in congenital adrenal hyperplasia. Clin Endo, 2016 Apr. DOI 10.1111/cen.13078.

G. Noppe, E.L. van den Akker, Y.B. de Rijke, J.W. Koper, V.W. Jaddoe, E.F. van Rossum, Long-term glucocorticoid concentrations as a risk factor for childhood obesity and adverse body fat distribution. Int J Obes. 2016 Jun. DOI 10.1038/ijo.2016.113.

N. Kieviet, S. de Groot, **G. Noppe**, Y.B. de Rijke, E.F. van Rossum, E.L. van den Akker, K. Dolman, A. Honig, Is poor neonatal adaptation after exposure to antidepressant medication related to fetal cortisol levels? An explorative study. Early Hum Dev. 2016 Jul; 98:37-43.

R. Chen, R.L. Muetzel, H. El Marroun, **G. Noppe**, E.F.C. van Rossum, V.W. Jaddoe, F.C. Verhulst, T. White, F. Fang, H. Tiemeier, No association between hair cortisol or cortisone and brain morphology in children. PNEC, 2016. Accepted for publication.