General introduction

Parts of this introduction are based on:

Wester VL, Lamberts SWJ, van Rossum EFC. Advances in the assessment of cortisol exposure and sensitivity. Curr Opin Endocrinol Diabetes Obes, 2014, 21:306–311.

Wester VL, van Rossum EFC. Clinical applications of cortisol measurements in hair. Eur J Endocrinol, 2015, 173:M1-10.

Wester VL, van Rossum EFC. 15, Obesity and metabolic syndrome: a phenotype of mild long-term hypercortisolism? Chapter in: The Hypothalamic Pituitary Adrenal Axis in Health and Disease (Editor: Geer EB). Springer, 2017. ISBN 978-3319459486.

1. OBESITY

Obesity is one of the biggest challenges in individual healthcare and public health policy of the 21st century. Obesity is associated with an increased risk of cardiovascular disease (CVD), diabetes mellitus, depression, osteoarthritis and certain cancers [1, 2]. An individual is considered obese when his or her body mass index (BMI) exceeds 30 kilograms per square meter, and by this definition more than 640 million people worldwide are obese [3]. This definition does not take into account body composition (i.e. the ratio between lean and fat mass), the distribution of fat tissue across the body (e.g., centripetal versus peripheral fat), nor the clinical consequences of increased weight and adiposity. Consequently, there have been attempts to create a definition of *clinically relevant obesity*.

One commonly used definition of clinically relevant obesity is the metabolic syndrome (MetS), which is focused on the cardiometabolic sequelae of central adiposity. MetS is a complex of five obesity-related risk factors that are associated with CVD: increased waist circumference, elevated blood pressure, elevated triglycerides, decreased high density lipoprotein (HDL) cholesterol and elevated fasting glucose. Although definitions and cut-off values vary slightly, an individual is considered to have MetS if he or she meets three out of five criteria. Approximately one in four adults in Europe fulfils criteria for MetS [4]. A large scale meta-analysis of prospective studies showed that MetS is associated with a 2.35-fold increased risk of CVD, and a 1.58-fold increased risk of all-cause mortality [5].

Combating obesity is challenging, for obese individuals as well as for the health care professionals taking care of them. Recently, a large cohort study in the United Kingdom showed that after exclusion of bariatric surgery, the probability that obese individuals attain normal weight is extremely low. Morbidly obese persons (BMI > 40) were even less likely to have clinically meaningful and sustained weight loss than obese persons with a BMI below 40 [6]. In most countries, access to behavioral interventions for obesity is limited. Bariatric surgery is by far the most effective intervention in obesity in terms of weight loss and glycemic control, but is associated with long-term sequelae such as dumping syndrome and nutritional deficiencies, and although the risk is low, a chance of potentially life-threatening postoperative complications [7-9].

The etiology of obesity is manifold and complicated. It is generally assumed that a strong genetic component underlies obesity, as exemplified by twin concordance studies which show an estimated heritability of approximately 40-70% [10]. However, this cannot explain the strong increase in obesity prevalence in the developed and undeveloped

world over the past decades. Presumably, a so-called *obesogenic* environment promotes obesity in genetically prone individuals. Well-recognized environmental influences on obesity include calorie-rich food consumption, physical activity, societal influences, short sleep duration and psychological factors [11]. Interestingly, several of these factors are known to increase cortisol. In particular consumption of carbohydrate-rich food, sleep deprivation and stress have been found to increase cortisol levels [12-14].

2. GLUCOCORTICOIDS

In the cortex of the adrenal glands, corticosteroids are produced. Corticosteroids are steroid hormones which are further subdivided into two classes: *glucocorticoids* and *mineralocorticoids*. In the blood, most of the corticosteroids are attached to binding proteins, mainly to corticosteroid binding globulin (CBG). According to the free hormone hypothesis, only the free, unbound fraction of steroid hormones can diffuse into the tissue and enter the cell, where they can activate their receptors [15]. Corticosteroids have effects through the activation of nuclear receptors: the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). Mineralocorticoids, the most important of which is aldosterone, activate the MR. Cortisol, the most important glucocorticoid hormone in humans, can activate both the GR and MR. After binding of cortisol, the nuclear receptors translocate to the nucleus where they influence gene expression [16].

Aldosterone is an effector in the Renin-Angiotensin-Aldosterone system (RAAS), and through activation of the MR in the kidney, regulates blood pressure and plasma sodium and potassium. Cortisol has effects throughout the body, and affects behavior, circulation, metabolism and immunity. High cortisol levels, such as occur in an acute stress response, rapidly lead to increases in gluconeogenesis, increased blood pressure, and suppression of inflammation. The increase in cortisol levels in response to stress is considered essential for survival [17]. In adrenocortical insufficiency (e.g. Addison's disease), cortisol levels do not adequately increase in response to acute illness and other stressors, and this may culminate in a life-threatening circulatory collapse (i.e. Addisonian crisis) [18]. On the other hand, long-standing increases in cortisol or other (exogenous) glucocorticoids are associated with a wide array of deleterious effects such as weight gain, insulin resistance, increased cardiovascular disease incidence, and osteoporosis (i.e. Cushing's syndrome) [19].

The production of cortisol is orchestrated by the hypothalamus-pituitary-adrenal (HPA) axis (see Figure 1). Under the influence of the diurnal rhythm and acute stressors, the hypothalamus produces corticotropin releasing hormone (CRH). CRH stimulates the

anterior pituitary to produce adrenocorticotropic hormone (ACTH), also known as corticotropin. ACTH stimulates the adrenal cortex to produce cortisol. Under normal circumstances, cortisol can decrease the production of both CRH and ACTH, thereby regulating its own production through a negative feedback loop [20]. Endogenous Cushing's syndrome can be caused by aberrations at the different levels of the HPA-axis. The majority of cases of Cushing's syndrome is caused by a pituitary adenoma which produces excessive ACTH, leading to increased glucocorticoid production by the adrenals: Cushing's disease. In other cases, Cushing's syndrome may be caused by adrenal diseases or by non-pituitary tumors which produce ACTH or CRH (ectopic Cushing's syndrome) [21].

The exposure to cortisol is further regulated at the tissue level. The enzyme 11β hydroxysteroid dehydrogenase (11β-HSD) type 2 converts cortisol into the inactive cortisone, while 11β-HSD type 1 activates cortisone into cortisol. A high local expression of 11β-HSD type 1 may therefore augment the local effects of cortisol, while a high expression of 11β-HSD type 2 may attenuate them. This is clinically relevant in the kidney, where a high expression of 11β-HSD type 2 exists, protecting the kidney from cortisol exerting its effects through the MR [17]. The sensitivity to glucocorticoids is variable, which further modulates the effects of cortisol on target tissues [16]. Glucocorticoid sensitivity is reviewed in section 7 of this introduction.

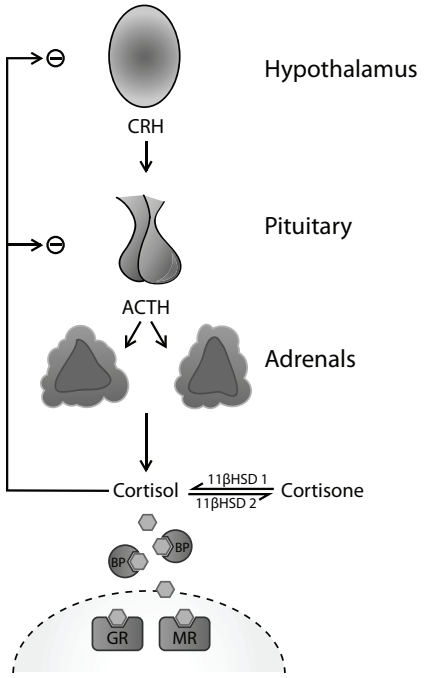


Figure 1. overview of the HPA axis.
The dashed line marks the intracellular environment. Abbreviations: 11βHSD, 11β-hydroxysteroid dehydrogenase; ACTH, andrenocorticotrophic hormone; BP, binding protein; CRH, corticotrophin releasing factor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor.

3. MEASURING CORTISOL IN CLINICAL PRACTICE AND RESEARCH

To assess cortisol status in humans, measurements can be performed in different matrices, such as blood, saliva, and urine (see Figure 4). Each matrix has its advantages and disadvantages. Blood and saliva cortisol represent time point measurements, which makes these matrices prone to the influence of short-term variations caused by the diurnal rhythm and acute stress. In blood, the total fraction of cortisol is usually measured, which is comprised mostly of CBG-bound cortisol [15]. This may create fallacious results in situations where CBG is increased, such as with the use of hormonal contraceptive medication where total cortisol is increased even though free cortisol levels are normal [19]. Salivary cortisol correlates well with free circulating cortisol, although approximately 30% of the cortisol is converted into cortisone before entering the saliva [22]. Salivary cortisol can be collected in virtually any setting, including at home, and during psychosocial stress challenges (e.g., Trier Social Stress Test). A potential pitfall in measuring salivary cortisol is gingival micro-trauma such as occurs with tooth brushing, which may cause small quantities of blood to mix through the saliva sample, leading to false increases. In urine, free cortisol is usually measured in 24 hour collections of urine. This provides an integrated measure of cortisol production in one day. However, urine collection may be experienced as cumbersome by individuals, and urinary free cortisol (UFC) may be confounded by renal insufficiency or polyuria [19].

The Endocrine Society currently recommends three first-line screening tests in patients suspected of endogenous Cushing's syndrome: late-night salivary cortisol (LNSC), the 1 mg dexamethasone suppression test (DST), in which serum cortisol is measured in the morning after an overnight 1 mg dose of dexamethasone, and 24 hour UFC [19]. Since none of these tests offers perfect diagnostic accuracy, at least two or three measurements are usually required to rule out or establish the diagnosis of Cushing's syndrome. This may be in part due to the pitfalls listed above, but also the fact that cortisol production is often variable in Cushing's syndrome: For instance, when tested repeatedly, the majority of patients with Cushing's syndrome have at least one normal UFC measurement [23, 24].

4. RATIONALE FOR A ROLE OF GLUCOCORTICOIDS IN OBESITY

One of the psychological factors that has most often been associated with obesity and an adverse cardiometabolic risk profile, is increased psychosocial stress. Studies investigating these relationships are widely divergent in terms of the populations investigated, and the way stress is measured. Unsurprisingly, reported results are not always consis-

tent. However, in a recent meta-analysis of longitudinal studies, increased psychosocial stress was associated with a small overall increase in adiposity [25]. Furthermore, in a meta-analysis which aggregated evidence from over a hundred thousand individuals who were on average followed for over a decade, high perceived stress significantly increased the incidence of coronary heart disease with a risk ratio of 1.27 [26]. One of the mechanisms that is suggested to explain these associations, is increased activity of the hypothalamus-pituitary-adrenal (HPA) axis associated with chronic stress, resulting in increased levels of cortisol.

Since many of the effects of the stress response are caused by increased cortisol levels, hypercortisolism (i.e. Cushing's syndrome) can be considered a biological model of extreme stress [27]. The majority of cases of endogenous Cushing's syndrome are caused by pituitary adenomas which produce excessive amounts of ACTH (Cushing's *disease*), resulting in increased release of cortisol from the adrenal glands [21].

All of the features of metabolic syndrome, including hypertension, abdominal obesity, dyslipidemia and insulin resistance, frequently occur in Cushing's (see Figure 2), either due to endogenous hypercortisolism or glucocorticoid therapy. As an expected result of the cardiometabolic derangements, cardiovascular causes of death are common in Cushing's syndrome [21]. It is therefore theoretically likely that part of the association between stress and cardiometabolic risk may be effected through activation of the HPA axis, and increased levels of cortisol. Obesity is a recognized cause of pseudo-Cushing's syndrome, however, until now it has been thought that most obese individuals do not have overt hypercortisolism [19].

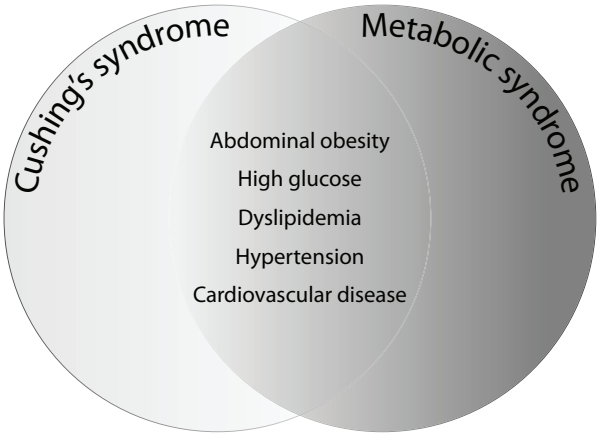


Figure 2. overlap between Cushing's syndrome and metabolic syndrome.

5. EVIDENCE FOR A LINK BETWEEN SYSTEMIC CORTISOL LEVELS AND OBESITY

There have been numerous attempts to unravel the association between obesity and exposure to systemic cortisol levels, using measurements in urine, saliva and blood. To interpret the results of these studies, it is important to take note of several situational and physiological factors that influence cortisol measurements. Cortisol follows a diurnal rhythm, characterized by a peak in the early morning (the cortisol awakening response, CAR), and generally declining levels during the day (see Figure 3). Cortisol rises in response to physical or psychological factors, which causes cortisol levels to be variable within and across days [20]. Saliva and blood measurements can be used to obtain information about time-point cortisol levels, while urinary free cortisol (UFC) is used to estimate the total cortisol output over a 24 hour period [19, 20].

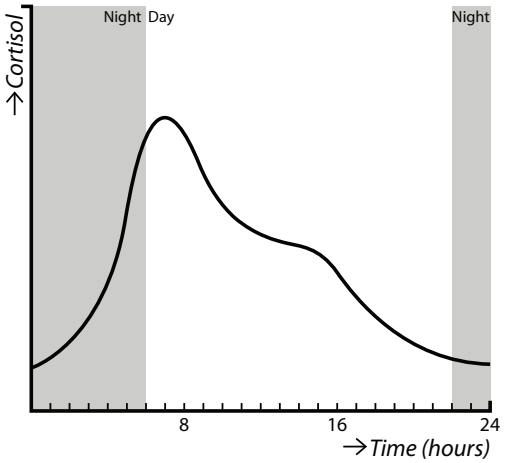


Figure 3. schematic representation of the cortisol diurnal rhythm.

A recent systemic review highlighted that studies investigating the associations between obesity and cortisol in body fluids provide inconsistent results [28]. Most published studies indicate that obesity is characterized by changes in the diurnal rhythm, with a blunted CAR, and a less sharp decline in cortisol levels over the course of the day. 24h-UFC tends to be higher in obese individuals, and the cortisol reactivity to acute stressors appears to be exaggerated. In most cases, negative studies or even opposing results have been reported as well [28]. These apparently inconsistent results may not be surprising, when we take into account the high variability of cortisol levels (Figure 3, Figure 4)

6. TOWARDS MEASUREMENT OF LONG-TERM GLUCOCORTICOID EXPOSURE: HAIR ANALYSIS

As explained above, measurements of cortisol in blood, saliva, and urine only represent time-point or short term cortisol exposure, which limits the representation of long-term circulating cortisol levels. Furthermore, these tests either rely heavily on patient adherence to collection instructions, or are perceived as invasive [19].

Scalp hair analysis has been used for decades to measure exposure to environmental toxins and drugs. Hair grows at a relatively constant rate of 1 cm per month. Several substances are retained in the hair strands, therefore hair can serve as a matrix to measure long-term exposure to a wide variety of toxins and hormones. Furthermore, by dividing hair samples in different segments and analyzing them separately, retrospective timelines of exposure can be created [29]. In past years, several laboratories have expanded hair analysis to measure long-term levels of cortisol and other steroid hormones [30-35]. This has allowed researchers to investigate cortisol exposure over much longer periods of time (months to years) than previously possible with samples of blood, saliva or urine (see Figure 4). Consequently, a large number of cross-sectional studies have been conducted and examined the associations between hair cortisol concentrations and a wide range of somatic and mental health measures [36, 37].

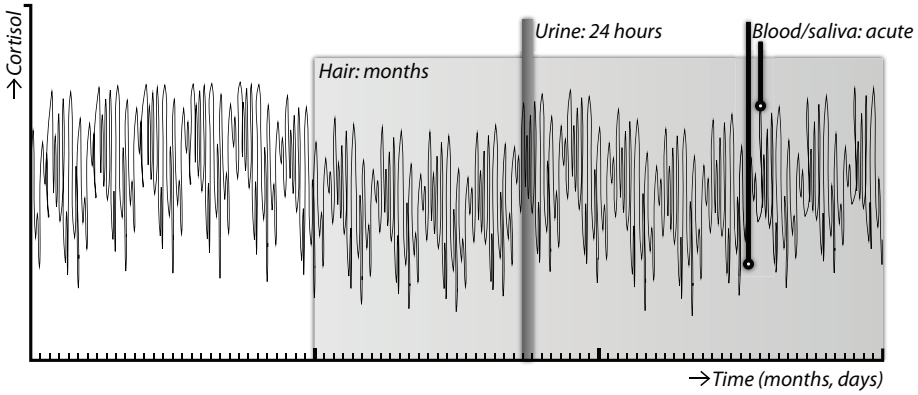


Figure 4. conceptual overview of the different matrices in which cortisol can be assessed. serum and saliva (time-point), urine (intermediate term output) and scalp hair (long-term cumulative levels). The line depicting circulating cortisol levels over a period of three months is fictional.

Scalp hair collection is straightforward and easily performed in any setting (see Figure 5). In accordance with guidelines published by the Society of Hair Testing, the hair sample is collected from the posterior vertex [29]. Scissors are used to cut a lock of hair as thick as a pencil, as close to the scalp as possible. Depending on the research question, hair

segments of one, up to multiple centimeters of length are used for analysis. Samples are weighed, and in most published methods washed. Steroids are extracted overnight in methanol and are then processed further depending on the type of analysis used. The extracted steroids can be measured using an immunoassay or liquid chromatography tandem-mass spectrometry (LC-MS/MS) [30-35].

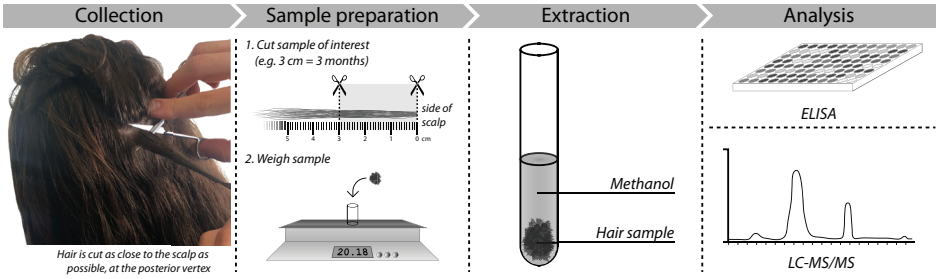


Figure 5. overview of hair sample collection, work-up and analysis.
Abbreviations: ELISA, enzyme-linked immunosorbent assay; LC-MS/MS, liquid chromatography - tandem mass spectrometry.

Recently, a direct comparison between hair cortisol analysis methods was published. Four different laboratories divided hair samples of the same persons and measured them using their different methods, comparing four immunoassay methods and two LC-MS/MS methods. Correlations between the different methods were high, with r^2 values ranging between 0.88 and 0.98 [38].

Apart from clinical states and stressors, several other factors can potentially affect hair cortisol. Hair cortisol has been shown to increase with age [39-42], and has been reported to be higher in men than in women [39, 41, 43, 44]. Hair treatments such as hair dyeing, permanent curling or straightening have been reported to decrease hair cortisol [34, 40, 44, 45], although other studies have not found this [31, 41, 42, 46-49]. Furthermore, the amount of cortisone decreased with higher hair washing frequency in large studies [40, 41].

Another hair-specific limitation is the phenomenon of wash-out, which means that hair cortisol is lower more distally in the hair. The mechanisms behind the wash-out phenomenon have not been fully clarified, but may include wear and tear, subsequent hair washings and exposure to ultraviolet light. Wash-out has been reported by multiple labs [31, 35, 50], and is an important consideration especially in studies involving retrospective timelines using segmental hair analysis.

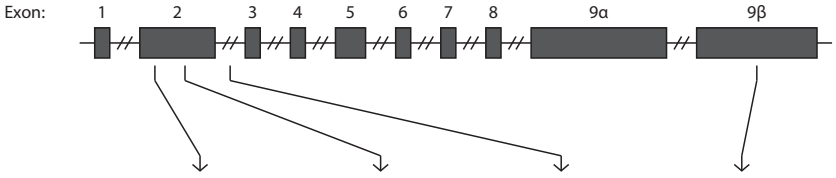
Medication use is known to influence different types of cortisol measurements [19], but until now this does not seem to be a major limiting factor in hair cortisol measurements. One study described that corticosteroid use, which was not further specified, increased hair cortisol [45]. It is important to consider however, that topical steroids may contaminate hair samples, and falsely increase hair cortisol levels through cross-reactivity in an immunoassay, as was used in the mentioned study. In general, topical or inhalation corticosteroids may also exert some systemic effects (see section 8), and thereby decrease hair glucocorticoids. Therefore, the influence of corticosteroid-containing medication on hair glucocorticoids needs further study, preferably by using LC-MS/MS measurements which are not limited by cross-reactivity.

7. COMMON VARIANTS IN THE GLUCOCORTICOID RECEPTOR AND THEIR INFLUENCE ON THE CARDIOMETABOLIC PHENOTYPE

As explained section 2, cortisol exerts its effects by binding to the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). Most of the metabolic effects of cortisol, including the effects on body composition leading to truncal obesity, are thought to arise from gene transactivation by the GR after ligand binding [51]. The sensitivity of the GR to cortisol and other glucocorticoids may vary between and within individuals, dependent on genetic variations in the GR and associated proteins, as well as dynamic changes in GR sensitivity [16].

Several polymorphisms in the GR gene may influence sensitivity to glucocorticoids. Research has mainly focused on four polymorphisms that have been associated with a distinct difference in glucocorticoid sensitivity, as assessed using dexamethasone suppression tests (DSTs), and/or changes in glucocorticoid receptor transactivation or transrepression activity [52-56]. Over the past years, these polymorphisms have been associated with subtle differences in clinical features, most of which were cardiometabolic in nature (see Figure 6). Approximately half of the general population carries a GR polymorphism associated with an increased sensitivity to glucocorticoids: *Bcl*I or N363S. The intronic *Bcl*I polymorphism (rs41423247) is associated with an increase in glucocorticoid sensitivity assessed using DST [54]. In line with this, *Bcl*I has been associated with insulin resistance, increased BMI and central adiposity [57]. The difference in insulin resistance disappeared after adjustment for BMI, indicating that the increased insulin resistance may be related to an increase in adiposity [58]. The less frequent N363S variation (rs56149945, formerly rs6195) has been associated with an increased glucocorticoid sensitivity in DST [52], as well as an increase in transactivational capacity [53]. Glucocorticoid receptor transactivation involves the activation of glucocorticoid

response elements (GREs), which is thought to be responsible for most of the metabolic effects of glucocorticoids [51]. In clinical studies, N363S has been associated with tissue effects of increased glucocorticoid receptor transactivation, including an increase in low-density lipoprotein (LDL) cholesterol in the very elderly [59], and a higher BMI [60].



Polymorphism	ER22/23EK	N363S	*Bcl*I	GR-9β
GC sensitivity (DST)	Decreased	Increased	Increased	-
In vitro activity	↓ Transactivation	↑ Transactivation	-	↓ Transrepression
Clinical associations	Increase in lean mass Lower LDL cholesterol Increase in insulin sensitivity Longevity Less cerebrovascular disease	Higher BMI Increased LDL cholesterol in the elderly	Higher BMI Central adiposity Lower HDL cholesterol Relative insulin resistance	Increase in proinflammatory markers Increased atherosclerosis and coronary heart disease risk

Figure 6. A schematic overview of functional glucocorticoid receptor polymorphisms and their key clinical associations.
Abbreviations: BMI, body mass index; DST, dexamethasone suppression test; GC, glucocorticoid; HDL, high-density lipoprotein; LDL, low-density lipoprotein

In contrast, the ER22/23EK (rs6189 and rs6190) polymorphism has been associated with a decreased glucocorticoid receptor transactivation [53] and an attenuated DST response [56]. Individuals carrying this polymorphism seem to be somewhat protected from adverse cardiometabolic glucocorticoid tissue effects. ER22/23EK carriage was associated with decreased total and LDL cholesterol levels, as well as a relative increase in insulin sensitivity [56], increased lean body mass and muscle strength in men [61], longevity [62], and decreased radiological signs of cerebrovascular disease [63].

Transrepression is the mechanism by which the glucocorticoid receptor may inhibit the activity of other transcription factors. The anti-inflammatory properties of glucocorticoids are attributed to glucocorticoid receptor transrepression [51]. The GR-9β polymorphism (rs6198) has been shown to reduce the transrepression potential of the glucocorticoid receptor in vitro, as indicated by a decreased dexamethasone-induced suppression of interleukin-2 [55]. In line with this, GR-9β carriage has been associated with increased C-reactive protein levels. In a large study comprising 7983 individuals, homozygosity for the GR-9β allele was associated with higher circulating C-reactive protein levels and an increase in carotid atherosclerosis and incident coronary heart disease

[64]. Since inflammatory processes are thought to be involved in atherosclerosis [65], these results are compatible with a decrease in glucocorticoid-mediated suppression of inflammation.

8. GLUCOCORTICOID CONTAINING MEDICATIONS

Systemic glucocorticoids such as prednisolone and dexamethasone have been used for decades for their potent anti-inflammatory effect, in inflammatory disorders and malignancies. The use of systemic glucocorticoids (often referred to as *corticosteroids*, although this term technically also includes steroids with mainly mineralocorticoid effects), frequently results in an iatrogenic Cushing's syndrome [19]. For many disorders, local glucocorticoids are now used, such as inhaled glucocorticoids for asthma, nasal glucocorticoids for allergic rhinitis, and topical glucocorticoids for skin disorders such as eczema and psoriasis. Millions of individuals in the Netherlands are prescribed local glucocorticoids on a yearly basis (GIP databank, Health Council of the Netherlands, https://www.gipdatabank.nl/). It is generally assumed that local glucocorticoids have negligible systemic effects, and only rarely result in features of Cushing's syndrome [66], such as in cases of excessive use or in prone individuals.

However, research suggests that normal use of local glucocorticoids has systemic effects. In children, the use of inhaled glucocorticoids in asthma is known to induce a slightly stunted linear growth [67]. A recent meta-analysis concluded that even in the lowest doses of every administration form, a suppression of the adrenocortical gland is sometimes observed [68]. This clearly indicates systemic effects. Given the widespread use of local glucocorticoids, and the fact that these agents are often used for extended periods of time for chronic conditions, they should be considered as a contributor to glucocorticoid exposure in the general population.

9. OUTLINE OF THE THESIS

The overarching aim of this thesis is to study the association between glucocorticoids and obesity. Three strategies were used to study this relationship. Long-term exposure to endogenously produced glucocorticoids was studied using measurement of glucocorticoids in scalp hair. Second, we studied the use of glucocorticoid containing medications. Third, we used genetic analysis to study variations in the glucocorticoid receptor which are known to change the sensitivity to glucocorticoids.

In **chapter 2**, we studied whether hair cortisol differs between obese patients and non-obese controls. In **chapter 3**, we studied hair cortisol levels in patients with structural heart disease who took part in a randomized clinical trial investigating mindfulness treatment. First, we studied which factors (clinical, demographic, subjective health status), influenced hair cortisol. Second, we studied whether hair cortisol levels changed in response to the mindfulness training intervention. In **chapter 4**, we studied hair cortisol and testosterone levels in patients with sarcoidosis, in association with psychological distress and fatigue. In **chapter 5**, we studied the diagnostic accuracy of hair cortisol for the diagnosis of endogenous Cushing's syndrome. In **chapter 6**, we investigated how exposure to natural sunlight, a potential confounder of hair measurements, influences hair glucocorticoids. In **chapter 7**, the association between functional variation in the glucocorticoid receptor and metabolic syndrome presence was studied in a large population based cohort. **Chapter 8** describes the result of a descriptive study, in which we extensively phenotyped and genotyped a cohort of obese outpatients with a focus on factors potentially contributing to weight gain. Factors studied include the use of weight gain inducing drugs, hormonal abnormalities, and the results of genetic testing. In **chapter 9**, we studied the use of glucocorticoid containing medications in a cohort of obese patients, and compared this to two non-obese cohorts. In **chapter 10**, we studied which factors influence hair glucocorticoids in a general population cohort, with a specific focus on the use of glucocorticoid containing medications, and the occurrence of recent stressful life events. In **chapter 11**, we studied the associations between the use of glucocorticoid containing medications and metabolic syndrome components in a large population based cohort. **Chapter 12** places the results of the studies described in this thesis in a broad context, discusses the implications of the findings, and provides recommendations for future research. **Chapter 13** provides a summary of this thesis.

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