Cortisol exposure and sensitivity in health and disease

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PhD Thesis, Erasmus University Rotterdam, The Netherlands

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Cortisol Exposure and Sensitivity in Health and Disease

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Chapter 1

Introduction

Parts of this introduction are based on:
Clinical features associated with Glucocoritoid Receptor Polymorphisms: An overview

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1.1 Regulation of Glucocorticoid secretion

Cortisol, the main glucocorticoid (GC) in man, is produced by the adrenal glands and its secretion is under control of the Hypothalamic-Pituitary-Adrenal (HPA) axis. Activation of the HPA-axis begins with the release of corticotrophin-releasing hormone (CRH) from neurons in the paraventricular nucleus (PVN) of the hypothalamus (1). CRH is released in the hypophysial-portal circulation and binds to the type 1 corticotrophin releasing hormone receptors (CRH-R1) on the anterior pituitary corticotroph cells. This stimulates the biosynthesis and secretion of adrenocorticotropic hormone (ACTH) (2). This hormone is produced by cleavage of pro-opiomelanocortin (POMC). ACTH is released in the peripheral circulation and stimulates glucocorticoid production by the zona fasciculate cells of the adrenal cortex, by binding to the type 2 melanocortin receptor (MC2-R). Binding of ACTH to its receptor results in elevated intracellular levels of cyclic AMP (cAMP), which ultimately leads to activation of steroidogenic enzymes, which are required for the cortisol synthesis from cholesterol (3-4). There are three regulatory mechanisms of cortisol secretion, namely 1) the inhibition of ACTH and CRH production by cortisol itself (negative feedback), 2) the circadian rhythm of ACTH and cortisol secretion and 3) the response to stress of the HPA-axis. A simplified version of the HPA-axis is shown in figure 1.

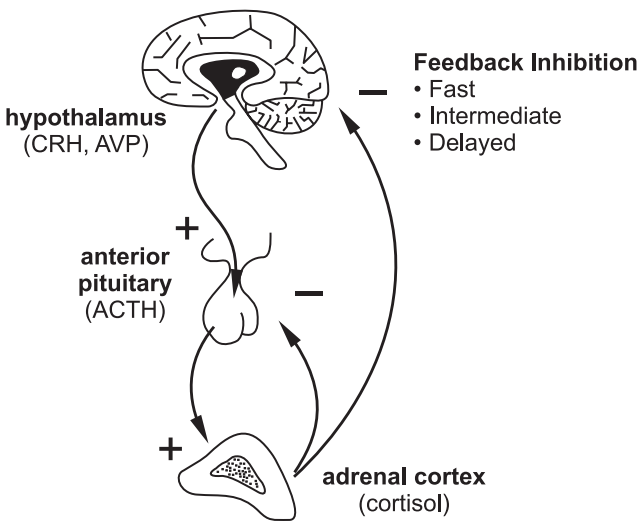

Figure 1. *A simplified overview of the regulation of cortisol by the HPA-axis. Under the influence of the circadian rhythm and stress, the hypothalamus secretes CRH into the hypophysial portal system and thereby stimulates the production of ACTH by the pituitary. In response to increased levels of ACTH the adrenal glands increase the secretion of cortisol. Cortisol inhibits its own production both at the hypothalamic and at the pituitary level, thereby completing a negative feedback loop.*

Negative feedback

The negative feedback action of cortisol is an important regulatory mechanism of the cortisol secretion. Cortisol feedback inhibition occurs at both the pituitary and the hypothalamic level and involves several time domains, namely fast, delayed (or intermediate) and slow feedback (2). The fast feedback or rate-sensitive feedback, is active in seconds to minutes and reads the rate of the GC increase in the plasma following stress-induced rises or administration of exogenous steroids (5). This feedback results in rapid inhibition of ACTH secretion. Rate-sensitive feedback can occur even when high levels of GCs are already present in the plasma, as long as there is a rise in GCs (6). Fast feedback does not require protein synthesis (2). Delayed or intermediate feedback occurs within 30 minutes to hours, affects corticotroph cells in the pituitary as well as hypothalamic cells and is proportional to the total dose of GCs administered (2, 6-8). The slow feedback, reflecting chronic exposure to GCs over days or weeks, affects both basal and stimulated HPA-axis activity and appears to act via the classic glucocorticoid receptor (GR) mechanism (9).

Circadian hypothalamic-pituitary-adrenal activity and pulsatility

Another regulatory mechanism of the HPA-axis is the circadian rhythm. In humans, cortisol levels have a diurnal rhythm, with levels rising steadily after 4 a.m., peaking within 30-45 minutes after wakening and then gradually decreasing during the day (Figure 2). The purpose of this circadian rhythm is helping the body to adjust its activities to the regular periodicity of day/night changes. In addition to the

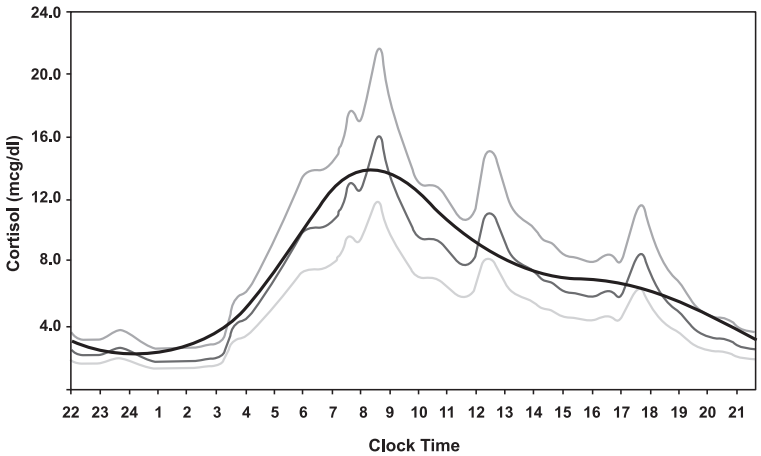

Figure 2. *Circadian rhythm of cortisol secretion. Adapted from Debono et al.(10)*

circadian rhythm, there is an ultradian rhythm (<24 hours), with a pulse of cortisol approximately every 1-2 hours. The frequency of these pulses is constant, however, the amplitude is variable, yielding the production of the circadian rhythm (11-12). One of the factors that is known to influence the circadian rhythm of cortisol secretion is shift work. Several studies have shown that shift work results in a decreased cortisol awakening response and elevated cortisol levels during the evening and night compared to individuals working during the day (13-15). Whether these changes in cortisol secretion result in long-term changes in cortisol levels has not been studied. In this thesis, we determined long-term cortisol levels in scalp hair of shift workers to assess whether shift work results in long-term changes in cortisol.

Stress-induced hypothalamic-pituitary-adrenal activity

The third regulatory mechanism is stress. Physical or psychological stress results in an increased cortisol secretion via an increase in hypothalamic CRH secretion, which results in an increase in ACTH biosynthesis and secretion, ultimately leading towards high circulating cortisol levels. Cortisol, together with catecholamines, causes the 'fight or flight' reaction. This reaction consists of rapid mobilization of energy from storage sites to provide extra fuel to the heart, muscles and brain. Furthermore, it increases blood pressure and cardiac output, which results in rapid transport of nutrients and oxygen to relevant tissues (1). Chronic stress is thought to lead to chronically elevated cortisol levels. This may ultimately lead to a detrimental body composition and an adverse metabolic profile.

1.2 The effects of Glucocorticoids

Cortisol has many effects throughout the body and is involved in numerous processes. The effects of cortisol on metabolism in the fed state are minimal, however, during fasting and stress, cortisol contributes to the maintenance (or increase) of plasma glucose levels. Cortisol influences glucose metabolism in two ways, namely by influencing the hepatic metabolism and affecting extrahepatic tissues such as skeletal muscle and adipose tissue in order to provide the liver with gluconeogenic precursors (16-17). The hepatic metabolism is influenced by increasing the expression of gluconeogenic enzymes, namely phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), the enzymes catalyzing the first and the last steps in the gluconeogenesis pathway (18). The effects of cortisol on skeletal muscle are catabolic, namely decreased glucose uptake, decreased protein synthesis and increased release of amino acids. These effects result in an increase in amino acid substrates for hepatic gluconeogenesis (19). Furthermore, cortisol stimulates lipolysis, resulting in increased fatty acid delivery to the liver, where lipid metabolism provides energy to support gluconeogenesis. With normal levels of cortisol, there is only limited lipolysis due to the direct effects of cortisol and the main role of cortisol is to permit the lipolytic activity of other hormones, such as catecholamines and growth hormone (20-21). However, at moderate to high concentrations, cortisol also

inhibits lipogenesis and reduces the sensitivity of adipose tissue to insulin (22). In addition, cortisol inhibits the glucose uptake in adipose tissue in order to maintain plasma glucose levels.

Other effects of cortisol are the inhibition of fibroblasts, leading to a loss of collagen and connective tissue (23); skeletal loss, due to direct stimulation of bone resorption via RANK-L expression and decreased osteoprotegerin (OPG) production and inhibition of osteoblast maturation and activity (24); inhibition of growth in children (25); suppression of both the innate and the acquired immune system and anti-inflammatory effects (26-28). Because of these anti-inflammatory and immune suppressive effects, GCs are frequently used in the treatment of inflammatory or auto-immune diseases. Furthermore, cortisol can affect renal function, brain function and mood as well as other tissues and organs (9). Long-term exposure to high levels of cortisol lead to changes in body composition, with an increase in visceral fat and muscle atrophy. Together with the changes in insulin sensitivity and lipid metabolism, this results in an increased risk of cardiovascular disease (29). However, whether long-term mildly elevated cortisol levels result in an increased cardiovascular risk is not quite known. In this thesis, we have studied long-term cortisol levels, measured in scalp hair, in older adults and determined whether physiologically higher hair cortisol levels are associated with an increased risk of cardiovascular disease.

1.3 Methods to measure cortisol

Cortisol is secreted from the adrenal cortex into the blood stream. In blood, cortisol is bound with high affinity to cortisol-binding globuline (CBG) (80-90%) and with low affinity to albumin (6-15%). The remainder (4-5%) is unbound and biologically active. There are several methods that can be used to measure cortisol levels (9). An overview of the advantages and disadvantages of the different methods is shown in Table 1.

Serum cortisol

The most common methods to measure serum cortisol levels are radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC) and liquid chromatography mass spectroscopy (LC-MS/MS) (30). A major disadvantage of the measurement of cortisol in serum is that no discrimination can be made between bound cortisol and free cortisol. Factors that increase CBG, such as pregnancy or the use of oral contraceptives, will result in elevated plasma cortisol levels (31). There are methods to assay serum free cortisol, but these methods are complex, time-consuming and expensive and, therefore, not often used. Furthermore, since cortisol is a stress hormone, acute stress, e.g. due to the venipuncture, may result in falsely elevated cortisol levels.
The utility of a single serum cortisol measurement is limited due to the circadian

rhythm and pulsatile secretion of cortisol, but the measurement in serum can provide useful information on the circadian cortisol rhythm. Furthermore, the measurement of midnight serum cortisol can be used as a second line screening test for hypercortisolism (29). However, this does require hospitalization, trained medical staff and painful venipunctures.

Table 1. *Advantages and disadvantages of different methods to measure cortisol levels*

Test	Advantages	Disadvantages
Serum	- One time point - Can be used to evaluate stress responses and circadian rhythmicity	- Invasive - No discrimination of free and bound cortisol - Requires trained personnel - Not representative of long-term cortisol levels - Influenced by acute stress - Influenced by time of the day
Saliva	- Non-invasive - Can be performed at home - Free cortisol - One time point - Can be used to evaluate stress responses and circadian rhythmicity	- Not representative of long-term cortisol levels - Influenced by acute stress - Influenced by time of the day - Influenced by blood contamination, licorice intake and smoking
24 hours urine collection	- Free cortisol - Production of 24 hours - No influence of time of the day - Minor influence of acute stress compared to saliva and serum	- Invasive - Cross-reactions in immune-assays with cortisol metabo lites - Falsely low when renal function is impaired (clearance< 60 ml/min)
Hair	- Non- invasive - Can be performed at home - Free cortisol? - Long-term cortisol levels (weeks to years) - Possibility to make a retrospective timeline of cortisol exposure - No influence of acute stress - No influence of time of the day	- Not possible in bald people and people with very short hair - New method - No measurement of circadian rhythmicity or stress response

Salivary cortisol

Similar to cortisol measurements in serum, salivary cortisol levels can be measured using RIA, ELISA, HPLC and LC-MS/MS (30). Cortisol profiles in serum and saliva are synchronous with equal amplitudes, therefore saliva can represent plasma cortisol levels across the 24h period (32). Salivary cortisol measurements provide advantages over serum cortisol. The sampling technique used is almost completely non-invasive, which avoids the stress-induced rise in cortisol associated with blood sampling. Furthermore, the cortisol concentration in saliva is independent of salivary flow rate and represents the free cortisol fraction (33-35). Therefore, salivary measurements are not influenced by changes in plasma CBG, for example due to oral contraceptive therapy and pregnancy. In contrast to serum cortisol measurements, saliva sampling can be performed at home and saliva samples can be mailed to the laboratory since salivary cortisol concentrations are stable for extended periods (36). Similarly as serum cortisol levels, salivary cortisol levels only represent one time point and can be used to evaluate stress responses and circadian rhythmicity. However, salivary cortisol levels, together with serum cortisol levels, are not very useful in the evaluation of long-term cortisol levels.

Urinary cortisol

One of the recommended first-line screening tests for hypercortisolism is the measurement of cortisol in 24-hours urine collections (29). Cortisol concentrations in 24h urine collections are not influenced by circadian rhythm and are useful in determining the total cortisol excretion of one day. Furthermore, it measures unbound cortisol, therefore this method is not influenced by CBG increasing conditions such as pregnancy and oral contraceptive use. One of the disadvantages of the measurement of cortisol in 24h urine collections is the cross-reaction that can occur when using immunoassays. Many steroid metabolites are excreted in urine as well and cross-reactions of these metabolites in immuno-assays may result in falsely elevated urinary cortisol levels. The effect of cross-reactions can be reduced by pre-treatment of the urine with organic solvent extraction prior to the measurement or by using LC-MS/MS. However, LC-MS/MS is relative expensive and pre-treatment of urine is time consuming and can induce additional variability in measured cortisol levels (37). When measuring cortisol in 24h urine collections, it is important to ensure that patients provide a complete 24h urine collection with appropriate total volume and urinary creatinine levels. Patients should be instructed not to drink excessive amounts of fluids during the collection, because this increases urinary cortisol levels (38). Furthermore, moderate to severe renal impairment can lead to falsely low urinary cortisol levels (39).

Hair cortisol

In the past few years a novel method to measure cortisol in scalp hair has been developed. This is currently the only method that provides the opportunity to measure long-term cortisol levels. The precise mechanism by which cortisol is incorporated

in the hairs is not known, but different incorporation routes have been proposed (40). The most likely route of cortisol incorporation is via passive diffusion from the blood stream into the growing hair cells at the base of the hair follicle. However, there might also be a contribution from cortisol out of sweat or sebum after the hair formation has completed or from external sources such as steroid-containing ointments (Figure 3). Furthermore, it has been proposed that the hair follicle itself displays a functional equivalent of the HPA-axis and is capable of synthesizing and secreting cortisol (41).

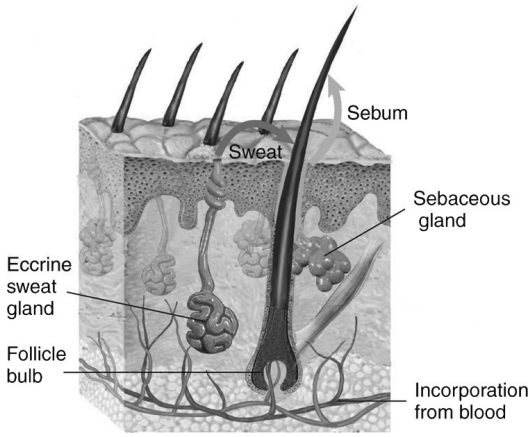

Figure 3. *Different mechanisms of cortisol incorporation into the hair shaft. Adapted from Meyer et al. (50).*

The first report of the measurement of endogenous cortisol in human hair was published in 2004 by Raul et al. (42). In this study, they measured cortisol levels by using HPLC-MS. In 2008 van Uum et al. developed an easier method in which an ELISA was used to measure cortisol levels in human hair (43). In this study, they found that hair cortisol levels and perceived stress were higher in patients with chronic pain. In the following years, several studies have shown that the measurement of cortisol in scalp hair is an easy and non-invasive method to assess long-term cortisol levels in relation to (psychological) stress, alcoholism, pregnancy and psychiatric illnesses (44-48). Furthermore, Thomson et al. have shown in 6 patients with hypercortisolism, that hair can be used to create a retrospective timeline of cortisol exposure (49). In this thesis we evaluated the measurement of cortisol in scalp hair in healthy individuals, described the factors that influence cortisol levels in scalp hair and showed the association of hair cortisol levels with waist-to-hip ratio and the use of hair as a method to measure cortisol exposure in retrospect for the previous months to years.

1.4 Hypercortisolism and hypocortisolism

Chronic cortisol excess leads to several symptoms and physical features known as Cushing's Syndrome (CS). Obesity is one of the most frequently seen manifestations of CS, in particular fat accumulation in the face (moon face), neck (buffalo hump), trunk and abdomen. Patients can also suffer from easy bruisability, hirsutism, menstrual irregularities, acne, hypertension, muscle weakness, osteoporosis, infections and psychiatric symptoms such as anxiety and depression (29, 51). The most common cause of CS is iatrogenic, as a result of chronic glucocorticoid therapy. Endogenous GC excess has an incidence of 2-3 cases per million individuals per year (52-53). In approximately 70% of the cases, CS is caused by a pituitary ACTH-producing adenoma (Cushing's Disease) (54). Other causes of endogenous hypercortisolism are primary adrenal tumors and ectopic ACTH production (55). Standard screening tests for CS are the measurement of cortisol in 24h urine collections, the measurement of cortisol in midnight saliva and the 1 mg overnight dexamethasone suppression test (DST) (29). Urinary and salivary cortisol levels should be measured at least on two occasions, since hypercortisolism can be variable in CS. The standard screening tests represent a timeframe of maximally 24 hours and do not provide historical information. Due to this short timeframe, these tests can be normal, in particular in case of cyclic CS. Cyclic CS is thought to be a rare disorder that is characterized by alternating episodes of endogenous cortisol excess and normal cortisol secretion. The cycles can occur regularly or irregularly with periods of normal cortisol secretion ranging from days to years (56). This makes cyclic CS a diagnosis that is difficult to make and easy to miss. The first-line treatment for CS is to surgically remove the basic lesion that causes hypercortisolism. In case of Cushing's Disease, the pituitary microadenoma will be removed via transsphenoidal surgery. However, when it is not possible to remove the pituitary adenoma or the ectopic ACTH producing tumor, or when hypercortisolism continues after surgery, radiotherapy or medical treatment can be used. There are several drugs used in the treatment of CS, namely steroid synthesis blockers such as ketoconazole and metyrapone, the dopamine agonist cabergoline, somatostatin analogues such as octreotide and pasireotide and the glucocorticoid receptor antagonist mifepristone (57). If hypercortisolism cannot be controlled with medical therapy or surgery, bilateral adrenalectomy can be performed as a definitive cure. However, this does have important implications in terms of lifelong dependence on hormone replacement therapy and the risk of future Addisonian crisis. In this thesis we investigated the usefulness of hair cortisol measurements in the diagnosis of Cushing's Syndrome and also specifically in cyclic CS. Furthermore, we studied whether hair cortisol measurements could be used to evaluate the efficacy of treatment of CS.

Hypocortisolism or adrenal insufficiency can be caused by destruction or dysfunction of the adrenal cortex (primary adrenal insufficiency) or secondary to deficient pituitary ACTH secretion (secondary adrenal insufficiency). Primary adrenal insufficiency, or

Addison's Disease, is rare and has a prevalence of 35-140 per million persons (58-60). The etiology of primary adrenal insufficiency has changed over time. Before 1920, the most common cause of adrenal insufficiency was tuberculosis. Since 1950, 80% of primary adrenal insufficiency is caused by autoimmune adrenalitis with adrenal atrophy (61). Other causes of primary adrenal insufficiency are adrenal hemorrhage or infarction, metastatic disease in both adrenals and various drugs that inhibit cortisol synthesis. Symptoms of chronic adrenal insufficiency are fairly unspecific, namely fatigue, weight loss, nausea and vomiting, hypotension, hyponatremia and hypoglycemia. Acute adrenal crisis is characterized by shock. This occurs most commonly in patients with primary adrenal insufficiency and can be precipitated by medical stress such as an infection. Patients with adrenal insufficiency should be treated with hydrocortisone. A novel method to evaluate disease course and treatment efficacy is the use of hair cortisol measurements to measure cortisol levels in retrospect. A previous study by Gow et al. has shown that hair cortisol measurements can be used to evaluate hydrocortisone replacement in patients with adrenal insufficiency at group level (62). Until now, it was not known whether pathologically low cortisol levels can be detected in retrospect in hair of an individual patient with hypocortisolism. In this thesis, we show hair cortisol levels of a woman who was diagnosed with Addison's Disease and was subsequently treated with hydrocortisone.

One of the drugs causing adrenal insufficiency is mitotane. Mitotane is used in the treatment of adrenocortical cancer and it is a steroidogenesis inhibitor (63). Mitotane affects all adrenocortical zones and therefore it is necessary to replace glucocorticoids and sometimes mineralocorticoids during mitotane treatment. However, mitotane is a strong inducer of hepatic CYP3A4 activity and it increases CBG, which results in an increased cortisol metabolism and a reduced free, biologically active, cortisol (64-67). Since the exact hydrocortisone dose needed is not well predictable, supraphysiological doses are often used in clinical practice to substitute the cortisol deficiency and to attempt prevention of Addisonian crises. The monitoring of hydrocortisone replacement therapy is difficult and relies on clinical symptoms. However, the clinical symptoms of adrenal insuffiency show extensive overlap with side effects of mitotane, which complicates the differentiation between adrenal insufficiency and mitotane induced side effects. The measurement of cortisol in serum is not useful since this reflects total cortisol and not the free fraction. In addition, serum and salivary measurements are also influenced by the time of intake of hydrocortisone. In this thesis we have evaluated the usefulness of hair cortisol measurements in the evaluation of hydrocortisone replacement therapy in adrenocortical cancer patients treated with mitotane.

1.5 Sensitivity to Glucocorticoids

In clinical practice, it is known that the sensitivity to exogenous GCs is extremely variable between patients. Some patients receiving GCs have an excellent response

with respect to their diseases, but suffer from severe side-effects, whereas others treated with similar doses do not respond at all. In vivo sensitivity to GCs can be tested with 0.25 mg DST . This test indeed showed a high variability in the sensitivity to GCs in healthy elderly individuals (68). Interestingly, the intra-individual GC sensitivity is rather stable, suggesting that there is a set point for the sensitivity to dexamethasone with respect to the feedback of the HPA-axis, which might be genetically determined. In general, the majority of the effects of GCs are mediated by the glucocorticoid receptor (GR). The GR gene (NR3C1) is located on chromosome 5 and is a member of the nuclear receptor family. It has a three-domain structure: 1) an amino-terminal transactivating domain, which directs transactivation of target genes, 2) a DNA-binding domain, interacting with glucocorticoid response elements (GRE) in the DNA and 3) a carboxy-terminal ligand-binding domain, which contains specific steroid and heat shock protein binding sites (69). In the unliganded form, the GR is present in the cytoplasm. After binding of cortisol a conformational change occurs, which leads to dissociation of the receptor from a large complex of proteins. This activated, ligand-bound receptor then translocates to the nucleus, where it acts as a transcription factor to regulate gene expression (70). Several main mechanisms of action are known. First, GR can activate gene expression by interacting with specific DNA sequences, the GREs, which are present in the promoter regions of steroid-responsive genes. This mechanism is called transactivation. When binding to negative GREs, transrepression of target genes occurs. The second mechanism of GR action is through interaction with other transcription factors such as activator protein 1 or nuclear factor κB, thereby repressing their transcriptional activity, which results in inhibition of pro-inflammatory transcription factors. A third mechanism involves nongenomic activation. GCs can have rapid effects on inflammation that are not mediated by changes in gene expression. This involves the activation of endothelial nitric oxide synthetase (71).

GR mutations leading to loss of function and generalized GC resistance are rare. Generalized cortisol resistance is characterized by hypercortisolism without Cushingoid features. In such condition, negative feedback on the HPA-axis is reduced due to diminished GC sensitivity, resulting in higher cortisol secretion by the adrenal glands to keep balance between need and production. However, due to increased ACTH levels, adrenal production of mineralocorticoids is also increased, causing the symptoms of hypertension and hypokalemia. In addition, due to the compensatory elevated cortisol levels, the capacity of the cortisol inactivating enzyme 11β-hydroxysteroid dehydrogenase II (11β-HSD II) is exceeded. This results in binding of GCs to the Mineralocorticoid Receptor (MR), which also significantly contributes to the development of hypertension and hypokalemia.

Additionally, as a result of the elevated ACTH levels, adrenal androgen levels are increased, causing -in particularly in women- acne, hirsutism, virilisation, male pattern baldness, menstrual irregularity and infertility (72).

1.6 Polymorphisms of the Glucocorticoid Receptor Gene

A large number of polymorphisms in the GR gene have been identified, dbSNP currently lists 2617. A few of these polymorphisms have been analyzed and are functionally relevant. These polymorphisms have not only been associated with changes in GC sensitivity or altered cortisol levels, but some are also associated with differences in body composition, metabolic parameters, autoimmune diseases and cardiovascular disease. A schematic overview of the GR gene including these polymorphisms is shown in Figure 4. The clinical features of these GR polymorphisms will be discussed briefly. An overview of the clinical features is shown in Table 2.

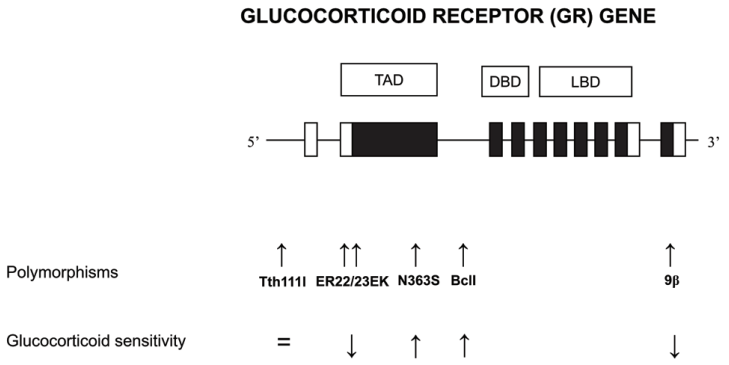

Figure 4. *Schematic overview of the Glucocorticoid Receptor Gene and its polymorphisms.*

The *Tth111*I polymorphism

The *Tth111*I (rs10052957) is a restriction fragment length polymorphism (RFLP), caused by a C to T change 3807 bp upstream of the GR mRNA start site (73). Rosmond et al. showed that this polymorphism is associated with elevated diurnal cortisol levels (74). However, this polymorphism is partially linked to the ER22/23EK polymorphism, with carriers of the latter polymorphism all carrying the *Tth111*I minor allele. In those individuals GC sensitivity appeared to be decreased, as measured by a low dose DST. In contrast, in carriers of the *Tth111*I minor allele without the ER22/23EK polymorphism, GC sensitivity was unaltered (73). Although this polymorphism may not be functional by itself, associations with unipolar and bipolar depression have been described (75-76). Also decreased size of the hippocampus and the amygdale was described in homozygous carriers of the *Tth111*I polymorphism (76). In this thesis we describe a new haplotype in HIV-infected African-Americans in which the *Tth111*I polymorphism is not linked to any of the other polymorphisms. In a group of African-American HIV-infected individuals, we studied whether this new haplotype was associated with metabolic status and body composition.

The ER22/23EK polymorphism

This polymorphism (rs6189 + rs6190) is located in the transactivation domain of the GR gene. In both of two adjacent codons (codon 22 and 23), a single nucleotide polymorphism (SNP) has been identified. The exact sequence alteration at the DNA level is GAG AGG to GAA AAG, which is translated in protein: glutamic acid-arginine (E-R) to glutamic acid-lysine (E-K). Since these SNPs are fully linked and may, due to their close locations, both be relevant for the underlying mechanism of an altered GR effect, these variants often have been studied as a combined polymorphism. It has been found that the ER22/23EK polymorphism is associated with relative GC resistance, as shown by less suppression of cortisol levels after 1 mg of dexamethasone and a reduction of transactivating capacity in peripheral blood mononuclear lymphocytes of heterozygous and homozygous carriers (77-78). The molecular mechanism in which the ER22/23EK polymorphism results in a relative resistance has been studied. In transfection experiments, carriage of the ER22/23EK polymorphism results in higher expression of the GR translational variant GR-A (79).

The GR-A variant results from translation of the GR mRNA from the first AUG codon. The second variant, GR-B, results from translation of the GR mRNA from the second AUG codon. The GR-B is transcriptionally more active than the GR-A protein (80). In ER22/23EK polymorphism carriers, the secondary structure of the GR-B is altered at the mRNA level. This probably results in ribosomal leakage yielding more of the GR-A translation variant. The higher intracellular concentrations of the less active variant of the GR could be (one of) the mechanism(s) underlying the relative GC resistance caused by the ER22/23EK polymorphism (79). In accordance with a relative systemic GC resistance, associations were found between the ER22/23EK polymorphism and lower fasting insulin levels, increased insulin sensitivity and lower total and low-density lipoprotein (LDL)-cholesterol levels (78). No associations with Body Mass Index (BMI) or fat mass were found, but a trend towards a smaller waist circumference was observed in female ER22/23EK-carriers and muscle mass and strength was significantly greater in male carriers (81). Associations between the ER22/23EK polymorphism and several autoimmune or inflammatory diseases were studied as well. A more aggressive disease course in patients with multiple sclerosis was associated with carriage of the ER22/23EK polymorphism (82). However, no associations were found between this polymorphism and Graves' ophthalmopathy and inflammatory bowel diseases (83-85). For rheumatoid arthritis (RA), contrasting studies have been published. Donn et al. described no association between ER22/23EK polymorphism carriage and RA, whereas van Oosten et al. found that carriers of the ER22/23EK polymorphism had a trend towards an increased risk of RA and all ER22/23EK carriers had erosive disease, whereas only 77% of the non-carriers had erosive disease (86-87).

Table 2. *Overview of the effects of the glucocorticoid receptor polymorphisms and their clinical features*

	In vitro			Response to dexamethasone		
	Transactivation	**Transrepression**	**Cortisol levels**	**1 mg**	**0.25 mg**	**Phenotype**
***Tth111*I**	Not tested	Not tested	↑	=	=	- Reduced risk of bipolar disorder - Lower volume of hippocampus and amygdale - Association with depression
ER22/23EK	↓	=	=	↓	=	- Healthy metabolic profile - Beneficial body composition - Longevity - Decreased risk of dementia - Increased risk of depression - More aggressive disease course in multiple sclerosis
N363S	↑	=	=	=	↑	- Increased BMI (in some studies) - Elevated cholesterol levels
***Bcl*I**	Not tested	Not tested	=	↑	↑	- More (abdominal) body fat - Less lean body mass - Increased risk of depression
9β	=	↓	=	=	=/↓	- Increased risk of myocardial infarction - Higher hs-CRP levels - Lower risk of S. aureus nasal carriage - Increased risk of auto-immune diseases, e.g RA and IBD

The N363S polymorphism

The N363S polymorphism (rs6195) is located in codon 363 of the GR-gene (exon 2) and results in an AAT to AGT nucleotide change. This change yields an amino acid alteration from asparagine (N) to serine (S). This polymorphism shows an increased trans-activating capacity in vitro and is associated with increased sensitivity in vivo, as was shown by increased cortisol suppression after a 0.25 mg DST in groups of Dutch elderly individuals and German individuals (77, 88-89). Several studies found that this polymorphism was associated with increased BMI and waist-to-hip ratio (88, 90-94). In addition, associations with higher cholesterol and triglycerides were found (90, 95-96). However, other studies could not confirm the association between the N363S polymorphism and BMI or WHR (97-98). The molecular mechanism concerning increased sensitivity to GCs is unknown. One study showed that there was an increased transactivation of the GC response element luciferase reporter gene by N363S variant GR (77). However, another study could not replicate this finding of increased transactivation (99). In addition, there was no difference between the N363S polymorphism and wildtype GR in the ability to undergo hormone-mediated down regulation. Interestingly, in the absence and presence of the synthetic glucocorticoid dexamethasone, micro-array data showed that the N363S polymorphism can regulate a novel set of genes in comparison to wild-type GR (99). This suggests that the N363S phenotype may be the result of differential effects on gene regulation. However, the exact mechanism and specific genes involved in this mechanism have not been clarified yet.

The *Bcl*I polymorphism

The *Bcl*I polymorphism (rs41423247) was first described as an RFLP consisting of a fragment of 2.3 kb and a fragment of 4.5 kb (100). The nucleotide alteration was identified as a C to G substitution, 646 nucleotides downstream from exon 2, yielding fragments of 2.2 kb and 3.9 kb (101). Carriers of the *Bcl*I polymorphism had lower cortisol levels after 0.25 mg dexamethasone, as well as after 1 mg dexamethasone, suggesting that this polymorphism results in an increased sensitivity to GCs. In line with an increased sensitivity to GCs, several studies have found positive associations between the *Bcl*I polymorphism and (abdominal) obesity (102-104). However, other studies found no association with obesity (95, 105-106). At present, the exact mechanism through which the *Bcl*I polymorphism may alter GC effects is unclear. This polymorphism is intronic and its location does not involve a coding, regulatory or splicing part of the GR gene. It is possible that this polymorphism is in linkage with other variations, e.g. in the promoter region, or linked to other functionally important polymorphisms.

The 9β polymorphism

The 9β polymorphism (rs6198) is an A to G substitution in an 'ATTTA motif' located in the 3' UTR of exon 9β. The 'ATTTA' motif is known to destabilize mRNA and decrease receptor protein expression in vitro. The ATTA-to-GTTTA change appears

to alter mRNA stability as well as protein expression in vitro (107). GR-β is produced by alternative splicing of the GR gene. This β isoform of the GR resides in the nucleus of cells and does not bind GCs or activate GC-responsive genes. It has been reported to function as a dominant negative inhibitor of the active receptor GR-α (108). It has been shown that the 9β polymorphism results in an increased expression and stabilization of the GR-β (107). It is possible that this increased expression and stability of the GR-β variant of the GR leads to a relative GC resistance. Indeed, in vitro it has been shown that transrepression in homozygous carriers of the 9β polymorphism was reduced. Transactivation was not influenced by this polymorphism (109).

1.7 Aims and outline of this thesis

Cortisol has many effects throughout the body and is involved in many processes. Elevated cortisol levels, as seen in patients with Cushing's Syndrome, result in a number of symptoms, including abdominal obesity, dyslipidemia, hypertension and insulin resistance, all leading to an increased cardiovascular risk (29). In addition, psychiatric symptoms such as depression, psychosis or mania can also be present in individuals with Cushing's Syndrome (110). Whether chronic mildly elevated cortisol levels would result in the same symptoms is still not clear. Several studies have investigated cortisol levels in relation to cardiovascular disease and psychiatric disorders, but no conclusive results were found. This might be caused by the way cortisol is measured in these studies. In almost all studies concerning cortisol, saliva or serum is used to measure cortisol levels. A major disadvantage of these methods is that it only represents a single time point and it does not provide information about long-term cortisol levels. Until a few years ago, there was no simple way of measuring long-term cortisol levels, and the only method to obtain long-term cortisol levels was by collecting multiple samples of e.g. urine. With the development of cortisol measurements in scalp hair, it seems possible to measure long-term cortisol levels and create retrospective timelines of cortisol exposure of months to years, depending on the length of the hair.

Although the actual concentration of cortisol in blood is very important for the effects of cortisol, the effect-size is also dependent on the sensitivity of the cells to cortisol. One of the factors that determines an individual's sensitivity to cortisol, is the presence of polymorphisms in the gene encoding the GR. It has been previously shown that some polymorphisms are associated with a relative glucocorticoid resistance, whereas others are associated with a relative glucocorticoid hypersensitivity.

In this thesis we have focused on two determinants of cortisol exposure, namely actual cortisol levels by using a novel method to measure cortisol in scalp hair (part 1) and polymorphisms that are known to alter cortisol sensitivity (part 2). We have used these two different ways of evaluating cortisol exposure in order to study

different aspects and diseases that are or might be related with changes in cortisol exposure. The following research aims were identified:

Part I: Cortisol in scalp hair

- To evaluate and validate the method to measure cortisol in scalp hair in our own group of healthy individuals (Chapter 2).

- To study whether long-term cortisol levels can be used as a diagnostic tool, and reflect clinical course in patients with Cushing's Syndrome and cyclic Cushing's Syndrome in order to find an easier and less invasive method to diagnose this syndrome (Chapter 3).

- To investigate whether hair cortisol measurements could be used to evaluate hydrocortisone replacement therapy in mitotane treated patients with adrenocortical cancer (Chapter 4).

- To study whether long-term cortisol levels in scalp hair are associated with S. aureus nasal carriage (Chapter 5). Since cortisol is known to suppress the immune system and carriage of the 9β polymorphism, which has been associated with a relative glucocorticoid resistance, results in less S. aureus nasal carriage, we hypothesized that higher long-term cortisol levels are associated with increased frequency of S. aureus nasal carriage.

- To study one of the factors that is known to influence the circadian rhythm of cortisol secretion, namely shift work, in order to investigate whether a disturbance in circadian rhythm of cortisol secretion results in long-term changes in cortisol levels (Chapter 6 and 7).

- To study whether physiologically higher cortisol levels are associated with an increased cardiovascular risk in a group of elderly individuals (Chapter 8). Since pathologically elevated cortisol levels result in an increased cardiovascular risk, we hypothesized that long-term mildly elevated cortisol levels are associated with an increased cardiovascular risk as well.

- To study whether long-term cortisol levels are altered in patients with psychiatric diseases, such as bipolar disorder (Chapter 9). Both in patients with hypercortisolism as in patients with hypocortisolism, psychiatric illnesses such as depression, mania, anxiety and psychosis, are frequently seen. We hypothesized that long-term cortisol levels are altered in patients with psychiatric illnesses.

Part II: The role of glucocorticoid receptor polymorphisms in cardiovascular risk and aging

- To study whether certain polymorphisms in the GR are more frequent in children born small for gestational age (SGA) and whether GR polymorphisms are associated with body composition and metabolic features in these children (Chapter 10). Children born small for gestational age are known to have an increased risk to develop metabolic disorders and cardiovascular disease at adult age. It has been suggested that fetal programming of the HPA-axis is one of the mechanisms resulting in this increased risk. We hypothesized that carriage of GR polymorphisms might be involved in fetal growth retardation resulting in being born small for gestational age and that these polymorphisms are associated with metabolic and body composition parameters in this group.

- To study whether GR polymorphisms are associated with body composition and metabolic features in HIV-infected patients (Chapter 11). HIV infection has been associated with features that resemble Cushing's syndrome. Therefore polymorphisms that result in an increased cortisol sensitivity might play a role in the development of these symptoms in HIV-infected patients.

- To study the gene-environment interaction of the GR polymorphisms with prenatal famine exposure in participants who were exposed to famine in utero during the Dutch Famine in World War II (Chapter 12). Maternal malnutrition results in detrimental effects in adult offspring which, in mice models, can be prevented by inhibition of cortisol synthesis. We hypothesized that carriage of GR polymorphisms will result in differences in body composition and metabolic parameters in adults that were prenatally exposed to famine.

- To study whether changes in cortisol sensitivity due to GR polymorphisms are associated with changes in body composition at older age (Chapter 13). It is known that high cortisol levels lead to an increase in visceral fat and a decrease in muscle mass. We studied whether GR polymorphisms are associated with changes in fat and muscle mass at older age.

- To study whether carriage of one of the GR polymorphisms would be protective or detrimental for developing a delirium at older age (Chapter 14). Hyperactivity of the HPA-axis and impairment of the negative feedback system have been suggested to be involved in the development of delirium at older age. We studied whether GR polymorphisms might be protective or detrimental for the development of delirium at older age.

In the general discussion (Chapter 15), the results of the various studies, together with the advantages and disadvantages of the hair cortisol measurements and polymorphism studies will be discussed.

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Part I:

Cortisol in scalp hair

Chapter 2

Evaluation of a method to measure long-term cortisol levels

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Abstract

Background

Elevated levels of cortisol are known to induce various symptoms and diseases, e.g. abdominal obesity, type 2 diabetes, osteoporosis and cardiovascular disease. Measuring serum, saliva and urine cortisol is limited to one time point. Measurement of cortisol in scalp hair is a recently developed method to measure long term cortisol levels. The aim of this study was to investigate whether hair cortisol is a feasible parameter to measure cortisol exposure.

Methods

We collected hair samples of 195 healthy individuals, 9 hypercortisolemic and one hypocortisolemic patient and measured hair cortisol levels. Cortisol was extracted from scalp hair using methanol and cortisol levels were measured using a salivary ELISA kit. Measurement of waist and hip circumferences and blood pressure was performed in 46 healthy subjects.

Results

We found a positive correlation between hair cortisol and both waist circumference ($r=0.392$, $p=0.007$) and waist-to-hip ratio (WHR) ($r=0.425$, $p=0.003$). No correlations were found between hair cortisol levels and BMI, blood pressure or age. There was no decline in cortisol levels in six consecutive hair segments. Hair cortisol levels were elevated in patients with known hypercortisolism ($p<0.0001$).

Conclusions

Hair cortisol was positively correlated with WHR, suggesting that hair cortisol reflects cortisol exposure at tissue level, which was also supported by elevated hair cortisol levels in hypercortisolemic patients and concordance between hair cortisol levels and clinical disease course. Cortisol levels in hair are slightly influenced by hair treatment but not by natural hair colour, use of hair products, gender or age.

Introduction

The stress hormone cortisol has a wide spectrum of physiological effects throughout the human body and is involved in glucose and lipid metabolism, body composition, immunosuppressive and anti-inflammatory actions (1). The effects of long term exposure to elevated cortisol levels comprise increased visceral fat mass, redistribution of body fat with accumulation of adipose tissue on abdomen and trunk and muscle atrophy. In addition, high levels of cortisol induce hypertension, insulin resistance and dyslipidemia, leading to an increased cardiovascular risk as is seen in Cushing's syndrome, which is a state of cortisol excess, or in use of high doses of exogenous glucocorticoids (2-3).

Until now, most studies addressed the relationship between total serum cortisol and symptoms or disease. Conflicting data have been published and the exact relationship between cortisol-mediated effects and symptoms or diseases remains to be established. Likely explanations for these conflicting data are the circadian rhythm, the pulsatile secretion of cortisol and the daily variation due to circumstances (e.g. stress or infection). This complicates the use of serum, saliva and urine cortisol in epidemiological studies. Recently, several studies have shown that endogenous cortisol can reliably be measured in scalp hair (4-10). Scalp hair grows with an average rate of one centimeter (cm) per month, therefore one cm of scalp hair could represent cortisol levels of one month. This suggests that hair cortisol can be used as a method to retrospectively measure cortisol exposure over the past weeks or months. This offers new potential ways to study the effects of e.g. chronic stress, which is thought to be accompanied by a hyperactive hypothalamic-pituitary-adrenal (HPA) axis. This has already been evaluated in several studies. In chronic pain patients, cortisol levels were elevated compared to healthy persons (9) and in healthy pregnant women hair cortisol levels were positively correlated with Perceived Stress Scale (PSS) scores (5). Furthermore, unemployment, which is a chronic stressor, was also associated with increased cortisol levels in hair (11). Recently, Pereg et al. showed elevated hair cortisol levels in patients with acute myocardial infarction (12).

Although the mechanism of cortisol incorporation into hair is not fully understood, measurement of cortisol levels in scalp hair is a very promising technique. Until now relatively small study populations were used and therefore replication is needed to evaluate the use of hair cortisol as a marker of long term endogenous cortisol levels. Hence, we determined cortisol levels in scalp hair of healthy individuals and patients with hyper- and hypocortisolism. The aim of this study was to investigate whether hair cortisol is a feasible parameter to measure cortisol exposure. We studied this by measuring cortisol levels in scalp hair of healthy individuals, patients with hypercortisolism and one patient with hypocortisolism. Factors that could influence hair cortisol concentrations were determined and we studied whether hair cortisol concentrations were associated with cortisol specific tissue effects such as body composition.

Table 1. *Baseline characteristics of healthy individuals.*

	Total group (n=195)	Men (n=90)	Women (n=105)
Age (years)	36 (18-63)	38(19-63)	35 (18-61)
Caucasian ethnicity	174 (90.6)	84 (94.4)	90 (87.4)
Natural hair colour			
Black	10 (5.2)	5 (5.6)	5 (4.8)
Brown	75 (38.7)	32 (36.0)	43 (41.0)
Blond	94 (48.5)	45 (50.6)	49 (46.7)
Red	6 (3.1)	1 (1.1)	5 (4.8)
Grey	9 (4.6)	6 (6.7)	3 (2.9)
Frequency of hair wash (per week)			
≤2 times per week	50 (25.9)	22 (24.7)	28 (26.9)
≥3 times per week	143 (74.1)	67 (75.3)	76 (73.1)
Use of hair products^			
No	104 (53.6)	42 (46.7)	62 (59.6)
Yes	90 (46.4)	48 (53.3)	42 (40.4)
Hair treatment^^			
No	152 (77.9)	90 (100.0)	62 (59.0)**
Yes	43 (22.1)	0 (0.0)	43 (41.0)
Body mass index (kg/m²)	24.1 (23.5-24.7)	24.6 (23.9-25.4)	23.5 (22.7-24.4)
Waist circumference (cm)	88.1 (83.7-92.6)	95.2 (90.8-99.7)	79.7 (72.8-86.5)**
Hip circumference (cm)	96.6 (92.7-99.5)	97.0 (94.4-99.7)	95.2 (89.9-100.8)
Waist-to-hip ratio	0.91 (0.88-0.94)	0.98 (0.95-1.00)	0.83 (0.80-0.85)**
Systolic blood pressure (mmHg)	135 (129-140)	140 (133-147)	128 (120-136)*
Diastolic blood pressure (mmHg)	82 (78-86)	83 (78.5-88.5)	80 (75-86)

Data shown as mean (95%CI) or number (%). Age is shown as mean (minimum-maximum).
** p 0.05, indicating significant differences between men and women*
*** p<0.01, indicating significant differences between men and women*
^ hair products concern spray, mousse, gel and wax
^^ hair treatment concerns dyeing, bleaching and permanent waving or straightening.
Waist circumference, hip circumference, waist-to-hip ratio and systolic and diastolic blood pressure are measured in the subgroup of 46 subjects.

Methods

Study population

In total 195 healthy individuals who did not use glucocorticoids participated in this study. All participants had to fill out a questionnaire concerning hair and medical conditions. In the initial group of 149 healthy individuals hair cortisol levels were determined as well as height and weight. This group was extended by an additional 46 persons, in whom also waist and hip circumferences and blood pressure were measured. BMI was calculated as kg/m2. To validate the measurement of cortisol in scalp hair, we also collected hair samples from ten adult patients, nine with hypercortisolism and one with hypocortisolism. Diagnosis of hypercortisolism was made if 24-hours urinary cortisol levels > 850 nmol/L. Hypocortisolism was diagnosed as serum cortisol levels below 200 nmol/L. Approval was given by the local Medical Ethics Committee and all participants gave written informed consent.

Hair collection

Around 100 strands of hair were collected from the posterior vertex of the scalp and were cut off as close to the scalp as possible. The hair was taped to a piece of paper and the scalp end was marked. The samples were stored in an envelope at room temperature until analysis.

Hair preparation

Hair samples were prepared as described previously by Sauvé et al. (7). A minimum of 10 mg of hair was weighed. In all participants, the three cm closest to the scalp end were used. In 28 women, in addition to the most proximal three cm, the remaining hair strands were divided in 5 segments of 3 cm, which resulted in analyses of 18 cm long hair, divided in six consecutive 3 cm segments. The different hair segments were put into separate glass vials and cut into small pieces, using small surgical scissors. 1 mL of methanol was added and the vial was sealed and incubated overnight for 16 hours at 52°C while gently shaking. After incubation, the methanol was removed, put into disposable glass tubes and was evaporated under constant stream of nitrogen. The samples were dissolved in 250 µL Phosphate Buffered Saline (pH 8.0) and samples were vortexed for one minute. Before analysis, the samples were vortexed again for 30 seconds.

Hair analysis

Cortisol levels were measured using a commercially available ELISA Kit for Salivary Cortisol (DRG International Inc, USA) as per manufacturer's directions with the reagents provided. Cross reactivity of other steroids with the kit's antibodies was reported as follows: Corticosterone (29.00%), Cortisone (3.00%), 11-Deoxycortisol (<1.00%), 17-OH Progesterone (<0.50%), other hormones (<0.10%). Intra-assay variation was below 5% and the inter-assay variation below 8% as stated by the manufacturer.

ELISA recovery

To validate the ELISA we created cortisol standards in PBS with concentrations of 5, 10, 20, 40, 80 and 160 nmol/L and measured the recovery in duplicate. We also spiked two hair samples with 20 nmol/L hydrocortisone, to measure recovery when hydrocortisone was dissolved in hair extract.

Statistical analysis

Statistical analyses were performed using SPSS 16.0 for Windows (SPSS Inc, Chicago, IL, USA). Differences in baseline characteristics were tested using Chi-square tests, Mann-Whitney U-tests and ANOVA. Variables that were not normally distributed were logarithmically transformed. The effects of the frequency of hair washing, natural hair colour, hair treatment and the use of hair products on cortisol levels were analyzed using univariate analysis of variance (UNIANOVA). Differences in hair cortisol levels between healthy individuals and patients with known hypercortisolism were analyzed with a Mann-Whitney U-test. Correlations of hair cortisol with blood pressure, BMI, waist and hip circumference and waist-to-hip ratio (WHR) were analyzed using Pearson's correlations. An ANOVA for repeated measurements was used for testing segment differences in healthy individuals with long hair.

Results

Assay recovery

The recovery of 5, 10, 20, 40, 80 and 160 nmol/L cortisol standards from PBS was 122.0%, 95.0%, 86.5%, 85.8%, 90.5% and 89.1% respectively. When hydrocortisone was added to hair extracts, the mean recovery was 84.5%.

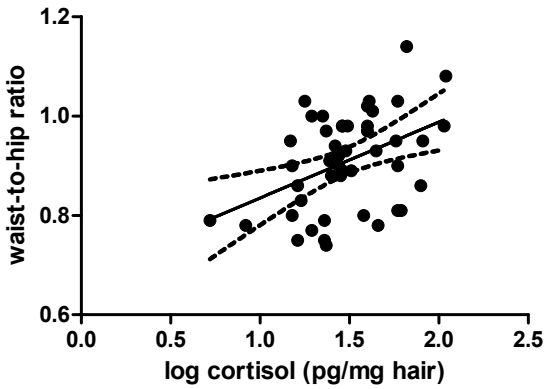

Figure 1. *Correlation of hair cortisol levels with waist-to-hip ratio. r=0.425, p=0.003. The line of best fit and its 95% Confidence Interval are shown.*

Healthy controls

Baseline characteristics of all healthy individuals are shown in Table 1. We found a significantly higher number of subjects who treated (dyed, bleached or permanent waved/straightened) their hair in women compared to men. Waist circumference, WHR and systolic blood pressure were all significantly lower in women. In the total group hair cortisol levels were normally distributed after log transformation (Kolmogorov-Smirnov p=0.200).

Interestingly, we found positive correlations between cortisol in hair and waist circumference (r=0.392, p=0.007) and WHR (r=0.425, p=0.003) (Figure 1). We found no correlation with BMI (p=0.646), hip circumference (p=0.096) or systolic and diastolic blood pressure (p=0.109 and p=0.365 respectively). In addition, we found no correlation between hair cortisol levels and age (p=0.388).

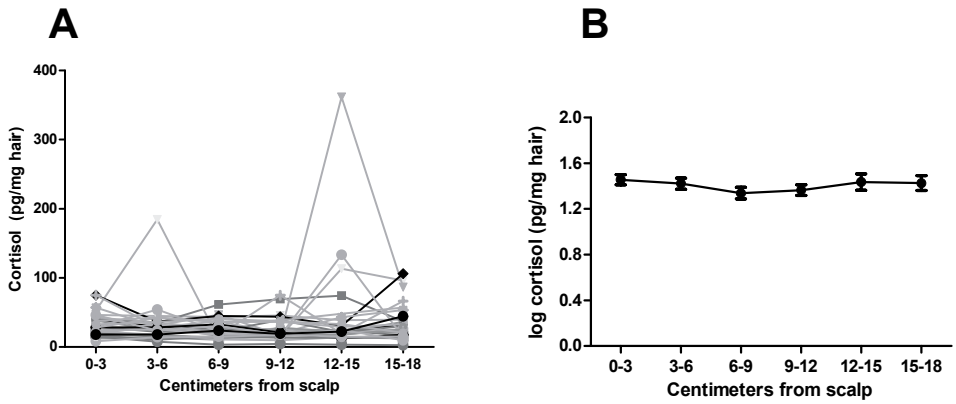

Figure 2. *Cortisol levels in six consecutive three cm segments of hair in 28 women. (A) Individual cortisol levels. Each line represents hair cortisol levels of an individual. The highest peak in this graph corresponds with a stressful period of final graduation exams. The peak in the line with grey round dots represents a stressful periof of graduation and a displeasing job. (B) Mean cortisol levels of 28 women in six consecutive three cm segments of hair. The first segment represents the segment closest to the scalp. There are no differences in cortisol levels between the different segments (p=0.249).*

In the total group cortisol levels in hair that was treated (dyed, bleached, permanent waved/straightened) were borderline significantly lower than in untreated hair (24.27 pg/mg hair versus 29.38 pg/mg hair, p= 0.051). Since there were only female participants in the group with treated hair, we compared mean cortisol levels between women with treated hair and women with untreated hair. We still found lower cortisol levels in treated hair (24.27 versus 29.44 pg/mg hair), although this was not statistically significant (p=0.079). The use of hair products, such as hair spray, mousse, gel and wax, on the day of hair sample collection was not significantly

associated with altered cortisol levels after adjusting for age, gender and hair treatment (24.83 pg/mg hair versus 28.44 pg/mg hair in hair with no hair products, p=0.109). No significant effects of frequency of hair washing (p=0.673) or gender (p=0.353) were observed. There was also no difference in hair cortisol levels between different hair colours (p=0.413). In 28 healthy women with long hair, hair strands were cut into six consecutive segments of 3 cm. Cortisol levels were determined in these segments. Cortisol levels were not statistically different in the consecutive segments (p=0.249) (Figure 2A and 2B).

Patients with hypercortisolism and hypocortisolism

Cortisol levels in scalp hair of patients with known hypercortisolism were significantly elevated compared to healthy individuals (Figure 3). In one patient with known Cushing's Disease (CD) and one patient with Addison's Disease (AD), we determined cortisol levels in consecutive hair segments, using the total length of the hair strands, to see whether cortisol in scalp hair reflected clinical course.

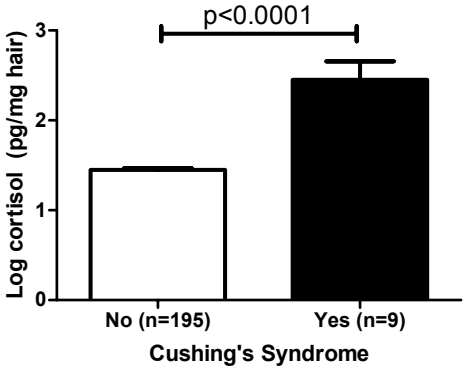

Figure 3. *Cortisol levels in healthy individuals and patients with hypercortisolism.*

The first patient is a 45 year old woman who was admitted to the hospital in September 2009 to confirm CD. She presented with symptoms of muscle weakness in upper arms and legs and weight gain. On examination she had hypertension, fat accumulation in the cheeks, the dorsocervical region and abdomen. Laboratory test revealed hypercortisolism with increased 24-hours UFC levels of 1951 and 1473 nmol/D. After 1 mg of dexamethasone, serum cortisol was insufficiently suppressed to 469 nmol/L. Magnestic Resonance Imaging (MRI) showed a pituitary microadenoma, and sinus petrosus sampling confirmed a pituitary source of ACTH overproduction. She started ketoconazole therapy in October 2009. At the end of February 2010, she underwent transsphenoidal resection of a ACTH-producing adenoma. Afterwards, she started hydrocortisone replacement therapy. Her hair was collected in August 2010. We divided the proximal hair segments in one cm

segments and the most distal segments in 2 cm segments (to have a minimal weight of 10 mg hair). Assuming one cm of hair corresponds with a period of one month, hair samples showed that cortisol levels were already elevated in December 2008. After the start of ketoconazole, cortisol levels decreased and after surgery, cortisol levels decreased further (Figure 4A).

The second patient is a 55 year old woman who had complaints of fatigue and constipation and was diagnosed with hypothyroidism in October 2008. After thyroid hormone replacement therapy, symptoms decreased and the patient started to feel better. However, since February 2009, her clinical state worsened. She suffered from slow movement, cold intolerance, muscle weakness and weight loss. At examination, she was hyperpigmented and had atrophic muscles. Her morning serum cortisol level was 44 nmol/L with an increased ACTH level of 289 pmol/L. Autoantibodies against the adrenals and thyroid were found and she was diagnosed with AD as part of the polyglandular syndrome type II. Her hair cortisol levels were within the normal range in the periode of November 2008- January 2009 (27.67 pg/mg hair). However, cortisol levels decreased to 12.55 pg/mg hair in July 2009. In August hydrocortisone replacement treatment was started, which resulted in higher levels of hydrocortisone (62.14 pg/mg hair) measured in the hair segment corresponding to the months of hydrocortisone use (Figure 4B).

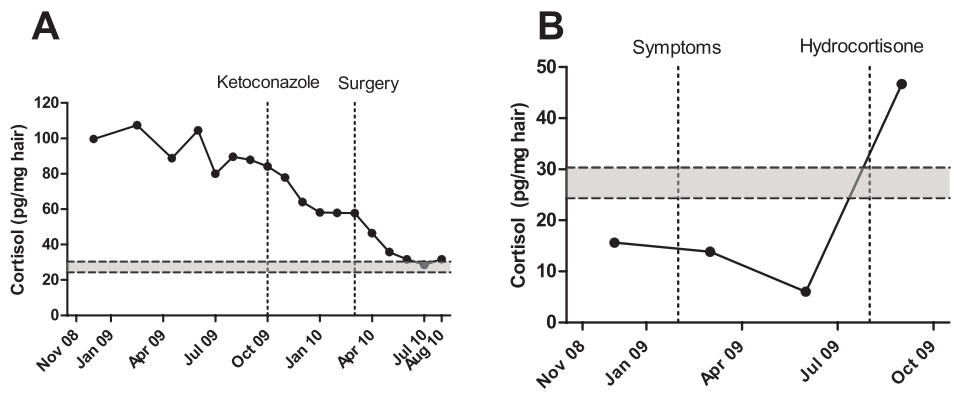

Figure 4. *Retrospective timeline of hair cortisol levels in a patient with hypercortisolism and a patient with hypocortisolism. The shaded bar reflects the 95% Confidence Interval of the mean hair cortisol levels in healthy women. (A) Patient with hypercortisolism. The patient started ketoconazole therapy in October 2009 and underwent transsphenoidal resection of the pituitary adenoma at the end of February 2010. (B) Patient with hypocortisolism. Symptoms started in February 2009 and in August 2009 hyodrocortisone replacement therapy was started.*

Discussion

The focus of this study was to evaluate whether cortisol in hair is a reliable long term cortisol parameter. One of the main findings of this study is the positive correlation between cortisol in hair and both waist circumference and WHR in healthy individuals. There was no correlation between hair cortisol and hip circumference or BMI. This observation is important, since one of the typical effects of cortisol is the accumulation of abdominal fat. BMI is a measure of body weight based on a person's weight and height and is an indicator of general obesity, but not specific for abdominal fat. Waist circumference and WHR are more specific measures for abdominal fat and therefore better indicators of cortisol exposure. The positive correlation between cortisol in hair and WHR indicates that hair cortisol reflects the effects of cortisol at the tissue level. To our knowledge, no other studies measured indicators of body composition in relation to hair cortisol levels.

We found no effect of gender and age on hair cortisol levels, which is in line with other studies (8, 13-14). There were also no differences in cortisol levels between different natural hair colours. In other studies, no effects of natural hair colour or melanin concentration on cortisol levels were found (7, 13, 15). Hair treatment seemed to be associated with slightly lower cortisol levels in hair, although this was not statistically significant. However, Sauvé et al., reported lower cortisol levels in treated hair (7). The mechanism in which dyeing or bleaching could influence cortisol levels in hair is not clear. Since most dye and bleach products penetrate through the hair matrix, there could be a direct effect of these products on incorporated cortisol. Furthermore, hair treatment could increase hair mass, which could result in decreased hair cortisol concentrations expressed per mg of hair.

Moreover, we found that cortisol levels in 18 cm long hair strands showed variation over time, but no overall decline. Our data support the finding of Thomson et al (8), where hair cortisol levels remained stable in 18 cm long hair strands of 8 healthy women. In addition, Davenport et al. showed that in rhesus macaques cortisol levels in the proximal segment were similar to the concentrations in the distal segment (16). For other steroid hormones, such as testosterone, progesterone and estradiol, no decline in hormone levels in 20-45 cm long hair strands was found either (17). In contrast, Kirschbaum et al. described a wash out effect of cortisol in healthy women (15). From the same research group, another study addressing cortisol levels in hair of unemployed individuals, showed a wash out effect of cortisol levels in the third three cm segment of hair (11). Also Gao et al. found a decrease in cortisol levels in the more distal hair segments measured with HPLC-FLU (13). A possible explanation for these contrary findings is the difference in sample preparation between the studies. In all studies that described a wash out effect, hair samples were washed with isopropanol or methanol before preparation (11, 15). In the study performed by Thomson et al. and in our study, the hair samples were not washed (8). Hair

segments more distal of the scalp, and therefore the oldest hair segments, are more exposed to environmental factors than the more proximal hair segments. It could be speculated that the older hair segments are more damaged due to UV irradiation, cosmetic products and frequent washing. Cortisol could escape from the more damaged distal hair segments, especially during the washing procedures. The effect of washing procedures on cortisol levels in hair was only tested in proximal hair segments (16) which gives no guarantee that washing does not affect cortisol levels in more distal hair segments. However, additional studies are needed to determine whether a wash out effect of cortisol in hair is present and whether this is influenced by the washing procedures prior to the sample preparation.

We also showed that hair cortisol was elevated in patients with known hypercortisolism and that hair cortisol corresponded with clinical course in a patient with CD and a patient with AD, which is also shown by Thomson et al. (8). This suggests that hair cortisol is indeed a feasible parameter of cortisol exposure and that it could give retrospective information about disease course. This method could also be very helpful to diagnose cyclic Cushing's syndrome, which is characterized by periodic hypersecretion of cortisol and a Cushingoid appearance and is often not recognized since standard biochemical tests can show normal cortisol concentrations between episodes of hypercortisolism.

In conclusion, we found a positive correlation between cortisol levels in hair and WHR, suggesting that cortisol in hair reflects cortisol exposure at the tissue level. This is also supported by elevated hair cortisol levels in hypercortisolemic patients and the concordance of hair cortisol levels with clinical course. Furthermore, we found that cortisol levels in hair might be slightly influenced by hair treatment, but not by other factors such as natural hair colour, use of hair products, gender and age.

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Chapter 3

A novel tool in the diagnosis and follow-up of (cyclic) Cushing's Syndrome: Measurement of long-term cortisol in scalp hair

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Journal of Clinical Endocrinology and Metabolism, 2012 Oct; 97 (10): E1836-1843

Abstract

Background

Measurement of cortisol in 24h urine collections and midnight saliva are standard screening tests for Cushing's Syndrome (CS). These tests reflect cortisol levels during a maximum of 24h and do not provide historical information. Therefore, they can yield normal results in case of cyclic CS, which is a rare disorder that is characterized by alternating episodes of endogenous cortisol excess and normal cortisol secretion. The measurement of cortisol in scalp hair is a novel tool that might be helpful to establish the diagnosis of (cyclic) CS. Our aim was to study whether hair cortisol timelines correspond with clinical course in patients with CS and whether we could create retrospective timelines of cortisol exposure that correspond with symptomatic periods in patients suspected of cyclic CS.

Methods

Scalp hair was collected in 14 patients with confirmed CS and 6 patients suspected of cyclic CS. Cortisol was extracted from the hair samples with methanol and an ELISA was used to measure cortisol levels in hair extracts. A group of 96 non-obese individuals were used as a control group.

Results

Hair cortisol levels were significantly elevated in CS patients ($p<0.0001$). Sensitivity and specificity of hair cortisol measurements for CS were 86% and 98% respectively. Hair cortisol timelines of patients with CS and cyclic CS corresponded with clinical course.

Conclusions

Hair samples can provide a historical timeline that corresponds with clinical course in patients with (cyclic) CS. This new diagnostic tool can contribute significantly to early recognition of patients suffering from cyclic CS.

Introduction

Cortisol, the most important glucocorticoid in man, is secreted by the adrenal glands and has many effects throughout the human body. It is e.g. involved in lipid and glucose metabolism, immune response and the development and functioning of numerous organs. Cortisol excess, as present in patients with Cushing's syndrome (CS), has many negative effects on the body. Symptoms caused by cortisol excess are weight gain, with accumulation of fat especially in the neck, face and abdomen, insulin resistance, muscle weakness, usually of the proximal muscles, bone loss and infections (1-2). Also psychological symptoms such as loss of emotional control, irritability and depression are common (1). The presence and severity of these symptoms depend on the level and duration of cortisol excess. Major causes of endogenous hypercortisolism are excessive ACTH production by a pituitary adenoma, excessive cortisol secretion from an adrenocortical tumor or hyperplasia and ectopic ACTH production by a non-pituitary tumor (1). Standard screening tests for CS are the measurement of cortisol in 24 hours urinary collections, the measurement of cortisol in midnight saliva and the 1 mg overnight dexamethasone suppression test (DST) (2). Urinary and salivary cortisol levels should be measured at least twice, since hypercortisolism in CS can be variable (2).

Cyclic CS is thought to be a rare disorder that is characterized by alternating episodes of endogenous cortisol excess and normal cortisol secretion. The cycles can occur regularly or irregularly with periods of normal cortisol secretion ranging from days to years (3). This makes cyclic CS a diagnosis that is difficult to make and easy to miss. The standard screening tests such as the measurement of cortisol in 24h urine collections or in saliva represent cortisol levels during a maximum of 24 hours and do not provide information on cortisol exposure in the previous weeks or months. Therefore, these methods are not optimal to diagnose cyclic CS or require multiple sample collections to confirm the diagnosis. The measurement of cortisol in scalp hair is a novel tool that might be helpful to establish the diagnosis of cyclic CS. Several studies have validated hair cortisol measurements and have shown that hair can be used to create a retrospective timeline of cortisol exposure, with every one cm of hair corresponding to a period of approximately 1 month (4-7). Thomson et al. already showed promising results of hair cortisol measurements in 5 patients with endogenous CS. In that study, hair cortisol levels corresponded with clinical course of the disease (7). The measurement of cortisol in scalp hair is currently the only method that provides information on cortisol exposure in the previous months to years (depending on the length of the hair) and could therefore be very valuable in patients suspected of cyclic CS.

The aim of the present study was to investigate whether hair cortisol timelines correspond with clinical course in patients with CS and whether we could create retrospective timelines of cortisol exposure that corresponded with symptomatic

periods in patients suspected of cyclic Cushing's Syndrome. Therefore, we measured hair cortisol levels in patients with confirmed Cushing's Syndrome and patients suspected of cyclic CS and compared our results with those of a large group of controls.

Methods

Patients

Patients admitted to the clinic or outpatient clinic of the Erasmus MC, the Sophia Children's Hospital, the Leeuwarden Medical Center or the St Radboud MC with confirmed CS or suspected of cyclic CS were included. Patients could not participate if there was insufficient hair growth at the posterior vertex of the scalp. This study was approved by the local Ethics committee and all patients gave written informed consent. In case the patients were younger than 18 years, informed consent was given by both the patients and their parents or guardians.

Healthy controls

To compare hair cortisol levels in CS patients with hair cortisol levels in healthy individuals and to calculate a reference range of normal hair cortisol levels, we selected healthy individuals with BMI between 18.5-24.9 kg/m2 and without abdominal obesity (n=96) (6). From the measurements in these 96 controls we calculated the reference range of normal hair cortisol levels defined as mean ± 1.96*SD and found a range of 9.9 - 75.9 pg/mg hair. We calculated sensitivity and specificity of the hair cortisol measurements for CS based on this reference range. In addition, we calculated specificity in overweight and obese individuals from our large group of healthy individuals and in the group of healthy individuals with abdominal obesity. The characteristics of the total group of healthy individuals are extensively described elsewhere (6).

Hair collection

A lock of approximately 100-150 hairs was cut from the posterior vertex of the scalp, as close to the scalp as possible. The hair samples were taped to pieces of paper and the proximal side of the hairs was marked. The hair samples were stored at room temperature in an envelope until analysis.

Hair preparation

From all controls and patients with CS we measured cortisol levels in hair segments of 1-3 cm, roughly corresponding with a period of 1-3 months. In addition, in a number of patients with long hair and in all patients suspected of cyclic CS, we divided the total length of the hair in segments of 1 cm, in order to create a retrospective timeline of monthly hair cortisol levels. Further preparation was performed as described by Sauvé et al (8). In brief, 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 methanol was used to extract cortisol from the hair samples. After incubation, the methanol was transferred to another vial and evaporated under a stream of nitrogen. Subsequently, the samples were redissolved in PBS and vortexed for one minute. Before analysis, the samples were vortexed again for 30 seconds.

Hair analysis

A commercially available ELISA Kit for Salivary Cortisol (DRG GmbH Marburg, Germany) was used to measure cortisol levels. Cross reactivity of other steroids with the kit's antibodies was reported as follows: Corticosterone (29%), Cortisone (3%), 11-Deoxycortisol (<1%), 17-OH Progesterone (<0.5%), other hormones (<0.1%). Intra-assay variation was below 5% and the inter-assay variation below 8%, as reported by the supplier. The recovery of the assay was tested and described previously (6).

Urine cortisol and serum cortisol

The measurement of cortisol in serum, saliva and urine was performed as part of the diagnostic process for the establishment of Cushing's Syndrome. Serum cortisol was determined using a competitive immunoassay (Immulite 2000, Siemens Healthcare Diagnostics B.V., Breda, The Netherlands). Serum cortisol levels of <718 nmol/L were considered as normal. Urinary cortisol was measured using an in house method, using UV detection after HPLC. Urinary cortisol levels were measured in 24h urine collections and levels of <119 nmol/24h were considered as normal.

Results

Cortisol levels were determined in scalp hair of 14 patients with Cushing's syndrome and 6 patients suspected of cyclic CS. Clinical data of all patients have been summarized in Table 1.

Long-term hair cortisol measurements

Mean hair cortisol levels were significantly elevated in patients with CS (cyclic CS patients excluded) compared to healthy individuals (399.7 pg/mg hair (95% Confidence Interval (CI):171.8-930.0) versus 27.3 pg/mg hair (95%CI: 24.6-30.4). ($p<0.0001$) (Figure 1). There was no significant effect of age, gender and hair treatment (dyeing and bleaching of hairs) on hair cortisol levels in the control group nor in the CS patients, although hair cortisol levels were slightly lower in healthy women who treated their hairs ($p=0.06$). Adjusting for these factors did not influence the results. Based on the upper limit of the reference range of non-overweight healthy controls (75.9 pg/mg hair), the sensitivity and specificity of hair cortisol measurements for CS were 86% and 98% respectively. The cut-off value of 75.9 pg/mg hair resulted in a specificity of hair cortisol measurements for CS of 98% and 93% in overweight and obese individuals, respectively, and 93% in individuals with abdominal obesity (Figure 1).

Table 1. *Overview of the patients included in this study*

	Gender	Age	Cause	Urinary cortisol at diagnosis (nmol/24h)
Cushing's Syndrome				
Patient 1	F	45	Pituitary microadenoma	332
Patient 2	F	35	Pituitary microadenoma	482
Patient 3	M	23	Pituitary microadenoma	973
Patient 4	M	36	Pituitary microadenoma	1156
Patient 5	F	45	Pituitary microadenoma	436
Patient 6	F	65	Pituitary macroadenoma	2061
Patient 7	F	48	Pituitary macroadenoma	10588
Patient 8	M	57	ACTH producing metastasized prostate carcinoma	45430
Patient 9	M	57	ACTH producing gastric carcinoid	51590
Patient 10	M	63	ACTH producing small cell lung carcinoma	697
Patient 11	F	48	ACTH producing neuro-endocrine tumour pancreas	31732
Patient 12	M	63	ACTH producing neuro-endocrine tumour pancreas	21831
Patient 13	F	50	ACTH producing metastasized mamma carcinoma	5213
Patient 14	M	76	Adrenal carcinoma	1955
Cyclic Cushing's Syndrome				
Patient 15	F	56	Unknown	6000
Patient 16	F	23	Unkown	
Patient 17	F	33	Thymus carcinoid	16009
Patient 18	F	26	Thymus carcinoid	47446
Patient 19	M	10	Pituitary microadenoma	563
Patient 20	M	35	Ectopic, source of ATCH production unkown	5132

Hair cortisol timelines in CS patients

We selected 3 CS patients with hair of sufficient length to evaluate the effect of treatment of their Cushing's Syndrome on hair cortisol levels. The hair samples of these patients were divided in one cm segments in order to measure cortisol exposure per month.

The hair cortisol timeline and 24h urinary cortisol levels of patient 1, a 45 year old woman, are presented in Figure 2A. This patient was diagnosed with Cushing's Disease in October 2009 and underwent transsphenoidal resection (TSR) of a microadenoma in February 2010. Three months before surgery she started with ketoconazole therapy (K). Urinary cortisol levels were elevated before the start of ketoconazole (332 nmol/24h, upper limit of normal: 119 nmol/24h) and were normalized after ketoconazole was started (69 nmol/24h). Her hair cortisol timeline showed elevated cortisol levels already a year before the start of ketoconazole and a decline in cortisol levels after the start of ketoconazole. After TSR, cortisol levels decreased even more (Figure 2A).

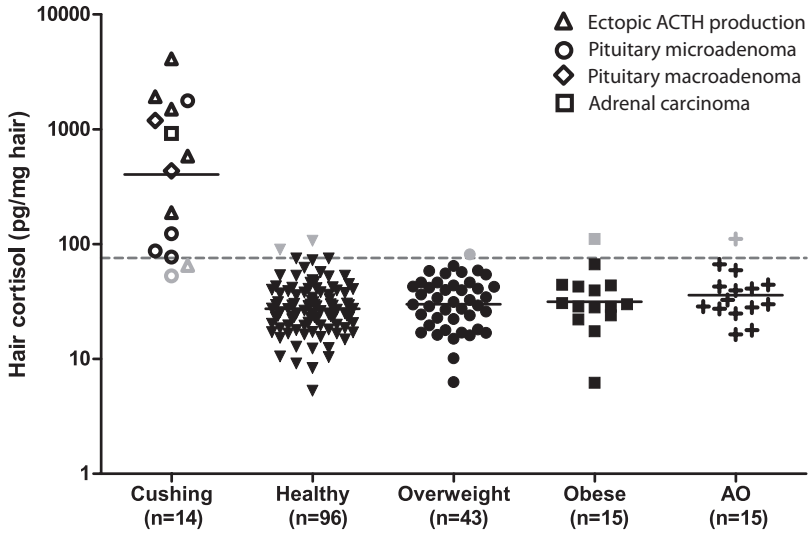

Figure 1. *Hair cortisol levels in patients with Cushing's Syndrome and healthy, overweight and obese controls and individuals with abdominal obesity.*
The dotted line represents the upper limit of normal hair cortisol levels (75.9 pg/mg hair). The grey symbols represent the individuals with cortisol levels below the upper limit of normal (in case of confirmed Cushing's Syndrome) or above the upper limit of normal (no Cushing's Syndrome). Based on this upper limit, the sensitivity for hair cortisol measurements in Cushing's Syndrome is 86%. This cut-off value of 75.9 pg/mg hair for the diagnosis of Cushing's Syndrome resulted in a specificity of 98% in healthy individuals, 98% in overweight individuals, 93% in obese individuals and 93% in individuals with abdominal obesity (AO). The nature of the Cushing's Syndrome is indicated by the different symbols used for the patients (see also Table 1).

Figure 2B shows the hair and urinary cortisol levels of Patient 2. This 35 year old woman was also diagnosed with Cushing's Disease in October 2009 (urinary cortisol level 482 nmol/24h, upper limit of normal: 119 nmol/24h). In January 2010 she started with ketoconazole (K). She underwent TSR at the end of March 2010. Despite surgery, she continued to have elevated 24h urinary cortisol levels (683 nmol/24h) and ketoconazole was restarted in June 2010. With this treatment, urinary cortisol levels decreased (188 nmol/24h), but remained above the upper limit of normal (119 nmol/24h). Hair cortisol levels were already elevated more than a year before the diagnosis of CS was established and decreased after ketoconazole treatment and surgery (85.4 pg/mg hair), but remained above the upper limit of normal, which is the same pattern as the multiple urinary collections show.

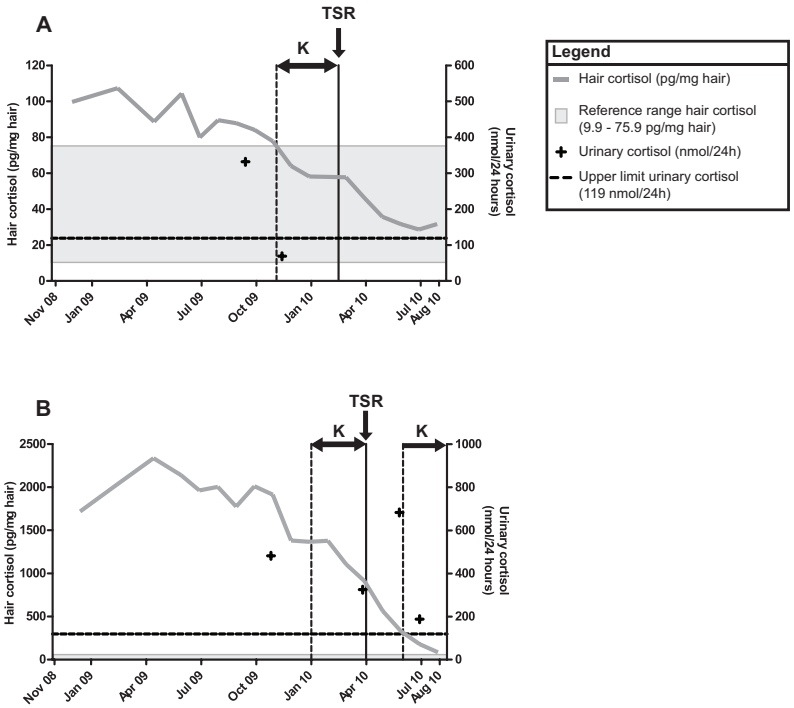

Figure 2. *Hair cortisol timelines of patients with Cushing's Syndrome before, during and after treatment. (A) Hair and urinary cortisol levels of patient 1; (B) Hair and urinary cortisol levels of patient 2. Cortisol was measured in adjacent 1 cm fragments derived from a single lock of hair. K= ketoconazole treatment; TSR= transsphenoidal resection.*

Patient 3 (Figure 4A), a 23 year old man, was diagnosed with medullary thyroid carcinoma during childhood, as part of Multiple Endocrine Neoplasia type 2A (MEN2A). In 2009 he developed Cushing's Syndrome, caused by a pituitary

microadenoma. After a three month period of ketoconazole treatment, the patient underwent TSR of the microadenoma in November 2010. Around the start of ketoconazole, his hair cortisol level was 123.4 pg/mg hair and his urinary cortisol levels ranged from 70-137 nmol/24h (upper limit of normal 119 nmol/24h). Four months after TSR, his hair cortisol level was decreased to 50.1 pg/mg hair (measured during hydrocortisone replacement therapy) (Figure 4A).

Hair cortisol timelines in patients suspected of cyclic CS

In the 6 patients with (suspected) cyclic CS we created timelines of cortisol exposure. Again, hair samples were divided in one cm segments to measure monthly cortisol exposure.

Patient 15 is a 56-year old woman who was recently diagnosed with cyclic CS. She noticed a moon face in August 2011 and was admitted to the hospital with hypertensive crisis in November 2011. In March – April 2011 and January 2012 she suffered from muscle pains. During the hypertensive crisis, her urinary cortisol level was extremely elevated (6000 nmol/24h). In the months January –March 2012 urinary cortisol measurements were repeated during several occasions and they were all found to be normal (54 nmol/24h, 80 nmol/24h and 44 nmol/24h, upper limit of normal: 119 nmol/24h). In March 2012, a hair sample was collected, which was of sufficient length to measure hair cortisol levels from April 2011 onwards. Her hair cortisol levels were elevated during the period in which she had a moon face and during the hypertensive crisis. In the periods with hair cortisol levels within the normal range, she suffered from muscle pain, which might be caused by the relative cortisol deficiency in that period (Figure 3A).

Patient 16, a 23-year old woman, was frequently seen at the outpatient clinic from January 2010 onwards. In the period of January-July 2009 she gained approximately 30 kg of weight, primarily located at the abdomen and face, despite being on a diet. On examination, she had an evident Cushingoid appearance. Urinary cortisol excretion was within the normal range on two occasions (January 2010: 23 nmol/24h; February 2010: 29 nmol/24h; upper limit of normal: 119 nmol/24h) and CS could not be confirmed. It was decided that urinary cortisol levels would be measured again upon return of the symptoms, but until now, no further episodes of weight gain or other Cushingoid symptoms have occurred. In April 2011, a hair sample was obtained that was of sufficient length to retrospectively measure cortisol levels from October 2008 onwards. In this hair sample we found a peak (94.1 pg/mg hair) in the hair segment corresponding with the symptomatic period in 2009. Hair cortisol levels during asymptomatic periods ranged from 12.1 to 29.7 pg/mg hair (Figure 3B).

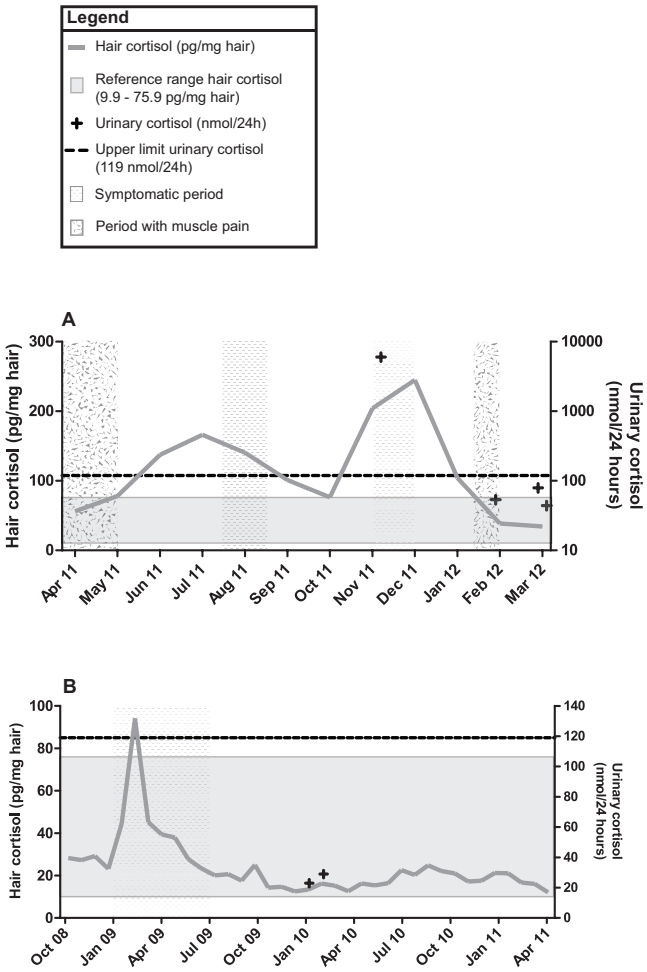

Figure 3. *Hair cortisol timelines of patients suspected of cyclic Cushing's Syndrome. (A) Hair cortisol levels of patient 15; (B) Hair cortisol levels of patient 16. Cortisol was measured in adjacent 1 cm fragments derived from a single lock of hair.*

Patient 17 (Figure 4B), a 33-year old woman, was diagnosed with CS due to an ACTH producing thymus carcinoid in September 2010. She had abdominal obesity, moon face, buffalo hump, hypertension and hypokalemia, all starting in August 2010. Laboratory examination showed an elevated urinary cortisol level (16009 nmol/24h; upper limit of normal: 119 nmol/24h) and absence of cortisol suppression after 1 mg dexamethasone. Her medical history suggested a cyclic pattern of hypercortisolism with previous symptoms of cortisol excess, such as moon face, proximal muscle weakness, acne and hirsutism from July to September

2009 and a period of minor hirsutism and acne in January 2010. Between these episodes, she had no complaints. Unfortunately, no urinary cortisol measurements were performed in the symptomatic and asymptomatic periods to confirm cyclic CS. However, the retrospective hair cortisol timeline of this patient, which reflected the period September 2009 – October 2010, showed a cyclic pattern with fourfold higher cortisol levels during symptomatic periods (around 80 pg/mg hair) compared to the asymptomatic periods (around 20 pg/mg hair, (Figure 4B). Hair cortisol levels in the symptomatic periods were above the upper limit of normal (75.9 pg/mg hair).

Patient 18 (Figure 4C), a 26-year old woman, was diagnosed with cyclic CS in October 2009. Since 2008 she suffered from repeated episodes of acne, hirsutism, low potassium levels, edema and weight gain. Her general practitioner found elevated serum cortisol levels during two of these episodes and referred the patient to an endocrinologist, who measured normal 24hours urinary cortisol excretion in December 2008 (58 nmol/24h, upper limit of normal: 119 nmol/24h), February 2009 (25 nmol/24h) and June 2009 (47 and 36 nmol/24h). In addition, there was normal suppression of cortisol after 1 mg of dexamethasone. In October 2009 she developed symptoms of cortisol excess again and her 24h urinary cortisol level was extremely elevated (47446 nmol/24h). Sinus petrosus sampling revealed a pituitary cause of hypercortisolism and MRI showed a small lesion in the pituitary. Despite transsphenoidal surgery in December 2009, the patient remained hypercortisolemic (urinary cortisol level 1103 nmol/24h) and was treated with ketoconazole and later with the combination of ketoconazole and cabergoline. During this period, urinary cortisol levels ranged from 73-181 nmol/24h. Additional examination revealed a small ACTH producing thymic carcinoid as the cause of hypercortisolism. Two hair samples were collected, one in October 2009 during a short period of hypercortisolism and one in August 2010, after a period of ketoconazole, cabergoline and hydrocortisone treatment. The first hair sample, reflecting the period August 2009 – October 2009, shows a timeline in which the cyclic pattern is very clear with elevated hair cortisol levels during the active episode (150.8 pg/mg hair) and normal cortisol levels in the asymptomatic period (24.4 and 36.7 pg/mg hair). The second hair sample, reflecting the period February 2010 – August 2010, shows continuously elevated hair cortisol levels from March 2010 until the moment of sample collection (ranging from 94.5 to 135.8 pg/mg hair), with no response to the addition of cabergoline to the treatment. This corresponds with the elevated urinary cortisol levels during combined treatment with ketoconazole and cabergoline (181 and 140 nmol/24h, upper limit of normal: 119 nmol/24h) (Figure 4C).

Patient 19 (Figure 4D), a 10-year old boy was first seen in 2009 because of growth retardation and weight gain. Serum cortisol was elevated, but this was attributed to his fear of blood and needles. After a repeated measure in December 2010, his serum cortisol level was normal (502 nmol/L, upper limit of normal 718 nmol/L). However, the patient had progressive symptoms and in June-July 2011, multiple elevated

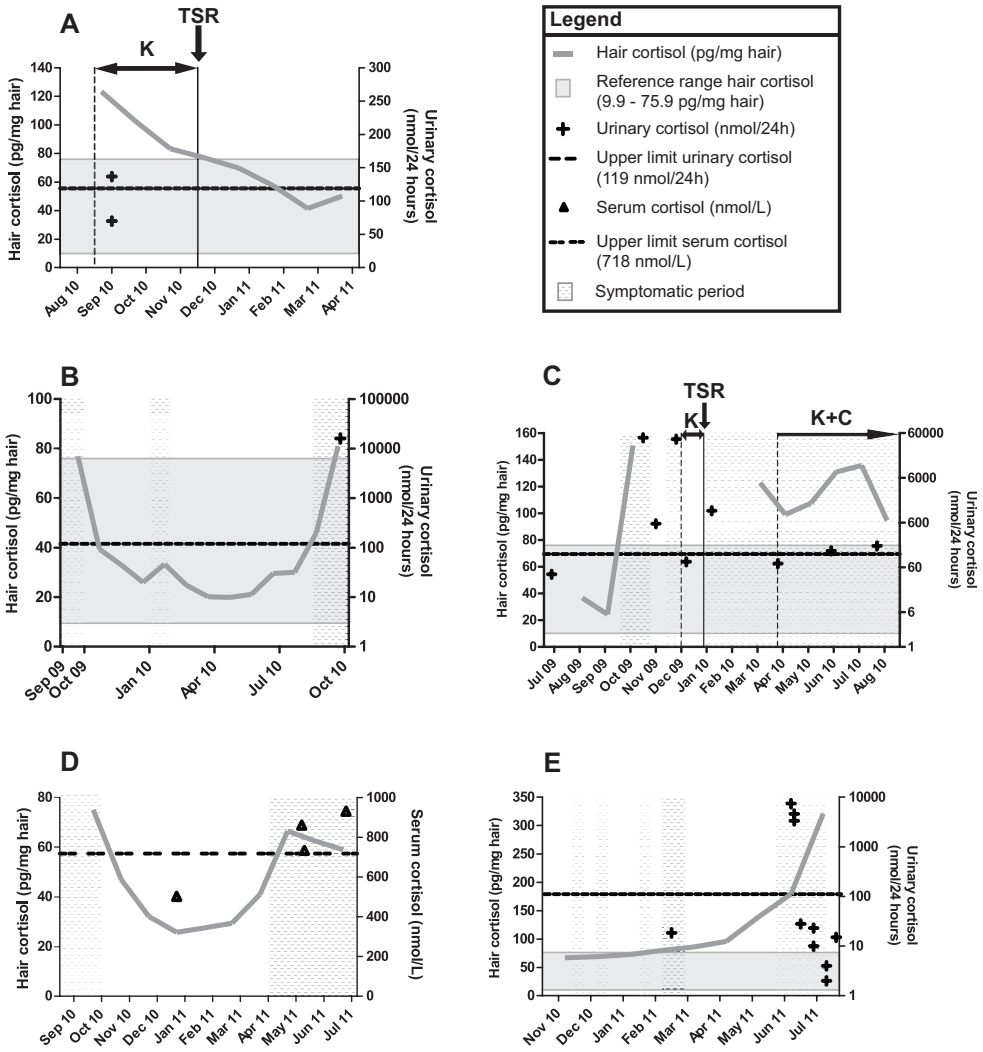

Figure 4. *Hair cortisol timelines of a patient with Cushing's Syndrome and 4 patients suspected of cyclic Cushing's Syndrome.*
(A) Hair and urinary cortisol levels of patient 3; (B) Hair and urinary cortisol levels of patient 17; (C) Hair and urinary cortisol levels of patient 18; (D) Hair and serum cortisol levels of patient 19; (E) Hair and urinary cortisol levels of patient 20. Cortisol was measured in adjacent 1cm fragments derived from a single lock of hair except in the case of patient 20, for whom two hair samples were used. K= ketoconazole treatment; K+C= combined treatment with ketoconazole and cabergoline; TSR= transsphenoidal resection.

serum (861, 734 and 931 nmol/L) and urinary cortisol levels (563 nmol/24h, upper limit of normal: 119 nmol/24h) were found in combination with absent suppression of serum cortisol after 1 mg of dexamethasone. Photographs of the patient revealed that he had developed a Cushingoid appearance in the period between 2009 and July 2011. The retrospective timeline of hair cortisol in this patient reflected the period September 2010 – July 2011 and revealed a cyclic pattern with threefold higher levels in September 2010 (75.1 pg/mg hair) and May-July 2011 (65.5 pg/mg hair) compared to the period in between (25.8 pg/mg hair) (Figure 4D).

Patient 20 (Figure 4E), a 35-year old man, had been frequently seen at the outpatient clinic for gastro-intestinal diseases because of chronic pancreatitis with endocrine and exocrine insufficiency, which was, at that time, attributed to marihuana abuse. During 2009-2011 the patient was admitted to the hospital several times because of severe abdominal pain and jaundice. During these admissions, examination revealed hypertension, hypokalemia, proximal muscle atrophia, easy bruisability and a moon face. CS was suspected, but urinary cortisol levels were normal (12 nmol/24h, upper limit of normal: 119 nmol/24h). In June 2011 the patient was admitted again and the diagnosis of CS was established by multiple elevated 24h urinary cortisol levels (3352, 4622 and 7422 nmol/24h). After one week of elevated urinary cortisol levels, cortisol levels returned to normal (28, 10, 23, 4, 2 and 15 nmol/24h) confirming cyclic CS. The cause of hypercortisolism was unknown and the patient underwent bi-adrenalectomy. Hair cortisol levels of this patient were increasing over time and above the normal range from February 2011 onwards. In contrast to his hair cortisol levels, his urinary cortisol levels were normal during hospitalization in February 2011. In total, seven out of ten urinary cortisol levels were normal, whereas hair cortisol levels were continuously elevated (Figure 4E).

Discussion

In this study we show that hair cortisol levels were significantly elevated in scalp hairs of patients with CS compared to healthy controls. In addition, timelines of cortisol exposure, constructed by measuring cortisol in consecutive hair segments, corresponded with clinical course of the disease, both in the non-cyclic and in cyclic CS patients. In recent literature, the measurement of cortisol in scalp hair has been well validated. Most studies have used this method to study long-term cortisol levels in patients with chronic pain and stress or in healthy individuals (6, 9-11). Two previous reports also showed that hair cortisol timelines corresponded with clinical course in patients with CS (6-7). Our study is the first study that also shows hair cortisol levels in patients suspected of cyclic CS and our cases illustrate that retrospectively obtained cortisol measurements can be of great value in the diagnosis of cyclic CS.

The sensitivity and specificity of cortisol levels in hair for the diagnosis of CS on basis of the upper limit of the reference range of our healthy individuals were 86% and 98% respectively. These percentages are comparable with values described when using 24h urinary cortisol or midnight salivary cortisol to screen for CS as described in a meta-analysis of Elamin et al (12), who found that the sensitivity of cortisol measurements in multiple 24h urine collections ranged from 38% to 100% (mean 84%) and specificity ranged from 44-100% (mean 92%), depending on the methods used and cutoff values set by the investigators. The sensitivity of midnight salivary cortisol ranged from 46% to 100% (mean 85%), with specificity ranging from 79-100% (mean 92%) (12). All of these values were based on the collection of a minimum of 2 samples. Furthermore, Friedman et al. showed that when collecting up to six 24h urinary samples, at least 88% of the patients with CS had at least one negative test (13). For midnight salivary cortisol, this was even 92% (13). This shows that it is required to collect multiple samples to establish or reject the diagnosis of CS. The advantage of measuring cortisol in hair is that only one sample needs to be collected to see whether cortisol levels were increased over longer periods of time. Furthermore, in cyclic CS or in patients with long hair, the creation of a hair cortisol timeline might provide the opportunity to evaluate an individual's baseline cortisol level. It might be potentially more useful to compare an individual's high cortisol levels with his/her own baseline cortisol levels than comparing it to the reference range from a group or population.

The diagnosis of cyclic CS is difficult and can be easily missed when standard tests are used during normocortisolemic periods. As shown in our cases, it may take several months to years to establish the diagnosis of cyclic CS using standard tests. The patients described here were finally diagnosed with cyclic CS by collection of multiple serum and 24h urine samples, which interferes with daily activities and is troublesome to obtain for prolonged periods of time. The collection of a single hair sample can provide cortisol levels at time-points reaching back months or even years, depending on the length of the hair samples. We have shown that clear cyclic patterns of cortisol excess were found in hair of patients suspected of cyclic CS. Only in one patient, with very short periods of cortisol excess (patient 20) no clear cyclic pattern was detected. However, in this patient hair cortisol measurements could have been of great value for earlier recognition of CS as well, since his hair cortisol levels were elevated from February 2011 onwards, whereas his urinary cortisol levels were normal in seven out of ten samples, indicating rapidly cycling episodes of hypercortisolism. This discrepancy between hair and urinary cortisol measurements can be attributed to the different timeframes that hair cortisol and urinary cortisol measurements represent. In our study, hair samples were measured in minimally 1 cm segments, corresponding to a period of 1 month, whereas urinary cortisol levels represent maximally 24 hours.

The use of hair samples as a historical record for cortisol exposure might be questioned if cortisol excess affects hair growth. Hair loss is a key feature of CS, but it is not known what the effect of cortisol excess is on hair growth. However, considering the clinical course in the patients described, hair cortisol levels seem to reflect treatment effect and the symptomatic periods very accurately when assuming a hair growth rate of one cm/month. This suggests that altered hair growth rate is not the case or at least does not play a major role in CS. In addition, in the study of Thomson et al, a growth rate of 1 cm/month seemed to apply to patients with CS as well (7). This suggests that cortisol excess does not play a major role in changes in hair growth rate. Furthermore, some studies reported a decrease in cortisol levels in the more distal hair segments indicating that hair could be used as a historical record for up to 6 months only (5). This is in contrast with the finding of Thomson et al. (7) and our own previous results in healthy individuals (6). We found no washout effect in hairs with a length of up to 18 cm. In addition, in this study our hair cortisol timelines showed variations corresponding with symptomatic periods in patients suspected of cyclic CS. Normal hair cortisol levels in asymptomatic periods were observed in hairs of more than 6 cm of length, suggesting that there is no washout effect of cortisol from the distal hair segments. Finally, at present the method to measure cortisol in scalp hair has been only used in a few laboratories worldwide and the normal values vary between these institutions. To implement this method as a diagnostic tool in the clinical setting further refinement and standardization of the technique would be valuable.

In conclusion, hair cortisol levels are increased in patients with CS and the sensitivity and specificity of hair cortisol measurements for CS is high. Furthermore, hair can provide a historical timeline that corresponds with clinical course in patients with Cushing's Syndrome, as well as in patients with cyclic CS. This new diagnostic tool can contribute significantly to earlier recognition of patients suffering from cyclic CS.

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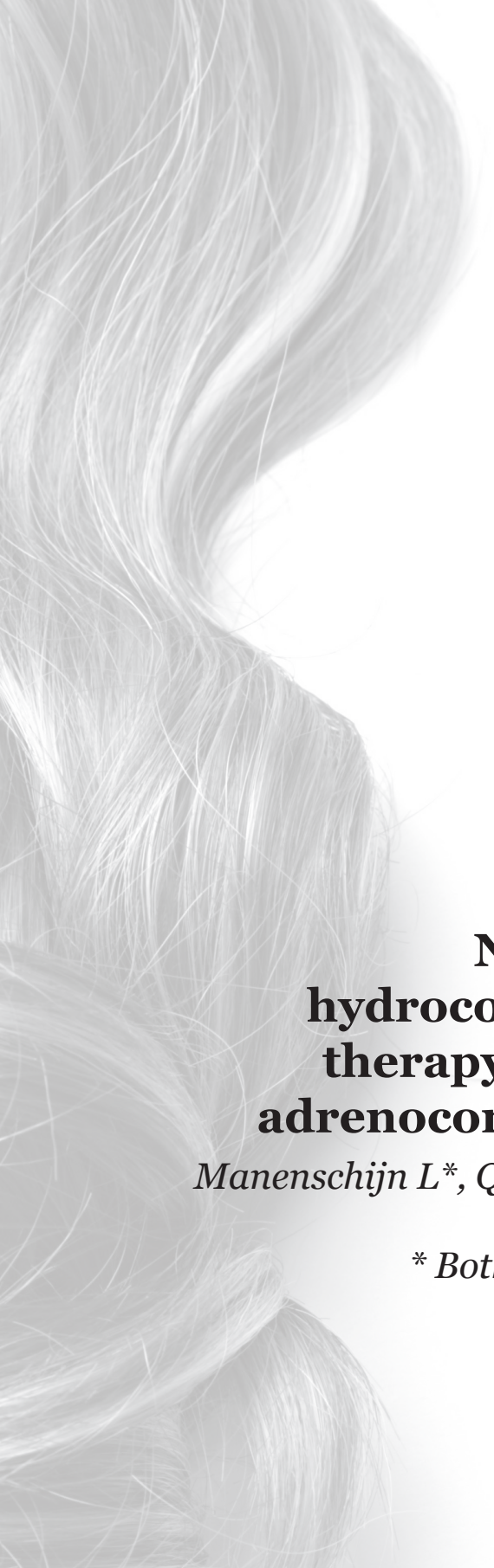

Chapter 4

Novel way to monitor hydrocortisone replacement therapy in mitotane-treated adrenocortical cancer patients

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** Both authors contributed equally*

Submitted

Abstract

Background

The only approved drug for the treatment of adrenocortical cancer (ACC) is mitotane. Mitotane is adrenolytic and therefore, hydrocortisone replacement therapy is necessary. Since mitotane increases cortisol binding globulin (CBG) and induces CYP3A4 activity, high doses of hydrocortisone are thought to be required. Evaluation of hydrocortisone therapy in mitotane-treated patients has been difficult since there is no good marker to evaluate hydrocortisone therapy. Measurement of cortisol in scalp hair is a relatively novel method that offers the opportunity to measure long-term cortisol levels. Our aim was to study whether hair cortisol measurements could be useful in evaluating hydrocortisone treatment in mitotane-treated ACC patients.

Methods

Hair cortisol levels were measured in 15 mitotane-treated ACC patients on hydrocortisone substitution and 96 healthy individuals. Cortisol levels were measured in 3 cm hair segments, corresponding to a period of 3 months.

Results

Hair cortisol levels were elevated in ACC patients compared to healthy individuals ($p<0.0001$). Seven ACC patients (47%) had hair cortisol levels above the reference range. Hair cortisol levels were associated with BMI ($\beta=0.53$, $p=0.042$). There was no correlation between hair cortisol levels and hydrocortisone doses ($\beta=0.41$, $p=0.13$) or hydrocortisone dosage and BMI ($\beta=-0.03$, $p=0.93$).

Conclusion

Almost half of the ACC patients had elevated cortisol levels, suggesting over-substitution of hydrocortisone. Hair cortisol levels, but not hydrocortisone dose, were positively associated with BMI, suggesting that hair cortisol levels may be a better indicator of cortisol exposure than hydrocortisone dose. Hair cortisol measurements can be used in the evaluation of hydrocortisone treatment in mitotane-treated ACC patients.

Introduction

Adrenocortical cancer (ACC) is a rare malignancy that occurs in approximately 0.5-2 per million persons per year (1-2). The only curative treatment is radical surgery (3). However, even after initial curative surgery, 40% of the patients have recurrent disease or distant metastases within 2 years (4). Adrenolytic therapy with mitotane (o'p'-DDD) is the primary treatment for patients with advanced ACC (3), either as monotherapy or in combination with cytotoxic chemotherapy. Mitotane is the only approved drug for ACC and has been shown to extend disease-free survival (5), although this has been questioned (6-9). Mitotane has also been used in an adjuvant setting and a panel of international experts recently recommended the administration of adjuvant mitotane in all patients at high risk of relapse (10). Mitotane is a steroidogenesis inhibitor at different enzymatic steps, which concentrates in the adrenal glands and is thought to provoke mitochondrial degeneration and destruction of the adrenal cortex (11). However, the precise mechanisms of action remain still unknown. It is suggested that serum concentrations of mitotane should exceed 14 mg/L to increase the likelihood for an anti-tumoral response, and kept below 20mg/l to avoid side effects (12-13). However, often this concentration is not achieved, due to the low bioavailability caused by its low absorption rate and its lipophilic nature (14). The aim of reaching target serum concentrations is also hampered by many side effects of mitotane treatment. Up to 80% of the patients have gastrointestinal side effects including nausea, vomiting, abdominal discomfort and diarrhoea. About 40% develop neurological symptoms such as dizziness, dysesthesia, sedation and ataxia (15-16).

Mitotane affects all adrenocortical zones, therefore it is necessary to replace glucocorticoids and sometimes mineralocorticoids during mitotane treatment. However, mitotane is a strong inducer of hepatic CYP3A4 activity (17) and increases cortisol binding globulin (CBG) (15), resulting in an increased cortisol metabolism and reduced free, active cortisol (18-22). Therefore normal hydrocortisone replacement therapy (approximately 20 mg/day) as used in adrenal insufficiency, is not sufficient in ACC patients on mitotane and higher dosages (40-80mg/day) are necessary. The monitoring of hydrocortisone substitution under mitotane treatment is difficult. One study suggested that free cortisol measurement may offer additional information in the follow-up of ACC patients on mitotane, especially in the subgroup of patients with Cushing's Syndrome (15). This marker indeed may overcome some of the complications in the interpretation of measured cortisol in serum; however, it only reflects acute free cortisol levels of minutes to hours. Therefore, the monitoring of hydrocortisone dose has relied on clinical symptoms until now. However, the clinical symptoms of cortisol deficiency show extensive overlap with side effects of mitotane. Therefore, the differentiation between symptoms of adrenal insufficiency and mitotane induced side effects is difficult. Measurement of cortisol in serum or urine is not helpful, since these methods measure total cortisol (excretion) and do

not reflect unbound, free cortisol levels. In addition, these measurements reflect only a short time frame, and serum levels are also influenced by the time of intake of hydrocortisone and may be affected by acute stress (e.g.venapuncture) in case of some residual adrenal function.

In the past few years, a novel method to measure long-term cortisol has been developed by extraction of cortisol from scalp hair (23). This measurement has been well validated by several groups (23-28). Since scalp hair grows with an average rate of 1 cm per month, a hair segment of 1 cm reflects mean cortisol levels of one month. Cortisol measurements in scalp hair have been shown to represent long-term cortisol levels in health and disease (29). Retrospective timelines of hair cortisol levels corresponded with clinical course in patients with Cushing's syndrome (26, 30-31), cyclic Cushing's Syndrome (30) and Addison's Disease (26, 32). In healthy individuals, hair cortisol levels were positively correlated with BMI and waist circumference, suggesting that hair cortisol measurements reflect cortisol exposure (26, 33-34). Furthermore, hair cortisol levels were elevated in individuals with chronic stress such as unemployment and chronic pain (28, 35). All these studies together show that the measurement of cortisol in hair is a reliable marker of long-term cortisol levels.

Our aim was to study whether hair cortisol measurements could be useful in the evaluation of hydrocortisone substitution in mitotane treated ACC patients and whether high dose hydrocortisone replacement as commonly used in clinical practice results in normal long-term cortisol levels in these patients.

Methods

Patients

Patients with histologically confirmed adrenocortical carcinoma, treated with mitotane, who were seen at the endocrine outpatient clinic of the Charité Campus Mitte, Berlin in the period July – December 2011, were asked to participate in this study. Data on tumor size and ENSAT stage at diagnosis and the current status of disease were collected, together with mitotane serum levels, body mass index (BMI) and hydrocortisone dosage of the period corresponding to the hair sample collection. This study was approved by the local medical ethics committee and all participants gave written informed consent.

Mitotane was given orally, and was started usually with an "intermediate dose regimen". Briefly, mitotane was administered at a starting dose of 1.0 g/day and increased in case of good gastrointestinal tolerance every two days by 0.5 g until a daily dose of 4.5 g/day was reached. Further adjustment of dosage was performed according to blood mitotane concentrations (aim: concentrations between 14 and 20 mg/l) and tolerability. All patients received the same mitotane formulation (Lysodren, 500 mg

tablets) that was purchased from Laboratoire HRA Pharma. Patients were followed up every 3 months including physical examination, routine laboratory evaluation, hormonal work-up, and monitoring of mitotane concentrations. For measurement of the latter plasma samples were shipped to Lysosafe Service (HRA Pharma) and plasma levels were determined by Atlanbio Bioanalysis (Saint-Nazaire, France).

Healthy individuals

We collected hair samples of 96 healthy individuals (age 18-65) with BMI 18.5-24.9 kg/m2 and no abdominal obesity. The reference range was calculated as mean ± 1.96*SD and was 9.9-75.9 pg/mg hair. Detailed information of this group of healthy individuals has been previously described (30, 33).

Hair collection

Hair samples were collected from the posterior vortex of the scalp. A lock of approximately 100-150 hairs was cut off as close to the scalp as possible. The 3 centimetres (cm) most proximal to the scalp were used in the measurements, which correspond to the 3-month period prior to hair sample collection.

Hair preparation

The hair preparation method has been extensively described in previous reports (23, 26). In brief, a minimum of 10 mg hair was weighed, put in a glass vial and cut into very small pieces. Methanol was added to the hairs to extract cortisol during an overnight extraction (16 hours) in a shaking waterbath at 52°C. Afterwards, the methanol was transferred to a clean glass tube and evaporated under a stream of nitrogen until completely dry, after which the samples were resolved in phosphate buffered saline (PBS pH 8.0).

Cortisol measurement

Cortisol was measured in the hair extract with a commercially available ELISA kit (DRG), which was originally developed to measure salivary cortisol. Cross reactivity of the kit's antibodies with other steroids was reported as follows: Corticosterone (29.00%), Cortisone (3.00%), 11-Deoxycortisol (<1.00%), 17-OH Progesterone (<0.50%), other hormones (<0.10%). Intra-assay variation was below 5% and inter-assay variation was below 8% as stated by the manufacturer. The low end detection limit for this assay is 1.5 nmol/L. Recovery of the assay was tested and described elsewhere (26).

Statistical analysis

Statistical tests were performed using SPSS version 17.0 and Graphpad version 5.0. A p-value <0.05 was considered statistically significant. The Mann-Whitney U-test was used to test for statistical significant difference between groups. Associations between hair cortisol levels, hydrocortisone dosage and BMI were tested using linear regression analysis.

Table 1. *Group characteristics of mitotane treated adrenocortical cancer (ACC) patients and healthy individuals.*

	ACC patients	Healthy individuals
Number	15	96
Number of women - n (%)	11 (73.3%)	53 (55.2%)
Age (years) – median (range)	49 (25-82)	32 (19-61)
Body mass index (kg/m2) – median (range)	26.0 (18.5-29.9)	22.5 (18.5-24.8)
Age at diagnosis (years) – median (range)	46 (24-74)	
ENSAT stage at diagnosis		
Stage 1 – n (%)	1 (6.7%)	
Stage 2 – n (%)	11 (73.3%)	
Stage 3 – n (%)	2 (13.3%)	
Stage 4 – n (%)	1 (6.7%)	
Tumor size at diagnosis (cm) – median (range)	10.5 (4.5-21.5)	
Hormone production at diagnosis		
No – n (%)	6 (40.0%)	
Cortisol – n (%)	3 (20.0%)	
Aldosterone – n (%)	1 (6.7%)	
Androgens (precursors) – n (%)	3 (20.0%)	
Multiple hormones – n (%)	2 (13.3%)	
Time since diagnosis (months) – median (range)	47 (2-187)	
Metastasis at hair collection – n (%)	8 (53.3%)	
Therapy at hair collection		
Mitotane monotherapy– n (%)	12 (80.0%)	
Streptozotocin + mitotane– n (%)	2 (13.3%)	
Gemcitabine + capecitabine + mitotane– n (%)	1 (6.7%)	
Serum mitotane levels (mg/L) – median (range)	11.5 (0.8-18.4)	
Daily hydrocortisone intake (mg) – median (range)	53.3 (0-60)	

Results

Clinical characteristics of the ACC patients and healthy individuals are shown in Table 1. Most patients were in ENSAT stage 2 (73.3%) at the moment of ACC diagnosis. Median time since diagnosis to the moment of hair sample collection was 47 months (range 2-187 months). None of the patients had a clear Cushingoid appearance at diagnosis, but 5 of them had slight cortisol overproduction at the time of diagnosis of ACC. Two patients had solely mild cortisol overproduction and three

patients had slight overproduction of multiple hormones including cortisol at the time of diagnosis. At the moment of hair sample collection, none of these patients had remaining cortisol overproduction and hair cortisol levels were not different between patients with and without a history of cortisol producing ACC (p=0.43).

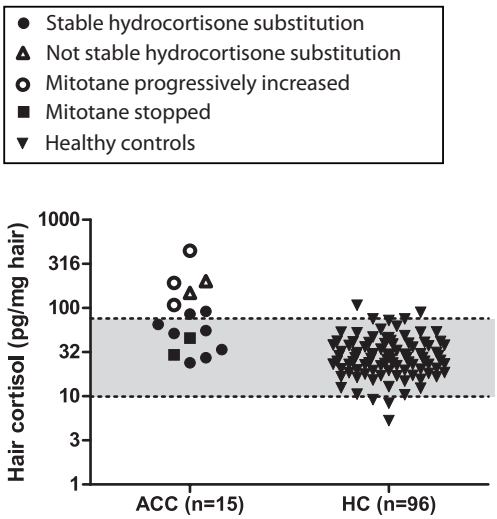

Figure 1. *Hair cortisol levels in mitotane treated adrenocortical cancer (ACC) patients with hydrocortisone substitution and healthy controls (HC). P<0.0001. The grey bar reflects the reference range of hair cortisol levels in healthy individuals.*

Hair cortisol levels were significantly elevated in mitotane treated ACC patients with hydrocortisone substitution (p<0.0001) compared to healthy individuals. Seven out of the 15 ACC patients (47%) had hair cortisol levels above the upper limit of normal (75.9 pg/mg hair), which ranged from 85.4 pg/mg hair to 445.2 pg/mg hair (Fig. 1). In three patients with elevated hair cortisol levels mitotane treatment was only recently started ("intermediate dose regimen") and the dosage was progressively increased in the three months period. In these patients increased hydrocortisone replacement therapy was started at the same time as mitotane, to prevent adrenal insufficiency (open circles Fig 1 and 2). Two patients were not on stable hydrocortisone substitution (closed squares Fig 1 and 2). One of these patients underwent surgery for recurrent disease with increased hydrocortisone dosage around the period of the surgery. The other patient had frequent symptoms of nausea and vomiting, which resulted in multiple short-term hydrocortisone dosage elevations. Two patients (closed circles Fig 1 and 2) were on stable hydrocortisone substitution and had stable mitotane serum levels. These patients had only mildly elevated cortisol levels. Six of the eight patients with normal hair cortisol levels were on stable hydrocortisone substitution and had stable mitotane serum levels (closed dots Fig. 1 and 2). The other two patients

with normal cortisol levels had stopped mitotane treatment before or during the three month period corresponding to the hair sample (closed squares Fig. 1 and 2).

Hair cortisol levels did not correlate with the mean hydrocortisone dosage of the three months corresponding with the hair cortisol timeframe (β=0.41, p=0.13) (Figure 2). In ACC patients, hair cortisol levels were positively correlated with BMI (β=0.53, p=0.04) (Figure 3). There was no correlation between hydrocortisone dosage and BMI (β=-0.03, p=0.93). During the hair cortisol timeframe (3 months) no adrenal crisis occurred, which might have required iv hydrocortisone application.

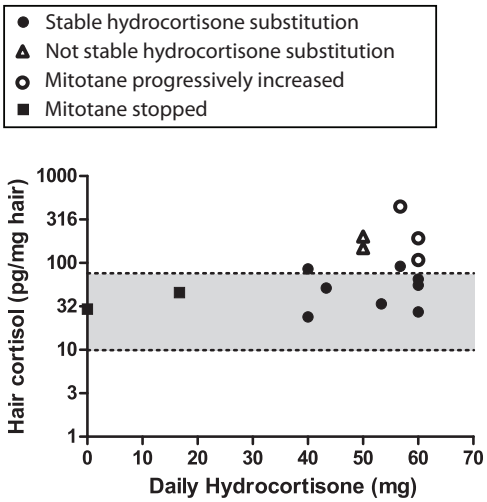

Figure 2. *Hair cortisol levels and daily hydrocortisone use (β=0.41, p=0.13). The grey bar reflects the reference range of hair cortisol levels in healthy individuals.*

Discussion

In this study we evaluated long-term cortisol levels in mitotane-treated ACC patients on hydrocortisone substitution by using a novel method to measure cortisol in scalp hair. We found that almost half of the patients had elevated hair cortisol levels and there was no correlation between hair cortisol levels and hydrocortisone dosage. Furthermore, we found a positive correlation between hair cortisol levels and BMI.

Monitoring hydrocortisone substitution during mitotane treatment is known to be a problem. Nowadays, the hydrocortisone dose is adjusted if patients have symptoms and clinical signs of hypo- or hypercortisolism. A marker to evaluate hydrocortisone substitution in these patients would be helpful for the physician. The traditional methods to measure cortisol are not optimal to use in the evaluation

of hydrocortisone substitution in mitotane treated ACC patients. Serum total cortisol levels cannot be used since mitotane raises CBG (15, 22). Measurement of cortisol excretion in 24h urine collection might be a potential useful method, but the collection of multiple 24h urine samples may be problematic and difficulties in 24h urine collections are well reported (36). In addition, mitotane induces hepatic CYP3A4 which results in a rapid inactivation of cortisol (21). Another method that has been considered as a useful marker is the measurement of ACTH in serum. However, ATCH is rapidly degraded by plasma proteases, which complicates the interpretation of serum values (37). Furthermore, patients with cortisol producing ACC, might have persistent suppression of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in suppressed ACTH levels. In these cases, suppressed serum ACTH levels do not reflect hydrocortisone over-replacement but a long-term suppressed HPA-axis (38). In our study, we used a novel method to measure cortisol in scalp hair to evaluate hydrocortisone substitution in mitotane treated ACC patients. Measurement of cortisol in scalp hair has been shown to reflect long-term cortisol exposure with one cm of hair corresponding to a period of one month (23, 26, 30-31). In this study we show that hair cortisol levels are significantly elevated in mitotane treated ACC patients on hydrocortisone substitution compared to physiological levels of hair cortisol in controls. Almost half of the patients (47%) had hair cortisol levels above the upper limit of normal, suggesting that these patients received too much hydrocortisone. The positive correlation between hair cortisol and BMI supports the notion that these patients might be over-substituted, since an increase in specifically abdominal fat is a key feature of hypercortisolism (39). Importantly, none of the patients had hair cortisol levels below the lower limit of normal, suggesting that no patient was undertreated on average and threatened by

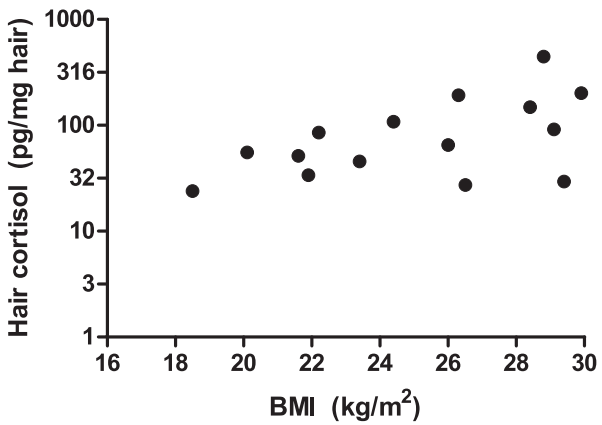

Figure 3. *Correlation between hair cortisol levels and BMI (β=0.53, p=0.042).*

adrenal crisis by underreplacement. A problem in the treatment of ACC patients with mitotane is to distinguish between mitotane side effects and cortisol deficiency, since both conditions result in similar symptoms. The measurement of cortisol in scalp hair may provide useful information regarding the cortisol exposure in these patients, and can be used to rule out or confirm hypocortisolism.

Interestingly, we did not find a correlation between hydrocortisone substitution and hair cortisol levels. In contrast, in patients with primary and secondary adrenal insufficiency it has been shown that hair cortisol levels corresponded with hydrocortisone dosage (32). The lack of correlation between hair cortisol levels and hydrocortisone substitution in mitotane treated patients can be explained by the CYP3A4 inducing effect of mitotane, resulting in an increased metabolism of hydrocortisone. However, the lack of correlation between hydrocortisone and hair cortisol levels might also be caused by the relatively low number of individuals with this rare disease. Hydrocortisone substitution did not correlate with BMI in our study, which supports the idea that hydrocortisone dosage in mitotane treated patients does not relate to tissue effects of cortisol. This underlines the need for a better tool to evaluate hydrocortisone substitution in these patients. The measurement of cortisol in scalp hair has been well validated in the past years and has been shown to be a reliable method to measure long-term cortisol exposure, both in health and disease (23, 26, 28, 30-35). Hair sample collection is easy and non-invasive, and samples can be stored in envelopes and send via mail, which makes it a simple and suitable method to use in patients. However, patients on chemotherapy with EDP (etoposid, doxorubicin and cisplatin) face the problem of chemotherapy-induced hair loss and this novel method cannot be used in these patients.

There are several limitations of our study. First, our patient group is small and heterogeneous. However, ACC is a rare disease and therefore it is difficult to obtain large homogeneous groups of patients. Still, there is a the need to validate our data in a future larger cohort. Second, although studies using hair cortisol measurements are emerging, there is still a lot unknown about the use of hair for cortisol measurements. It is not clear how and where cortisol is incorporated in the hairs. In addition, the effects of chronic disease and mitotane treatment on hair growth rate are not known. However, in patients with Cushing's Syndrome and Addison's Disease, hair cortisol timelines corresponded very well to the clinical course when assuming a hair growth rate of 1 cm per month (26, 30-31). This suggests that there is not a major influence of hyper- or hypocortisolism on hair growth rate.

In conclusion, our study is the first study that shows that hair cortisol measurements can be used to evaluate hydrocortisone substitution in mitotane treated ACC patients. We showed that almost half of the patients had hair cortisol above the upper limit of normal, which suggests that they are oversubstituted. Furthermore, hair cortisol levels, but not hydrocortisone dose, were positively associated with BMI, suggesting

that hair cortisol levels may be a better indicator of cortisol exposure in mitotane treated ACC patients than hydrocortisone dose. This novel method can contribute significantly in the evaluation of hydrocortisone substitution in ACC patients on mitotane; especially ruling out previous under-replacement and thus giving some clue for adjusting replacement in the future.

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Chapter 5

Long-term cortisol levels are not associated with *staphylococcus aureus* nasal carriage

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Abstract

Background
Staphylococcus aureus (S. aureus) colonizes the anterior nares in a part of the population and persistent carrier state is associated with increased infection risk. Knowledge concerning the determinants of S. aureus nasal carriage is limited. Previously, we found that glucocorticoid receptor polymorphisms influence carrier risk, suggesting involvement of glucocorticoids. Our aim was to study long term cortisol levels in non-carriers, intermittent and persistent carriers of S. aureus. We hypothesized that cortisol levels are higher in carriers, since cortisol-induced immune suppression would enhance S. aureus colonization.

Methods
We determined nasal carrier state and long term hair cortisol levels in 72 healthy subjects. Nasal swabs were collected twice with an interval of two weeks. Cortisol levels were determined in hair segments of three centimeter, which corresponds to a period of roughly three months.

Results
Of all 72 participants, 38 were non-carriers, 10 were intermittent carriers and 24 were persistent carriers of S. aureus. Cortisol levels did not differ between these carrier groups (p=0.638).

Conclusions
Long term cortisol levels are not associated with S. aureus nasal carriage.

Introduction

Staphylococcus aureus (S. aureus) can colonize the skin and mucosae of humans and is most frequently located in the anterior nares (1). Carriage of this pathogen is a major risk in the development of various S. aureus infections (2). Worldwide, the prevalence of meticillin-resistant strains is increasing (MRSA). Therefore, it is important to know why some people are more vulnerable to S. aureus colonization and what determines carrier state. Historically, three patterns of carriage are recognized in humans: persistent carriage, intermittent carriage and no carriage. Persistent carriers have a higher risk of developing S. aureus infections and the density of the colonization is higher compared to intermittent carriers (3). Furthermore, persistent carriers are usually colonized by one specific strain, while intermittent carriers may switch strains over time (4). However, recently we found that intermittent and noncarriers share similar S. aureus nasal elimination kinetics and antistaphylococcal antibody profiles, suggesting that there are only two types of nasal carriers, namely persistent carriers and others (5).

The pathogenesis of S. aureus nasal colonization is not completely understood. Host characteristics of the immune system seem to play a role in determining carrier status, as well as environmental and humoral factors in nasal secretions (2). Another factor that seems to influence carrier status is glucocorticoid (GC) sensitivity. Polymorphisms of the glucocorticoid receptor gene, which are associated with differences in GC sensitivity, have been associated with S. aureus carrier state (6). Homozygous carriers of the 9β polymorphism (rs6198), which is associated with a relative GC resistance with respect to transrepressional effects, had a 68% decreased risk of persistent nasal carriage. In contrast, subjects with an ER22/23EK+9β (rs6189+rs6190 and rs6198) polymorphism, which is also associated with a relative resistance, in particular with respect to the negative feedback mechanism and in various tissues, showed an 80% increased risk of being persistent carriers. The main glucocorticoid in humans is cortisol. Since cortisol is capable to suppress the immune system and polymorphisms affecting GC sensitivity seem to influence S. aureus carriage state, long term cortisol levels may differ between the different carrier states.

In this study we determined cortisol levels in scalp hair to measure long term cortisol levels. Measurement of cortisol in scalp hair is a recently developed method to measure long term cortisol levels (7-11). The important advantage of this type of cortisol measurement is that there are no limitations caused by the pulsatile secretion of cortisol, the circadian rhythm of cortisol secretion and the variability due to acute circumstances (e.g. physical or emotional stress). Hair grows at approximately 1 cm per month (12), therefore one cm of hair represents the cortisol level in one month. Previous studies showed that endogenous cortisol can be measured reliably in scalp hair (7-11). We hypothesize that higher hair cortisol levels are associated with persistent nasal carriage of S. aureus.

Methods

Study population

Eighty healthy individuals participated in this study. All subjects filled out a questionnaire regarding gender, age, the use of hair products, hair treatment (perm, coloring, bleaching) and medical status (autoimmune disease and glucocorticoid use). Participants were excluded if they used glucocorticoids during the last three months or had an autoimmune disease. The study was approved by the Medical Ethics committee of the Erasmus Medical Center and all participants gave informed consent.

S. aureus nasal carriage

Nasal swabs were collected twice with an interval of two weeks and were analyzed as previously described (3). Subjects were defined as persistent carriers when both cultures were positive, intermittent carriers when one of the cultures was positive and non-carriers when none of the cultures was positive for S. aureus.

Hair collection and preparation

From all participants, approximately 100 hairs were cut from the posterior vertex. The 3 cm proximal to the scalp was used for analysis. Since hair grows with an average rate of 1 cm per month, this corresponds roughly to cortisol levels in the last three months. A detailed description of hair preparation has been given by Sauvé et al. (9). In brief, the 3 cm hair segment was weighted and cut into small pieces in a glass vial with surgical scissors. 1 mL of methanol was added and the vial was incubated for 16 hours overnight at 52 °C, while gently shaking. The methanol was then transferred into a glass tube and evaporated under nitrogen. When dry, the samples were dissolved in 250 µL Phosphate Buffered Saline (pH 8.0) and vortexed for 1 minute followed by a 30 second vortex before analysis.

Hair analysis

Cortisol levels were measured using a commercially available ELISA Kit for Salivary Cortisol (DRG International Inc, USA) as per manufacturer's directions with the reagents provided. Cross reactivity of other steroids with the kit's antibodies was reported as follows: Corticosterone (29.00%), Cortisone (3.00%), 11-Deoxycortisol (<1.00%), 17-OH Progesterone (<0.50%), other hormones (<0.10%). Intra assay variation was below 5% and the inter-assay variation below 8% as stated by the manufacturer.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL, USA). Differences in baseline characteristics between the different carrier state groups were tested using Chi-square tests and ANOVA. Cortisol levels

were log transformed to obtain a normal distribution. ANOVA was used to test the effects of gender, hair treatment and the use of hair products on cortisol levels. With an ANCOVA the relationship between cortisol levels and S. aureus carrier state was tested, while controlling for gender, age and hair treatment.

Table 1. *Group characteristics*

	Non carriers n=38	Intermittent carriers n=10	Persistent carriers n=24	p-value
Age (years)	35.42 (21-60)	41.00 (22-58)	33.71 (19-55)	.272
Gender (% women)	24 (63.2%)	6 (60%)	13 (54.2%)	.781
Hair treatment	8 (21.1%)	3 (30%)	8 (33.3%)	.543
Use of hair products	17 (44.7%)	8 (80%)	8 (34.8%)	.054

Data are shown number (%). Age is shown as mean (minimum-maximum). Hair treatment includes dyeing, bleaching and permanent waving. Hair products used were spray, mousse, gel and wax.

Results

After exclusion of subjects who used glucocorticoids in the last three months or had an autoimmune disease, 72 individuals were analyzed. Carrier group characteristics are presented in Table 1. There were no significant differences in group characteristics.

After log transformation, hair cortisol levels were normally distributed (Kolmogorov-Smirnov p=0.200). Hair cortisol levels were significantly lower in the hair treatment group compared to the untreated hair group (23.77 pg/mg hair versus 33.42 pg/mg hair, $F(1,70)=8.295$, $p=0.005$). No significant effects of gender $F(1,70)=2.428$, $p=0.124$) or use of hair product ($F(1,69)=0.106$, $p=0.746$) on cortisol levels was found. There was no correlation between age and hair cortisol ($r_s=-0.028$, $p=0.816$).

The mean cortisol levels of the carrier groups are shown in Figure 1. Hair cortisol levels did not significantly differ in the last 3 months between carrier groups ($F(2,66)=0.425$, $p=0.638$, corrected for hair treatment, age, gender). In addition, cortisol levels in the combined group of non-carriers and intermittent carriers were not significantly different from cortisol levels in persistent carriers ($F(1,67)=0.837$, $p=0.363$).

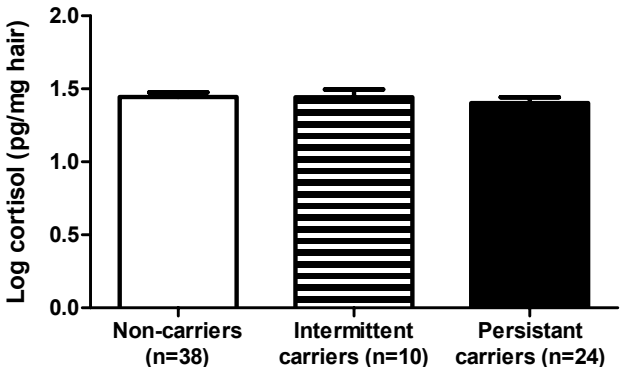

Figure 1. *Mean cortisol levels in the carrier groups. Error bars represent SEM.*

Discussion

Our study shows that there are no differences in long term cortisol levels, as measured in scalp hair, between non-carriers, intermittent carriers and persistent carriers of S. aureus. To our knowledge, this is the first study that determined hair cortisol levels with regard to S. aureus nasal carriage status. Measurement of cortisol in scalp hair is a recently developed method to measure long term cortisol levels and seems a reliable measure of long term cortisol exposure, as shown in several other studies (7-11). Higher cortisol levels during a longer period of time could lead to an immunosuppressive effect and subsequently to increased S. aureus colonization. Theoretically, higher cortisol levels in scalp hair could be associated with persistent nasal carriage of S. aureus. However, in the present study we did not observe chronically higher cortisol levels in persistent nasal S. aureus carriers.

Interestingly, polymorphisms in the glucocorticoid receptor gene seemed to influence S. aureus carrier state. We previously showed that the 9β polymorphism was associated with a 68% decreased risk of S. aureus carriage (6). This polymorphism is thought to increase the stability of the GR-β isoform, which is a dominant negative inhibitor of the active GR-α, and therefore causes a relative GC resistance. This relative resistance could lead to decreased suppression of the immune system by GCs, which would result in a more active immune system, yielding a decreased risk of S. aureus colonization. This was indeed the main finding of that study. Furthermore, the ER22/23EK polymorphism of the GR gene was found to be associated with an increased risk of S. aureus carriage. The ER22/23EK polymorphism is associated with a relative resistance for GCs for its transactivating effect, but this polymorphism may not affect the immune system since normal transinhibition of the proinflammatory nuclear factor NF-κB was observed (13). Although numerous effects throughout the body have been reported for all these GR polymorphisms, no differences in serum cortisol levels have been described (14), suggesting that the

mildly altered GC sensitivity is mostly present at the tissue level with a potential cell-specific effect. Since GR polymorphisms only slightly affect GC sensitivity with respect to the negative feedback mechanism, no evident differences in cortisol levels may be observed. This would explain why we did not find any differences in long term cortisol levels between carrier states despite the fact that there was a clear effect of GR polymorphisms. In our study population, GR polymorphisms were not determined, therefore an interaction between long term cortisol levels and GR polymorphisms could not be studied.

Since cortisol levels seem not to significantly influence risk of S. aureus colonization, possibly genetics and other host factors are more important. This is also supported by the finding that non-carriers when inoculated with different S. aureus strains quickly become non-carriers again, while persistent carriers select the strain they were carrying before (15). These findings suggest that individuals are quite stable regarding their carrier state. Cortisol levels can change over time due to emotional or physical stress and are therefore not necessarily stable and possibly a less important host factor for S. aureus colonization. Nevertheless, we cannot rule out that cortisol may indirectly play a role in S. aureus colonization by interacting with host factors of the immune system and an individual's genetic make-up. Interestingly, persistent S. aureus colonization does not seem to lead to greater physical stress since cortisol levels would then be increased in persistent carriers.

In conclusion, we found no differences in long term cortisol levels, as measured in scalp hair between non-carriers, intermittent carriers and persistent carriers of S. aureus in the anterior nares. Thus, cortisol levels seem not to significantly affect S. aureus carriage state.

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Chapter 6

Shift work at young age is associated with elevated long-term cortisol levels and body mass index

Manenschijn L, van Kruysbergen RGPM, de Jong FH, Koper JW, van Rossum EFC

Journal of Clinical Endocrinology and Metabolism, 2011 Nov; 96 (11): E1862-1865

Abstract

Background
The incidence of obesity and other features of the metabolic syndrome is increased in shift workers. This may be due to a misalignment between the internal circadian rhythm and the behavioral rhythm. The stress-hormone cortisol could play a role in this phenomenon since it is secreted in a circadian rhythm and long-term elevated cortisol leads to components of the metabolic syndrome. We compared cortisol levels in scalp hair of shift and day workers to study changes in long-term cortisol due to shift work.

Methods
Hair samples were collected from 33 shift workers and 89 day workers. Cortisol was extracted from the hair samples with methanol and cortisol levels were measured using ELISA. Height and weight were measured and BMI was calculated.

Results
Shift workers had higher hair cortisol levels than day workers (47.32 pg/mg hair (95% Confidence Interval (CI): 38.37-58.21) versus 29.72 pg/mg hair (95% CI: 26.18-33.73), $p< 0.001$). When divided in age groups based on the median age, elevated cortisol levels were only present in younger shift workers (48.53 pg/mg hair (95% CI: 36.56-64.29) versus 26.42 pg/mg hair (95% CI: 22.91-30.55), $p<0.001$). BMI was increased in younger shift workers as well (27.2 (95% CI: 25.5-28.8) versus 23.7 (95% CI: 22.8-24.7) in young day workers, $p=0.001$). Hair cortisol and BMI were positively correlated ($\beta= 0.262$, $p=0.005$).

Conclusions
Shift work at young adult age is associated with elevated long-term cortisol levels and increased BMI. Elevated cortisol levels and BMI may contribute to the increased cardiovascular risk found in shift workers.

Introduction

Shift work, defined as work performed primarily outside standard working hours, has been associated with increased incidences of obesity and other features of the metabolic syndrome, such as hypertension, hyperlipidemia and insulin resistance, ultimately leading to an increased incidence of cardiovascular disease (1-3). It is hypothesized that these health problems in shift workers are caused by misalignment between the endogenous circadian system and the behavioral cycles (4-5). One of the factors that could play a role in the development of the metabolic syndrome in shift workers is the stress-hormone cortisol. Cortisol is secreted in a circadian rhythm with high levels in the early morning and low levels in the evening and night. Pathologically high levels of cortisol are associated with abdominal obesity, insulin resistance, hypertension and dyslipidemia, all features of the metabolic syndrome (6-7). Changes in behavioral cycles due to shift work could result in disruption of the circadian rhythm of cortisol secretion, resulting in hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, leading to long term elevated cortisol levels. Several studies have investigated cortisol rhythms in shift workers and found that the cortisol awakening response is decreased and evening cortisol levels are increased during shift work (8-10). Whether these changes in the cortisol secretion result in long term changes in cortisol exposure has not been studied, probably because there was no suitable method to measure long term cortisol levels. Recently, a novel method was developed to measure long term cortisol levels in scalp hair, with one cm of hair representing a period of approximately one month (11-13). This method is very suitable to evaluate changes in cortisol levels in shift workers over a prolonged period of time. Our aim was to investigate long term hair cortisol levels in shift workers in comparison with individuals working only during the day. In addition, we hypothesized that BMI is higher in shift workers and is correlated to long term cortisol levels.

Methods

Subjects

Fifty male shift workers from a textile factory in the Netherlands were asked to participate in this study. Seventeen of them were excluded due to glucocorticoid use or insufficient hair growth at the posterior vertex. All 33 remaining participants worked in the same factory and were working in a fast forward rotating shift schedule. The morning shift started at 6:00 and continued to 14:00, the afternoon shift started at 14:00 until 22:30 and the night shift started at 22:30 until 6:00. All participants worked continuously 2 days in morning shift, two days in evening shift and two days in night shift with four days of rest after the night shift. After the rest days, they started again with two days of day shift etc. Age at the start of working in shifts and the number of years in shift work were documented.

As a control group, we used the 89 healthy men from our previous study (13). All subjects worked only during the day and did not use glucocorticoids. From both shift and day workers height and weight were measured and Body Mass Index (BMI) was calculated. This study was approved by the Medical Ethical Committee and all participants gave written informed consent.

Hair collection, preparation and analysis

A detailed description of the methods used to measure cortisol in scalp hair can be found elsewhere (12-13). In brief, hair samples were cut from the posterior vertex of the scalp, as close to the scalp as possible. The most proximal 3 cm of the hair strands were used, corresponding roughly to a period of three months. Cortisol was extracted from the hair samples by overnight incubation in methanol. After incubation, methanol was evaporated under a stream of nitrogen and the samples were dissolved in Phosphate Buffered Saline (PBS pH 8.0). Cortisol was measured using a salivary ELISA cortisol kit (DRG Instruments GmbH, Marburg, Germany).

Statistical analysis

Statistical analyses were performed with SPSS 17.0 and GraphPad Prism 5.0. Mann-Whitney-U tests and Chi-square tests were used to determine differences in group characteristics. Cortisol levels were log transformed to obtain a normal distribution. ANCOVA was used to investigate differences in cortisol levels between shift workers and day workers. Linear regression was used to determine correlations between cortisol and BMI.

Table 1. *Group characteristics*

	Day workers (n=89)	Shift workers (n=33)
Age (years)	33 (19-63)	41 (27-62)[a]
Caucasian ethnicity	83 (94.3%)	25 (75.8%)[b]
Frequency of hair wash		
≤ 2 times per week	22 (25%)	2 (6.3%)[a]
≥ 3 times per week	66 (75%)	30 (93.8%)[a]
Use of hair products	47 (52.8%)	13 (40.6%)
BMI (g/m^2)	24.6 (18.4-34.6)	27.0 (19.2-33.9)[b]
Duration of shift work (years)		7 (2-42)

Data are shown as median (range) or number (percentage)
[a] *p<0.05*
[b] *p<0.01*

Results

Group characteristics are shown in table 1. Shift workers were significantly older, washed their hair more frequently and had a higher BMI than day workers. Mean hair cortisol levels were significantly higher in the shift workers than in the day workers (47.32 pg/mg hair (95% Confidence Interval (CI): 38.37-58.21) versus 29.72 pg/mg hair (95% CI: 26.18-33.73), p< 0.001). After adjustment for age, BMI and frequency of hair wash this difference remained significant (p=0.01). Since the age of shift workers and day workers was significantly different, we divided both groups in two subgroups based on the median age (41 years) of the shift workers. With no individuals aged 40, the subgroups were <40 years of age and >40 years of age. In the group <40 years of age, hair cortisol levels were significantly higher in the shift workers (n=14) than in the day workers (n=54) (48.53 pg/mg hair (95% CI: 36.56 – 64.29) versus 26.42 pg/mg hair (95% CI: 22.91-30.55), p<0.001), also after adjustment for age, BMI and frequency of hair wash (p=0.004). However, in the older group, there were no significant differences in hair cortisol levels between shift workers (n= 19) and day workers (n= 35) (p=0.171 unadjusted) (Figure 1). We found no correlations between cortisol and age at the start of working in shifts (p=0.376) and duration of working in shifts (p=0.476).

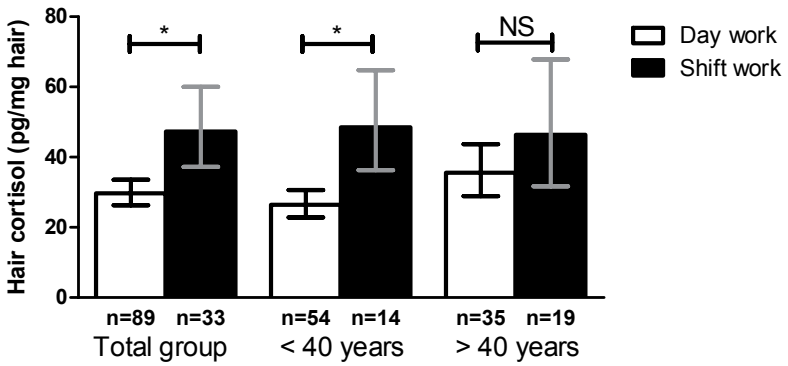

Figure 1. *Long-term cortisol levels in shift workers and day workers in total and divided in age groups, based on the median age of shift workers. * p< 0.001 (unadjusted); n= number; NS= not significant. Data are shown as geometric mean with 95% Confidence Interval.*

BMI was significantly higher in the shift workers compared to day workers. When divided in age subgroups this difference was only present in the younger group. Young shift workers had a mean BMI of 27.2 (95% CI: 25.5-28.8), whereas young day workers had a mean BMI of 23.7 (95% CI: 22.8-24.7) (p=0.001). At older age, there was no difference in BMI between both groups (p=0.508). Since we found a similar pattern for BMI and hair cortisol, with the main difference between young day and

shift workers, we investigated whether there was an association between hair cortisol and BMI in this group. We found a significant positive correlation between cortisol and BMI with β=0.262 (p=0.005 unadjusted, p=0.01 after adjustment for age). Furthermore, when the total group was divided in groups based on BMI classification with normal individuals having a BMI <25 kg/m2, overweight individuals with a BMI of 25-30 kg/m2 and obese individuals with a BMI >30 kg/m2, we found that cortisol increased significantly through the groups, with a mean cortisol level of 31.26 pg/mg hair (95% CI: 26.79–36.48) in individuals with a normal BMI, a cortisol of 36.06 pg/mg hair (95% CI: 30.48–42.76) in overweight individuals and 60.95 pg/mg hair (95% CI: 43.95–84.72) in obese individuals (p=0.002).

Discussion

The main finding of our study is that long-term cortisol levels, measured in scalp hair, are significantly increased in individuals working in shifts, especially in the group younger than 40 years of age. At older age, there is no difference in hair cortisol levels between shift and day workers. The same pattern was found for BMI, with higher BMI in young shift workers compared to young day workers and no difference in BMI at older age. Hair cortisol levels and BMI were positively correlated.

Our results indicate that the previously documented changes in circadian rhythm of cortisol secretion in shift workers (8-10) result in a long term elevation of cortisol levels. This appears to be especially the case in the younger group of shift workers, which may be important, since long term elevated cortisol levels and obesity at younger age can contribute significantly to increased cardiovascular risk at older age. Hair cortisol levels in shift and day workers at older age were similar, suggesting that older individuals working in shifts suffer less from stress or adjust better to shift work than younger individuals, resulting in a habituation to the alterations in the HPA-axis. This contrasts with the general hypothesis that the tolerance to shift work decreases with age. Several studies investigating this issue report inconclusive results. Some studies found no effect of age on the tolerance to shift work (14-15) and other studies have shown a decreased tolerance in older shift workers (16-17). These contrasting findings may be caused by the differences in study population and outcome measurements used in these studies. In our study we measured long term cortisol levels, a biological marker for chronic stress, and we did not find differences between older day workers and shift workers. This may theoretically be explained by selection of individuals who have a higher tolerance for shift work and therefore still work in shifts at older age, whereas individuals who cannot adjust to shift work already stopped working in shifts at a younger age. A negative correlation between hair cortisol and number of years working in shifts would support this hypothesis. However, we did not see this in our study group. Another explanation for the lack of increased cortisol levels in older shift workers could be that the impact of shift work on the circadian rhythm is not as strong in older individuals as in younger individuals, since the circadian rhythm and sleep pattern change during aging.

Our finding of increased long-term hair cortisol levels in shift workers is of particular importance, since cortisol may contribute to the increased prevalence of obesity and cardiovascular risk that is found in shift workers. This finding is supported by our observation of an increased BMI in shift workers and the positive correlation between hair cortisol and BMI. Interestingly, our shift workers all worked in a fast forward shift schedule, which is supposed to be less deleterious than backward shift schedules (18-19). The changes in long term cortisol might be even more abundant in shift workers working in other schedules.

Unravelling the role of cortisol in the health problems found in shift workers could result in new approaches to prevent cardiovascular damage in this specific group. However, our study was limited by a relatively small number of shift workers and lack of data concerning diet, exercise, education level, social economic status, perceived stress and different shift schedules. Nevertheless, the differences found between day workers and shift workers may be of significance and more research is needed to truly reveal the role of cortisol and its potential negative effects in shift workers.

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Chapter 7

Shift work at older age is not associated with changes in long-term cortisol levels, body mass index and perceived stress

Manenschijn L, van Kruysbergen RGPM, Rodenburg W, van Steeg H, van Rossum EFC

Submitted

Abstract

Background
Shift work is associated with an increased incidence of the metabolic syndrome and cardiovascular disease. Cortisol might play a role in this association. In a previous study we found that shift work at young age was associated with elevated hair cortisol levels, a measure of long-term systemic cortisol levels, and higher BMI. The aim of this study was to replicate these previous findings and to study additional potentially influencing factors such as physical activity, diet patterns, type of shift schedule and perceived stress.

Methods
Hair samples were collected in 59 male employees of a textile factory in the Netherlands: 19 day workers, 19 shift workers in a fast forward rotating shift schedule (schedule 1) and 21 shift workers in a slow backward rotating shift schedule (schedule 2). Cortisol was extracted from the hair samples with methanol and an ELISA was used to measure cortisol levels. Height, weight, waist and hip circumferences were measured and data regarding physical activity, diet patterns and perceived stress were collected by means of questionnaires.

Results
We found no differences in age, BMI, waist circumference, waist-to-hip ratio (WHR) and perceived stress. Mean age was 50 years in day workers and 48 years in shift workers. The shift workers were more physically active than day workers and drank more soda drinks per week. Hair cortisol levels were 22.1 pg/mg hair (95% CI=17.5-28.0) in day workers, 23.9 pg/mg hair (95% CI=19.0-30.3) in shift workers in shift schedule 1 and 29.4 pg/mg hair (95% CI= 23.6-36.7) in shift workers working in shift schedule 2. These hair cortisol levels were not significantly different between day workers and shift workers in shift schedule 1 or between shift workers in schedule 1 and schedule 2. There was a tendency towards higher hair cortisol levels in shift workers in schedule 1 compared to day workers (p=0.08)

Conclusions
Shift work at older age does not result in evident changes in long-term cortisol levels and body composition, although a slow backward rotating shift schedule seems to result in slightly higher long-term cortisol levels than day work or a fast forward rotating schedule, despite no differences in perceives stress. Future studies should focus on evaluating changes in long-term cortisol levels, body composition and life style factors in young adult shift workers.

Introduction

Shift work has been associated with an increased risk of developing the Metabolic Syndrome and cardiovascular disease (1-3). The exact pathway through which this develops is not known, but it has been hypothesized that the hormone cortisol might play a role in this. Cortisol is secreted in a circadian rhythm with high levels during the early morning and low levels during the evening and night. Furthermore, pathologically high levels of cortisol are associated with abdominal obesity, insulin resistance, hypertension and dyslipidemia (4-5). These factors are all features of the metabolic syndrome. Several studies have found that shift work influenced the circadian rhythm of cortisol secretion, leading to a decreased cortisol awakening response and increased cortisol levels during the evening and night (6-8). Whether these changes in circadian rhythm result in changes in long-term cortisol levels was not known since it was difficult to study long-term cortisol levels. In the past few years a novel method to measure long-term cortisol levels has been developed. By measuring cortisol in scalp hair, it is possible to measure long-term cortisol levels with one centimetre of hair corresponding to a period of roughly one month. This method has been well validated, and it has been shown that cortisol levels in scalp hair reflect endogenous cortisol exposure in healthy individuals, chronic stress and individuals with hypercortisolism (9-19). In our previous study, we used this novel method to measure long-term cortisol levels in shift workers working in a fast-forward rotating shift schedule and day workers (20). We found that shift workers had elevated hair cortisol levels compared with day workers, and body mass index (BMI) was higher in shift workers as well. Interestingly, when stratified by age, we found that the increase in cortisol and BMI was only present in the younger adult shift workers (< 40 years of age) and not in older shift workers (20). Since our previous study is the first and only study that has measured long-term cortisol levels in shift workers, it is necessary to replicate the findings. Furthermore, in our previous study, we only included shift workers working in a fast-forward rotating shift schedule. This type of shift schedule is thought to be the least detrimental (21-22). This suggests that the effects of other types of shift schedules might even be more abundant. Therefore, we collected hair samples in other groups of shift workers with different working schedules and day workers and measured long-term cortisol levels. In addition, we measured anthropometric parameters and collected data concerning physical activity, diet patterns and perceived stress.

Methods

Subjects

Male shift workers and day workers from a textile factory in the Netherlands were asked to participate in this study. In total, 83 men were willing to participate and had sufficient hair growth at the posterior vertex of the scalp for hair cortisol analysis.

Twenty-four participants were excluded due to glucocorticoid use. Of the 59 remaining participants, 19 men worked only during the day, 19 men worked in the fast forward rotating shift schedule (schedule 1) and 21 men worked in a slow backward rotating shift schedule (schedule 2). Individuals working in the fast forward rotating shift schedule worked continuously 2 days in the morning shift (6:00 – 14:00h), 2 days in the evening shift (14:00-22:30h) and 2 days in the night shift (22:30-6:00h). After the night shift, they had 4 days of rest and then started again with the morning shift, evening shift etc. Individuals working in the slow backward rotating shift schedule worked only on week days and not in weekends. They first worked 5 days in the morning shift, from 6:00 to 14:00, then 5 days in the night shift, from 22:30 to 6:00 and then 5 days in the evening shift, from 14:00 until 22:30. After the evening shift they started again with the morning shift, followed by the night shift and then the evening shift, etc. The study was approved by the Medical Ethical Committee and all participants gave written informed consent.

Data collection

From all participants height, weight, waist and hip circumference was measured and BMI and waist-to-hip ratio (WHR) was calculated. Questionnaires were used to asses physical activity (International Physical Activity Questionnaire (IPAQ) short form) (23), diet pattern, alcohol use (Garretsen alcohol index) (24), smoking status (current, former and never) and perceived stress (PSS) (25).

Hair collection, preparation and analysis

Hair sample collection and preparation was done in accordance with a previously described method (12, 14). In brief, hair samples of approximately 100-150 hairs were collected from the posterior vertex of the scalp and cut of as close to the scalp as possible. In this study, we used the 3 cm of hair closest to the scalp, which reflects the period 3 months prior to hair sample collection. Hair samples were weighed and cut into small pieces of 1-2 mm and methanol was added to extract cortisol from the hair samples. After an extraction period of 16 hours at 52°C the methanol was transferred into clean vials and the methanol was evaporated under a stream of nitrogen. The samples were dissolved in Phosphate Buffered Saline (PBS, pH 8.0) and an salivary ELISA cortisol kit (DRG Instruments GmbH, Marburg, Germany) was used to measure cortisol levels in hair extracts.

Statistical analyses

Statistical analyses were performed with SPSS 17.0 and GraphPad Prism 5.0. Mann-Whitney U tests and Chi-square tests were used to determine differences between shift workers and day workers. Hair cortisol levels were logarithmically transformed to obtain a normal distribution. ANCOVA was used to investigate differences in cortisol levels between shift schedule 1, shift schedule 2 and day workers. Linear regression was used to determine correlations between cortisol levels and other continues variables such as BMI, WHR, PSS score, age, duration of shift work etc.

Table 1. *Group characteristics*

	Day workers (n=19)	**Shift schedule 1 (n=19)**	**Shift schedule 2 (n=21)**
Age (years) – median (IQR)	50 (47-53)	48 (41-58)	48 (42-53)
Shift duration (years) – median (IQR)	13 (5-25)	15 (12-23)	19 (10-23)
Alcohol			
No – n(%)	4 (21.1%)	3 (15.8%)	3 (14.3%)
Light – n(%)	9 (47.4%)	9 (47.4%)	7 (33.3%)
Moderate – n(%)	5 (26.3%)	6 (31.6%)	8 (38.1%)
Excessive – n(%)	1 (5.3%)	1 (5.3%)	3 (14.3%)
Smoking status			
Never – n(%)	7 (36.8%)	10 (52.6%)	4 (19.0%)
Former – n(%)	4 (21.1%)	2 (10.5%)	5 (23.8%)
Current – n(%)	8 (42.1%)	7 (36.8%)	12 (63.2%)
Physical activity			
Low – n(%)	1 (6.3%)	0 (0.0%)	0 (0.0%)
Moderate – n(%)	12 (75.0%)	4 (25.0%)	7 (36.8%)
High – n(%)	3 (18.8%)	12 (75.0%)**	12 (63.2%)*
Number of snacks per week – median (IQR)	9 (6-12)	11 (8-17)	8 (6-14)
Number of soda drinks per week – median (IQR)	2 (2-8)	6 (3-13)*	14 (9-17)**
BMI (kg/m2) – median (IQR)	26.0 (25.0-29.2)	26.0 (24.3-27.4)	26.8 (24.4-28.1)
Waist (cm) – median (IQR)	98.5 (94.8-103.0)	94.0 (84.0-101.0)	96.0 (90.5-105.5)
WHR – median (IQR)	0.96 (0.93-1.02)	0.94 (0.91-0.99)	0.95 (0.92-1.02)
Perceived Stress Scale score	10 (5-13)	11 (9-16)	12 (8-15)

**p<0.05, **p<0.01*

Results

Group characteristics are shown in Table 1. There were no significant differences in age, duration of work, BMI, WHR, alcohol use, smoking status and perceived stress between the groups (Table 1). With regards to diet pattern, we found no differences in frequency of breakfast and warm meals per week, no differences in the amount of fruit and vegetables per week and no difference in number of snacks per week

between day workers and shift workers in two types of shift schedules. However, the number of soda drinks per week was significantly higher in individuals working in shift schedule 1 compared to day workers ($p<0.001$) and shift workers in shift schedule 2 ($p=0.03$). Shift workers were more physically active than day workers ($p=0.02$ and $p=0.006$ for shift schedule 1 and 2 respectively versus day workers) (Table 1). Shift workers were in particular more physically active during working hours compared to day workers.

As shown in figure 1 hair cortisol levels were 22.1 pg/mg hair (95% CI=17.5-28.0) in day workers, 23.9 pg/mg hair (95% CI=19.0-30.3) in shift workers working in shift schedule 1 and 29.4 pg/mg hair (95% CI= 23.6-36.7) in shift workers in shift schedule 2. There was no significant difference in hair cortisol levels between day workers and shift workers in schedule 1 ($p=0.64$), or between shift workers in schedule 1 and shift workers in schedule 2 ($p=0.21$). There was a tendency towards higher hair cortisol levels in shift workers in schedule 2 compared to day workers ($p=0.08$) (Fig 1).

We found no correlations between hair cortisol levels and age ($p=0.74$), BMI ($p=0.58$), waist circumference ($p=0.70$), WHR ($p=0.93$) and perceived stress ($p=0.09$) in the total group nor after stratification based on their (shift) work schedule.

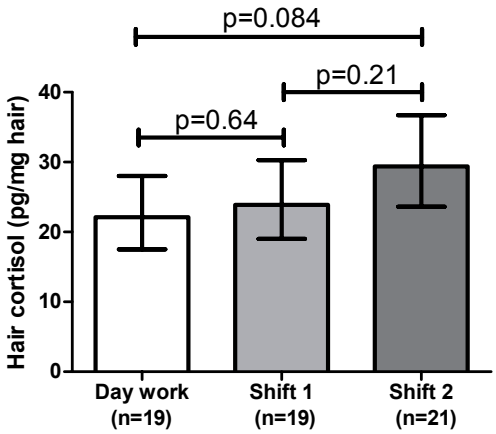

Figure 1. *Hair cortisol levels in day workers and shift workers in a fast forward rotating shift schedule (shift 1) and a slow backward rotating shift schedule (shift 2).*

Discussion

In this study, we found no differences in hair cortisol levels, BMI, WHR and perceived stress between day workers and shift workers in two different shift schedules. Furthermore, hair cortisol levels were not correlated to BMI, WHR of perceived stress.

The intention of our study was to further explore our previous finding of elevated long-term cortisol levels in scalp hair of shift workers. In this study we included two groups of shift workers with different shift work schedules. The first working in fast forward rotating shift schedule and the second working in a slow backward rotating shift schedule. The fast forward rotating shift schedule was similar to the shift schedule of the participants in our previous study. In contrast to our previous study, we did not find any differences in hair cortisol levels between day workers and shift workers in a fast forward rotating shift schedule (20). However, in our previous study the finding of elevated hair cortisol levels was only statistically significant in the group <40 years of age. The group of shift workers aged >40 years had similar hair cortisol levels as day workers aged >40 years (20). The mean age of participants in the current study was 50 years (48 years in shift workers) and only 10 participant of the total group were aged <40 years (2 in day work, 4 in shift schedule 1 and 4 in shift schedule 2). Hence, this study confirms that there are no differences in long-term cortisol levels between shift workers in a fast forward rotating shift schedule and day workers at older age.

Interestingly, we found a tendency towards higher hair cortisol levels in shift workers working in a slow backward rotating shift schedule compared to day workers. Several studies have suggested that slow backward rotating shift schedules lead to more detrimental effects, such as increased fatigue, sickness absence and decreased alertness, than fast forward rotating shift schedule (21-22). The tendency towards higher long-term cortisol levels in shift workers in a slow backward rotating shift schedule suggests that this type of shift schedule might lead to a larger misalignment between the biological circadian rhythm and the behavioural rhythm, resulting in higher long-term cortisol levels. This trend is already present in our group of 21 older aged shift workers. These effects of this type of shift schedule might be even more distinct in younger shift workers. Additional studies are necessary to study the effects of a slow backward rotating shift schedule on long-term cortisol levels in younger adults.

BMI, waist circumference and WHR were not significantly different between day workers and the two groups of shift workers. This is in line with our previous study in which we did not find a difference in BMI between older day and shift workers (20). Our results suggest that shift work at older age does not have the same detrimental effects as shift work at younger age. There are only a few studies which investigated

the effect of age on the tolerance to shift work and as far as we know, none of these studies studied cortisol levels. Most studies evaluated sleep quality, insomnia and work related injuries or accidents and some studies reported a decreased tolerance to shift work at older age, whereas others did not find this age-related decrease in shift work tolerance (26-28). An explanation for these contrary findings might be the differences in study population, outcome measurements and type of shift schedules. Our finding that shift work at older age does not affect long-term cortisol levels or BMI might be explained by a selection bias. Individuals with the highest tolerance for shift work will continue to work in shifts during a longer period than shift workers with a low tolerance for shift work. When studying a group of older shift workers, it might be that selection of individuals with a high tolerance to shift work has occurred. Hence, the effects of shift work could be more evident in younger shift workers and it would therefore be more important to study cortisol, body composition, diet patterns, physical activity and perceived stress in younger adult shift workers. The effects of shift work at younger age could be more detrimental, since elevated cortisol levels and obesity induced at younger age will result in earlier onset of cardiovascular damage and development of type 2 diabetes.

We also found no differences in perceived stress between shift workers and day workers and physical activity was even higher in shift workers than in day workers. The higher physical activity in shift workers is probably due to the differences in work. Day workers sit mainly behind a desk, whereas shift workers have a more active job. Our results suggest that the detrimental effects of shift work cannot be explained by more perceived stress or less physical activity in shift workers, since perceived stress was similar as in day workers and shift workers were more physically active.

In conclusion, we confirmed our previous finding that shift work at older age does not result in significant changes in long-term cortisol levels and body composition. Furthermore, there are no clear differences in physical activity, diet patterns and perceived stress between older shift and day workers that can explain the detrimental effects of shift work. However, we found a trend towards elevated long-term cortisol levels in employees working in a slow backward rotating shift schedule, which supports the previous studies reporting that this type of schedule induces more adverse health effects than day work or a fast forward schedule. Future studies focusing on evaluating the changes in long-term cortisol levels, type of working schedule, body composition and life style factors in young adult shift workers are needed.

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Chapter 8

High long-term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease

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Abstract

Background
Stress is associated with an increased incidence of cardiovascular disease. The impact of chronic stress on cardiovascular risk has been studied by measuring cortisol in serum and saliva, which are measurements of only 1 time point. These studies yielded inconclusive results. The measurement of cortisol in scalp hair is a novel method that provides the opportunity to measure long-term cortisol exposure. Our aim was to study whether long-term cortisol levels, measured in scalp hair, are associated with cardiovascular diseases.

Methods
A group of 283 community-dwelling elderly participants were randomly selected from a large population-based cohort study (median age 75 years, range 65-85 years). Cortisol was measured in 3 cm hair segments, corresponding roughly with a period of 3 months. Self-reported data concerning coronary heart disease, stroke, peripheral arterial disease, diabetes mellitus, and other chronic non-cardiovascular diseases were collected.

Results
Hair cortisol levels were significantly lower in women than in men (21.0 pg/mg hair vs. 26.3 pg/mg hair, $p<0.001$). High hair cortisol levels were associated with an increased cardiovascular risk (OR 2.7, $p=0.01$) and an increased risk of type 2 diabetes mellitus (OR 3.2, $p=0.04$). There were no associations between hair cortisol levels and non-cardiovascular diseases.

Conclusions
Elevated long-term cortisol levels are associated with a history of cardiovascular disease. The increased risk we found is equivalent to the effect of traditional cardiovascular risk factors, suggesting that long-term elevated cortisol may be an important cardiovascular risk factor.

Introduction

Stress is thought to be one of the main factors negatively impacting health, resulting in an increased incidence of obesity, type 2 diabetes mellitus and cardiovascular disease (1-3). The effects of stress are mediated by the stress hormone cortisol. Cortisol is involved in the regulation of glucose and lipid metabolism, body composition and the immune system (4). Long-term pathologically elevated cortisol levels, as extensively described in patients with extreme endogenous cortisol production (Cushing's Syndrome) and in patients using glucocorticoids (5-7), are associated with increased visceral fat mass, atrophy of the proximal muscles, hypertension, insulin resistance, and dyslipidemia, which all results in an increased cardiovascular risk (8-9). The impact of slightly elevated cortisol levels, e.g. due to chronic stress, is less clear. Several studies reported that higher cortisol levels were associated with a higher incidence of cardiovascular disease or cardiovascular risk factors (10-12). However, other studies have not shown associations between cortisol and cardiovascular risk factors (13-14) or reported that low cortisol levels were associated with cardiovascular risk factors (15). In most of these studies, cortisol was measured in serum or saliva. Because cortisol is secreted in a circadian rhythm and with pulses, the timing of sample collection is crucial when measuring cortisol in serum or saliva. In addition, cortisol is a stress hormone, and acute stress, e.g. caused by the research setting or venipuncture, will influence the measurement. A single measurement of cortisol in serum or saliva therefore poorly reflects long-term cortisol levels. An alternative method to measure cortisol is in scalp hair. This method offers long-term measurements of cortisol levels, with 1 cm of hair representing cortisol levels of 1 month. In the last few years the measurement of cortisol in scalp hair has been well validated (16-19). Previous studies have already shown that chronic stress is associated with elevated hair cortisol levels (20-23). Therefore, the measurement of long-term cortisol levels in scalp hair may be a more accurate tool to study the association between cortisol and cardiovascular disease. The aim of our study was to investigate whether there is an association between long-term cortisol levels and cardiovascular disease in community-dwelling older persons. We hypothesized that high long-term cortisol levels, measured in scalp hair, are associated with an increased cardiovascular risk.

Methods

Participants

A randomly selected group of 574 individuals participating in the Longitudinal Aging Study Amsterdam (LASA) were asked to participate in this study. LASA is a large population based cohort study on predictors and consequences of changes in physical, cognitive, emotional and social function in older persons. Detailed information concerning data collection and sampling have been described previously (24-25).

The current study was performed as a substudy of LASA and was performed in the period October 2010 to June 2011. Of the 574 individuals asked to participate in this substudy, 244 refused to participate or did not have sufficient hair growth at the posterior vertex. Of the 330 individuals who were willing to participate, 47 were excluded because of glucocorticoid use in the 6 months prior to hair sample collection. This study was approved by the local ethics committee and all participants gave written informed consent.

Data collection

Body height was measured using a stadiometer, body weight was measured using a calibrated balance beam scale (without wearing upper clothing and shoes) and body mass index (BMI) was calculated. Waist circumference was measured twice and the mean waist circumference was calculated. Smoking status (current, former and never) and alcohol consumption were assessed with a questionnaire. Alcohol consumption was categorized as no alcohol consumption, light, moderate, excessive and very excessive alcohol consumption, according to the alcohol consumption index adapted from the Garretsen alcohol index (26). The prevalence of chronic non-specific lung disease (including asthma and chronic obstructive pulmonary disease), cancer, osteoporosis, diabetes mellitus, coronary heart disease (CHD), peripheral arterial disease and stroke were based on self-report. The presence of cardiovascular disease was scored positive if 1 of the following diseases were present: CHD, stroke or peripheral arterial disease. The presence of other chronic diseases was scored positive if patients were suffering from non-specific lung disease, osteoporosis or cancer.

Hair collection, preparation and cortisol measurement

Hair samples of approximately 150 hair strands were cut from the posterior vertex, as close to the scalp as possible. The proximal three cm of hair, reflecting roughly the three months prior to hair sample collection, were used for the measurement of cortisol. Hair sample preparation has been described in detail (18-19). In brief, a minimum of 15 mg of hair was weighted and cut into small pieces in a glass vial. Methanol was added to extract cortisol from the hair samples during an overnight incubation (16 hours) at 52°C. Afterwards, the methanol was transferred into a clean glass vial and was evaporated under a nitrogen stream until completely dry. The samples were dissolved in PBS (pH 8.0) and, before analysis vortexed until thoroughly mixed. Cortisol levels in the hair extracts were measured using a commercial ELISA kit for salivary cortisol (DRG Instruments GmbH, Marburg, Germany). Cross reactivity of the kit's antibodies with other steroids was reported as follows: Corticosterone (29.00%), Cortisone (3.00%), 11-Deoxycortisol (<1.00%), 17-OH Progesterone (<0.50%), other hormones (<0.10%). Intra-assay variation was below 5% and inter-assay variation was below 8% as stated by the manufacturer. The low end detection limit for this assay is 1.5 nmol/L. Recovery of the assay was tested and described elsewhere (18).

Statistical Analyses

All statistical analyses were conducted using SPSS version 16.0 (SPSS Inc, Chicago, IL, USA). Since hair cortisol levels were not normally distributed, hair cortisol levels were divided in quartiles and logistic regression was used to investigate the relationship between hair cortisol levels and individual chronic diseases as well as total cardiovascular and total other chronic diseases. All analyses were adjusted for age, gender, smoking status, alcohol consumption and physical activity. Correlations between hair cortisol, age, BMI and waist circumference were tested using Spearman's correlation test. A p-value smaller than 0.05 was considered to indicate statistically significant differences.

Results

Characteristics of the study group are shown in Table 1. The age ranged from 65 to 85 years with a median age of 75 years, and 66.1% was female. There were significant differences between men and women in alcohol consumption, smoking status and prevalence of osteoporosis and type 2 diabetes (Table 1). Hair cortisol levels were significantly higher in men than in women (median 26.3 pg/mg hair [IQR 20.6-35.5 pg/mg hair] vs. 21.0 pg/mg hair [IQR: 16.0-27.0 pg/mg hair]).

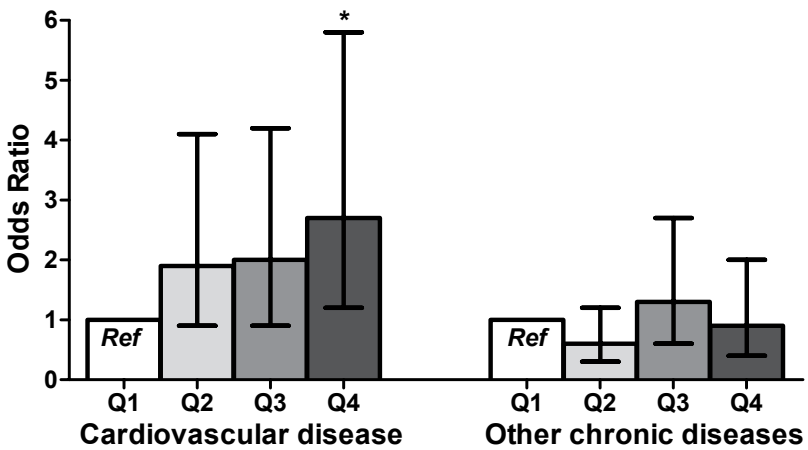

Figure 1. *Odds ratios for cardiovascular and other chronic diseases according to quartiles of hair cortisol.*
*Odds ratio and 95% confidence interval are adjusted for age, gender, smoking status, alcohol consumption and physical activity. Cardiovascular disease includes coronary heart disease, stroke and peripheral arterial disease. Other chronic diseases include non-specific lung disease, osteoporosis and cancer. Ref= reference quartile; Q= quartile. * p<0.05.*

Hair cortisol levels were divided in quartiles, and the lowest quartile was considered the reference quartile. Cut-off points for hair cortisol quartiles were: 16.9 pg/mg hair, 22.1 pg/mg hair and 30.6 pg/mg hair. Compared with participants from the lowest quartile, the odds ratios (OR) for cardiovascular disease (including CHD, stroke and peripheral arterial disease) were 1.9 (p=0.09) for the second, 2.0 (p=0.08) for the third and 2.7 (p=0.01) for the highest hair cortisol quartiles (Figure 1). The ORs and 95% confidence intervals (CIs) of the individual cardiovascular diseases are shown in Figure 2. Higher hair cortisol levels were associated with an increased risk of CHD (p=0.07, p=0.14, p=0.03 for the second, third and fourth quartile respectively).

Table 1. *Group characteristics*

	Total group	**Women**	**Men**
Number – n (%)	283	187 (66.1%)	96 (33.9%)
Age (years) – median (IQR)	74.8 (70.1-79.6)	75.3 (70.8-79.7)	72.7 (69.1-79.4)
BMI (kg/m2) – median (IQR)	27.1 (24.9-30.3)	27.8 (24.9-30.7)	26.8 (25.0-29.4)
Waist circumference (cm) – median (IQR)	99.0 (92.8-105.8)	96.9 (90.5-103.8)	102.5 (97.2-110.5)
Alcohol			
Never – n (%)	49 (17.4%)	40 (21.4%)	9 (9.5%)
Light- – n (%)	149 (52.8%)	106 (57.0%)	43 (44.8%)
Moderate – n (%)	74 (26.2%)	38 (20.4%)	36 (37.5%)
(very) Excessive – n (%)	10 (3.6%)	3 (1.6%)	7 (7.3%)
Smoking			
Never – n (%)	108 (38.2%)	88 (47.1%)	20 (21.1%)
In the past – n (%)	149 (52.7%)	88 (47.1%)	61 (64.2%)
Current smoker – n (%)	25 (8.8%)	11 (5.9%)	14 (14.7%)
Chronic diseases			
Cardiovascular disease			
Coronary heart disease – n (%)	75 (26.5%)	44 (23.5%)	31 (32.3%)
Stroke – n (%)	16 (5.7%)	9 (4.8%)	7 (7.3%)
Peripheral arterial disease – n (%)	57 (20.1%)	38 (20.5%)	19 (19.8%)
Diabetes Mellitus – n (%)	43 (15.2%)	22 (11.8%)	21 (21.9%)
Non Cardiovascular Disease			
Non-specific chronic lung disease – n (%)	22 (7.8%)	16 (8.6%)	6 (6.3%)
Cancer – n (%)	40 (14.1%)	29 (15.5%)	11 (11.5%)
Osteoporosis – n (%)	53 (18.7%)	48 (25.7%)	5 (5.2%)
Cortisol (pg/mg hair) – median (IQR)	22.1 (16.9-30.5)	21.0 (16.0-27.0)	26.3 (20.6-35.5)

In addition, there was a higher risk of peripheral arterial disease in participants with higher hair cortisol levels (OR 1.4 [p=0.47], 1.5 [p=0.36] and 2.5 [p=0.05] for the second, third and fourth quartile respectively). Furthermore, participants in the third and fourth hair cortisol quartile had an increased risk of stroke, although this was not statistically significant (OR= 3.6, p=0.16 for the third quartile and OR=3.3, p=0.17 for the fourth quartile). However, combining the third and the fourth quartile resulted in a significantly increased OR of the upper half of the group compared to the lower part of the group (first and second quartile) (OR=3.5; 95% CI=1.0-12.1; p=0.05). Participants in the highest quartile also had an increased risk of type 2 diabetes mellitus (OR 3.2, p=0.04). We found similar results after correction for BMI in all analyses. There were no significant associations between hair cortisol levels and the total group of non-cardiovascular diseases (Figure 1) and the individual non-cardiovascular diseases such as cancer, osteoporosis and chronic non-specific lung diseases (Figure 2).

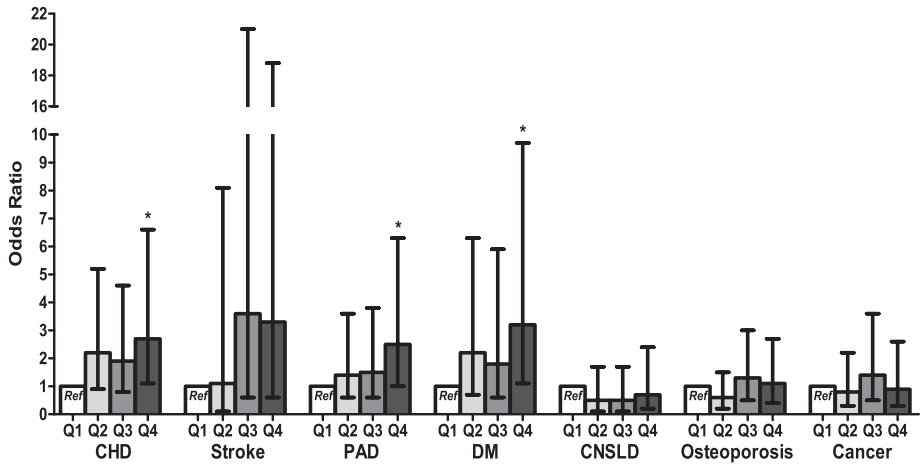

Figure 2. *Odds ratios for chronic diseases according to quartiles of hair cortisol. Odds ratio and 95% confidence interval are adjusted for age, gender, smoking status, alcohol consumption and physical activity. CHD= coronary heart disease; PAD= peripheral arterial disease; DM=type 2 diabetes mellitus; CNSLD= chronic non-specific lung disease; Ref= reference quartile; Q= quartile. *p<0.05.*

In addition, we found no correlation between hair cortisol levels and BMI (r=0.06, p=0.36), waist circumference (r=0.11, p=0.08) or age (r=-0.02, p=0.76) in the total group, and also not when studied in men and women separately. There were also no differences in hair cortisol levels between participants based on their smoking status. Hair cortisol levels were 21.2 pg/mg hair (IQR: 16.6-26.9) in participants who never smoked, 22.1 pg/mg hair (IQR: 17.4-33.6) in former smokers and 26.3 pg/mg

hair (IQR: 18.7-32.3) in current smokers (p=0.22). We found significantly higher hair cortisol levels in the group of participants with higher alcohol consumption. Hair cortisol levels were 21.2 pg/mg hair (IQR= 15.2-29.4) in participants who did not drink alcohol at all, 21.7 pg/mg hair (IQR=16.9-28.9) in participants with light alcohol consumption, 24.5 pg/mg hair (IQR=17.1-36.3) in moderate alcohol consumers and 30.4 pg/mg hair (IQR=25.0-45.0) in participants with (very) excessive alcohol consumption (p=0.05).

Discussion

We investigated the association between long-term cortisol levels, measured in scalp hair and a history of cardiovascular diseases in a community-dwelling older population. The major finding of our study is the increased cardiovascular risk in participants with high long-term cortisol levels, whereas no associations were found between long-term cortisol levels and chronic non-cardiovascular diseases.

The relationship between cortisol and cardiovascular diseases has been extensively studied, but with conflicting results. Some studies have shown that high cortisol levels are associated with an increased cardiovascular risk, or increased cardiovascular mortality, whereas others cannot confirm this association (10-15, 27). These inconclusive results might be due to the methods used to measure cortisol levels. Our study is the first study that used the measurement of cortisol in scalp hair to investigate the association between cortisol and cardiovascular disease in a population based group of elderly individuals. Over the past few years, several studies have documented that the measurement of cortisol in scalp hair is a reliable method to measure cortisol exposure over prolonged periods of time and that it is a potential biomarker for chronic stress (20, 22-23). Furthermore, it has been shown that hair cortisol levels correlates with typical cortisol tissue effects (18, 28). The measurement of cortisol in scalp hair is the reflection of the mean cortisol level over a prolonged period (in our study 3 months) and is therefore not influenced by the time of sample collection and acute stress. For this reason the measurement of cortisol in scalp hair is a very suitable method to study the effects of long-term cortisol levels, and therefore chronic stress, in population-based studies.

We found a 2.7-fold increased risk of cardiovascular disease in our participants in the highest hair cortisol quartile compared to participants in the lowest quartile. This OR is in the same range as previously described ORs of traditional cardiovascular risk factors such as hypertension (OR 2.5) and abdominal obesity (OR 2.2) and is only slightly lower than the increased risk of cardiovascular disease caused by diabetes mellitus (OR 3.1) and dyslipidemia (OR 3.3) (29). This suggests that high long-term cortisol levels might be an important risk factor for cardiovascular disease as well, which is also supported by a study from Pereg et al. (30), who measured hair cortisol levels in patients admitted to the emergency room with and without acute myocardial

infarction. They showed that hair cortisol levels in the 3 months before admission to the emergency department were significantly higher in the patients with an acute myocardial infarction than in patients who were admitted for other reasons such as non-myocardial chest pain, infections, and syncope. In contrast to the study of Pereg et al. (30), our study is cross-sectional and can therefore only show an association between hair cortisol levels and a history of cardiovascular disease. However, because Pereg et al. (30) showed that high cortisol levels are present before the onset of a cardiovascular event, the association we found may reflect a causative association. This is also supported by the well-known increased cardiovascular disease risk in patients suffering from Cushing's syndrome, which is characterized by pathologically elevated endogenous cortisol (31). Because our study and the study by Pereg et al. are the only 2 studies that have investigated the association between long-term cortisol levels and cardiovascular disease, and currently no studies have evaluated changes in cortisol after cardiovascular events, replication in larger studies is necessary to reveal the true significance of high long-term cortisol levels in the development of cardiovascular disease.

Interestingly, we did not find a correlation between hair cortisol levels and BMI, and there was only a tendency towards a correlation between hair cortisol levels and waist circumference in our group of elderly subjects. This is in contrast with our previous reports in younger adults (18, 28). However, several studies have shown that at older age, a change in body composition occurs, with an increase in body fat and a decrease in fat free mass, despite a stable BMI (32-33). Therefore, a lower BMI might also be the result of lower muscle mass rather than fat mass. Due to these changes in body composition at older age, BMI may not be a reliable marker of body fat and therefore not an accurate predictor of cardiovascular disease and mortality at older age. Furthermore, it might be that at older age, the effects of cortisol on BMI and abdominal obesity are not as clear as in younger adults and that other factors, such as activity level and health status, affect BMI more than slightly elevated cortisol levels. In addition, several studies have shown that BMI at older age is not predictive of mortality risk (both all-cause mortality and cardiovascular mortality) and that higher BMI at older age is even associated with an increased survival in elderly (34-36).

The lower hair cortisol levels in women than in men may be explained by differences in age, number of participants that dyed and bleached their hair and the number of participants that used hair products (18,19). However, we did not find a correlation between hair cortisol levels and age, and after exclusion of women with dyed and bleached hair and all participants using hair products, hair cortisol levels remained significantly higher in men (data not shown). This suggests that the difference in hair cortisol levels is a true gender difference, not caused by gender differences in external influences. In our previous study in healthy adults (age 18-63 years), we did not find any gender differences in hair cortisol levels (18). In addition, other

hair cortisol studies have reported no gender differences in hair cortisol levels (37-38). However, these studies have been performed in participants aged 20-51 (38) or have compared hair cortisol levels of women aged >40 years to hair cortisol levels of men aged <40 years (37). Furthermore, these studies had only small sample sizes. Interestingly, Dettenborn et al. (39) found higher cortisol levels in men aged 18-49 compared to women of the same age, and no difference in hair cortisol levels between men and women at age 50-90. A limitation of that study was that there were only 31 participants (19 men, 12 women) in the age group 50-90 year. Our study is the first study in which hair cortisol levels were measured in a large group of older adults. Replication is needed to confirm our findings of gender differences in long-term cortisol levels at older age. In our study, we did not find a correlation between hair cortisol levels and age. This is in contrast with the study performed by Dettenborn et al. (39). Dettenborn et al. described a quadratic relationship between hair cortisol levels and age, with higher hair cortisol levels in (very) young children and old adults. However, the studied groups was rather small, with only 31 participants in the age group 50-90 years, and the association was weak. We studied a large group of elderly subjects, with an age range of 65-85 years. In this range, we did not find a correlation between hair cortisol and age.

We studied the association between long-term cortisol levels, measured in scalp hair, and cardiovascular disease in a group of 283 elderly individuals. The population studied is representative for the community-dwelling older population in the Netherlands. However, a few limitations need to be discussed. First, the prevalence of chronic diseases was based on self-report. Nevertheless, self-report was found to be valid after comparing it to general practitioners' information (40). Second, the prevalence of some chronic diseases, such as stroke and non-specific lung disease, was low (<10%), which resulted in limited statistical power. Third, we did not have any data concerning blood pressure or lipid status. These factors might be confounders in the association between hair cortisol levels and increased cardiovascular risk. Fourth, data on other comorbidities that might influence cortisol levels, such as psychiatric illnesses or sleep disorders, and psychological stress were not available.

In conclusion, our results suggest that higher long-term cortisol levels are associated with an increased risk of cardiovascular disease. The odds of cardiovascular disease in elderly individuals with increased hair cortisol levels were similar to the odds reported for established cardiovascular risk factors. This suggests that long-term elevated cortisol may be an important risk factor for cardiovascular disease.

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Chapter 9

Long-term cortisol in Bipolar Disorder: Associations with age of onset and psychiatric co morbidity

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Abstract

Background
Dysregulation of the hypothalamic-pituitary-adrenal (HPA-)axis is hypothesized to play a role in the pathogenesis of bipolar disorder (BD). Conflicting results have been reported when saliva or serum was used to measure cortisol levels. A recently developed method is to measure cortisol in scalp hair, with one cm of scalp hair representing one month. We studied whether there are differences in long-term hair cortisol levels between BD patients and healthy individuals and whether there are associations between hair cortisol and disease characteristics.

Methods
Hair samples were collected in 100 BD patients and 195 healthy controls. Long-term cortisol levels were determined in 3 cm hair segments. Saliva samples were collected on two consecutive evenings. Documented disease characteristics were disease state, age of onset and psychiatric co-morbidity.

Results
Hair cortisol levels were not statistically different in BD patients compared to healthy controls ($p=0.233$) and were not associated with the disease state at the moment of sample collection ($p=0.978$). In the subgroup of patients with age of onset ≥30 years, hair cortisol levels were significantly elevated compared to the subgroup with age of onset <30 years and to healthy controls ($p=0.004$). Psychiatric co-morbidity was associated with elevated cortisol levels (44.87 versus 31.41 pg/mg hair; $p=0.021$), with the exclusion of panic disorder, which was associated with decreased cortisol levels (22.13 versus 34.67 pg/mg hair; $p=0.019$).

Conclusions
Elevated long-term cortisol levels might play a role in a subgroup of patients with BD. There may be differences in pathogenesis of younger and older onset BD suggesting two different disease entities.

Introduction

Cortisol, the main glucocorticoid in humans, is released in response to stress as part of the hypothalamic-pituitary-adrenal (HPA) axis that affects mineralocorticoid and glucocorticoid receptors throughout the brain. Dysregulation of the HPA-axis is known to occur in several psychiatric disorders, such as anxiety disorder (e.g. post traumatic stress disorder) (1) and mood disorders including bipolar disorder (BD) (2-8). In addition, depression and mania are common psychiatric symptoms seen in patients treated with corticosteroids or suffering from Cushing's Syndrome, a disorder caused by cortisol excess (9-10). Dysregulation of the HPA-axis in BD is also supported by several studies that have found more non-suppression after dexamethasone (dex) administration and increased cortisol secretion after corticotrophin releasing hormone (CRH) in patients with BD (11-12). However, conflicting data have been published concerning HPA-axis dysfunction during episodes and during euthymic phases of the disease. Furthermore, there are only a few studies reporting dexamethasone suppression tests (DSTs) or combined dex/CRH tests in patients with bipolar disorder (11-12).

In the majority of previous studies salivary or serum cortisol levels have been used to evaluate HPA-axis functioning. However, these studies yielded conflicting findings. Some studies reported no differences in basal salivary or serum cortisol levels in patients with BD (2, 13-15), whereas others described elevated cortisol levels (3-4). These conflicting findings might be the result of methodological differences between the studies. Some studies reported cortisol levels in patients in remission, while others measured cortisol levels during active depression or mania. Furthermore, there is a large discrepancy in the methods used to evaluate cortisol levels in BD patients. Some studies evaluated the cortisol awakening response, whereas others investigated diurnal rhythms of cortisol secretion or cortisol levels at single time points. Cortisol is secreted in a circadian rhythm and with a pulsatile pattern, which complicates the interpretation of serum and salivary cortisol levels. Furthermore, in blood, only 10% of the circulating cortisol is free, 75% is bound to cortisol binding globulin (CBG) and 10% to albumin (16). In most studies describing serum cortisol levels, the concentration of CBG is not taken into account. Therefore the concentrations of unbound, biologically active, cortisol are not known. In addition, since cortisol is a stress hormone, acute psychological or physical stress will also influence serum and saliva cortisol levels. A recently developed method to measure cortisol levels in scalp hair is a feasible method to determine long-term cortisol levels and appears to yield a reliable estimate of long-term HPA-axis activity (17-18). Since hair grows with an average rate of 1 cm per month, a hair segment of e.g. 3 cm would reflect mean cortisol levels over a period of approximately 3 months. This long-term cortisol measurement is therefore not influenced by the time of sample collection or acute stress due to daily circumstances or the research setting. Steudte et al. (2010) used hair cortisol levels to measure HPA-axis activity in generalized

anxiety disorder (GAD) and found decreased cortisol levels in hair of patients with GAD, but no differences in salivary cortisol levels between GAD patients and healthy controls (19). This suggests that hair cortisol levels may reflect the chronic cortisol exposure, whereas the results found with saliva or serum cortisol levels might also include acute responses to the measurement circumstances. Several other studies have shown that hair cortisol is indeed a marker of long-term cortisol exposure (17, 20-23).

The aim of this study was to explore the role of long-term endogenous cortisol exposure by comparing long-term cortisol levels in BD patients with healthy controls using cortisol measurements in scalp hair. We aim to obtain insight in the long-term assessment of cortisol in patients with bipolar disorder, since chronic subtle changes in HPA-axis functioning appear to be involved in the pathophysiological processes leading to mood episodes. We hypothesized that hair cortisol levels are higher in BD patients compared to healthy individuals. In addition, we explored the relation between cortisol levels in hair and clinical course of the disease, with characteristics like disease state, age of onset, and psychiatric co-morbidity. Furthermore, we measured saliva cortisol levels in order to compare the observed findings of hair cortisol.

Methods

Study design

This is a cross-sectional study involving outpatients with BD. The study was approved by the local medical ethics committee and is carried out in accordance with the declaration of Helsinki. After complete description of the study to the participants, all participants gave informed consent.

Participants

Bipolar Disorder patients

Patients with BD (type I, type II and Not Otherwise Specified) included in our previous study (24) concerning the role of glucocorticoid and mineralocorticoid receptor polymorphisms were asked to participate and the first 100 eligible patients were included in this study. Patients were eligible for inclusion if they had not been using glucocorticoids in the six months prior to hair sample collection and if they had sufficient hair growth at the posterior vertex. Detailed description of the assessment methods of the patients has been described elsewhere (24-25). In brief, participants were interviewed by trained psychologist to collect socio-demographic data and disease characteristics. Diagnoses of BD and psychiatric co-morbidities were based on DSM-IV criteria and were assessed with a standardized diagnostic interview developed by Sheehan et al. (26) using the Dutch version of the MINI International Neuropsychiatric Interview Plus (version 5.00-R; MINI-PLUS) (27).

The Questionnaire for Bipolar Illness, Dutch translation (28-29) was used to specify subtypes of BD, its course over time and detailed information about age of onset of first symptoms regarding hypomanic, manic and depressive episodes. The Questionnaire for Bipolar Illness, Dutch translation is a widely used questionnaire, developed by the National Institute of Mental Health. In addition, detailed information was gathered to define whether patients suffered from depression, mania or a combined episode during the three months prior to hair sample collection (this timeframe corresponds with cortisol measurements in 3 cm hair segments). Based on this information, disease state was categorized as stable disease, depressive episode, manic episode or mixed episode. Data on age of onset were present in 84 patients and data concerning psychiatric co-morbidities were present in 99 patients.

Healthy controls
A group of 195 healthy controls were used as control group. Detailed information of this study group has been reported previously (17). Both patients and healthy controls filled out a questionnaire concerning hair treatment (dyeing/bleaching/permanent waving/straightening), the use of hair products (gel, wax, hair spray) and frequency of hair wash.

Sample collection

In all patients and healthy controls, a lock of hair of approximately 100-150 hairs was cut of from the posterior vertex as close to the scalp as possible. Hair was taped to a paper and stored until preparation. In addition, in 90 patients with BD also saliva samples were collected on two consecutive evenings at 2200h. Collection at 2200h was chosen since several studies showed that saliva samples collected in the late evening on separate days show the lowest variation in cortisol levels compared to diurnal cortisol measurements and the cortisol awakening response (30-32). This suggests that late evening cortisol levels are slightly less influenced by acute stressors and daily influences than e.g. the cortisol awakening response, and may thereby be a better reflection of basal cortisol status. Participants were instructed not to eat, drink or brush their teeth 30 minutes prior to the saliva collection. Saliva was collected by spitting into a Salicap plastic tube. Saliva samples were stored at -20°C until analysis. Cortisol levels were measured separately in both saliva samples and we calculated the mean salivary cortisol levels afterwards. We used the mean salivary cortisol levels in the statistical analyses.

Hair sample preparation

Hair samples were measured, weighted and put into separate glass vials. The 3 cm segment most proximal to the scalp was used in the analysis, which corresponds roughly to a period of three months. In the glass vial, hair segments were cut into small pieces and 1 mL of methanol was added to extract cortisol from the hair samples. After overnight incubation for 16 hours at 52°C while gently shaking, the methanol was transferred to another glass vial and was evaporated under a nitrogen stream and the samples were dissolved in 250 µL phosphate buffered saline (pH 8.0).

Cortisol measurement

Saliva samples were thawed and vortexed. After this, saliva samples were centrifuged for 5 minutes to separate the mucins. Cortisol in saliva as well as in the hair extracts was measured using a commercially available salivary ELISA cortisol kit (DRG Instruments GmbH, Marburg, Germany). Cross reactivity of the kit's antibodies with other steroids was reported as follows: Corticosterone (29.00%), Cortisone (3.00%), 11-Deoxycortisol (<1.00%), 17-OH Progesterone (<0.50%), other hormones (<0.10%). Intra-assay variation was below 5% and the inter-assay variation below 8% as stated by the manufacturer. The low end detection limit for this assay is 1.5 nmol/L. To test the recovery of the assay, we created cortisol standards in PBS with concentrations of 5, 10, 20, 40, 80 and 160 nmol/L and measured the recovery in duplicate. We also spiked two hair samples with 20 nmol/L hydrocortisone, to measure recovery when hydrocortisone was dissolved in hair extract. The recovery of 5, 10, 20, 40, 80 and 160 nmol/L cortisol standards from PBS was 96.0%, 94.0%, 94.0%, 95.0%, 96.8% and 89.9% respectively. When hydrocortisone was added to hair extracts, the mean recovery was 101.3%.

Statistical analysis

SPSS 17.0 for Windows and GraphPad 5.0 were used for statistical analysis. Differences between group characteristics were tested with Chi-square and Kruskal Wallis tests. After log transformation hair and saliva cortisol levels were normally distributed. Differences in cortisol levels between healthy controls and BD patients and between disease characteristics of BD patients were tested with ANCOVA and linear regression. The association between saliva cortisol and hair cortisol levels was tested using linear regression analysis. All hair cortisol analyses were adjusted for gender, age, frequency of hair wash, hair treatment (dyeing, bleaching, permanent straightening or waving) and the use of hair products. Saliva cortisol analyses were adjusted for gender and age.

Results

Hair cortisol measurements were completed in 100 BD patients and 195 healthy controls. Saliva cortisol levels were available in 90 BD patients. Group characteristics are shown in Table 1. All BD patients were treated in the outpatient Department of Mood Disorders in The Hague, the Netherlands. BD patients were significantly older than healthy controls and had significantly higher BMI. Furthermore, BD patients washed their hairs less frequently and used less hair products, but dyed/bleached their hairs more often compared to the healthy control group (Table 1). The number of patients on lithium, antidepressants, antipsychotics and other mood stabilizers are shown in Table 1. There were only 2 (2.0%) BD patients that did not use anti-depressants, antipsychotics, lithium or other mood stabilizers, 30 patients (30.0%) used only 1 type of medication, 31 patients (31.0%) used 2 types of medication and 19 patients (19.0%) used 3 types of medication. There was no correlation between log-transformed saliva cortisol and hair cortisol measurements (β=0.157, p=0.140) (Figure 1).

Table 1. *Group characteristics*

	Bipolar disorder (n=100)	Healthy controls (n=195)	p-value
Age (years) – median (range)	52.0 (20-82)	32.0 (18-63)	<0.001
Number of women – n (%)	62 (62.0%)	103 (52.8%)	0.18
BMI (kg/2) – median (IQR)	25.3 (23.5-28.0)	23.7 (21.7-26.5)	<0.001
Frequency of hair wash – n (%)			
≤ 2 times/week	53 (53.0%)	50 (25.6%)	
≥ 3 times/week	47 (47.0%)	143 (73.3%)	<0.001
Hair treatment*	40 (40.0%)	43 (22.1%)	0.001
Use of hair products**	32 (32.0%)	90 (46.2%)	0.018
Age of onset Bipolar Disorder			
median (IQR)	21.0 (15.5-35.0)		
< 30 years of age – n (%)	55 (55%)		
> 30 years of age – n (%)	29 (29%)		
One or more psychiatric co morbidities – n (%)	42 (42.0%)		
Co morbid panic disorder	14 (14.0%)		
Co morbid anxiety disorder	16 (16.0%)		
Co morbid agoraphobia	21 (21.0%)		
Co morbid pain disorder	1 (1.0%)		
Co morbid somatoform disorder	2 (2.0%)		
Medication			
Lithium – n (%)	68 (68.0%)		
Other mood stabilizers – n (%)	17 (17.0%)		
Antidepressants – n (%)	31 (31.0%)		
Antipsychotics – n (%)	34 (34.0%)		
Disease state in the period covered by the hair sample			
Stable disease– n (%)	43 (43.0%)		
Depressive episode – n (%)	29 (29.0%)		
Manic episode – n (%)	3 (3.0%)		
Mixed episode – n (%)	8 (8.0%)		

** Hair treatment: dyeing, bleaching, permanent waving, permanent straightening of hairs*
*** Hair products: includes hair spray, wax, mouse and gel and other not wash-related hair products.*

Hair cortisol

There were no correlations between hair cortisol levels and age in healthy controls (r=0.062, p=0.39) or BD patients (r=-0.047, p=0.64). There were also no differences in hair cortisol levels between men and women in healthy controls (p=0.87) or BD patients (p=0.12) and hair cortisol levels did not correlate with BMI in BD patients (r=0.163, p=0.11) or in healthy controls (r=0.042, p=0.59). Hair cortisol levels in the total group of BD patients were not different from hair cortisol levels in healthy controls (31.8 pg/mg hair (95% Confidence interval (CI): 28.4 – 35.8) versus 28.2 pg/mg hair (95% CI: 25.9 – 30.6); p=0.233, adjusted for gender, age, hair treatment, hair products and frequency of hair wash). Since there was variability in the state of disease at the moment of sample collection, the group of BD patients was split up into groups with a stable period (n=43), depressive episode (n=29), manic episode (n=3) or combined episode (n=8) in the 3 months prior to sample collection. There were no significant differences between hair cortisol levels in the groups with various disease states in the three months prior to hair sample collection (unadjusted p=0.868; adjusted for gender, age, hair treatment, hair products and frequency of hair wash p=0.978). In addition, there was no effect of lithium (p=0.357), other mood stabilizers (p=0.388), antidepressants (p=0.816) or antipsychotics (p=0.278) on cortisol levels.

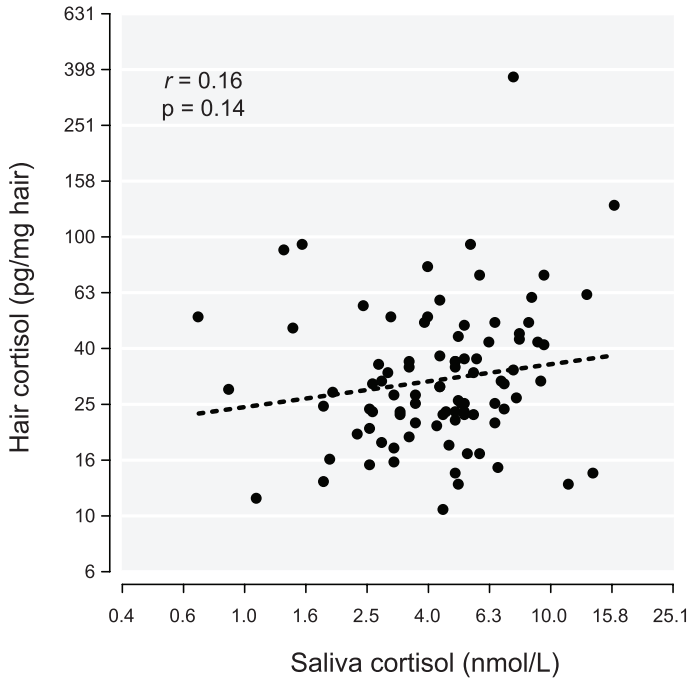

Figure 1. *Relationship between the mean cortisol level in two evening saliva samples (collected at 22.00h) and the hair cortisol levels on logarithmic scales. The Pearson's correlation coefficient and its accompanying p-value are given with a regression line.*

We found no significant correlation between age of onset of BD (age at which the first episode of (hypo)mania or depression presented) and hair cortisol levels (β =0.156, p=0.179). However, when assessing the distribution of hair cortisol throughout the different age of onset groups divided in decades, we found significantly elevated hair cortisol levels in the group of patients with their first depression or mania ≥ 30 years of age compared to the group of patients with their first depression or mania before 30 years of age (p=0.004) (Figure 2). Hair cortisol levels in the group with onset of BD < 30 years of age were similar to those in the healthy controls (p=0.886, adjusted for gender, age, hair treatment, hair products and frequency of hair wash) (Figure 2). There were no differences in use of lithium (p=0.536), other mood stabilizers (p=0.384), antidepressants (p=0.397) and antipsychotics (p=0.278) between the group of patients with an older age of onset of BD compared to the group of patients with an age of onset <30 years. Furthermore, there was no correlation between duration of the disease and hair cortisol levels (r=-0.105, p=0.34).

Interestingly, in patients with co-morbid panic disorder (n=14) hair cortisol levels were significantly lower than in BD patients without panic disorder (22.1 versus 34.7 pg/mg hair, p=0.019, adjusted for gender, age, hair treatment, hair products and frequency of hair wash) (Figure 3). These hair cortisol levels in patients with co-morbid panic disorder were even lower than hair cortisol levels in healthy individuals (p=0.05, adjusted for gender, age, hair treatment, hair products and frequency of hair wash). Furthermore, we found elevated hair cortisol levels in the group of BD patients with other psychiatric co-morbidities (e.g. anxiety disorders, agoraphobia,

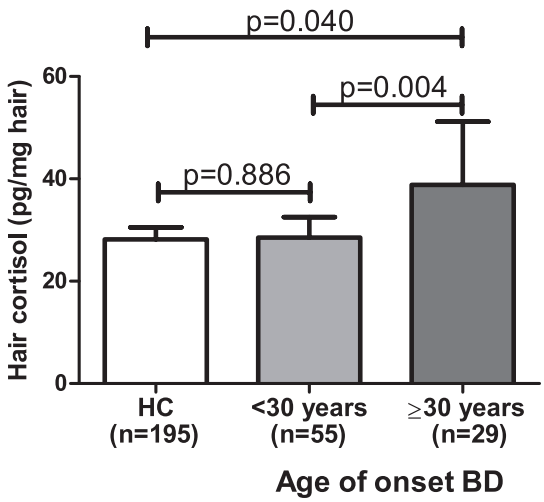

Figure 2. *Hair cortisol levels in healthy controls, bipolar disorder patients with younger and older age of onset. HC, healthy controls; p-values are adjusted for age, gender, use of hair products, hair treatment and frequency of hair wash. Error bars represent SEM.*

pain disorder, somatoform disorder) compared to the BD patients without psychiatric co-morbidities (44.9 pg/mg hair (95% CI: 34.4-58.6) versus 31.4 pg/mg hair (95% CI: 26.1-37.8) in patients without co-morbidities, p=0.021). When patients with co-morbid panic disorder were included in the group of total co-morbidities, there was no significant difference in hair cortisol levels between BD patients with and without psychiatric co-morbidities anymore (34.7 versus 31.3 pg/mg, p=0.448). Additional adjustment for BMI did not change any of the results.

Saliva cortisol

There were no significant differences in salivary cortisol levels between BD patients with a depressive episode, manic episode or stable disease at the moment of saliva collection (p=0.304). In addition, we found no differences in salivary cortisol levels between age of onset of disease before the age of 30 years and after the age of 30 years (p=0.155). In contrast to the results with hair cortisol levels, co-morbid panic disorder was associated with a trend towards increased salivary cortisol levels (5.5 (95% CI: 4.0-7.5) nmol/L versus 4.1 (95% CI: 3.6-4.7) nmol/L, unadjusted p=0.094). However, this was not statistically significant after adjustment for gender and age (p=0.245) (Figure 3). Furthermore, we found no differences between the group of BD patients with psychiatric co-morbidities (panic disorder excluded) and without psychiatric co-morbidity (p=0.562). Also, when panic disorder was included in the total group of psychiatric co-morbidities, there were no significant differences in salivary cortisol levels between the BD patients with and without co-morbidities (p=0.306). An overview of all results for hair and salivary cortisol is given in Table 2.

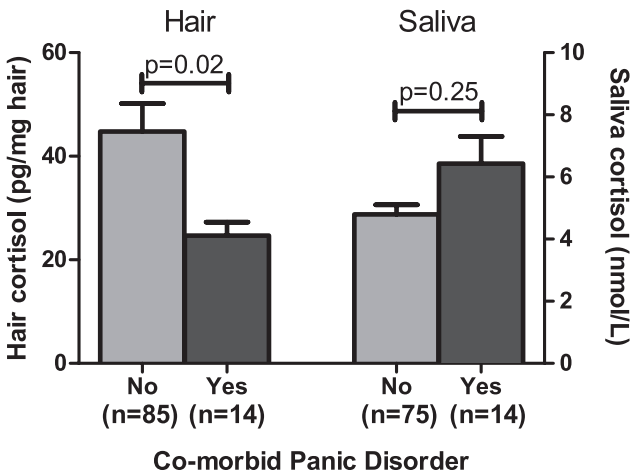

Figure 3. *Hair cortisol and salivary cortisol levels in bipolar disorder patients with and without co-morbid panic disorder. Hair cortisol analyses are adjusted for age, gender, use of hair products, hair treatment and frequency of hair wash. Saliva cortisol analyses are adjusted for gender and age. Error bars represent SEM.*

Discussion

In this study, we measured long-term hair cortisol levels of patients with bipolar disorder and healthy individuals. In addition, we measured evening salivary cortisol levels in bipolar disorder patients. The main finding of this study is the elevated hair cortisol level in patients with older age of onset BD compared to the group with a younger age of onset and healthy controls. We also found that BD patients with co-morbid psychiatric disorders (excluding panic disorder) had higher hair cortisol levels than patients with only BD. Interestingly, in patients with co-morbid panic disorder, hair cortisol levels were significantly decreased, whereas saliva cortisol levels were slightly elevated. No further correlations between hair and salivary cortisol measurements were found.

Table 2. *Overview of the hair cortisol and saliva cortisol results of disease characteristics in patients with Bipolar Disorder*

	Hair cortisol				**Salivary cortisol**			
	crude β	**p**	**adjusted** β	**adjusted p**	**crude** β	**p**	**adjusted** β	**adjusted p**
Age of onset Bipolar Disorder (<30 versus ≥ 30 years)	0.249	0.022	0.335	0.004	0.166	0.146	0.163	0.155
Co morbid panic disorder	-0.211	0.036	-0.243	0.019	0.187	0.094	0.122	0.245
Psychiatric co morbidity (excl. panic disorder)	0.227	0.036	0.253	0.021	0.020	0.865	0.068	0.562
Psychiatric co morbidity	0.087	0.389	0.078	0.448	0.153	0.329	0.142	0.306

The Beta was calculated with linear regression. Hair cortisol analyses were adjusted for age, gender, use of hair products, hair treatment and frequency of hair wash. Saliva cortisol analyses were adjusted for gender and age.

This is the first study that measured long-term hair cortisol levels of patients with BD. Several studies have measured cortisol levels in serum and saliva and results have been inconclusive with reports of increased (3-4) and normal cortisol levels (2, 13-15) compared to healthy individuals. Serum and saliva cortisol measurements reflect cortisol levels at one time point and are influenced by the pulsatile pattern and circadian rhythm of cortisol secretion, as well as by acute stress due to daily circumstances. These limitations do not apply to hair cortisol measurements which makes hair cortisol a better marker of HPA-axis functioning on the long-term. Recently, two studies measured hair cortisol levels and related them to mood disorders (33-34). Dettenborn et al. showed that hair cortisol levels were elevated in medicated patients with major depression (33) and Dowlati et al. showed that there was no difference in hair cortisol levels between depressed and non-depressed patients with coronary artery disease (34). However, hair cortisol levels of both

depressed and non-depressed patients with coronary artery disease were higher than those in healthy controls. The possible psychological stress associated with suffering from coronary artery disease and the presence of coronary artery disease itself could have abolished the differences in hair cortisol between depressed and non-depressed patients, resulting in different results than the observations of Dettenborn et al. Since both studies only included patients with major depression and not BD, it is difficult to compare their results to our findings.

In our study, we found that patients with younger (<30 years of age) and older (≥30 years of age) onset BD clearly differed in their mean hair cortisol levels. Our finding of elevated hair cortisol levels in BD patients with older age of onset supports the hypothesis of dysfunction and hyperactivity of the HPA-axis which has been described in BD patients and their offspring (2, 6-7, 35-38). However, cortisol levels in patients with earlier age of onset BD were comparable to hair cortisol levels in healthy individuals. In the past decade, several studies have found that there might be differences in disease characteristics and pathogenesis of early and late onset BD. Early onset of BD seems to be associated with greater severity and a poorer long term outcome (39-40). In addition, several genetic differences have been described between early and late onset BD (41-42). Differences in cortisol levels and HPA-axis functioning between subtypes of BD based on age of onset have not been studied previously. Our hair cortisol data suggest that elevated cortisol levels may play a role in late onset BD, but not in early onset BD. This suggests that BD onset at younger age may be less influenced by dysregulation of the HPA-axis, but more by other mechanisms e.g. changing regulation of sex hormones or differences in genetics of the disease. Later onset of the disease might be influenced by dysregulation of the HPA-axis through e.g. stressful life events, which are thought to be a trigger for manifestation of the disease (43). Furthermore, it can also be hypothesized, that long-term elevations in cortisol affect proneness for mood disorders by neuronal damage in the brain (44), which may lead to a higher risk of developing mood disorders. As seen in patients with endogenous hypercortisolism, psychopathology continues even after cure (9-10), suggesting that long-term elevations in cortisol can have irreversible effects on the brain, resulting in mood disorders.

We did not observe these differences in age of onset with saliva cortisol, suggesting that saliva cortisol may be an acute marker of cortisol rather than an estimate of the HPA-axis activity on the long-term. It supports the additional value of measuring hair cortisol levels. Furthermore, we found no differences in hair cortisol levels between patients in different disease states. The same applied to the observed findings of salivary cortisol in relation to BD, which is consistent with several other studies (2-3). This suggests that the increased HPA-axis activity may be a trait phenomenon rather than a state phenomenon in the subgroup of patients with older age of onset.

Moreover, we found that psychiatric co-morbidity was associated with elevated hair cortisol levels within the group of BD patients. With respect to the different types of co-morbidities, we found significantly lower cortisol levels in BD patients with co-morbid panic disorder. In contrast to this, evening saliva cortisol levels tended to be higher in this group. Our study is the first study that describes both hair and saliva cortisol levels in patients with panic disorder. These data again suggest that hair cortisol levels may reflect cortisol levels over a larger time frame (about three months is this study, since we used three cm length of hair), whereas saliva cortisol may provide an estimate of one time point including potential acute effects of the circumstances. Steudte et al. (19) previously demonstrated similar results in a group of patients with generalized anxiety disorder. In this group they found that hair cortisol levels were decreased compared to healthy individuals, whereas there was no difference between saliva samples of GAD patients and healthy individuals (19). The increased saliva cortisol levels in our BD patients with panic disorder compared to those without comorbid panic disorder, might be explained by more perceived stress in the participants with panic disorder prior to a visit to the research center than BD patients without panic disorder. Reports of saliva, serum and urine cortisol levels in patients with panic disorder have shown conflicting results (45-50), which emphasizes the need for a new reliable measurement of long-term cortisol exposure.

There are several limitations of this study. First, the healthy individuals were significantly younger than the BD patients, which may have affected our findings. However, there was no effect of age on cortisol levels in the control group (17) or in the bipolar disorder group. In addition, other studies reported no effect of age on hair cortisol levels (18, 51-52), and all analyses were adjusted for age. Second, several studies have shown that other factors, such as suicide attempts and childhood trauma can have a significant effect on the HPA-axis (53-54). In our study population, there was no significant effect of childhood abuse (sexual, physical and verbal) on hair cortisol levels (data not shown), but we did not collect data concerning suicide attempts. In future studies, other factors that might influence the HPA-axis such as childhood trauma and suicide attempts should be taken into account as well.

Third, our study lacked data concerning smoking status and somatic health. However, we found no differences in hair cortisol levels between smokers and non-smokers in our group of healthy individuals (data not shown), and a recent study of Dettenborn et al. (33) did not find differences in hair cortisol levels between smokers and non-smokers as well. Furthermore, our results of possible increased saliva cortisol levels with decreased hair cortisol levels in patients with panic disorder were found in a group of only 14 patients with co-morbid panic disorder in addition to bipolar disorder. The pathogenesis of panic disorder in BD patients might be different from the pathogenesis of panic disorder in patients without BD or another psychiatric disorder. Therefore, these results have to be interpreted carefully and it is not certain whether the results can be extrapolated to patients with panic disorder

without other psychiatric disorders. Finally, measurement of cortisol in scalp hair is a relatively new and promising method but many details concerning hair growth rate and incorporation of cortisol in hair are still unknown.

Despite these limitations, our results support the hypothesis that elevated long-term cortisol levels play a role in a subgroup of patients with bipolar disorder. Since we found that cortisol levels were only elevated in the group of patients with a relatively late age of onset of BD, we hypothesize that there may be differences in pathogenesis among patients with early and late onset BD yielding two different disease entities. Future research should focus on exploring these subtypes of BD based on age of onset with potentially a differential neuro-endocrine background.

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Part II:

The role of glucocorticoid receptor gene polymorphisms in cardiovascular risk and aging

Chapter 10

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

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Abstract

Background

Smaller size at birth has been associated with an increased risk of metabolic and cardiovascular disorders in adult life. Fetal programming of the hypothalamic-pituitary-adrenal (HPA)-axis has been suggested as a possible explanation. Fetal glucocorticoid (GC) overexposure has effects that suggest a role of GCs in this programming. The effects of GCs are mediated through the GC receptor (GR). Several functional polymorphisms have been described, which are associated with relative GC resistance or hypersensitivity. Our aim was to compare frequencies of GR haplotypes, characterized by the ER22/23EK, N363S, *Bcl*I or 9β polymorphisms, in subjects born small for gestational age (SGA) and associate birth anthropometry data, response to growth hormone (GH) treatment, blood pressure, glucose and insulin concentrations and body composition with these haplotypes.

Methods

418 SGA subjects and 697 healthy controls were enrolled in this study. Anthropometry data were obtained, as well as blood samples to determine fasting glucose and insulin concentrations. Dual Energy X-ray Absorptiometry scans were used to measure the amount of fat and lean mass.

Results

No differences were found between GR haplotype frequencies in SGA children compared to healthy controls. No associations were found between GR haplotypes and birth length and birth weight, growth response during GH treatment, blood pressure, glucose and insulin concentrations and body composition.

Conclusion

Glucocorticoid receptor haplotypes and their effect on GC sensitivity do not seem to play a significant role in GH-induced catch-up growth and the risk factors of developing metabolic and cardiovascular disorders in adult life of SGA children.

Introduction

Small size at birth has been associated with an increased risk of metabolic and cardiovascular disorders in adult life, such as hypertension, hyperlipidaemia, insulin resistance, type 2 diabetes mellitus and osteoporosis (1-2). Therefore, children born small for gestational age (SGA), defined as birth length or birth weight < -2 SD for gestational age, might have an increased risk of metabolic and cardiovascular disorders in adult life. Only 2.3% of all live born children is SGA (3). Postnatal catch-up growth in weight or BMI is also a risk factor for hypertension, ischaemic heart disease, insulin resistance and obesity in later life (4-6). Altered fetal programming of the hypothalamic-pituitary-adrenal(HPA)-axis has been suggested as an explanation for this association (7). There are several effects of fetal glucocorticoid (GC) overexposure that suggest a role in early life programming of adult cardiovascular and metabolic disorders. Exogenous GCs lead to fetal growth retardation and a lower birth weight in humans. Human fetal blood cortisol concentrations are increased in infants after intrauterine growth retardation and also in pre-eclampsia, implicating higher endogenous cortisol concentrations in retarded fetal growth (1, 7). Also, the major systems affected by early life programming are GC sensitive, namely blood pressure and blood glucose/insulin resistance (1). Disorders such as hypertension and insulin resistance are also present in Cushing's syndrome, which is caused by GC excess (8). All these findings together suggest a role of GCs in the early life programming of adult disorders.

The effects of GCs are mediated through the GC receptor (GR). The GR is a member of the nuclear receptor family and is expressed in most fetal tissues from the early embryonic stages (1, 7). The individual GC sensitivity differs between subjects and might be partly explained by polymorphisms of the GR gene. Four GR polymorphisms are thought to be physiologically functional: ER22/23EK (rs6189 and rs6190), N363S (rs6195), *Bcl*I (rs41423247) and the 9β (rs6198) polymorphism. They are associated with either GC hypersensitivity or resistance. GC hypersensitivity is associated with clinical signs like visceral obesity, type 2 diabetes mellitus, higher BMI and hypertension and GC resistance is associated with increased length, increased muscle mass and a healthier metabolic profile.

The ER22/23EK polymorphism consists of two linked single nucleotide polymorphisms in codons 22 and 23 in exon 2. The alteration at the DNA level is GAG AGG to GAA AAG change, which leads to a glutamic acid-arginine (E-R) to glutamic acid- lysine (E-K) change (9). This polymorphism is associated with a relative GC resistance, a healthier metabolic profile and increased insulin sensitivity (10-11).

The N363S and the *Bcl*I polymorphisms are associated with increased sensitivity to GCs, visceral obesity and type 2 diabetes. The N363S polymorphism is located in codon 363 and causes an AAT to AGT nucleotide change. This change results

in an amino acid change from aspargine to serine. The *Bcl*I polymorphism is a C to G substitution located in intron 2 (12). The N363S and *Bcl*I polymorphism are both associated with higher BMI, higher waist-to-hip ratio, visceral adiposity, hypertension and higher cholesterol and triglyceride concentrations.

The GR-9β polymorphism is an ATTTA to GTTTA change in exon 9β, resulting in an increased expression and stability of GR-β in vivo, consequently leading to GC resistance (13-15). GR-9β has been associated with decreased GC transrepressive activity (16), decreased microbial colonization (17), and with increased inflammatory mediators leading to an increased risk to cardiovascular disease (18).

We hypothesized that there could be an association between the GR polymorphisms, small size at birth and risk factors for metabolic and cardiovascular disorders in later life. We set out to study this in a group of subjects born SGA compared to controls.

Methods

Subjects

This study included 418 subjects (196 males and 222 females) born SGA. All subjects fulfilled the following inclusion criteria: 1) Birth length and/or birth weight standard deviation score (SDS) below -2.0 SD for gestational age (19); 2) an uncomplicated neonatal period, without signs of severe asphyxia, sepsis or long-term complications of respiratory ventilation. Exclusion criteria were: twins, non-Caucasian ethnicity, endocrine or metabolic disorders and chromosomal defects or syndromes. The cause of SGA in this study population is unknown. Catch-up growth was defined as an achieved height SDS for age above -2.0 SD according to Dutch standards at last visit (20). SGA children who did not show catch –up growth (SGA short, n=191), started with growth hormone (GH) treatment. The mean age at start of treatment was 7.51 (2.9) years. Children were treated with biosynthetic growth hormone at a dose of 1 mg/m2 body surface area/day. The GH dosage was adjusted to the calculated body surface every three months. For present study the growth during 1 year of GH treatment was analysed. Written informed consent was obtained from the parents/ guardians of each child. This study was approved by the Medical Ethics Committee of the Erasmus Medical Center.

Control group

The control group consisted of 165 healthy subjects who were randomly collected from hospitals in the Netherlands, where they had been seen because of a minor accidental health problem, but otherwise they were normal. All participants were born appropriate for gestational age and had normal adult height. To compare polymorphism frequencies we also used blood samples from 532 healthy subjects who were randomly collected from the Rotterdam blood donation bank. Written informed consent was obtained from all the participants.

Anthropometric analysis

Data on gestational age, birth length and birth weight were obtained using primary health care records and hospital records. In all subjects, standing height was measured to the nearest 0.1 cm by a Harpenden stadiometer and weight was measured to the nearest 0.1 kg by scale (Servo-Balance KA-20-150S) wearing underwear only. Standard height was expressed as SDS adjusting for sex and age using Dutch standards (20). Body weight was expressed as SDS adjusting for sex and height using Dutch standards (20).

Blood pressure, insulin and glucose analysis

In 317 SGA subjects, systolic and diastolic blood pressure was measured every five minutes during a period of one hour in the morning. Mean systolic and diastolic blood pressure were calculated from these measurements. In 255 SGA subjects fasting glucose concentrations were measured and in 155 SGA subjects, fasting insulin concentrations were also determined.

Body composition analysis

Fat and lean body mass distributions were measured with Dual Energy X-ray Absorptiometry scans (type Lunar DPX-L; GE Healthcare Madison, WI) in 243 SGA subjects. Total lean body mass, total fat body mass and fat percentages were determined.

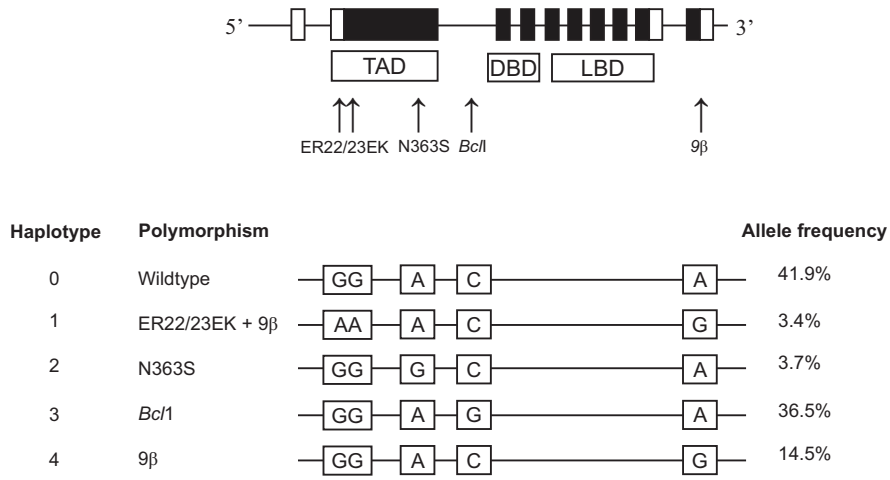

Figure 1. *Schematic overview of the glucocorticoid receptor (GR) gene polymorphisms and genotypes. The alleles were defined as haplotypes such as G-A-C-A (wildtype) representing a Guanidine (G) nucleotide at the 200 G>A polymorphic site, an Adenosine (A) nucleotide at the 1220 A>G polymorphic site, a Cytidine (C) nucleotide at the C>G BclI polymorphic site and a Adenosine (A) at the A>G 9Beta polymorphic site; TAD= transactivating domain, DBD= DNA binding domain, LBD= ligand binding domain.*

Genetic analysis

DNA was isolated from peripheral blood leucocytes using standard techniques and dissolved in double-distilled water and stored at -20°C. Allelic discrimination was performed to genotype the subjects, using TaqMan Universal PCR master mix, primers and probes (Applied Biosystems) and a Taqman ABI Prism 7900HT Sequence Detection System as previously described (17). Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60°C and optimized concentrations for primers and probes of 400 nmol/L for each polymorphism. The polymorphisms used in this study were chosen on the basis of their reported functionality, and not as tagging SNPs. In addition, we constructed haplotypes as previously described (17). Haploview showed that four of the five haplotypes that were constructed cover around one third of the GR gene variability (19 out of 57 haplotypes). The *Bcl*I polymorphism is not available in the HapMap database, therefore the haplotype characterized by this polymorphism could not be taken into account. However, the minor allele of this polymorphism has the highest minor allele frequency. This suggests that a considerable part of the variation in the GR gene is covered by the haplotypes we constructed (21). The GR gene and haplotypes are shown in Figure 1.

Table 1. *Baseline characteristics.*

	SGA
Number	418
Male (%)	46.9
Gestational age (weeks)	38.00 (4.00)
Birth length SDS	-2.74 (1.55)
Birth weight SDS	-2.35 (1.16)
Age (years)	10.53 (13.22)
Height SDS	-2.65 (1.55)
Weight SDS	-2.76 (1.83)
Body mass index SDS*	-1.11 (1.61)
Diastolic blood pressure (mmHg)	62.54 (11.46)
Systolic blood pressure (mmHg)	107.92 (16.00)
Insulin (mmol/L)	7.33 (6.00)
Glucose (mmol/L)	4.80 (0.63)
Fat percentage	15.03 (16.33)
Fat mass (g)	6221 (12856)
Lean mass (g)	36119 (28074)

Data are expressed as median (IQR).
** Body mass index SDS adjusted for age*

Statistical analysis

All statistical analyses were performed with SPSS (version 16.0.1 for Windows). Comparison of GR haplotypes frequencies between the SGA-group and the control group was performed with the Chi-Square test. Comparison of the means of anthropometric measures, blood pressures, fasting glucose and insulin concentrations, total fat mass, fat percentage and total lean mass in the SGA group with the genotypes was performed using the ANCOVA test. All analyses were corrected for age and sex. A p-value < 0.05 was considered to indicate a significant difference.

Table 2. *Glucocorticoid Receptor gene haplotype frequencies in SGA subjects and healthy controls.*

Genotype	SGA		Controls*		p-value
	N	%	N	%	
Haplotype 0					0.098
0	154	38.4	245	35.8	
1	160	39.9	317	46.3	
2	87	21.7	123	18.0	
Haplotype 1					0.648
0	385	93.2	642	92.2	
1	28	6.8	53	7.6	
2	0	0.0	1	0.1	
Haplotype 2					0.330
0	380	91.3	646	92.9	
1	36	8.7	49	7.1	
Haplotype 3					0.251
0	175	42.2	261	37.4	
1	188	45.3	333	47.8	
2	52	12.2	103	14.8	
Haplotype 4					0.265
0	286	71.3	519	75.8	
1	103	25.7	150	21.9	
2	12	3.0	16	2.3	

*P-values are calculated with Chi-square test; 0= non-carriers, 1= heterozygous carriers, 2= homozygous carriers, N= number of SGA subjects; * Controls include samples from the Rotterdam Blood bank.*

Results

Baseline characteristics of 418 SGA subjects are shown in Table 1. In SGA subjects and controls, the distribution of genotypes for all individual GR gene polymorphisms were in Hardy-Weinberg equilibrium (p>0.05). No significant differences were found in allele frequencies (Table 2). No significant differences in birth length and birth weight in the total SGA group were found between the four GR haplotypes, except for haplotype 4. Carriers of this haplotype had lower birth weight SDS (p=0.044) (Table 3). Analysis of the effect of GH treatment in SGA subjects who did not show catch-up growth (SGA short), measured as gain in height SDS after one year of GH treatment, did not show differences in height gain between GR haplotypes (Table 3).

Mean systolic and diastolic blood pressure and fasting insulin and glucose concentrations did not show any significant differences between the GR haplotypes (Table 4). In 243 SGA subjects, fat percentage, total fat mass and total lean mass were measured using DEXA scans. No differences were found in mean fat percentage, mean total fat mass and mean total lean mass between the five GR haplotypes (Table 5). These results were adjusted for age, sex and height.

When divided in SGA subjects who did not reach normal height (SGA short) and subjects who showed catch-up growth (SGA-CU), also no differences in allele frequencies were found: Haplotype 0 showed a heterozygous frequency of 37.6% in SGA short versus 41.9% in SGA-CU and a homozygous frequency of 22.4% versus 16.2% (p=0.230). Haplotype 1 showed a heterozygous frequency of 6.4% in SGA short versus 8.3% in SGA-CU (p= 0.518). Haplotype 2 showed a heterozygous frequency of 7.8% versus 10.1% (p= 0.470) and haplotype 3 showed a heterozygous frequency of 43.7% versus 52.3% and a homozygous frequency of 10.4% versus 13.8% (p= 0.100). Haplotype 4 showed a heterozygous frequency of 27.5% versus 22.9% and a homozygous frequency of 3.5% versus 2.9% (p= 0.668).

Discussion

In this study, we found no differences in five GR haplotype frequencies in SGA subjects compared to healthy controls. Also, between GR haplotypes, no difference was found in mean birth weight and birth length, except for carriers of haplotype 4, characterized by 9β, who showed lower birth weight SDS compared to non-carriers. We found no differences in growth response during one year of growth hormone treatment between the GR haplotypes in SGA short subjects. In addition, no significant differences were found in blood pressure, fasting insulin and glucose concentrations, percentage fat and total fat and lean mass between the GR haplotypes.

Table 3. *Differences in mean birth length SDS, birth weight SDS in the total group of SGA subjects and height gain SDS after one year of growth hormone treatment in SGA short subjects between the glucocorticoid receptor gene haplotypes.*

Genotype	N	Birth length	p-value	N	Birth weight	p-value	N	Height gain	p-value
Haplotype 0			0.468			0.337			0.162
0	153	-3.20		158	-2.49		71	0.98	
1	148	-3.04		156	-2.43		67	1.17	
2	84	-3.21		87	-2.30		46	1.27	
Haplotype 1			0.070			0.178			0.684
0	371	-3.10		385	-2.40		176	1.14	
1	26	-3.57		28	-2.65		13	1.04	
Haplotype 2			0.695			0.678			0.291
0	365	-3.12		380	-2.42		174	1.10	
1	35	-3.21		36	-2.35		17	1.32	
Haplotype 3			0.362			0.563			0.168
0	168	-3.13		175	-2.38		92	1.23	
1	181	-3.19		188	-2.46		77	1.05	
2	50	-2.90		52	-2.32		21	0.90	
Haplotype 4			0.545			0.044			0.376
0	273	-3.12		286	-2.36		126	1.15	
1	101	-3.22		103	-2.62		54	1.02	
2	11	-2.81		12	-2.29		4	1.54	

Data are expressed as means, p-value was calculated using ANCOVA; 0= non-carriers, 1= heterozygous carriers, 2= homozygous carriers, N= number of SGA subjects.

GR gene polymorphisms are known to play a role in the sensitivity to GCs (12). Several studies suggest that fetal programming of the hypothalamic-pituitary-adrenal axis might result in pathology in later life in SGA children. Subjects born with low birth weight have higher plasma cortisol concentrations in adult life, suggesting altered HPA-axis programming (7). Individual differences in GC sensitivity might play a role in fetal programming of the HPA-axis. Based on the reported higher cortisol concentrations, one could expect that SGA subjects have a higher frequency of GR haplotypes associated with relative GC resistance (haplotype 1, characterized by the ER22/23EK and the 9β polymorphism and haplotype 4, characterized by the 9β polymorphism), since this relative GC resistance would be compensated by increased cortisol concentrations. We did not find this association. Our pilot study in a group of 119 SGA children, showed a lower frequency of the *Bcl*I polymorphism compared to

Table 4. *Differences in systolic and diastolic blood pressure (mmHg), fasting glucose (mmol/L) and fasting insulin (mmol/L) concentrations between glucocorticoid receptor gene haplotypes in SGA subjects.*

Genotype	N	SBP	p	DBP	p	N	Fasting glucose	p	N	fasting insulin	p
Haplotype 0			0.734		0.434			0.377			0.134
0	119	107.92		61.94		98	4.94		60	9.20	
1	186	107.69		62.27		149	4.83		91	8.08	
Haplotype 1			0.313		0.647			0.603			0.918
0	294	107.53		62.13		238	4.88		145	8.43	
1	20	110.07		62.81		16	4.98		10	8.90	
Haplotype 2			0.142		0.131			0.758			0.743
0	288	108.04		62.36		234	4.89		140	8.43	
1	29	104.53		59.76		21	4.82		15	8.74	
Haplotype 3			0.858		0.672			0.990			0.570
0	134	107.63		62.08		100	4.87		64	8.16	
1	182	107.87		62.14		155	4.89		91	8.68	
Haplotype 4			0.668		0.470			0.379			0.331
0	217	107.64		61.97		185	4.90		112	8.31	
1	88	108.11		62.57		62	4.80		39	9.17	

Data are expressed as means, p-value was calculated using ANCOVA; data are adjusted for age and gender; 0= non-carriers, 1= heterozygous and homozygous carriers, N= number of SGA subjects, SBP= systolic blood pressure, DBP= diastolic blood pressure.

controls (22). A previous cohort study in the general population showed that carrying a GR haplotype including the *Bcl*I polymorphism was associated with a smaller size at birth corrected for gestational age. Carriers of this haplotype had both a lower birth weight and birth length compared to non-carriers (2). In contrast, Bertalan et al. found that the *Bcl*I polymorphism was associated with higher gestational-age-adjusted birth weight in preterm neonates (23). We studied a large group of SGA subjects and did not find an association between the haplotype 3, which includes the *Bcl*I polymorphism, and size at birth corrected for gestational age. This is in line with the results published by Geelhoed et al. They showed that in a large population based cohort GR polymorphisms were not associated with birth weight, birth length and postnatal growth (21). Interestingly, we found an association between heterozygous carriers of haplotype 4, characterized by the 9β polymorphism and lower birth weight corrected for gestational age. The 9β polymorphism is located in the 3'-UTR end of the GR gene, leading to increased stability of the GR-β splice variant at the mRNA level. This variant functions as a dominant negative inhibitor of the active GR-α isoform, resulting in a relative GC resistance. Carriers of the 9β polymorphism seem to have a decreased GC transrepression with a normal transactivation (16). For haplotype 4, characterized by the 9β polymorphism, no associations were found with cortisol response in dexamethasone-suppression test, BMI, waist-to-hip ratio, insulin sensitivity and lipid profile (16). Haplotype 4 was associated with decreased transrepressive effects on the immune system and inflammation. Carriers of haplotype 4 had increased cardiovascular risk factors, namely elevated sensitive C-reactive protein and Interleukin-6 concentrations and increased intima media thickness, resulting in increased risk of myocardial infarction and coronary heart disease (18). Our finding of lower birth weight in heterozygous carriers of haplotype 4 compared to non-carriers is in line with the a priori hypothesis based on mechanistic background and phenotype of haplotype 4. However, the difference in birth weight between carriers and non-carriers of haplotype 4 is not highly significant and shows no allele-dossage effect. Therefore, we do not interpret this finding as a realistic association until future studies can reproduce this.

SGA children showing postnatal catch-up in BMI have a higher risk of developing insulin resistance, central obesity and cardiovascular diseases (24-26). Those with catch-up in BMI had lower insulin sensitivity than SGA children without catch-up (26-28). Therefore, we expected a higher frequency of GR haplotypes leading to glucocorticoid hypersensitivity (haplotype 2, characterized by the N363S polymorphism and haplotype 3, characterized by the *Bcl*I polymorphism) in SGA children with catch-up growth. Our subgroup analysis of SGA children with short stature versus those with catch-up growth showed no differences between the GR haplotypes. Surprisingly, we did not find an association between GR haplotypes and catch-up growth or insulin resistance. This might be the result of the relatively small study groups. However, other studies did find associations in smaller subgroups than ours (11, 29-31).

Table 5. *Differences in fat percentage, total fat mass (g) and total lean mass (g) between glucocorticoid receptor gene haplotypes in SGA subjects.*

Genotype	N	Fat %	p	Total fat mass	p	Total lean mass	p
Haplotype 0			0.798		0.544		0.716
0	90	19.13		4808		30761	
1	141	17.27		5082		30620	
Haplotype 1			0.446		0.716		0.468
0	225	17.81		4932		30479	
1	17	16.75		4645		29854	
Haplotype 2			0.995		0.816		0.353
0	223	17.68		4932		30339	
1	20	18.79		5117		31046	
Haplotype 3			0.369		0.494		0.844
0	93	16.54		5164		30549	
1	149	18.58		4864		30479	
Haplotype 4			0.647		0.971		0.615
0	174	17.99		4977		30620	
1	57	18.00		4966		31477	

Data are expressed as means; p-value was calculated using ANCOVA; data are adjusted for age, gender and height; 0= non-carriers, 1= heterozygous and homozygous carriers, N= number of SGA subjects.

The growth response during 1 year of growth hormone treatment was not different between carriers of the various GR polymorphisms. In a previous study, preterm born carriers of the 23K variant of the ER22/23EK polymorphism showed complete catch-up growth to an adult height similar to the reference mean (32), indicating that the ER22/23EK polymorphism might be beneficial for growth. As a result, we expected more gain in height SDS during growth hormone treatment in carriers of haplotype 1, including the ER22/23EK and the 9β polymorphism, but we could not confirm this expectation. This suggests that GR gene polymorphisms do not have a substantial effect on the sensitivity to growth hormone in this group. The growth retarding effect of GCs, known from corticosteroid treatment, is caused by the inhibition of endogenous growth hormone secretion, collagen synthesis, cartilage sulfation, chondrocyte mitosis, GH receptor binding and insulin-like growth factor I (IGF-I) activity (33). It should, however, be noted that growth retardation is only seen when supraphysiological doses of corticosteroids are used.

We also found no differences in systolic and diastolic blood pressure, fasting glucose and insulin concentrations and body composition between the GR haplotypes in SGA subjects. In the study published by Finken et al. preterm born carriers of the ER22/23EK polymorphism had lower fasting insulin concentrations than non-carriers (32), but systolic and diastolic blood pressure and body composition measured by waist-hip-ratio, absolute fat mass and fat percentage showed also no differences between carriers and non-carriers of the ER22/23EK or N363S polymorphism in preterm born children (32).
Recently, subgroups within the SGA group have been described in a study by Ester et al (34). Differences in catch-up growth were found between SGA children with only small birth length, SGA children with a small birth length and a low birth weight and SGA children with a small birth length, low birth weight and a small head circumference (34). In the future it might be interesting to investigate whether GR gene polymorphisms have different effects in these subgroups.

In conclusion, we found no differences in allele frequencies of the GR gene haplotypes between SGA subjects and healthy controls and no associations were found with birth anthropometry, response to GH treatment, blood pressure, fasting glucose and insulin concentrations and body composition. Therefore, GR haplotypes do not seem to play a major role in body composition, growth hormone induced growth, glucose and insulin concentrations and blood pressure in SGA subjects.

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Chapter 11

Associations of glucocorticoid receptor haplotypes with body composition and metabolic parameters in HIV-infected patients from the FRAM study

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Submitted

Abstract

Background
HIV infection has been associated with dyslipidemia, insulin resistance and changes in body composition, including loss of subcutaneous fat and skeletal muscle, with relative sparing of upper trunk and visceral fat. Because of resemblance to Cushing's syndrome, caused by glucocorticoid (GC) excess, we hypothesized that variations in the glucocorticoid receptor (GR) gene, associated with changes in sensitivity to GCs, may be associated with such abnormalities in HIV-infected patients.

Methods
GR polymorphisms were determined in HIV-infected participants from the study of Fat Redistribution and Metabolic Change in HIV infection (FRAM). We created haplotypes in 754 participants and assessed the associations with fasting metabolic parameters and body composition by MRI.

Results
After stratification for ethnicity, we found no consistent pattern of associations between described GR haplotypes and body composition or metabolic parameters in HIV-infected patients. However, we found a new haplotype comprising the *Tth111*I polymorphism in African Americans. Heterozygous carriers of this haplotype (n=24) had significantly higher levels of HDL-cholesterol compared with age- and gender-matched non-carriers (n=96) (median 55 vs. 44 mg/dL, p=0.026) and a tendency towards lower glucose (-5 mg/dl) and triglyceride (-21 mg/dl) levels and lower visceral adipose tissue mass (-0.22 L). CD4 count, as well as skeletal muscle mass were also lower in carriers of this haplotype (-154 cells/uL and -1.6 L respectively).

Conclusions
Although our cohort included only a small number of carriers of the new *Tth111*I haplotype, these results are suggestive that this GR haplotype may be associated with healthier metabolic profile in African Americans with HIV-infection.

Introduction

Human immunodeficiency virus (HIV) infection and treatment with highly active antiretroviral therapy (HAART) have been associated with loss of subcutaneous fat mass and skeletal muscle, with retention of visceral fat (1-3). In addition, HIV-infected patients also develop insulin resistance and dyslipidemia (4-6). These findings resemble the symptoms seen in patients with Cushing's syndrome, which is caused by glucocorticoid (GC) excess (7). However, in HIV-infected patients with HAART-associated lipodystrophy, basal serum cortisol levels are not elevated, cortisol levels are suppressed into the normal range with 1 mg of dexamethasone (8) and 24-hour urinary free cortisol excretion is normal (9-10).

The effects of cortisol, the main GC in humans, are mediated by the glucocorticoid receptor (GR). This receptor is a member of the nuclear receptor family, and the gene encoding it is located on chromosome 5. The large variation in sensitivity to GCs between individuals is partly explained by polymorphisms in the GR gene (Figure 1). A number of these polymorphisms are associated with changes in GC sensitivity (11-12).

The *Tth111*I polymorphism (rs10052957) (13) is associated with elevated diurnal cortisol levels (14). The functionality of this polymorphism is unknown and in Caucasians the *Tth111*I polymorphism is always linked to the ER22/23EK, the *Bcl*I or the 9β polymorphism. The ER22/23EK polymorphism (rs6189 and rs6190) in codons 22 and 23 in exon 2 is associated with a relative resistance to GCs, a healthier metabolic profile and increased insulin sensitivity (15-16). The *Bcl*I polymorphism (rs41423247) is, just like the N363S polymorphism (rs6195), associated with increased sensitivity to GCs, higher BMI, higher waist-to-hip ratio, visceral adiposity, hypertension, and higher cholesterol and triglyceride concentrations (12). The 9β polymorphism is hypothesized to result in increased expression and stability of the splice variant GR-β in vivo (17). GR-β functions as a dominant negative inhibitor of the active receptor GR-α, and increased expression of GR-β may therefore lead to GC resistance (17).

Our aim was to study the associations of these GR gene polymorphisms with MRI-measured regional fat and skeletal muscle distribution and metabolic parameters in HIV-infected participants from the first examination of the study of Fat Redistribution and Metabolic Change in HIV Infection (FRAM). We hypothesized that polymorphisms associated with relative GC hypersensitivity would be associated with subcutaneous fat loss, muscle loss, and other metabolic abnormalities frequently seen in HIV-infected patients. While previous studies (12) have documented polymorphisms in the GR gene in relation to body composition and metabolic factors, no study has examined these associations of GR gene polymorphisms in the setting of HIV infection.

Methods

Subjects

We determined GR polymorphisms in HIV-infected patients who were participants of the study of Fat Redistribution and Metabolic changes in HIV infection who consented to have DNA analyzed. A detailed description of the FRAM study has been reported elsewhere (18). From all participants written informed consent was obtained. The study protocol was approved by the institutional review board of each participating institution. For this study, all subjects with current use of GCs were excluded.

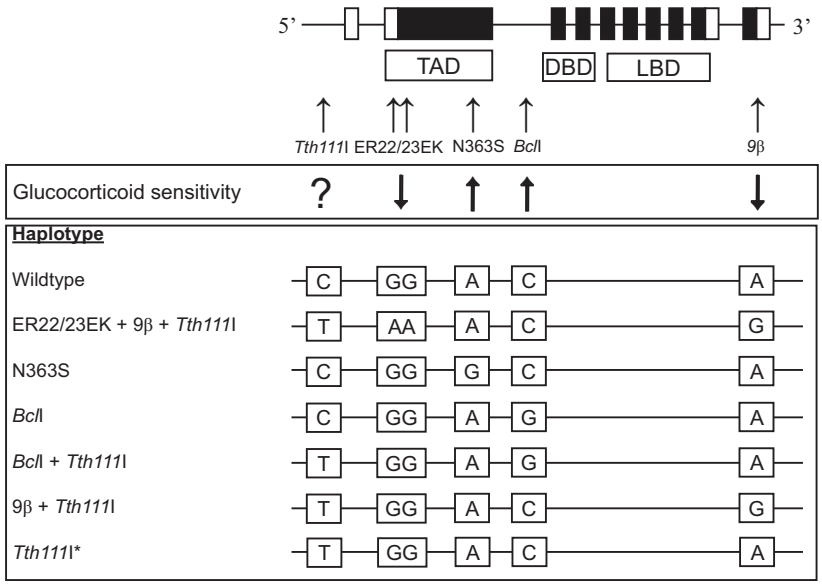

Figure 1. *Glucocorticoid receptor gene and its haplotypes Nucleic acid changes are indicated.*
*A = adenosine; C = cytidine; DBD = DNA-binding domain; G = guanidine; LBD = Ligand-binding domain; T = thymine; TAD = transactivating domain. * We only found this haplotype in African-Americans.*

Data collection

Standardized questionnaires were used to obtain a history of medication use and medical history and data on sociodemographic factors. Fasting serum glucose and lipids (total cholesterol, triglycerides, direct low density lipoprotein (D-LDL) and high density lipoprotein (HDL) cholesterol) were measured centrally (Covance Central Lab Service, Indianapolis, Indiana), as were fasting insulin concentration

(Linco Research Inc, St Louis, MO). Insulin resistance was assessed using HOMA, which was derived as insulin x glucose/22.5, with insulin measured in μIU/mL and glucose in mmol/L. Anthropometric data were collected by centrally trained research associates. Height, weight, waist and hip circumference were measured and waist-to-hip ratio (WHR) and body mass index (BMI) were calculated. Whole-body MRI was performed to evaluate regional and total body composition quantitatively using a standard protocol (1). Adipose tissue and skeletal muscle volumes were normalized in all analyses by dividing by height squared, with summaries back-transformed to 1.75 m of height.

Genetic analysis

DNA was isolated from peripheral blood leucocytes using standard techniques and dissolved in double-distilled water and stored at -20°C. Allelic discrimination was performed to genotype the subjects, using TaqMan Universal PCR master mix, primers and probes (Applied Biosystems) and a Taqman ABI Prism 7900HT Sequence Detection System as previously described (19). Reaction components and amplification parameters were based on the manufacturer's instructions. Five polymorphisms in the GR gene were determined and haplotypes were constructed as previously described (19). The GR gene and its haplotypes are shown in Figure 1. Haplotypes were successfully constructed in 754 HIV infected patients.

Statistical analysis

We compared characteristics of HIV-infected carriers of the haplotypes versus non-carriers using Fisher's exact test and Chi-squared test for categorical variables and the Wilcoxon rank sum test for continuous variables. Our genetic analysis identified 27 HIV-infected carriers of the *Tth111*I haplotype, of whom 24 were African-American, 1 Hispanic and 2 with another ethnicity (Supplemental data 2). We compared characteristics of the 24 African-American carriers to the 257 African-American non-carriers who were HIV-infected, using Fisher's exact test for categorical variables and the Wilcoxon rank sum test for continuous variables. We also compared characteristics of carriers and non-carriers by identifying 96 African-Americans without the *Tth111*I haplotype who were age and gender matched to the carriers. All analyses were conducted using the SAS system, version 9.2 (SAS Institute, Inc., Cary, NC).

Results

Among the 754 HIV-infected participants with successfully constructed haplotypes, median age at examination was 42 years (range 19-76 years), 70% were male, and 37% were African-American. The median duration of HIV infection was 8.1 years (range 1.4-21.1 years).

Table 1. *Hapotype frequency and ethnicity*

	Caucasian	African American	Hispanic	Asian	Other	P-value
Wildtype						<0.001
Non-carrier	123 (31.4%)	33 (11.7%)	12 (18.5%)	0 (0.0%)	3 (37.5%)	
Heterozygous carrier	186 (47.4%)	129 (45.9%)	30 (46.2%)	6 (75.0%)	4 (50.0%)	
Homozygous carrier	83 (21.2%)	119 (42.3%)	23 (35.4%)	2 (25.0%)	1 (12.5%)	
ER22/23EK + 9β + *Tth111*I						0.12
Non-carrier	375 (95.7%)	278 (98.9%)	64 (98.5%)	8 (100.0%)	8 (100.0%)	
Heterozygous carrier	17 (4.3%)	3 (1.1%)	1 (1.5%)	0 (0.0%)	0 (0.0%)	
Homozygous carrier	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
N363S						<0.01
Non-carrier	365 (93.1%)	278 (98.9%)	60 (92.3%)	7 (87.5%)	8 (100.0%)	
Heterozygous carrier	26 (6.6%)	3 (1.1%)	5 (7.7%)	1 (12.5%)	0 (0.0%)	
Homozygous carrier	1 (0.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
*Bcl*I						<0.001
Non-carrier	255 (65.1%)	239 (85.1%)	43 (66.2%)	7 (87.5%)	6 (75.0%)	
Heterozygous carrier	116 (29.6%)	40 (14.2%)	21 (32.3%)	1 (12.5%)	1 (12.5%)	
Homozygous carrier	21 (5.4%)	2 (0.7%)	1 (1.5%)	0 (0.0%)	1 (12.5%)	
*Bcl*I + *Tth111*I						0.31
Non-carrier	284 (72.4%)	194 (69.0%)	55 (84.6%)	4 (50.0%)	6 (75.0%)	
Heterozygous carrier	100 (25.5%)	79 (28.1%)	9 (13.8%)	4 (50.0%)	2 (25.0%)	
Homozygous carrier	8 (2.0%)	8 (2.8%)	1 (1.5%)	0 (0.0%)	0 (0.0%)	
9β + *Tth111*I						<0.001
Non-carrier	294 (75.0%)	255 (90.7%)	53 (81.5%)	8 (100.0%)	6 (75.0%)	
Heterozygous carrier	83 (21.2%)	26 (9.3%)	11 (16.9%)	0 (0.0%)	1 (12.5%)	
Homozygous carrier	15 (3.8%)	0 (0.0%)	1 (1.5%)	0 (0.0%)	1 (12.5%)	
*Tth111*I						<0.001
Non-carrier	392 (100.0%)	257 (91.5%)	64 (98.5%)	8 (100.0%)	6 (75.0%)	
Heterozygous carrier	0 (0.0%)	24 (8.5%)	1 (1.5%)	0 (0.0%)	2 (25.0%)	
Homozygous carrier	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	

We found significant differences in haplotype frequencies between the different ethnicities (Caucasian, African-American, Hispanic, Asian and other) (Table 1). Caucasians were significantly more likely to be carriers of the N363S, the *Bcl*I and 9β + *Tth111*I haplotypes and significantly less likely to be carriers of the wildtype haplotype, compared with African-Americans. Interestingly, in African-Americans, we found 24 heterozygous carriers (8.5% of all African-Americans) of a haplotype with only the *Tth111*I polymorphism, without the presence of one of the other polymorphisms (either *Bcl*I, 9β, or 9β+ER2223EK) to which it is usually linked in Caucasians. This novel haplotype was not present in the Caucasian participants of this study.

In the total group, we found significantly lower visceral adipose tissue (VAT) in homozygous carriers of the wildtype haplotype, and higher triglyceride levels and lower HDL levels in carriers of the *Bcl*I haplotype compared to non-carriers (Supplemental data Table 1A and 1D). Furthermore, we found significantly higher VAT, lower insulin and lower HDL levels in homozygous carriers of the 9β + *Tth111*I haplotype (Supplemental data Table 1F). In contrast, heterozygous carriers of the 9β + *Tth111*I haplotype had higher insulin levels compared to non-carriers and homozygous carriers.

Since we found significant differences in haplotype frequency between the ethnicity groups, we stratified for ethnicity. The number of Hispanics, Asian and other ethnicity groups was low, therefore, we only performed statistical analyses in Caucasians (Supplemental data Table 2) and African Americans (Supplemental data Table 3). In both the Caucasians and African-Americans, we combined heterozygous and homozygous carriers in the statistical analysis to increase the statistical power. In Caucasians, we found no association between the wildtype haplotype and body composition or metabolic parameters. In Caucasian carriers of the ER22/23EK + 9β + *Tth111*I haplotype, we found significant lower level of triglycerides (113 vs. 212 mg/dL, p=0.04). None of the other metabolic parameters was statistically different. Caucasian carriers of the N363S haplotype had significantly higher volume of upper trunk subcutaneous adipose tissue (SAT) (p=0.03). There were no differences in leg SAT, arm SAT, lower trunk SAT and total SAT volume between Caucasian carriers and non-carriers of the N363S haplotype. Caucasian carriers of the *Bcl*I haplotype had borderline significant lower HDL-cholesterol levels. No other statistically significant changes in metabolic parameters were observed.

Furthermore, we found a statistically significant lower total SAT volume in Caucasian carriers of the *Bcl*I + *Tth111*I haplotype (9.0 vs. 11.1 L, p=0.03) and carriers of the 9β + *Tth111*I haplotype were using less HAART compared to non-carriers (71.4% vs. 80.3%, p=0.04). In African-Americans, we only found a slightly lower volume of upper trunk skeletal muscle in carriers of the *Bcl*I + *Tth111*I haplotype (5.5 vs. 5.2 L, p=0.04) compared to non-carriers and a higher frequency of detectable HIV RNA copies in carriers of the 9β + *Tth111*I haplotype (p=0.04).

Among the 281 African-American HIV-infected participants with known haplotype status, 24 were found to be carriers of the novel *Tth111*I haplotype and 257 were non-carriers (Table 1). In carriers of this haplotype, we found significantly higher HDL-cholesterol levels (median 55 vs. 44 mg/dL, p=0.008), and a trend towards lower triglycerides (106 vs 125.5 mg/dL, p=0.09) and lower visceral fat volume (0.87 vs. 0.93 L, p=0.08). In addition, we found lower skeletal muscle volume in the limbs (both $p<0.05$) and a trend towards less lower trunk and total skeletal muscle volume. Furthermore, CD4 count was significantly lower in heterozygous *Tth111*I carriers. After matching carriers and non-carriers based on age and gender, the finding of higher HDL levels, lower leg and total skeletal muscle volume and lower CD4 count remained statistically significant. In addition, there was a trend towards lower visceral fat volume, lower triglycerides and lower glucose levels and HOMA in *Tth111*I carriers in the age and gender matched analysis (Table 2).

Discussion

A major finding of this study is the presence of a novel haplotype comprising the *Tth111*I polymorphism without the presence of the ER22/23EK, 9β or *Bcl*I polymorphism in African-Americans. Although there is extensive literature documenting polymorphisms in the GR gene in different study populations, this specific haplotype has never been found before. Furthermore, among HIV-infected African Americans, we found significantly higher HDL cholesterol levels, lower CD4 count and lower skeletal muscle in (heterozygous) carriers compared with non-carriers of this haplotype. There was also a trend towards lower triglycerides, lower glucose levels, less insulin resistance and lower visceral fat volume in carriers.

A previous study of uninfected Swedish persons found that the *Tth111*I polymorphism is associated with elevated diurnal cortisol levels (14). However, that study did not look at haplotypes; therefore the possible influence of other polymorphisms to which *Tth111*I is usually linked in Caucasians (ER22/23EK, *Bcl*I or 9β polymorphism) cannot be assessed. To our knowledge, this is the first report of this GR gene haplotype which was only present in African Americans. Our results suggest that this unique haplotype might be associated with a healthier metabolic profile, since HDL levels were higher and triglycerides and glucose levels were somewhat lower. However, carriers of the *Tth111*I haplotype also had lower CD4 counts and lower skeletal muscle mass, which might suggest a detrimental effect of this haplotype in the setting of HIV infection. Lower CD4 count and skeletal muscle mass would ordinarily be associated with lower HDL levels. Therefore, replication in a large cohort of healthy African-Americans is necessary to evaluate the effect of this haplotype on GC sensitivity.

The other 6 haplotypes showed some associations with body composition or metabolic parameters in HIV-infected patients. When stratified by ethnicity, we found lower triglycerides levels in Caucasian ER22/23EK + 9β + *Tth111*I haplotype,

Table 2. *Demographic and clinical characteristics of African-American HIV-infected carriers and non-carriers of the Tth111I haplotype*

Parameter	Carrier (n=24)	All non-carriers (n=257)	p-value	Carrier (n=24)	Matched (4:1) non-carriers (n=96)	p-value
Age (years)	43.0 (37.0-45.5)	43.0 (37.0-48.0)	0.44	43.0 (37.0-45.5)	43.0 (37.0-45.0)	0.95
Gender						
Female	12 (50%)	104 (40.5%)	0.39	12 (50%)	48 (50%)	>0.99
Male	12 (50%)	153 (59.5%)		12 (50%)	48 (50%)	
BMI (kg/m2)	23.4 (22.0-28.6)	25.0 (22.0-29.5)	0.20	23.4 (22.0-28.6)	25.5 (22.5-31.3)	0.10
Current CD4 (cells/uL)	215.0 (134.5-428.0)	342.0 (206.5-517.5)	0.04	215.0 (134.5-428.0)	369.0 (202.0-528.0)	0.05
Detectable HIV RNA	15 (62.5%)	147 (57.2%)	0.67	15 (62.5%)	62 (64.6%)	0.81
HAART use	16 (66.7%)	176 (68.5%)	>0.99	16 (66.7%)	61 (63.5%)	0.64
Leg SAT (L)	4.9 (2.6-8.7)	4.4 (2.7-7.1)	0.55	4.9 (2.6-8.7)	5.6 (3.3-8.9)	0.86
VAT (L)	0.87 (0.21-1.06)	0.93 (0.45-1.99)	0.08	0.87 (0.21-1.06)	1.09 (0.46-2.01)	0.06
Arm SAT (L)	1.4 (0.8-1.9)	1.2 (0.9-2.0)	0.92	1.4 (0.8-1.9)	1.5 (0.9-2.2)	0.43
Upper Trunk SAT (L)	3.6 (1.3-5.5)	2.8 (1.8-5.7)	0.78	3.6 (1.3-5.5)	3.5 (2.1-6.5)	0.33
Lower Trunk SAT (L)	5.2 (2.1-10.5)	4.8 (2.7-9.1)	0.99	5.2 (2.1-10.5)	6.2 (2.9-10.2)	0.52
Total SAT (L)	19.7 (10.1-31.7)	15.1 (8.4-25.5)	0.62	19.7 (10.1-31.7)	16.9 (10.4-30.0)	0.70
Leg SM (L)	10.0 (8.9-10.7)	10.9 (9.3-12.4)	0.03	10.0 (8.9-10.7)	11.4 (9.9-12.9)	0.01
Arm SM (L)	3.2 (2.7-3.7)	3.5 (3.0-4.2)	0.05	3.2 (2.7-3.7)	3.5 (2.9-4.3)	0.09
Upper Trunk SM (L)	5.6 (4.5-6.2)	5.4 (4.5-6.4)	0.83	5.6 (4.5-6.2)	5.5 (4.5-6.4)	0.92
Lower Trunk SM (L)	7.2 (6.2-7.9)	7.6 (6.7-9.0)	0.07	7.2 (6.2-7.9)	7.6 (6.4-9.0)	0.17
Total SM (L)	26.0 (22.9-28.0)	28.0 (24.3-32.6)	0.06	26.0 (22.9-28.0)	27.6 (25.2-33.8)	0.05
Glucose (mg/dL)	87.0 (81.0-95.0)	91.0 (85.0-99.0)	0.13	87.0 (81.0-95.0)	92.0 (85.5-99.5)	0.06
Insulin (µU/mL)	13.0 (10.0-18.5)	15.0 (10.0-23.0)	0.33	13.0 (10.0-18.5)	16.0 (12.0-26.0)	0.11
HOMA	3.0 (2.1-4.7)	3.4 (2.3-6.0)	0.31	3.0 (2.1-4.7)	3.8 (2.6-6.8)	0.09
Triglycerides (mg/dL)	106.0 (53.5-162.5)	125.5 (85.0-194.0)	0.09	106.0 (53.5-162.5)	127.0 (86.0-192.0)	0.09
Total Chol (mg/dL)	186.0 (158.0-211.5)	188.0 (159.0-227.5)	0.47	186.0 (158.0-211.5)	187.0 (158.5-215.0)	0.55
D-LDL (mg/dL)	96.5 (74.0-123.5)	104.0 (75.0-138.0)	0.54	96.5 (74.0-123.5)	97.0 (72.0-127.0)	>0.99
HDL (mg/dL)	55.0 (44.5-65.5)	44.0 (37.0-55.5)	0.008	55.0 (44.5-65.5)	44.0 (37.0-56.0)	0.026

Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = Cholesterol. Matching was done by age and gender. All participants are HIV-infected African-Americans. MRI data are adjusted for height.

higher volume of upper trunk SAT in N363S carriers, lower total SAT volume in *Bcl*I + *Tth111*I haplotype carriers and more HAART use in 9β + *Tth111*I haplotype carriers. In African-Americans, we found a lower volume of upper trunk skeletal muscle in *Bcl*I + *Tth111*I haplotype carriers and a higher frequency of detectable HIV RNA copies in 9β + *Tth111*I haplotype carriers. There was no consistent pattern of changes in body composition or metabolic parameters in any of these haplotypes and the statistically significant differences found were small. Therefore, we think that these findings are the result of multiple testing, rather than representing true findings. Since we found no major effect of GR gene haplotypes in our study, it seems unlikely that polymorphisms in the GR gene play an important role in the metabolic changes that are observed in HIV-infected patients.

However, the clinical features of HIV-infected patients, such as muscle wasting, dyslipidemia and visceral obesity-associated insulin resistance, are suggestive of a possible hypercortisolemic state or an increased sensitivity to GCs (20). Additionally, the cytokine profile seen in HIV-infected patients (decreased interleukin (IL)-2, IL-12 and IFN-γ together with an increase in IL-4) is observed in patients with GC excess (21). Since cortisol levels are not elevated in HIV-infected patients with lipodystrophy (9-10), GC sensitivity at the tissue level might be altered. The polymorphisms we studied are associated with changes in GC sensitivity (11), but were not associated with differences in body composition or metabolic parameters in HIV-infected patients in our study (except for the *Tth111*I haplotype in African-Americans). Other mechanisms with more localized effects might be involved in increasing sensitivity to GCs in specific tissues. One of the possible key factors in the development of the metabolic and body composition changes may be the increase in TNF-α, IL-1β and IL-6 that is seen during HIV infection (20). These cytokines can act directly on the insulin signalling system and stimulate the expression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in target tissues. This enzyme converts cortisone into active cortisol (9, 20) and is therefore the local regulator of cortisol bioavailability. It has been shown that the mRNA expression of 11β-HSD1 is increased in adipose tissue of patients with HAART associated changes and that the mRNA expression correlates with the severity of intra-abdominal fat accumulation and metabolic disturbances (22). Another mechanism resulting in increased GC effects at the cellular level is via the HIV-1-encoded accessory protein Vpr. Vpr has multiple functions, including nuclear translocation, induction of apoptosis and host cell arrest in the G2/M phase of the cell cycle (23). Vpr also directly increases glucocorticoid sensitivity in target tissues. The exact mechanism is unclear, but it is known that the LXXLL motif sequence of the Vpr molecule can bind directly to the GR and it is thought that this binding is essential in the interaction between the nuclear receptors and the coactivator molecules (24).

Despite the other mechanisms that change GC sensitivity, the presence or absence of polymorphisms in the GR gene might influence the changes in body composition and metabolic parameters. Our study revealed no effect of 6 haplotypes on body composition and metabolic parameters after adjustment for ethnicity in HIV-infected patients, but we did find an association of the newly discovered *Tth111*I haplotype. Although our cohort included only a small number of carriers of the *Tth111*I haplotype, our results are suggestive that this novel GR haplotype may be associated with higher levels of HDL cholesterol and possibly a healthier metabolic profile, despite lower CD4 count and lower skeletal muscle mass, in African-Americans in the setting of HIV infection. Larger studies are needed in order to further our understanding of the relationship of the GR gene with metabolic abnormalities.

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***Supplementary data 1A**. Associations of GR haplotypes with body composition and metabolic parameters in the total group of HIV-infected patients*

	Wildtype			
Parameter	**non-carriers (n=171)**	**Heterozygous carriers (n=355)**	**Homozygous carriers (n=228)**	**p-value**
Age (years)	42.0 (36.0-47.0)	43.0 (37.0-48.0)	42.5 (37.0-48.0)	0.65
Gender				
Female	45 (26.3%)	105 (29.6%)	68 (29.8%)	0.69
Male	126 (73.7%)	250 (70.4%)	160 (70.2%)	
BMI (kg/m2)	24.2 (22.1-27.4)	24.6 (22.1-27.4)	24.7 (22.1-28.0)	0.61
Current CD4 (cells/uL)	413.0 (243.0-600.0)	379.5 (238.0-559.0)	385.0 (213.5-524.0)	0.41
Detectable HIV RNA	76 (44.4%)	173 (48.7%)	111 (48.7%)	0.58
HAART use	136 (79.5%)	256 (72.1%)	170 (74.6%)	0.24
Leg SAT (L)	3.1 (2.0-5.6)	3.4 (2.2-5.7)	3.2 (2.1-5.9)	0.55
VAT (L)	1.9 (0.8-3.0)	1.6 (0.8-2.8)	1.4 (0.6-2.5)	0.04
Arm SAT (L)	1.1 (0.9-1.5)	1.1 (0.8-1.7)	1.0 (0.8-1.6)	0.90
Upper Trunk SAT (L)	2.9 (1.9-4.7)	2.8 (1.9-4.6)	2.8 (1.8-4.7)	0.94
Lower Trunk SAT (L)	4.1 (2.2-6.2)	4.1 (2.3-7.0)	3.7 (2.5-7.2)	0.74
Total SAT (L)	12.3 (7.5-16.9)	12.3 (7.9-19.3)	11.4 (8.2-18.7)	0.73
Leg SM (L)	10.6 (9.2-12.0)	10.4 (9.1-11.9)	10.8 (9.3-12.1)	0.64
Arm SM (L)	3.5 (2.9-4.1)	3.4 (2.9-4.2)	3.5 (3.0-4.1)	0.94
Upper Trunk SM (L)	5.4 (4.5-6.4)	5.4 (4.6-6.3)	5.3 (4.5-6.4)	0.97
Lower Trunk SM (L)	7.4 (6.5-8.9)	7.6 (6.6-8.9)	7.6 (6.8-9.0)	0.57
Total SM (L)	27.4 (23.9-31.5)	27.3 (23.5-30.9)	27.4 (24.5-31.2)	0.82
Glucose (mg/dL)	92.0 (86.0-101.0)	93.0 (87.0-100.0)	93.0 (86.0-100.0)	0.86
Insulin (μU/mL)	15.0 (11.0-23.0)	16.0 (11.0-23.0)	16.0 (11.0-23.0)	0.86
HOMA	3.4 (2.4-5.6)	3.6 (2.5-5.4)	3.7 (2.3-5.9)	0.93
Triglycerides (mg/dL)	182.0 (111.0-288.5)	159.5 (98.0-271.0)	162.0 (94.0-270.0)	0.28
Total Chol (mg/dL)	192.0 (165.5-235.0)	193.5 (162.0-226.0)	191.0 (162.0-229.5)	0.60
D-LDL (mg/dL)	105.0 (85.5-133.5)	106.0 (77.5-135.5)	108.0 (76.0-130.0)	0.94
HDL (mg/dL)	39.0 (33.0-49.0)	40.5 (32.0-51.0)	43.0 (35.0-54.0)	0.10

Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height.

***Supplementary data 1B**. Associations of GR haplotypes with body composition and metabolic parameters in the total group of HIV-infected patients*

	ER22/23EK + 9beta + *Tth111*I			
Parameter	**non-carriers (n=733)**	**Heterozygous carriers (n=21)**	**Homozygous carriers (n=0)**	**p-value**
Age (years)	42.0 (37.0-48.0)	39.0 (35.0-44.0)		0.12
Gender				
Female	211 (28.8%)	7 (33.3%)		0.40
Male	522 (71.2%)	14 (66.7%)		
BMI (kg/m2)	24.5 (22.1-27.4)	26.1 (22.9-27.3)		0.63
Current CD4 (cells/uL)	385.0 (235.0-559.5)	416.0 (297.0-670.5)		0.38
Detectable HIV RNA	352 (48.0%)	8 (38.1%)		0.39
HAART use	547 (74.6%)	15 (71.42%)		0.80
Leg SAT (L)	3.3 (2.1-5.7)	2.8 (1.6-6.4)		0.51
VAT (L)	1.6 (0.7-2.7)	1.9 (0.7-3.1)		0.69
Arm SAT (L)	1.1 (0.8-1.6)	1.1 (0.8-1.8)		0.92
Upper Trunk SAT (L)	2.8 (1.9-4.7)	2.4 (1.7-3.8)		0.68
Lower Trunk SAT (L)	4.0 (2.4-6.8)	2.8 (2.1-7.8)		0.35
Total SAT (L)	12.1 (8.0-18.6)	9.2 (7.2-20.9)		0.55
Leg SM (L)	10.6 (9.2-12.0)	11.1 (8.8-12.1)		0.83
Arm SM (L)	3.5 (2.9-4.2)	3.6 (2.6-4.1)		0.68
Upper Trunk SM (L)	5.4 (4.6-6.3)	5.3 (4.4-6.4)		0.67
Lower Trunk SM (L)	7.5 (6.6-8.9)	7.6 (6.9-8.5)		0.74
Total SM (L)	27.4 (24.2-30.4)	27.4 (24.7-30.2)		0.76
Glucose (mg/dL)	93.0 (86.0-100.0)	91.0 (85.0-98.0)		0.59
Insulin (μU/mL)	16.0 (11.0-23.0)	15.0 (12.5-22.0)		0.98
HOMA	3.7 (2.4-5.7)	3.5 (2.9-4.3)		0.86
Triglycerides (mg/dL)	166.0 (101.0-277.5)	122.0 (90.0-335.0)		0.38
Total Chol (mg/dL)	192.0 (163.0-230.5)	199.0 (171.0-214.0)		0.60
D-LDL (mg/dL)	107.0 (79.0-135.0)	100.0 (82.0-130.0)		0.51
HDL (mg/dL)	41.0 (33.0-52.0)	41.0 (35.0-49.0)		0.69

Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height.

***Supplementary data 1C**. Associations of GR haplotypes with body composition and metabolic parameters in the total group of HIV-infected patients*

	N363S			
Parameter	**non-carriers (n=718)**	**Heterozygous carriers (n=35)***	**Homozygous carriers (n=1)***	**p-value**
Age (years)	42.0 (37.0-48.0)	45.5 (36.0-49.0)		0.58
Gender				
Female	206 (28.7%)	12 (33.3%)		0.57
Male	512 (71.3%)	24 (66.7%)		
BMI (kg/m2)	24.6 (22.1-27.6)	24.0 (22.2-26.3)		0.47
Current CD4 (cells/uL)	384.0 (230.5-557.5)	479.5 (268.0-729.5)		0.08
Detectable HIV RNA	341 (47.5%)	19 (52.8%)		0.61
HAART use	533 (74.2%)	29 (80.6%)		0.56
Leg SAT (L)	3.3 (2.1-5.8)	2.8 (2.0-3.5)		0.11
VAT (L)	1.6 (0.7-2.7)	1.4 (0.6-3.1)		0.77
Arm SAT (L)	1.1 (0.8-1.6)	1.0 (0.8-1.4)		0.42
Upper Trunk SAT (L)	2.8 (1.9-4.7)	3.6 (2.0-5.3)		0.34
Lower Trunk SAT (L)	4.0 (2.4-6.8)	3.6 (2.2-5.1)		0.48
Total SAT (L)	12.1 (7.9-18.7)	11.1 (8.0-14.4)		0.79
Leg SM (L)	10.6 (9.1-12.0)	9.9 (9.3-10.8)		0.09
Arm SM (L)	3.5 (2.9-4.2)	3.1 (2.9-3.8)		0.08
Upper Trunk SM (L)	5.4 (4.5-6.4)	5.1 (4.6-5.9)		0.14
Lower Trunk SM (L)	7.6 (6.6-8.9)	7.3 (5.8-8.2)		0.06
Total SM (L)	27.5 (23.8-31.3)	25.1 (23.2-28.2)		0.06
Glucose (mg/dL)	93.0 (86.0-100.0)	94.0 (89.5-101.5)		0.40
Insulin (μU/mL)	15.0 (11.0-23.0)	16.5 (11.0-26.0)		0.53
HOMA	3.6 (2.4-5.7)	4.0 (2.6-6.1)		0.59
Triglycerides (mg/dL)	166.0 (99.0-278.5)	148.0 (105.5-239.5)		0.94
Total Chol (mg/dL)	193.0 (163.0-229.5)	176.5 (164.0-232.5)		0.77
D-LDL (mg/dL)	106.0 (79.0-134.0)	112.0 (90.0-141.5)		0.51
HDL (mg/dL)	41.0 (33.0-52.0)	41.0 (36.5-49.5)		0.98

*Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height. * heterozygous and homozygous carriers are combined in the statistical analyses.*

***Supplementary data 1D**. Associations of GR haplotypes with body composition and metabolic parameters in the total group of HIV-infected patients*

	***Bcl*I**			
Parameter	**non-carriers (n=550)**	**Heterozygous carriers (n=179)**	**Homozygous carriers (n=25)**	**p-value**
Age (years)	43.0 (37.0-48.0)	40.0 (36.0-47.0)	44.0 (41.0-51.0)	0.04
Gender				
Female	161 (29.3%)	51 (28.5%)	6 (24.0%)	0.84
Male	389 (70.7%)	128 (71.5%)	19 (76.0%)	
BMI (kg/m2)	24.7 (22.1-27.9)	24.2 (22.1-26.3)	24.7 (22.7-27.7)	0.29
Current CD4 (cells/uL)	385.0 (230.5-554.5)	377.0 (238.0-573.0)	520.0 (303.0-597.0)	0.35
Detectable HIV RNA	269 (48.9%)	82 (45.8%)	9 (36.0%)	0.37
HAART use	400 (72.7%)	141 (78.8%)	21 (84.0%)	0.15
Leg SAT (L)	3.3 (2.1-5.8)	3.5 (2.1-5.6)	3.2 (2.5-4.5)	1.00
VAT (L)	1.6 (0.7-2.6)	1.9 (0.7-2.9)	1.7 (0.9-2.8)	0.36
Arm SAT (L)	1.1 (0.8-1.6)	1.1 (0.9-1.5)	1.2 (0.9-1.4)	0.88
Upper Trunk SAT (L)	2.8 (1.9-4.7)	2.8 (1.8-4.4)	2.9 (2.1-5.0)	0.66
Lower Trunk SAT (L)	3.9 (2.3-7.1)	4.2 (2.3-6.4)	4.6 (3.2-6.0)	0.61
Total SAT (L)	11.7 (7.8-18.9)	12.7 (7.8-17.4)	13.5 (10.2-15.5)	0.78
Leg SM (L)	10.6 (9.2-12.1)	10.4 (9.2-11.7)	9.8 (8.9-12.6)	0.84
Arm SM (L)	3.5 (2.9-4.2)	3.4 (2.9-4.0)	3.1 (2.5-4.2)	0.51
Upper Trunk SM (L)	5.4 (4.5-6.3)	5.5 (4.6-6.4)	5.4 (4.3-6.5)	0.83
Lower Trunk SM (L)	7.6 (6.7-9.0)	7.3 (6.4-8.6)	7.7 (6.1-8.9)	0.28
Total SM (L)	27.4 (23.8-31.0)	27.2 (23.8-31.2)	27.7 (21.4-32.3)	0.75
Glucose (mg/dL)	93.0 (86.0-100.0)	92.0 (86.0-99.0)	94.0 (84.0-106.0)	0.54
Insulin (μU/mL)	15.5 (11.0-24.0)	16.0 (11.0-21.0)	15.0 (11.0-23.0)	0.95
HOMA	3.7 (2.4-5.9)	3.5 (2.5-5.2)	3.3 (2.4-5.1)	0.80
Triglycerides (mg/dL)	152.0 (97.0-268.0)	191.0 (112.0-303.0)	174.0 (91.0-275.0)	0.05
Total Chol (mg/dL)	192.0 (164.0-226.0)	194.0 (160.0-239.0)	205.0 (168.0-233.0)	0.48
D-LDL (mg/dL)	108.0 (79.0-132.0)	103.0 (79.0-136.5)	99.0 (83.0-148.0)	0.87
HDL (mg/dL)	42.0 (34.0-53.0)	38.0 (32.0-48.0)	38.0 (32.0-47.0)	0.01

Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height.

Supplementary data 1E. *Associations of GR haplotypes with body composition and metabolic parameters in the total group of HIV-infected patients*

	BclI + Tth111I			
Parameter	**non-carriers (n=543)**	**Heterozygous carriers (n=194)**	**Homozygous carriers (n=17)**	**p-value**
Age (years)	42.0 (37.0-48.0)	42.0 (37.0-48.0)	42.0 (36.0-46.0)	0.94
Gender				
Female	160 (29.5%)	52 (26.8%)	6 (35.3%)	0.66
Male	383 (70.5%)	142 (73.2%)	11 (64.7%)	
BMI (kg/m2)	24.6 (22.2-27.4)	24.1 (21.9-27.4)	26.6 (24.9-29.3)	0.13
Current CD4 (cells/uL)	389.0 (230.5-560.5)	380.0 (254.5-557.5)	417.0 (237.0-624.0)	0.91
Detectable HIV RNA	267 (49.2%)	85 (43.8%)	8 (47.1%)	0.40
HAART use	402 (74.0%)	147 (75.8%)	13 (76.5%)	0.88
Leg SAT (L)	3.2 (2.1-5.7)	3.3 (2.0-5.8)	3.9 (1.8-6.4)	0.87
VAT (L)	1.6 (0.7-2.7)	1.5 (0.7-2.6)	2.1 (1.0-3.4)	0.49
Arm SAT (L)	1.1 (0.9-1.6)	1.1 (0.8-1.6)	1.4 (0.7-2.0)	0.72
Upper Trunk SAT (L)	2.9 (1.9-4.8)	2.7 (1.8-4.2)	4.5 (2.0-5.80	0.25
Lower Trunk SAT (L)	4.0 (2.4-6.6)	3.7 (2.1-6.9)	6.3 (2.5-9.6)	0.19
Total SAT (L)	12.2 (8.3-18.3)	11.5 (6.9-17.9)	16.7 (6.1-20.9)	0.28
Leg SM (L)	10.5 (9.1-11.8)	10.6 (9.1-12.1)	11.8 (9.3-12.5)	0.52
Arm SM (L)	3.5 (2.9-4.1)	3.6 (3.0-4.2)	3.6 (3.0-3.9)	0.57
Upper Trunk SM (L)	5.4 (4.5-6.3)	5.4 (4.6-6.4)	5.0 (4.6-6.7)	0.91
Lower Trunk SM (L)	7.5 (6.6-8.8)	7.6 (6.6-9.0)	8.0 (7.0-9.6)	0.31
Total SM (L)	27.3 (23.8-30.7)	27..9 (23.6-31.8)	27.6 (25.9-33.4)	0.71
Glucose (mg/dL)	93.0 (86.0-100.0)	92.0 (87.0-101.0)	93.0 (88.0-98.0)	0.84
Insulin (μU/mL)	16.0 (11.0-23.0)	15.0 (11.0-22.0)	19.0 (11.0-28.0)	0.78
HOMA	3.7 (2.4-5.6)	3.4 (2.5-5.9)	3.8 (2.6-7.3)	0.76
Triglycerides (mg/dL)	165.0 (97.0-280.0)	168.5 (106.0-283.0)	145.0 (109.0-204.0)	0.74
Total Chol (mg/dL)	193.0 (163.0-231.0)	192.0 (164.0-229.0)	194.0 (153.0-213.0)	0.76
D-LDL (mg/dL)	106.0 (77.0-135.0)	107.5 (85.0-133.0)	110.0 (74.0-150.0)	0.92
HDL (mg/dL)	40.0 (33.0-51.0)	41.0 (33.0-52.0)	44.0 (29.0-53.0)	0.75

Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height.

***Supplementary data 1F**. Associations of GR haplotypes with body composition and metabolic parameters in the total group of HIV-infected patients*

	9beta + *Tth111*I			
Parameter	**non-carriers (n=616)**	**Heterozygous carriers (n=121)**	**Homozygous carriers (n=17)**	**p-value**
Age (years)	42.0 (37.0-48.0)	44.0 (38.0-49.0)	40.0 (38.0-46.0)	0.40
Gender				
Female	183 (29.7%)	34 (28.1%)	1 (5.9%)	0.10
Male	433 (70.3%)	87 (71.9%)	16 (94.1%)	
BMI (kg/m2)	24.6 (22.1-27.4)	24.5 (22.7-27.7)	24.4 (22.0-25.7)	0.60
Current CD4 (cells/uL)	384.5 (229.0-555.0)	408.5 (256.5-602.0)	317.0 (164.0-558.0)	0.52
Detectable HIV RNA	287 (46.6%)	66 (54.5%)	7 (41.2%)	0.26
HAART use	465 (75.5%)	83 (68.6%)	14 (82.4%)	0.18
Leg SAT (L)	3.3 (2.1-5.8)	3.1 (2.2-5.4)	2.3 (1.9-3.6)	0.30
VAT (L)	1.5 (0.7-2.6)	2.0 (1.0-2.9)	2.2 (1.3-4.4)	<0.01
Arm SAT (L)	1.1 (0.8-1.6)	1.1 (0.9-1.6)	1.1 (0.9-1.3)	0.71
Upper Trunk SAT (L)	2.8 (1.8-4.8)	2.9 (2.0-4.6)	2.8 (2.1-3.6)	0.62
Lower Trunk SAT (L)	3.9 (2.3-7.0)	4.3 (2.5-6.6)	3.4 (2.1-4.50	0.41
Total SAT (L)	12.0 (7.8-18.7)	12.7 (8.7-19.1)	10.2 (8.9-12.3)	0.41
Leg SM (L)	10.6 (9.1-12.0)	10.6 (9.3-11.9)	10.6 (9.4-11.5)	0.92
Arm SM (L)	3.5 (2.9-4.1)	3.5 (2.9-4.3)	3.8 (3.2-4.2)	0.48
Upper Trunk SM (L)	5.4 (4.5-6.3)	5.6 (4.6-6.5)	5.8 (4.5-6.3)	0.51
Lower Trunk SM (L)	7.6 (6.6-8.9)	7.6 (6.7-9.0)	7.4 (6.5-7.7)	0.94
Total SM (L)	27.3 (23.6-31.0)	27.6 (24.5-31.4)	27.4 (24.4-30.1)	0.69
Glucose (mg/dL)	93.0 (86.0-100.0)	93.0 (86.5-101.5)	93.0 (82.0-97.0)	0.87
Insulin (µU/mL)	15.0 (11.0-23.0)	19.0 (12.0-26.0)	13.5 (8.5-18.0)	0.04
HOMA	3.6 (2.4-5.5)	3.9 (2.5-6.3)	3.0 (1.9-4.4)	0.08
Triglycerides (mg/dL)	165.0 (97.0-272.0)	155.0 (111.0-288.0)	277.0 (159.0-454.0)	0.07
Total Chol (mg/dL)	191.5 (161.0-230.0)	198.0 (170.0-224.0)	191.0 (173.0-244.0)	0.43
D-LDL (mg/dL)	106.0 (79.0-134.0)	111.0 (88.0-134.0)	91.0 (72.0-117.0)	0.35
HDL (mg/dL)	41.0 (33.0-53.0)	38.0 (33.0-49.0)	34.0 (30.0-44.0)	0.04

Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height.

Supplementary data 2A. *Associations of GR haplotypes with body composition and metabolic parameters in Caucasian HIV-infected patients*

	Wildtype			
Parameter	**non-carriers (n=123)**	**Heterozygous carriers (n=186)**	**Homozygous carriers (n=83)**	**p-value**
Age (years)	41.0 (36.0-48.0)	44.0 (38.0-51.0)	41.0 (38.0-48.0)	0.09
Gender				0.88
Female	24 (19.5%)	40 (21.5%)	16 (19.3%)	
Male	99 (80.5%)	146 (78.5%)	67 (80.7%)	
BMI (kg/m2)	24.1 (22.1-26.9)	24.5 (22.2-26.8)	24.1 (21.9-26.3)	0.38
Current CD4 (cells/uL)	419.0 (273.0-625.0)	423.0 (279.0-585.0)	403.0 (199.5-563.3)	0.53
Detectable HIV RNA	49 (39.8%)	79 (42.5%)	30 (36.1%)	0.62
HAART use	98 (79.7%)	139 (74.7%)	69 (83.1%)	0.27
Leg SAT (L)	2.8 (1.9-4.3)	2.9 (1.8-4.5)	2.5 (2.0-3.6)	0.79
VAT (L)	2.02 (0.79-3.31)	2.40 (1.17-3.43)	1.86 (0.81-3.13)	0.07
Arm SAT (L)	1.0 (0.8-1.4)	1.0 (0.8-1.4)	1.0 (0.8-1.2)	0.42
Upper Trunk SAT (L)	2.7 (1.8-4.4)	2.7 (2.0-4.4)	2.8 (1.8-3.8)	0.61
Lower Trunk SAT (L)	3.4 (2.0-5.4)	3.7 (2.2-5.6)	3.4 (2.1-5.3)	0.43
Total SAT (L)	10.7 (6.8-14.5)	11.0 (7.6-15.6)	10.5 (7.8-13.1)	0.69
Leg SM (L)	10.6 (9.3-12.1)	10.4 (9.0-11.6)	10.1 (8.4-11.6)	0.16
Arm SM (L)	3.6 (2.9-4.2)	3.4 (2.9-4.1)	3.4 (2.8-4.1)	0.50
Upper Trunk SM (L)	5.6 (4.6-6.5)	5.4 (4.6-6.2)	5.1 (4.5-6.4)	0.45
Lower Trunk SM (L)	7.4 (6.5-8.9)	7.7 (6.6-8.8)	7.3 (6.5-8.9)	0.85
Total SM (L)	27.8 (24.6-31.7)	27.0 (23.5-30.2)	26.4 (23.1-30.2)	0.27
Glucose (mg/dL)	87.0 (79.8-93.0)	88.0 (84.0-95.0)	85.0 (78.8-92.0)	0.22
Insulin (µU/mL)	15.0 (10.8-23.5)	16.0 (11.0-23.8)	16.0 (11.0-23.0)	0.74
HOMA	3.4 (2.4-5.7)	3.9 (2.6-6.0)	3.7 (2.3-5.4)	0.61
Triglycerides (mg/dL)	210.0 (115.0-343.0)	203.5 (118.5-311.3)	211.0 (133.0-317.0)	0.94
Total Chol (mg/dL)	200.0 (167.5-235.0)	202.0 (169.8-244.0)	193.0 (170.0-228.0)	0.74
D-LDL (mg/dL)	104.0 (85.0-130.0)	115.0 (87.0-141.0)	111.0 (79.0-139.0)	0.26
HDL (mg/dL)	38.0 (32.0-47.0)	38.5 (30.0-47.0)	37.0 (32.0-51.0)	0.95

Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height.

Supplementary data 2B. *Associations of GR haplotypes with body composition and metabolic parameters in Caucasian HIV-infected patients*

	ER22/23EK + 9beta + *Tth111*I			
Parameter	**non-carriers (n=375)**	**Heterozygous carriers (n=17)**	**Homozygous carriers (n=0)**	**p-value**
Age (years)	43.0 (38.0-50.0)	39.0 (35.0-45.0)		0.07
Gender				0.12
Female	74 (19.7%0	6 (35.3%)		
Male	301 (80.3%)	11 (64.7%)		
BMI (kg/m2)	24.3 (22.1-26.6)	26.1 (22.5-29.3)		0.42
Current CD4 (cells/uL)	415.0 (273.0-583.0)	445.0 (322.0-704.0)		0.29
Detectable HIV RNA	151 (40.3%)	7 (41.2%)		0.94
HAART use	293 (78.1%)	13 (76.5%)		0.82
Leg SAT (L)	2.8 (1.9-4.1)	3.1 (1.6-7.1)		0.91
VAT (L)	2.14 (1.02-3.33)	1.95 (0.82-2.91)		0.48
Arm SAT (L)	1.0 (0.8-1.3)	1.0 (0.8-2.1)		0.59
Upper Trunk SAT (L)	2.7 (1.9-4.2)	2.8 (1.5-5.8)		0.93
Lower Trunk SAT (L)	3.5 (2.1-5.4)	3.0 (1.6-9.3)		0.80
Total SAT (L)	10.8 (7.4-14.9)	9.9 (6.5-24.4)		0.95
Leg SM (L)	10.4 (9.0-11.7)	10.9 (8.4-11.9)		0.83
Arm SM (L)	3.4 (2.9-4.1)	3.7 (2.5-4.2)		0.91
Upper Trunk SM (L)	5.3 (4.6-6.4)	4.7 (4.3-5.8)		0.15
Lower Trunk SM (L)	7.5 (6.6-8.9)	7.5 (6.5-8.1)		0.41
Total SM (L)	27.2 (23.3-30.9)	27.4 (22.7-30.0)		0.63
Glucose (mg/dL)	94.0 (87.8-102.0)	91.0 (84.0-97.5)		0.24
Insulin (µU/mL)	16.0 (11.0-23.0)	14.0 (10.5-20.5)		0.59
HOMA	3.7 (2.5-5.7)	3.3 (2.6-4.2)		0.42
Triglycerides (mg/dL)	212.0 (123.0-321.0)	113.0 (90.0-300.5)		0.04
Total Chol (mg/dL)	201.0 (170.0-235.0)	199.0 (159.5-214.0)		0.33
D-LDL (mg/dL)	110.0 (86.0-139.0)	105.0 (75.0-131.5)		0.50
HDL (mg/dL)	38.0 (31.0-47.0)	39.0 (35.0-48.5)		0.29

Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height.

***Supplementary data 2C**. Associations of GR haplotypes with body composition and metabolic parameters in Caucasian HIV-infected patients*

	N363S			
Parameter	**non-carriers (n=365)**	**Heterozygous carriers (n=26)***	**Homozygous carriers (n=1)***	**p-value**
Age (years)	42.0 (37.0-49.5)	46.0 (36.0-51.0)		0.25
Gender				0.22
Female	72 (19.7%)	8 (29.6%)		
Male	293 (80.3%)	19 (70.4%)		
BMI (kg/m2)	24.3 (22.1-26.7)	25.2 (22.2-26.6)		0.84
Current CD4 (cells/uL)	412.0 (272.5-582.0)	503.0 (344.0-877.0)		0.06
Detectable HIV RNA	145 (39.8%)	13 (48.1%)		0.39
HAART use	285 (78.1%)	21 (77.8%)		0.91
Leg SAT (L)	2.8 (1.9-4.2)	2.4 (1.9-3.4)		0.48
VAT (L)	2.16 (1.03-3.32)	1.81 (0.77-3.60)		0.87
Arm SAT (L)	1.0 (0.8-1.3)	1.0 (0.8-1.5)		0.88
Upper Trunk SAT (L)	2.7 (1.8-4.1)	3.6 (2.4-5.8)		0.03
Lower Trunk SAT (L)	3.5 (2.1-5.4)	3.8 (2.2-5.4)		0.62
Total SAT (L)	10.8 (7.0-15.1)	11.1 (8.2-14.5)		0.36
Leg SM (L)	10.4 (9.0-11.7)	10.0 (9.3-11.1)		0.48
Arm SM (L)	3.5 (2.9-4.1)	3.3 (2.9-3.9)		0.26
Upper Trunk SM (L)	5.4 (4.6-6.4)	5.1 (4.6-6.1)		0.24
Lower Trunk SM (L)	7.5 (6.6-8.9)	7.6 (6.0-8.5)		0.44
Total SM (L)	27.3 (23.4-30.9)	25.6 (23.2-29.1)		0.33
Glucose (mg/dL)	94.0 (87.0-101.8)	94.0 (91.0-102.0)		0.43
Insulin (μU/mL)	16.0 (11.0-23.0)	18.0 (11.0-27.0)		0.30
HOMA	3.7 (2.4-5.5)	4.1 (2.5-6.5)		0.33
Triglycerides (mg/dL)	205.0 (117.5-321.5)	215.0 (135.0-303.0)		0.88
Total Chol (mg/dL)	201.0 (170.0-235.0)	206.0 (163.0-259.0)		0.97
D-LDL (mg/dL)	110.0 (84.5-136.0)	117.0 (88.0-143.0)		0.51
HDL (mg/dL)	38.0 (31.0-47.0)	40.0 (33.0-46.0)		0.47

*Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height. * heterozygous and homozygous carriers are combined in the statistical analyses.*

***Supplementary data 2D**. Associations of GR haplotypes with body composition and metabolic parameters in Caucasian HIV-infected patients*

	BclI			
Parameter	**non-carriers (n=255)**	**Heterozygous carriers (n=116)***	**Homozygous carriers (n=21)***	**p-value**
Age (years)	43.0 (38.0-50.0)	42.0 (36.5-49.5)		0.48
Gender				0.78
Female	51 (20.0%)	29 (21.2%)		
Male	204 (80.0%)	108 (78.8%)		
BMI (kg/m2)	24.2 (22.0-26.8)	24.4 (22.1-26.6)		0.67
Current CD4 (cells/uL)	410.5 (274.5-591.0)	432.0 (258.0-581.5)		0.57
Detectable HIV RNA	100 (39.2%)	58 (42.3%)		0.55
HAART use	200 (78.4%)	106 (77.4%)		0.80
Leg SAT (L)	2.6 (1.9-4.0)	2.9 (2.0-4.9)		0.11
VAT (L)	2.13 (1.05-3.32)	2.12 (0.94-3.35)		0.80
Arm SAT (L)	1.0 (0.8-1.3)	1.0 (0.8-1.4)		0.20
Upper Trunk SAT (L)	2.7 (1.9-4.3)	2.7 (1.9-4.2)		0.99
Lower Trunk SAT (L)	3.4 (2.1-5.4)	3.6 (2.2-5.6)		0.73
Total SAT (L)	10.7 (6.9-15.1)	11.1 (7.7-15.0)		0.55
Leg SM (L)	10.3 (8.9-11.7)	10.6 (9.2-11.9)		0.37
Arm SM (L)	3.5 (2.8-4.1)	3.4 (2.9-4.2)		0.89
Upper Trunk SM (L)	5.2 (4.5-6.3)	5.5 (4.7-6.4)		0.32
Lower Trunk SM (L)	7.6 (6.6-8.9)	7.4 (6.5-8.8)		0.63
Total SM (L)	27.0 (23.2-30.4)	27.5 (23.9-31.3)		0.64
Glucose (mg/dL)	95.0 (87.0-101.0)	93.0 (88.0-102.0)		0.51
Insulin (μU/mL)	16.0 (11.0-23.0)	16.0 (11.0-23.0)		0.92
HOMA	3.8 (2.4-5.9)	3.6 (2.5-5.4)		0.92
Triglycerides (mg/dL)	190.0 (116.0-300.0)	219.0 (129.0-356.0)		0.07
Total Chol (mg/dL)	201.0 (171.0-229.0)	200.0 (166.5-244.0)		0.97
D-LDL (mg/dL)	113.0 (86.5-139.0)	103.0 (79.0-134.0)		0.14
HDL (mg/dL)	39.0 (32.0-48.5)	37.0 (30.0-45.0)		0.05

*Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height. * heterozygous and homozygous carriers are combined in the statistical analyses.*

***Supplementary data 2E**. Associations of GR haplotypes with body composition and metabolic parameters in Caucasian HIV-infected patients*

	BclI + Tth111I			
Parameter	**non-carriers (n=284)**	**Heterozygous carriers (n=100)***	**Homozygous carriers (n=8)***	**p-value**
Age (years)	44.0 (37.3-50.0)	41.0 (37.0-46.0)		0.16
Gender				0.39
Female	61 (21.5%)	19 (17.6%)		
Male	223 (78.5%)	89 (82.4%)		
BMI (kg/m2)	24.4 (22.1-26.6)	24.0 (21.9-26.9)		0.50
Current CD4 (cells/uL)	424.0 (268.5-591.5)	385.0 (279.0-558.0)		0.98
Detectable HIV RNA	120 (42.3%)	38 (35.2%)		0.20
HAART use	218 (76.8%)	88 (81.5%)		0.29
Leg SAT (L)	2.8 (2.0-4.3)	2.8 (1.7-4.1)		0.34
VAT (L)	2.05 (1.00-3.27)	2.19 (1.10-3.64)		0.59
Arm SAT (L)	1.0 (0.8-1.3)	0.9 (0.7-1.4)		0.13
Upper Trunk SAT (L)	2.8 (2.0-4.3)	2.5 (1.6-4.1)		0.07
Lower Trunk SAT (L)	3.5 (2.2-5.4)	3.3 (1.8-5.3)		0.18
Total SAT (L)	11.1 (8.2-15.0)	9.0 (6.0-15.2)		0.03
Leg SM (L)	10.3 (9.0-11.7)	10.7 (9.2-12.1)		0.22
Arm SM (L)	3.4 (2.8-4.1)	3.6 (2.9-4.1)		0.27
Upper Trunk SM (L)	5.3 (4.5-6.3)	5.4 (4.7-6.5)		0.39
Lower Trunk SM (L)	7.4 (6.5-8.8)	7.6 (6.8-9.0)		0.27
Total SM (L)	27.0 (23.2-30.5)	27.9 (24.1-31.7)		0.24
Glucose (mg/dL)	94.0 (87.0-101.0)	95.0 (89.0-103.0)		0.24
Insulin (μU/mL)	16.0 (11.0-230)	16.0 (11.0-230)		0.99
HOMA	3.7 (2.4-5.5)	3.6 (2.6-6.1)		0.77
Triglycerides (mg/dL)	203.5 (115.3-324.0)	217.5 (120.0-293.0)		0.81
Total Chol (mg/dL)	199.0 (169.0-233.0)	204.0 (175.0-236.0)		0.51
D-LDL (mg/dL)	109.0 (79.0-139.0)	114.0 (89.3-135.8)		0.48
HDL (mg/dL)	38.0 (31.0-47.0)	39.5 (31.3-47.8)		0.39

*Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height. * heterozygous and homozygous carriers are combined in the statistical analyses.*

***Supplementary data 2F**. Associations of GR haplotypes with body composition and metabolic parameters in Caucasian HIV-infected patients*

	9beta + *Tth111*I			
Parameter	**non-carriers (n=294)**	**Heterozygous carriers (n=83)***	**Homozygous carriers (n=15)***	**p-value**
Age (years)	42.0 (37.0-49.0)	45.0 (38.0-50.0)		0.29
Gender				0.56
Female	62 (21.1%)	18 (18.4%)		
Male	232 (78.9%)	80 (81.6%)		
BMI (kg/m2)	24.3 (22.1-26.9)	24.2 (22.1-27.0)		0.69
Current CD4 (cells/uL)	415.0 (272.0-578.0)	418.0 (273.5-638.5)		0.67
Detectable HIV RNA	114 (38.8%)	44 (44.9%)		0.29
HAART use	236 (80.3%)	70 (71.4%)		0.04
Leg SAT (L)	2.8 (1.9-4.2)	2.6 (1.9-4.7)		0.95
VAT (L)	2.13 (1.02-3.34)	2.06 (1.04-3.29)		0.72
Arm SAT (L)	1.0 (0.8-1.4)	1.1 (0.8-1.3)		0.42
Upper Trunk SAT (L)	2.7 (1.8-4.2)	2.7 (2.0-4.3)		0.93
Lower Trunk SAT (L)	3.5 (2.1-5.4)	3.5 (2.1-5.4)		0.94
Total SAT (L)	10.8 (7.1-15.0)	10.9 (7.9-15.2)		0.49
Leg SM (L)	10.3 (9.0-11.8)	10.6 (9.3-11.7)		0.66
Arm SM (L)	3.4 (2.8-4.1)	3.6 (3.0-4.3)		0.20
Upper Trunk SM (L)	5.3 (4.6-6.3)	5.7 (4.6-6.4)		0.35
Lower Trunk SM (L)	7.5 (6.6-8.8)	7.5 (6.6-8.9)		0.80
Total SM (L)	27.1 (23.2-30.3)	27.4 (23.9-31.1)		0.30
Glucose (mg/dL)	94.0 (87.0-101.0)	95.0 (88.0-102.5)		0.45
Insulin (μU/mL)	16.0 (11.0-23.0)	17.0 (10.0-25.0)		0.72
HOMA	3.7 (2.5-5.4)	4.3 (2.4-5.9)		0.58
Triglycerides (mg/dL)	212.5 (122.0-314.5)	192.0 (115.8-338.5)		0.79
Total Chol (mg/dL)	200.5 (169.0-235.0)	201.0 (170.0-234.5)		0.76
D-LDL (mg/dL)	109.5 (82.8-137.3)	110.5 (87.8-135.3)		0.79
HDL (mg/dL)	38.0 (31.0-47.0)	38.0 (31.8-47.3)		0.86

*Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height. * heterozygous and homozygous carriers are combined in the statistical analyses.*

Supplementary data 3A. *Associations of GR haplotypes with body composition and metabolic parameters in African-American HIV-infected patients*

	Wildtype			
Parameter	**non-carriers (n=33)**	**Heterozygous carriers (n=129)**	**Homozygous carriers (n=119)**	**p-value**
Age (years)	43 (36-47)	42 (37-47)	43 (37-48)	0.67
Gender				0.67
Female	16 (48.5%)	52 (40.3%)	48 (40.3%)	
Male	17 (51.5%)	77 (59.7%)	71 (59.7%)	
BMI (kg/m2)	23.7 (22.2-28.7)	24.7 (21.6-28.3)	25.2 (22.5-30.1)	0.19
Current CD4 (cells/uL)	299.0 (187.0-562.8)	330.0 (183.0-500.0)	362.5 (209.8-526.8)	0.66
Detectable HIV RNA	20 (60.6%)	71 (55.0%)	71 (59.7%)	0.64
HAART use	26 (78.8%)	85 (65.9%)	81 (68.1%)	0.39
Leg SAT (L)	5.8 (2.9-7.1)	3.9 (2.5-7.6)	4.4 (2.6-8.7)	0.76
VAT (L)	1.11 (0.54-2.16)	0.93 (0.40-1.85)	0.88 (0.46-1.90)	0.63
Arm SAT (L)	1.3 (1.0-2.0)	1.3 (0.8-1.9)	1.2 (0.8-2.1)	0.78
Upper Trunk SAT (L)	3.5 (2.0-5.5)	2.8 (1.7-5.5)	2.8 (1.7-5.8)	0.59
Lower Trunk SAT (L)	6.3 (4.0-9.5)	4.0 (2.3-9.0)	5.0 (2.7-9.7)	0.37
Total SAT (L)	16.6 (12.1-23.7)	15.0 (8.1-26.6)	15.1 (8.4-27.5)	0.79
Leg SM (L)	10.3 (8.9-11.6)	10.5 (9.5-12.4)	11.1 (9.3-12.4)	0.39
Arm SM (L)	3.5 (2.7-4.0)	3.4 (2.9-4.3)	3.5 (3.0-4.3)	0.49
Upper Trunk SM (L)	5.1 (4.4-5.8)	5.5 (4.5-6.4)	5.4 (4.5-6.4)	0.45
Lower Trunk SM (L)	7.0 (6.5-8.1)	7.5 (6.5-8.9)	7.6 (6.9-9.1)	0.26
Total SM (L)	26.3 (22.7-30.2)	27.6 (23.6-31.3)	28.0 (25.0-32.3)	0.41
Glucose (mg/dL)	82.0 (77.2-89.0)	82.5 (77.0-92.0)	85.8 (78.9-93.0)	0.18
Insulin (μU/mL)	14.5 (10.3-27.0)	15.0 (11.0-22.0)	15.0 (10.0-24.0)	0.88
HOMA	3.5 (2.3-7.1)	3.3 (2.2-4.9)	3.5 (2.3-6.0)	0.74
Triglycerides (mg/dL)	120.0 (92.5-171.5)	118.0 (77.0-190.5)	132.0 (80.3-201.0)	0.89
Total Chol (mg/dL)	192.0 (161.0-233.0)	188.0 (156.5-218.0)	186.0 (156.0-236.0)	0.36
D-LDL (mg/dL)	121.0 (85.0-144.5)	95.0 (73.0-133.5)	107.0 (75.8-130.0)	0.40
HDL (mg/dL)	47.0 (38.5-63.5)	45.0 (36.0-58.5)	45.5 (37.8-55.0)	0.80

Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height.

***Supplementary data 3B**. Associations of GR haplotypes with body composition and metabolic parameters in African-American HIV-infected patients*

	BclI			
Parameter	**non-carriers (n=239)**	**Heterozygous carriers (n=40)***	**Homozygous carriers (n=2)***	**p-value**
Age (years)	43.0 (37.0-47.0)	40.5 (35.5-47.0)		0.21
Gender				0.82
Female	98 (41.0%)	18 (42.9%)		
Male	141 (59.0%)	24 (57.15)		
BMI (kg/m2)	25.1 (22.0-29.6)	24.2 (21.9-25.9)		0.14
Current CD4 (cells/uL)	325.0 (194.0-509.5)	360.0 (234.0-554.0)		0.49
Detectable HIV RNA	140 (58.6%)	22 (52.4%)		0.42
HAART use	160 (66.9%)	32 (76.2%)		0.25
Leg SAT (L)	4.3 (2.7-8.1)	5.4 (2.4-6.2)		0.65
VAT (L)	0.93 (0.45-1.86)	0.71 (0.22-2.19)		0.84
Arm SAT (L)	1.3 (0.8-2.1)	1.2 (1.0-1.8)		0.97
Upper Trunk SAT (L)	2.9 (1.7-5.7)	2.8 (1.6-4.5)		0.69
Lower Trunk SAT (L)	4.9 (2.6-9.6)	4.8 (2.9-7.6)		0.79
Total SAT (L)	15.2 (8.3-26.7)	15.3 (11.2-21.9)		0.92
Leg SM (L)	10.9 (9.3-12.3)	10.7 (9.5-11.9)		0.78
Arm SM (L)	3.5 (3.0-4.3)	3.6 (2.9-4.0)		0.94
Upper Trunk SM (L)	5.4 (4.5-6.3)	5.4 (4.4-6.5)		0.78
Lower Trunk SM (L)	7.6 (6.7-9.0)	7.2 (6.5-8.5)		0.44
Total SM (L)	27.6 (24.0-31.3)	26.7 (23.8-31.1)		0.92
Glucose (mg/dL)	91.0 (84.0-99.3)	90.5 (83.0-98.3)		0.65
Insulin (μU/mL)	15.0 (10.0-24.0)	14.0 (9.5-20.5)		0.65
HOMA	3.3 (2.3-6.0)	3.4 (2.0-4.4)		0.50
Triglycerides (mg/dL)	127.0 (81.8-197.3)	112.0 (68.0-172.0)		0.17
Total Chol (mg/dL)	187.0 (159.0-225.0)	192.5 (154.3-240.0)		0.35
D-LDL (mg/dL)	104.0 (75.0-130.0)	114.0 (71.8-145.8)		0.35
HDL (mg/dL)	46.0 (37.0-56.3)	45.0 (38.8-58.5)		0.90

*Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height. * heterozygous and homozygous carriers are combined in the statistical analyses.*

***Supplementary data 3C**. Associations of GR haplotypes with body composition and metabolic parameters in African-American HIV-infected patients*

	BclI* + *Tth111I			
Parameter	**non-carriers (n=194)**	**Heterozygous carriers (n=79)***	**Homozygous carriers (n=8)***	**p-value**
Age (years)	43.0 (37.0-47.0)	43.0 (37.0-48.0)		0.49
Gender				0.78
Female	79 (40.7%)	37 (42.5%)		
Male	115 (59.3%)	50 (57.5%)		
BMI (kg/m2)	24.9 (22.4-29.6)	24.6 (21.4-28.2)		0.19
Current CD4 (cells/uL)	336.0 (205.8-517.3)	324.0 (189.0-501.0)		0.76
Detectable HIV RNA	114 (58.8%)	48 (55.2%)		0.51
HAART use	132 (68.0%)	60 (69.0%)		0.92
Leg SAT (L)	4.3 (2.5-7.6)	4.8 (2.9-7.5)		0.50
VAT (L)	0.93 (0.44-1.98)	0.90 (0.41-1.88)		0.97
Arm SAT (L)	1.2 (0.9-2.1)	1.3 (0.9-2.0)		0.98
Upper Trunk SAT (L)	2.8 (1.7-5.8)	2.9 (1.9-5.3)		0.99
Lower Trunk SAT (L)	4.8 (2.7-9.1)	5.0 (2.6-9.6)		0.99
Total SAT (L)	15.1 (8.5-26.3)	15.4 (8.2-25.9)		0.93
Leg SM (L)	11.0 (9.5-12.3)	10.5 (9.1-12.3)		0.38
Arm SM (L)	3.5 (3.0-4.2)	3.4 (2.8-4.3)		0.66
Upper Trunk SM (L)	5.5 (4.5-6.4)	5.2 (4.3-6.1)		0.04
Lower Trunk SM (L)	7.6 (6.7-9.0)	7.4 (6.3-8.9)		0.35
Total SM (L)	27.8 (25.1-31.7)	26.5 (23.3-31.0)		0.31
Glucose (mg/dL)	91.0 (84.5-101.0)	90.0 (84.0-97.0)		0.42
Insulin (μU/mL)	15.0 (10.0-24.0)	15.0 (11.0-22.0)		0.74
HOMA	3.5 (2.2-6.0)	3.2 (2.3-5.1)		0.52
Triglycerides (mg/dL)	125.0 (76.5-193.0)	120.0 (86.0-193.0)		0.67
Total Chol (mg/dL)	188.0 (155.5-232.5)	188.0 (161.0-217.0)		0.57
D-LDL (mg/dL)	104.0 (73.5-132.0)	105.0 (77.0-133.0)		0.97
HDL (mg/dL)	45.0 (37.5-55.0)	45.0 (36.0-61.0)		0.83

*Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height. * heterozygous and homozygous carriers are combined in the statistical analyses.*

Supplementary data 3D. *Associations of GR haplotypes with body composition and metabolic parameters in African-American HIV-infected patients*

	9beta + *Tth111*I			
Parameter	**non-carriers (n=255)**	**Heterozygous carriers (n=26)**	**Homozygous carriers (n=0)**	**p-value**
Age (years)	43.0 (37.0-47.0)	43.5 (37.8-47.0)		0.92
Gender				0.91
Female	105 (41.2%)	11 (42.3%)		
Male	150 (58.8%)	15 (57.7%)		
BMI (kg/m2)	24.9 (21.9-29.2)	24.1 (22.7-30.2)		0.66
Current CD4 (cells/uL)	339.0 (204.0-514.5)	266.5 (159.3-488.5)		0.37
Detectable HIV RNA	142 (55.7%)	20 (76.9%)		0.04
HAART use	175 (68.6%)	17 (65.4%)		0.71
Leg SAT (L)	4.4 (2.6-7.6)	4.7 (3.4-6.0)		0.77
VAT (L)	0.88 (0.42-1.91)	1.38 (0.65-2.14)		0.21
Arm SAT (L)	1.3 (0.9-2.0)	1.2 (1.0-2.3)		0.52
Upper Trunk SAT (L)	2.8 (1.7-5.5)	3.5 (2.0-6.5)		0.28
Lower Trunk SAT (L)	4.8 (2.6-9.5)	5.3 (3.7-8.9)		0.56
Total SAT (L)	15.4 (8.2-26.6)	13.9 (11.3-23.3)		0.79
Leg SM (L)	10.8 (9.3-12.3)	11.0 (9.1-12.9)		0.69
Arm SM (L)	3.5 (3.0-4.2)	3.7 (2.9-4.2)		0.69
Upper Trunk SM (L)	5.4 (4.5-6.3)	5.6 (4.8-6.5)		0.48
Lower Trunk SM (L)	7.5 (6.6-8.9)	7.7 (6.7-9.1)		0.96
Total SM (L)	27.3 (23.9-31.1)	28.6 (25.0-34.0)		0.61
Glucose (mg/dL)	91.5 (85.0-99.0)	88.0 (79.8-96.8)		0.18
Insulin (μU/mL)	15.0 (10.0-22.0)	17.0 (11.8-32.3)		0.09
HOMA	3.3 (2.2-5.6)	3.8 (2.3-7.9)		0.18
Triglycerides (mg/dL)	123.0 (77.0-191.3)	128.0 (101.3-225.0)		0.30
Total Chol (mg/dL)	188.0 (159.0-227.3)	196.0 (154.8-227.8)		0.75
D-LDL (mg/dL)	104.0 (75.0-133.3)	94.0 (71.0-131.3)		0.53
HDL (mg/dL)	46.0 (38.0-57.3)	42.0 (35.0-53.0)		0.22

Data are presented as Median (IQR) or numbers (%). VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SM = skeletal muscle; Chol = cholesterol. MRI data are adjusted for height.

Chapter 12

The effects of glucocorticoid receptor polymorphisms and prenatal exposure to famine interact

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Submitted

Abstract

Background
The effects of genes may be modified by the prenatal environment. We assessed whether glucocorticoid receptor (GR) gene polymorphisms interacted with prenatal exposure to famine on body composition and metabolic parameters.

Methods
Among a group of 776 58-year old men and women who were born as term singletons around the time of the 1944-1945 Dutch famine, we measured height, weight, waist and hip circumference and systolic (SBP) and diastolic blood pressure (DBP), glucose and insulin levels and fasting lipid profiles. We also determined GR polymorphisms.

Results
The effects of the GR polymorphisms depended on exposure to famine. Compared to non-carriers exposed to famine during late gestation, carriers of the GR-9β polymorphism had significantly lower weight (-8.2 kg, p=0.005), lower BMI (-3.2 kg/m2, p<0.001), smaller waist (-9.9 cm, p=0.001) and hip circumference (-5.7 cm, p=0.005). Also carriers of the ER22/23EK polymorphism exposed to famine during late gestation had lower weight (-14.6 kg, p=0.036), lower hip circumference (-10.8 cm, p=0.024) and lower BMI (-4.3 kg/m2, p=0.024) than non-carriers. GR-9β carriers exposed to famine during early gestation had a higher DBP (+6 mmHg, p=0.049) and SBP (+11 mmHg, p=0.061) compared to non-carriers.

Conclusions
Our findings suggest that the effects of GR polymorphisms on body composition and blood pressure are modified by nutrition during gestation and provide further evidence that genetic influences can be modified by nutrition of the human fetus in utero.

Introduction

In the past decades, many studies have suggested that fetal adaptations to adverse environmental circumstances affect long-term health outcomes, resulting in an increased risk of obesity, type 2 diabetes mellitus and cardiovascular disease (1-6). This led to the 'fetal origins of adult disease' hypothesis, which proposed that an adverse fetal environment leads to permanent physiological, neuroendocrine and metabolic changes that have pathological consequences in adult life (7). The hypothalamic-pituitary-adrenal (HPA) axis is one of the systems that is hypothesized to be involved in this relationship as a potential underlying mechanism. Studies in rats have shown that maternal protein restriction during pregnancy resulted in high blood pressure in the offspring and that this effect can be prevented by inhibition of corticosterone biosysnthesis during pregnancy (8). In addition, treatment of pregnant rats with synthetic glucocorticoids (GCs) resulted in a lower mean birth weight, persistent elevated arterial blood pressure and fasting hyperglycemia in adult rat offspring (9). Furthermore, it has been shown that malnutrition of pregnant rats leads to lower placental 11β- hydroxysteroid dehydrogenase type2 (11β-HSD2) activity (10-12). The enzyme 11β-HSD2 converts active cortisol into its inactive form cortisone. Inhibition of placental 11β-HSD2 activity results in lower fetal weight both in rats and in humans (13-16), which is probably due to overexposure of the fetus to maternal GCs.

The effects of GCs are mediated by the Glucocorticoid Receptor (GR). The GR is a member of the nuclear receptor family and the gene encoding this receptor is located on chromosome 5. The GR is expressed in most fetal tissues from the early embryonic stages (17-18). The sensitivity to GCs differs between individuals, but is rather stable within an individual. This inter-individual difference but intra-individual stability can be partially explained by polymorphisms in the GR gene. There are several polymorphisms known that alter GC sensitivity in vitro and in vivo and these polymorphisms have been associated with changes in body composition and metabolic parameters (19-20). The ER22/23EK polymorphism (rs6189 and rs6190) is associated with a relative glucocorticoid resistance (21-22) and consists of two linked single nucleotide polymorphisms in codons 22 and 23 of exon 2. Carriers of this polymorphism have a healthier metabolic profile and increased insulin sensitivity compared to non-carriers, as well as more lean body mass and a greater adult height (23-24). The 9β polymorphism is thought to lead to a relative GC resistance by increasing the expression and stability of the GR-β variant, a dominant negative inhibitor of the active GR-α (25). This polymorphism does not affect transactivation of target genes, but is associated with a significant reduction of transrepressive effects of GCs, resulting in a more active immune system (26-28). The N363S (rs6195) and the *Bcl*I (rs41423247) polymorphism are both associated with a relative glucocorticoid hypersensitivity and higher BMI, higher waist-to-hip ratio, visceral adiposity, hypertension and higher cholesterol and triglyceride concentrations (19-20, 29-34).

It has previously been shown that the effects of polymorphisms may be modulated by prenatal undernutrition (35-36). Since GR polymorphisms are associated with altered GC sensitivity and body compositional and metabolic changes, prenatal exposure to undernutrition might also modulate the effect of these polymorphisms. The Dutch famine birth cohort study provides the unique opportunity to study the effects of maternal malnutrition and gene-environment interactions on adult diseases. This cohort consists of men and women born as term singletons in the Wilhelmina Gasthuis in Amsterdam around the time of the Dutch famine. The effects of prenatal famine exposure on adult diseases depend on the time of gestation at the moment of famine exposure and are independent of birth weight (37). Therefore, the purpose of this study is to determine whether the effects of GR polymorphisms on anthropometric and metabolic parameters are modulated by famine exposure during gestation. We hypothesize that exposed persons carrying the "hypersensitive" GR variants (N363S and *Bcl*I) have an unfavourable body composition and adverse metabolic outcomes, as they are more sensitive to the possible effects of increased levels of GCs as a consequence of maternal malnutrition. In contrast, we hypothesize that exposed individuals with a genotype consistent with a relative resistance to GCs (ER22/23EK and9β) will have a beneficial body composition and a healthier metabolic profile at adult age.

Methods

Participants

The Dutch famine birth cohort consists of 2414 men and women born between 1 November 1943 and 28 February 1947 as term singletons in the Wilhelmina Gasthuis in Amsterdam. Detailed information about the selection procedure is described elsewhere (38). On 1 September 2002, 1423 members of the cohort were still alive and living in the Netherlands and were asked to participate in this study. Of the cohort of 1423 members, 810 people agreed to participate in this study. This study was approved by the local medical ethics committee and has been carried out in accordance with the Declaration of Helsinki. All participants gave written informed consent.

Exposure to famine

The official daily food rations for the general population aged ≥21 years was used to define exposure to famine (39). If the average daily food ration of the mother contained <1000 calories during any 13-week period of gestation, the individual was considered to be prenatally exposed to famine. With this definition, babies born between 7 January 1945 and 8 December 1945 were exposed in utero. We used periods of 16 weeks to differentiate between those who were exposed in early gestation (born between 19 August and 8 December 1945), in mid gestation (born between 29 April and 18 August 1945) and in late gestation (born between 7 January and 28 April 1945). Individuals born before 7 January 1945 and conceived and born after 8 December 1945 were considered as unexposed to famine in utero.

Study parameters

Information about the mother, the course of the pregnancy and the size of the baby at birth was retrieved from the medical birth records (38). Standardized interviews and measurements were performed by trained nurses. In standardized interviews, information was gathered concerning socio-economic status, medical history, lifestyle and use of medication. Socio-economic status was defined according to International Socioeconomic Index of Occupational Status 92, which is based on the participant's, or their partner's occupation, whichever status was highest (40). Height was measured using a fixed or portable stadiometer and weight was measured with Seca and portable Tefal scales. Waist circumference was measured with a flexible tape midway between the costal margin and the iliac crest. Blood pressure was measured in duplo on two occasions using an automated device (Omron 705 CP/IT; Omron Healthcare UK, West Sussex, UK) and mean systolic and diastolic blood pressure were calculated. An oral glucose tolerance test (OGTT) with a standard load of 75 g was performed after an overnight fast. Participants taking glucose-lowering medication were excluded from the OGTT. Venapuncture was performed at 0, 30, 60 and 120 min after glucose administration and glucose and insulin concentrations were measured. Furthermore, blood was drawn for analysis of total cholesterol, High Density Lipoprotein (HDL)-cholesterol and triglycerides. Low Density Lipoprotein (LDL)- cholesterol was calculated using the Friedewald formula.

Genotyping

Genomic DNA was extracted from fasting blood samples. The GR polymorphisms ER22/23EK, N363S, *Bcl*I and 9β were genotyped with Taqman allelic discrimination assays. The assays were designed and optimized by Applied Biosystems (Foster City, CA). The analyses were performed as described previously (41). Assays were run on 90 blood bank samples to test for adequate cluster separation. Genotypes were determined in 2 ng genomic DNA. Reactions were performed on the Taqman Prism 7900HT platform. Genotyping for at least one polymorphism was successful in 776 subjects.

Statistical analysis

All statistical analyses were performed using SPSS version 19.0. Logarithmic transformations were applied to variables with skewed distributions. Linear regression models were used to compare body composition, blood pressure and glucose, insulin and cholesterol levels between polymorphism groups and exposure groups. Possible interactions of the effects of prenatal famine exposure and the GR gene polymorphisms were assessed by adding an interaction term (genotype*exposure) to the regression equation. In all analyses heterozygous and homozygous carriers were combined to increase the statistical power. Furthermore, all statistical analyses were adjusted for sex, BMI, birth weight, placental size, smoking, and current socioeconomic status, with exception of the statistical analysis with BMI as outcome measurements. Those measurements were only adjusted for sex, birth weight, placental size, smoking, and current socioeconomic status. Differences were considered to be statistically significant if p-values were <0.05.

***Table 1**. Descriptives according to prenatal famine exposure.*

	Exposure to famine					
	Born before	**In late gestation**	**In mid gestation**	**In early gestation**	**Conceived after**	**All**
N	239	140	114	72	211	776
Men (%)	47.3	43.6	37.7*	41.7	50.7	45.6
Birth weight (g)	3380	3191*	3204*	3528	3482	3361 (470)
Placental size (cm2)	295	268*	267	279	273	279 (67)
Adult outcomes						
Smoking (%)	21.3	27.1	26.5	33.3*	22.6	24.6
Socio-economic status	48	51	52	47	50	50 (14)
Clinical outcomes						
Height (cm)	170.2	169.8	168.8	170.7	170.4	170.0 (9.1)
Weight (kg)	83.3	81.4	80.0	81.9	84.3	82.6 (15.9)
BMI (kg/m2)	28.7	28.2	28.0	27.9	29.0	28.5 (4.9)
Waist circumference (cm)	97.4	96.0	94.5	95.3	97.6	96.6 (13.3)
Hip circumference (cm)	104.1	103.8	104.6	103.8	104.9	104.3 (10.3)
SBP (mmHg)	138	136	136	136	136	137 (18)
DBP (mmHg)	81	81	80	82	82	81 (10)
Total cholesterol (mmol/l)	5.8	5.9	5.7	6.1	5.8	5.8 (1.1)
HDL cholesterol (mmol/l)	1.6	1.5	1.5	1.5	1.5	1.5 (0.4)
LDL cholesterol (mmol/l)	3.7	3.7	3.5	3.9	3.6	3.6 (1.0)
2h glucose (mmol/l) [a]	5.8	6.2	6.2	6.1	5.9	6.0 (1.4)
2h insulin (pmol/l) [a]	243	266	255	270	239	250 (2.1)

Data are given as means (SD) except where given as percentages and [a]geometric means (geometric SD).
** Statistically significant difference compared to those unexposed to famine during gestation.*

Results

Genotypes of the GR polymorphisms were available in 776 participants. We first studied the effects of prenatal exposure to famine independent of the GR polymorphisms. Second, we studied the effects of GR polymorphisms in the total group and third, we have studied the interactions between prenatal exposure to famine and carriage of GR polymorphisms.

Effect of prenatal exposure to famine

Table 1 shows the characteristics of participants according to timing of famine exposure during gestation. 239 (30.8%) were born before the famine, 140 (18.0%) were exposed during late gestation, 114 (14.7%) were exposed during mid gestation, 72 (9.3%) were exposed to famine during early gestation and 211 (27.2%) were conceived and born after famine. Those exposed to famine in mid or late gestation were lighter at birth. At adult age, those exposed to famine prenatally tended to have higher glucose and insulin levels, while those exposed to famine in early gestation tended to have higher cholesterol levels. None of these differences were statistically significant. Those exposed to famine in early gestation were more often smokers.

Effect of GR polymorphisms in the total group

In all analyses, heterozygous and homozygous carriers were combined because of the low number of homozygous carriers. We found no differences in body composition, blood pressure, total, HDL- and LDL-cholesterol levels and glucose and insulin levels after OGTT between carriers and non-carriers of the ER22/23EK, 9β, N363S or *Bcl*I polymorphism. The results according to carriership of GR polymorphisms are shown in Table 2.

Interactions between GR polymorphisms and prenatal exposure to famine

The prevalence of the GR polymorphisms was not different between the different exposure groups (Table 3).

ER22/23EK polymorphism

We found significant interactions of exposure to famine during late gestation and ER22/23EK carriage on weight, BMI and hip circumference (p=0.04, p=0.05 and p=0.04 respectively). When exposed to famine during late gestation, carriers of the ER22/23EK had a significantly lower weight (65.8 kg (95% CI= 52.4-79.2) versus 80.4 kg (95% CI= 77.6-83.2), p=0.036) and BMI (23.0 kg/m2 (95%CI= 19.9-26.6) versus 27.3 kg/m2 (95% CI= 26.5-28.1), p=0.024) at the age of 58 compared to non-carriers. In addition, there was a significant lower hip circumference in ER22/23EK carriers (92.1 cm (95% CI=82.9-101.3) versus 102.9 cm (95% CI= 100.9-104.8), p=0.024). In individuals exposed to famine during mid and early gestation, we observed no differences between carriers and non-carriers of the ER22/23EK polymorphism with regards to body composition and metabolic parameters.

9β polymorphism

We found significant interactions of famine exposure in late gestation and 9β carriage for weight (p=0.02), BMI (p=0.001), waist circumference (p=0.002) and hip circumference (p=0.01). When exposed to famine during late gestation, carriers of the 9β polymorphism had significantly lower weight (74.1 kg (95% CI= 69.1-79.1) versus 82.3 kg (95% CI= 79.1-85.4), p=0.005), lower BMI (24.9 kg/m2 (95% CI= 23.6-26.2) versus 28.1 kg/m2 (95% CI= 27.2-29.0), p<0.001), smaller waist (88.8 cm (95% CI= 84.2-93.3) versus 98.7 cm (95% CI= 94.9-100.6), p=0.001) and hip circumference (98.4 cm (95% CI= 95.0-101.9) versus 104.1 cm (95% CI= 102.0-106.3), p=0.005) (Figure 1).

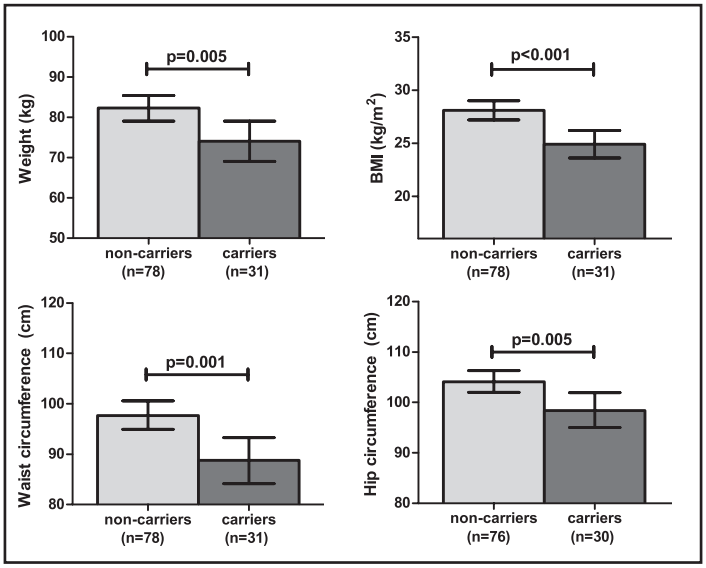

Figure 1. *Weight, body mass index (BMI), waist and hip circumference in individuals exposed to famine during late gestation, stratified by 9β carriage status.*

We did not find a significant interaction of famine exposure in mid gestation and 9β carriage. The interaction of famine exposure during early gestation and 9β carriage was statistically significant for systolic blood pressure (p=0.031) and diastolic blood pressure (p=0.015). Systolic blood pressure was higher in 9β carriers compared to non-carriers (142 mmHg (95% CI= 132-151) versus 131 mmHg (95% CI=124-138), p=0.061). In addition, diastolic blood pressure was higher in 9β carriers as well (85 mmHg (95% CI= 80-90) versus 79 mmHg (95% CI= 75-82), p=0.049).

N363S polymorphism
The interaction of exposure to famine during late gestation and carriage of N363S on height was statistically significant (p=0.003). N363S carriers exposed to famine during late gestation were significantly taller than non-carriers (174.6 cm (95% CI= 170.3-179.0) versus 169.8 cm (95% CI= 168.5-171.2), p=0.04). No other differences in body composition or metabolic parameters were observed between carriers and non-carriers exposed to famine during late gestation. In the groups exposed to famine during early and mid gestation, and in the groups born before and conceived after famine, there were no differences between N363S carriers and non-carriers.

BclI polymorphism
We did not find any significant interactions of famine exposure and *Bcl*I carriage.

Table 2. *Clinical outcomes according to carriership of GR polymorphisms.*

	ER22/23EK		**9β**		**N363S**		***Bcl*I**	
	C	**NC**	**C**	**NC**	**C**	**NC**	**C**	**NC**
Height (cm)	168.9	170.1	169.9	169.7	169.8	170.0	170.0	169.9
Weight (kg)	80.7	82.7	82.7	82.3	82.3	82.6	82.8	82.2
BMI (kg/m2)	28.2	28.5	28.6	28.5	28.5	28.5	28.7	28.4
Waist circumference (cm)	94.9	96.6	96.6	96.5	96.0	96.7	96.9	96.2
Hip circumference (cm)	104.3	104.3	104.4	104.3	103.9	104.4	104.8	103.8
SBP (mmHg)	137	137	136	137	136	137	136.8	137.0
DBP (mmHg)	81	81	81	81	80	81	81	81
Total cholesterol (mmol/L)	5.6	5.9	5.9	2.7	6.0	5.8	5.8	5.9
HDL cholesterol (mmol/l)	1.5	1.5	1.5	1.5	1.6	1.5	1.5	1.5
LDL cholesterol (mmol/l)	3.5	3.7	3.7	3.6	3.8	3.6	3.7	3.7
2h glucose (mmol/l)[a]	6.2	6.0	6.0	6.0	6.1	6.0	6.0	6.0
2h insulin (pmol/l)[a]	245	249	240	269	241	250	241	255

Data are given as mean, except where given as percentage and [a]geometric means.
C, carriers; NC, non-carriers.

Discussion

We found significant interaction of prenatal exposure and GR polymorphism carriage. Compared with non-carriers who were also exposed to famine in late gestation, 9β carriers had a significantly lower weight, BMI, waist and hip circumference. Similar effects were found for the ER22/23EK polymorphism, although there were only 6 carriers of this polymorphism exposed to famine during late gestation. Interestingly, these are the only two polymorphisms of the GR gene, which have previously been associated with GR resistance. Our results may suggest that genetic influences of GR polymorphisms can be modified by nutrition of the human fetus in utero.

Table 3. *Polymorphism frequencies in the famine exposure groups*

	Exposure to famine				
	Born before	**In late gestation**	**In mid gestation**	**In early gestation**	**Conceived after**
GR resistant polymorphisms					
ER22/23EK					
Non-carriers	220 (94.0%)	129 (95.6%)	102 (91.1%)	66 (94.3%)	194 (94.6%)
Heterozygous carriers	14 (6.0%)	6 (4.4%)	10 (8.9%)	4 (6.0%)	10 (4.9%)
Homozygous carriers	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.5%)
9β					
Non-carriers	164 (71.9%)	94 (71.2%)	69 (63.9%)	48 (68.8%)	120 (63.8%)
Heterozygous carriers	58 (25.4%)	36 (27.3%)	34 (31.5%)	21 (30.0%)	60 (31.9%)
Homozygous carriers	6 (2.6%)	2 (1.5%)	5 (4.6%)	1 (1.4%)	8 (4.3%)
GR hypersensitive polymorphisms					
N363S					
Non-carriers	219 (93.2%)	127 (92.7%)	106 (94.6%)	62 (88.6%)	192 (94.1%)
Heterozygous carriers	16 (6.8%)	10 (7.3%)	6 (5.4%)	8 (11.4%)	12 (5.9%)
Homozygous carriers	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
*Bcl*I					
Non-carriers	80 (34.2%)	52 (39.1%)	41 (36.1%)	33 (47.8%)	85 (43.8%)
Heterozygous carriers	119 (50.9%)	61 (45.9%)	55 (49.5%)	29 (42.0%)	84 (43.3%)
Homozygous carriers	35 (15.0%)	20 (15.0%)	15 (13.5%)	7 (10.1%)	25 (12.9%)

The 9β polymorphism is an A to G substitution in exon 9β of the GR gene which results in a relative GC resistance (25). This relative GC resistance seems to result in a more active immune system (25-27, 42). With regards to body composition and metabolic parameters, we previously found no differences between elderly carriers and non-carriers of the 9β polymorphism (28). However, Syed et al found a 2.7% lower WHR in female and a more favourable lipid profile in male carriers of the 9β polymorphism (43). In the current study, we found no effects of the 9β polymorphism on body composition in the total group. However, individuals exposed to famine during late gestation carrying the 9β polymorphism had a 10 cm smaller waist compared to individuals who were also exposed to famine during late gestation, but did not carry the 9β polymorphism. The BMI of 9β carriers was normal (mean 24.9 kg/m2) whereas non-carriers were on average classified as overweight (mean BMI of 28.1 kg/m2). These effects are quite large and only present in individuals who were exposed to famine during late gestation. One explanation might be that the relative

GC resistance of the 9β polymorphism might protect carriers from the detrimental effects of GC excess in utero, ultimately resulting in a healthier body composition at older age. During pregnancy, cortisol serum levels increase to three times non-pregnant levels by the third trimester. Most of this increase can be explained by higher corticosteroid-binding globulin (CBG) concentrations due to increased estrogens. However, the net effect of these changes lead to approximately doubled plasma-free cortisol levels during late pregnancy (44). Maternal malnutrition leads to a reduced expression of placental 11β-HSD2, which probably leads to increased cortisol exposure of the fetus (10-12, 45). If the HPA-axis of the exposed individuals is reprogrammed in utero to adapt to the higher cortisol exposure in utero, a relative glucocorticoid resistance will prevent individuals from developing a more adipose body composition in adult life. Carriers of the relative glucocorticoid resistant 9β polymorphism, who are exposed to famine in late gestation might be a little bit more resistant to glucocorticoids, ultimately leading to a more healthy body composition at adult age.

The other GR polymorphism that is associated with a relative glucocorticoid resistance is the ER22/23EK polymorphism. Similarly as 9β carriage and famine exposure during late gestation, carriers of the ER22/23EK polymorphism that were exposed to famine during late gestation seem to have a beneficial body composition compared to non-carriers.

Furthermore, we found a statistically significant interaction of 9β carriage and famine exposure in early gestation on blood pressure. Both systolic and diastolic blood pressure were higher in individuals carrying the 9β polymorphism who were exposed to famine during early gestation. This might be explained by a diminished negative feedback due to a relative GC resistance, which leads towards higher cortisol levels. These higher cortisol levels might lead to high blood pressure via binding of cortisol to the mineralocorticoid receptor in the kidneys. This mechanism of hypertension due to glucocorticoid resistance has also been described in patients with severe generalized GC resistance (46).

Although the results show at least some evidence for our hypothesis regarding the hyposensitive GR polymorphisms and prenatal famine exposure, we could not show any evidence for our hypothesis on the hypersensitive GR polymorphisms in relation to famine exposure. The two polymorphisms that are associated with a relative hypersensitivity to GCs (N363S and *Bcl*I) did not seem to have any modulating effect in the relationship between prenatal undernutrition and body composition and metabolic parameters at older age. We did find a taller stature in N363S carriers who were exposed to famine during late gestation compared to non-carriers exposed in late gestation, but this was the only significant finding and there were no other body compositional or metabolic changes associated with N363S carriage. Furthermore, the number of N363S carriers was very small. Since there was no clear pattern of changes in metabolic parameters or body composition in carriers of the

N363S polymorphism, but only a single finding, we do not interpret this finding as realistic association until replication in other studies can confirm our finding. In addition, we found no significant interactions between famine exposure and *Bcl*I carriage. The N363S and *Bcl*I polymorphism have previously been associated with (abdominal) obesity and elevated cholesterol levels in adult populations (19-20). We could not replicate these findings in the current group, also not when the total group, independent of famine exposure, was studied. The lack of association between body composition and metabolic parameters and the interaction of these polymorphisms and prenatal famine exposure suggests that these factors do not affect body composition and metabolic parameters at older age at all.

Although the Dutch famine birth cohort is a unique cohort, there are some limitations. First, the study group is rather small to study the effects of polymorphisms. In our study, the number of ER22/23EK and N363S carriers was very low. Second, we performed a large number of statistical tests, yielding a risk of false positive results. However, we have looked specifically at consistent patterns of significant changes in polymorphism carriers, and found a clear pattern of the 9β variant, which is in line with previous data and the biological background of this polymorphism (19, 25).

In conclusion, our study is the first study showing interactions between GR polymorphisms and prenatal exposure to famine. This provides further evidence that genetic influences can be modified by nutrition of the human fetus in utero.

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Chapter 13

Glucocorticoid receptor gene haplotype is associated with beneficial changes in fat mass in older persons

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Submitted

Abstract

Background
High levels of glucocorticoids (GCs) are associated with increased visceral fat mass, muscle atrophy, features of the metabolic syndrome and cardiovascular disease. The inter-individual sensitivity to GCs is highly variable which can be partly explained by polymorphisms in the glucocorticoid receptor (GR) gene. The aim of this study was to determine whether GR polymorphisms play a role in changes in body composition in older adults and in the risk of developing the metabolic syndrome and cardiovascular disease.

Methods
GR gene polymorphisms were determined in 757 participants of the Longitudinal Aging Study Amsterdam, a population-based cohort study in individuals aged >65 years. Data on body composition (anthropometry and dual energy x-ray absorptiometry (DEXA) scans), the metabolic syndrome and cardiovascular disease were collected in 1995-1996, 1998-1999 and 2005-2006.

Results
Body mass index (-0.7 kg/m2), waist circumference (-0.6 cm) and hip circumference (-3.9 cm) decreased significantly in ER22/23EK + 9β + *Tth111*I haplotype carriers, whereas these parameters increased in non-carriers (+0.1 kg/m2, +2.8 cm and +0.4 cm, respectively) over a 9-year period. In addition, DEXA scans showed decreased fat mass (-2179 g), particularly trunk fat mass (-1100 g), in carriers during a 3-year period compared with increased fat mass (+234 g) and trunk fat mass (+66 g) in non-carriers. Homozygous 9β + *Tth111*I haplotype carriers had higher odds of metabolic syndrome compared with non-carriers (OR 2.7, 95% CI= 1.1-6.7, p=0.015).

Conclusions
Carriers of the ER22/23EK + 9β + *Tth111*I haplotype, which is associated with a relative GC resistance, have a beneficial change in body composition during longitudinal follow-up compared with non-carriers. Relative GC resistance at older age might protect against the detrimental effects of aging on body composition.

Introduction

Cortisol, the main glucocorticoid (GC) in humans, is the end product of the hypothalamic-pituitary-adrenal axis and has numerous effects throughout the body, including the regulation of lipid and glucose metabolism, immunosuppressive and anti-inflammatory actions, as well as vascular effects (1). It is known that long-term pathologically elevated cortisol levels, e.g. due to extreme endogenous cortisol production (Cushing's Syndrome) or prolonged use of exogenous glucocorticoids, leads to increased visceral fat mass, atrophy of the proximal muscles, hypertension, dyslipidaemia and insulin resistance, which results in an increased cardiovascular risk (2-4).

Between individuals, there is a high variability in the effects of exogenous GCs. Some individuals respond very well with regard to their (auto-immune) disease, whereas others do not respond at all. This indicates that there is a large variability in the sensitivity to exogenous GCs in the population, which also has been shown in older adults (5). An individual's sensitivity to GCs can be tested with a very low dose (0.25 mg) dexamethasone suppression test (DST). Previous research in a large group of healthy individuals showed great inter-individual variability in GC sensitivity in response to the very low dose DST, but with rather stable intra-individual responses (5).

The Glucocorticoid Receptor (GR) plays an important role in the cascade of GC action. Upon binding of cortisol to the GR, the GR translocates into the nucleus where it binds to GC response elements of target genes and activates or inhibits gene transcription (6). We previously demonstrated that several polymorphisms in the GR gene are associated with altered GC sensitivity and changes in body composition and metabolic parameters (7-8). The *Tth111*I polymorphism (rs10052957) has been associated with elevated diurnal cortisol levels (9). However, this polymorphism is always linked to another polymorphism, such as the ER22/23EK polymorphism, the *Bcl*I or the 9β polymorphism. The functionality of the *Tth111*I variant, located in the promotor region of the GR gene is not known. However, the molecular mechanism of the linked ER22/23EK polymorphism (rs6189 + rs6190), which induces a relative GC resistance, characterized by less suppression of cortisol after dexamethasone in vivo and a reduction in transactivation capacity in vitro (10), has previously been elucidated (11). Carriers of this polymorphism have a healthier metabolic profile, with lower fasting insulin levels, increased insulin sensitivity and lower total and low-density lipoprotein (LDL) cholesterol (12), as well as a beneficial body composition at young adult age (13). Two polymorphisms that are associated with a relative GC hypersensitivity are the N363S polymorphism (rs6195) and *Bcl*I polymorphism (rs41423247). The N363S polymorphism is associated with an increased insulin response after dexamethasone, a tendency towards lower bone mineral density, increased Body Mass Index (BMI) and increased waist-hip-ratio

(WHR) (14-16). The *Bcl*I polymorphism is associated with abdominal obesity in middle-aged adults and with a lower lean body mass at older age (17-18). The 9β polymorphism (rs6198) is associated with a relative glucocorticoid resistance. This polymorphism is thought to result in an increased expression and stability of the GR-β, which is an active inhibitor of the active form of the GR. This polymorphism does not seem to influence transactivation, but leads to a significant reduction of the transrepressive effects of GCs, resulting in a more effective immune status (19). This polymorphism is associated with a higher risk of rheumatoid arthritis and elevated levels of interleukin-6 (IL-6) and high sensitive (hs)-C-reactive protein (CRP) (20-21), leading to a subtle pro-inflammatory status. Homozygous carriers of this polymorphism had increased intima media thickness (IMT) and an almost three-fold increased risk of cardiovascular disease, probably due to this subtle, but chronic pro-inflammatory state (21). An overview of the GR gene, its polymorphisms and the haplotypes is shown in Figure 1.

Since these GR polymorphisms alter GC sensitivity, they may play a role in changes in body composition during aging and the development of the metabolic syndrome and cardiovascular disease. Therefore, we studied the effects of these polymorphisms in a large group of older adults participating in the Longitudinal Aging Study Amsterdam (LASA). We hypothesized that haplotypes associated with a hypersensitivity to GCs are related to an increase in fat mass during aging and an increased risk of the metabolic syndrome and cardiovascular disease. In contrast, we hypothesized that GR variants, which are known to be related to a relative glucocorticoid resistance, are associated with beneficial changes in body composition and a reduced risk of the metabolic syndrome and cardiovascular disease during aging.

Methods

Participants

This study was conducted using data from the Longitudinal Aging Study Amsterdam (LASA), which is an ongoing multidisciplinary cohort study on predictors and consequences of changes in physical, cognitive, emotional and social functioning in older people. A random sample of men and women aged 55 years and over was drawn from the population registry of 11 municipalities in three regions of the Netherlands. The sample represents the older Dutch population with respect to degree of urbanization and geographical region. Detailed information concerning sampling and data collection has been described previously (22). This study was approved by the local medical ethics committee and all participants gave written informed consent.

The sample for this study included participants who took part in the main and medical interview in the second, third and fifth cycles of LASA (1995-1996, 1998-1999 and 2005-2006, respectively). Anthropometric data from the second and fifth cycle

were available for 333 participants. DEXA scans were completed in the Amsterdam population only and data from both the second and third cycles were available for 295 individuals. Data regarding the metabolic syndrome were available for 855 participants in the second cycle and data regarding the presence of cardiovascular diseases were available for 757 participants in the third cycle.

Body composition

Height, weight, waist circumference and hip circumference were measured in the second and fifth cycles. BMI was calculated as weight (kg) divided by height (m2). Waist-to-hip ratio (WHR) was calculated as waist circumference (cm) divided by hip circumference (cm). Fat (arm, leg, head, trunk and total fat) and total lean body mass were measured with whole body Dual Energy X-ray Absorptiometry (DEXA) scans.

Metabolic syndrome and cardiovascular disease

Presence of the metabolic syndrome was scored in the second cycle (1995-1996) and based on the presence of three or more of the following criteria:

- Triglycerides > 150 mg/dl
- HDL cholesterol < 40 mg/dl for men, < 50 mg/dl for women
- Blood pressure > 160/90 mmHg or antihypertensive medication
- Waist circumference > 102 cm for men and >88 cm for women
- Fructosamine > 247 mmol/L or antidiabetic medication

Because the instructions before blood sampling allowed participants to drink tea and eat plain toast, fasting blood samples could not be guaranteed. Therefore, measures of triglycerides and to a lesser extent HDL cholesterol could have been affected. In addition, fructosamine was used instead of glucose, as fasting blood samples could not be guaranteed. A cut-off of 247 mmol/L for fructosamine corresponds to a cut-off of 6.1 mmol/L for fasting glucose with regards to sensitivity and specificity in discriminating participants with glucose intolerance from those with normal glucose tolerance. Presence of cardiovascular disease (including peripheral arterial disease, coronary heart disease and stroke) was based on self-report and collected in the third cycle (1998-1999).

SNPs and haplotypes

Genomic DNA was extracted from the samples of peripheral venous blood collected at the second cycle (1995-1996) following standard procedures. Genotypes were determined using the Taqman allelic discrimination assay. The Assay-by-Design service (www.appliedbiosystems.com) was used to set up a Taqman allelic discrimination assay for *Tth111*I (rs10052957), ER22/23EK (rs6189 + rs6190), N363S (rs6195), *Bcl*I (rs41423247) and 9β (rs6198). The exact procedure has been described previously (23). Results were analysed by the ABI Taqman 7900HT using

the sequence detection system 2•22 software (Applied Biosystems Inc., Nieuwerkerk aan den IJssel, the Netherlands). Accuracy of genotyping results was tested by re-genotyping 80 randomly selected samples. Haplotypes were created as previously described by using the program PHASE (24).

Statistical analyses

All statistical analyses were performed using SPSS version 17.0. Logarithmic transformations were applied to variables with skewed distributions. ANCOVA was used to examine cross-sectional (at all cycles) and longitudinal (change between cycles) differences in body composition (anthropometry and DEXA scans) between genotypes. Logistic regression was used to calculate the odds of having (components of) the metabolic syndrome and cardiovascular disease for carriers of a particular genotype compared with non-carriers. In all analyses, heterozygous and homozygous carriers of the ER22/23EK + 9β + TthIIII haplotype were combined. All analyses were adjusted for age, gender, region, education, smoking status and alcohol consumption. Differences were considered to be statistically significant if p-values were <0.05.

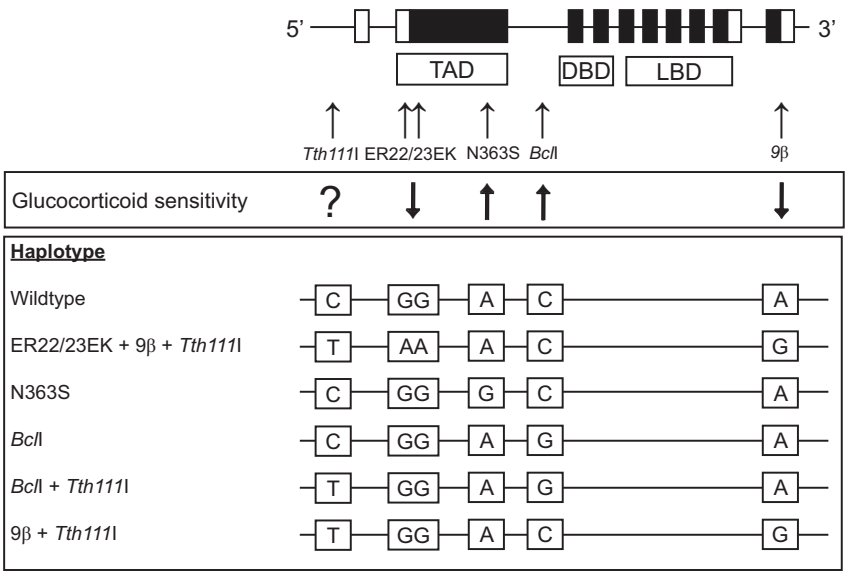

Figure 1. *Overview of the glucocorticoid receptor gene, the polymorphisms and the haplotypes.*

Results

This study was conducted in participants of the second (1995-1996), third (1998-1999) and fifth (2005-2006) cycles of LASA. In the second cycle, the median age was 75 years (range 65-89 years) and 51% were female. In the third cycle, the median age was 76 years (range 68-90) and 52% were female and in the fifth cycle, the median age was 81 years (range 75-97) and 58% were female. The numbers of participants included in each of the models varied per outcome, as certain outcomes were measured in subsamples only (Table 1).

ER22/23EK + 9β + Tth111I haplotype
In cross-sectional analyses, body height, weight, BMI, waist and hip circumference and WHR were not statistically significant different between carriers and non-carriers of the ER22/23EK + 9β + *Tth111*I haplotype in the second or the fifth LASA cycle. However, in the 9 year period between the second and the fifth cycles, haplotype carriers showed a greater decrease in BMI (-0.7 kg/m2, 95% CI=-1.7 to +0.2 kg/m2), waist circumference (-0.6 cm, 95% CI= -4.1 to +2.9 cm) and hip circumference (-3.9 cm, 95% CI=-6.5 to -1.3 cm) compared with non-carriers (BMI +0.1 kg/m2, 95% CI =-0.2 to +0.4, p=0.089; waist circumference +2.8 cm, 95% CI= +1.8 to +3.7 cm, p=0.083; hip circumference +0.4 cm, 95% CI= -0.3 to +1.0 cm, p=0.002). No cross-sectional differences in fat or lean mass between carriers and non-carriers were found at either the second and third cycles. However, during the three years of follow-up, carriers lost 1541 g (95% CI= -3187 - +106 g) of total body mass, whereas non-carriers gained 234 g (95% CI= -197- +665 g, p=0.016). During this 3-year period, carriers lost 2179 g of total fat mass (95% CI= -3707 to -652 g), while non-carriers lost significantly less fat mass (-85 g, 95% CI= -485 to +315 g, p=0.004). This difference resulted mainly from changes in trunk fat mass: carriers lost 1100 g of trunk fat (95% CI= -2121 to -78 g), whereas non-carriers gained 66 g trunk fat (95% CI= -202 to +333 g, p=0.018). Gain in lean mass was not significantly different between non-carriers (+319 g, 95%CI= +126 to +512) and carriers (+639 g, 95% CI= -99 to +1376 g, p=0.56). The changes in body composition are depicted in Figure 2. There were no significant differences in the odds of metabolic syndrome, or features of the metabolic syndrome, in carriers of the ER22/23EK + 9β + *Tth111*I haplotype compared with non-carriers. In addition, cardiovascular disease risk was not different in carriers compared with non-carriers.

N363S haplotype
No significant cross-sectional or longitudinal differences were found in body composition as measured with DEXA or anthropometry between carriers and non-carriers. Carriage of the N363S haplotype did not result in differences in the odds of having the metabolic syndrome, features of the metabolic syndrome, or cardiovascular disease.

Table 1. *Glucocorticoid Receptor gene haplotypes and their frequencies in LASA.*

	Anthropometry (n=333)	**DEXA scan (n=295)**	**MetS (n=855)**	**CVD (n=757)**
ER22/23EK + 9β + *Tth111*I*				
Non-carriers	308 (92.5%)	276 (95.6%)	797 (93.2%)	706 (93.3%)
Heterozygous carriers	24 (7.2%)	18 (6.1%)	56 (6.5%)	49 (6.5%)
Homozygous carriers	1 (0.3%)	1 (0.3%)	2 (0.2%)	2 (0.3%)
N363S				
Non-carriers	310 (93.1%)	274 (92.9%)	797 (93.2%)	704 (93.0)
Heterozygous carriers	23 (6.9%)	21 (7.1%)	58 (6.8%)	53 (7.0%)
Homozygous carriers	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
*Bcl*I				
Non-carriers	210 (63.1%)	179 (60.7%)	550 (64.3%)	487 (64.3%)
Heterozygous carriers	112 (33.6%)	103 (34.9%)	271 (31.7%)	239 (31.6%)
Homozygous carriers	11 (3.3%)	13 (4.4%)	34 (4.0%)	31 (4.1%)
*Bcl*I + *Tth111*I				
Non-carriers	229 (68.8%)	210 (71.2%)	613 (71.7%)	539 (71.2%)
Heterozygous carriers	97 (29.1%)	84 (28.5%)	227 (26.5%)	204 (26.9%)
Homozygous carriers	7 (2.1%)	1 (0.3%)	15 (1.8%)	14 (1.8%)
9β + *TthIII*I				
Non-carriers	250 (75.1%)	223 (75.6%)	645 (75.4%)	572 (75.6%)
Heterozygous carriers	70 (21.0%)	64 (21.7%)	190 (22.2%)	167 (22.1%)
Homozygous carriers	13 (3.9%)	8 (2.7%)	20 (2.3%)	18 (2.4%)

Presented are the numbers of participants included in the analyses for each of the outcomes.
** Heterozygous and homozygous carriers were combined in all statistical analyses.*

BclI haplotype

No cross-sectional differences were found in anthropometry between carriers and non-carriers in the second cycle. However, after 9 years of follow-up, heterozygous carriers of the *Bcl*I haplotype had a higher WHR (mean 0.96, 95% CI=0.95-0.98), whereas homozygous carriers had a lower WHR (mean 0.92, 95% CI= 0.87-0.96) compared with non-carriers (mean 0.94, 95% CI=0.93-0.95, p=0.037). The longitudinal anthropometric data did not show any difference in changes between non-carriers and carriers of the *Bcl*I haplotype. There were no cross-sectional differences between non-carriers and carriers with regard to body composition measured with DEXA in the second or the third cycle. However, differences were found in changes in arm fat over time, with an increase in arm fat for both

heterozygous carriers (+20 g, 95% CI= -213 to +253 g) and homozygous carriers (+896 g, 95% CI= +239 to +1552 g), and a decrease in non-carriers (-247 g, 95% CI= -423 to -72 g, p=0.002). No significant differences were found in changes over time with regard to other fat depots such as trunk fat, leg fat or head fat. There were no significant differences in the odds of (features of) the metabolic syndrome and cardiovascular disease between carriers and non-carriers of the *Bcl*I haplotype.

BclI + Tth111I haplotype
No significant cross-sectional or longitudinal differences were found in any of the outcomes between carriers and non-carriers.

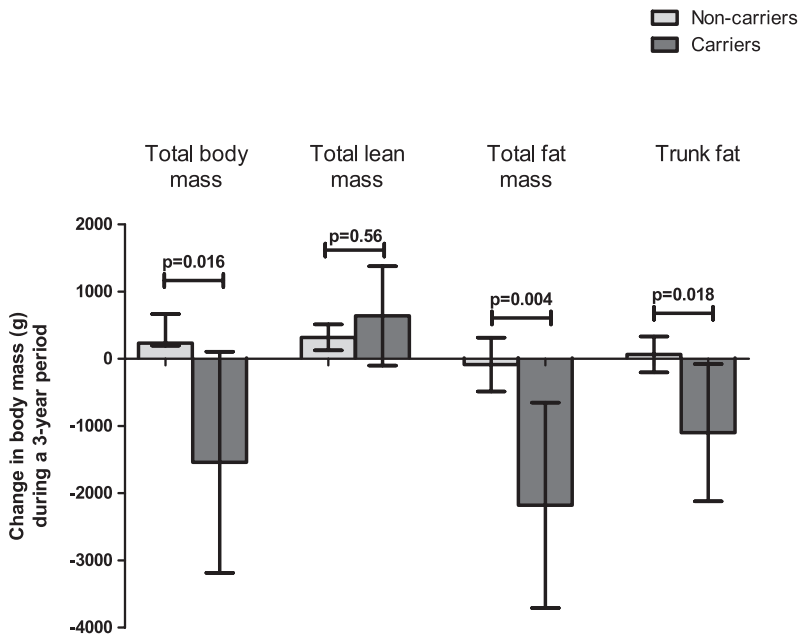

Figure 2. *Change in body composition in the period 1995/1996 - 1998/1999 in carriers and non-carriers of the ER22/23EK + 9β + Tth111I haplotype.*

9β + Tth111I haplotype
No cross-sectional differences in height, weight, BMI, waist and hip circumference and WHR were found between carriers and non-carriers of the 9β + *Tth111*I haplotype. Furthermore, no significant cross-sectional differences were found between carriers and non-carriers in body composition. In addition, there was no significant difference in change in body composition over time as measured with DEXA scans between carriers and non-carriers, however, homozygous carriers lost weight (-3.2 kg, 95% CI= -6.7 to +0.3 kg), whereas heterozygous carriers gained weight (+0.6 kg, 95% CI= 0.0 to +1.2 kg, p=0.027). In accordance, BMI decreased in homozygous carriers

with 0.7 kg/m2 (95% CI= -5.0 to +3.7 kg/m2)and increased in heterozygous carriers with 0.6 kg/m2 (95% CI= 0.0 to +1.2 kg/m2, p=0.044). Furthermore, homozygous carriers had higher odds of metabolic syndrome compared with non-carriers (OR 2.7, 95% CI= 1.1-6.7, p=0.015). Heterozygous carriers had no significant difference in odds of the metabolic syndrome (OR 0.8, 95% CI= 0.6-1.1, p=0.25) compared with non-carriers. There was no statistically significant difference in the odds of cardiovascular disease in heterozygous carriers compared with non-carriers. The number of homozygous carriers with cardiovascular disease was too small to calculate the OR.

Discussion

The main findings of this study were the beneficial changes in body composition during aging found in ER22/23EK + 9β + *Tth111*I carriers compared with the non-carriers. The longitudinal data showed that ER22/23EK + 9β + *Tth111*I carriers had a decrease in BMI whereas non-carriers had an increase in BMI over time. This decrease in BMI was caused by a decline in fat mass, in particular in trunk fat mass, whereas non-carriers gained fat mass over time. Lean body mass remained unchanged in ER22/23EK + 9β + *Tth111*I carriers. Furthermore, we found that homozygous carriers of the 9β + *Tth111*I haplotype had a 2.7-fold higher risk of the metabolic syndrome.

The ER22/23EK polymorphism consists of two linked single nucleotide polymorphisms in codon 22 and 23 of the transactivating domain of the GR gene. Carriers of the ER22/23EK polymorphism have a higher expression of the GR-A (11). The GR-A is one of the translational variants of the GR and has shown to be less functionally active than the shorter GR-B (20). This altered GR-A/GR-B expression ratio may explain the previously observed decrease in GC sensitivity in ER22/23EK carriers. Decreased sensitivity to GCs in vivo has been shown by a low dose dexamethasone suppression test, during which carriers of the ER22/23EK polymorphism showed significantly less suppression of cortisol levels after 1 mg of dexamethasone than non-carriers (12). Also in vitro transactivational capacity of the GR with the ER22/23EK variant has shown to be reduced (10). In accordance with a relative GC resistance, our previous studies demonstrated that carriers of the ER22/23EK polymorphism had lower insulin levels, better insulin sensitivity (as determined by lower HOMA-IR values) and lower total and LDL cholesterol levels (12). Furthermore, young adult male ER22/23EK carriers were found to be taller and to have a significantly higher lean body mass and more muscle strength (13). In the present group of older adults, we found no significant differences in body composition between carriers and non-carriers of the haplotype containing the ER22/23EK polymorphism. However, we found that ER22/23EK carriers had beneficial changes in body composition during aging in contrast with non-carriers. In carriers, the total amount of body fat significantly decreased over time, while the lean body mass

increased. In non-carriers there was an increase in fat mass over time, while there was no increase in lean body mass. During aging, body compositional changes occur, leading to a decrease in lean body mass and an increase in body fat at older age (25-26). Carriers of the ER22/23EK polymorphism seem to be relatively protected to the detrimental effects of aging on body composition, which might ultimately result in an increased lifespan. Actually, in a previous study, we observed a better survival in elderly male carriers of the ER22/23EK polymorphism (27). In that study, none of the 21 ER22/23EK carriers died, whereas 19% of the non-carriers died during a 4-year follow-up. This potential association with longevity was supported by the observation that the frequency of ER22/23EK carriers doubled in the oldest half of another large cohort study in the elderly (12). In the longevity study, we observed no differences in body composition between carriers and non-carriers at baseline. This is in line with our current study, since we found no cross-sectional differences between carriers and non-carriers. However, the longitudinal data showed significant differences between carriers and non-carriers. In the longevity study, no longitudinal data concerning body composition were available. It is known that greater body mass index, and in particular greater fat mass, is related to higher levels of CRP (28-29). High CRP levels are known to directly affect vascular vulnerability and increase cardiovascular risk (30-32). One of the possible mechanisms of a better survival in ER22/23EK carriers might be that the greater loss of (trunk) fat during aging in ER22/23EK carriers results in lower CRP levels, which might protect the ER22/23EK carriers from cardiovascular damage.

The observed differences in the longitudinal data for carriers of the ER22/23EK polymorphism, did not result in significant differences after 3 and 9 years of follow-up. Apparently, the changes on body composition over time are too small to lead to statistically significant differences cross-sectionally. Therefore, the clinical relevance of these findings can be questioned. However, in previous studies we have found that carriage of the ER22/23EK polymorphism was associated with longevity. Our finding of minor beneficial changes in body composition during aging might contribute to a healthier cardiometabolic status, which might result in increased longevity. Although the observed changes are rather small, they contribute to the understanding why ER22/23EK carriers live longer.

Furthermore, we found that homozygous carriers of the 9β + *Tth111*I haplotype had a 2.7-fold increased risk of the metabolic syndrome. The 9β polymorphism, similarly as the ER22/23EK polymorphism, is associated with a relative GC resistance. However, it has been shown that the 9β polymorphism affects mainly the transrepressive effects of GCs, whereas the ER22/23EK polymorphism affects transactivation (10, 19). The influence of GCs on the immune system is mainly regulated through transrepression. In line with a relative GC resistance, carriers of the 9β polymorphism had a reduced risk of bacterial colonization with Staphylococcus aureus and are more susceptible for rheumatoid arthritis, which suggests that this polymorphism leads to a more active immune system (33-34). In a previous study, we have shown that homozygous

carriers of the 9β polymorphism had an increased cardiovascular risk. This may be related to a lifelong exposure to a more active pro-inflammatory system, as supported by observed higher CRP and interleukin-6 levels (21). Interestingly, in the current study, we found an increased risk of the metabolic syndrome in homozygous carriers of the 9β polymorphism. Previous research suggests that the metabolic effects of GCs may be mainly caused by the GR transactivating effects and might be less influenced by the 9β polymorphism (21). However, metabolic syndrome is associated with a state of chronic inflammation, elevated hsCRP levels and cardiovascular disease. The same associations have been found in homozygous carriers of the 9β polymorphism. Chronic inflammation is known to lead to insulin resistance and metabolic syndrome. Therefore, the chronic pro-inflammatory state of the carriers may underlie the higher risk on developing the metabolic syndrome.

For the N363S and the *Bcl*I + *Tth111*I haplotype, we did not find any effects. The *Bcl*I (without *Tth111*I) haplotype showed a lower WHR in homozygous carriers with a higher WHR for heterozygous carriers in the fifth cycle. However, there were no differences in WHR in the second cycle and these data were not supported by body compositional differences on DEXA scans. Furthermore, we found no allele-dosage effect. Therefore, this may be a type I error, and should be interpreted with caution.

The population we studied is part of a large population that is representative for the community dwelling older population in the Netherlands. However, a few limitations need to be discussed. First, the number of participants with both cardiovascular disease and homozygous carriage of any of the haplotypes was small. Therefore, the results of the logistic regression analysis for homozygous carriers of all haplotypes have to be interpreted very cautiously, and replication in larger study populations is needed to confirm our findings in the homozygous carriers. Second, it should be noted that the prevalence of cardiovascular diseases was based on self report. However, self-report was found to be valid when compared with general practitioners information in this sample (35). Third, when conducting genetic association studies, the issue of multiple testing needs to be addressed. Due to the large number of statistical tests, the rate of false positive results is high and adjustment for multiple testing should be considered. In this study, we did not adjust for multiple testing, because of the low numbers of participants in some of the categories. However, we carefully reviewed all our statistically significant findings and only interpreted significant findings as true differences if a consistent, biologically plausible, pattern was found across outcomes. Our main finding of beneficial changes in body composition of ER22/23EK + 9β + *Tth111*I carriers is of interest since this was observed in two independent type of measurements (anthropometric data and DEXA scans) and this finding is in line with our a priori hypothesis and previous literature, as well as biologically plausible.

In conclusion, in a sample of older adults, GR variants, which are known to be related to a relative glucocorticoid resistance, are associated with beneficial changes in body composition and a reduced risk of the metabolic syndrome. These beneficial changes might protect the carriers of this haplotype for the detrimental effects of aging on body composition, resulting in healthier aging and a survival benefit as was observed previously (27).

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Chapter 14

Glucocorticoid receptor gene haplotype is associated with a decreased risk of delirium in the elderly

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Abstract

Background
Delirium is the most common mental disorder at older age in hospitals after acute admission. The pathogenesis of delirium is largely unknown. Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, leading to increased cortisol levels, has been suggested to play a role in the development of delirium. The effects of cortisol, the most important glucocorticoid (GC) in humans, are mainly mediated by the glucocorticoid receptor (GR). Several polymorphisms in the GR gene that alter the GC sensitivity are known. The aim of this study was to study the role of these GR polymorphisms in delirium in elderly patients.

Methods
Patients aged 65 years and older admitted to the medical department or scheduled for hip surgery were included. Delirium was diagnosed using the Confusion Assessment Method. Five single nucleotide polymorphisms in the glucocorticoid receptor gene were genotyped and haplotypes were constructed.

Results
Delirium was associated with impaired cognitive ($p<0.001$) and functional function ($p<0.001$), as well as with older age ($p<0.001$). Homozygous carriers of haplotype 4, characterized by the presence of the *Bcl*I and *Tth111*I minor alleles, had a 92% decreased risk of developing delirium ($p=0.02$), independent of age, cognitive and functional state.

Conclusions
Homozygous carriage of the *Bcl*I - *Tth111*I haplotype of the GR gene is related to a reduced risk of developing delirium. This suggests that altered glucocorticoid signalling may be involved in the pathogenesis and development of delirium in the elderly.

Introduction

Delirium, which is characterized by disturbance of consciousness, change in cognition, psychomotor behavioural disturbances and variable emotional disturbances, including fear, depression or euphoria, is one of the most common mental disorders at older age after acute hospital admission. Delirium develops over a short period of time, fluctuates during the day and is mostly caused by a medical condition like infection, postoperative state, substance intoxication or withdrawal (1). The pathogenesis of delirium is largely unknown, but increasing evidence suggests a role for the hypothalamic-pituitary-adrenal (HPA) axis. Hyperactivity of the HPA-axis has been described, with elevated plasma cortisol levels in delirious patients compared with non-delirious patients (2-3). Also in cerebrospinal fluid, cortisol levels were higher in patients with delirium compared to patients who did not develop delirium (4). In patients with Cushing's syndrome, characterized by GC excess, delirium has been reported as well (5-7). In addition, glucocorticoid therapy has been associated with the delirium (8). Dexamethasone suppression tests (DSTs) have been conducted in patients with and without delirium in order to test the HPA-axis feedback mechanism. In patients with delirium, there were significantly more non-suppressors than in the non-delirious patients, suggesting an impaired feedback mechanism of the HPA-axis in delirium (9-10). These results suggest that hyperactivity and impairment of the negative feedback system of the HPA axis may be involved in the pathogenesis of delirium.

The effects of glucocorticoids (GCs) are mainly mediated by the Glucocorticoid Receptor (GR). In the past decade, several polymorphisms have been described which are known to alter the sensitivity to GCs (11-12). The *Tth111*I polymorphism (rs10052957) of the GR gene has been associated with elevated diurnal cortisol levels. However, this polymorphism is linked to the ER22/23EK polymorphism, the 9β polymorphism and the *Bcl*I polymorphism. At present its functionality is unknown. The ER22/23EK polymorphism (rs6189 and rs6190) and the 9β polymorphism (rs6198) are associated with a relative GC resistance. In elderly subjects, the ER22/23EK polymorphism was associated with longevity and decreased risk of dementia (13-14). The N363S (rs6195) and the *Bcl*I (rs41423247) polymorphism are associated with increased sensitivity to GCs, resulting in a clinical picture with subtle Cushingoid features, e.g. increased abdominal obesity, hypertension and lower bone mineral density (11).

The GR gene and its polymorphisms and haplotypes are shown in Figure 1 (15). No previous studies on the relationship of GR variants, associated with altered GC sensitivity, and delirium have been conducted, although relationships with major depression and dementia have previously been found (16). Our aim is to study the role of GR gene polymorphisms in the development of delirium in medical and surgical hospitalized older patients. We hypothesize that increased sensitivity to GCs

due to variation in the GR gene could lead to a more active negative feedback system, which in case of acute illness could result in a faster decline in GC levels which could have a protective effect in the development of delirium. In addition, we hypothesize that variations in the GR gene which are related to GR resistance with respect to the negative feedback action, could increase the risk of a delirium.

Methods

Participants

From 2003 through 2008 all medical patients aged 65 years and older attending the Emergency Room of Academic Medical Center, Amsterdam with an acute illness were invited to participate. Also hip fracture patients who were scheduled for surgery were asked to participate. Informed consent was obtained from all participants or closest proxy in cases of cognitive impairment. The institutional Medical Ethics Committee approved the study.

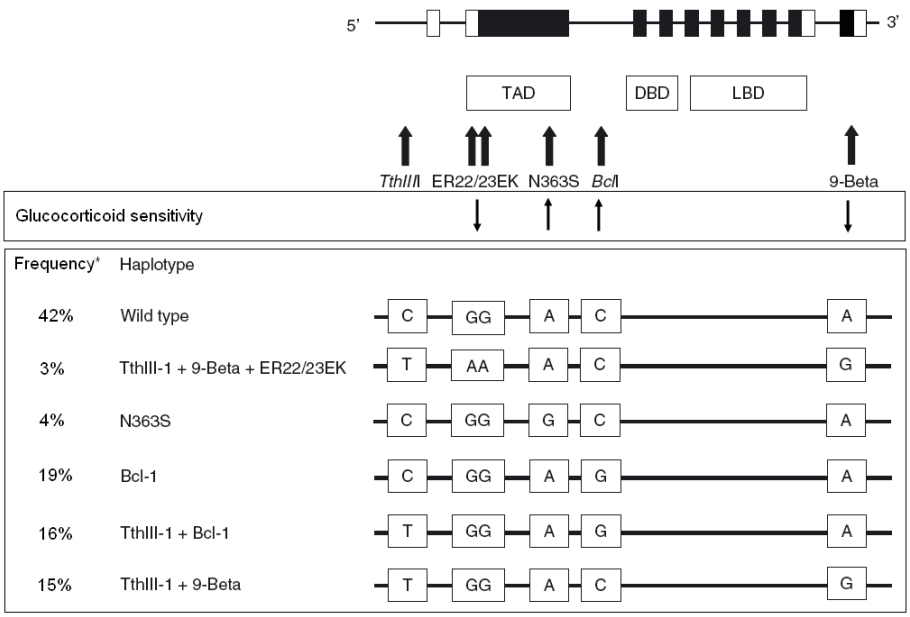

Figure 1. *Haplotypes of the glucocorticoid receptor gene. Nucleic acid changes are indicated. A, adenosine; C, cytidine; DBD, DNA-binding domain; G, guanidine; LBD, ligand-binding domain; T, thymine; TAD, transactivating domain. * frequencies are adapted from Derijk 2009.*

Procedures

Procedures are described in detail elsewhere (17). In brief, the presence or absence of delirium was scored using the Confusion Assesment Method within 48 hours of admission (18). Information for the diagnosis was based on psychiatric examination, medical and nursing records including the Delirium Observation Screening Scale (19) and information given by the patient's representative. For all patients, possible confounding factors, including demography, cognitive and functional functioning, were registered. Pre-existing global cognition was scored to be impaired when participants had a medical history of dementia of any cause or had a score above 3.9 at the Informant Questionnaire on Cognitive Decline Short Form (IQCODE-sf) (20). For the IQCODE-sf, the informant was asked to recollect the situation two weeks prior to the start of the illness for which the patient was admitted. The informant had to compare this situation with the situation ten years before. In case of missing IQCODE-sf we used a Mini-Mental State Examination score <24 (21) for patients without delirium. To measure functional functionality, patients or their closest relative in cases of cognitive impairment were asked to complete the 15-item Katz Index of Activities of Daily Living based on the situation two weeks prior to admission (22). Patients with a score of 7 and more were considered as functional impaired.

Genotyping

DNA was isolated from 10 ml whole blood on an AutopureLS apparatus according to a protocol provided by the manufacturer (Gentra Systems, Minneapolis, USA). DNA was genotyped by allelic discrimination using TaqMan Universal PCR master mix, primers and probes (Applied Biosystems) and the Taqman ABI Prism 7900HT Sequence Detection System. Reaction components and amplification parameters were based on the manufacturer's instructions, using an annealing temperature of 60 °C and optimized concentrations for primers and probes of 400 nmol/L for each polymorphism. Haplotypes were constructed as previously described (23). The GR gene and haplotypes are shown in Figure 1.

Table 1. *Patient characteristics*

	Delirium (n=299)	**No delirium (n=508)**	**P-value**
Median age (range)	83.0 (65.0-102.0)	77.7 (65.0-98.0)	<0.001
Male (%)	114 (38.1%)	229 (45.1%)	0.054
Caucasian ethnicity (%)	267 (85.9%)	494 (88.4%)	0.702
Cognitive impairment (%)	245 (83.1%)	128 (25.6%)	<0.001
Functional impairment (%)	185 (64.2%)	134 (26.9%)	<0.001
Diagnosis (%)			0.002
Surgery (Hip fracture)	76 (47.5%)	84 (52.5%)	
Internal Medicine	223 (34.5%)	424 (65.5%)	

Statistical analysis

All statistical analyses were performed with SPSS Version 16.0 for Windows. Differences in characteristics of patients with and without delirium were tested with Mann-Withney U tests and Chi-square tests. The association between delirium and the GR haplotypes was investigated by logistic regression analysis, with adjustment for independent risk factors, which were determined by backward selection procedure of multivariable logistic regression analyses. A p-value below 0.05 was considered significant.

Table 2. *Frequencies of GR gene haplotypes in patients with and without delirium*

	Total group (n=807)	**Delirium (n=299)**	**No delirium (n=508)**	**P-value**
Wildtype				0.41
0	244 (30.2%)	82 (27.4%)	162 (31.9%)	
1	396 (49.1%)	153 (51.2%)	243 (47.8%)	
2	167 (20.7%)	64 (21.4%)	103 (20.3%)	
ER22/23EK - 9β - *Tth111*I				0.36
0	765 (94.8%)	282 (94.3%)	483 (95.1%)	
1	41 (5.1%)	16 (5.4%)	25 (4.9%)	
2	1 (0.1%)	0 (0.0%)	1 (0.3%)	
N363S				0.24
0	744 (92.2%)	281 (94.0%)	463 (91.1%)	
1	62 (7.7%)	18 (6.0%)	44 (8.7%)	
2	1 (0.1%)	1 (0.2%)	0 (0.0%)	
*Bcl*I				0.81
0	518 (64.2%)	196 (65.5%)	322 (63.4%)	
1	254 (31.5%)	91 (30.4%)	163 (32.1%)	
2	35 (4.3%)	12 (4.0%)	23 (4.5%)	
*Bcl*I – *Tth111*I				0.12
0	583 (72.2%)	219 (73.2%)	364 (71.7%)	
1	208 (25.8%)	78 (26.1%)	130 (25.6%)	
2	16 (2.0%)	2 (0.7%)	14 (2.8%)	
9β - *Tth111*I				0.54
0	610 (75.6%)	223 (74.6%)	387 (76.2%)	
1	181 (22.4%)	68 (22.7%)	113 (22.2%)	
2	16 (2.0%)	8 (2.7%)	8 (1.6%)	

0= non-carries; 1= heterozygous carriers; 2= homozygous carriers.

Results

GR gene polymorphisms were determined in 881 patients and complete data sets of 870 patients were available. Haplotypes could be successfully constructed in 807 patients. Of these patients, 299 had been diagnosed with delirium. Baseline characteristics of patients with and without delirium are shown in Table 1. Delirious patients were significantly older (p<0.001) and had more frequently pre-existing cognitive (p<0.001) or functional (p<0.001) impairment than patients without delirium. When all patients were divided in two different diagnosis groups (surgical patients versus internal medicine patients), we found a significant difference in prevalence of delirium between these groups (p=0.002). The prevalence of delirium in the group of patients admitted to the department of internal medicine was 34.5% versus 47.5% in patients with hip fractures.

All GR gene polymorphisms and haplotypes were in Hardy Weinberg equilibrium in the total study population. Frequencies of GR haplotypes in patients with and without delirium are presented in Table 2. No significant differences in haplotype frequency were found between patients with and without delirium. Delirium was independently associated with cognitive impairment (Odds Ratio (OR) = 9.34, 95% CI 6.30-13.83, p<0.001), functional impairment (OR 1.91, 95% CI 1.31-2.79, p=0.001) and age (OR 1.03, 95% CI 1.01-1.06, p=0.012) in logistic regression analyses. After adjusting for these risk factors, we found a more than 90% lower risk of delirium in homozygous carriers of the *Bcl*I – *Tth111*I haplotype (Table 3). After adjustment for diagnosis group (internal and surgical patients), this lower risk of delirium in homozygous carriers of the *Bcl*I – *Tth111*I haplotype remained significant (OR 0.089 (95% CI 0.010-0.784), p=0.029). There were no significant differences of heterozygous and homozygous carriage of the *Bcl*I – *Tth111*I haplotype between the different diagnosis groups (p=0.363).

Discussion

The major finding in this study is the significantly lower risk of developing delirium in homozygous carriers of the haplotype comprising the *Bcl*I and *Tth111*I polymorphisms, independent of age, cognitive and functional impairment and diagnosis.

The functionality of the haplotype characterized by the *Bcl*I and *Tth111*I polymorphisms, is not exactly known. To our knowledge, only three studies have described two separate haplotypes which both included the *Bcl*I polymorphism (23-25). Comparing haplotype frequencies and using information on linkage disequilibrium of GR polymorphisms available in the HapMap database, we deduce that the *Bcl*I and the *Bcl*I – *Tth111*I haplotype in our study correspond to the *Bcl*I containing haplotypes in these studies. Stevens et al. showed that the haplotype

Table 3. *Odd ratio's of GR gene haplotypes, adjusted for age, cognitive and functional impairment*

	OR (95% CI)	P-value
Wildtype		
0	1	
1	1.239 (0.819-1.874)	0.311
2	1.096 (0.660-1.819)	0.724
ER22/23EK - 9β - Tth111I		
0	1	
1*	1.289 (0.586-2.836)	0.528
N363S		
0	1	
1*	0.853 (0.426-1.707)	0.653
*Bcl*I		
0	1	
1	0.988 (0.671-1.454)	0.951
2	0.691 (0.292-1.635)	0.400
*Bcl*I – Tth111I		
0	1	
1	1.003 (0.668-1.507)	0.987
2	0.081 (0.009-0.710)	0.023
9β - Tth111I		
0	1	
1	1.068 (0.700-1.629)	0.761
2	2.884 (0.734-11.253)	0.127

*0= non-carries; 1= heterozygous carriers; 2= homozygous carriers; *= heterozygous and homozygous carriers are combined.*

corresponding with the *Bcl*I – *Tth111*I haplotype in our study, was associated with low post dexamethasone cortisol levels, suggesting glucocorticoid hypersensitivity, whereas the other haplotype including the *Bcl*I polymorphism, which is corresponding with our *Bcl*I haplotype, was not (25). Glucocorticoid hypersensitivity, which is characterized by an increased response of the negative feedback system to GCs resulting in lower cortisol levels could be protective in the development of delirium. In contrast, Rautanen et al. showed that the haplotype corresponding to our *Bcl*I – *Tth111*I haplotype was associated with higher salivary and basal serum cortisol levels, suggesting a relative GC resistance (24). However, Rautanen et al also showed that this haplotype was associated with lower birth weight and length for gestational age, which could be an effect of GC hypersensitivity. In addition, most studies studying the

*Bcl*I polymorphism independently showed that this polymorphism was associated with increased GC sensitivity (11-12, 26). The effect of the *Tth111*I polymorphism is not known. In one study, elevated diurnal cortisol levels were found (27). However, the *Tth111*I polymorphism is partially linked to the ER22/23EK polymorphism, which is associated with a relative GC resistance (27). In carriers of the *Tth111*I polymorphism without the ER22/23EK polymorphism, GC sensitivity was unaltered (28). This may implicate that the *Tth111*I polymorphism itself may be not functional.

In the present study we found a protective effect of the *Bcl*I – *Tth111*I haplotype on the development of delirium in elderly patients. This is in line with the findings of impaired negative feedback of the HPA-axis in patients with delirium. Impairment of the negative feedback system could result in increased cortisol levels during stress situations, whereas a relative hypersensitivity to GCs could result in a faster decline in cortisol levels in stress situations. This would result in lower cortisol levels during acute stress, which might have a protective effect on the development of delirium. The reduction of delirium risk was only present in homozygous, but not heterozygous carriers of the *Bcl*I – *Tth111*I haplotype. Homozygous carriage of this haplotype may activate alternative signaling pathways which are not activated in the presence of only one minor allele. This could explain why we did not find an effect of heterozygous carriage on delirium risk. Our finding of the protective effect of a GR haplotype contributes to the hypothesis that GCs and GC sensitivity are involved in the development of delirium. Further research is needed to clarify the mechanism in which GCs are involved in the development of delirium.

We found no increased risk of delirium in carriers of polymorphisms associated with relative GC resistance, such as the ER22/23EK and the 9β polymorphism. However, in this study the number of ER22/23EK polymorphism carriers was low, therefore the lack of association could be due to the small study group. Replication in a larger study group is therefore necessary.

In conclusion, we found that homozygous carriers of the *Bcl*I – *Tth111*I haplotype of the GR gene have a decreased risk of developing delirium, independent of age, cognitive and functional state. This suggests that the glucocorticoid receptor might be involved in the pathogenesis and development of delirium in the elderly. However, replication in an independent validation group is necessary to confirm our finding. The effect of the *Bcl*I – *Tth111*I haplotype on the negative feedback system could be verified by examining the HPA-axis regulation under stress in carriers of this haplotype. Our present finding indicates that an individual's genetic make-up might partially determine the vulnerability to develop a delirium during acute disease. Early detection of patients at risk for delirium is of paramount importance to facilitate preventive measures, as well as early recognition and intervention. Our finding suggests that glucocorticoids might play a role in the pathogenesis of delirium and that future research on other genes involved in glucocorticoid signaling needs to be conducted.

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Part III:

General Discussion and Summary

Chapter 15

General Discussion

15.1 Rationale

The research described in this thesis can be divided into two strongly related, but distinct themes. Both themes concern the effects of cortisol and cortisol sensitivity. The difference is that the first part describes the effects of exposure to cortisol on parameters such as body composition and metabolism in a number of study groups; the second part investigates the influence of genetic variation in the glucocorticoid receptor gene on the effects of cortisol. All studies are performed in groups in which it was hypothesized that cortisol, or changes in cortisol or cortisol sensitivity might play a role in the pathogenesis of the disorder, or that cortisol might increase the risk of developing the disorder. In this chapter these two aspects will be discussed separately, in the interest of clarity.

15.2 Cortisol in scalp hair

General remarks on measuring cortisol in scalp hair

In the first part of this thesis, we focused on a recently developed method to measure cortisol in scalp hair. Since systemic cortisol seems to be incorporated in hair, it can be used as a measure of long-term cortisol exposure. We first described hair cortisol measurements in a large group of healthy individuals in which we have shown that hair cortisol levels are only mildly influenced by hair treatment (dyeing and bleaching) and correlate with waist-to-hip ratio (WHR). Furthermore, we have shown that hair cortisol levels remain stable in the hair shaft along a minimum of 18 cm (which corresponds to approximately 18 months), suggesting that it is possible to create a retrospective timeline of hair cortisol levels of at least 18 months. In addition, we have shown the usefulness of hair cortisol measurements in the diagnosis and follow-up of patients with (cyclic) Cushing's Syndrome and in the evaluation of hydrocortisone replacement therapy in patients treated with mitotane for adrenocortical cancer. Finally, we have used this method to study changes in cortisol levels due to shift work and long-term cortisol levels in patients with Bipolar Disorder or cardiovascular diseases.

Cortisol in scalp hair in patients with Cushing's Syndrome

Although there is a difference in studies between the length of the hairs that can be used, all studies agree that segmental hair cortisol measurements provide retrospective timelines of cortisol exposure. This advantage of hair cortisol measurement is of great value to study disorders in which changes in the hypothalamic-pituitary-adrenal axis (HPA-axis) might be involved. In (endogenous) Cushing's Syndrome there is hypercortisolism, usually caused by a pituitary microadenoma or, less often, an adrenal adenoma, carcinoma or ectopic ACTH producing tumor. The first study of hair cortisol measurements in patients with Cushing's Syndrome was by Thomson et al. (1). This pilot study showed that hair cortisol measurements could

be used to create retrospective timelines of cortisol exposure in patients with Cushing's Syndrome, which corresponded with clinical course and treatment effects. We collected hair samples in 14 patients with confirmed Cushing's Syndrome. In these patients we tested the sensitivity and specificity of hair cortisol measurements for the diagnosis of Cushing's Syndrome. The sensitivity that we found was 86%. In a meta-analysis of Elamin et al. the sensitivity of cortisol measurement in multiple 24-hour urine collections ranged from 38-100%, with a mean sensitivity of 84% (2). This is comparable with our hair cortisol measurements. However, in case of hair cortisol, we collected only one hair sample, whereas the sensitivity of urinary cortisol was based on multiple urinary samples. For patients, collecting urine is inconvenient, and in clinical practice the 24 hour urine collection is often incomplete, which can complicate the interpretation of the results. The sensitivity of midnight salivary cortisol ranged from 46% to 100% (mean 85%). Specificity of hair cortisol measurements (98%) was also similar to the specificity of urinary (44-100%, mean 92%) and salivary (79-100%, mean 92%) cortisol for Cushing's Syndrome (2). The hair sample collection is easy and non-invasive, and is not influenced by the fluctuations during the day. These advantages, together with the good sensitivity and specificity suggest that hair cortisol measurements might be of great value in the diagnosis of Cushing's Syndrome. An overview of the sensitivity and specificity of different diagnostic tests for Cushing's Syndrome is shown in Table 1.

Table 1. *Sensitivity and specificity of diagnostic tests for Cushing's Syndrome.*

Test	Sensitivity	Specificity	Min. number of samples needed	Free/total cortisol	Reference
Midnight Serum cortisol	90-92%	96%	2	Total	(3)
Midnight Salivary cortisol	85% (46-100%)	92% (79-100%)	2	Free	(2)
24-hour urinary cortisol	82% (38-100%)	92% (44-100%)	2	Free (unconjugated)	(2)
DST	93-96%	80%	1	Total	(3-4)
Hair cortisol	86%	98%	1	Free	(5)

DST, dexamethasone suppression test.

Cortisol in scalp hair in patients with cyclic Cushing's Syndrome

One of the most important aspects of the hair cortisol measurement in the diagnosis of Cushing's Syndrome, is the ability to create retrospective timelines. As we show in this thesis, it can be difficult to establish the diagnosis of cyclic Cushing's Syndrome with the standard screening tests. We have shown 6 cases of patients suspected of cyclic Cushing's Syndrome and in all these cases, the initial standard screening tests failed to confirm the suspicion of Cushing's Syndrome. In all patients, it took several

months to even years and multiple urine collections to establish the diagnosis. By cutting one hair sample, as close to the scalp as possible, we were able to create timelines which showed the presence of cyclic hypercortisolism. The episodes of hypercortisolism in the hair samples corresponded to symptomatic periods. This suggests that hair cortisol measurements might be very useful to establish the diagnosis of cyclic Cushing's Syndrome. However, there are a few limitations in case of cyclic Cushing's Syndrome. First, it is only possible to create timelines when hair is long enough to cover the period of interest. Furthermore, if a patient has very short periods of hypercortisolism (e.g. one week), it will be difficult to process the hair sample in such a way that a cyclic pattern will appear. We showed one case of a man with cyclic Cushing's Syndrome with cycles of 1 week. In this case urinary cortisol levels were normal in 7 out of the 10 urinary cortisol measurements. However, hair cortisol levels were continuously elevated. Although a clear cyclic hair cortisol pattern may be not present in such very rapidly cycling patients, hair cortisol levels can still be of value since they show a mean cortisol level over e.g. one month. If there was a week of hypercortisolism within that month, hair cortisol levels would still be higher than compared to the months without hypercortisolism.

Cortisol in scalp hair as a tool to evaluate hydrocortisone substitution

Another clinical setting in which the hair cortisol measurement can be of great value, is the evaluation of the treatment of adrenocortical cancer with mitotane. Mitotane is adrenolytic and an inhibitor of steroidogenesis which affects all adrenocortical zones (6). Therefore it is necessary to replace cortisol and sometimes also mineralocorticoids during mitotane treatment. However, mitotane is also a strong inducer of hepatic CYP3A4 activity and increases the concentration of cortisol binding globulin, which results in an increased cortisol metabolism and reduced free, active cortisol (7-10). Therefore, normal hydrocortisone replacement (around 20 mg/day) is not sufficient in adrenal insufficiency and higher dosages are necessary. In clinical practice, the evaluation of hydrocortisone replacement therapy in patients treated with mitotane is based on the clinical symptoms, since there is no good tool to measure long-term cortisol levels. Mitotane-treated patients frequently suffer from symptoms which could be due to adverse effects of mitotane, but also resemble symptoms of adrenal insufficiency. Therefore, discrimination of these condition would be valuable. In this thesis, we have used the hair cortisol measurement to evaluate the use of hydrocortisone in patients treated with mitotane with adrenocortical cancer. In our study, we found that hair cortisol levels were above the normal range in almost half of these patients. Importantly, none of the patients seemed undersubstituted. Furthermore, hair cortisol levels correlated positively with BMI. Hypercortisolism results in abdominal obesity and higher BMI. The positive correlation between hair cortisol and BMI might suggest that hair cortisol measurements in patients treated with mitotane reflect the cortisol exposure at the tissue level. Importantly, in this study the used dosage of hydrocortisone was not correlated with BMI. Therefore,

our study suggests that hair cortisol measurements can be valuable in titrating the dosage of hydrocortisone in patients on mitotane.

Cortisol in scalp hair and body composition

In the mitotane treated patients on hydrocortisone substitution, we found a positive correlation between body composition, measured in BMI, and hair cortisol levels. In literature, the relationship between cortisol levels and body composition, measured as BMI as well as waist-to-hip ratio (WHR), has been extensively studied. Several studies have shown that there is an increased adrenal cortisol production and that 24-hour urinary cortisol levels are increased in obese individuals (11-13). However, other studies have shown that there is an increased rate of metabolic clearance of cortisol in obesity and found normal or even decreased plasma and salivary cortisol levels in obese individuals (14-16). Plasma and salivary cortisol levels reflect only minutes to hours and do therefore not provide information about long-term cortisol levels in obesity. In our study in healthy individuals, we found a positive correlation between hair cortisol levels and waist-to-hip ratio. In our shift workers, we found a positive correlation between hair cortisol levels and BMI. Recently, our finding of a positive correlation between hair cortisol and BMI was replicated by Stalder et al. in two different studies (17-18). In two independent study groups consisting of healthy students from the Technical University of Dresden, Germany, they found a positive correlation between hair cortisol levels and BMI. It is known that elevated cortisol levels, as seen in Cushing's Syndrome, lead to central obesity. The results of the study by Stalder et al., together with the positive correlations between hair cortisol and BMI or WHR suggests that the measurement of cortisol in scalp hair is a adequate marker of long-term cortisol exposure, since central obesity is one of the key features of hypercortisolism.

Cortisol in scalp hair and cardiovascular disease

Along with central obesity, other effects of hypercortisolism are insulin resistance, dyslipidemia and hypertension. These features are all part of the metabolic syndrome and are associated with an increased cardiovascular risk. Long-term highly elevated cortisol levels, as seen in Cushing's syndrome, are associated with an increased incidence of cardiovascular diseases. However, whether long-term slightly elevated cortisol levels are associated with cardiovascular diseases is not clear. Many studies have investigated this relationship, but since only serum, saliva and 24-hour urinary cortisol levels were available, the true relationship between long-term cortisol levels and cardiovascular disease was burdensome to study and contradictory results have been published (19-24). The measurement of cortisol in scalp hair may contribute significantly to reveal the true role of cortisol in the development of cardiovascular disease. In order to study whether there is a relationship between hair cortisol levels and cardiovascular disease, we collected hair samples in a group of 283 older adults aged from 65-85 years. Together with hair samples, we collected data concerning their medical history. In this study, we found a significant relationship between

hair cortisol levels and a history of cardiovascular disease. The highest hair cortisol quartile had an Odds Ratio (OR) of 2.7 for having a history of cardiovascular disease. We found no significant relationship between hair cortisol levels and other diseases, such as non-specific chronic lung diseases, osteoporosis and cancer. This suggests that high cortisol levels are specifically associated with cardiovascular diseases and not with a history of chronic diseases in general. However, the study we performed was a cross-sectional study in which we collected information about cardiovascular diseases in retrospect. The ideal way to study the relationship between hair cortisol levels and cardiovascular disease would be a prospective study. In that way, it would be possible to study whether cortisol levels are elevated prior to the development of cardiovascular disease or the result of cardiovascular diseases. One study that supports the hypothesis that high cortisol levels play a role in the development of cardiovascular disease is the study by Pereg et al. (25). In this study, men who presented at the Emergency Department with chest pain were included. At the moment of presentation, a hair sample was collected and retrospective long-term hair cortisol levels were measured in the three months prior to the onset of chest pain. They found that hair cortisol levels were significantly higher in the men with chest pain caused by cardiovascular damage than in the group of men with non-cardial chest pain (including infections, non-myocardial chest pain and syncope). This study suggests that hair cortisol levels were already elevated prior to the acute myocardial infarction. This may indicate that high cortisol levels may contribute to the development of cardiovascular disease. However, a prospective study including hair cortisol measurements and measurements of the classic cardiovascular risk factors is needed to reveal the true significance of long-term slightly elevated cortisol levels in the development of cardiovascular disease.

Cortisol in scalp hair and psychiatric disorders

Another field in which changes in cortisol might contribute to disease development is psychiatry. In patients with Cushing's Syndrome, as well as in patients with cortisol deficiency, psychiatric symptoms such as depressive symptoms and anxiety, are commonly seen. In severely hypercortisolemic patients also mania and psychoses can occur. In addition, changes in serum and salivary cortisol levels and response to dexamethasone suppression tests have been observed in patients with bipolar disorder, major depression, schizophrenia and anxiety disorders (26-28). In parallel to the studies on cortisol and cardiovascular disease, the use of serum, salivary and urinary cortisol levels in psychiatric disorders resulted in contradictory findings with regard to cortisol levels in these disorders (29-33). Furthermore, the state of the disease (manic, depressive, psychosis, remission) seems to affect cortisol levels in serum and saliva as well. In this thesis, we have studied long-term cortisol levels in scalp hair of patients with bipolar disorder. We found that in the total group of bipolar disorder patients, hair cortisol levels were comparable to those in healthy individuals. However, in patients who developed bipolar disorder at older age, hair cortisol levels were significantly higher compared to a younger age of onset and

healthy individuals. These findings suggest that distinct subtypes of bipolar disorder patients exist with different ages of onset and cortisol profiles, and potentially a different pathophysiological background. This needs additional investigation to further explore whether this indeed concerns different disease entities, and whether treatment response is different between these subgroups. Furthermore, we found that in BD patients with panic disorder, hair cortisol levels were significantly lower than in BD patients without panic disorder and healthy individuals. Interestingly, salivary cortisol levels were not decreased and were even a little bit elevated, although this was not statistically significant. This finding is in line with the results of a study by Steudte et al., who found decreased hair cortisol levels in generalized anxiety disorder, together with normal salivary cortisol levels (34). Hair cortisol levels in both studies represented a three month period, whereas salivary cortisol levels reflect only minutes to hours. In psychiatric disorders such as generalized anxiety disorder and panic disorder, the acute stress due to the research setting might influence the salivary cortisol levels enormously. This effect is not present in the hair samples, since cortisol levels at the time of the hair sample collection are not yet incorporated in the hair shaft and do therefore not influence the mean cortisol levels over a longer period. Therefore, it seems more reasonable to use hair cortisol levels to study long-term changes in cortisol levels, whereas salivary cortisol levels can be used as a non-invasive method to study the acute changes in circadian rhythm and stress response during different phases of the disorders. The combination of these two measurements to study both the long-term cortisol levels as well as stress reactivity might lead to more insight into the regulation of the hypothalamic-pituitary-adrenal axis in psychiatric disorders.

Cortisol in scalp hair and altered day-night rhythm

Cortisol is secreted in a circadian rhythm with high levels in the early morning and low levels during the evening and night. Disturbances in this rhythm might result in detrimental effects on body composition and metabolic parameters. One of the factors that influences the circadian rhythm of cortisol secretion is shift work. A few studies have shown that the cortisol rhythm changes during shift work, with a lower early morning peak and higher levels during the evening and night (35-37). Whether these changes in cortisol secretion result in long-term changes in cortisol secretion has not been studied, since there was no suitable method to study this. In this thesis, we studied hair cortisol levels in male shift workers, working in a fast forward rotating shift schedule. This shift schedule consisted of 2 days of morning shift (6.00-14.00), followed by 2 days afternoon shift (14.00-22.30) and then 2 days of night shift (22.30-6.00). After the night shift there were 4 days of rest, after which the shift workers started again with 2 days of morning shift, etc. We found that hair cortisol levels, together with BMI, were significantly elevated in shift workers compared to day workers. Interestingly, this difference was specifically present in shift workers younger than 40 years of age. In the group of shift workers above the age of 40, we found no significant differences in hair cortisol levels nor BMI

compared to day workers over 40 years of age. In a second study, we confirmed this finding in the older group of shift workers. However, in that study in older adults, we did find a trend towards higher hair cortisol levels in shift workers working in a slow backward rotating shift schedule, in which shift workers first worked 5 days in the morning shift, then 5 days in the night shift, followed by 5 days in the afternoon shift. Participants in this shift schedule only worked during weekdays and not in the weekends. Several studies have suggested that slow backward rotating shift schedules lead to even more detrimental effects, such as increased fatigue, sickness absence and decreased alertness than fast forward rotating shift schedules. The tendency of higher hair cortisol levels in shift workers in a slow backward rotating shift schedule may suggest that this type of shift schedule might lead to a larger misalignment between the biological circadian rhythm and the behavioral circadian rhythm. The measurement of cortisol in scalp hair can contribute significantly to studies investigating the long-term effects of shift work. Because shift work results in changes in circadian rhythm of cortisol secretion, the timing of saliva and serum sample collection is difficult. Because hair grows approximately one cm per month, the time point of hair collection does not influence the hair cortisol measurement, and therefore it is possible to collect hair samples throughout the day without adjusting to shift schedules. This offers the opportunity to collect samples in large study groups in order to reveal the true significance of long-term cortisol changes in shift workers. Since the differences in hair cortisol levels and BMI were specifically present in younger shift workers, future studies should focus on the detrimental effects of shift work in younger adults.

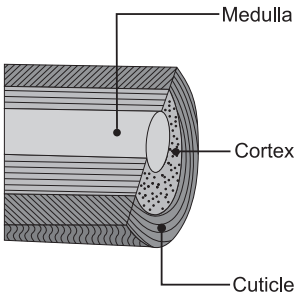

Figure 1. *Structure of the hair shaft.*

Technical aspects and limitations of the hair cortisol measurement

Our studies, and studies of other groups, have shown promising results concerning hair cortisol measurements. However, as with every novel method, many uncertainties still need to be investigated. First of all, it is not known how cortisol is incorporated in the hair shaft. In order to better understand the mechanisms of cortisol incorporation, it would be helpful to know where cortisol is incorporated in

the hair shaft. The hair shaft consists of three parts, namely the medulla, the cortex and the cuticle (Figure 1). The inner layer, the medulla, consists of polygonal cells with a sponge-like appearance. The cortex, which surrounds the medulla, consists of cornified fibrous cells. These cells are packed with keratin filaments and the cortex contains melanosomes, which determine the color of the hair. The outer layer of the hair is the cuticle, which is thin and translucent. The cuticle consists of multiple layers of corneocytes. Where cortisol is located in the hair shaft is not known and has not been studied. However, a number of studies have investigated the incorporation of drugs in hair. It has been suggested that drugs may bind to the hair cell components such as melanin (38-39). However, since drugs can also be found in unpigmented hair of albino animals, the binding of drugs with melanin does not seem to be an important mechanism (40). In one study in which the authors have use infrared microscopy to study the presence of several drugs in hair, they found that several drugs are present in the medulla of the hair shaft, which suggests that the most prominent route of incorporation is via diffusion from the capillary system directly into the hair root, with the drugs binding to the medulla material (41).

However, other possible mechanisms are incorporation via sebum or sweat. Every hair follicle has its own sebaceous gland and sweat glands are distributed all over the skin surface. It is known that cortisol is present in sweat and in a recent study by the group of Kirschbaum, it was shown that hair cortisol levels were higher in healthy participants who claimed to sweat very often (unpublished data, orally presented by T. Stalder at the Annual Meeting of the Society of Hair Testing, Toronto, Canada, June 2012). Furthermore, van Uum et al. showed that exposure of hair samples to a hydrocortisone solution for 60 minutes resulted in significantly higher hair cortisol levels (42). In addition, they found that even with thorough washing procedures, the hair cortisol levels of the exposed hairs were still higher than cortisol levels of non-exposed hairs. With this experiment, they tried to mimic sweat and they claim that exposure of hair to sweat around the time of hair collection will lead to increased hair cortisol levels, which cannot be effectively decreased with standard washing procedures (42). Whether the contribution of cortisol from sweat will interfere with the hair cortisol results has not been studied. The studies from Kirschbaum and van Uum suggest that excessive sweating might result in elevated cortisol levels in hair, however, van Uum only showed in vitro data. It is not clear whether 60 minutes exposure to a hydrocortisone solution is a equivalent to sweat exposure in vivo. Furthermore, in the study from Kirschbaum et al. participants who claimed to sweat a lot had higher hair cortisol levels. However, it is not known whether these participants had other factors that might have led to higher hair cortisol levels and excessive sweating, such as stress and sports. Therefore, at this moment, the higher hair cortisol levels cannot be fully assigned to contribution from cortisol in sweat. Moreover, a substantial number of studies (from our group, as well as from other researchers) have now shown that hair cortisol levels seem to represent systemic (circulating) cortisol levels, and the magnitude of the influence of extreme transpiration seems to be minor (5, 43-45).

Studying where and how cortisol is incorporated in the hair shaft is important, since this will provide information about possible confounding external factors. One of the possible influencing factors is hair treatment (dyeing or bleaching of hairs). In our study in healthy individuals, we found a trend towards lower hair cortisol levels in women with treated hair. A study from Sauvé et al. even found a statistically significant lower concentration of cortisol in treated hair. A possible explanation for this finding could be that dye-molecules that are incorporated in the hair shaft have a direct interaction with the cortisol molecules. It could also be that the weight of dyed hair samples is higher, resulting in lower cortisol concentrations when calculated as cortisol pg/mg hair. However, Sauvé et al. found, that dyeing of hair samples after they were cut from the scalp, did not result in lower cortisol levels. This suggests that the effect of dyeing on hair sample weight might be not of influence in lowering hair cortisol levels.

Hair cortisol measurements, in contrast to serum, salivary and urinary cortisol measurements are able to provide long-term cortisol levels. This is a great advantage, as it offers the opportunity to measure retrospective cortisol levels by collecting only one sample. Serum and saliva cortisol values only represent the last minutes to hours and urinary samples represent maximally 24 hours. These methods are very suitable to study the acute stress responses and circadian rhythms of cortisol, but do not offer the possibility to study cortisol levels of months or even years. In hair, each centimeter (cm) represents approximately 1 month of growth, therefore, depending on the hair length, it is possible to measure cortisol levels of the past several years. In our study in healthy individuals, we tested whether cortisol remained stable along the hair shaft over 18 cm. We found no decline in cortisol levels, suggesting that cortisol remains stable along the hair shaft. Furthermore, in patients with Cushing's Syndrome and Addison's Disease, we were able to create timelines that corresponded with clinical course of the past 2-3 years. In addition, Thomson et al showed that hair cortisol levels remained stable over 14 cm, which corresponds to a period of 14 months, in 8 healthy individuals (1). In the same study, they showed that it was possible to create retrospective timelines in Cushing's patients up to 18 cm (1). However, Kirschbaum et al. have shown that in their studies, there is a decline in cortisol levels after 6 cm of hair, suggesting that there is a washout of cortisol from the distal hair segments (44, 46). An explanation for the differences in the ability to create retrospective timelines might be the different methods that are used to measure cortisol levels. In the study by Thomson et al., and in our studies, the hair samples were not washed prior to analysis. Furthermore, hair samples were cut into small pieces of 1-2 mm, whereas Kirschbaum et al. used a Retsch ball mill to powder the hairs (44). Since hair fibers can be easily damaged by e.g. UV irradiation, hair treatment such as dyeing, bleaching, permanent waving and permanent straightening and chlorine (swimming pool water), it might be that the more damaged distal hair segments are more vulnerable to a certain "washout" effect. Washing of the hair samples with isopropanol or methanol prior to extraction might result in lower cortisol levels in the more distal hair segments for this reason.

Another issue, which is in particularly important when creating timelines, is the hair growth rate. Generally, it is assumed that hair grows with an average rate of 1 cm per month, with a range of 0.6-1.4 cm/month (47). There are only a few studies that have investigated hair growth rate, but what has been shown, is that the growth rate is influenced by a number of factors. It has been shown that hair growth rate differs between ethnicities, with Asian hairs growing the fastest (about 0.48 mm per day) and African hairs growing the slowest (0.26 mm per day) (48-49). Caucasian hair grows with an average of 0.40 mm per day (48-49). However, a recent study by Saint Olive Baque et al. showed that it was not ethnicity that influenced the hair growth rate, but the thickness of the hair fiber. Thicker hair, which is mostly present in Asians, corresponded to a faster growth, whereas thin hair corresponded with a lower growth rate. This was independent of ethnicity (48). The differences in hair growth rate between ethnicities or thickness of hair fibers is not of great importance if for example hair samples are used to measure one long-term hair cortisol level of one period. In this case, the cortisol levels will be measured in e.g. one 3 cm long hair segment. In general, it is assumed that this will represent cortisol levels of approximately 90 days (3 months). In Asians however, this would represent an average of 62 days, whereas in Africans this will represent an average of 115 days. In this case, the difference in time frame between Asians and Africans is quite large. However, it has been shown that intra-individual hair cortisol levels are rather stable over several months to even years.(50). This suggests that the collection of one hair sample, independent of whether it corresponds to approximately 2 months (e.g. in Asians) or 4 months (e.g. in Africans), reflects individual long-term cortisol exposure. Therefore, in the setting of evaluating long-term cortisol levels, without the need for a retrospective timeline, the time frame difference of the hair sample between ethnicities might be of less importance.

With regard to creating timelines, the effects of ethnicity or hair fiber thickness are of great importance. Since hair grows slower in Africans and faster in Asians due to differences in hair thickness, we would recommend to adjust the timelines to the average hair growth rate per ethnicity. Since Asian hair grows almost twice as fast as African hair, adjustment of the hair cortisol timeline is needed in order to create a timeline that truly reflects cortisol levels per month.

Furthermore, seasonal changes and illnesses might affect hair growth rate as well. However, as far as we known, no studies have investigated hair growth rate during different seasons and therefore, the effect of e.g. temperature changes and sunlight exposure on hair growth rate are not known. In our Cushing patients, of whom only 1 was not Caucasian, but from Egyptian descent, we assumed a hair growth rate of 1 cm per month and in all patients, this resulted in hair cortisol timelines that nicely reflected clinical course and treatment effects. The timelines were ranging from 3 months up to 21 months, which included multiple different seasons. The good fitting of the timelines with clinical course suggests that the seasonal effect on hair growth rate does not result in major changes in hair growth rate. Furthermore,

suffering from an illness does not seem to affect hair growth rate in our Cushing patients, since all timelines corresponded with clinical course and treatment effects during the active disease state as well as the remitted state when assuming a standard hair growth rate of 1 cm per month.

Although there might be a number of factors influencing hair growth rate, the most important one seems to be ethnicity (explained by differences in hair thickness). Adjustment for hair growth between the different ethnicities should be done and more research is needed to study the effects of seasonal changes and suffering from illnesses on hair growth rate.

Another point of discussion is the suggestion of the presence of a functional equivalent of the HPA-axis in the hair follicle (51). In one study Ito et al. shows that microdissected, organ-cultured human scalp hair follicles respond to CRH stimulation by up-regulation of proopiomelanocortin (POMC) transcription and immunoreactivity for ACTH and α-MSH. ACTH also up-regulated cortisol immunoreactivity in hair follicles. Furthermore, they showed that isolated human hair follicles secreted substantial levels of cortisol into the culture medium and that this secretion was further up regulated by CRH. In addition, CRH production was down regulated by addition of hydrocortisone to the medium, suggesting that there is a negative feedback system in the hair follicle (51). Two other reports of cortisol production by the hair follicle are from Sharpley et al. (52-53). In these studies, a small number of healthy participants are exposed to a brief stressor, namely the cold pressor test (CPT), in which they have to put their hand in ice cold water for 1 minute. In the first study, with 3 participants, they collected hair samples from the CPT arm and opposite leg plus 8 saliva samples. Hair and saliva samples were collected every 5 minutes from 5 minutes before the CPT to 30 minutes after the CPT. Salivary cortisol levels showed a significant increase 10-15 minutes after the CPT. Hair cortisol levels from the CPT arm increased to 500 pg/mg during the CPT, but were normalized 5minutes after the CPT. Hair cortisol levels from the opposite leg did not show any changes at all (52). In the second study, consisting of 5 participants, hair cortisol levels were collected from three sites, namely the wrist, the elbow and halfway between the wrist and the elbow. There was no consistent pattern of hair cortisol increase during or following the CPT at any location (52). Furthermore, the lowest concentration of cortisol measured in the arm hairs was around 350 pg/mg, which is much higher that the concentrations we (and other groups) have measured in scalp hair. The authors of both studies conclude that these data provide some support on the responsivity of hair cortisol concentrations to relatively brief stressors. Furthermore, they claim that their data show that peripheral cortisol production in hairs is independent of the central HPA cortisol production in saliva (52-53). In our studies in patients with Cushing's Syndrome and Addison's Disease, we find hair cortisol levels that correspond with clinical course of the disease and the effect of treatment (5, 45). In patients with cyclic Cushing's Syndrome, we found high cortisol levels during symptomatic periods and normal cortisol levels during asymptomatic periods.

If hair follicles would produce cortisol and have a negative feedback mechanism that is sensitive to the addition of hydrocortisone to the culture medium, it would not be logical that hair cortisol levels are high during an active period of Cushing's disease, as we observed. The high serum cortisol levels would activate the negative feedback in the hair follicle, which should result in low hair cortisol levels. However, this is not what we found. Furthermore, in Addison's Disease, patients have antibodies against the steroidogenic enzyme 21-hydroxylase in the adrenal cortex (54). We measured low cortisol levels in our patient with Addison's Disease, which increased after treatment with hydrocortisone. Since the antibodies in Addison's disease are directed against the enzyme 21-hydroxylase (54), and there is no evidence of the presence of this enzyme in the hair follicle, the assumed "HPA-axis" in the hair follicle should not be affected. Furthermore, treatment with hydrocortisone should result in activation of the negative feedback system in the hair follicle leading towards lower levels of cortisol measured in scalp hair. If the hair follicle would have its own HPA-axis, then the results found in our Addison patient would have to be the opposite of what we found. Hair cortisol levels should have been normal during the disease and should have been suppressed during hydrocortisone treatment. In addition, we measured hair cortisol levels in patients treated with mitotane and hydrocortisone replacement therapy. Mitotane is adrenolytic and therefore hydrocortisone replacement therapy is necessary (6). If there was a HPA-axis in the hair follicle, it would not be affected by mitotane. Treatment with hydrocortisone would therefore lead to activation of the negative feedback, resulting in suppressed cortisol levels in hairs, unless passive diffusion of hydrocortisone directly to the hair matrix is overruling this potential negative feedback. In our group of patients on mitotane treatment, no patient had cortisol levels below the reference range. Contrary, almost half of the patients had hair cortisol levels above the reference range, suggesting over replacement of hydrocortisone. Our results in these patients groups suggest that the cortisol levels measured in scalp hair resemble serum cortisol levels. Although there might be a functional equivalent of the HPA-axis in the hair follicle, our results suggest that the diffusion of cortisol from blood (and sweat or sebum) is more important than the production of cortisol by the hair follicle.

Although there are a number of unresolved questions with regard to the incorporation of cortisol in hair and the effects of hair growth rate, influences of hair treatment, racial differences and contribution of a possible HPA-axis equivalent in the hair follicle, we have shown in this thesis that the measurement of cortisol in scalp hair is a reliable and non-invasive method to measure chronic cortisol levels. Because of the non-invasive, easy collection and the ability to create retrospective timelines, the hair cortisol measurement has proven itself to be of great value in the diagnosis and follow-up of patients with abnormalities in cortisol levels, such as patients with Cushing's Syndrome and patients treated with mitotane on hydrocortisone substitution. Furthermore, we have shown that the hair cortisol measurement can be easily used in large studies to investigate the role of cortisol in the development of cardiovascular disease, psychiatric diseases and to study the effect of changes in

circadian rhythm on long-term cortisol levels. Although more research is needed to further validate the hair cortisol measurement, our results suggest that this method is a very suitable method to use in clinical practice and research settings.

15.3 Genetic variations of the Glucocorticoid Receptor Gene

In the second part of this thesis, we studied whether changes in cortisol sensitivity due to genetic variations, are associated with different clinical characteristics and diseases that might be related with changes in cortisol exposure. Since cortisol has many effects on body composition and metabolic parameters, in most studies, we focused on the role of Glucocorticoid Receptor (GR) Gene polymorphisms in changes in body composition and metabolic parameters. The groups we have studied included individuals born small for gestational age, HIV-infection and older adults. Furthermore, we studied the gene-environment interaction of GR polymorphisms and prenatal starvation. In addition, we studied whether relative hypersensitivity or relative resistance to cortisol might affect the risk of developing delirium at older age.

Polymorphisms are minor changes in the DNA sequence with a prevalence of more than 1 % in the general population. In contrast to rare mutations with usually large clinical effects, polymorphisms are more frequent and the effects are usually rather small. In this thesis, we have studied five polymorphisms in the gene encoding the Glucocorticoid Receptor which have been associated with changes in cortisol sensitivity in the past. An overview of the results of the polymorphism studies in this thesis is shown in Table 2.

Polymorphisms in the Glucocorticoid Receptor Gene that are associated with a relative glucocorticoid hypersensitivity

The N363S and the *Bcl*I polymorphism have both been associated with a relative hypersensitivity to cortisol. In previous studies, it has been shown that the N363S polymorphism was associated with an increased trans-activating capacity in vitro and an increased sensitivity to dexamethasone in vivo (55-57). In line with the increased sensitivity to glucocorticoids, several studies have found associations between the N363S polymorphism and higher BMI and waist-to-hip ratio, higher prevalence of type 2 diabetes and higher cholesterol and triglyceride levels in healthy and overweight or obese individuals of different ethnicities (55, 58-65). However, in other studies, these results could not be confirmed (66-67). In this thesis, we have studied the N363S polymorphism with regard to body composition and metabolic parameters in a group of children born small for gestational age, a group of HIV-infected adults, a group of older adults and as interaction with prenatal malnutrition and we found no associations at all. This suggests that the effect of this polymorphism

is rather small, and in case of adverse conditions, such as born small for gestational age, HIV-infection or prenatal malnutrition, the effect might be completely overruled. However, the number of N363S carriers in the studied populations in this thesis was usually rather small, with in most studies no homozygous carriers. The effect of the N363S polymorphism might be more abundant in homozygous carriers.

Table 2. *Overview of the results described in part 2 of this thesis.*

Haplotype	Study Group	Result
Glucocorticoid hypersensitive haplotypes		
N363S	All studies in this thesis	• No effects found
*Bcl*I	All studies in this thesis	• No effects found
*Bcl*I + *Tth111*I	Older adults, age 65-85 years	• Decreased risk of developing delirium
Glucocorticoid resistant haplotypes		
ER22/23EK + 9β + *Tth111*I	Individuals exposed to prenatal undernutrition during late gestation	• Lower weight • Lower BMI • Smaller hip circumference
	Older adults, age 65-85 years	• Decrease in BMI, waist and hip circumferences during aging • Decrease in total fat mass and trunk fat mass during aging
9β + *Tth111*I	Individuals exposed to prenatal undernutrition during late gestation	• Lower weight • Lower BMI • Smaller waist circumference • Smaller hip circumference
	Individuals exposed to prenatal undernutrition during early gestation	• Higher systolic and diastolic bloodpressure
	Older adults, age 65-85 years	• Increased risk of metabolic syndrome
Unknown		
*Tth111*I	African American HIV-infected patients	• Higher levels of HDL cholesterol • Tendency towards lower glucose levels • Tendency towards lower triglyceride levels • Lower CD4 count • Lower skeletal muscle mass

BMI, Body Mass Index; HDL, High Density Lipoprotein.

A polymorphism that is more frequent and also associated with a relative hypersensitivity to glucocorticoids is the *Bcl*I polymorphism of the Glucocorticoid Receptor gene. This polymorphism has been associated with lower levels of cortisol after dexamethasone, both 0.25 mg as well as 1 mg (68). In line with this, several studies have found positive associations with (abdominal) obesity (69-72). However, other studies could not confirm these associations (60, 73-74). The studies in this thesis revealed no associations between the *Bcl*I polymorphism and body composition or metabolic parameters in a variety of study groups. Even with sufficient numbers of homozygous carriers, no associations were found, suggesting that the effect of this polymorphism on body composition and metabolic parameters might be small and totally diminished in the setting of being born small for gestational age, HIV-infection or prenatal exposure to malnutrition.

Interestingly, we found an association between *Bcl*I carriage and the risk of developing delirium. Homozygous carriers of the *Bcl*I + *Tth111*I haplotype had a 92% decreased risk of developing a delirium during hospital admission for various reasons. Delirium is a disturbance of consciousness, change in cognition, psychomotor behavioral disturbances and variable emotional disturbances which develops after acute hospital admission. It is mostly caused by a medical condition like infection, postoperative state, substance intoxication of withdrawal (75). During physical and emotional stress, such as infection, postoperative state and substance withdrawal, cortisol levels increase. It has been hypothesized that the negative feedback system is impaired during delirium and several studies have shown that cortisol levels are relatively high in patients with delirium compared to non-delirious patients (76-77). Furthermore, more non-suppressors to dexamethasone were present in delirious patients compared to non-delirious patients (78-79). Impairment of the negative feedback system during acute situations such as infection and surgery could result in higher levels of cortisol. Exposure of the brain to higher cortisol levels can result in delirium, as seen in Cushing's Syndrome and patients on glucocorticoid therapy (80-83). Homozygous carriage of the *Bcl*I + *Tth111*I haplotype might result in a more sensitive negative feedback system and therefore a faster decline in cortisol levels during the acute situation. This might have a protective effect on the brain, preventing the development of delirium. To test this hypothesis, it would be interesting to measure cortisol levels in carriers and non-carriers of the *Bcl*I + *Tth111*I haplotype during delirium.

Polymorphisms in the Glucocorticoid Receptor Gene that are associated with a relative glucocorticoid resistance

Two polymorphisms that have been associated with an relative glucocorticoid resistance are the ER22/23EK and the 9β polymorphism. The ER22/23EK polymorphism is located in the transactivating domain of the GR gene and was shown to be associated with a decreased transactivating capacity (57, 84). It has been shown in transfection experiments, that carriage of the ER22/23EK polymorphism results

in higher expression of the GR translational variant GR-A (85). The GR-A variant results from translation of the GR mRNA from the first AUG codon. The second variant, GR-B, results from translation of the GR mRNA from the second AUG codon. The GR-A is transcriptionally less active than the GR-B protein (86). In ER22/23EK carriers, the structure of the GR-B at the mRNA level is altered, probably resulting in more of the GR-A translation variant (85). In vivo, less suppression of cortisol after 1 mg of dexamethasone was found in ER22/23EK carriers (84). In accordance with this relative resistance, associations were found between the ER22/23EK polymorphism and lower fasting insulin levels, increased insulin sensitivity and lower total and low-density lipoprotein cholesterol levels (84). However, previous studies showed no associations with BMI or fat mass, except for a tendency towards a smaller waist circumference in female ER22/23EK carriers (84). In this thesis, we found associations between the ER22/23EK polymorphism and body composition, both in older adults as well as in individuals exposed to famine during late gestation. In older adults, during a 9-year old follow-up, ER22/23EK carriers decreased their BMI, as well as their waist and hip circumference, whereas non-carriers increased BMI and waist and hip circumference. This change in body composition was also observed by DEXA scans, showing in a three year follow-up, that ER22/23EK carriers lost significantly more total fat and specifically trunk fat compared to non-carriers. However, this did not result in significant differences between carriers and non-carriers cross-sectionally. Apparently, the observed changes on body composition over time are too small to lead to statistically significant differences cross-sectionally. Although the effects might be small with a follow-up of 3 and 9 years, in the long term, they might contribute to a healthier body composition and longevity. In line with this, it has been shown that carriage of ER22/23EK is associated with longevity (84, 87).

In adults who had been exposed to famine prenatally during late gestation, we found effects of the ER22/23EK polymorphism on body composition as well. Carriers that were exposed had a significantly lower weight, lower BMI and smaller hip circumference than individuals who were exposed to famine during late gestation but were not carriers of this polymorphism. The same effects were found in carriers of the 9β polymorphism. Carriers exposed to famine during late gestation had significantly lower weight, BMI and smaller waist and hip circumference compared to exposed non-carriers. This suggests that the effects of the polymorphisms that conferred a relative resistance can be modified by environmental factors such as prenatal nutritional state. Prenatal malnutrition might lead to higher cortisol levels and reprogramming of the HPA-axis in utero to adapt to the higher cortisol levels. A relative glucocorticoid resistance might prevent these adaptations, which might result in prevention of developing a more adipose body composition in adult life.

A relative glucocorticoid resistance seems beneficial with regard to body composition, however, we also found an increased risk of having the metabolic syndrome at older age in carriers of the 9β polymorphism. In this thesis, we described a 2.7-

fold increased risk of having the metabolic syndrome in homozygous carriers of the 9β polymorphism. In line with this, van den Akker et al. found an increased risk of myocardial infarction and coronary heart disease in homozygous 9β carriers (88). Since this polymorphism has not been associated with detrimental body compositional changes or metabolic parameters in the general (elderly) population, in contrast to the ER22/23EK polymorphism, these factors cannot explain the described increased risk (89). It is known, that the 9β polymorphism seems to affect transrepression and the effects of GCs on the immune system are mainly regulated through transrepression (89). In 9β carriers, there is an increased expression and stabilization of the GR-β isoform (90). This isoform, produced by alternative splicing of the GR gene, resides in the nucleus of cells and does not bind GCs or activate GC-responsive genes. Therefore, it probably functions as a dominant negative inhibitor of the active receptor GR- α (91). Increased expression and stability of the GR- β isoform might result in a relative GC resistance. A relative glucocorticoid resistance of the transrepressive effects of GCs would result in a more active immune system. Previous findings of higher CRP and interleukin-6 levels support this hypothesis (88). Metabolic syndrome is associated with a state of chronic inflammation, elevated hsCRP levels and cardiovascular disease. Therefore, the chronic pro-inflammatory state of homozygous carriers of the 9β polymorphism may underlie the higher risk of developing the metabolic syndrome.

Ethnical differences in Glucocorticoid Receptor Gene polymorphisms

In this thesis, we also found a new haplotype which contains only the *Tth111*I polymorphism. In previous studies in Caucasians, this polymorphism was always linked to other polymorphisms such as the ER22/23EK, the 9β or the *Bcl*I polymorphism and it has been hypothesized that this polymorphism is not functional by itself. However, in our study in HIV-infected patients, we found 24 carriers of a haplotype only containing the *Tth111*I polymorphism without the presence of one of the other polymorphisms. All carriers were of African-American origin, suggesting that this haplotype ethnicity dependent and probably not present in Caucasians. We found that this new haplotype was associated with higher HDL cholesterol levels, a tendency towards lower glucose and triglyceride levels and lower CD4 count and skeletal muscle mass compared to gender and age matched controls. In the presence of HIV-infection, this haplotype seems to have a beneficial effect on the metabolic profile. However, we studied this haplotype in the setting of HIV-infection, and therefore replication in healthy African-American individuals is needed to study the true effects of this new haplotype on body composition and metabolic parameters.

Remarks concerning genetic association studies

This thesis contains five studies in which we have investigated associations between body composition and metabolic parameters and GR polymorphisms. The study populations ranged from children born small for gestational age to HIV-infected adults, older adults and adults exposed to famine during gestation. In all these studies,

we found no associations between the polymorphisms associated with a relative hypersensitivity (N363S and *Bcl*I) and body composition and metabolic parameters. In literature, however, many associations have been described concerning body composition with these two polymorphisms in healthy or obese adults (55, 58-65, 69-72). Apparently, the effects of these polymorphisms are only present in healthy or obese subjects, but when there is an additional detrimental situation, the effects of these polymorphisms are overruled, suggesting that the contribution of these GR polymorphisms to the development of cardiometabolic diseases in individuals with an underlying disorder is rather small. For the polymorphisms that are associated with a relative glucocorticoid resistance (ER22/23EK and 9β), we did find some effects on body composition. However, in case of the ER22/23EK polymorphism, the effects on body composition during aging did not result in cross-sectional differences in body composition, suggesting that the effects are rather small. Only in the case of prenatal exposure to famine, we found large difference between carriers and non-carriers of the resistant polymorphisms. This suggests that the effects of polymorphisms can be modified by (extreme) environmental disturbances such as prenatal malnutrition.

In the past years, many genetic studies have been performed with different approaches ranging from candidate gene studies to genome wide associations studies (GWAS). The main difference between candidate gene studies and GWAS is that in GWAS there is no hypothesis, whereas in candidate gene studies (including the studies described in this thesis), the studies are hypothesis driven. In candidate gene studies usually a clear hypothesis is established in advance due to the fact that there is more known about the function of the gene, the molecular mechanism of the polymorphism or its in vitro and in vivo effects. Although many genes have been found to be associated to some kind of disorder or disease, the effects are usually very small and very large populations are needed to find statistically significant results. And even with very large numbers, the genes found usually only explain a small fraction of the population variation, suggesting that other factors, such as e.g. gene-environment interactions, might be very important in the development of diseases. Furthermore, the large samples sizes that are needed require inclusion of individuals from different ethnicities into the same study. As seen in our study in HIV-infected patients, the polymorphism frequency is highly different between ethnicities and we even found a new haplotype in African-Americans, which we had not found before in Caucasians. This shows that genetic studies in large populations are rather difficult and can result in quite a few fallacies. Another problem with genetic studies is the multiple testing, resulting in false positive or false negative results and replication is therefore always needed in another cohort. Adjusting for multiple testing might be a solution for a part of this problem, but can also lead to rejection of true positive results. Replication of the results in other cohorts is necessary, but can also lead to false rejection of the first observed findings. Furthermore, replication in different cohorts in different environments might result in totally different effects of this polymorphisms since the effects of polymorphisms can be modified by environmental factors, as can be seen in our study in adults prenatally exposed to famine.

With regard to the GR polymorphisms, it seems that the effects of these polymorphisms on body composition and metabolic parameters are only clearly present in healthy individuals without any comorbid disorder or disease. It might be also interesting to study the effects of these polymorphisms in combination with adverse environmental situations in order to find larger effects on the cardiometabolic profile.

15.4 Conclusion and future perspectives

In this thesis, we have focused on two determinants of cortisol exposure, namely actual long-term cortisol levels by using a novel method to measure cortisol in scalp hair and polymorphisms that are known to alter cortisol sensitivity. We have shown that the measurement of cortisol in scalp hair is a promising tool to evaluate long-term cortisol levels, both in health and disease. Because it is non-invasive and easy to collect, this new method is very promising for use in the clinical setting for the diagnosis and follow-up of patients with disorders of the HPA-axis, such as Cushing's Syndrome and Addison's Disease, as well as for monitoring therapy in patients on hydrocortisone substitution therapy. In addition, this method is valuable in scientific research, shedding a new light on many conditions in which cortisol may be of importance, since this hair cortisol measurement provides the opportunity to determine cumulative cortisol levels in retrospect for prolonged periods of time. We have shown that higher hair cortisol levels are associated with higher BMI and WHR and with an increased cardiovascular risk. Furthermore, we have shown that shift work resulted in higher long-term cortisol levels and that cortisol levels were higher in patients with late onset bipolar disorder.

Altered cortisol sensitivity, due to polymorphisms in the GR gene, did not result in differences in body composition or metabolic parameters in children born small for gestational age and HIV-infected patients. In older adults we found beneficial changes in body composition in carriers of the ER22/23EK polymorphism, and a higher risk of developing the metabolic syndrome in homozygous carriers of the 9β polymorphism. This suggests that the effects of the GR polymorphism are in particularly present in normal individuals without any co-morbid disorder such as HIV-infection. Furthermore, we found that the effect of GR polymorphisms can be modified by an adverse environment, namely prenatal exposure to famine. This was specifically the case for the polymorphisms (ER22/23EK and 9β) conferring a relative resistance to glucocorticoids.

Cortisol has many effects throughout the body and as shown in this thesis, changes in cortisol exposure are associated with changes in cardiometabolic profile. In this thesis, we studied long-term cortisol levels and cortisol sensitivity separately. In the future, it would be interesting to study the combination of long-term cortisol levels and cortisol sensitivity to get a more complete view of the total cortisol exposure. The effects of high long-term cortisol levels on body composition might be even

more abundant in carriers of glucocorticoid hypersensitive polymorphisms such as the N363S and the *Bcl*I polymorphism, whereas the effects might be only mild in the more glucocorticoid resistant genotypes. Studying cortisol exposure by combining the two methods might result in a more complete view of the role of cortisol exposure in the development of different diseases. In particular with regard to cardiovascular diseases such a "cortisol profile" may in the future lead to classification of individuals at risk for developing cardiovascular disease or other diseases based on the combination of their genetic profile and long-term glucocorticoid levels.

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Chapter 16

Summary
Samenvatting

Summary

Glucocorticoids are hormones produced by the adrenal gland. The most important glucocorticoid in man is cortisol. The effects of cortisol are mainly mediated by the glucocorticoid receptor. This thesis is focused on the effects of cortisol exposure in health and disease, which is studied in two ways. First by measuring long-term cortisol levels in scalp hair and second, by studying changes in cortisol sensitivity due to variations in the glucocorticoid receptor gene.

As introduced in **chapter 1**, cortisol is secreted in a circadian rhythm, with pulses and its secretion is influenced by acute stress. This complicates the use of standard specimens such as serum, saliva and urine for the measurement of cortisol. In this chapter, the disadvantages of the standard cortisol measurements in healthy individuals and patients suspected of hypo- or hypercortisolism are described and the background of the novel method to measure cortisol in scalp hair is introduced. Furthermore, in this chapter, the background of glucocorticoid sensitivity and individual differences in glucocorticoid sensitivity due to polymorphisms in the glucocorticoid receptor gene is described. The aims of the first part of this thesis were to evaluate the method to measure cortisol in scalp hair, as a measure of systemic long-term cortisol, and to study whether this method is can be used in clinical practice in patients with hypo- and hypercortisolism and patients on hydrocortisone replacement therapy. Furthermore, we studied long-term changes in cortisol levels in psychiatric disorders, cardiovascular disease and in shift workers. The second part of this thesis focused on the role of glucocorticoid receptor gene polymorphisms. The aims of this part were to study whether polymorphisms, associated with changes in glucocorticoid sensitivity, are associated with changes in body composition and metabolic parameters in different study populations, such as children born small for gestational age, HIV-infected patients, adults who had been exposed to famine in utero and elderly.

In **chapter 2** we studied whether the measurement of cortisol in scalp hair is a feasible method to measure long-term cortisol exposure. We measured hair cortisol levels in a group of 195 healthy individuals en found that hair cortisol levels were positively correlated with waist-to-hip- ratio, suggesting that indeed hair cortisol levels reflect long-term cortisol exposure. Furthermore, we found that hair treatment (dyeing and bleaching of hairs) slightly lowered cortisol levels in scalp hair and that cortisol levels remained stable in long hair, suggesting that it is possible to create retrospective timelines of cortisol exposure in individuals with long hair.

In **chapter 3** we studied whether the measurement of cortisol in scalp hair could contribute in the diagnosis and follow-up of patients with Cushing's syndrome and cyclic Cushing's syndrome. Standard screening tests for Cushing's syndrome are the measurement of cortisol in multiple 24h urine collections, late night saliva, and dexamethasone suppression test. However, these tests can be normal in case of cyclic Cushing's Syndrome. In this chapter, we show that the measurement of cortisol in one hair sample has a similar sensitivity and specificity for Cushing's

syndrome as the measurement of cortisol in multiple urinary or saliva samples. Furthermore, we show that hair cortisol measurements provide the opportunity to create a retrospective timeline of cortisol exposure which can be helpful to diagnose patients with cyclic Cushing's syndrome.

In **chapter 4** we studied whether hair cortisol can serve as a monitoring tool of hydrocortisone replacement therapy in patients with adrenocortical cancer on mitotane treatment. Mitotane is known to increase cortisol binding globuline (CBG) and induces cortisol metabolism. Therefore, high doses of hydrocortisone are necessary in patients on mitotane treatment. In clinical practice, it is difficult to evaluate the effect of hydrocortisone replacement therapy, since side-effects of mitotane are similar to symptoms of cortisol deficiency. In chapter 4 we show that hair cortisol measurements can be used to evaluate hydrocortisone replacement therapy in mitotane treated patients with adrenocortical cancer. We show that almost half of the patients have hair cortisol levels above the normal range, and that hair cortisol levels are positively correlated with BMI, suggesting that these patient are oversubstituted. Importantly, none of the patients had hair cortisol levels below the normal range.

In **chapter 5** we investigated whether long-term cortisol levels are associated with Staphylococcus Aureus nasal carriage. Since cortisol induces immune suppression, and a previous study has shown that a relative glucocorticoid resistance due to a glucocorticoid receptor gene polymorphism results in a decreased risk of nasal carriage, we hypothesized that higher cortisol levels are associated with persistant Staphylococcus Aureus nasal carriage. However, we show that there is no difference in long-term cortisol levels between non-carriers, intermittent carriers and persistant carriers, suggesting that cortisol levels do not significantly affect Staphylococcus Aureus carriage state.

In **chapter 6** we studied the effects of shift work on long-term cortisol levels. Shift work is known to lead to changes in the natural rhythm of cortisol secretion. Furthermore, shift work is associated with an increased incidence of obesity and cardiovascular disease. In this chapter, we showed that long-term hair cortisol levels were higher in male shift workers in a fast forward rotating shift schedule compared to male day workers. Interestingly, this was in particular the case in shift workers below the age of 40. Furthermore, BMI was also higher in shift workers compared to day workers. Again, this difference was in particular significantly present in the group of shift workers below the age of 40. Hair cortisol levels were positively correlated with BMI in this study. These results suggest that cortisol might play a role in the association between shift work and cardiovascular disease, and that the detrimental effect of shift work might be more abundant in younger shift workers.

In **chapter 7** we described the second study in shift workers. In this chapter, we show again that there is no difference in hair cortisol levels and BMI between shift workers and day workers at older age when working in a fast forward rotating shift schedule. However, we did find a tendency towards higher hair cortisol levels in shift workers in a slow backward rotating shift schedule, suggesting that this type of

shift schedule might have more detrimental effects than a fast forward rotating shift schedule.

In **chapter 8** we have studied the association between long-term cortisol levels and cardiovascular disease in a group of 283 community dwelling older adults. We found that the individuals in the highest hair cortisol quartile had a 2.7-fold increased risk of cardiovascular disease. Furthermore, this group also had an increased risk of type 2 Diabetes Mellitus compared to individuals in the lowest cortisol quartile. The increased cardiovascular risk we found for higher cortisol levels is comparable to the effects of traditional risk factors such as high blood pressure and abdominal obesity. Excessive alcohol usage was also associated with increased hair cortisol levels. This study suggests that higher cortisol levels might be an important risk factor for cardiovascular disease as well.

In **chapter 9** we investigated whether cortisol levels were different in patients with bipolar disorder (manic depression) compared to healthy individuals. We found that hair cortisol levels were significantly elevated in bipolar disorder patients with an older age of onset. This suggests that elevated long-term cortisol levels might play a role in a subgroup of bipolar disorder patients and that there might be a difference in pathogenesis between younger and older onset bipolar disorder. Furthermore, we found that hair cortisol levels, but not salivary cortisol levels, were significantly lower in bipolar disorder patient with panic disorder compared to healthy individuals and bipolar disorder patients without panic disorder. In contrast, bipolar patients with other types of psychiatric comorbidity have increased hair cortisol levels. Thus, this study shows that within bipolar disorder, subtypes might exist with altered systemic cortisol exposure.

In **chapter 10** we studied whether polymorphisms in the glucocorticoid receptor gene are associated with birth anthropometry, blood pressure, glucose and insulin levels and body composition in individuals born small for gestational age. We found no difference in allele frequency between children born small for gestational age and healthy controls. Furthermore, in these children, no associations were found with birth anthropometry, response to growth hormone treatment, blood pressure, glucose and insulin levels and body composition. Apparently, the glucocorticoid receptor gene polymorphisms do not play a major role in the development of detrimental body compositional and metabolic changes in children born small for gestational age.

In **chapter 11** we identified a new haplotype in HIV-infected African-American patients. HIV-infection and HAART therapy have been associated with body compositional and metabolic changes that resemble Cushing's syndrome. We studied whether polymorphisms in the glucocorticoid receptor gene, which are known to alter cortisol sensitivity, were associated with the development of these Cushing-like symptoms. In this study, we found a new haplotype in African-Americans in which the *Tth111*I polymorphism was found without the presence of the ER22/23EK, the 9β or the *Bcl*I polymorphism. This new haplotype was not present in Caucasians. In African-American HIV-infected patients, this new haplotype was associated with

higher HDL-cholesterol levels and a possibly healthier metabolic profile. However, research in healthy African-Americans has to be performed to evaluate the true effects of this haplotype. The other glucocorticoid receptor gene haplotypes were not associated with any body compositional or metabolic changes.

In **chapter 12** the gene-environment interaction of glucocorticoid receptor gene polymorphisms and prenatal exposure to famine was studied. We found an interaction of the polymorphisms associated with a relative glucocorticoid resistance (ER22/23EK and 9β) and exposure to famine during late gestation. These interactions resulted in a beneficial body composition with lower BMI and smaller waist and hip circumference at older age. This suggests that the effect of the glucocorticoid receptor gene polymorphisms on body composition can be modified by nutrition during gestation.

In **chapter 13** we studied associations between glucocorticoid receptor gene polymorphisms and body composition, metabolic parameters and cardiovascular disease cross-sectionally and longitudinally in a group of community dwelling older adults. We found that carriage of the ER22/23EK polymorphism was associated with beneficial longitudinal changes in body fat at older age. Total body fat, and in particular trunk fat decreased over time, whereas muscle mass remained stable. These beneficial changes might protect carriers of this haplotype for the detrimental effects of aging on body composition, resulting in healthier aging and a possible survival benefit, as has been shown previously.

In **chapter 14** we investigated whether glucocorticoid receptor gene polymorphisms influence the risk of developing a delirium at older age. Hyperactivity of the HPA-axis and impairment of the negative feedback system have been suggested to be involved in the development of delirium at older age. We studied 881 older patients of whom 299 developed a delirium during hospital admission. We found that carriers of the *Bcl*I – *Tth111*I haplotype had a 92% lower risk of developing delirium during hospital admission. This was independent of age, cognitive and functional state. This suggests that the glucocorticoid receptor might be involved in the pathogenesis and development of delirium in elderly.

Chapter 15 contains a general discussion in which the findings described in this thesis are put into a broader perspective. The benefits and disadvantages of the measurement of cortisol in scalp hair are discussed, as well as our results of the polymorphism studies and general remarks concerning polymorphism studies. We conclude that although there are a number of unresolved questions concerning the methods and incorporation of cortisol in human hair, our studies suggest that this method is a very suitable method to use in clinical practice and research settings. With regard to the GR polymorphisms, we conclude that it seems that the effects of these polymorphisms on body composition and metabolic parameters are predominantly present in healthy individuals, and may be only a subtle modifier in some (pathological) conditions.

Samenvatting

Glucocorticoïden zijn hormonen die geproduceerd worden door de bijnier. Het belangrijkste glucocorticoïd in mensen is cortisol. De effecten van cortisol worden met name gemedieerd door de glucocorticoïd receptor. In dit proefschrift bestuderen we de effecten van cortisol blootstelling in gezonde personen en individuen met een aandoening. Deze blootstelling aan het hormoon cortisol hebben we op twee verschillende manieren onderzocht. In het eerste deel van dit proefschrift hebben we lange termijn cortisol waarden gemeten in hoofdhaar en in het tweede deel hebben we naar veranderingen in de gevoeligheid voor cortisol gekeken door variaties in het gen dat voor de glucocorticoïd receptor codeert te onderzoeken.

Zoals beschreven in **hoofdstuk 1** wordt cortisol in een circadiaan ritme afgegeven aan de bloedbaan. De cortisol productie is tevens pulsatiel en wordt beïnvloed door acute stress. Hierdoor is het lastig om standaard methodes, zoals het meten van cortisol in speeksel, bloed of urine, te gebruiken om lange termijn cortisol waarden te meten. In dit hoofdstuk worden de nadelen van de standaard cortisol metingen in gezonde mensen, maar ook in patiënten die verdacht worden van hypo- of hypercortisolisme weergegeven en wordt de nieuwe methode om cortisol in hoofdhaar te meten geïntroduceerd. Tevens worden in dit hoofdstuk de achtergronden van glucocorticoïd gevoeligheid en individuele verschillen in gevoeligheid door polymorfismen in het glucocorticoïd receptor gen beschreven. Het doel van het eerste deel van dit proefschrift was om de methode van het meten van cortisol in hoofdhaar te valideren en te bestuderen of deze methode in de kliniek gebruikt kan worden bij patiënten met hypo- en hypercortisolisme en bij patiënten die behandeld worden met hydrocortison. Verder hebben we ook gekeken naar lange termijn cortisol waarden bij psychiatrische en cardiovasculaire ziekten en bij mensen die in ploegendienst werken. In het tweede deel van het proefschrift ligt de focus op de rol van glucocorticoïd receptor polymorfismen. Het doel van dit deel van het proefschrift was om te kijken of polymorfismen, die geassocieerd zijn met veranderingen in de gevoeligheid voor glucocorticoïden, geassocieerd zijn met veranderingen in lichaamssamenstelling en metabole parameters. We hebben dit onderzocht in verschillende populaties, namelijk kinderen die te klein geboren zijn, HIV-positieve patiënten, volwassenen die in utero bloot zijn gesteld aan ondervoeding en ouderen.

In **hoofdstuk 2** hebben we onderzocht of het meten van cortisol in hoofdhaar een goede methode is om lange termijn cortisol waarden te meten. We hebben cortisol waarden gemeten in hoofdhaar van 195 gezonde mensen en vonden dat haar cortisol waarden positief gecorreleerd waren met de buik-heup ratio. Gezien het feit dat van cortisol bekend is dat het een toename geeft van de buikomvang, suggereert dit dat hoofdhaar cortisol waarden inderdaad lange termijn cortisol waarden reflecteren. Verder vonden we dat het verven of bleken van hoofdhaar resulteerde in iets lagere cortisol waarden en dat cortisol stabiel bleef in lang haar. Hierdoor blijkt het mogelijk te zijn om retrospectieve tijdlijnen van cortisol waarden te maken bij mensen met lang haar.

In **hoofdstuk 3** hebben we onderzocht of het meten van cortisol in hoofdhaar kan bijdragen bij de diagnose en het vervolgen van mensen met het syndroom van Cushing en mensen met de cyclische variant hiervan. Standaard testen om te screenen voor het syndroom van Cushing zijn het meten van cortisol in meerdere 24-uurs urine collecties, speeksel verzameld op de late avond of om middernacht en een dexametason suppressie test. In het geval van cyclisch syndroom van Cushing kunnen deze testuitslagen normaal zijn. In dit hoofdstuk beschrijven we dat het meten van cortisol in hoofdhaar dezelfde sensitiviteit en specificiteit heeft voor het diagnosticeren van het syndroom van Cushing als de standaard testen waarbij meerdere malen getest moet worden. Ook laten we zien dat het mogelijk is tijdlijnen te creëren bij patiënten met lang haar. Deze tijdlijnen zouden kunnen bedragen tot een sneller diagnose bij patiënten met het cyclische syndroom van Cushing.

In **hoofdstuk 4** hebben we het effect van hydrocortison behandeling bij patiënten met een bijnierschorscarcinoom die behandeld worden met mitotaan geëvalueerd. Van mitotaan is het bekend dat het het cortisol bindend globuline (CBG) verhoogt en dat dit middel het cortisol metabolisme versnelt. Patiënten die behandeld worden met mitotaan hebben daarom hogere doseringen hydrocortison nodig. Klinisch is het lastig om te evalueren of de dosering van hydrocortison voldoende is, omdat de bijwerkingen van mitotaan grotendeels hetzelfde zijn als de symptomen van een tekort aan cortisol. In dit hoofdstuk laten we zien dat het meten van cortisol in hoofdhaar gebruikt kan worden om hydrocortison behandeling te monitoren bij patiënten die mitotaan gebruiken. We laten zien dat bijna de helft van de patiënten cortisol waarden hebben die hoger zijn dan de bovengrens van normaal en dat haar cortisol waarden positief gecorreleerd zijn met BMI. Dit suggereert dat deze patiënten te hoge doseringen hydrocortison krijgen. Ook een belangrijke bevinding was dat geen van de patiënten cortisol waarden onder de ondergrens van normaal bleek te hebben.

In **hoofdstuk 5** hebben we gekeken naar lange termijn cortisol waarden en dragerschap van Staphylococcus Aureus. Eén van de effecten van cortisol is immuun suppressie en een eerdere studie heeft aangetoond dat een relatieve glucocorticoïd resistentie door een polymorfisme in het glucocorticoïd receptor gen leidt tot een verlaagd risico op S. Aureus dragerschap. Onze hypothese was, dat hogere haar cortisol waarden geassocieerd zouden zijn met persisterend dragerschap van S. Aureus. Echter, in onze studie vonden we geen verschil in haar cortisol waarden tussen niet-dragers, intermitterende dragers en persisterende dragers. Dit suggereert dat cortisol waarden geen significant effect hebben op S. Aureus dragerschap.

In **hoofdstuk 6** hebben we gekeken naar het effect van het werken in ploegendienst op lange termijn cortisol waarden. Het is bekend dat het werken in ploegendiensten leidt tot veranderingen in het ritme van cortisol secretie en dat het werken in ploegendienst is geassocieerd met obesitas en cardiovasculaire ziekten. In dit hoofdstuk laten we zien dat lange termijn cortisol waarden bij ploegendienst medewerkers, die in een snel voorwaarts roterend schema werken, significant hoger zijn dan bij mensen die in dagdienst werken. Dit was specifiek het geval voor

mensen onder de 40 jaar. Ook de BMI was hoger in ploegendienst medewerkers vergeleken met dagdienst medewerkers. Wederom was dit verschil voornamelijk aanwezig in de groep werknemers jonger dan 40 jaar. Hoofdhaar cortisol waarden waren positief gecorreleerd met BMI. Deze resultaten suggereren dat cortisol een rol speelt in de associatie tussen het werken in ploegendiensten en het ontwikkelen van cardiovasculaire ziekten. De nadelige effecten van ploegendiensten lijken het meest aanwezig te zijn in jongere ploegendienst medewerkers.

In **hoofdstuk 7** beschrijven we de 2e studie bij ploegendienst medewerkers. In dit hoofdstuk laten we wederom zien dat er geen verschil is in haar cortisol en BMI tussen ploegendienst medewerkers en dagdienst medewerkers op oudere leeftijd. Echter, er was wel een trend zichtbaar met hogere cortisol waarden bij mensen die in een langzaam achterwaarts roterend ploegendienst schema werkten. Dit suggereert dat dit type schema mogelijk meer nadelige effecten heeft dan een snel voorwaarts roterend schema.

In **hoofdstuk 8** hebben we de associatie tussen lange termijn cortisol waarden en cardiovasculaire ziekten bij 283 ouderen onderzocht. We vonden dat ouderen met de hoogste haar cortisol waarden een 2.7 keer verhoogd risico hadden op het hebben van hart- en vaatziekten. Ook het risico op het krijgen van diabetes mellitus was verhoogd. Het verhoogde risico dat we vonden voor lange termijn cortisol waarden is vergelijkbaar met het verhoogde risico dat gevonden wordt bij traditionele risicofactoren zoals hoge bloeddruk en abdominale obesitas. Dit suggereert dat hoge cortisol waarden mogelijk ook een belangrijke risicofactor zijn voor het ontwikkelen van hart- en vaatziekten.

In **hoofdstuk 9** hebben we onderzocht of lange termijn cortisol waarden anders zijn in patiënten met een bipolaire stoornis (manische depressiviteit) in vergelijking met gezonde mensen. We hebben gevonden dat haar cortisol waarden significant hoger zijn bij mensen waarbij de bipolaire stoornis pas op latere leeftijd ontstond. Dit suggereert dat hoge cortisol waarden een rol spelen bij een deel van de patiënten met een bipolaire stoornis en dat er mogelijk een verschil is in pathogenese tussen het ontstaan van een bipolaire stoornis op jonge en op latere leeftijd. Verder vonden we dat haar cortisol waarden, maar niet speeksel cortisol waarden, significant lager waren bij patiënten met een paniekstoornis naast de bipolaire stoornis. Bipolaire patiënten met andere vormen van psychiatrische comorbiditeit bleken juist een hogere haar cortisol waarde te hebben. Deze studie suggereert dat er binnen het ziektebeeld van een bipolaire stoornis subtypes zouden bestaan met veranderde systemische blootstelling aan cortisol.

In **hoofdstuk 10** hebben we gekeken of polymorfismen in het glucocorticoïd receptor gen geassocieerd zijn met geboorte lengte en gewicht, bloeddruk, glucose en insuline waarden en lichaamssamenstelling bij kinderen die te klein geboren zijn. Er werd geen verschil gevonden in de frequentie van het voorkomen van de polymorfismen tussen gezonde controles in kinderen die te klein geboren zijn. Tevens werden er geen associaties gevonden tussen de polymorfismen en geboorte lengte en gewicht, respons op groeihormoon behandeling, bloeddruk, glucose en

insuline waarden en lichaamssamenstelling. Het lijkt erop dat polymorfismen in het glucocorticoïd receptor gen geen belangrijke rol spelen in de nadelige effecten op lichaamssamenstelling en metabole parameters bij kinderen die te klein geboren zijn.

In **hoofdstuk 11** beschrijven we de vondst van een nieuw haplotype in HIV-positieve patiënten met een Afrikaans-Amerikaanse achtergrond. HIV-infectie en HAART behandeling zijn geassocieerd met veranderingen in lichaamssamenstelling en metabole parameters die sterke overeenkomsten vertonen met het syndroom van Cushing. In dit hoofdstuk hebben we gekeken of polymorfismen in het glucocorticoïd receptor gen geassocieerd zijn met het ontwikkelen van deze Cushing-achtige symptomen. In deze studie vonden we een nieuw haplotype in Afrikaans-Amerikaanse patiënten, waarbij het *Tth111*I polymorfisme gevonden werd zonder de aanwezigheid van ER22/23EK, *Bcl*I of 9β. Dit nieuwe haplotype was niet aanwezig in Caucasische patiënten. Dit nieuwe haplotype was geassocieerd met hogere HDL-cholesterol waarden en mogelijk een gezonder metabool profiel. Echter, meer onderzoek in gezonde Afrikaans-Amerikaanse mensen is nodig om het echte effect van dit haplotype te bestuderen. De andere glucocorticoïd receptor gen haplotypes bleken niet geassocieerd te zijn met lichaamssamenstelling of metabole veranderingen bij HIV- positieve patiënten.

In **hoofdstuk 12** hebben we de gen-omgevingsinteractie van glucocorticoïd receptor polymorfismen en prenatale blootstelling aan ondervoeding bekeken. We vonden dat er een interactie was tussen de polymorfismen die geassocieerd zijn met een relatieve resistentie (ER22/23EK en 9β) en blootstelling aan ondervoeding in het laatste trimester van de zwangerschap op lichaamssamenstelling. Deze interacties resulteerden in een gezondere lichaamssamenstelling, met een lager BMI en een smallere buik en heup omtrek. Dit suggereert dat de effecten van de glucocorticoïd receptor polymorfismen op lichaamssamenstelling veranderd kunnen worden door de voedingsstatus tijdens de zwangerschap.

In **hoofdstuk 13** hebben we gekeken naar de associatie tussen glucocorticoïd receptor polymorfismen en lichaamssamenstelling, metabole parameters en cardiovasculaire ziekten bij een groep ouderen. Dit is zowel cross-sectioneel als longitudinaal gedaan. We hebben gevonden dat het ER22/23EK polymorfisme resulteerde in longitudinale veranderingen in lichaamssamenstelling. De totale vet massa en voornamelijk het buik vet verminderden over de tijd, terwijl de spiermassa gelijk bleef. Deze veranderingen zouden de dragers van dit haplotype mogelijk kunnen beschermen tegen de nadelige effecten van veroudering op de lichaamssamenstelling, wat kan resulteren in gezonder ouder worden en mogelijk een overlevingsvoordeel, zoals bekend is van deze variant.

In **hoofdstuk 14** hebben we onderzocht of polymorfismen in het glucocorticoïd receptor gen invloed hebben op het risico om een delirium te ontwikkelen. Hyperactiviteit van de hypothalamus-hypofyse-bijnier-as en verminderde werking van de negatieve feedback zouden een rol kunnen spelen bij het ontwikkelen van een delirium op oudere leeftijd. We hebben bij een groep van 881 oudere patiënten, van wie 299 patiënten een delirium ontwikkelden tijdens

ziekenhuisopname, de glucocorticoïd receptor polymorfismen onderzocht. We vonden dat dragers van het *Bcl*I – *Tth111*I haplotype een 92% lager risico hadden op het ontwikkelen van een delirium. Dit was onafhankelijk van leeftijd en de cognitieve en functionele status van de patiënt. Dit suggereert dat de glucocorticoïd receptor mogelijk een rol speelt in het ontstaan van een delirium bij ouderen patiënten.

Hoofdstuk 15 bevat een algemene discussie waarin de resultaten die beschreven zijn in dit proefschrift in een breder perspectief worden geplaatst. De voor- en nadelen van het meten van cortisol in hoofdhaar worden besproken, tezamen met de resultaten van de glucocorticoïd receptor polymorfisme studies en de algemene voor- en nadelen van polymorfisme studies. Tot slot wordt geconcludeerd dat, ondanks dat er nog veel (methodologische) zaken onbekend zijn bij het meten van cortisol in hoofdhaar, onze studies laten zien dat het meten van cortisol in hoofdhaar een goede en simpele manier is om lange termijn cortisol waarden te meten. Deze methode kan zowel klinisch als in de onderzoekswereld gebruikt worden. Met betrekking tot de genetische studies concluderen we dat het erop lijkt dat de effecten van deze glucocorticoïd receptor polymorfismen op lichaamssamenstelling en metabole parameters met name aanwezig zijn bij gezonde individuen en van minder belang zijn bij de onderzochte aandoeningen.

Chapter 17

Curriculum Vitae
List of Publications
PhD Portfolio
Dankwoord

Curriculum Vitae

Laura Manenschijn werd op 23 september 1985 geboren in Hellendoorn. Zij volgde het voortgezet wetenschappelijk onderwijs aan de Christelijke Scholengemeenschap Reggesteyn te Nijverdal, alwaar zij in 2003 haar diploma haalde. In 2003 werd zij decentraal toegelaten voor de studie geneeskunde aan de Erasmus Universiteit in Rotterdam. In 2006 begon ze, naast haar studie geneeskunde, te werken in het laboratorium endocrinologie, afdeling inwendige geneeskunde van het Erasmus MC onder begeleiding van Dr. E.F.C. van Rossum, Dr. J.W. Koper en Prof. Dr. S.W.J. Lamberts. In 2007 startte Laura met haar afstudeeronderzoek waarbij ze 7 maanden onderzoek deed op het endocrinologisch laboratorium van de afdeling Inwendige Geneeskunde, Erasmus MC, Rotterdam. Dit resulteerde in het verslag 'The role of glucocorticoid receptor gene polymorphisms in neurological, psychiatric and infectious disease and intra-uterine growth retardation' en het behalen van haar doctoraal Geneeskunde. In 2009 startte Laura met haar promotie onderzoek op de afdeling Endocrinologie, Inwendige Geneeskunde, Erasmus MC, onder supervisie van dr. E.F.C. van Rossum en prof. Dr. S.W.J. Lamberts en in 2010 haalde ze haar arts-examen. Gedurende haar promotietraject heeft zij meerdere prijzen gewonnen, waaronder 5 travel awards, 2 young investigator awards, de presidential poster award van de Endocrine Society en de prijs voor het beste klinische artikel van de Nederlandse Vereniging voor Endocrinologie. Per 1 januari 2013 is Laura begonnen met de opleiding Interne Geneeskunde (opleiders Prof. Dr. J.L.C.M. van Saase en Dr. A. Rietveld) in het Sint Franciscus Gasthuis in Rotterdam.

List of Publications

- Functional polymorphism of the glucocorticoid receptor gene associates with mania and hypomania in bipolar disorder.
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- Clinical features associated with glucocorticoid receptor polymorphisms: An overview.
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- Measuring cortisol levels in hair: potential clinical applications in Cushing's Syndrome.
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- Long term cortisol in Bipolar Disorder: Associations with age of onset and psychiatric co-morbidity.
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- A novel tool in the diagnosis and follow-up of (cyclic) Cushing's Syndrome: Measurement of long-term cortisol in scalp hair.
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- High long-term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease.
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  J Clin Endocrinol Metab. 2013 May; 98(5): 2078-2083.

- Glucocorticoid sensitivity in health and disease.
  Quax RAM, ***Manenschijn L****, Koper JW, Hazes JMW, Lamberts SWJ, van Rossum EF, Feelders RA.*
  Nature Reviews Endocrinology, in press.

- Novel way to monitor hydrocortisone replacement therapy in mitotane-treated adrenocortical cancer patients
  Manenschijn L*, Quinkler M, van Rossum EFC.*
  Submitted

- Long-term cortisol levels in scalp hair in recent-onset and established rheumatoid arthritis
  Quax RAM, ***Manenschijn L****, Huisman AM, Han K, Hazes JMW, Feelders RA, Koper JW, van Rossum EFC.*
  Submitted

- Glucocorticoid receptor haplotypes and changes in body composition and metabolic parameters in HIV-infected patients from the FRAM study.
  Manenschijn L*, Scherzer R, Koper JW, Danoff A, van Rossum EFC, Grunfeld C.*
  Submitted

- Glucocorticoid receptor gene haplotype is associated with beneficial changes in fat mass in older persons
  Manenschijn L*, van Schoor NM, Peeters GMEE, Lips P, van den Akker ELT, Koper JW, van Rossum EFC.*
  Submitted

- The effects of glucocorticoïd receptor polymorphisms and prenatal exposure to famine interact.
  Manenschijn L*, de Rooij SR, Noppe G, van den Akker ELT, Bruining GJ, van Rossum EFC, Roseboom TJ.*
  Submitted

- Cushing's syndrome in an HIV-infected patient due to the use of fake skin cosmetics.
  Bosma JW, Deckers MML, Touw DJ, ***Manenschijn L****, van der Poest Clement EH, Veenstra J.*
  Submitted.

- Validation and reference ranges of hair cortisol measurements in healthy children
  *Noppe G, van Rossum EFC, Koper JW, **Manenschijn L**, de Rijke YB, van den Akker ELT.*
  Submitted

- Recent major life events increase hair cortisol concentrations in patients with Bipolar Disorder.
  *Staufenbiel DM, Spijker AT, Koenders MA, **Manenschijn L**, Elzinga BM, van Rossum EFC.*
  In preparation

PhD Portfolio

Name PhD student:	Laura Manenschijn
Erasmus MC Department:	Internal Medicine
Research School:	Molecular Medicine
PhD period:	August 2009 – September 2012
Promotor:	S.W.J. Lamberts
Co-promotor:	E.F.C. van Rossum

General courses

2010	Short introductory course on statistics & survival analysis for MD's
2010	CPO minicursus (Methodologie van patiëntgebonden onderzoek en voorbereiding van subsidie aanvragen
2011	Didactic Skills

Specific courses

2010	Basic course in human genetics
2010	SNP course VII
2010	Symposium 'Op het grensvlak van de vasculaire geneeskunde en nefrologie'
2011	Basic and Translational Endocrinology
2011	Erasmus MC course in clinical neuro-endocrinology

Seminars and workshops

2009	Marius Tausk Masterclass in honour of prof. Cidlowski
2010	Photoshop and Illustrator CS4
2011	Adobe Indesign
2011	Endocrine Trainee Day, Boston, USA

Oral Presentations

2009	Marius Tausk Masterclass in honour of prof. Cidlowski. **Hair cortisol as a method to measure long-term cortisol levels: Relation to features of the metabolic syndrome and psychiatric disease.** Oestgeest, The Netherlands.
2011	Science days Department of Internal Medicine. **Evaluation of a method to measure long-term cortisol levels in health and bipolair disorder.** Antwerp, Belgium.
2011	Geriatric Days. **Glucocorticoid Receptor haplotype is associated with a decreased risk of delirium in elderly patients.** Den Bosch, The Netherlands.
2011	Clinical Endocrinology Days. **Hair cortisol – a parameter of long-term cortisol levels? Evaluation in health and disease.** Noordwijkerhout, The Netherlands.

2011 European Congress of Endocrinology. **Evaluation of a method to measure long-term cortisol levels in health and disease.** Rotterdam, The Netherlands.

2011 Annual Meeting of Young Active Research in Endocrinology. **Long-term cortisol levels measured in scalp hair in health and disease.** Stockholm, Sweden.

2012 Dutch Endocrinology Days. **Evaluation of a new tool to measure cortisol in the diagnosis and follow-up of patients with (cyclic) Cushing's Syndrome.** Noordwijkerhout, The Netherlands.

2012 Dutch Endocrinology Days. **Shift work at young age is associated with elevated long-term cortisol levels and body mass index.** Noordwijkerhout, The Netherlands.

2012 Annual Meeting of the American Psychosomatic Society. **Long-term cortisol levels in Bipolair Disorder: Associations with age of onset and psychiatric co-morbidity.** Athens, Greece.

2012 Spring Conference Psychiatry. **Long-term cortisol levels in Bipolair Disorder: Associations with age of onset and psychiatric co-morbidity.** Maastricht, The Netherlands.

2012 University of Maryland, Baltimore, research lecture. **Novel tool to measure long-term cortisol levels: Relationship with obesity and cardiovascular disease.** Baltimore, USA.

2012 65th Anniversary of the Dutch Endocrine Society. **Shift work at young age is associated with elevated long-term cortisol levels and body mass index.** Amsterdam, The Netherlands.

2013 Wetenschapscafé Sint Franciscus Gasthuis. **Cortisol in hoofdhaar: een nieuwe methode om lange termijn cortisol waarden te meten.** Rotterdam, The Netherlands.

Poster presentations

2010 Science days Department of Internal Medicine. **Cortisol in hair as a method to measure long-term cortisol levels.** Antwerp, Belgium.

2011 Spring Congress Psychiatry. **Evaluation of a method to measure long-term cortisol levels in health and bipolar disorder.** Amsterdam, The Netherlands.

2011 European Congress of Endocrinology. **Evaluation of a method to measure long-term cortisol levels in health and disease.** Rotterdam, The Netherlands.

2011 95th Annual Meeting of the Endocrine Society. **Evaluation of a new method to measure long term cortisol levels in health and bipolar disorder.** Boston, USA.

2011	Targeting the Pituitary, Expert knowledge forum. **Evaluation of a new tool to measure cortisol in the diagnosis and follow-up of patients with (cyclic) Cushing's Syndrome.** Berlin, Germany. Awarded with the Young Investigators Award.
2012	Science Days Department of Internal Medicine. **Evaluation of a new tool to measure cortisol in the diagnosis and follow-up of patients with (cyclic) Cushing's Syndrome.** Antwerp, Belgium.
2012	Annual Molmed Day. **Evaluation of a new tool to measure cortisol in the diagnosis and follow-up of patients with (cyclic) Cushing's Syndrome.** Rotterdam, The Netherlands.
2012	Annual Molmed Day. **Shift work at young age is associated with elevated long-term cortisol levels and body mass index.** Rotterdam, The Netherlands.
2012	European Congress of Endocrinology. **Shift work at young age is associated with elevated long-term cortisol levels and body mass index.** Florence, Italy.
2012	European Congress of Endocrinology. **Measurement of cortisol in scalp hair can be a new tool in the diagnosis and follow-up of patients with Addison's Disease and (cyclic) Cushing's Syndrome.** Florence, Italy.
2012	96th Annual Meeting of the Endocrine Society. **Measurement of cortisol in scalp hair can be a new tool in the diagnosis and follow-up of patients with Addison's Disease and (cyclic) Cushing's Syndrome.** Houston, USA. Awarded with the Presidential Poster Award of the Endocrine Society.
2012	96th Annual Meeting of the Endocrine Society. **Shift work at young age is associated with elevated long-term cortisol levels and body mass index.** Houston, USA.
2012	96th Annual Meeting of the Endocrine Society. **Long-term cortisol levels measured in scalp hair are associated with type 2 diabetes and cardiovascular disease in the elderly.** Houston, USA.
2013	Science Day Sint Franciscus Gasthuis. **Long-term cortisol levels, measured in scalp hair, are inadequately normal in rheumatoid arthritis.** Rotterdam, The Netherlands.

Other (inter)national conferences

2009	2nd Benelux Nuclear Receptor Meeting held jointly with the Marius Tausk Guest Professorship Symposium. Oestgeest, The Netherlands.
2011	Lustrum congress Pituitary Center Rotterdam. Rotterdam, The Netherlands

Lecturing

2010	Presentation for 2nd year Medical Students about doing research at the Department of Internal Medicine, Section of Endocrinology

Supervising practicals and excursion, tutoring

2011-2012	Journal Clubs
2011-2012	Endocrinology practical lectures for Medical Students
2011	Tutoring 1st years Medical Students

Supervising Master's Theses

2010	Andrea Jetten, Biological Psychology student

Awards

2011	Goodlife Healthcare Travel Award of the Dutch Endocrine Society. Rotterdam, The Netherlands.
2011	Travel award of the Endocrine Society. Boston, USA.
2011	Young Investigators Poster Award. Targeting the pituitary: Expert Knowledge Forum, Berlin, Germany.
2012	Award for best article in clinical endocrinology 2011 from the Dutch Endocrine Society. Noordwijkerhout, The Netherlands.
2012	Basic Science Meeting Grant of the European Society of Endocrinology. Florence, Italy.
2012	Science Meeting Grant of the European Society of Endocrinology. Florence, Italy.
2012	Goodlife Healthcare Travel Award of the Dutch Endocrine Society. Florence, Italy.
2012	Women in Endocrinology Young Investigators Award. Houston, USA.
2012	Presidential Poster Award from the Endocrine Society. Houston, USA.

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Cortisol exposure and sensitivity in health and disease
Laura Manenschijn