Long-Term Cortisol Exposure in Stress-Related Diseases

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Long-Term Cortisol Exposure in Stress-Related Diseases

Blootstelling aan lange-termijn cortisol
in stress-gerelateerde ziektes

Thesis

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GENERAL INTRODUCTION

Parts of this introduction are based on:
Hair cortisol, stress exposure, and mental health in humans: A systematic review.
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1   THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS

The hypothalamic-pituitary-adrenal axis (HPA axis) is a complex and dynamic system of direct influences and feedback interactions between three endocrine glands: the hypothalamus, the pituitary gland, and the adrenal glands. In response to stimuli from the central nervous system, neurons in the paraventricular nucleus of the hypothalamus secrete corticotrophin releasing hormone (CRH) and vasopressin [1]. CRH and vasopressin stimulate the biosynthesis and secretion of adrenocorticotropic hormone (ACTH), which in turn acts on the adrenal cortex and stimulates production and release of glucocorticoid (GC) hormones. In humans, the main GC is cortisol [1]. Figure 1 gives a schematic overview of this mechanism.
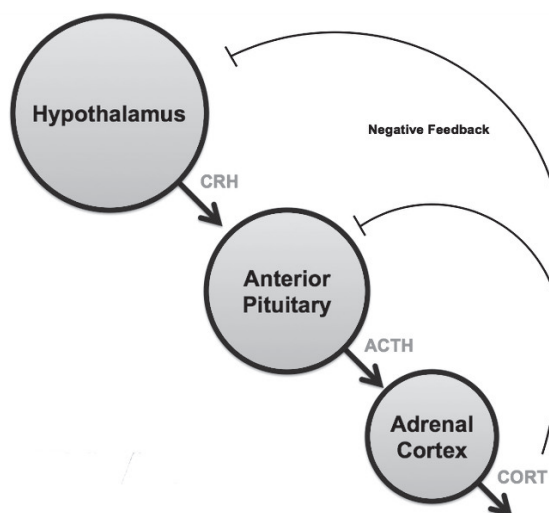

Figure 1. *Schematic overview of the regulation of the HPA axis activity including the negative feedback loop. Picture adapted from Sweis, 2012 [2]. Abbreviations: CRH, corticotrophin releasing hormone; ACTH, adrenocorticotropic hormone; CORT, cortisol.*

1.1   Regulation of the HPA Axis

In daily life, the regulation of the HPA axis is crucial for proper body and brain functioning as cortisol regulates numerous basal processes such as fat and glucose metabolism, blood pressure, inflammatory and immune responses, and thereby aids the organism to flexibly adjust to environmental challenges [3]. The three main regulatory mechanisms will be discussed in the subsequent sections.

1.1.1   Chronobiological Rhythms

Circulating cortisol levels of healthy persons are known to be influenced by different chronobiological rhythms, i.e. the ultradian rhythm, the circadian rhythm, and seasonal rhythms. The ultradian rhythm is caused by oscillatory neurons in the hypothalamus, releasing CRH (and thereby stimulating ACTH and cortisol release) approximately

every 1-2 hours in a pulsatile fashion [4]. The pulsatile secretion is thought to prevent down-regulation of the HPA axis while maintaining its ability to respond to stressors [5]. While the frequency of these pulses is constant, the amplitude can vary and thereby allows the production of the subsequent circadian rhythm [6]. In the circadian rhythm, the cortisol levels rise steadily after 4.00 am, and peak approximately 30 to 40 minutes after awakening. This phenomenon of a morning rise is called the cortisol awakening response (CAR). The purpose of the CAR is uncertain, however, it has been suggested to be linked to the hippocampus' preparation of the HPA axis in regard to the upcoming day [7]. The cortisol levels then decrease throughout the day, with a small second peak at early afternoon. The nadir is reached around 3.00 am. The circadian rhythm serves the purpose of helping the body adjust to its activities of the day and night cycles. Figure 2 depicts the circadian rhythm of hourly measured plasma cortisol in healthy individuals. The seasonal rhythm presents itself by higher morning and evening salivary cortisol levels during winter months [8], however, reasons behind the effect of season are not well understood until now [8, 9].
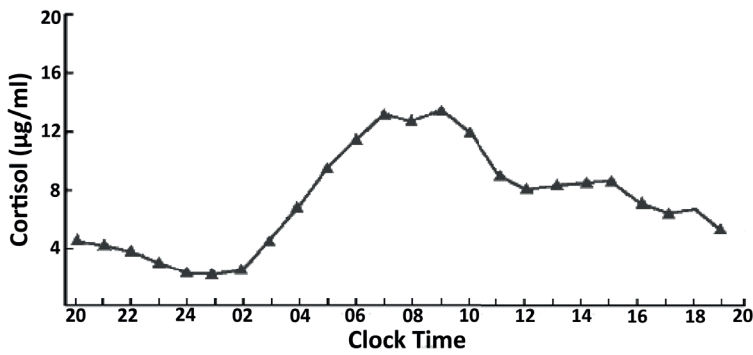
Figure 2. *Circadian rhythm of cortisol secretion in healthy individuals. Picture adapted from Kandel, 2012 [10].*

1.1.2   Negative Feedback

Apart from stimulatory regulation as described above, the HPA axis also knows inhibitory regulation, i.e. negative feedback. The negative feedback is initiated by the circulating cortisol levels and occurs at both the pituitary and the hypothalamic levels, demonstrating an inhibitory action on CRH and ACTH production and thereby decreasing cortisol production and secretion. The so-called negative feedback loop is also shown in Figure 1. This negative feedback can be fast, delayed, or slow feedback [11]. The fast (or rate-sensitive) feedback occurs within seconds to minutes and depends on the rate of GC change in plasma, e.g. following stress-induced rises or administration of exogenous steroids, and results in rapid inhibition of ACTH secretion. The delayed (or intermediate) feedback acts within 30 minutes to hours and can affect responses in pituitary and hy-

pothalamic cells. The suppression of secretion of CRH and ACTH is directly proportional to the concentration of GCs previously reached in the plasma. The slow feedback results from constant GC exposure for days to weeks and affects both basal as well as stimulated pituitary and hypothalamic activity [11]. Which kind of feedback is activated seems to depend on the rise of cortisol and the timing and duration of the stressor.

1.1.3 Stress

The third regulatory mechanism is stress. Upon appearance of a physical or psychosocial stressor (e.g. infection, pain, or fear), the HPA axis increases its activity to maintain the adaptive ability required for dealing with the stressors.

The concept of stress has evolved since the late 19th century. The term "stress" is often used for both a stressor and the reaction of the body to the stressor. In 1936, Selye coined the concept of stress and later defined stress as "a non-specific response of the body to any demand placed in it" [12]. He further differentiated the concept of stress into *eustress* and *distress,* which are positively and negatively connoted interpretations of a stressor, respectively; eustress is mostly short-termed and enhances performance, whereas distress can go on for longer periods and most often has an adverse influence on body and mind. Therefore, it is usually "distress" what is commonly meant when talking about "stress". Indeed, a stressor is regarded harmful when there is a discrepancy between the demands of a stressor, and the perceived ability to cope with it. Lazarus developed a 2-stage appraisal view of stress and suggested that an individual's stress level is directly affected by their cognitive appraisal of a situation [13]. During the primary appraisal, the situation is assessed to be a threat or not, and during the secondary appraisal, the individual evaluates whether they have the ability to effectively cope with the situation. In this way, secondary appraisal interacts with the primary appraisal to determine the emotional reaction to the situation. Indeed, the cognitive appraisal has been found to account for 20—30% of the stress response [14].

The stress response consists of three phases: stress reaction, recovery, and adaptation [15]. In reaction to basically all stressors, two classes of hormones are released; catecholamines and glucocorticoids, and the speed and magnitudes of both parts depend on the specific stressor [16]. Catecholamines such as noradrenaline or adrenaline exert their effects via the nervous system within seconds, thereby enabling immediate physical reactions associated with the flight-or-fight response (also known as acute stress reaction). Glucocorticoids such as cortisol act via the hormonal route and support the activity of catecholamines over the course of minutes or hours. The increased secretion of catecholamines and cortisol can result in temporarily increased availability of energy by increased muscle strength, increased memory function, increased immunity, and decreased sensitivity to pain [3]. This increased release is under normal circumstances terminated by cortisol itself via the negative feedback-loop. During the recovery phase,

containment of the stress reaction takes place, as well as encoding of information. During the adaptive phase, the experience is consolidated in memory to ensure adaptive behavior in the future. The adaptive stress response can turn maladaptive when chronically stimulated and can become more damaging than the stressor itself (especially if the stressor is purely psychological) [17]. The initiation as well as the termination of the stress response is susceptible to dysregulation; as these processes can be delayed, excessive, flattened, or prolonged [15, 18].

1.2. Dysregulation of the HPA Axis

As described above, there are several regulatory mechanisms regarding the HPA axis; however, dysregulations can also occur. The HPA axis dysregulation can result in hypocortisolism and hypercortisolism. Dysregulation can vary from extreme dysregulation to subtle dysregulation, and it can be caused both endogenously and exogenously.

Two well-known, extreme examples of hypo- and hypercortisolism are Addison's Disease (AD) and Cushing's Disease (CD), in which there is an under- and an overproduction of cortisol, respectively. In AD, also known as primary adrenal insufficiency, the destruction or dysfunction of the adrenal cortex leads to adrenal insufficiency and hence, hypocortisolism. Adrenal insufficiency can also be secondary (caused by impairment of the pituitary) or tertiary (due to impairment of the hypothalamus). Symptoms of adrenal insufficiency are unspecific, involving severe abdominal pains, vomiting, profound muscle weakness and fatigue, weight loss, hypotension, kidney failure, but also depression, and changes in mood and personality [19, 20]. In CD, a pituitary adenoma increases the secretion of ACTH, stimulating the synthesis of cortisol by the adrenal glands. The extremely high levels of cortisol lead to Cushing`s Syndrome (CS), which is a collection of signs and symptoms due to prolonged exposure to cortisol. Hypercortisolism can be primary, secondary, or tertiary, depending on whether it originates from the adrenal glands, the pituitary, or the hypothalamus. These signs and symptoms include weight gain, striae, fat accumulation in the face (moon face) and/or the neck (buffalo hump), hypertension, hirsutism, acne, menstrual irregularities, fatigue, osteoporosis, impaired immunological function, and also memory and attention dysfunction and symptoms of anxiety and (psychotic) depression [21-23].

The above described conditions are diseases with extreme HPA axis dysregulations that can present with psychopathological comorbidities. A subtle chronic dysregulation of the HPA axis is thought to play a role in several psychopathological disorders; however, it is not known whether the dysregulation is cause or consequence of the psychopathological disorders. Both hypo- and hyperactivity of the HPA axis have been found in different psychiatric populations, for example in patients with depressive and anxiety disorders [24-26], or with personality disorders [27]. Abnormal CAR and/or disturbed negative feedback mechanisms as shown by the dexamethasone suppres-

sion test (DST) have been linked to diagnoses of depressive and anxiety disorders [25, 26, 28]. These interactional findings are furthermore emphasized by the intriguing fact that, in general, a normalization of the HPA axis activity is considered a prerequisite for convalescence [29].

As mentioned above, apart from endogenous dysregulations, the HPA axis is also susceptible to exogenous influences. The most common cause of CS is iatrogenic CS, caused by treatment with GC medications. These medications are prescribed e.g. for their anti-inflammatory effects, for their immunosuppressive effects, as well as physiological replacement in case of insufficient cortisol production. They can be applied in different forms, including pills, injections, nasal sprays, inhalation medication, topical ointments, and eye drops.

2.   MEASUREMENT OF CORTISOL

As mentioned before, cortisol is produced in the adrenal cortex. From there, it is released into the systemic circulation. In blood, a high amount of cortisol (80-90 %) is bound to cortisol-binding globuline (CBG) and to albumin (6-15 %). The remaining, unbound cortisol (4-5 %) is biologically active. Several methods have been developed to measure cortisol levels.

One important distinction in this area is the measurement of short-term cortisol levels from the measurement of long-term cortisol levels, as both methods capture different aspects of cortisol. Research using short-term circulating cortisol levels provides valuable information about cortisol dynamics and stress reactivity, while studies on hair cortisol assess a completely different phenomenon of the HPA axis, namely that of long-term (i.e. months to years) total cortisol exposure.

2.1.   Short-Term Measurements of Cortisol

The short-term cortisol measurements consist of the measurement of cortisol in blood serum, saliva, or urine. These analyses offer the possibility to explore the dynamics and the concentrations of acutely (serum, saliva) or short-term (urine) circulating cortisol concentrations. The cortisol levels obtained by these techniques show considerable intra- and interindividual differences, which are caused by cortisol's circadian rhythm as well as its pulsatile secretion [30], daily variation, and reactivity to acute transient stress, such as nervousness or rush [31]. These techniques further call for invasive or frequent sampling and in particular urinary and salivary samples are prone to measurement error and sloppiness as the samples are often collected by the participants themselves, without supervision [32]. These are confounding variables that hamper the comparability of

existing studies in this area. In addition, these techniques do not cover the long-term effect of stress exposure very well.

2.2.   Long-Term Measurement of Cortisol

The short-comings of the measurement of short-term cortisol have led to the development of a new method to measure cortisol exposure in humans; the extraction of cortisol from human hair, with first evidence provided in 2004 [33]. Since then, several research groups have been focusing on this promising technique with some of its numerous advantages being the non-invasiveness, the standardized sampling, and, maybe most intriguing, the possibility to use hair as a retrospective biomarker of cortisol exposure. As hair grows approximately one centimeter per month [34], hair analysis offers the possibility to show the average long-term activity of the HPA axis, and to compare several hair segments/months with each other, including segments *before* the presence of a stressful event.

For the analysis, a strand of hair is cut from the posterior vertex position of the scalp. Cortisol of the hair segment of interest is extracted by methanol and further analyzed by either immunoassays or liquid chromatography tandem-mass spectrometry (LC-MS/MS) [35, 36]. The analysis using immunoassays such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) is limited to the measurement of one steroid of interest in question, whereas analysis with LC-MS/MS allows the measurement of several steroids in one sample, thereby enabling the creation of steroid profiles. Both methods are depicted in Figure 3.

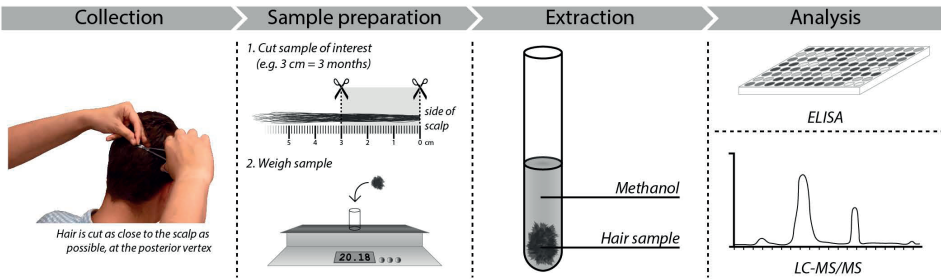**Figure 3.** *The process of sample collection, preparation, and analysis. With permission from Wester [37].*

3.   APPLICABILITY OF LONG-TERM CORTISOL RESEARCH

Hair cortisol research is a rapidly emerging research area and has successfully been applied to studies of patients with varying physical and psychological symptoms, i.e. chronic stress and stress-related disorders and diseases (e.g. obesity and mental illness).

Hair cortisol research has also shown useful as a diagnostic tool for (cyclic) CS, as it offers the opportunity to create timelines in hair and therefore catch episodes of disease that are otherwise difficult to detect [38].

3.1.　Chronic Stress

When a stressor regularly resurfaces and/or does not disappear, the stress response cannot be terminated but continues. Individuals that undergo this prolonged stress reaction are therefore hypothesized to have higher cortisol concentrations in their body than individuals who are not exposed to chronic stress. As the increased release of cortisol for a prolonged period is associated with allostatic load and its deleterious effects, the objective assessment of chronic stress(ors) is an important prerequisite to help identify individuals at risk for the adverse effects of elevated cortisol, to help them reduce stress and to prevent further adversities.

Chronic stress is supposed to be reflected in hair cortisol concentrations. Indeed, a number of studies have examined the association between long-term stress exposure and hair cortisol, covering a range of different stressors such as endurance sport [39], shift work [40], unemployment [41], recent major life events (e.g. death of a close relative, serious illness, etc) [42, 43], or severe chronic pain [44], and have shown increased long-term cortisol levels in individuals subjected to chronic stress. Therefore, in a broad area of research, recent and/or ongoing stress generally seems to be associated with increased hair cortisol. This is in line with research on short-term cortisol measured with the known methods by blood, saliva, and urine, which has also reported increased cortisol levels in chronically stressed individuals [45, 46].

3.2.　Stress-Related Disorders: Mental Health Aspects

Stress-related disease or stress-related disorder is a general term for any pathological condition caused by somatic or mental stress, i.e. by a period of relative hypercortisolism. Common examples of stress-related diseases and disorders are hypertension, bruxism, gastrointestinal problems, migraine, asthma, depression, anxiety, and obesity. Related to the presence of stress-related diseases is the assessment of stress-related psychological measures, which rely on individual experiences and interpretations. In general, the inclusion of the personal perception is an important part of stress research. It is an established assumption that the impact of stressful events is not only caused by the intensity of this event but also influenced by individual and contextual factors [47, 48]. Coping style, composed of temperament, personality, and acquired skills, has been shown to influence HPA axis reactivity [49, 50]. Therefore, the assessment of these factors is important in stress research. For the assessment of stress, either objective criteria (such as the presence or absence of a predefined stressor) or subjective criteria can be

applied. The subjective impression of stress is often assessed by means of questionnaires which patients fill in on their own.

Previous reports on stress-related disorders have consistently associated more stress-related physical features (such as increased BMI) with higher long-term cortisol concentrations [51-53]. Interestingly, the results are inconsistent when it comes to stress-related psychological aspects. This "lack of psychoendocrine covariance" is explainable by the divergence in time due to lag effects caused by different onsets and offsets of the observed stress responses [54], by the fact that most studies used a hair sample reflecting the average cortisol exposure of three months whereas the commonly used perceived stress scale (PSS) includes only the previous four weeks [47], or by problems that arise with retrospective subjective assessment like recall bias or social desirability bias [55]. Furthermore, it has been proposed that psychoendocrine covariance may change over time in response to severe stressors, thereby reflecting the long-term alterations of HPA axis activity and individual psychological responses associated with a specific disorder [54]. However, the lack of association could also mean that perceived stress has less validity to increase hair cortisol concentrations than a medical diagnosis.

The role of the HPA axis in mental disorders has been explored thoroughly for several decades, with studies both contradicting each other as well as adding to better understanding. Research in the context of acutely circulating cortisol levels has also yielded inconsistent results concerning unipolar and bipolar depression. Increased serum [56] and saliva [26, 57] cortisol levels as well as decreased serum and saliva cortisol levels [58] and no difference in salivary cortisol levels [59] were reported, differences which could partly be due to different subtypes of these disorders [60]. A normalization of the HPA axis activity is considered a prerequisite for clinical improvement [29], and a normalization of acutely circulating cortisol has indeed been found in patients who remit [61-63]. First studies on long-term cortisol levels have looked into this association [53, 64, 65].

Anxiety disorders present in a variety of manifestations and are considered a blanket term for disorders including generalized anxiety disorder (GAD), panic disorder (with or without agoraphobia), agoraphobia, specific phobia, social phobia, obsessive-compulsive disorder (OCD), posttraumatic stress disorder (PTSD), and acute stress disorder [66]. Anxiety disorders have often been linked with abnormal dynamics of the HPA axis and cortisol concentrations [25, 67]. First studies on the association between long-term cortisol concentrations and anxiety disorders have been conducted [68-70].

3.3.   Cortisol-Related Diseases: Somatic Health Aspects

Diseases that are characterized by extremely high or low cortisol levels, such as CD and AD, have been described above. Increased cortisol levels have also been reported in other medical conditions. A high level of cortisol (as seen in CS) is often marked by (abdominal) obesity. This observation led to the hypothesis that also a modest increase in

cortisol exposure may contribute to overweight and obesity in the general population. Obesity has been associated with increased cortisol output, as determined by urinary free cortisol. Positive correlations have been reported between long-term cortisol values and body mass index (BMI) as well as waist circumference [51, 71, 72], in adult and paediatric cohorts [73]. Obesity is in some cases accompanied by other cardiometabolic risk factors such as elevated blood pressure, elevated fasting plasma glucose, high serum triglycerides, and low high-density lipoprotein (HDL) levels. If three or more of these five medical conditions cluster, it is generally referred to as Metabolic Syndrome (MetS). The presence of MetS, in turn, is associated with the risk of developing cardiovascular disease (CVD) and diabetes mellitus [74]. First cross-sectional studies have shown that higher long-term cortisol levels were associated with increased presence of MetS [72] and with an adverse cardiovascular risk profile [75].

Apart from diseases associated with endogenously increased or decreased cortisol levels, cortisol can also be used in the treatment of different diseases. Hydrocortisone, prescribed for adrenal insufficiency (AI), is chemically identical to cortisol and is taken orally three times daily to mimic the circadian rhythm. First studies have used long-term cortisol levels as a measure to assess long-term systemic levels of administered hydrocortisone in AI patients [76-78], with the results indicating that long-term cortisol levels may be used to identify overtreatment with hydrocortisone.

4.   AIM AND OUTLINE OF THIS THESIS

The main aim of this thesis was to examine the associations between long-term cortisol as measured in scalp hair and different aspects of stress-related diseases and disorders. For this, the thesis could incorporate data from various smaller and large-scaled patient cohort samples, including the Netherlands Study of Depression and Anxiety (NESDA), the Bipolar Stress Study, patients from the Obesity Center CGG of the Erasmus Medical Center, and patients from the outpatient clinic of the department of Endocrinology of the Leiden University Medical Center.

Our first objective was to examine which factors influence long-term corticosteroid levels in persons without current psychopathology to determine which covariates need to be taken into account when studying long-term corticosteroid measures. In **Chapter 2**, we therefore describe which sociodemographic, health and lifestyle, and hair (treatment) characteristics affect long-term corticosteroid levels measured in scalp hair. In **Chapter 3**, we investigate the associations between long-term corticosteroid levels and depressive and anxiety disorders and their characteristics. In **Chapter 4**, we describe the effects of stressful life events, mood, and social support on long-term cortisol levels in patients with a bipolar disorder. In **Chapter 5**, we describe our results of a study

designed to evaluate the long-term cortisol levels in three groups, i.e. in normal-weight, over-weight, and obese participants. In **Chapter 6**, we compare long-term cortisol levels of patients with primary and secondary adrenal insufficiency on hydrocortisone replacement therapy with long-term cortisol levels of control patients with a pituitary disease but no hydrocortisone replacement therapy, and with long-term cortisol levels of healthy controls. We also investigate possible determinants of long-term cortisol levels in patients treated with hydrocortisone, i.e. sociodemographic, health and lifestyle, and hair (treatment) characteristics. In **Chapter 7**, we analyze whether long-term cortisol levels of patients with primary and secondary adrenal insufficiency on hydrocortisone replacement therapy correlate with quality of life (QoL). In the general discussion (**Chapter 8**), the results of the various study are discussed and placed within the context of other research groups working on the subject of long-term corticosteroids and stress-related diseases and disorders. **Chapter 9** provides a summary.

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02

Determinants of Hair Cortisol and Hair Cortisone Concentrations in Adults

ABSTRACT

Background

The analysis of hair cortisol concentrations (HairF) is a promising new tool for the assessment of long-term cortisol. With the development of multiple steroid analyses by means of liquid chromatography tandem-mass spectrometry (LC-MS/MS), the analysis of cortisone in hair (HairE) has also been facilitated. However, the influence of various types of determinants on HairF and HairE is still largely unknown. This study systematically assesses the influence of sociodemographic, health, lifestyle, and hair (treatment) characteristics on HairF and HairE.

Methods

Data of 760 psychiatrically healthy participants (71.8% female, mean age 45.89 years) of the Netherlands Study of Depression and Anxiety (NESDA) were used. HairF and HairE were measured in the proximal 3 cm of scalp hair, using LC-MS/MS.

Results

HairF and HairE strongly correlated. In simple linear regressions, HairF and HairE were higher in older age, in presence of diabetes mellitus, and in men compared to women. More frequent washing of the hair was associated with lower HairF and HairE. Darker hair colours were associated with higher HairF and HairE. An effect of season and of use of oral contraceptives was found for HairF. After full mutual adjustment, only age, presence of diabetes mellitus, hair washing frequency, and season remained significant determinants of HairF.

Conclusions

This large-scale study shows that HairF and HairE are upregulated in older age and in the presence of diabetes mellitus. This suggests that these levels are important for somatic health and should be taken into account when using hair corticosteroid analysis in future studies.

INTRODUCTION

The hypothalamic-pituitary-adrenal (HPA) axis has been subjected to a wide array of research questions, with one of the main interest areas being one of its end products, the hormone cortisol.

Until a decade ago, cortisol had predominantly been measured in saliva, serum or urine, which allows analyzing the dynamics of cortisol production for up to 24 hours. Then, a new technique was developed to measure long-term cortisol levels in scalp hair, allowing quantifying the average cortisol production for a period ranging from one month to several months or even years [1, 2]. The number of studies on hair cortisol concentrations (HairF) substantially increased throughout the past years [3]. While HairF tend to remain stable in health, increased as well as decreased HairF have been associated with a range of pathological and psychological conditions such as cardiovascular disease, obesity, Cushing's disease, and mood and anxiety disorders [4-8].

Determinants of HairF have been described before [9-11]. Two of these studies [9, 10] investigated simple associations of different socioeconomic and health variables with HairF, whereas one study [11] reported socioeconomic and health-related determinants of HairF in older adults using partial and full mutual adjustment. The first two studies showed associations of HairF with age (higher HairF in young and old age), sex (men show higher HairF than females), metabolic syndrome (MetS) (higher HairF in presence of MetS), and weight-related anthropometric measures (increasing HairF with increasing body mass index (BMI). The third study confirmed these simple associations and reported positive associations with smoking, diabetes mellitus, mental health, daytime sleeping, working status, and negative associations with diastolic blood pressure. However, in full mutual adjustment, only age and smoking remained independent predictors of HairF. These studies have produced important results, however, a systematic study with a large sample size and partial as well as mutual adjustment for predictors of HairF in a younger age group is still lacking. Furthermore, in different publications, the effects of sociodemographic and health and lifestyle variables on HairF show a noticeable discrepancy. Existing literature aiming at summarizing the effects of influencing factors have reported inconsistent and conflicting results for age, sex, alcohol and nicotine use, physical activity, and for hair (treatment) characteristics. Factors that could account for these incongruent findings are the relatively small sample sizes, therefore possibly rendering power problems. Furthermore, most studies varied in the extent to which they took other covariates into account. Disentangling these effects is of paramount importance to enable future studies to adjust their analyses for potential confounding factors. This seems especially important for research on stress and mental illness, as the effects of these conditions on the HPA-axis have been shown to be small [12], rendering them vulnerable to being overshadowed by stronger effects when these are not

controlled for. This could be one of the reasons that for example self-reported stress, has shown divergent relations with HairF, with positive, inverse, and no correlations [see 12, for review].

Until now, most studies on HairF have been conducted using immunoassays [13]. With the recent development and validation of HairF measurements with liquid chromatography tandem-mass spectrometry (LC-MS/MS) [14, 15], the quantification of other steroids in scalp hair has become possible. As cortisol can be converted into inactive cortisone, the assessment of hair cortisone (HairE) in parallel to HairF may give even more insight into the cumulative amount of active and inactive corticosteroids in the body. Following the argumentation of Stalder et al. [16], as recent research suggested that under specific circumstances, salivary cortisone may provide a better reflection of systemic cortisol levels than salivary F [17]. The authors showed that salivary cortisone closely reflected free serum cortisol after adrenal stimulation and hydrocortisone administration, and that it was unaffected by changes in corticosteroid-binding globulin (CBG). Furthermore, because HairE has been shown to be approximately 3-4-fold higher than HairF [16], it might also be more readily measureable than HairF. To further investigate the relevance of HairE, we also calculated the F/E-ratio, and an additive F+E measure as an indication of total glucocorticoid supply.

Results on both HairF and HairE have been reported in first publications [2, 16, 18, 19]. In all studies, HairF and HairE strongly correlated. However, all reports on HairE have been exploratory, and as of yet no study has set out to systematically assess determinants of HairE. The relevance of these determinants follows the same argumentation as for the determinants of HairF described above [19].

We therefore set out to examine the wide range of potential determinants of both HairF and HairE levels within one well-studied cohort. This seems necessary for future research as it aids in deciding which factors need to be assessed apart from the variable of interest in order to control for their influence on HairF and HairE. The present study uses detailed information of 760 subjects participating in the Netherlands Study of Depression and Anxiety (NESDA) to examine which 1) sociodemographic, 2) health & lifestyle, and 3) hair (treatment) characteristics affect long-term HairF and HairE levels measured in scalp hair.

MATERIALS AND METHODS

Study Sample

Data were derived from the Netherlands Study of Depression and Anxiety (NESDA), an ongoing longitudinal cohort study on the predictors, course and consequences of depressive and anxiety disorders. The NESDA sample consists of 2981 participants aged

18–65 years at inclusion, comprising persons with no depressive or anxiety disorder, persons who have had a disorder in the past, and persons with a current depressive and/or anxiety disorder. A detailed description of the NESDA study can be found elsewhere [20]. The research protocol was approved by the Ethical Committee of the participating research centers, and all participants provided written informed consent. Hair data collection was added to NESDA's 6-year follow-up assessment, in which 2256 participants of the initial 2981 sample participated (75.68%). All determinants used for the present analysis were measured during this 6-year follow-up wave. For the present study, we only selected persons without recent (1-year recency) psychopathology in order to obtain an indication of the main determinants of HairF and HairE unbiased by potential HPA-axis altering effects by underlying psychopathology. A total of 1536 were considered 'psychiatrically healthy' as they had no recent depressive disorder, bipolar disorder, or anxiety disorder, as defined on the basis of the Composite International Diagnostic Interview (CIDI) [21]. Further inclusion criteria were willingness to provide a hair sample, sufficient hair growth at the posterior vertex position of the head, and a hair sample weight of at least 5 mg, rendering an initial sample of n = 1141 participants. Exclusion criteria for the present study were the use of antidepressants and (frequent use of) benzodiazepines (n = 190), and use of corticosteroids (n = 126, of which n = 88 confirmed and n = 38 unclear), resulting in a sample of 825 subjects.

HairF and HairE measurement

During the visit at the research facility, hair strands of approximately 100 hairs were cut as close as possible from the scalp from a posterior vertex position. The most proximal three cm of hair were used for analysis. Based on a mean hair growth rate of one cm per month [22], the hair samples reflect the cumulative cortisol and cortisone secretion of the previous three months. Hair samples were weighted, finely cut with surgical scissors, and washed with 1.0mL of LC-MS grade isopropanol for two minutes. The extraction of cortisol and cortisone was achieved by overnight incubation with 1.4 mL LC-MS grade methanol and in presence of 100 μL internal standard (cortisol-d4) for 18 hours at 25 ^{0}C while gently shaking. The analysis using LC-MS/MS including matrix interferences has extensively been described elsewhere [15]. The inter-day variation in the present study was <8.3% for cortisol and <4.8% for cortisone. Steroid peak integrations were reviewed and manually integrated by two independent persons when automated peak integration feature incorrectly or partially integrated peaks. The analyses were successfully performed in 760 participants, who comprised the final sample. In 65 individuals, HairF and HairE levels could not be determined due to interference in the sample by unknown compounds.

Sociodemographic variables, health and lifestyle factors, and hair (treatment) characteristics

The sociodemographic factors included sex, age, educational level (years of attained education), and ancestry (North-European or not North-European). Health and lifestyle factors included alcohol consumption, smoking behaviour, physical activity levels, as well as waist and hip circumference, presence of diabetes mellitus, hypertension, and other chronic disease information. Waist and hip circumference were each determined twice with a measuring tape, and the average of both measurements was used. Diabetes mellitus and hypertension were ascertained using self-report. Other self-reported chronic diseases (lung disease, cancer, rheumatic, intestinal and liver disease) were grouped in an "other disease" indicator. For subgroup analyses in women, information on the use of oral contraceptives and pregnancy was obtained. Alcohol consumption was categorized into non-drinker, moderate drinker, and hazardous drinker according to the Alcohol Use Disorders Identification Test (AUDIT) [23], and also expressed as number of units of alcohol per week. Smoking status was categorized into current smoker or non-smoker, and the number of cigarettes per week was recorded. The average number of cigarettes per week was calculated on all subjects. Non-smokers are represented in all analyses with zero cigarettes per week. Physical activity was assessed with the International Physical Activity Questionnaire (IPAQ) and expressed per 1000 metabolic equivalent of task minutes (MET-min) a week. A self-developed questionnaire was used to assess hair characteristics. Participants were asked to indicate their natural hair color (blond, brown, red, black, or grey) and whether their hair was dyed and/or bleached and/or permed in the previous three months. The hair washing frequency (<3 times a week or ≥3 times per week) was assessed, as was the use of any products except for shampoo on the scalp on the day of sample collection. The day of sample collection was used for the calculation of the season that was represented in the most proximal three cm of hair.

Statistical analysis

SPSS 20.0 for Windows (IBM, Chicago, Illinois) was used for statistical analysis. HairF and HairE values were logarithmically transformed to achieve normal distribution. For descriptive purposes, HairF and HairE are provided in pg/mg and are reported as median (Mdn) and interquartile range (IQR). Other data, unless otherwise indicated, are presented in original units with mean (M) and standard deviation (SD), or in sample size (n) and percentage (%). In a first step, associations between HairF, HairE, and socio-demographic, health and lifestyle factors, and hair treatment) characteristics were examined using simple linear regression analyses. In the next step, partially adjusted regression models with adjustment for sex and age were conducted. In a final step, fully adjusted regression analyses with all variables entered simultaneously were performed

to examine the independence of HairF and HairE predictors. Variables that were valid for women only (i.e. hormonal contraceptives and pregnancy) were not included in the fully adjusted model, however, the analyses were stratified and rerun for women with these variables included. The results of the linear regression are reported as standardized coefficients (beta), and p-value. The significance level was set at p = 0.05.

Of the 760 participants, 616 provided a full dataset without any missing values. The missing information were as follows: 1.1% alcohol consumption, 0.1% smoking, 6.8% physical activity, 3.2% waist circumference, 3.0% hip circumference, 3.0% natural hair color, 3.2% hair treatment, 3.0% hair washing frequency, and 3.0% use of hair styling products. Multiple imputation was used to complete the dataset, assuming a missing at random approach and creating five imputed datasets. All characteristics were used as predictors to impute missing values, and the observed minimum and maximum values of from the original full dataset were used as constraints. The pooled estimates of the five imputed datasets are reported.

RESULTS

Sample characteristics

The sample characteristics of the 760 participants (71.8 % female) are presented in Table 1. The median HairF of this sample was 3.18 (2.16 – 5.58) pg/mg hair, whereas the median HairE of this sample was 10.52 (7.41 – 15.22) pg/mg hair.

Table 1. Descriptive information of the study population

Characteristics	All (n = 760)	Women (n = 546)	Men (n = 214)
Sociodemographics			
Age (in years)	45.89 (13.80)	45.45 (13.33)	47.00 (14.91)
Educational level (in years)	13.35 (3.29)	13.42 (3.28)	13.18 (3.30)
North-European ancestry	736 (96.8 %)	526 (96.3 %)	210 (98.1 %)
Health and lifestyle indicators			
Alcohol consumption (units/week)	5.84 (7.44)	4.69 (6.07)	8.80 (9.54)
No alcohol consumption (categories)	114 (15.0 %)	92 (16.9)	22 (10.3 %)
moderate	539 (70.9 %)	401 (73.4 %)	137 (64.0 %)
hazardous	107 (14.1 %)	53 (9.7 %)	54 (25.2 %)
Smoking (cigarettes/week)	16.04 (41.03)	14.94 (39.00)	18.87 (45.77)
Smoking (current)	117 (23.3 %)	122 (22.3 %)	55 (25.7 %)
No use of hormonal contraceptives*	n/a	401 (73.4 %)	n/a
Oral contraceptives	n/a	80 (14.7 %)	n/a
Other	n/a	65 (11.9 %)	n/a
Pregnancy*	n/a	23 (4.2 %)	n/a

Table 1. Descriptive information of the study population (continued)

Characteristics	All (n = 760)	Women (n = 546)	Men (n = 214)
Physical activity (1000 MET-min/week)	3.99 (3.37)	3.88 (3.20)	4.27 (3.78)
Waist circumference (in cm)	90.36 (13.41)	87.73 (12.34)	97.06 (13.72)
Hip circumference (in cm)	104.27 (10.22)	104.09 (10.75)	104.73 (8.72)
Diabetes mellitus	28 (3.7 %)	12 (2.2 %)	16 (7.5 %)
Hypertension	115 (15.1 %)	72 (13.2 %)	43 (10.1 %)
Other chronic disease	154 (20.3 %)	113 (20.7 %)	41 (19.2 %)
Hair (treatment) characteristics			
Natural hair color black	38 (5.0 %)	22 (4.0 %)	17 (7.9 %)
Natural hair color brown	233 (30.7 %)	175 (32.1 %)	58 (27.1 %)
Natural hair color blond	331 (43.6 %)	254 (46.5 %)	78 (36.5 %)
Natural hair color red	26 (3.4 %)	22 (4.0 %)	4 (1.9 %)
Natural hair color grey	132 (17.4 %)	74 (13.6 %)	58 (27.1 %)
No hair treatment in past 3 months	496 (65.3 %)	286 (52.4 %)	210 (98.1 %)
Hair dyed	69 (9.1 %)	65 (11.9 %)	4 (1.9 %)
Hair bleached	223 (29.3 %)	221 (40.5 %)	2 (0.9 %)
Hair permed	20 (2.6 %)	19 (3.5 %)	1 (0.5 %)
Hair washing frequency (≥ 3/week)	538 (70.8 %)	381 (69.8 %)	156 (73.1 %)
Use of hair styling products on day of sampling	333 (43.8 %)	249 (45.6 %)	85 (39.7 %)
Season represented in hair sample			
Winter	168 (22.1 %)	127 (23.3 %)	41 (19.2 %)
Spring	184 (24.2 %)	126 (23.1 %)	58 (27.1 %)
Summer	200 (26.3 %)	143 (26.2 %)	57 (26.2 %)
Fall	208 (27.4 %)	150 (27.5 %)	58 (27.1 %)

*Data are presented as pooled mean (pooled standard deviation), and as pooled n (pooled percentage). Met-min, minutes of metabolic energy turnover; *, in women only; n/a, not applicable; other chronic diseases, lung disease, cancer, rheumatic, intestinal and liver disease.*

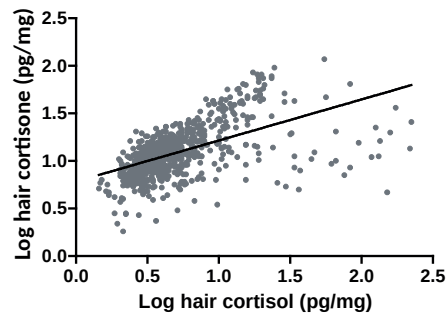HairF and HairE showed a significant positive linear correlation with each other, r = .55, p <.001 (Figure 1).

Figure 1. *The association between HairE and HairF. Both are expressed as log-transformed values of the respective hormone in pg/mg hair.*

Associations with HairF concentrations

In partially adjusted regression analyses, men showed significantly higher HairF than women (3.57 (2.42 – 7.89) vs. 2.98 (2.07 – 5.19)), β = .104, p = 0.003 (Table 2). Older age was significantly associated with higher HairF (β = .184, p < 0.001). Higher waist circumference was related to higher HairF (β = .089, p < 0.03). HairF were also higher in case of diabetes mellitus (β = .092, p = 0.01). Natural hair color showed an association with HairF, indicating that persons with black hair had increased HairF compared to persons with other hair colors (β = .084, p = .02). The hair washing frequency was also associated with altered HairF (β = -.118, p = 0.002), demonstrating that persons who wash their hair three or more times a week have lower HairF (2.97, 2.05 – 4.86) than persons who wash their hair twice a week or less (3.81, 2.54 – 8.64). An effect of season was detected, in that hair samples representing covering winter months show lower HairF than hair samples covering spring and summer (β = -.120, p = .007, and β = -.097, p = .03, respectively). In women, use of oral contraceptives was associated with higher HairF (β = .107, p = .01).

Table 2. Regression analyses on HairF and HairE

Characteristics	Standardized simple linear regression coefficients	p-value	Standardized multiple linear regression coefficients (adjusted for sex and age)[1]	p-value	Standardized multiple linear regression coefficients (mutual adjustment)[2]	p-value	
Sex (female)	**-.114**	**.002**	**-.104**	**.003**	-.070	.117	HairF
	-.173	**<.001**	**-.160**	**<.001**	-.057	.186	HairE
Age (years)	**.189**	**<.001**	**.184**	**<.001**	.108	**.028**	HairF
	.264	**<.001**	**.255**	**<.001**	**.155**	**.001**	HairE
Educational level (years)	-.035	.332	.005	.883	.029	.470	HairF
	-.068	.060	-.012	.735	-.011	.786	HairE
North-European ancestry	-.021	.562	-.031	.383	-.020	.594	HairF
	-.017	.647	-.031	.369	-.020	.583	HairE
Alcohol consumption (units/week)	**.078**	**.032**	.011	.774	.015	.708	HairF
	.154	**<.001**	.061	.100	.059	.123	HairE
No alcohol consumption (categories)	Ref		Ref		Not included		
moderate	-.031	.520	-.039	.402			HairF
	-.019	.724	-.069	.539			HairE
hazardous	.041	.384	.018	.698			HairF
	.061	.226	.071	.619			HairE
Smoking (cigarettes/week)	.026	.482	.022	.534	.021	.562	HairF
	.066	.068	.061	.078	.054	.132	HairE
Smoking (current)	.046	.207	.049	.166	Not included		HairF

Table 2. Regression analyses on HairF and HairE (continued)

Characteristics	Standardized simple linear regression coefficients	p-value	Standardized multiple linear regression coefficients (adjusted for sex and age)[1]	p-value	Standardized multiple linear regression coefficients (mutual adjustment)[2]	p-value	
	.075	**.039**	**.079**	**.022**	Not included		HairE
No use of hormonal contraceptives*	Ref		Ref		Not included		
Oral contraceptives	.017	.684	**.107**	**.014**			HairF
	-.093	**.027**	.004	.923			HairE
Other	-.047	.257	.013	.749			HairF
	-.053	.210	.012	.777			HairE
Pregnancy*	-.031	.449	.016	.692	Not included		
	-.040	.353	.021	.629			
Physical activity (1000	.025	.488	.006	.876	.023	.549	HairF
MET-min/week)	-.008	.833	-.036	.299	-.027	450	HairE
Waist circumference (cm)	**.166**	**<.001**	**.089**	**.026**	.107	.107	HairF
	.202	**<.001**	**.082**	**.036**	.116	.074	HairE
Hip circumference (cm)	**.085**	**.020**	.062	.083	-.005	.927	HairF
	.067	.066	.034	.323	-.038	.494	HairE
Diabetes mellitus	**.134**	**<.001**	**.092**	**.011**	**.095**	**.011**	HairF
	.137	**<.001**	**.075**	**.034**	.056	.120	HairE
Hypertension	**.086**	**.017**	.009	.815	-.019	.648	HairF
	.148	**<.001**	.044	.244	.012	.763	HairE
Other chronic disease	.043	.235	-.002	.957	-.001	.980	HairF
	.102	**.005**	.042	.237	.037	.309	HairE
Natural hair color blond	Ref		Ref		Ref		
Natural hair color brown	.023	.561	.013	.744	-.002	.959	HairF
	.090	**.021**	**.076**	**.045**	.056	.141	HairE
Natural hair color black	**.106**	**.004**	**.084**	**.023**	.064	.110	HairF
	.099	**.010**	.066	.081	.062	.125	HairE
Natural hair color red	-.003	.937	-.001	.986	-.028	.525	HairF
	.032	.404	.036	.334	.034	.406	HairE
Natural hair color grey	**.110**	**.005**	.007	.874	.004	.927	HairF
	.179	**<.001**	.033	.441	.012	.779	HairE
No hair treatment in past	.003	.946	-.041	.317	.050	.675	HairF
3 months	**.134**	**<.001**	**.097**	**.013**	-.010	.932	HairE
Hair dyed	-.017	.644	.014	.701	.006	.922	HairF
	-.076	**.042**	-.032	.373	-.040	.532	HairE

Table 2. Regression analyses on HairF and HairE (continued)

Characteristics	Standardized simple linear regression coefficients	p-value	Standardized multiple linear regression coefficients (adjusted for sex and age)[1]	p-value	Standardized multiple linear regression coefficients (mutual adjustment)[2]	p-value	
Hair bleached	.014	.704	.051	.193	.093	.398	HairF
	-.125	**.001**	**-.094**	**.014**	-.082	.468	HairE
Hair permed	.014	.701	.018	.634	.033	.479	HairF
	-.037	.364	-.032	.431	-.032	.507	HairE
Hair washing frequency (≥ 3/week)	**-.157**	**<.001**	**-.118**	**.002**	**-.135**	**.001**	HairF
	-.179	**<.001**	**-.121**	**.002**	**-.116**	**.003**	HairE
Use of hair styling products on day of sampling	-.061	.093	-.006	.880	.004	.924	HairF
	-.155	**<.001**	**-.083**	**.022**	-.053	.149	HairE
Season Winter	Ref		Ref		Ref		
Season Spring	**.099**	**.031**	**.120**	**.007**	**.138**	**.002**	HairF
	.030	.516	.057	.191	.079	.074	HairE
Season Summer	.086	.061	**.097**	**.032**	**.115**	**.012**	HairF
	.045	.333	.057	.191	.077	.079	HairE
Season Fall	.069	.113	.078	.086	**.101**	**.028**	HairF
	-.025	.591	-.015	.740	.005	.916	HairE

*Met-min, minutes of metabolic energy turnover; *, in women only; other chronic disease, lung disease, cancer, rheumatic, intestinal and liver disease.*
[1]*Age and sex themselves were only adjusted for each other.*
[2]*Multiple linear regression was performed including all variables listed in the table except for use of hormonal contraceptives and pregnancy (due to sex-specificity) and except for categories of alcohol consumption and smoking, as the continuous variable was already included.*

A fully adjusted regression was conducted with all variables entered (Table 2). This analysis revealed that with mutual adjustment, the effects of age, diabetes mellitus, hair washing frequency, and season remained statistically significant. Using only these significant variables in a regression analyses resulted in a model fit of .07 (adjusted r^2). The analyses were stratified for sex, and in analyses on women, use of oral contraceptives and pregnancy were included in the model. In this model, full adjustment resulted in a model in which only age, diabetes mellitus, and use of oral contraceptives remained significant, with a resulting model fit of .06 (adjusted r^2). Age remained the strongest predictor of HairF. Its relation with HairF is depicted in Figure 2, illustrating a rather linear association between age and HairF both in men and women.
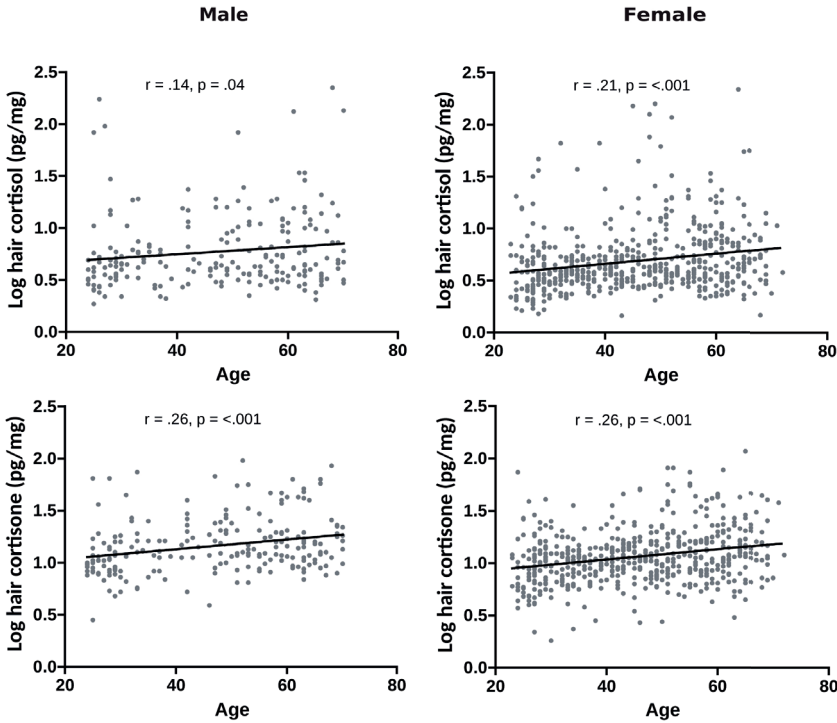
Figure 2. *Upper row: The correlation between age and HairF (log-transformed), stratified for sex. Lower row: The correlation between age and HairE (log-transformed), stratified for sex.*

Associations with HairE concentrations

As for HairF, men showed significantly higher HairE than women (11.57 (8.49 – 18.28) vs. 10.15 (7.02 – 14.27)), $\beta = .160$, $p < .001$ (Table 2). Older age was also significantly associated with higher HairE ($\beta = .255$, $p < 0.001$). Current smokers demonstrated higher HairE than non-smokers ($\beta = .079$, $p = .02$). Higher waist circumference and the presence of diabetes mellitus were associated with higher HairE ($\beta = .082$, $p = .04$, and $\beta = .075$, $p = .03$, respectively). The natural hair color showed an association with HairE, indicating that persons with brown hair had higher HairE compared to persons with blond hair ($\beta = .076$, $p = .05$). Bleaching of the hair as well as frequently washing of the hair and use of hair products was all associated with lower HairE ($\beta = -.094$, $p = .01$, $\beta = -.121$, $p = .002$, and $\beta = -.083$, $p = .02$, respectively).

A mutually adjusted regression was run with all variables. In this analysis, age and hair washing frequency remained significant. The model fit was .08 (adjusted r^2). Age remained the strongest predictor of HairE, and both in men and women, age showed a significant positive linear association with HairE (Figure 2).

Associations with HairF/HairE ratio and sum of HairF+HairE

In adjusted analyses, the ratio HairF/HairE showed associations with use of oral contraceptives in women (β = .119, p = .01), as well as with season. The sum of HairF+HairE was significantly associated with age (β = .150, p = .002), presence of diabetes mellitus (β = .082, p = .03), hair washing frequency (β = -.132, p = .001), and season. No other associations reached significance with mutual adjustment (Table 3).

Table 3. Regression analyses on the ratio HairF/HairE and the sum HairF+HairE

Characteristics	Standardized simple linear regression coefficients	p-value	Standardized multiple linear regression coefficients (adjusted for sex and age)[1]	p-value	Standardized multiple linear regression coefficients (mutual adjustment)[2]	p-value	
Sex (female)	.010	.783	.011	.772	-.035	.450	Ratio
	-.165	**<.001**	**-.152**	**<.001**	-.079	.073	Sum
Age (years)	.011	.770	.011	.759	.004	.932	Ratio
	.257	**<.001**	**.250**	**<.001**	**.150**	**.002**	Sum
Educational level (years)	.013	.722	.015	.678	.043	.297	Ratio
	-.068	.059	-.014	.696	.000	.992	Sum
North-European ancestry	-.009	.813	-.008	.816	-.003	.927	Ratio
	-.013	.727	-.027	.443	-.011	.756	Sum
Alcohol consumption (units/week)	-.031	.397	-.035	.370	-.029	.468	Ratio
	.124	**.001**	.032	.397	.032	.404	Sum
No alcohol consumption (categories)	Ref		Ref		Not included		
moderate	-.015	.759	-.014	.769	Not included		Ratio
	-.040	.416	-.053	.274	Not included		Sum
hazardous	.000	1.000	.003	.950	Not included		Ratio
	.049	.311	.015	.755	Not included		Sum
Smoking (cigarettes/week)	-.024	.512	-.023	.521	-.020	.602	Ratio
	.047	.192	.042	.223	.038	.293	Sum
Smoking (current)	-.006	.878	-.005	.895	Not included		Ratio
	.068	.060	**.073**	**.036**	Not included		Sum
No use of hormonal contraceptives*	Ref		Ref		Not included		
Oral contraceptives	**.090**	**.035**	**.119**	**.010**	Not included		Ratio
	-.056	.185	.048	.277	Not included		Sum
Other	-.014	.740	.006	.899	Not included		Ratio
	-.045	.281	.023	.577	Not included		Sum
Pregnancy*	-.017	.682	-.010	.827	Not included		Ratio

Table 3. Regression analyses on the ratio HairF/HairE and the sum HairF+HairE (continued)

Characteristics	Standardized simple linear regression coefficients	p-value	Standardized multiple linear regression coefficients (adjusted for sex and age)[1]	p-value	Standardized multiple linear regression coefficients (mutual adjustment)[2]	p-value	
	-.016	.702	.045	.291	Not included		Sum
Physical activity (1000	.041	.258	.041	.260	.054	.157	Ratio
MET-min/week)	.016	.658	-.011	.746	.000	1.000	Sum
Waist circumference (cm)	.031	.396	.038	.351	.032	.636	Ratio
	.208	**<.001**	**.095**	**.015**	.114	.080	Sum
Hip circumference (cm)	.047	.202	.046	.209	.026	.658	Ratio
	.085	**.019**	.054	.123	-.021	.711	Sum
Diabetes mellitus	.043	.239	.044	.236	.063	.098	Ratio
	.153	**<.001**	**.093**	**.008**	**.082**	**.025**	Sum
Hypertension	-0.19	.593	-.027	.498	-.033	.433	Ratio
	.142	**<.001**	.040	.289	.009	.821	Sum
Other chronic disease	-.033	.370	-.038	.311	-.032	.396	Ratio
	.086	**.018**	.027	.457	.022	.539	Sum
Natural hair color blond	Ref		Ref		Ref		
Natural hair color brown	-.039	.318	-.040	.314	-.042	.297	Ratio
	.059	.132	.045	.236	.029	.449	Sum
Natural hair color black	.046	.236	.046	.236	.028	.510	Ratio
	.117	**.002**	**.085**	**.019**	.076	.055	Sum
Natural hair color red	-.030	.460	-.030	.451	-.059	.198	Ratio
	.013	.734	.017	.653	-.002	.965	Sum
Natural hair color grey	-.016	.680	-.021	.645	-.005	.906	Ratio
	.164	**<.001**	.022	.608	.007	.866	Sum
No hair treatment in past	**-.113**	**.002**	**-.134**	**.001**	.041	.683	Ratio
3 months	**.081**	**.029**	.035	.376	.056	.676	Sum
Hair dyed	.039	.296	.039	.300	.033	.550	Ratio
	-.044	.239	-.001	.982	.000	.997	Sum
Hair bleached	**.126**	**.001**	**.144**	**<.001**	.175	.061	Ratio
	-.067	.070	-.028	.472	.021	.857	Sum
Hair permed	.048	.214	.047	.224	.067	.118	Ratio
	-.018	.638	-.013	.731	-.009	.855	Sum
Hair washing frequency	-.039	.288	-.039	.314	-.064	.101	Ratio
(≥ 3/week)	**-.183**	**<.001**	**-.127**	**.001**	**-.132**	**.001**	Sum
Use of hair styling products	.051	.164	.058	.129	.044	.266	Ratio
on day of sampling	-.123	.001	-.049	.172	-.031	.404	Sum

Table 3. Regression analyses on the ratio HairF/HairE and the sum HairF+HairE (continued)

Characteristics	Standardized simple linear regression coefficients	p-value	Standardized multiple linear regression coefficients (adjusted for sex and age)[1]	p-value	Standardized multiple linear regression coefficients (mutual adjustment)[2]	p-value	
Season Winter	Ref		Ref		Ref		
Season Spring	**.102**	**.025**	**.106**	**.021**	**.110**	**.019**	Ratio
	.051	.263	.079	.074	**.098**	**.027**	Sum
Season Summer	.057	.101	.078	.093	.082	.077	Ratio
	.059	.199	.072	.102	**.090**	**.042**	Sum
Season Fall	**.110**	**.017**	**.112**	**.015**	**.122**	**.009**	Ratio
	.020	.664	.030	.492	.053	.239	Sum

*Met-min, minutes of metabolic energy turnover; *, in women only; other chronic disease, lung disease, cancer, rheumatic, intestinal and liver disease.*
[1] Age and sex themselves were only adjusted for each other.
[2] Multiple linear regression was performed including all variables listed in the table except for use of hormonal contraceptives and pregnancy (due to sex-specificity) and except for categories of alcohol consumption and smoking, as the continuous variable was already included.

DISCUSSION

The current study examined HairF and HairE levels in a large sample and assessed the influence of different sociodemographic, health and lifestyle, and hair (treatment) characteristics on these hormones. Both HairF and HairE showed robust linear increases with age. Lower HairF and HairE levels were found in subjects who wash their hair more frequently. Elevated HairF levels were related to the presence of diabetes mellitus. Furthermore, hair samples representing the winter months showed lower HairF. This is the first study to show these effects in a large middle-aged sample with full adjustment. This approach enabled the investigation of independent effects of all considered determinants and showed that for above mentioned results, the associations with HairF and HairE are indeed distinct.

The strongest influence on HairF and HairE was age of the participant. Age has often [9, 11, 16], but not consistently [1, 10], been associated with HairF in earlier research. However, studies that found curvilinear [9] or linear [11, 16] effects comprised a wider age range, and may therefore be more suitable to detect endocrine changes related to age. Thus, these studies indicate that age is a strong predictor of hair corticosteroids and needs to be included as possible covariate in studies that use participants with a range

of age. Hair washing frequency also influenced HairF. Previous research has provided inconsistent results, with some studies reporting effects [24], whereas others could not detect any influence [1, 10]. Frequent washing of the hair might damage the hair structure and thereby lead to an increased wash-out of cortisol. This reasoning is consistent with the often replicated result that cortisol content decreases the more distal the hair segment is from the scalp [9, 25, 26]. The current study also found HairF to be associated with the presence of diabetes mellitus. This result is consistent with other research on this topic [5, 11], which was conducted in older adults. In middle-aged adults, this relationship has until now only been approximated. Stalder and colleagues provided evidence for a positive relationship of HairF with glycated haemoglobin, as an index of long-term glucose levels [16]. Taken together, these results suggest that higher HairF are associated with long-term increased glucose levels and presence of diabetes mellitus across different age spans. One unexpected finding was the effect of season on HairF. Recent studies approached aspects of this issue by investigating effects of temperature, humidity and sweating on HairF [27, 28]. An inverse correlation with temperature and positive correlation with relative humidity for HairF is reported. Our results of higher HairF in spring and summer contradict their findings regarding temperature but may support their result on humidity. Another study, examining determinants of maternal hair cortisol concentrations at delivery reflecting the last trimester of pregnancy, also reported a seasonal effect. Mothers showed significantly higher HairF in summer and autumn as opposed to winter [29]. These finding support our results on higher HairF in summer months. Grass et al showed that sweating did not seem to be of major influence on HairF. Thus, we cannot rule out that seasonal influences affect HPA- axis activity and thereby alter HairF.

In the present study, with partial adjustment, men showed higher HairF and HairE concentrations than women. Our results concur with three previous studies [5, 9, 11] but are at variance with others [1, 2]. A possible explanation for higher levels in men might be that men seem to have lower corticosteroid binding globulin (CBG) levels while total cortisol levels are comparable to women's total cortisol [30]. This may result in higher free cortisol levels in men. It has been thought that the free fraction of cortisol is incorporated in hair [31], which might explain the observed sex differences. However, the gender effect was not independent of other covariates as it lost significance in full adjustment, which is in line with the study that used partial and full adjustment in older adults [11]. Higher waist circumference was associated with increased HairF in analyses with partial-adjustment. Significant results have been reported in most studies that investigated this association [1, 16]. The correlation between waist circumference, or better, visceral fat, and long-term cortisol is well supported by literature [see 32, for review]. A darker natural hair color was associated with higher HairF in analyses with partial adjustment. However, this effect was not independent of other covariates. A

non-significant trend for higher HairF in darker hair has been reported in one previous study [9]; however, in a direct comparison between light-blond and dark-brown hair, no differences were observed. This corresponds to our finding of no difference between blond and brown hair; however, no conclusion can be drawn from their study on the association between HairF and black hair as they did not investigate this specific association. Furthermore, no other study has been able to replicate this result. This hair color effect was not caused by differences in socio-economic status (as approximated by educational level), body composition or Northern-European ancestry status in our study. This study is also the first to find a positive effect of oral contraceptives on HairF. These are surprising results, since HairF are generally considered to reflect the free fraction of cortisol which should not be influenced by use of oral contraceptives [9]. Other studies examining the association between HairF and the use of oral contraceptives did not report significant results [9, 10, 33]. In this study, we could not unravel whether this effect was caused by a specific hormone combination.

The present study did not detect effects of alcohol and nicotine use on HairF levels. Smoking was not related to HairF in most studies [9, 28, 34], however, contradicting results have also been reported [11, 35]. No significant effect of alcohol use on HairE was found in this study. Previous studies were inconsistent regarding the effect of alcohol on HairF. Some studies found no effect [11, 16], whereas others reported increased HairF in excessive alcohol users [5, 35] and alcohol-dependant individuals [36].

Previous research has examined covariates of HairE [16, 19]; however, these factors not been systematically assessed until now. This is the first study to systematically assess the effect of different variables on HairE. HairE seems to be influenced by factors quite consistent with influences of HairF, namely age and hair washing frequency, and (although not independent of other covariates) also gender, waist circumference, presence of diabetes mellitus, and natural hair color. In contrast to HairF, HairE were not affected by use of oral contraceptives or by season. They were, however, affected by smoking status, hair treatment, and use of hair styling products. Interestingly, current smokers had higher HairE, independent of their average number of cigarettes. This pattern has been reported before for HairF [35]. Hair treatment, i.e. bleaching of the hair, was associated with lower HairE. Reports on hair treatment on HairF have been inconsistent so far; however, in case of reported effects they usually tend to lower the hormone content [1, 16, 37]. The use of hair styling products also influenced HairE. As the hair samples were washed before extraction and analysis, it seems unlikely that the actual product used on the day of sample collection caused this effect, however, repeated cosmetic treatment could affect and lower hormone concentrations, as has been suggested before [2].

HairE were 3.4-fold higher than HairF, a ratio that is comparable to the ratio reported by other studies [2, 16, 18, 19]. The ratio itself was generally not associated with the factors that influenced HairF and HairE. This corroborates previous results of Stalder et

al [16]. The sum of HairF and HairE as an indicator of a general glucocorticoid supply (i.e. active cortisol and inactivated cortisol that had been active once) showed associations with factors comparable to the associations of only HairF and/or HairE. Visual inspection of our data also showed that high outlying values were particularly present in HairF but less so in HairE levels (data not shown); a tendency also observed in Stalder et al..

Several limitations have to be acknowledged for the present study. The nature of the current study is cross-sectional, which prevents the interpretation of causal inferences. Due to the exploratory nature of this study, we did not correct for multiple testing.

In conclusion, the current study presents results of the first large scale study to systematically assess determinants of both HairF and HairE levels. Based on our results, age, sex, waist circumference, diabetes mellitus, hair washing frequency, natural hair color, and depending on the hormone in question, also use of oral contraceptives, season represented in the hair sample, smoking status, hair treatment, and use of hair styling products have been confirmed and identified as important influences and should be considered as potential confounding influences in future research.

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03

LONG-TERM CORTICOSTEROID LEVELS MEASURED IN HAIR ARE RELATED TO SEVERITY OF DEPRESSIVE AND ANXIETY DISORDERS

ABSTRACT

Background

Depressive and anxiety disorders have been linked to a disturbed hypothalamus-pituitary-adrenal (HPA)-axis, shown by altered short-term cortisol concentrations. Hair cortisol levels (HairF) and hair cortisone levels (HairE) have been shown to reflect integrated long-term cortisol and cortisone levels, and are promising endocrine markers of chronic (psychological and physical) stress. We aimed to use hair corticosteroid analysis to assess corticosteroid exposure of a three-month period in persons with a depressive and/or anxiety disorder and to compare their levels with those of healthy controls and remitted persons.

Methods

Data from 1166 participants of the Netherlands Study of Depression and Anxiety (NESDA) were used. A total of 266 participants had a recent (1-month) diagnosis of a depressive and/or anxiety disorder, 655 had remitted disorders, and 245 had no lifetime diagnosis (healthy controls). HairF and HairE were measured in the proximal three cm of scalp hair, using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results

The presence of a comorbid depressive and anxiety disorder was significantly associated with increased HairF levels (p = .031), as was the severity of depressive symptoms (p = .029) and, with trend significance, the severity of anxiety symptoms p = .069). Remitted disorders were not associated with altered HairF or HairE levels.

Conclusions

This study demonstrates that persons with current severe symptoms of depression and/or anxiety, but not those with remitted symptoms, show higher levels of cortisol in scalp hair, which is indicative of a chronic overactivation of the HPA-axis.

INTRODUCTION

Dysfunction of the hypothalamus-pituitary-adrenal (HPA)-axis may result in increased or decreased levels of its end-products, cortisol, commonly referred to as "the stress hormone". A disproportionate change in cortisol levels is one of the main hypothesized pathophysiological mechanisms underlying stress-related disorders. Indeed, depressive and anxiety disorders have been linked to altered cortisol concentrations using point measures taken from urine, blood or saliva [1-5]. However, findings have been inconsistent regarding relative hyper- or hypocortisolism in different (subtypes of) disorders. This problem coincides with one shortcoming of these methods, i.e. that they only reflect cortisol levels over a relative short time period, i.e. minutes to several days, thereby not reflecting chronic exposure to cortisol excretion, which likely is more relevant in terms of health effects. Related, a question that remains to be answered is whether altered cortisol levels represent a trait or state effect in depression [6] and anxiety. One possibility to shed more light on these issues is the use of a relatively new method to assess long-term cortisol concentrations, i.e. the analysis of cortisol in hair.

Hair cortisol concentrations (HairF) are assumed to reflect integrated long-term cortisol levels, and have been proposed as a promising endocrine marker of chronic stress. Higher HairF in patients with depression compared to healthy controls have been reported [7, 8], however, other studies showed no difference [9, 10]. Depressive symptoms were in some [11, 12] but not other [13, 14] studies associated with HairF. These studies used depressive symptoms rather than the diagnosis of a major depressive disorder (MDD) in diverse populations with various comorbidities. Research on HairF and anxiety disorders has also produced first results; posttraumatic stress disorder (PTSD) has been associated with increased HairF in the months after the trauma, followed by decreased HairF in the longer run [15-17]. HairF are lower in generalized anxiety disorder (GAD) [18], unchanged in social phobia [19], and were not associated with anxiety symptoms or severity of anxiety in patients with various other primary diagnoses [8, 20, 21].

To summarize, first studies have been published, but there still is a considerable lack of consistent information on the relationship with depressive and anxiety disorder characteristics such as age of onset, duration of disorder, severity, and use of antidepressants. Furthermore, most studies involved small samples (many less than n = 50), which may partially explain discrepant results across studies. The development of new techniques to measure HairF have led to the possibility to measure other steroid hormones in scalp hair as well. One of these other hormones is cortisone, the inactive form of cortisol. The enzyme 11β-hydroxysteroid dehydrogenase type 1 (11βHSD-1) converts cortisone into active cortisol, whereas its counterpart 11β-hydroxysteroid dehydrogenase type 2 (11βHSD-2) converts active cortisol to inactive cortisone. The assessment of hair cortisone (HairE) in parallel to HairF has been postulated to provide even more insight into

the cumulative amount of active and inactive corticosteroids in the body, and that HairE levels may provide a useful and robust marker of long-term HPA-axis activity [22]. Apart from the absolute levels of HairF and HairE, the cortisol-to-cortisone ratio is also of interest, as it reflects the 11βHSD-activity [23]. The importance of this ratio in stress-related research has been emphasized by studies using urinary measures showing an altered ratio under stress [24] and demonstrating an enhanced ratio in depressed patients [25, 26], suggestive of an increased conversion to active cortisol by 11βHSD-1.

Measurement of hair cortisone concentrations (HairE) have been shown by our group and by others to be easily measurable and to correlate with HairF [22, 23, 27, 28]. Until now, no study has investigated the associations between HairF, HairE, the ratio HairF/HairE, and (characteristics of) depressive and anxiety disorders while accounting for diverse covariates and using a large sample. Insight into the amounts of long-term cortisol and cortisone, as well as their ratio, may give new insights into the underlying pathophysiology of the HPA-axis and its role in depressive and anxiety disorders.

In the present study, we used data from the Netherlands Study of Depression and Anxiety (NESDA) to examine whether there are differences in HairF, HairE and their ratio (HairF/HairE) between healthy control subjects and subjects with a depressive disorder or an anxiety disorder. As the NESDA project comprises a large sample size, the unique set-up allows us to run statistical analyses on patients with current, remitted, and no disorders which provide insight into state versus trait associations and to examine the impact of specific characteristics, such as symptom severity and medication use, on the association between psychopathology and hair corticosteroid levels.

MATERIALS AND METHODS

Study Design

Data were used from the Netherlands Study of Depression and Anxiety (NESDA), an ongoing longitudinal cohort study on the predictors, course and consequences of depressive and anxiety disorders. The NESDA sample consisted of 2,981 participants aged 18–65 years at inclusion, comprising persons with no depressive or anxiety disorder, persons who have had a disorder in the past, and persons with a current depressive and/or anxiety disorder. A detailed description of the NESDA study rationale, design, and sampling procedure can be found elsewhere [29]. The research protocol was approved by the Ethical Committee of the participating centres, and all participants provided written informed consent. Hair data collection was added to NESDA's 6-year follow-up assessment, in which 2256 participants of the initial 2981 sample participated (75.68%). Therefore, the 6-year follow-up wave is considered baseline for the present analysis, and all determinants considered were measured at that wave. For the present study, from

the 2256 participants, we selected all participants that had sufficient hair on the posterior vertex position of the head, the willingness to participate, and a hair sample weight of at least 5 mg (n = 1677). The participants filled in a self-developed questionnaire on their hair (treatment) characteristics. Psychiatric diagnoses were defined on the basis of the Composite International Diagnostic Interview (CIDI, versions 2.1) [30], which classifies diagnoses according to the DSM-IV criteria. Exclusion criteria were the diagnosis of bipolar disorder within the past year (n = 22), use of lithium (n = 11), and current use of systemic or local corticosteroids in the past three months (n = 320), resulting in a sample of 1264 subjects.

HairF and HairE measurement

During the visit at the research facility, hair strands of approximately 100 hairs were cut as close as possible from the scalp from a posterior vertex position. The most proximal three cm of hair were used for analysis. Based on a mean hair growth rate of one cm per month [31], hair samples reflect the cumulative cortisol and cortisone secretion of the previous three months. Hair samples were weighted, finely cut with surgical scissors, and washed with 1.0 mL of LC-MS grade isopropanol for two minutes. The extraction and subsequent analysis of cortisol and cortisone by liquid chromatography-tandem mass spectrometry has extensively been described elsewhere [32]. The inter-day variation in the present study was <8.3% for cortisol and <4.8% for cortisone [27]. Steroid peak integrations were reviewed and manually integrated by two independent persons when automated peak integration feature incorrectly or partially integrated peaks. In 98 individuals, HairF or HairE levels could not be determined due to interference in the hair sample. The analyses were successfully performed in 1166 participants, rendering our final study sample.

Psychiatric characteristics

The DSM-IV Composite International Diagnostic Interview (CIDI) was used to assess diagnoses of depressive disorders (dysthymia, MDD) and anxiety disorders (social phobia, panic disorder, agoraphobia, panic disorder with agoraphobia, and GAD).

To investigate whether HairF and HairE levels were different between participants with a current diagnosis, participants with a remitted diagnosis, and healthy controls, we first created three groups of subjects. The first group consisted of participants with a current (1 month) depressive and/or anxiety disorder diagnosis (n = 266). The second group consisted of participants with no current (1 month), but a remitted diagnosis of depressive and/or anxiety disorder (n = 655). The third group comprised psychologically healthy subjects with no lifetime history of either depressive or anxiety disorders and served as control group (n = 245).

Chapter 3

The total score of the 30-item self-report version of the Inventory for Depressive Symptomatology (IDS) was used to measure the severity of depressive symptoms (range: 0 - 84). The total score of the Beck Anxiety Inventory (BAI) was used to assess the severity of anxiety symptoms (range: 0 - 63). Additionally, the Fear Questionnaire (FQ) was used as an indicator of phobic symptoms (range: 0 - 120) and the total score of the childhood trauma questionnaire (CTQ) was used as a measure of childhood traumatization (range: 0 - 100).

To account for the use of antidepressants, we distinguished tricyclic antidepressants (TCA, ACT-code N06AA), selective serotonin reuptake inhibitors (SSRI, ATC-code N06AB), and other antidepressants (ATC-code N06AF/N06AX). Whether respondents used antidepressants was based on drug container inspection of all drugs used in the past month at baseline and classified according to the World Health Organization Anatomical Therapeutic Chemical (ATC) classification. Duration of symptoms within the previous two years was assessed with a lifechart.

Covariates

We previously described the effects of age, diagnosis of diabetes mellitus, hair washing frequency (0-3 times/week, > 3 times/week), and season (winter, spring, summer, fall) reflected in hair sample on HairF and HairE levels [27]. These will therefore be used as covariates in this study. Additionally, we included sex and hair treatment as covariates, as these factors were associated with HairF and/or HairE in other studies [33, 34]. Other covariates such as waist circumference, smoking, and alcohol intake were not found to be related to HairF and HairE levels in our previous study [27] therefore not considered in our analyses.

Statistical analyses

For descriptive analyses, HairF and HairE are provided in pg/mg and are reported as median (Mdn) and interquartile range (IQR). For inferential analyses, HairF and HairE values were subjected to a logarithmic transformation to achieve normal distribution. Other data, unless otherwise indicated, is presented in original units with mean (M) and standard deviation (SD), or in sample size (n) and percentage (%). Baseline characteristics of the three groups were compared using χ^2 and analysis of variance statistics. Differences between HairF and HairE levels of groups of participants were conducted with univariate analyses of covariance (ANCOVA) and with linear regression analyses. Results are derived from partially-adjusted (adjusted for sex and age) and as fully-adjusted (adjusted for all covariates) analyses. The significance level was set at p <0.05. All analyses were conducted using SPSS 20.0 for Windows (IBM, Chicago, Illinois). For significant findings, effect sizes were calculated with Cohen's d [35] between healthy controls and the group in question.

Of the 1166 participants, n = 1075 provided a full dataset without any missing values on covariates. Multiple imputation was used to complete the dataset, assuming a missing at random approach and creating five imputed datasets. All characteristics were used as predictors to impute missing values, and the observed minimum and maximum values of the original full dataset were used as constraints. The pooled estimates of the five imputed datasets are reported.

RESULTS

Sample characteristics
Characteristics across groups are presented in Table 1. A total of 245 subjects had no current or lifetime diagnoses (healthy controls), 655 participants had remitted depressive and/or anxiety disorders, and 266 participants presented with a current (past one month) diagnosis of depressive disorder (n = 78), anxiety disorder (n = 122), or comorbid both depressive and anxiety disorder (n = 66).

As expected, participants with a current diagnosis had higher scores on the psychiatric questionnaires and higher use of antidepressants than had remitted participants, who in turn scored higher on psychiatric questionnaires and use of antidepressants than healthy controls.

HairF and HairE
The median HairF of the sample was 3.26 (2.20 – 5.47) pg/mg hair, whereas the median HairE of this sample was 10.68 (7.58 – 15.36) pg/mg hair. HairF and HairE showed a significant positive linear correlation with each other, r = .58, p <.001.

Long-term corticosteroids in relation to remitted and current diagnoses
Comparing HairF, HairE and the ratio HairF/HairE between the three groups (healthy controls, remitted diagnoses, current diagnoses) showed no difference in any of these parameters between the groups (F (2, 1153) = 1.217, p = .296 for HairF, F (2, 1153) = 1.366, p = .255 for HairE, and F (2, 1153) = .355, p = .715 for HairF/HairE, respectively). The presence of a remitted disorder, analyzed as one group and as subgroups of remitted depressive disorder, remitted anxiety disorder and remitted comorbid depressive and anxiety disorder, was not significantly or trend associated with HairF, HairE, or HairF/HairE (p > .10). Regarding current diagnoses, a current depressive disorder (n = 78) and a current anxiety disorder (n = 122) were not associated with altered HairF, HairE, or HairF/HairE (all p > .10). A current comorbid depressive and anxiety diagnosis (n = 66) was associated with elevated HairF (β = .067, p = .031, d = .33), see Figure 1, and trend-associated with HairF/HairE (β = .055, p = .086, d = .34) but not with HairE (p > .10).

Table 1. Descriptive information of the study population

Characteristics	Healthy controls (n = 245)	Remitted diagnosis (n = 655)	1-month diagnosis (n =266)	p-value
Sociodemographics				
Sex (female)	153 (62.4 %)	513 (78.3 %)	198 (74.4 %)	**<.001**
Age (in years)	45.77 (15.05)	46.81 (12.95)	48.17 (12.78)	.122
Health and lifestyle indicators				
Diabetes mellitus	5 (2.0 %)	25 (4.1 %)	11 (4.1 %)	.306
Hair (treatment) characteristics				
Hair treatment in past 3 months	74 (30.2 %)	253 (38.6 %)	105 (39.5 %)	**.041**
Dyed	12 (4.9 %)	62 (9.5 %)	17 (6.4 %)	
Bleached	63 (25.7 %)	206 (31. 5 %)	90 (33.8 %)	
Permed	2 (.1 %)	8 (1.2 %)	6 (2.3 %)	
Hair washing frequency (> 3/week)	176 (71.8 %)	460 (70.2 %)	184 (69.2 %)	.802
Season represented in hair				.076
Winter	43 (17.6%)	173 (26.5%)	75 (28.2%)	
Spring	59 (24.1%)	154 (23.5%)	65 (24.4%)	
Summer	65 (26.5%)	164 (25.5%)	61 (22.9%)	
Fall	78 (31.8%)	164 (25.5%)	65 (24.4%)	
Psychiatric aspects				
IDS	5.53 (5.53)	13.02 (8.53)	26.32 (12.98)	**<.001**
BAI	2.99 (4.05)	6.95 (6.11)	15.57 (1.07)	**<.001**
FQ	7.71 (8.77)	15.28 (13.77)	30.03 (18.76)	**<.001**
CTQ	33.59 (10.20)	39.94 (12.56)	45.07 (14.89)	**<.001**
Age of onset	n/a	22.41 (12.98)	16.98 (11.28)	**<.001**
Duration of depressive symptoms in prior 2 years (months)	n/a	3.24 (6.88)	13.12 (10.49)	**<.001**
Duration of anxiety symptoms in prior 2 years (months)	n/a	5.59 (9.43)	16.91 (9.83)	**<.001**
Use of antidepressants				**<.001**
TCA	0	16 (2.4 %)	16 (6.0 %)	
SSRI	1 (0.4 %)	88 (13.44 %)	48 (18.0 %)	
Other antidepressants	0	27 (4.1 %)	35 (13.2 %)	

Data are presented as pooled mean (pooled standard deviation), and as pooled n (percentage). IDS, Inventory of Depressive Symptomatology: BAI, Beck Anxiety Inventory; FQ, Fear Questionnaire; CTQ, Childhood Trauma Questionnaire; n/a, not applicable; TCA, Tricyclic Antidepressant; SSRI, Selective Serotonin Reuptake Inhibitor.

The partially and fully adjusted models for HairF, HairE, and HairF/HairE in relation to disorder status are shown in Table 2.

In subgroup analyses (not in the Table), we explored current subtypes of depressive disorders (major depressive disorder, n = 43; dysthymia, n = 17; both major depressive disorder and dysthymia, n = 18) and of anxiety disorders (social phobia, n = 29;
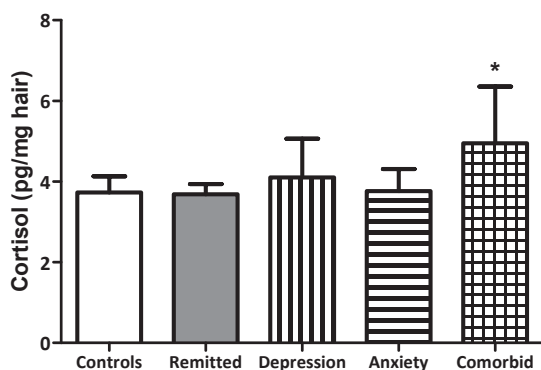
Figure 1. *HairF values for healthy controls, participants with a remitted diagnoses, a current depressive disorder, a current anxiety disorder, and a current comorbid disorder. HairF levels are expressed as geometric mean with 95% CI. *, p < .05 in the fully-adjusted analyses.*

panic disorder with agoraphobia, n = 19; panic disorder without agoraphobia, n = 10; agoraphobia, n = 28; generalized anxiety disorder, n = 15; and more than one anxiety disorder, n = 21). The presence of current social phobia and the presence of more than one current anxiety disorder simultaneously were associated with increased HairE (β = .074, p = .01, d = .53, and β = .058, p = .041, d = .24, respectively). No other significant or trend associations emerged (all p > .10).

Long-term corticosteroids and severity measures

The severity of depressive symptoms (indicated by the IDS total score) was associated with HairF in the whole sample of participants (β = .63, p = .029), whereas the severity of anxiety (indicated by the BAI total score) was only trend-associated with HairF (β = .52, p = .069) (Figure 2). HairE showed no association with any of the severity scores (all p > .10). The ratio HairF/HairE was trend-significantly associated with the severity of depression (IDS), anxiety (BAI), and phobic symptoms (FQ) (β = .52 – .57, all p < .10).

Long-term corticosteroids in relation to disorder characteristics

In participants with a current diagnosis of a depressive and/or anxiety disorder (n = 266), age of onset and duration of psychopathology was not associated with HairF, HairE, and the ratio HairF/HairE (all p > .10) (not in the Table).

Examining the relation of antidepressant medication revealed that the use of SSRIs was associated with increased HairF (β = .074, p = .010, d = .28) and trend-significantly associated with the ratio HairF/HairE (β = .054, p = .066, d = .27). No other associations between HairF, HairE, the ratio HairF/HairE and use of antidepressants reached significance (all p > .10). The use of SSRIs and the severity of depression were independently associated with elevated HairF (use of SSRIs: β = .070, p = .015; severity depression: β = .062, p = .038) when analyzed within one model. The use of SSRIs and the severity of anxiety also retained their independent (trend-) association with HairF (use of SSRIs : β = .072, p = .012; severity anxiety: β = .049, p = .098) when analysed within one model (not in the Table).

Table 2. Associations between depression and/or anxiety diagnosis status and HairF and HairE.

	HairF: Partially adjusted analyses β	HairF: Fully adjusted analyses β	HairE: Partially adjusted analyses β	HairE: Fully adjusted analyses β	Ratio: Partially adjusted analyses β	Ratio: Fully adjusted analyses β
Long-term corticosteroids in relation to remitted and current diagnoses						
Healthy controls (n=245)	Ref		Ref		Ref	
Remitted depressive and/or anxiety disorder (n=655)	<.001	.003	-.006	-.002	.030	.029
Current depressive and/or anxiety disorder (n=266)	.043	.046	.040	.044	.027	.025
Long-term corticosteroids and current diagnoses						
Healthy controls (n=245)	Ref		Ref		Ref	
Current depressive disorder (n = 78)	.018	.022	.017	.020	.023	.024
Current anxiety disorder (n = 122)	.002	.008	.026	.033	-.019	-.019
Current comorbid disorder (n=66)	.072*	.067*	.033	.032	.059†	.055†
Long-term corticosteroids and severity						
IDS	.064*	.063*	.015	.018	.057†	.054†
BAI	.054†	.052†	.019	.023	.056†	.052†
FQ	.049†	.040	.005	.001	**.062***	.057†
CTQ	.043	.038	.043	.040	-.001	-.002
Long-term corticosteroids and antidepressant medication						
No medication	Ref		Ref		Ref	
TCA	-.025	-.022	-.027	-.023	-.010	-.011
SSRI	**.072***	**.074***	.040	.046	**.058***	.054†
Other AD	-.029	-.029	-.034	-.037	-.015	-.012

Data are presented as standardized coefficients (β). *, p < .05; †, p <.10.

IDS, Inventory of Depressive Symptomatology; BAI, Beck Anxiety Inventory; FQ, Fear Questionnaire; CTQ, Childhood Trauma Questionnaire; TCA, Tricyclic Antidepressant; SSRI, Selective Serotonin Reuptake Inhibitor; AD, antidepressants.

Partially adjusted, adjusted for age and sex. Fully adjusted, adjusted for age, sex, diagnosis of diabetes mellitus, hair washing frequency, season, and hair treatment.
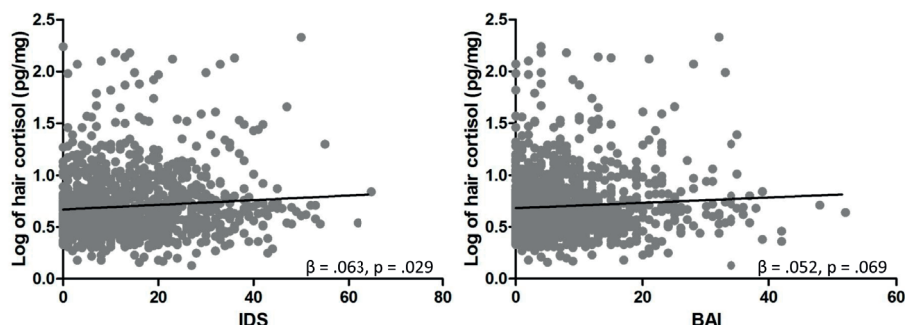
Figure 2. *Log-transformed HairF values and their association with the severity indexes for depression and anxiety. IDS, Inventory of Depressive Symptomatology: BAI, Beck Anxiety Inventory. Values are derived from fully-adjusted models.*

DISCUSSION

The current study examined HairF and HairE levels as well as the ratio of HairF/HairE in a large sample with and without depression and anxiety. One aim of our study was to gain insight into the amounts of long-term cortisol and cortisone, as well as their ratio, in depressive and anxiety disorders. The 3.3-fold increase of HairE over HairF corresponds to previously reported research [10, 22, 23, 28]. We found that the current severity of symptoms was associated with higher long-term HairF levels rather than the presence of a specific current or remitted diagnosis per se. One explanation for this result could be the associated disease burden and daily impairments, and therefore increased stress that may come with a more severe disorder. This hypothesis would be supported by research showing that increased stress has often been associated with increased HairF [12, 36, 37]. This might also be reflected by the association between HairF and the presence of a current comorbid depressive and anxiety disorder, in which a comorbid disorder might be interpreted as a more severe disorder than one single disorder. Indeed, the comorbid group had significantly higher scores on the depressive and anxiety severity scales than the other participants (data not shown). A more technical explanation could be that the severity data had higher variation and thus more statistical power compared to analyses on the diagnostic groups.

We also explored whether subtypes of depressive and anxiety disorders were differently associated with HairF, HairE, or HairF/HairE. However, these results have to be interpreted cautiously due to the small sample sizes. We found indications that the diagnosis of social phobia may be associated with increased HairE. The association between social phobia and HairF was subject to one other study and the authors found no differences in HairF between healthy controls and patients with social phobia [19]. This is in line with our results. The association between social phobia and HairE has not been

described yet. We did not find associations of HairF, HairE, or HairF/HairE with any other anxiety disorder. Until now, one study that looked into HairF and GAD found decreased HairF in patients and one study on bipolar patients found lower HairF for patients with a comorbid panic disorder [38]. The presence of (only a) current depressive disorder did not show a relationship with HairF, HairE, or the ratio HairF/HairE. For MDD, conflicting results regarding the association between diagnosis and HairF have been reported [7-9, 13]. To our knowledge, dysthymia has not been assessed in other studies until now.

Another aim was to use the information on the level of HairF, HairE, and the ratio HairF/ HairE to gain new insights into the underlying pathophysiology of the HPA-axis and its role in depressive and anxiety disorders. This association seems to be less straightfor- ward than initially assumed. The significant results were found in participants with a current diagnosis, whereas remitted patients did not show altered long-term cortico- steroid levels. This is an interesting result, as it poses a possible answer to the question whether a dysregulated HPA-axis in mentally ill patients is a state or trait phenomenon. A stronger association with active cortisol rather than inactivated cortisol, as found in our study, may indicate that the alterations in HairF associated with psychopathological characteristics seem to be a state (reflected by HairF) rather than a trait (reflected by HairE). This would imply that during the episode of a disorder, the HPA axis is dysregu- lated, but that the cortisol levels return to normal once the patient remits. This could be supported by the results that remitted patients did show long-term corticosteroid levels comparable to healthy controls. Interestingly, a study that investigated differences in HairF between healthy controls, patients with a recurrent depressive disorder and patients with a first-episode disorder found that only patients with a first episode had increased HairF, whereas patients with a recurrent depressive episode had comparable HairF as healthy controls [8]. The authors hypothesize that the duration or the number of recurring episodes of the depressive episode might alter the sensitivity of HPA axis of the patients with a recurrent depressive episode. This gives rise to the question whether the underlying pathophysiology is different for persons with a first/single episode com- pared to persons with recurrent episodes. In our study, the duration of mental disorders showed no association with long-term corticosteroid levels. Future research will have to further shed light into these associations.

To our knowledge, this is the first study to assess relationships between the ratio HairF/ HairE and psychopathological characteristics. The ratio did not reach significance in as- sociations; however, several trend-associations emerged. These trends were achieved in analyses showing significant associations with HairF but not HairE, which could mean that the trends are driven by the associations with HairF, making it likely that the ratio and the 11βHSD-activity do not provide additional information in hair research.

Another interesting finding is that the use of SSRIs is associated with higher HairF. The use of antidepressants has been shown to influence the HPA-axis in many ways, from

regulation of glucocorticoid receptor (GR) expression to post-translational modifications, which may result in differences in GR nuclear translocation and GR-dependent gene transcription [39, 40]. Two other studies reported associations between HairF and psychotropic drugs [34, 41], whereas a third study did not find differences in HairF between users and non-users [42]. Apart from the working mechanisms of SSRIs on the HPA axis, another explanation could be that patients with more severe depressive symptoms may tend to use medication as opposed to patients with minor symptoms. However, the use of SSRIs and the severity of depression were independently associated with elevated HairF, rendering this hypothesis unlikely. Other explanations have yet to be found.

For significant effects, effect sizes were calculated, with effects ranging from .24 - .53 and thereby reflecting small to moderate effects. This is in line with previously calculated effect sizes in long-term corticosteroid research [36], in which for long-term cortisol and mental illness, most effect sizes were small to medium. Our effect sizes are also comparable to those of a study assessing salivary cortisol levels, in which persons with a comorbid anxiety and depressive disorder had higher morning cortisol levels than healthy controls, with effect sizes ranging from .25 - .30) [2].

This study has several strengths and limitations that need to be acknowledged. The current study is the first study that included participants from a large, longitudinal cohort study on depression and anxiety. This allowed us to additionally study associations between long-term glucocorticoids and pure diagnostic subgroups free from other comorbid psychological disorders while still retaining sample sizes comparable to other studies. Furthermore, extensive information on covariates and psychological characteristics were available. However, the cross-sectional nature of the study did not allow us to draw causal conclusions about the direction of associations between long-term glucocorticoids and depressive as well as anxiety symptoms. Another limitation is that although our study comprised a large sample size, the size of the diagnostic (sub)groups was rather small for some subgroups. Another factor that needs to be mentioned is that the time frame represented by the hair (i.e. three months) did not correspond to the time frame of the diagnosis used here (i.e. one month). This unequal time frame was caused by practical reasons, in that we had too little hair samples per person to allow for hair measurements of one cm. However, therefore we cannot exclude the possibility that we underestimate the effects of depressive and anxiety disorders on HairF and HairE, as also a healthy/diagnosis-free period may be covered by the hair sample.

To conclude, this study demonstrates that persons with current severe symptoms of depression or anxiety, but not those with remitted symptoms, show higher long-term levels of cortisol, as measured in scalp hair, which is indicative of chronic overactivation of the HPA-axis.

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04

RECENT NEGATIVE LIFE EVENTS INCREASE HAIR CORTISOL CONCENTRATIONS IN PATIENTS WITH BIPOLAR DISORDER

ABSTRACT

Background

Life events induce stress, which is considered to negatively impact the course of disease in patients with bipolar disorder (BD), its effects being predominantly mediated by cortisol. Cortisol in scalp hair has been identified as a biomarker for assessing long-term cortisol levels, and allows clarifying the relation between life events, hair cortisol concentrations (HCC), and clinical course over time.

Methods

In 71 BD patients, we analyzed the proximal 3 cm of hair, reflecting 3 months of cortisol production, and investigated the association between HCC, the number of life events, the amount of social support, and mood in the 3 months prior to the hair assessment and between HCC and mood in the subsequent 3 months.

Results

Although the total number of life events was not associated with HCC ($p > 0.05$), the number of negative life events was associated with increased HCC ($r^2 = 0.04$, $p = 0.02$). Social support showed an inverse association with HCC in patients reporting negative life events ($r^2 = 0.07$, $p = 0.03$). HCC and mood were neither associated in the 3 months prior to hair sampling nor in the subsequent 3 months.

Conclusions

This study indicates that patients who experienced recent negative life events have increased hair cortisol levels, which seem to be attenuated by social support.

INTRODUCTION

Evidence for stress as cause or amplifier of a wide range of somatic and mental disorders has been firmly established [1]. Individuals with a mental illness such as bipolar disorder (BD) are more likely to report the experience of a stressful life event and may also be more affected by the consequences of stress than healthy persons [2]. BD is a chronic mental illness that causes people to have one or more episodes of high (manic) and low (depressed) mood. The occurrence of life events as well as the hypothalamic-pituitary-adrenal (HPA) axis has been reported to play a potential role in the induction of a new affective episode [3-4]. A life event is defined as a 'dateable occurrence representing discrete changes in the subject's social or personal environment that is external and verifiable rather than internal or psychological' [5-6]. The availability or lack of social support is one of those environmental factors that have been widely recognized as a factor that may promote psychological well-being and physical health [7-8]. Social support has been defined as the actuality and the perception that one is cared for and valued, has assistance available from other people, and that one is part of a supportive social network. It can involve emotional support as well as instrumental support, information and companionship [9-11]. Social support can buffer the impact of stressful experiences and it can function as a coping mechanism [12].

BD has been associated with a dysregulation of the endocrine stress system, the HPA axis [3]. Conflicting data have been published on cortisol levels, the end product of the HPA axis. Normal as well as elevated basal salivary and serum cortisol levels have been reported [13-16]. These inconsistent findings could partly be caused by methodological differences between the studies but also by differential exposure to stressful life events.

The possibility to measure cortisol in scalp hair has the advantage of being non-invasive and, unlike measurements in blood, saliva or urine, is insensitive to daily variation, short-term transient stress, and oral contraceptives [17], and to reflect the cortisol concentration of several weeks or months [18].

In our previous study on long-term cortisol in bipolar patients, no difference in hair cortisol concentrations (HCC) between mood episodes was found, however, increased HCC were observed in patients with a late age of onset (>30 years) [19]. Recently, evidence was provided that the occurrence of stressful life events increases HCC in healthy young adults as well as in crack cocaine users [20-21].

Until now, it is not known whether patients with BD show increased HCC after a major life event, and whether increased HCC after a life event are associated with mood symptoms. Initial results in a longitudinal study on our cohort showed an effect of life events on mood symptoms and functional impairment, especially in patients with BD I as compared to BD II [22].

Chapter 4

We aimed to explore the effects of life events and social support on hair cortisol levels in bipolar patients and to investigate whether HCC are associated with mood in the subsequent three months.

MATERIALS AND METHODS

Study Design

This is a cross-sectional and 3-month prospective study among outpatients with a diagnosis of BD. The current project is part of "The Bipolar Stress Study", which is a cross-sectional and 24 month longitudinal study that aims on identifying risk factors that have an impact on the clinical course of BD and treatment of patients with BD. In the present study, we focused on the association between life events and HCC in patients with BD, and explore whether HCC in patients with life events are predictive of mood in the subsequent three months The study was approved by the local medical ethics committee and carried out in accordance with the declaration of Helsinki. After complete description of the study, all patients gave their informed consent.

Participants

Patients with BD from the same cohort whose hair cortisol characteristics have previously been published in our article concerning cross-sectional data [19] were included in this study. All patients were participants of The Bipolar Stress Study and were treated in the local outpatient Department of Mood Disorders in The Hague, the Netherlands. During their regular visits every three months at the outpatient clinic, information on mood and life events was assessed. The hair sample was obtained at the seventh measurement (after 21 months of participation); the second last assessment in this study. Detailed description of the assessment methods of the patients has been described elsewhere [19, 23]. Patients were eligible for inclusion if they had not been using glucocorticoids in the 6 months prior to hair sample collection and if they had sufficient hair growth at the posterior vertex. The first 100 patients that fulfilled these requirements were asked to participate and to provide a hair sample.

Patients were interviewed by trained psychologists 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 [24] using the Dutch version of the MINI International Neuropsychiatric Interview Plus (version 5.00-R; MINI-PLUS; [25]). Patients were classified in subtypes according to the DSM-IV-TR: bipolar I disorder, bipolar II disorder, cyclothymia, and bipolar disorder NOS, which vary in severity and frequency of mood episodes. BD I is characterized by one or more manic episodes, whereas BD II is defined by no manic episodes, but one or more hypomanic

episodes and one or more major depressive episode. The Questionnaire for Bipolar Illness, Dutch translation [26-27] was used to identify subtypes of BD, its course over time and detailed information about age of onset of first symptoms regarding hypomanic, manic and depressive episodes.

Hair sample collection, preparation, and cortisol measurement

From all patients, approximately 100—150 hairs were cut from the posterior vertex as close to the scalp as possible. Hair sample preparation has been described in detail elsewhere [18]. In short, the hair was taped to a paper and stored until preparation. The 3 cm hair segments most proximal to the scalp were weighted in separate glass vials and then minced with small surgical scissors. One mL of methanol was added to extract cortisol from the hair samples and incubated for 16 h at 52°C while gently shaking. Afterwards, the methanol was transferred to a clean glass vial and was evaporated under a constant nitrogen stream until completely dry. The samples were then dissolved in 250 microliter phosphate buffered saline (pH 8.0) and 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 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 reported by the supplier. The low-end detection limit for this assay is 1.5 nmol/L. HCC are reported as median and interquartile range (IQR).

Life events

The occurrence of life events in the previous three months was assessed with *Paykel's self-report questionnaire* consisting of 61 life events [28]. Patients indicated which events on the list occurred within the preceding three months. The 61 single life event items were a priori grouped into three main categories: negative life events, positive life events, and ambiguous life events. Events were categorized by independent raters, since patients' rating of an event may be influenced by their current mood state. A total of 39 different negative life events consisted of life events such as increasing arguments with the spouse, relationship break-up, business failure, serious illness of a family member, failure of an important exam, demotion at work, and unemployment for one month. A total of eleven positive life events consisted of life events such as promotion at work, engagement, marriage, and a desired pregnancy. Eleven life events were rated as neutral or ambiguous events (change of work field, change of work hours, moving).

Social support

The presence and degree of social support was assessed with the Social Support List (*Sociale Steun Lijst* (SSL; [9]). This list quantifies two aspects of social support: 'frequency of social support' measures the frequency of social support that the patient receives; and 'perceived lack of social support' is the perceived difference in social support between that which is desired and what is received by the patient. Patients rated items from the three subscales using a 4-point scale to indicate the frequency in which they received social support ("frequency"). For the "perceived" score, patients rated the perceived discrepancy between the desired and received level of social support on a 4-point scale, ranging from too little to too much. The scores were summed for each variable, with a higher score indicating a higher amount of social support.

Mood

Illness severity was assessed in two ways. Symptom severity as well as the functional impact of the mood disturbances was measured. The observer based *Young Mania Rating Scale* (YMRS, [29]) was used to assess the number and severity of mania symptoms. The *Quick Inventory of Depressive Symptoms* (QIDS, [30]) was administered to assess the number and severity of depression symptoms. Both the YMRS and the QIDS were administered during the visit at the outpatient clinic. The functional impact of mood disturbances was measured with the monthly retrospective life chart method (LCM-r) by the National Institute of Mental Health (NIMH) [31]. This instrument was used to assess medication use and monthly functional impairment resulting from mania or depression symptoms in the subsequent three months after collecting the hair sample and was administered by the research psychologist during every visit at the outpatient clinic. Based on the life chart data, the mean severity of functional impairment was calculated by averaging the monthly severity scores for depression and mania of every three-month period between the visits. A similar method has been used previously [32].

Statistical analysis

SPSS 20.0 for Windows was used for statistical analysis. Hair cortisol levels, the number of life events, social support, age, and BMI were continuous variables, and gender, dyeing of hair, bleaching of hair, age of onset before/after the age of 30, comorbidities, and medication use were used as dichotomous variables. Pearson Chi-Square tests were used to compare frequency distributions. After log transformation, hair cortisol levels were normally distributed. Linear regression analyses were performed to analyze the association between the number of life events and HCC, between social support measures and HCC, and between HCC and mood. Backward stepwise regression was used to identify the best predictors of the following variables: gender, age, BMI, natural hair color, frequency of hair wash, use of hair products, use of lithium, use of antiepileptics, use

of antidepressants, use of antipsychotics, use of benzodiazepines, mood phase, bipolar disorder subtype, psychiatric comorbid disorders (except for panic disorder), comorbid panic disorder, age of onset, the presence of metabolic syndrome, and the presence of an endocrine disease. The final model consisted of the age of onset, presence of comorbid disorders, hair treatments, and use of benzodiazepines to account for their influences, and the variable in question, such as number of life events. The results of the linear regression are reported as standardized coefficients (beta), t-value of the life event variable, and r^2 change (increase in explained variation that the life event variable adds to the model). The association between life events and mood was analyzed using non-parametric (Spearman) correlation. The temporal relations are illustrated in Figure 1.
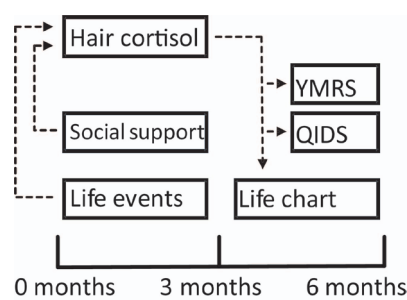

Figure 1. *Schematic overview of the assessment moments and the analyzed relationships between the different measurements. We investigated the influence of life events, social support, stability (life chart) and mood (QIDS, YMRS) on hair cortisol levels that were assessed at the 3-month assessment, and whether hair cortisol levels from the 3-month assessment had an influence on mood and stability as reported at the 6-month assessment.*

RESULTS

Hair cortisol measurements were available in 100 patients. For 96 of these patients, life event data were available. Of these 96 patients, 71 patients had complete information on the final set of covariates and constituted our final study sample. Ten patients reported a treated endocrine disease (n = 2 diabetes, n = 7 hypothyroidism, n = 1 diabetes and hypothyroidism), and a total of 17 patients presented with metabolic syndrome. Group characteristics are shown in Table1. The median HCC was 30.28 with an interquartile range from 22.73 to 48.96. Hair cortisol levels of patients with either metabolic syndrome or an endocrine disease were not different from the hair cortisol concentrations of the other patients (F(1,66) = 0.87, p = 0.35 and F(1,69) = 0.03, p = 0.86) respectively, and controlling for these variables did not alter the results.

Table 1. Group characteristics (n = 71)

Age (years) – median (IQR)	52 (43 – 62)
Number of women – n (%)	41 (57.7 %)
BMI (kg/m^2) – median (IQR)	25.5 (23.5 – 27.75)
HCC (pg/mg) – median (IQR)	30.28 (22.73 – 48.96)
Hair treatment	
Hair dyed	30 (42.3 %)
Hair bleached	8 (11.3 %)
Frequency of hair wash	
< 2 times/week	39 (54.9 %)
≥ 3 times/week	32 (45.1 %)
Age of onset BD	
< 30 years of age – n (%)	45 (63.4 %)
> 30 years of age – n (%)	26 (36.6 %)
Psychiatric comorbidities	
Anxiety disorder without panic disorder – n (%)	19 (26.8 %)
Somatoform disorder – n (%)	3 (4.2 %)
Pain disorder – n (%)	2 (2.8 %)
Panic disorder – n (%)	13 (18.3 %)
Medication	
Lithium – n (%)	59 (83.1 %)
Antiepileptics – n (%)	14 (19.7 %)
Antidepressants – n (%)	25 (35.2 %)
Antipsychotics – n (%)	24 (33.8 %)
Benzodiazepines – n (%)	18 (25.4 %)
Diagnosis	
Bipolar disorder type 1	54 (76.1 %)
Bipolar disorder type 2 and NOS	17 (23.9 %)

IQR, interquartile range; BMI, body mass index; HCC, hair cortisol concentrations; BD, bipolar disorder; NOS, not otherwise specified.

Life events and hair cortisol

Descriptive information of the life events is provided in Table 2. The total number of life events was not associated with HCC (Table 3). The number of negative life events was associated with increased HCC (β = 0.22, t(64) = 2.07, r^2 = 0.04, p = 0.04). Selecting the patients that only experienced negative life events (excluding all patients that reported any positive or ambiguous life events in addition to negative events), the results became even more significant (β = 0.31, t(64) = 2.54, r^2 = 0.08, p = 0.02), as illustrated in Figure 2. For positive life events and ambiguous life events, the analyses could not be performed separately due to small sample sizes.

Table 2. Reported life events by BD patients (n = 71)

Occurrence of at least 1 life event – n (%)	49 (69.0 %)
At least 1 negative event – n (%)	45 (63.4 %); only negative events – 32 (45.1 %)
At least 1 positive event – n (%)	12 (16.9 %); only positive events – 3 (4.2 %)
At least 1 ambiguous event – n (%)	7 (9.9 %); only ambiguous events – 1 (1.4 %)
Number of all events – M (SD)	1.70 (1.84)
Number of negative events – M (SD)	1.34 (1.56)
Number of positive events – M (SD)	0.24 (0.60)
Number of ambiguous events – M (SD)	0.13 (0.41)

Descriptive information on the occurrence of 61 life events. M, mean; SD, standard deviation.

Table 3. The effect of life events on HCC for the whole group and stratified for subgroups

All BD patients (n = 71)	
All life events	$\beta = 0.18$, $t(64) = 1.65$, $r^2 = 0.03$, $p = 0.10$
Negative life events	$\beta = 0.22$, $t(64) = 2.07$, $r^2 = 0.04$, $p = 0.04$
Only negative life events	$\beta = 0.31$, $t(64) = 2.54$, $r^2 = 0.08$, $p = 0.02$
BD I (n = 54)	
All life events	$\beta = 0.25$, $t(47) = 1.91$, $r^2 = 0.05$, $p = 0.06$
Negative life events	$\beta = 0.28$, $t(47) = 2.18$, $r^2 = 0.06$, $p = 0.03$
Only negative life events	$\beta = 0.31$, $t(47) = 2.18$, $r^2 = 0.07$, $p = 0.04$
BD II/NOS (n = 17)	
All life events	$\beta = -0.09$, $t(10) = -0.37$, $r^2 = 0.007$, $p = 0.72$
Negative life events	$\beta = -0.09$, $t(10) = -0.32$, $r^2 = 0.005$, $p = 0.76$
Only negative life events	$\beta = 0.36$, $t(10) = 1.11$, $r^2 = 0.06$, $p = 0.35$
Age of onset < 30 (n = 45)	
All life events	$\beta = 0.19$, $t(39) = 1.58$, $r^2 = 0.03$, $p = 0.12$
Negative life events	$\beta = 0.23$, $t(39) = 2.04$, $r^2 = 0.05$, $p = 0.05$
Only negative life events	$\beta = 0.28$, $t(39) = 2.28$, $r^2 = 0.07$, $p = 0.03$
Age of onset > 30 (n = 26)	
All life events	$\beta = 0.11$, $t(20) = 0.47$, $r^2 = 0.008$, $p = 0.64$
Negative life events	$\beta = 0.20$, $t(20) = 0.85$, $r^2 = 0.03$, $p = 0.41$
Only negative life events	$\beta = 0.39$, $t(20) = 1.47$, $r^2 = 0.11$, $p = 0.17$

HCC, Hair cortisol concentrations; BD, bipolar disorder; all life events, the total number of life events; negative life events, the number of negative life events in presence of positive and/or ambiguous life events; only negative life events, the number of negative life events in presence of no positive and/or ambiguous life events. The table shows the results of linear regression analyses. All p-values were adjusted for dyeing and bleaching of the hair, use of benzodiazepines, comorbidities except for panic disorder, and comorbid panic disorder. Except for the analysis stratified for age of onset, all values were also adjusted for age of onset before or after 30 years. R^2 indicates the change in r^2 of the model by adding the variable in question to the model.
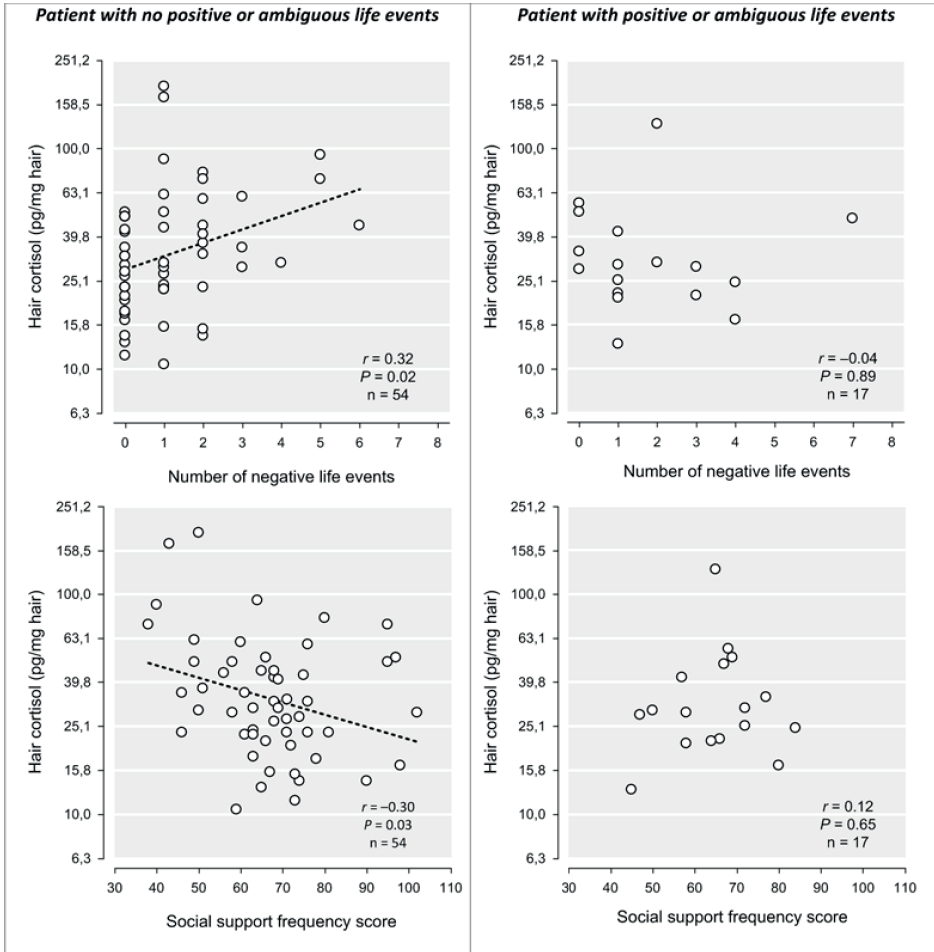
Chapter 4

Figure 2. *First row: The number of negative life events is associated with an increase in hair cortisol concentrations in patients with only negative life events, whereas in presence of positive or neutral life events, this association was not significant. This graph shows the inverse log-transformed cortisol values. Second row: The score of the frequency measurement of the Social Support List is associated with a decrease in hair cortisol concentrations in the presence of negative life events, whereas no association was found between social support and hair cortisol concentrations in patients with also positive or neutral life events.*

Based on the recent finding that life events relate to mood in BD I but not in BD II patients [22], analyses were stratified for subtype of BD. We found a trend for an effect of the total number of life events on hair cortisol for patients with BD I ($\beta = 0.25$, t(47) = 1.91, $r^2 = 0.05$, p = 0.06) whereas this was not significant for patients with BD II/NOS. More specifically, an increase in HCC was only seen in patients with BD I for the number of only negative events ($\beta = 0.31$, t(47) = 2.18, $r^2 = 0.07$, p = 0.04) but not in patients with BD II.

As our group showed before that age of onset before or after 30 years of age is associated with different HCC [19], analyses were also stratified for age of onset before or after 30. The total number of life events was not associated with HCC in either the early age of onset or the late age of onset patients. The number of negative life events was associated with increased HCC in patients with an early age of onset (β = 0.23, t(39) = 2.04, r^2 = 0.05, p = 0.05; only negative events: β = 0.28, t(39) = 2.28, r^2 = 0.07, p = 0.03), but not in patients with a late age of onset. Age of onset was not associated with BD subtype, and the frequency distribution was not different for the groups (χ (1, N=71) = 0.02, p = 0.90).

Hair cortisol and mood

Patients reported a mean score of 0.54 (SD = 1.94) on the YMRS (range of potential scores: 0 - 60) and a mean score of 6.90 (SD = 5.18) on the QIDS (range of potential scores: 0 - 27). The mean functional impairment as assessed with the LCM-r was 0.52 (SD = 0.67) for the depressive symptoms and 0.17 (SD = 0.39) for the manic symptoms. For both LCM-r measurements, the range of potential scores is 0 to 4). Hair cortisol was neither associated with mood symptoms in the three months prior to the hair collection and therefore corresponding to the time frame captured by the hair sample nor predictive of mood in the three months after taking the hair sample (all p > 0.05). The disorder state in the corresponding three months has earlier been shown to not be associated with HCC [19].

Life events and mood

The number of all life events correlated significantly with the QIDS score in the corresponding three months, ρ = 0.26, p = 0.03. The number of negative life events in presence of other life events also showed this association; ρ = 0.29, p = 0.02. Reducing the sample to the patients with only negative life events made the association even stronger, ρ = 0.33, p = 0.02. The relation between life events (all, negative, only negative), the YRMS score and the functional impairment for depressive symptoms and manic symptoms did not reach significance (all p > 0.05).

Hair cortisol, life events, and social support

The frequency of social support and the perceived social support showed a mean score of 66.58 (SD = 13.81) and -34.68 (SD = 12.14), respectively. The frequency of social support was inversely associated with HCC (β = -0.21, t(64) = -2.04, r^2 = 0.04, p = 0.05), as was the perceived social support (β = -0.22, t(64) = -2.06, r^2 = 0.04, p = 0.04). Further analyses revealed that the decreasing effect of the frequency of social support on hair cortisol concentrations was only present in patients who experienced negative life events (β = -0.28, t(38) = -2.21, r^2 = 0.07, p = 0.03), as illustrated in Figure 2, and not in patients who did not experience life events (Table 4). Perceived social support did not reach significance when stratifying for the occurrence of negative life events.

Table 4. The effect of social support on HCC in presence and absence of life events

All BD patients (n = 71)	
Frequency of social support	$\beta = -0.21$, $t(64) = -2.04$, $r^2 = 0.04$, $p = 0.05$
in no life events	$\beta = 0.46$, $t(15) = 0.46$, $r^2 = 0.006$, $p = 0.65$
in life events	$\beta = -0.27$, $t(42) = -2.21$, $r^2 = 0.07$, $p = 0.03$
in negative life events	$\beta = -0.28$, $t(38) = -2.21$, $r^2 = 0.07$, $p = 0.03$
Perceived social support	$\beta = -0.22$, $t(64) = -2.06$, $r^2 = 0.04$, $p = 0.04$
in no life events	$\beta = -0.11$, $t(14) = -0.55$, $r^2 = 0.30$, $p = 0.60$
in life events	$\beta = -0.18$, $t(41) = -1.45$, $r^2 = 0.03$, $p = 0.15$
in negative life events	$\beta = -0.18$, $t(37) = -1.39$, $r^2 = 0.03$, $p = 0.17$
BD I	
Frequency of social support	$\beta = -0.19$, $t(47) = -1.48$, $r^2 = 0.03$, $p = 0.15$
in no life events	$\beta = 0.14$, $t(9) = 0.87$, $r^2 = 0.02$, $p = 0.40$
in life events	$\beta = -0.29$, $t(31) = -1.98$, $r^2 = 0.07$, $p = 0.06$
in negative life events	$\beta = -0.30$, $t(29) = -1.97$, $r^2 = 0.08$, $p = 0.06$
in only negative life events	$\beta = -0.40$, $t(20) = -2.29$, $r^2 = 0.13$, $p = 0.03$
BD II	
Frequency of social support	$\beta = -0.34$, $t(10) = -1.46$, $r^2 = 0.09$, $p = 0.18$
in no life events	-
in life events	$\beta = -0.57$, $t(4) = -1.69$, $r^2 = 0.18$, $p = 0.19$
in negative life events	$\beta = -0.03$, $t(2) = -0.05$, $r^2 < 0.001$, $p = 0.97$
in only negative life events	-
Age of onset < 30	
Frequency of social support	$\beta = -0.09$, $t(39) = -0.72$, $r^2 = 0.007$, $p = 0.48$
in no life events	$\beta = 0.22$, $t(15) = 1.51$, $r^2 = 0.05$, $p = 0.17$
in life events	$\beta = -0.18$, $t(25) = -1.23$, $r^2 = 0.03$, $p = 0.23$
in negative life events	$\beta = -0.18$, $t(24) = -1.23$, $r^2 = 0.03$, $p = 0.23$
in only negative life events	$\beta = -0.28$, $t(16) = -1.85$, $r^2 = 0.07$, $p = 0.08$
Age of onset > 30	
Frequency of social support	$\beta = -0.64$, $t(20) = -3.96$, $r^2 = 0.33$, $p = 0.001$
in no life events	$\beta = -1.18$, $t(4) = -1.44$, $r^2 = 0.27$, $p = 0.25$
in life events	$\beta = -0.56$, $t(12) = -2.80$, $r^2 = 0.28$, $p = 0.02$
in negative life events	$\beta = -0.62$, $t(9) = -2.85$, $r^2 = 0.33$, $p = 0.02$
in only negative life events	$\beta = -1.18$, $t(4) = -3.02$, $r^2 = 0.45$, $p = 0.04$

HCC, Hair cortisol concentrations; BD, bipolar disorder; in negative life events, in presence of negative life events irrespective of concurrent positive and/or ambiguous life evens; only negative life events, in presence of negative life events while in absence of positive and/or ambiguous life events. The table shows the results of linear regression analyses. All p-values were adjusted for dyeing and bleaching of the hair, use of benzodiazepines, comorbidities except for panic disorder, and comorbid panic disorder. Except for the analysis stratified for age of onset, all values were also adjusted for age of onset before or after 30 years. R² indicates the change in r² of the model by adding the variable in question to the model.

As with life events, stratification for type of bipolar disorder showed that the frequency of social support in presence of negative life events was trend-associated with decreased HCC in patients with BD I (β = -0.30, t(29) = -1.97, r^2 = 0.08, p = 0.06, only negative events: β = -0.40, t(20) = -2.29, r^2 = 0.13,p = 0.03) but not in patients with BD II/NOS or in patients with no negative life events. Stratification for age of onset showed that the frequency of social support in presence of negative life events showed no effect on HCC in patients with an early age of onset, but a trend in patients with only negative events: β = -0.28, t(16) = -1.85, r^2 = 0.07, p = 0.08) and decreased HCC in patients with a late age of onset and with negative life events (β = -0.62, t(9) = -2.85, r^2 = 0.33, p = 0.02, only negative events: β = -1.18, t(4) = -3.02, r^2 = 0.45, p = 0.04).

DISCUSSION

The focus of our research project "The Bipolar Stress Study" is to identify the influence of biological and psychological stress on characteristics and course of bipolar disorder. In the current study we focused on the association between life events and HCC in patients with BD, and to explore whether HCC in patients with life events are predictive of mood in the subsequent three months. For this purpose, we benefited from earlier findings regarding the same cohort [19, 22] as we could account for the previously reported relations such as age of onset and subtype of bipolar disorder, and extended these findings.

In this study, we found an association between the number of negative life events and increased HCC, in particular in patients with the BD I subtype or in those who had an age of onset before 30 years. The observed increased HCC after life events are consistent with current literature on the influence of life events on hair cortisol levels [20-21]. However, we showed that it is not the total number of life events but in particular the number of negative life events, which significantly increases HCC in the corresponding months. Negative life events might aggravate rumination that may exacerbate the already adverse experience [33], thereby potentiating the effect of life events. Interestingly, it has previously been reported that bipolar patients show normal initial cortisol responses to stressful life events, but that they have more difficulty in terminating the stress response and returning to a stable baseline mood. Whereas healthy participants reported that it took them on average 2.3 days to return to pre-event mood and behavioral levels, cyclothymic and dysthymic patients stated that their average recovery time was 3.9 days and 7.7 days, respectively [34]. It is essential that an individual is able to terminate the cortisol response appropriately to prevent hypercortisolemic effects [35]. A failure to do so would reflect in higher mean cortisol levels over a prolonged period of time, which would contribute to higher HCC levels. Regarding social support, our first analyses indicated that the frequency

of social support and perceived social support were associated with decreases in HCC. Further analyses revealed that the attenuating effect of the frequency of social support on HCC was only present in patients with BD I that experienced (negative) life events and not in patients without any life events, suggesting that social support could partially attenuate the increasing HCC after negative life events. One might speculate that mitigating against increased long-term cortisol levels might protect from new mood episodes, as the "social zeitgeber theory" has proposed that life events might induce a new mood episode through disruptions in social and biological rhythms [36]. Through this mechanism, increased social support may stabilize the social and thereby also the biological rhythm.

Stratifying the analyses led to interesting findings, i.e. that the number of negative life events leads to HCC increases in BD I patients and in patients with an early age of onset. Stronger effects of life events on HCC in patients with BD I and an early age of onset could be due to various reasons. One reason might be a power problem that reflects the smaller number of participants in BD II and with a late age of onset rather than an underlying difference between the subtypes. This seems probable, as the standardized coefficients of BD II and a late age of onset are greater than the standardized coefficients of BD I and early age of onset, but fail to reach significance. However, other possibilities need to be acknowledged. A stronger effect of life events on mood symptoms and functional impairment on patients with BD I patients as compared to BD II patients has already been found in a longitudinal study on this cohort [22]. One possibility might be the different course trajectory of the subtypes. BD II is associated with a more chronic course and fluctuations in mood [37]. This is also consistent with the kindling theory, suggesting that stress triggers the initial episodes and recurrences, but that successive episodes are progressively induced independently of stressors [38]. With more episodes and a more chronic course, the association between stress and a new mood episode may already be too weak to be detected. For age of onset, an early onset BD has been suggested to be associated with a genetic vulnerability [39], whereas late onset BD is thought to be initially triggered by a major life event preceding the first episode [40]. A late onset is therefore more likely associated with a chronic HPA axis disturbance, which is in line with the earlier reported higher HCC in patients with a late age of onset compared to patients with a younger age of onset in this cohort [19]. Therefore, another major life event might not influence HCC as much as it would in patients with early age of onset and hypothetically more normal HPA axis functioning.

The question arises, how these results have to be interpreted, i.e. whether this increase reflects a healthy reaction of the HPA axis to a stressor, or whether it is a risk factor for a new mood episode. The same holds true for the potential absent HCC increase in BD II patients and patients with a late age of onset, and whether this reflects the failure to start a stress reaction to a life event, or whether it is a protective property that these patients have. An answer to this question would need a more frequent monitoring of the patients to assess exactly when a life event has happened, and a smaller time frame

covered by the hair sample, for example 1 cm instead of 3 cm. This would yield insight in whether an increase in HCC happens in all patients and that some patient groups are faster in ending the prolonged stress response than others, or whether the increase is a feature associated with different patient characteristics. In our study, this frequent monitoring was not possible due to logistic reasons.

In the current study, we found no association between hair cortisol concentrations and mood. However, for investigating the influence of HCC on mood and vice versa, the presence of distinct mood symptoms is required. Our well-treated sample only showed minor mood fluctuations and reported few clinical symptoms, thereby questioning the validity of our negative findings.

Several strengths and limitations of the present study need to be mentioned. The patients that participated in this research projects were seen regularly and frequently, i.e. every 3 months, and the occurrence of life events and presence of mood disorder symptoms were inquired upon every visit. For research on life events this is of high value, as in long periods between visits, patients may either forget to report life events, or may aggravate the meaning of events in order to explain for example a new mood episode, a process called "effort after meaning" [4]. However, we have to acknowledge the already mentioned small sample size of patients with either only positive or only ambiguous events, and the lack of a control group, i.e. HCC of healthy controls who also provided information on life events and mood. Furthermore, our sample presented with sparse mood symptoms which could be related to the high number of patients that used psychotropic medication. This treatment might be another limitation for this study as the effects of psychotropic medication on HCC have not been extensively examined until now; however, it seems an almost inevitable factor when dealing with this patient group. A related point is that patients with serious mood problems at the moment of assessment might not come to the outpatient department [41]. Our timeframe of observation amounted to six months, one period of three months that comprised the hair sample and the information on mood and life events of the corresponding three months, and the period of the subsequent three months, that comprised information on mood. Seeing that manic patients report on average 0.4 - 0.7 mood episodes per year [42], our design was likely too short to appropriately capture major mood changes.

To summarize, in the present study we showed that the number of negative life events is associated with increased hair cortisol concentrations in bipolar patients. These results indicate that even in the presence of a disorder that is associated with a dysregulation of the HPA-axis, exposure to negative life events might induce further HPA-axis disturbances. This relationship seems to be modified by social support, pointing towards the flexibility of the HPA-axis in both directions. Furthermore, these are first indications that there might be subtypes of bipolar patients that react differently to life events in terms

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of cortisol secretion, which might on the long term warrant different approaches to coping with stress or treatment of these patients.

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05

Long-Term Cortisol levels Measured in Scalp Hair of Obese Patients

ABSTRACT

Background

In obese subjects a relatively high cortisol output in urine has been observed compared to non-obese individuals. However, cortisol levels in blood, saliva and urine in association with obesity have been inconsistent across studies, possibly due to the high variability of systemic cortisol levels. Cortisol levels measured in scalp hair provide a marker for long-term cortisol exposure, and have been associated with cardiovascular disease in an elderly population and to disease course in Cushing's disease. We aimed to compare hair cortisol levels between obese patients and non-obese controls.

Methods

We measured hair cortisol levels of 47 obese patients (median BMI 38.8, range 31.1 - 65.8), 41 overweight and 87 normal-weight subjects using an enzyme-linked immunosorbent assay (ELISA).

Results

Obese patients had higher hair cortisol levels than overweight and normal weight subjects (respectively 30.8 vs. 8.5 and 8.4 pg/mg hair, p < 0.001). No significant difference in hair cortisol levels was found between normal weight and overweight subjects.

Conclusions

Our results suggest a higher long-term cortisol exposure in obese patients, which may contribute to cardiovascular disease risk. Future research will determine whether long-term cortisol levels provide a novel treatment target in the management of cardiovascular disease risk in obesity.

INTRODUCTION

Cortisol, the main glucocorticoid hormone in humans, is produced by the adrenal cortex under the influence of pituitary adrenocorticotropic hormone. An extreme excess of cortisol, as seen in Cushing's syndrome, is often marked by obesity and features of the metabolic syndrome (MetS), including dyslipidemia, hypertension, hyperglycemia and insulin resistance [1-2]. This observation has given rise to the hypothesis that a modest increase in cortisol exposure may contribute to obesity and MetS in the general population.

In line with this, obesity has been associated with increased cortisol output, as determined by urinary free cortisol. However, these results were inconsistent across studies, and this increased cortisol production does not seem to be reflected by serum and salivary cortisol [3]. These inconsistencies can be partially explained by the high variability in systemic cortisol levels, caused by pulsatile secretion, a diurnal rhythm and day-to-day fluctuations [4]. A relatively novel method to measure cortisol exposure is through scalp hair analysis. Several laboratories have successfully validated hair cortisol concentrations (HCC) as a marker of long-term cortisol exposure for periods of up to several months, thereby avoiding the limitations of time-point measurements [5-7]. With the use of HCC, increased long-term cortisol levels have recently been linked to disease course in Cushing's disease, presence of MetS and cardiovascular disease [8-11].

Previously, a positive correlation has been reported between HCC and body mass index (BMI) and waist circumference [5, 9, 12]. Furthermore, we recently reported increased HCC in obese children [13]. However, in an elderly cohort and a group of healthy adults we found no association between HCC and BMI [5, 10]. Until now, no study has been published which reported long-term cortisol levels in a patient population being evaluated for obesity. Therefore, we devised this study to examine whether obese individuals who visited our obesity center have higher hair cortisol concentrations than non-obese controls.

METHODS AND PROCEDURES

Participants

Obese patients were recruited from an outpatient academic obesity center at the Erasmus MC, Rotterdam, The Netherlands. Inclusion criteria were age above 18 years, BMI above 30 kg/m^2, ability to take part in physical exercise and at least one minor complication related to obesity, such as dyslipidemia, impaired glucose tolerance or hypertension. Patients were excluded when a secondary cause of the obesity was found, such as Cushing's syndrome, hypothyroidism or syndromal obesity, or if they took

glucocorticoid-containing medication. Between October 2011 and September 2013, we approached all patients who fulfilled the inclusion criteria (n = 60). In total, 141 healthy normal weight (BMI 18.5 - 24.9) and non-obese overweight (BMI 25.0 - 29.9) subjects from our previous validation study served as controls [5]. This study was approved by the local medical ethics committee. Written informed consent was obtained from all participants. All study procedures were conducted in accordance with the declaration of Helsinki.

Hair sample collection and analysis

From all subjects, a lock of approximately 100 hairs was collected from the posterior vertex prior to treatment for obesity, cut as close to the scalp as possible. Preparation and analysis of hair samples was performed as described previously [5]. In brief, approximately 15 mg of the proximal 3 cm of hair was weighed and finely cut. Extraction took place in 1 ml of methanol at 52 °C for 16 hours. After extraction, methanol was transferred into glass tubes, and evaporated under constant nitrogen stream. Next, 250 μL of phosphate buffered saline (PBS, pH 8.0) were added. Samples were vortexed prior to analysis, which was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit for cortisol in saliva (DRG Instruments GmbH, Marburg, Germany).

Laboratory diagnostics

In obese patients, blood was drawn after an overnight fast. HbA1c, glucose, triglycerides and total, LDL and HDL cholesterol were measured using routine laboratory procedures. Patients were coded as having MetS according to ATPIII criteria [2].

Statistical analysis

IBM SPSS version 21 and GraphPad Prism version 5.01 were used for analysis. Differences in characteristics were tested using Chi-square and Kruskall-Wallis tests. HCC values were logarithmically transformed to attain normal distribution. HCC differences between groups of subjects were analyzed using analyses of (co)variance. Correlations between HCC and metabolic parameters were tested using Spearman's rho. Statistical significance was defined as a *P*-value < 0.05.

RESULTS

Sixty obese patients were approached. Of these, 53 had sufficient scalp hair to obtain a sample. One patient was excluded, because she was diagnosed with an obesity-related syndrome. We successfully measured HCC in 47 out of 52 obese patients, and in 128

out of 141 non-obese controls. Baseline characteristics of all subjects are summarized in Table 1. Compared to controls, obese patients were on average older and comprised more women. The majority of obese patients (n = 39, 75%) fulfilled ATPIII criteria for diagnosis of MetS, while all of the 13 remaining patients had at least one component of MetS.

Table 1. Baseline, hair characteristics, and hair cortisol levels for normal weight, overweight and obese subjects

	Normal weight N=87	Overweight N=41	Obese N=47	P value of difference
Baseline characteristics				
Male	37 (43%)	25 (61%)	11 (23%)	0.002
Age	33 (20 – 61)	34 (19 – 63)	47 (18-68)	0.024
BMI (kg/m^2)	22.5 (19.3 – 24.8)	27.2 (25.0 – 29.7)	38.8 (31.1 – 65.8)	<0.001
Hair characteristics				
Hair washings >3 week	65 (76%)	30 (73%)	31 (66%)	0.491
Use of hair products [a]	41 (48%)	20 (49%)	19 (40%)	0.666
Hair coloring [b]	17 (20%)	6 (15%)	14 (30%)	0.170
Hair bleaching [b]	6 (7%)	0 (0%)	5 (11%)	0.116
Hair cortisol levels				
Hair cortisol (pg/mg hair)	8.4 (6.5 – 10.9)	8.5 (5.9 – 12.4)	30.8 (21.8 – 43.5)	<0.001

Values of baseline and hair characteristics represent median (range) or number (percentage). Hair cortisol values represent mean (95% CI). [a]Hair products concern hairspray, mousse, gel and wax used on the day of sample collection. [b]Concerns coloring or bleaching in the 3 months before hair sample collection.
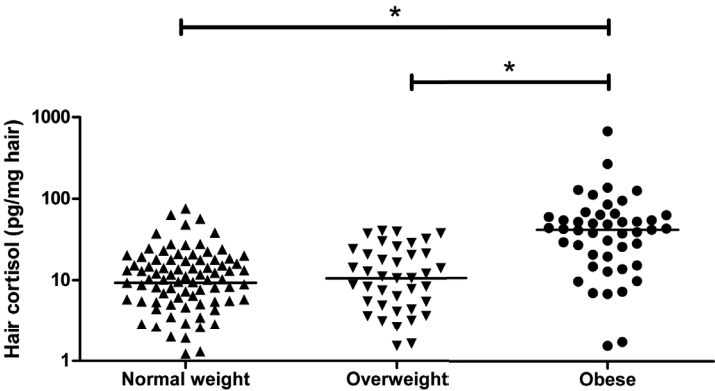

Figure 1. *Hair cortisol levels (pg/mg hair, logarithmic scale) in normal weight and overweight controls, and in obese patients. Horizontal lines represent the group median. *P<0.001*

Obese patients had significantly higher HCC than normal-weight and overweight controls (F(2, 172) = 19.788, $P < 0.001$, see Figure 1). However, HCC did not significantly differ between normal weight and overweight controls (post hoc comparison, $P = 0.96$; Figure 1, Table 1). Additional stepwise adjustment for age, sex, hair treatment and use of hair products did not significantly change our results. Within obese patients, HCC did not correlate significantly with BMI, waist circumference, HbA1c, LDL cholesterol, HDL cholesterol, triglycerides and glucose (data not shown).

DISCUSSION

In this case-control study, we found that obese patients had higher cortisol levels in scalp hair compared to non-obese controls. Our results are in agreement with results from Stalder *et al*. and our own results in a population of shift work employees, as both studies reported a positive correlation between HCC and BMI [9, 12]. In our previous studies in healthy adults and in a group of elderly subjects, we did not find this correlation, however we observed a correlation with waist/hip ratio in healthy adults [5, 10].

Notably, obese individuals had higher HCC than overweight and normal weight individuals, but HCC was similar between normal weight and overweight subjects. Possibly, the association between increased weight and long-term cortisol only reveals itself in a more extreme phenotype of adiposity. BMI does not discriminate between muscle and fat mass. Although it stands to reason that our obese group (with a median BMI of 38.8) has a higher fat mass than non-obese controls, the same may not be true for overweight versus normal-weight individuals. This may also explain why we did not find a correlation between BMI and HCC in our previous study, which comprised individuals mainly of the normal to overweight BMI group [5].

It would be of interest to study the correlation with features of MetS, however, metabolic data were only available for obese patients, and we found no significant correlation between HCC and cardiometabolic risk factors in this group. Previously, Stalder *et al*., reported significant associations between HCC and all separate components of MetS [9]. However, obese patients in our study represent a relatively metabolically unhealthy population, with a 75% prevalence of MetS. Presumably, a wider range of risk profiles is needed to reveal associations.

Due to the cross-sectional design of this study, our results do not provide evidence for a causal relationship between high cortisol exposure and obesity. Further research is warranted to investigate whether relatively high cortisol exposure precedes weight gain and other cardiometabolic risk factors. We recently reported an increased HCC in obese children, indicating that long-term cortisol exposure may influence weight early in life [13]. An explanation for the increase in long-term cortisol in obese patients may be

that obesity has been associated with psychosocial stress and mental health disorders [14-15]. This may increase long-term cortisol levels [16]. In this context, it is of interest that shift workers who have an altered diurnal rhythm have increased HCC [12]. Alternatively, one might speculate that liver steatosis associated with obesity affects cortisol breakdown, inducing a vicious circle with elevated long-term systemic cortisol levels, which may in turn promote adiposity [17].

In follow up studies of these patients we will investigate whether therapies for weight loss are associated with a decrease in long-term cortisol levels. The associations between HCC and cardiovascular disease and MetS that recently have been reported provide a first indication that strategies that lower long-term cortisol levels may reduce cardiovascular risk [9-11].

In conclusion, we found that obese patients who visit an outpatient obesity clinic have significantly higher cortisol levels in scalp hair than normal-weight and overweight subjects. Future research will show whether long-term cortisol provides a novel treatment target in the reduction of cardiovascular disease risk in obese patients.

Chapter 5

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06

INCREASED HAIR CORTISOL CONCENTRATIONS AND BMI IN PATIENTS WITH PITUITARY-ADRENAL DISEASE ON HYDROCORTISONE REPLACEMENT

ABSTRACT

Background

Intrinsic imperfections and lack of reliable biomarkers preclude optimal individual dosing of hydrocortisone replacement in adrenal insufficiency (AI). However, the clinical relevance of optimal dosing is exemplified by frequently occurring side effects of over-replacement and the dangers of under-replacement. Cortisol in scalp hair has been identified as a retrospective biomarker for long-term cortisol exposure. We compared hair cortisol concentrations ($CORT_{hair}$) of patients with primary or secondary AI on replacement therapy with those of patient controls with a pituitary disease without AI (PC) and of healthy controls (HC).

Methods

In this cross-sectional study, hair samples and anthropometric data were collected in 132 AI patients (52 males), 42 PC (11 males), and 195 HC (90 males). The proximal 3 cm of hair were used. $CORT_{hair}$ were measured using ELISA.

Results

$CORT_{hair}$ were higher in AI patients than in HC and PC ($p < 0.001$), and hydrocortisone dose correlated with $CORT_{hair}$ ($p = 0.04$). Male AI patients demonstrated higher $CORT_{hair}$ than female patients ($p < 0.001$). AI patients had higher body mass index (BMI) than HC ($p < 0.001$), and BMI correlated with $CORT_{hair}$ in the whole sample ($p < 0.001$).

Conclusions

Physiological hydrocortisone replacement is associated with increased $CORT_{hair}$. The association between $CORT_{hair}$ and BMI could suggest a mild overtreatment that may lead to adverse anthropomorphic side effects, especially in males. $CORT_{hair}$ measurements may be a promising additional tool to monitor cumulative hydrocortisone replacement in AI.

INTRODUCTION

Adrenal insufficiency (AI) in which the adrenal corticosteroid synthesis, i.e. cortisol production, is insufficient can be primary in case of pathology of the adrenal glands, or secondary in case of hypopituitarism. Patients with AI need replacement therapy with exogenous glucocorticoids, preferably hydrocortisone, which is synthetically produced cortisol [1]. In persons with intact adrenal function around 5 to 10 mg of cortisol per m^2 of body surface area per day is produced [2], with increased requirements during stress. The corresponding chronic oral replacement dosage is 15–25 mg per day, usually divided in three dosages in an attempt to mimic the circadian rhythm of natural cortisol secretion, with a peak in the morning and a gradual decrease during the day and evening [1, 3]. It is recommended that hydrocortisone replacement should be individualized, taking into account blood pressure, metabolic derangements and sense of well-being [4]. Various maintenance dosing strategies have been published [5]. However, it is likely that there will be large individual variation in substitution requirements in view of differences in cortisol sensitivity due to polymorphisms of the glucocorticoid receptor gene [6]. Currently available cortisol measurements in plasma, urine or saliva do not reflect cortisol action at tissue level. In accordance, plasma and salivary cortisol concentrations vary considerably between patients receiving hydrocortisone replacement, limiting the possibility to titrate individual hydrocortisone doses upon single plasma, or salivary measurements [7]. A method to retrospectively assess cortisol for longer periods of time is the analysis of cortisol in scalp hair [8]. As hair grows approximately one cm per month [9], a hair sample of for example three cm represents the long-term cortisol concentration of three months. Hydrocortisone is identical to human cortisol and has been shown to be measureable in scalp hair [8, 10]. Hair cortisol levels ($CORT_{hair}$) have repeatedly been associated with body mass index (BMI) and increased risk of metabolic syndrome and cardiovascular disease in populations with endogenous cortisol metabolism [11-15]. Until now, there is very limited data on the clinical utility of $CORT_{hair}$ measurements in patients with hydrocortisone replacement as is the case in AI. A recent study by Gow et al. demonstrated that hydrocortisone dose was significantly positively associated with $CORT_{hair}$ in patients with primary AI [10]. Furthermore, they demonstrated a significant difference in $CORT_{hair}$ in male subjects between patients and controls, but no statistically significant difference in females. In addition, they did not observe a difference between male and female patients' $CORT_{hair}$. Thus, this study provided data indicative of a potential gender dependent effect in $CORT_{hair}$ in patients on hydrocortisone replacement. However, it should be acknowledged that this study included only 13 male patients *vs.* 80 female patients, which limits the generalizability of the results. Therefore, we aimed to compare $CORT_{hair}$ in a large cohort of patients with primary and secondary AI on hydrocortisone replacement therapy (AI patients) with $CORT_{hair}$ of control patients

with a pituitary disease but no hydrocortisone replacement therapy (PC) and healthy controls (HC). Furthermore, we aimed to explore possible determinants of $CORT_{hair}$ in hydrocortisone treated AI patients, i.e. self-reported hydrocortisone intake, sex, age, and weight. We hypothesized that AI patients would have higher $CORT_{hair}$ than PC and HC, and that AI patients show side effects associated with high cortisol levels. Moreover, we hypothesized that $CORT_{hair}$ are associated with doses of hydrocortisone replacement and BMI.

METHODS AND PROCEDURES

Study design

This study was designed as a cross-sectional assessment of patients seen at the out-patient clinic of the department of Endocrinology of the Leiden University Medical Center. This study was conducted between July 2012 and January 2014. Hair samples were collected and patients were asked to fill out two short self-developed question-naires: one questionnaire about their hair treatment, and one questionnaire about their hydrocortisone intake (i.e. self-reported daily dose, time of intake, frequency of increas-ing/decreasing hydrocortisone dose) and/or the potential usage of other exogenous glucocorticoids. Clinical data of patients were obtained from their medical records.

Participants

Patients

We included two groups of patients: group I) patients with primary or secondary adrenal insufficiency using hydrocortisone (AI patients), and group II) patient controls (PC) with a pituitary disease not using hydrocortisone. A total of 184 patients were willing to par-ticipate. Patients could not participate in case of insufficient hair growth at the posterior vertex of the scalp. Ten patients were excluded from the analysis because of interpreta-tive difficulty of their chronic steroid replacement scheme; three had high levels prob-ably due to a hydrocortisone stress scheme for Addison's crisis in the three months prior to hair collection, three patients were excluded because of $CORT_{hair}$ >3 SD with no clear explanation, and four patients were excluded due to debatable AI diagnosis and incon-sistent hydrocortisone use. The final sample comprised a total of 174 patients (i.e. 132 AI patients and 42 PC). Primary AI had been diagnosed by very low early morning cortisol concentrations (<120 nmol/l) or insufficient stimulation following ACTH test (below 550 nmol/l) usually in the presence of positive adrenal auto-antibodies or an alternative explanation. Secondary adrenal insufficiency was preferably diagnosed using an insulin tolerance test (ITT), or if contra-indicated, a CRH test using the same cut-off as for ACTH stimulation. Pituitary hormone replacement was prescribed dependent on the results of

the annual evaluation of pituitary functions. In case of AI, hydrocortisone was prescribed (usually 20 mg/d divided into 3 dosages, with adjustments if clinically judged necessary by the treating physician) together with advices to increase the hydrocortisone dose in case of exposure to severe somatic and/or psychological stressors. In case of other hormone deficiencies, patients were substituted accordingly.

Healthy controls

To compare $CORT_{hair}$ between patients and healthy individuals, we used a group of 195 healthy controls (HC) previously described elsewhere [8]. The study was approved by the local ethics committee. All patients and controls gave written informed consent.

Hair cortisol assessment

A lock of approximately 150 hairs was cut as close to the scalp as possible from the posterior vertex. For analysis, the most proximal three cm of hair were used, correspond-ing to the most recent three months. Hair sample preparation and analysis has been described previously [8]. In short, a minimum of 10 mg of hair was weighed and cut into small pieces in a glass vial. Extraction of cortisol took place in 1 mL of methanol for 16h at 52^{0}C while gently shaking. After extraction, the methanol was transferred to another vial and evaporated under a constant stream of nitrogen. The samples were dissolved in 250 µL of phosphate buffered saline (PBS, pH 8.0) for analysis. A commercially available ELISA Kit for salivary cortisol (DRG GmbH, Marburg, Germany) was used to measure cortisol levels. A correction factor was applied to the results to account for the potential influ-ence of different hair weights. 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 reported by the supplier. The recovery of the assay was described previously [8].

Statistical analysis

SPSS 20.0 for Windows was used for statistical analysis. Differences in demographic in-formation between groups were tested with One-Way-ANOVAs and Pearson Chi Square tests. After logarithmic transformation, $CORT_{hair}$ were normally distributed. Analyses on $CORT_{hair}$ and differences between groups were performed by means of univariate gen-eral linear models. If groups differed on age, sex, BMI, or hair treatment (see Table 1 and Table 2), analyses on group differences were adjusted accordingly. For analyses of the etiologies of hydrocortisone use, post-hoc tests were applied. Pearson and spearman correlations were used for correlation analyses, depending on normality of the distribu-tion. $CORT_{hair}$ are provided in pg/mg and are reported as median (Mdn) and interquartile range (IQR).

Role of the funding source

This study was sponsored by the Leiden University Medical Center and the Erasmus Medical Center, the Netherlands. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Participant characteristics (Table 1)

132 AI patients, 42 PC, and 195 HC were included in the analysis. The frequency of using glucocorticoid containing medication (other than hydrocortisone or maintenance dose) was 14.9% and did not differ between AI patients and PC (p = 0.26). Of these, 12.6% used one, and 2.3% used two kinds of glucocorticoid containing medication. The most frequent used products were ointments (n = 8) and inhalation aerosols (n = 8). Five patients used nasal spray, and one patient had received an injection into a joint. Patients that used externally applied glucocorticoid containing medication did not show different CORT$_{hair}$ than non-applying patients and were therefore not excluded (p = 0.75). The mean disease duration was not significantly different between AI patients (18.33 ± 13.54

Table 1. Baseline characteristics of patients, patient controls and HC

	AI patients (n = 132)	PC (n = 42)	HC (n = 195)	P-value[1]	P-value[2]	P-value[3]
Age	54.84 (14.99)	49.07 (12.94)	36.17 (12.23)	**0.05**	**0.001**	**0.001**
Sex (male)	52 (39.4%)	11 (26.2%)	90 (46.2%)	0.12	0.23	**0.02**
BMI	27.80 (5.12)	28.70 (7.45)	24.34 (3.85)	0.95	**0.001**	**0.001**
Use of exogenous glucocorticoids #	15 (11.5%)	2 (4.9%)	NA	0.21	NA	NA
Hypertension	56 (43.8%)	24 (60.0%)	NA	0.07	NA	NA
Diabetes mellitus	21 (9.2%)	4 (9.8%)	5 (2.6 %)	0.92	**0.008**	**0.029**
Hair dyed	42 (36.2%)	16 (42.1%)	37 (19.0%)	0.52	**0.001**	**0.002**
Hair bleached	13 (9.9%)	9 (22.0%)	13 (6.7%)	**0.04**	0.29	**0.002**
Hair permed	2 (1.5%)	3 (7.3%)	2 (1.0%)	**0.05**	0.69	**0.01**
Use hairproduct	66 (50.0%)	22 (53.7%)	90 (46.4%)	0.68	0.52	0.40
Frequency hair wash > 3 times/week	46 (35.1%)	17 (41.5%)	143 (74.1%)	0.46	**0.001**	**0.001**

Data are presented as mean (standard deviation), and as n (valid percentage). AI, adrenal insufficiency; PC, patient control group; HC, healthy control group; BMI, Body Mass Index; NA, not applicable; #, use of other external glucocorticoids (besides hydrocortisone).
P-value1: comparison between AI patients and PC, P-value2: comparison between AI patients and HC, P-value3: comparison between PC and HC.

years) and PC (15.06 ± 10.30 years), p = 0.16). Presence of hypertension (defined as either blood pressure above 140/90 or use of antihypertensive medication) and presence of diabetes mellitus (defined as use of oral medication and/or insulin injection) was not different between AI patients and PC, and in AI patients, frequencies of hypertension and diabetes mellitus were comparable between genders. Both AI and PC showed higher frequencies of diabetes mellitus than HC (both p < 0.005). Hypertension data were not available for HC.

CORT$_{hair}$ in AI patients, PC, and HC (Figure 1a-b)

Analyses showed a significant difference in CORT$_{hair}$ between the three groups, $F(2, 343) = 35.39$, p < 0.001, adjusted for age, gender, and dyeing of the hair. Post-hoc tests indicated that AI patients had higher CORT$_{hair}$ (33.89, 14.82 – 89.29) than PC (13.66, 6.22 – 26.58), p = 0.001, and HC (10.07, 3.52 – 17.83), p < 0.001, and that PC had higher CORT$_{hair}$ than HC, p = 0.04. In AI patients, 35.6% (61.5% males, 18.8% females) presented with CORT$_{hair}$ above our lab-internal cut-off for normal, as did 7.1% (9.1% males, 6.5% females) of PC and 3.1% (5.6% males, 1.0% females) of HC. Our lab-internal upper limit of normal is 52 pg/mg. For determination, we restricted our group of healthy controls to the ones with a BMI between 18.5 and 30.0, and used the 97.5 percentile as cut-off value.

In AI patients, men had significantly higher CORT$_{hair}$ (75.25, 28.91 – 159.81) than women (19.59, 11.49 – 38.49), $F(1, 112) = 8.17$, p = 0.00 5), adjusted for age and dyeing of the hair. No gender differences were observed in CORT$_{hair}$ in PC. In HC, females showed higher CORT$_{hair}$ than males, $F(1, 191) = 5.45$, p = 0.02. Stratified analysis for gender revealed that male AI patients had higher CORT$_{hair}$ than male PC and HC (p = 0.02 and p < 0.001, respectively), whereas for female AI patients, CORT$_{hair}$ was trend-significantly higher than in female PC (p = .07) and significantly higher than in female HC (p < 0.001). Males in the
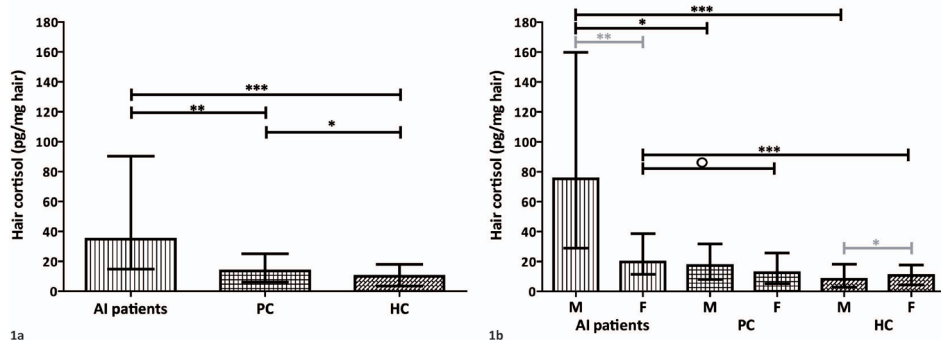
Figure 1. *Median and IQR of CORT$_{hair}$. AI, adrenal insufficiency; PC, patient control group; HC, healthy control group. Untransformed data are shown. 1a) CORT$_{hair}$ of AI patients, PC, and HC. 1b) CORT$_{hair}$ for AI patients, PC, and HC, stratified for sex. *** = p < 0.001; ** = p < 0.01; * = p < 0.05, ○ = p < 0.1. Black lines represent differences between the participant groups, whereas grey lines represent sex differences within each participant group.*

PC group did not show different $CORT_{hair}$ from males in the HC group, and females in the PC group had not different $CORT_{hair}$ compared to females in the HC group (all p > 0.1). Within AI patients, no difference in $CORT_{hair}$ was found for the various etiologies of AI.

Correlation between hydrocortisone dose and hair cortisol levels (Figure 2)

Self-reported daily hydrocortisone maintenance dose correlated with $CORT_{hair}$ ($\rho = 0.18$, p = 0.04). Stratification for gender showed that this correlation was primarily driven by the female AI patients ($\rho = 0.24$, p = 0.04), whereas the correlation was not significant in male AI patients. Neither incidental higher and/or lower hydrocortisone dosages nor the morning (peak) dose of hydrocortisone ($\rho = 0.15$, p = 0.09) were related to $CORT_{hair}$. The self-reported daily hydrocortisone dose in mg/kg or mg/m^2 was not related to $CORT_{hair}$ and stratification for sex did not render different results.
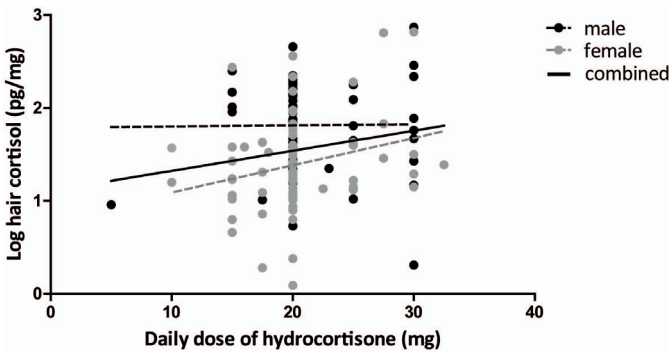

Figure 2. *The relationship between daily hydrocortisone dose (mg/day) and CORT$_{hair}$ (pg/mg), $\rho = 0.18$, p = 0.04, as indicated with the black solid line, which is the regression line of the group analysis. Analyses stratified for sex show that this effect was driven by the female AI patients (grey; $\rho = 0.24$, p = 0.04), whereas no effect was observed for the male AI patients (black; $\rho = -0.04$, p = 0.79). CORT$_{hair}$ are shown on a log scale.*

Correlations between anthropometrics and hair cortisol levels (Figure 3)

As indicated in Table 2, BMI differed significantly between AI patients, PC and HC ($F_{2, 327}$) = 23.90, p < 0.001. Post-hoc tests revealed that the BMI of AI and PC patients was significantly higher compared to HC (p < 0.001), but there was no significant difference between AI and PC. For the whole group of participants, BMI showed a significant correlation with $CORT_{hair}$ ($\rho = 0.24$, p < 0.001). Stratification for sex and participant group revealed a significant correlation between BMI and $CORT_{hair}$ for male AI patients ($\rho = 0.34$, p = 0.02), but not for female AI patients nor for male or female PC or HC. Waist-to-hip ratio (WHR) information was only available in a subset of 50 AI patients, 12 PC, and 45 HC. WHR was not different between the groups. In the whole sample of participants, WHR and CORT correlated significantly (r = 0.20, p = 0.04). WHR and waist circumference were related to self-reported dose in mg/kg (r = -0.3, p = 0.04, and r = -0.58, p < 0.001,

respectively) and to self-reported dose in mg/BSA (r = -0.36, p = 0.01, and r = -0.64, p < 0.001, respectively), but were not related to the total self-reported daily hydrocortisone maintenance dose (r = 0.11, p = 0.46, and r = 0.19, p = 0.17, respectively).
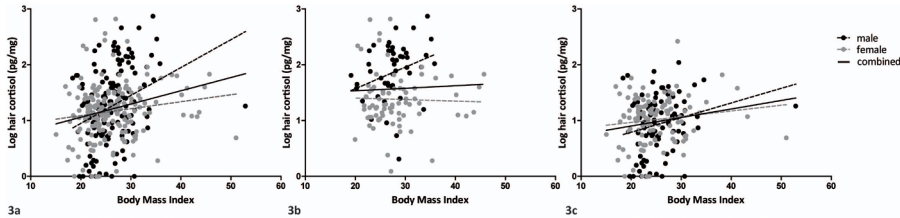

Figure 3. *The relationship between BMI and $CORT_{hair}$. 3a) The association between BMI and $CORT_{hair}$ for all participants was significant ($\rho = 0.24$, p < 0.001, black solid line); stratification for sex did not change the results ($\rho = 0.35$, p < 0.001 for male participants (black); $\rho = 0.18$, p = 0.02 for female participants (grey). 3b) In only the adrenal insufficiency (AI) patients, the association between BMI and $CORT_{hair}$ was not significant; $\rho = 0.11$, p = 0.23 (black solid line). Stratification for sex rendered a significant correlation for male AI patients ($\rho = 0.34$, p = 0.02, black) but no association for female AI patients ($\rho = 0.04$, p = 0.73, grey). 3c) In the control persons, the association did reach significance ($\rho = 0.14$, p = 0.04, black solid line). Stratified analyses showed that this effect was driven by the male controls ($\rho = 0.24$, p = 0.02, black) but was not significant for female controls ($\rho = 0.10$, p = 0.31, grey).*

Table 2. Baseline characteristics of male and female AI patients.

	Males (n = 52)	Females (n = 80)	P-value
Age	55.94 (16.01)	54.13 (14.35)	0.50
BMI	27.57 (3.85)	27.95 (5.80)	0.68
Duration of follow-up (years)	17.87 (12.69)	18.62 (14.11)	0.76
Daily hydrocortisone dose (mg)	21.58 (4.98)	20.39 (4.21)	0.15
Daily hydrocortisone dose mg/kg	0.25 (0.07)	0.27 (0.07)	0.12
Daily hydrocortisone dose mg/BSA	10.35(2.54)	10.84 (2.27)	0.26
Use of external glucocorticoids #	5 (10.0%)	10 (12.5%)	0.66
Hypertension	23 (46.9%)	33 (41.8%)	0.57
Diabetes Mellitus	4 (8.0%)	8 (10.0%)	0.70
Hair dyed	0	42 (58.3%)	**0.001**
Hair bleached	0	13 (16.5%)	**0.002**
Hair permed	1 (1.9%)	1 (1.3%)	0.76
Use hairproduct	19 (36.5%)	47 (58.8%)	**0.01**
Frequency hair wash			**0.03**
< 2 times/week	28 (53.8%)	57 (72.2%)	
> 3 times/week	24 (46.2%)	22 (27.8%)	

Data are presented as mean (standard deviation), and as n (valid percentage). #: use of other external glucocorticoids (besides hydrocortisone); BMI, Body Mass Index; BSA, body surface area.

DISCUSSION

The present study showed that patients using hydrocortisone replacement for AI demonstrate higher $CORT_{hair}$ than pituitary patients and healthy controls with an intact HPA-axis. Furthermore, a gender-effect was identified, with male patients with AI demonstrating higher $CORT_{hair}$ than females, without differences in self-reported hydrocortisone intake. Intriguingly, this gender effect seems to be specific for hydrocortisone use, since it is not present in controls with an intact HPA-axis. In female patients, higher self-reported hydrocortisone intake was associated with higher $CORT_{hair}$, whereas this association was not found in male patients who demonstrated on average higher $CORT_{hair}$ even in the lower dose range.

In male, but not female AI patients, higher $CORT_{hair}$ were associated with higher BMI. This relation suggests that high $CORT_{hair}$ may reflect chronic overexposure to hydrocortisone, at least in male patients. However, further study is required to understand the role of gender in the determination of cortisol levels in hair and to confirm whether $CORT_{hair}$ are indeed representative for corticosteroid exposure in the rest of the organs. Furthermore, it is still unclear how exactly cortisol, and hence hydrocortisone, is incorporated into scalp hair [16]. Therefore, the question remains whether it is the cumulative amount of cortisol or the cortisol peak that is most influential on $CORT_{hair}$. In our study, total dose appears to be associated with $CORT_{hair}$, and not a single/maximum dose. In contrast, the three patients who received a hydrocortisone bolus for an Addison crisis had extremely high values and were excluded from the study (data not shown), suggesting a role for a supraphysiological peak in determination of $CORT_{hair}$. In contrast to the positive correlation between the absolute hydrocortisone dose and $CORT_{hair}$, we found no relation between body weight-adjusted dose and $CORT_{hair,.}$ This is an interesting finding, since previous research has reported that clearance of hydrocortisone in serum is faster in obese patients, and that adjusting the dose for body weight may be beneficial for the patient [5, 17]. However, the current study may imply that tissue exposure following ingestion of hydrocortisone (at a physiological level) is independent of distribution volume, i.e. weight, at least for these patient groups as measured by $CORT_{hair}$, and thus questions the need to increase the hydrocortisone dose in obese patients. This is in accordance with recent guidelines pointing to no adjustment for weight (except for children) [3]. Male patients reached higher $CORT_{hair}$ with considerably lower hydrocortisone dosages than female patients. This "higher sensitivity" to hydrocortisone is in accordance with the positive association between $CORT_{hair}$ and BMI in male patients. A possible explanation for this higher sensitivity in male patients might be that men seem to have lower corticosteroid binding globulin (CBG) levels while total cortisol levels are comparable to women's total cortisol [18]. This may result in higher free cortisol levels in men upon hydrocortisone intake. As free cortisol is thought to be the cortisol fraction

which is incorporated in hair [19], this might explain the sex difference found in our study and in the study of Gow and colleagues [10].

However, the clearly increased CORT$_{hair}$ suggest that in general patients with AI are chronically over-replaced, despite prescribed hydrocortisone replacement dosages aiming at mimicking a "physiological" level. A higher daily hydrocortisone dose has been previously linked to a more adverse cardiometabolic risk profile, characterized by higher BMI [20]. Steroid excess-related morbidity is well known from AI cohorts treated with higher doses, resulting in the awareness to replace hydrocortisone with the lowest dose possible, generally regarded to as a daily hydrocortisone dose of 20 mg [21-22]. It is intriguing that AI patients treated with the currently advised low hydrocortisone dose have clearly increased CORT$_{hair}$ and additionally present with steroid-related side effects, such as increased BMI.

It appears unlikely that an increased perceived stress of being a patient influences CORT$_{hair}$ in this study. Some AI patients are known to occasionally increase their hydrocortisone doses in situations of increased psychological stress [23], which might result in higher CORT$_{hair}$. In population studies, CORT$_{hair}$ has been associated with perceived stress [24-26], but if this was the case in the present study, the increased stress of being a patient should then also be present in our PC group. However, CORT$_{hair}$ between PC and HC were comparable.

Several strengths and limitations of the present cross-sectional study need to be mentioned. In total, we included a considerable number of patients, which enabled us to examine CORT$_{hair}$ of patient groups with pituitary diseases due to different etiologies. Furthermore, we included a patient control group with a pituitary disease and normal adrenal function. All concurrent pituitary insufficiencies were treated, but we do acknowledge that, such as hydrocortisone replacement therapy, intrinsic imperfections of hormone replacement are also an important issue for gonadal steroids, thyroid hormone, and growth hormone replacement.

Besides the demonstrated association between CORT$_{hair}$ and anthropometrics, a considerable amount of studies demonstrated the association between high CORT$_{hair}$ and psychological symptoms [25]. Furthermore, in a recent study it was demonstrated that a higher hydrocortisone intake in patients with primary adrenal insufficiency was associated with more impairments in quality of life, psychological morbidity, and maladaptive personality traits [27]. For future research, it would be interesting to assess patients' perceived well-being in relation to CORT$_{hair}$.

In conclusion, patients on hydrocortisone replacement therapy have elevated CORT$_{hair}$, a finding which is predominantly present in male patients. Despite a low dose of on average 21 mg/day only 64.4% of patients had CORT$_{hair}$ in the normal range. This study provides important data on the fact that contemporary steroid replacement still results in clear supraphysiological (hair) cortisol levels, especially in males. However, it needs to

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be confirmed that $CORT_{hair}$ reflects cortisol (over)exposure in other organs of the body in exogenously treated patients, or that the incorporation of $CORT_{hair}$ is different from the reference population with normal HPA-axis. Next, it needs to be established which are safe, gender-specific $CORT_{hair}$ for patients to allow for the monitoring of hydrocortisone dose while avoiding the dangers of under- and over-replacement.

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QUALITY OF LIFE IN PATIENTS WITH ADRENAL INSUFFICIENCY CORRELATES STRONGER WITH HYDROCORTISONE DOSAGE, THAN WITH LONG-TERM SYSTEMIC CORTISOL LEVELS

ABSTRACT

Background

In patients with adrenal insufficiency (AI) a higher hydrocortisone intake has been associated with more impairment in Quality of Life (QoL). Irrespective of age, sex and severity of AI the dosage of hydrocortisone is titrated around 20 mg/D in all patients with AI based on physical and mental signs and symptoms. However, until now it is unknown whether these QoL impairments are related to increased systemic cortisol exposure. Measurement of hair cortisol levels ($CORT_{hair}$) can be used to assess chronic systemic cortisol exposure. This study aimed to explore whether QoL in patients with AI is associated with $CORT_{hair}$ and daily hydrocortisone intake.

Methods

We performed a cross-sectional study in 120 patients with AI on stable hydrocortisone replacement, in whom hair samples and QoL data were collected. $CORT_{hair}$ were measured with ELISA, and QoL was assessed with validated questionnaires (SF-36, EQ-5D, HADS, MFI-20).

Results

Patients reported impairments in 14 of 15 QoL subscales ($p < .001$). More impairments in physical aspects of QoL correlated with higher $CORT_{hair}$ and higher daily hydrocortisone intake ($p < .05$), an effect that was more pronounced in female patients. Regression analyses including both $CORT_{hair}$ and hydrocortisone intake revealed a significant negative contribution of higher hydrocortisone intake on physical aspects of QoL ($p < .046$), whereas no significant contribution was found for $CORT_{hair}$.

Conclusions

The present study showed that patients with AI report several impairments in QoL which are associated with hydrocortisone intake, and to a lesser extent reflected by chronic systemic cortisol exposure as measured by hair cortisol. This suggests that QoL impairments in patients with AI are not per se the effect of prolonged exposure to elevated systemic cortisol levels.

INTRODUCTION

Adrenal insufficiency (AI) is treated with glucocorticoid replacement therapy, usually 20 to 30 mg of hydrocortisone daily, divided in three dosages (10-15 mg in the morning, 5-10 mg in the afternoon, 4-5 mg in the evening), in order to mimic the natural circadian secretion of cortisol [1]. However, even when patients with primary AI are in a stable medical condition, they report impaired quality of life (QoL) [2-6]. In addition, in patients with secondary AI due to pituitary disease, hypopituitarism was found to be an important predictor of QoL impairments [7-9]. It has been suggested that these QoL impairments are associated with intrinsic imperfections in glucocorticoid replacement therapy, and therefore, it is advised that hydrocortisone replacement should be individualized [10]. For instance, there is large individual variation in sensitivity to cortisol, which is partly explained by polymorphisms of the glucocorticoid receptor gene [11]. However, determining an optimal hydrocortisone replacement dose is complicated by the lack of reliable chronic parameters, and as a result many patients may be chronically under- or overtreated with potential paramount consequences for well-being and health.

Until now, it is not well established whether QoL is affected by the degree of cortisol exposure (i.e., adequacy of hydrocortisone replacement) in patients with AI. In a single study, authors investigated plasma cortisol day curves and well-being in a small sample of seven patients with AI and demonstrated that subphysiological cortisol levels cor-related with lower well-being [12]. Other studies examined the relation between the dosage and intake scheme of glucocorticoid replacement therapy and QoL, and demonstrated that in patients with AI, QoL was inversely correlated with the hydrocortisone dose [6, 13]. Importantly, associations between hydrocortisone intake and QoL do not provide any information about causality, since it might be that high cortisol levels cause QoL impairments, but it might also be that patients with worse QoL need more hydrocortisone.

Addressing this relationship is further complicated by the difficulty of adequately measuring cortisol levels throughout the day, since cortisol levels vary depending on different treatment regimens (i.e., varying hydrocortisone doses, as well as differences in timing, absorption, and metabolism of hydrocortisone), and currently available cortisol measurements (i.e., plasma, urinary, salivary) are limited to short-term assessments.

A promising method to assess cortisol for prolonged periods of time is the analysis of cortisol levels in scalp hair (CORThair) [14-15]. We (and others) recently assessed the use of this measure in AI patients treated with exogenous hydrocortisone. Patients with AI have increased levels and hydrocortisone intake has been found to correlate with COR-Thair [16-17]. A significant gender effect has been reported in CORThair in patients with AI treated with glucocorticoid replacement therapy, with male patients demonstrating higher CORThair than females while using the same dose of hydrocortisone [16-17].

In the present study, we aimed to explore whether CORThair is correlated with QoL. We first compared QoL in patients with stable treatment for AI with QoL in healthy controls. Second, we examined potential correlations between QoL, CORThair, and daily hydrocortisone intake as another parameter to assess cortisol exposure.

METHODS AND PROCEDURES

Participants

Scalp hair samples were collected of 132 patients with primary or secondary AI on hydrocortisone replacement from the Endocrinology out-patient clinic of the Leiden University Medical Center (cohort previously described in [17]). Of this group, nine patients did not fill out QoL questionnaires and three patients filled out less than 75% of the questionnaires and were therefore excluded from the analysis. Thus, 120 patients with longstanding AI on a stable dose were included in the present study. Primary AI had been diagnosed by very low early morning cortisol concentrations (<120 nmol/l) or insufficient stimulation following ACTH test (below 550 nmol/l) usually in the presence of positive adrenal auto-antibodies or an alternative explanation. Secondary adrenal insufficiency was preferably diagnosed using an insulin tolerance test, or if contra-indicated, a CRH test using the same cut-off as for ACTH stimulation. Pituitary hormone replacement was prescribed dependent on the results of the annual evaluation of pituitary functions. In case of AI, hydrocortisone was prescribed (usually 20 mg per day divided into three dosages, adjusted at the discretion of the treating physicians) together with the advice to increase the hydrocortisone dose in case of exposure to severe somatic and psychological stressors. Comparison QoL data of 437 healthy controls were derived from a previous study from our department [18].

The local ethics committee approved this study. All patients gave written informed consent.

QoL assessment

QoL was assessed with the following four validated questionnaires:
The Short-Form 36 (SF-36) assesses functional status and general well-being and consists of 36 items covering nine health concepts: 1) physical functioning, 2) social functioning, 3) role limitation (physical), 4) role limitation (emotional), 5) mental health, 6) vitality, 7) pain, 8) general health perception, and 9) general perception of change in health. Scores are expressed on a 0–100 scale, and higher scores indicate better QoL [19].

The EuroQoL-5D (EQ-5D) assesses the current health status reflected in five health dimensions; 1) mobility, 2) self-care, 3) usual activities, 4) pain/discomfort, and 5) anxiety/depression. Scores are expressed on a 1-3 scale per dimension, with higher scores

indicating worse QoL. Also a visual analogue scale is included ranging from 0 to 100 for recording an individual's rating for their current health-related well-being, with higher scores indicating a better health status [20].

The Hospital Anxiety and Depression Scale (HADS) assesses both anxiety and depressive symptoms and consists of 14 items on a 4-point scale. Higher scores indicate more severe anxiety and depressive symptoms [21-22].

The Multidimensional Fatigue Inventory (MFI-20) consists of 20 statements assessing fatigue on a five-point scale covering five dimensions; 1) general fatigue, 2) physical fatigue, 3) reduced activity, 4) reduced motivation, and 5) mental fatigue. Scores vary from 0-20; with higher scores indicating greater fatigue [23].

QoL of healthy controls

QoL data of healthy controls were previously collected at our department [18]. The EuroQoL-5D and two subscales of the Short-Form 36 (i.e., mental health, vitality) were not assessed in this group of healthy controls. QoL data of 437 healthy controls (136 males) with a mean age of 50.9 ± 13.6 years were available and the total group was used for comparison.

Hair collection, preparation, and analysis

A lock of approximately 150 hairs from the posterior vertex was cut as close to the scalp as possible. The hair samples were taped to paper and stored in the dark at room temperature until further analysis. One cm represents the average cortisol concentrations of one month [15], since it is assumed that hair grows one cm per month, with a range of 0.6 – 1.4 cm/month [16]. Hair samples are specifically taken from the vertex region of the scalp because its most uniform growth pattern and phase [17-18], and importantly, has been specifically been validated for cortisol with the lowest mean coefficient of intra-individual variation [19]. For analyses, the most proximal 3 cm of hair was used, corresponding to the most recent 3 months. A minimum of 10 mg of hair was weighed and cut into small pieces. For extraction, 1 mL of methanol was added and the samples were incubated for 16h at 52^0C. After extraction, the methanol was transferred to another vial and evaporated under a constant stream of nitrogen. The samples were dissolved in 250 µL of phosphate buffered saline (PBS, pH 8.0). A commercially available ELISA Kit for salivary cortisol (DRG GmbH, Marburg, Germany) was used to measure cortisol levels. The procedure has been described in detail elsewhere [14]. Our laboratory internal upper limit of normal is 52 pg/mg.

Statistical analyses

Data were analyzed using PASW Statistics version 20.0 (SPSS Inc., Chicago, IL). $CORT_{hair}$ were reported as median and interquartile ranges (IQR). Other data were presented as

Mean ± SD, unless mentioned otherwise. After logarithmic transformation, $CORT_{hair}$ were normally distributed. The primary analysis comprised the comparison of QoL of patients with AI to healthy controls by using independent sample t-tests when data were normally distributed and Mann-Whitney U tests when data were not normally distributed. In order to evaluate whether the previously found gender effect in $CORT_{hair}$ is reflected in QoL, this analysis was also performed after stratification for gender.

The secondary analysis comprised the assessment of the potential association between QoL, $CORT_{hair}$, and daily hydrocortisone intake. Partial correlations were calculated between QoL and $CORT_{hair}$ and daily hydrocortisone intake, adjusted for age and gender. Subsequently, groups were stratified for gender and partial correlations were calculated between QoL and $CORT_{hair}$ and daily hydrocortisone intake, adjusted for age. Regression analyses including linear and quadratic terms were used to examine possible u-shaped associations. Furthermore, regression analyses including both $CORT_{hair}$ and daily hydrocortisone intake were used to differentiate between the contributions of these two factors. Because of the exploratory nature of these analyses, adjustment of the level of significance for multiple testing was not performed, and the level of significance was set at $p < .05$.

RESULTS

Patient characteristics

A total of 120 patients with longstanding AI (46 males) with a mean age of 55.0 ± 14.7 years were included in the analyses. The duration of follow-up was on average 18.5 ± 13.3 years, with a median of 15.8 years (IQR: 8.1-28.9). Patients used a mean daily dose of 21.1 ± 4.5 mg. In the whole group of patients, 34% presented with $CORT_{hair}$ above our lab-internal cut-off for normal (52 pg/mg). Of the males, 59% demonstrated $CORT_{hair}$ higher than the lab-internal cut-off, in contrast to 19% of the females ($p < .001$). As previously reported [17], also in the present study male patients demonstrated higher $CORT_{hair}$ than female patients (75.3 (26.2 - 150.1) vs. 19.7 (11.6 - 38.5), $p < .001$). Furthermore, female patients dyed or bleached their hair more and used hair products more frequently than male patients (all $p < .03$) (Table 1).

Daily hydrocortisone intake and CORThair showed a significant, but modest correlation ($r = 0.185$, $p = .047$). To evaluate whether there were differences in $CORT_{hair}$ between different etiologies of AI, five groups were formed: 1) AI due to previous treatment for Cushing's disease (n = 18), 2) other functioning pituitary adenomas (n = 14, including acromegaly (n = 5), prolactinoma (n = 8), and FSH producing adenoma (n = 1)), 3) non-functioning pituitary adenoma+craniopharyngioma (n = 48, nonfunctioning pituitary adenoma (n = 35) and craniopharyngioma (n = 13)), 4) primary AI (n = 18), and 5) other

Table 1. Clinical characteristics of AI patients (males vs. females)

	Patients with AI (n = 120)	Males with AI (n = 46)	Females with AI (n = 74)	*p* value
Age (years)	55.0 ± 14.7	57.2 ± 14.8	53.6 ± 14.6	.167[b]
BMI (kg/m^2)	28.0 ± 5.1	27.5 ± 3.5	28.3 ± 5.9	.799[b]
Duration of follow-up (years)	18.5 ± 13.3	18.2 ± 12.6	18.7 ± 13.8	.995[b]
Use of external glucocorticoids #	15 (13%)	5 (11%)	10 (14%)	.702[c]
Hair cortisol levels	23.7 (14.0 – 84.7)	75.3 (26.2 – 150.1)	19.7 (11.6 - 38.5)	**<.001[a]**
Hair cortisol above our lab-internal cut-off (52 pg/mg)	41 (34%)	27 (59%)	14 (19%)	**<.001[c]**
Daily hydrocortisone dose (mg)	21.1 ± 4.5	21.5 ± 5.1	20.8 ± 4.2	.334[b]
Daily hydrocortisone dose (mg/kg)	0.26 ± 0.07	0.2 ± 0.07	0.3 ± 0.07	.065[b]
Daily hydrocortisone dose (mg/BSA)	10.7 ± 2.4	10.3 ± 2.5	11.0 ± 2.3	.077[b]
Hair dyed	44 (37%)	0 (0%)	44 (60%)	**<.001[c]**
Hair bleached	18 (15%)	0 (0%)	18 (24%)	**<.001[c]**
Hair permed	3 (3%)	1 (2%)	2 (3%)	.848[c]
Use of hair product	60 (50%)	17 (37%)	43 (58%)	**.024[c]**
Frequency hair wash > 3 times/week	42 (35%)	21 (46%)	21 (28%)	.061[c]

Data are presented as mean (standard deviation), and as n (valid percentage). [a] Independent samples t-test, [b] Mann-Whitney U-test, [c] Chi-square test. AI, adrenal insufficiency; BMI, body mass index; BSA, body surface area; #: use of other external glucocorticoids (in addition to their regular hydrocortisone substitution). p value: AI males vs. AI females.

causes of hypopituitarism (n=22, including congenital hypopituitarism (n = 6), hypopituitarism after radiotherapy/surgery/traumatic brain injury (n = 7), and other causes such as pituitary inflammation or Sheehan's syndrome (n = 9)). CORT$_{hair}$ was lowest in patients with CD, but group differences did not reach statistical significance (p = .126) (Figure 1). The self-reported hydrocortisone dose was significantly different between groups (p = .003), with patients with primary AI using a higher dose (24.7 ± 4.5 mg) compared to patients with CD (20.0 ± 4.8), NFA+CP (20.9 ± 3.8) or other causes of hypopituitarism (19.2 ± 5.4).

QoL

Compared to healthy controls, patients with AI reported worse QoL on all subscales (except general health perception, SF-36) (p < .05) (Table 2). After stratifying for gender, male patients reported worse QoL on 12 of the 15 subscales (p < .05) and female patients reported worse QoL on 14 of the 15 subscales (p < .001) in comparison to controls (Table 2). Comparing QoL between the different etiology groups revealed significantly more depressive symptoms (HADS) in patients with CD (7.3 ± 4.0) relative to patients with NFA+CP (4.3 ± 3.5) (p = .022) (Figure 2). Furthermore, patients with CD reported more physical fatigue (14.7 ± 2.0), more reduced activity (12.9 ± 1.2) (MFI-20), and worse
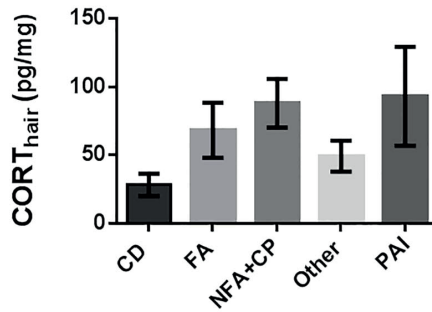
Figure 1. *Comparison of CORT$_{hair}$ between patient groups. Mean hair cortisol levels (CORT$_{hair}$) +/- standard error to the mean, stratified per patient category as follows: 1. CD: Cushing's disease (n = 18); 2. FA: other functioning pituitary adenomas (n = 14, including acromegaly (n = 5), prolactinoma (n = 8), FSH producing adenoma (n = 1)); 3. NFA+CP: non-functioning pituitary adenomas (n = 48, including craniopharyngeoma (n = 13) and NFA (n = 35)); 4. PAI: primary adrenal insufficiency (n = 18); 5. Other: other causes of hypopituitarism (n = 22, including congenital hypopituitarism (n = 6), hypopituitarism after radiotherapy/surgery/traumatic brain injury (n=7), other causes such as pituitary inflammation or Sheehan's syndrome (n = 9)). The figure shows a difference in CORT$_{hair}$ between patients with AI due to previous treatment for CD and the other groups, but this difference was not found to be statistically significant (p = .126).*

mental health (58.9 ± 20.2) (SF-36) compared to patients with PAI (11.7 ± 4.7; 10.7 ± 4.2; 77.7 ± 18.2) (p = .004, p = .009, p = .044, respectively). Considering that only patients with CD differed from the other groups, QoL analyses were corrected for etiology of CD.

Relations between CORT$_{hair}$ and QoL

As shown in Table 3, correlations between CORT$_{hair}$ and QoL, adjusted for age, gender, and etiology CD revealed that in the whole group, higher CORT$_{hair}$ correlated at trend level with more limitations in daily activities (EQ-5D) (r = 0.180, p = .059). After stratification for gender, it was observed that in male patients higher CORT$_{hair}$ was associated with more physical fatigue (r = .355, p = .018). In female patients higher CORT$_{hair}$ was associated with more limitations in daily activities (r = 0.239, p = .046) and more pain (r = 0.269, p = .024) (EQ-5D).

In the whole group, QoL of patients with CORT$_{hair}$ above the lab-internal cut-off for normal was not different from patients with CORT$_{hair}$ in the normal range (p > .05). However, female patients with CORT$_{hair}$ levels above the lab-internal cut-off (n = 14 (19%)) reported lower physical functioning (52.9 ± 28.7, p = .025) and more pain on the SF-36 (52.8 ± 29.7, p = .033), as well as on the EQ-5D (2.2 ± 0.8, p = .049) relative to females with CORT$_{hair}$ within the normal range. No differences in QoL were found between male patients with CORT$_{hair}$ above the lab-internal cut-off (n = 27 (59%)), and male patients with CORT$_{hair}$ within the normal range (p > .05). Regression analyses including age, gender, and etiology CD, as well as CORT$_{hair}$ as a quadratic term did not render significant results.

Table 2. Quality of life scores in patients with AI vs. healthy controls.

	Patients with AI (n=120)	Healthy controls (n=437)	p value[1]	Males with AI (n=46)	Healthy males (n=136)	p value[2]	Females with AI (n=74)	Healthy females (n=301)	p value[3]
SF-36									
Physical functioning	73.3±24.7	88.2±16.6	**<.001**	82.6±20.3	90.1±15.9	**.003**	67.4±25.5	87.3±16.9	**<.001**
Social functioning	67.4±28.8	88.4±18.7	**<.001**	75.3±24.4	91.7±15.4	**<.001**	62.5±30.4	86.9±19.8	**<.001**
Role limitation (physical)	53.4±44.5	84.5±31.3	**<.001**	66.3±41.2	87.8±26.2	**<.001**	45.4±44.8	83.1±33.0	**<.001**
Role limitation (emotional)	72.8±40.5	86.5±29.5	**<.001**	82.6±32.0	90.1±24.4	.102	66.7±44.1	84.8±31.4	**<.001**
Pain	73.0±26.8	85.8±18.5	**<.001**	82.0±21.1	88.0±16.4	.106	67.4±28.5	84.8±19.3	**<.001**
General health perception	46.8±22.2	71.6±18.7	**<.001**	50.7±22.7	74.5±17.1	**<.001**	44.4±21.7	70.4±19.3	**<.001**
General perception of change in health	50.3±22.7	53.6±17.9	.140	49.4±18.8	54.4±18.0	.210	50.9±24.9	53.2±17.9	.344
HADS									
Anxiety	5.5±3.9	4.1±3.2	**<.001**	3.9±2.7	3.0±2.7	**.036**	6.4±4.2	4.5±3.3	**<.001**
Depression	5.1±4.1	2.8±2.8	**<.001**	4.2±3.9	2.7±2.5	**.015**	5.6±4.1	2.8±3.0	**<.001**
Total score	10.5±7.3	6.8±5.3	**<.001**	8.2±6.0	5.7±4.4	**.016**	12.0±7.6	7.3±5.6	**<.001**
MFI-20									
General fatigue	11.7±2.2	8.5±4.0	**<.001**	11.0±2.2	7.5±3.5	**<.001**	12.1±2.1	8.9±4.2	**<.001**
Physical fatigue	13.1±2.6	7.6±3.7	**<.001**	12.3±2.6	7.3±3.5	**<.001**	13.5±2.3	7.7±3.8	**<.001**
Reduced activity	12.2±2.3	7.2±3.5	**<.001**	11.8±2.5	7.1±3.3	**<.001**	12.4±2.2	7.2±3.5	**<.001**
Reduced motivation	11.4±2.6	7.3±3.4	**<.001**	11.6±2.9	7.2±3.3	**<.001**	11.3±2.5	7.3±3.4	**<.001**
Mental fatigue	11.3±2.3	7.8±3.9	**<.001**	10.9±2.2	6.9±3.4	**<.001**	11.5±2.3	8.2±4.0	**<.001**

Mann-Whitney U-test s. Data are presented as mean (standard deviation). AI: adrenal insufficiency. p value[1]: patients with PAI vs. Healthy controls; p value[2]: AI males vs. healthy males; p value[3]: AI females vs. healthy females.
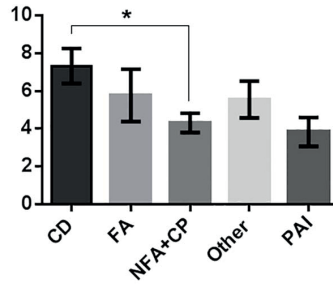
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Figure 2. *Comparison of depressive symptoms (HADS) between patient groups. Depressive and Anxiety symptoms as measured by the Hospital Anxiety and Depression Scale (HADS), stratified by the following patient categories: 1. CD: Cushing's disease (n = 18); 2. FA: functioning pituitary adenomas (n = 14, including acromegaly (n = 5), prolactinoma (n = 8), FSH producing adenoma (n = 1)); 3. NFA+CP: non-functioning pituitary adenomas (n = 48, including craniopharyngeoma (n = 13) and NFA (n = 35)); 4. PAI: primary adrenal insufficiency (n = 18) 5. Other: other causes of hypopituitarism (n = 22, including congenital hypopituitarism (n = 6), hypopituitarism after radiotherapy/surgery/traumatic brain injury (n=7), other causes such as pituitary inflammation or Sheehan's syndrome (n = 9)).*

Relationships between daily hydrocortisone intake and QoL

Correlations between hydrocortisone intake and QoL, adjusted for age, gender, and etiology CD, revealed that in the whole group, higher hydrocortisone intake was associated with more impairments in physical functioning (r = -0.208, p = .027), less vitality (r = -0.251, p = .007), a greater decrease in perceived health (change in health) (r = -0.317, p = .001) (SF-36), more limitations in daily activities (r = 0.206, p = .032), and a worse perceived health status (r = -0.297, p = .002) (EQ-5D). After stratification for gender, it was observed that higher hydrocortisone intake was associated with a worse perceived health status in male patients (r = -0.319, p = .048). In female patients, higher hydrocortisone intake was associated with less vitality (r = -0.334, p = .005), a greater change in health (r = -0.349, p = .003) (SF-36), more limitations in daily activities (r = 0.264, p = .028), and a worse perceived health status (r = -0.307, p = .012) (EQ-5D). Regression analyses including age, gender, and etiology CD, as well as daily hydrocortisone dose as a quadratic term revealed a significant quadratic contribution of hydrocortisone dose to depressive symptoms (HADS) (β = 1.150, p = .019), mental fatigue (MFI-20) (β = -1.079, p = .033), physical functioning (β = -0.946, p = .046), social functioning (β = -1.232, p = .012), change in health (SF-36) (β = -1.031, p = .039), pain (β = 1.413, p = .004) and perceived health status (EQ-5D) (β = -1.022, p = .036), indicating that relatively low, as well as relatively high hydrocortisone intake was associated with more depressive symptoms, more limitations in physical functioning and social functioning, more pain, and lower perceived health, but less mental fatigue.

Table 3. Correlations between QoL, CORT$_{hair}$ and hydrocortisone dose

		Patients with AI[a]		Males with AI[b]		Females with AI[b]	
		CORT$_{hair}$	HC dose	CORT$_{hair}$	HC dose	CORT$_{hair}$	HC dose
SF-36	Physical functioning	-.097	-.208*	-.019	-.200	-.122	-.164
	Vitality	-.068	-.251**	.067	-.085	-.164	-.334***
	Change in health	.015	-.317***	.011	-.258	.031	-.349***
MFI-20	Physical fatigue	.120	.136	.335*	.203	-.028	.066
EQ-5D	Activity	.180	.206*	.016	.153	.239*	.264*
	Pain	.131	.134	-.054	.013	.269*	.215
	VAS	-.071	-.297***	.171	-.319*	-.164	-.307*

*[a] partial correlations correcting for age and gender; [b] partial correlations correcting for age. *p < .05; **p < .01; ***p < .005. AI: adrenal insufficiency. CORT$_{hair}$ (pg/mg); hydrocortisone dose (mg). Only significant correlations are shown.*

Regression analysis including CORT$_{hair}$ and hydrocortisone intake

Regression analysis including both CORT$_{hair}$ and daily hydrocortisone dose, as well as age, gender, and etiology CD, revealed a significant contribution of daily hydrocortisone dose to physical functioning (β = -0.182, p = .046) change in health (SF-36) (β = -0.254, p = .008), limitations in physical activities (β = 0.204, p = .034) perceived health status (EQ-5D) (β = -0.277, p = .004). No significant contribution of CORT$_{hair}$ was found in this by using this regression model. Post-hoc analyses on these significant results using the same regression analyses, but without CORT$_{hair}$, resulted in slightly increased beta's (increases ranging from .008 to .022), indicating that part of the variation of CORT$_{hair}$ was explained by hydrocortisone intake, which is not surprising considering the association between daily hydrocortisone intake and CORT$_{hair}$ (r = 0.185, p = .047).

DISCUSSION

The present exploratory study confirmed that patients with AI report more impairments in QoL compared to healthy controls [2-4, 6], which is dependent on the cause of AI and demonstrated that daily hydrocortisone intake was inversely correlated with QoL (physical aspects). This is in accordance with some [6, 13, 28], but not all studies [2-3, 29]. Interestingly, this association was not found with systemic cortisol exposure, since only a few aspects of QoL were associated with CORT$_{hair}$, suggesting that QoL impairments are not per se due to chronic overtreatment with hydrocortisone. Nevertheless, CORT$_{hair}$ did explain a part of the variation of the observed associations between daily hydrocortisone intake and QoL, indicating that the actual cumulative cortisol exposure should also be taken into account.

Previous QoL studies in patients with AI identified several influencing factors, such as autoimmune co-morbidity [300], delay of diagnosis [30], higher age at manifestation, and female gender [30]. Furthermore, it is suggested that intrinsic imperfections in replacement therapy also play a role [10]. The present study is the first to examine the relation between actual chronic cortisol tissue exposure and QoL in patients with AI as measured by $CORT_{hair}$. Based on this first explorative study it seems that associations between hydrocortisone intake and QoL are not (directly) influenced by cortisol exposure. This suggests that QoL impairments in patients with AI are not per se related to higher cortisol exposure, but it might be more obvious that the relation between hydrocortisone intake and QoL is (at least partly) explained by that patients who take more hydrocortisone basically need more hydrocortisone. Furthermore, assessing the potential effect of occasionally taking higher hydrocortisone doses did not reveal a significant effect (data not shown).

Cortisol acts in the central nervous system by binding to mineralocorticoid- and glucocorticoid receptors. The current notion is that the effects of cortisol binding to mineralocorticoid and glucocorticoid receptors follow an inverted u-shaped dose response curve, with both pathological low and high cortisol levels negatively affecting the mediating function of these receptors [31]. This mechanism might underlie the observed impairments in QoL in female patients with $CORT_{hair}$ above the lab-internal cut-off. Since there is no explicit lower limit of $CORT_{hair}$, there is no evidence that under-replacement negatively affects QoL. Interestingly, this inverted u-shaped dose response curve was identified in the quadratic associations found between hydrocortisone intake and physical, mental and social aspects of QoL.

Despite the heterogeneous origin of AI in this cross-sectional analysis, we found that $CORT_{hair}$ correlated with two physical aspects of QoL (physical activities and pain) in female patients, and one physical aspect in male patients (physical fatigue). In addition, female patients with $CORT_{hair}$ above the lab-internal cut-off reported more impairment in QoL relative to females with $CORT_{hair}$ below the lab-internal cut-off. This difference was not found for male patients. The found gender difference in $CORT_{hair}$ in the present sample with males demonstrating higher $CORT_{hair}$ than females, was previously described [21] and may be due to, among other factors, sex-specific differences in levels of circulating cortisol binding globulin. Therefore, analyses of the present study were stratified for gender. Furthermore, in male patients higher $CORT_{hair}$ was associated with higher BMI [21], suggesting a metabolic effect of overexposure. Recently, Quinkler et al. demonstrated in patients with AI using conventional hydrocortisone replacement that switching to once-daily hydrocortisone dual release tablets did not ameliorate QoL, although BMI and HbA1c improved [32]. Together with the results of the present study, this would suggest that more adequate cortisol exposure predominantly affect somatic outcome, and to a lesser extent patient-perceived well-being, in particular in

males. Furthermore, it was observed that patients with CD showed lower CORT$_{hair}$ relative to the other groups (although not significant). We postulate that this observation is also related to the gender effect since ninety-four percent of the patients with CD were females, and comparing CORT$_{hair}$ between female patients with CD and other female patients revealed no significant results (data not shown). Furthermore, it can be speculated that low CORT$_{hair}$ found in patients with CD might be explained by irreversible changes in cortisol metabolism (e.g. more efficient breakdown of cortisol) related to the previous exposure to elevated cortisol levels.

In addition, the observation that patients AI due to previous treatment for CD reported more QoL impairments compared to the other diagnostic groups, while also having the lowest CORT$_{hair}$ (not significant), could potentially be explained by the fact that these patients have been exposed to excessive cortisol levels in the past. Previous literature reported that potential damage caused by this excessive exposure to cortisol might only be party reversible [7, 9]. Therefore, it might be that QoL impairments in the CD group are to a larger extent explained by the previous hypercortisolism, than due to current cortisol levels as measured by CORT$_{hair}$.

As previous studies show, the assessment of CORT$_{hair}$ is a useful tool in the diagnosis of Cushing's syndrome or AI [20, 33] or as indicator of somatic disease and distress [34-36]. Furthermore, the assessment of CORT$_{hair}$ in the present study enabled us to discriminate between cause and consequences, since impairments in QoL were associated with a higher hydrocortisone intake, but were not reflected by higher CORT$_{hair}$. Several small studies on the relation between CORT$_{hair}$ and depressive symptoms, anxiety or general well-being in subjects without AI have been published, however at present no other study primarily focused to this extent on QoL in relation to CORT$_{hair}$ in AI [34, 37-39]. Younge and colleagues assessed CORT$_{hair}$, as well as QoL and psychological parameters (i.e., SF-36, HADS) in patients with structural heart disease. They demonstrated that higher CORT$_{hair}$ was correlated with lower self-reported physical functioning, which remained significant after adjustment for age, gender and BMI. No significant correlations were found on other aspects [40]. Similarly, in the present study, physical aspects of QoL were associated with QoL, while no correlations were found with other aspects of QoL.

In the present study, CORT$_{hair}$ and some physical aspects of QoL were associated with each other in a heterogeneous group of patients with AI. It is important to acknowledge that we studied correlations within a group of patients with AI with impairments in QoL [2-6]. This group is potentially yielding a relatively small variation of QoL, thereby impeding finding associations between CORT$_{hair}$ and QoL. Other aspects that should be taken into account while interpreting the results are the multidimensional character of QoL [41], and the possibility that the used generic and domain-specific QoL questionnaires might have been not sensitive enough. Although a disease-specific QoL questionnaire for primary adrenal insufficiency (i.e., AddiQoL [42-43]) could have been more sensitive

and suitable, it was not used because it has not yet been translated and validated into the Dutch language. In addition, it should be acknowledged that although the present sample was heterogeneous regarding etiology of AI and that both patients with primary and secondary AI were included, it provides a representative sample of everyday clinical practice. Finally, no conclusions can be drawn about causality due to the cross-sectional design of this study. Future studies using a longitudinal design could provide more information about the time course of QoL impairments, as well as the contribution of $CORT_{hair}$.

In conclusion, this is the first report that further explored the relation between QoL, hydrocortisone intake and actual cortisol exposure in AI patients by measuring hair cortisol, a marker of long-term systemic cortisol exposure. Patients with AI demonstrated several impairments in QoL which were sex-specifically associated with hydrocortisone intake, but were to a lesser extent reflected by chronic cortisol exposure as measured by hair cortisol, suggesting that QoL impairments in patients with AI are not explained by the effect of prolonged exposure to elevated systemic cortisol levels.

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Chapter 7

08 GENERAL DISCUSSION

Parts of this discussion are based on:
Hair cortisol, stress exposure, and mental health in humans: A systematic review.
Staufenbiel, S.M., Penninx, B.W.J.H., Spijker, A.T., Elzinga, B.M., & van Rossum, E.F.C. (2013).
Psychoendocrinology, 38(8), 1220-1235.

8   RATIONALE

This thesis contains five studies on the relationship between long-term cortisol (and cortisone) as measured in scalp hair – as indicator of the chronic activity of the hypothalamus-pituitary-adrenal (HPA) axis – and various factors of stress-related diseases and disorders. The research described can be divided into two main areas, i.e. long-term cortisol and cortisone in psychology and psychiatry, and long-term cortisol and cortisone in somatic medicine.

In this general discussion, implications of the main findings will be reviewed, methodological considerations will be discussed, and suggestions for future research directions will be provided.

8.1   Implications of Main Findings

8.1.1   *Sociodemographic, Health and Lifestyle, Hair (Treatment) Characteristics, and Long-Term Corticosteroid Levels*

Already in the first publication that used hair as a measurement of long-term corticosteroids in 2004 [1], the question arose which factors - other than the variable of interest - could influence long-term corticosteroid levels. In subsequent publications, researchers investigated associations between their variable of interest and diverse hypothetical covariates and confounders. Disentangling the effects of covariates seemed of paramount importance to enable studies to adjust their analyses for potential confounders. This was the focus of our study described in **Chapter 2**. For this study, we used data from 760 respondents participating in the research cohort of the Netherlands Study of Depression and Anxiety (NESDA), an ongoing longitudinal cohort study on the predictors, course and consequences of depressive and anxiety disorders. The analysis indicated that both long-term cortisol and long-term cortisone levels showed robust linear increases with age. Age has often been associated with long-term cortisol [2-4] in earlier research. However, studies that found curvilinear [4] or linear [2, 3] effects comprised a wider age range, and may therefore be more suitable to detect endocrine changes related to age. Thus, our study and others indicate that age is a strong predictor of hair corticosteroids and needs to be included as possible covariate in studies that use participants with a range of age. Reasons why higher age coincides with higher cortisol levels are manifold. A higher production could be due to more 'triggers' of the HPA axis due to psychological stress [5], to more inflammation [6] and/or more metabolic and cardiovascular diseases [7]; factors that are biologically associated with higher cortisol levels.

Lower long-term cortisol and long-term cortisone levels were found in subjects who wash their hair more frequently. This effect has also been reported earlier in other research [8]. Frequent washing of the hair might damage the hair structure and thereby lead to an increased wash-out of cortisol. This reasoning is consistent with the often

replicated result that cortisol content decreases the more distal the hair segment is from the scalp [4, 9, 10]. Other studies have reported no effect of hair washing frequency on long-term cortisol levels [10, 11]. It is undetermined yet how clinically relevant this factor is as relationships between biological parameters and long-term cortisol levels are also apparent when not accounting for the hair washing frequency; however, accounting for this effect in group comparisons is advised.

We further found that elevated long-term cortisol levels were related to the presence of diabetes mellitus. This result is consistent with other research on this topic [2, 3, 12]. The association was to be expected and is biologically sound, as already in subclinical Cushing's Syndrome, characterized by elevated cortisol levels, a high prevalence of diabetes mellitus is noted [13, 14]. Taken together, these results suggest that higher long-term cortisol levels are associated with long-term increased glucose levels and presence of diabetes mellitus across different age spans.

Furthermore, in our study, hair samples representing the winter months showed lower long-term cortisol. A seasonal effect on long-term cortisol has also been reported by another group, in which participants had higher long-term cortisol in summer and autumn as opposed to winter [15]. As mentioned in the Introduction (Chapter 1), seasonal effects on the HPA axis have been reported before [16], however, the mechanisms behind that mechanisms are not well understood [17]. Apart from that regulatory mechanism, another explanation for the seasonal effect observed in our study may be that participants tend to sweat more in warmer months and there may be an effect of cortisol in sweat on the hair in the proximity of the scalp. However, results of a study in which the influence of sweating on long-term cortisol levels was examined show no effect of sweating [18], therefore rendering this explanation unlikely. Other explanations have yet to be found.

Weaker effects were found for sex. Men showed higher long-term cortisol and long-term cortisone levels than women, which is in line with previous studies [2, 4, 12] but at variance with others [1, 11]. However, the sex effect was not independent of other covariates. Higher waist circumference was associated with increased long-term cortisol levels, which concurs with most studies that investigated this association [3, 11]. The correlation between waist circumference, or better, visceral fat, and long-term cortisol is well supported by literature [see 19, for review]. A darker natural hair colour was associated with higher long-term cortisol. Other studies also observed this trend [4].

Our study was the first to report a positive effect of oral contraceptives on long-term cortisol. These are surprising results, since long-term cortisol levels are generally considered to reflect the free fraction of cortisol which should not be influenced by use of oral contraceptives [4]. Other studies examining the association between long-term cortisol levels and the use of oral contraceptives did not report significant results [4, 20, 21]. As we had no information on the type of oral contraceptives (i.e., which hormone

combination), we could not further unravel whether this effect was caused by a specific hormone combination.

Long-term cortisone seems to be influenced by factors quite consistent with influences of long-term cortisol, namely age and hair washing frequency, and weaker with sex, waist circumference, presence of diabetes mellitus, and natural hair colour. In contrast to long-term cortisol, long-term cortisone levels were additionally affected by smoking status, hair treatment, and use of hair styling products. Current smokers had higher long-term cortisone, independent of their average number of cigarettes. Hair treatment, i.e. bleaching of the hair, was associated with lower long-term cortisone. Furthermore, the use of hair styling products also influenced HairE. As the hair samples were washed before extraction and analysis, it seems unlikely that the actual product used on the day of sample collection caused this effect; however, repeated cosmetic treatment could affect and lower hormone concentrations, as has been suggested before [1].

Based on our results, age, sex, waist circumference, diabetes mellitus, depending on the hormone in question also use of oral contraceptives, season and smoking status have been confirmed and identified as potential influencing factors, while hair washing frequency, natural hair colour, hair treatment, and use of hair styling products have been confirmed and identified as potential confounding factors and should be considered in future research when examining long-term corticosteroid levels.

8.1.2   Long-Term Corticosteroids in Mental Health Aspects

We then studied the associations between long-term corticosteroids and psychopathological characteristics. We found that participants with a current comorbid depressive and anxiety disorder presented with higher long-term cortisol, while long-term cortisol was not different in patients with a remitted disorder or with a current single diagnosis (**Chapter 3**). For this study, we again used the NESDA cohort, including this time 1166 participants. Additional analyses revealed that the severity of current symptoms was associated with higher long-term cortisol levels. Long-term cortisone was associated with the presence of social phobia, but not with other current or remitted diagnoses. The interesting fact that the significant results were found in participants with a current diagnosis, whereas remitted persons did not show altered long-term corticosteroid levels, poses a possible answer to the question whether a dysregulated HPA axis in mentally ill patients is a state or trait phenomenon. A stronger association with active cortisol rather than inactivated cortisol, as found in our study, may also support the concept that the alterations in long-term cortisol associated with psychopathological characteristics are a state (reflected by long-term cortisol) rather than a trait (reflected by long-term cortisone). This would imply that during the episode of a disorder, the HPA axis is dysregulated, but that the cortisol levels return to normal once the patient remits. This is also supported by the findings that remitted patients showed long-term

corticosteroid levels comparable to healthy controls. The state vs trait phenomenon has also been examined using salivary cortisol levels with somewhat different results. For example, the cortisol awakening curve (CAR) has been shown to be increased in patients with a current as well as a remitted major depressive disorder [22] and a current as well as remitted panic disorder with agoraphobia [23] as compared to control subjects, which does support the notion of a trait rather than a state marker [22], and which was hypothesized to reflect either a specific biological vulnerability or a biological scar [24]. As the assessments of salivary cortisol and long-term cortisol capture different aspects of HPA axis functioning, the integration of both methods will likely further the quest for an answer regarding state or trait factors of HPA axis dysregulation in psychopathology.

In a different group of patients with a mental illness, i.e. bipolar disorder, we showed an association between long-term cortisol and the number of negative life events (**Chapter 4**). For this study, we included 71 patients from The Bipolar Stress Study, a cross-sectional and 24 month longitudinal study that aims on identifying risk factors that have an impact on the clinical course of bipolar disorder and treatment of patients with bipolar disorder. The effect of elevated long-term cortisol levels after life events is consistent with studies in other populations [25, 26]. Negative life events might aggravate rumination that may exacerbate the already adverse experience [27], thereby potentiating the effect of life events. It has previously been reported that bipolar patients show normal initial cortisol responses to stressful life events, but that they have more difficulty in terminating the stress response. It is essential that an individual is able to terminate the cortisol response appropriately to prevent hypercortisolemic effects [28], as a failure to do so would reflect in higher mean cortisol levels over a prolonged period of time. This would contribute to higher long-term cortisol levels. Interestingly, social support showed an inverse association with long-term cortisol in patients reporting negative life events. This indicates that social support may attenuate the adverse effects of negative life events. As the "social zeitgeber theory" has proposed that life events might induce a new mood episode through disruptions in social and biological rhythms [29], increased social support during a difficult time may stabilize social rhythm, sleep pattern, and thereby also the biological rhythm.

8.1.2.1 Long-Term Corticosteroids in Mental Health Aspects: Conclusion
To conclude, the research in this thesis conducted on the association between long-term cortisol and psychiatric aspects show that long-term cortisol is higher in patients with severe symptoms of depression and anxiety, and in patients with major negative events. This is in line with the idea that chronic stress as measured in hair cortisol shows a more pronounced effect, whereas the altered HPA axis activity in mental illness is more subtle. The chronic dysregulation of the HPA axis might therefore begin in the transition of the stress reaction to the (unsuccessful) recovery. Alternatively, the dysregulation might

prevent an appropriate stress reaction. These and other hypotheses have to be explored in quest of the starting point of mental illness. Until this issue is resolved, the combination of long-term cortisol levels and short-term cortisol levels may provide the most thorough information on HPA axis activity and dynamics. Consensus between those kinds of measurements is neither desired nor required to add up to a more complete understanding of the role of cortisol concentrations in the stress system. If anything, integrating both methods to display both baseline activity and stress reactivity of the HPA axis seems the most promising approach to understand the neurobiological components of development and remission of (mental) illnesses.

8.1.3   Long-Term Corticosteroids in Somatic Health Aspects
In addition to psychopathophysiology, we also studied associations between long-term cortisol and somatic factors. Herefore, we recruited 47 obese patients from the Obesity Center CGG of the Erasmus Medical Center, a multidisciplinary referral center for diagnostic testing and tailored treatment of obesity. We compared their data to that of 41 overweight and 87 normal weight controls. One of the results is that obese participants had higher long-term cortisol levels than overweight and normal weight participants, while between overweight and normal weight participants, no difference n long-term cortisol was detected (**Chapter 5**). Our results are in line with other studies that reported a positive correlation between HCC and BMI [3, 30]. Results on an association between long-term cortisol and increased (central) adiposity/obesity have also been reported before [11, 12]. Interestingly, long-term cortisol was similar between normal weight and overweight participants. BMI does not discriminate between muscle and fat mass, and although it is to assume that our obese group (with a median BMI of 38.8) has a higher fat mass than non-obese controls, this might not apply for overweight individuals. This may also explain null results of correlations between BMI and long-term cortisol in studies comprising participants with normal to overweight BMI [e.g. 11]. A reason as to why the obese individuals have higher long-term cortisol levels may be due to metabolic processes in the liver. The liver is the major site of cortisol clearance. Obesity (and cortisol excess) is known to be associated with non-alcoholic fatty liver disease (NAFLD) [31], which first manifests with liver steatosis and gradually progresses to steato-hepatitis. The activity of hepatic enzymes responsible for cortisol clearance and regeneration has been shown to be decreased and increased, respectively, in patients with steato-hepatitis [32]. It therefore could be speculated that the hypercortisolism we observed in obese patients is due to liver steato-hepatitis yielding dysfunction of liver enzymes.

Two of our studies focussed on patients with adrenal insufficiency, conducted in patients from the outpatient clinic of the department of Endocrinology of the Leiden University Medical Center. In the first study, we compared long-term cortisol levels of 132 patients with primary or secondary adrenal insufficiency on replacement therapy

with those of 42 patient controls (with a pituitary disease without adrenal insufficiency) and of 195 healthy controls, and demonstrated that long-term cortisol levels were higher in patients with primary or secondary adrenal insufficiency on replacement therapy than in patient controls and healthy controls (**Chapter 6**). This result is in line with all other studies on this association [33-35]. On the one hand, this finding might imply a possible chronic overtreatment with hydrocortisone. On the other hand, the intake of hydrocortisone medication is associated with peaks of cortisol shortly after ingestion, and it may be that the peaks are integrated into the hair, thereby increased the long-term cortisol levels measured in hair. We also showed that the hydrocortisone dose correlated with long-term cortisol. This result is in line with one other study with a comparable patient group [33], supporting the assumption that long-term cortisol levels reflect hydrocortisone intake. Additional analyses revealed that male patients with adrenal insufficiency had higher long-term cortisol levels than female patients, an effect that was not observed in the other study on this patient group [33]. Furthermore, we showed that patients with adrenal insufficiency had higher body mass index (BMI) than healthy controls. In male, but not in female patients with adrenal insufficiency, higher long-term cortisol levels were associated with higher BMI. The association between long-term cortisol and BMI could also suggest a mild overtreatment that may lead to adverse anthropomorphic side effects, especially in males. The results further indicate that long-term cortisol in combination with clinical symptoms of hydrocortisone over-treatment may be used to first identify overtreatment with hydrocortisone and then to improve refinement of individualized hydrocortisone substitution therapy in patients with adrenal insufficiency. In this study, we also assessed whether the increased stress of being chronically ill might influence long-term cortisol levels and might result in need of dose adjustment (as some patients with adrenal insufficiency known to occasion-ally increase their hydrocortisone doses in situations of increased psychological stress [36], which might result in higher long-term cortisol). In population studies, long-term cortisol has been associated with perceived stress [37-39], but if this was the case in the present study, the increased stress of being a patient should then also be present in our patient control group. However, long-term cortisol levels between patient controls and healthy controls were comparable. Besides the demonstrated association between long-term cortisol and anthropometrics, a considerable amount of studies demonstrated the association between high long-term cortisol and psychological symptoms [38]. We therefore examined the association between long-term cortisol, hydrocortisone intake, and quality of life (QoL) in 120 patients with stable adrenal insufficiency (**Chapter 7**). We confirmed that patients with adrenal insufficiency report more impairments in QoL compared to healthy controls [40-43], which is dependent on the cause of adrenal insuf-ficiency. Patients reported impairments in 14 of 15 QoL subscales. We demonstrated that daily hydrocortisone intake was inversely correlated with physical aspects of QoL.

This is in accordance with some [43-45] but not all studies [40, 41, 46]. Interestingly, this association was to a lesser extent found with systemic cortisol exposure, since only a few aspects of QoL were associated with long-term cortisol. Nevertheless, long-term cortisol did explain a part of the variation of the observed associations between daily hydrocortisone intake and QoL, indicating that the actual cumulative cortisol exposure should also be taken into account. The correlation of more impairment in physical aspects of QoL and higher daily hydrocortisone intake was more pronounced in female patients. These results show that patients with adrenal insufficiency report several impairments in QoL which are sex-specifically associated with hydrocortisone intake, and to a lesser extent reflected by chronic systemic cortisol exposure as measured by long-term cortisol. This suggests that QoL impairments in patients with adrenal insufficiency are not per se due to chronic overtreatment with hydrocortisone.

8.1.3.1 Long-Term Corticosteroids in Somatic Health Aspects: Conclusion
To conclude, the associations between long-term cortisol and somatic aspects show that also in weight and BMI, long-term cortisol is higher in the more severe forms than in the normal range. In patients with adrenal insufficiency, long-term cortisol correlates with the dose of hydrocortisone and in males also with higher BMI. Hair cortisol analysis may provide a new method for identifying chronic hydrocortisone overtreatment in patients with adrenal insufficiency, which may improve current glucocorticoid replacement quality and limit the health effects of previously unobserved overtreatment.

8.2   Technical Aspects and Considerations

The use of scalp hair as a method to study long-term cortisol levels has received increasing attention from several research groups around the world. Until 2012, when work on this thesis began, according to PubMed, 24 original articles had been published on "hair cortisol" in humans. Four years later, the number of articles had increased to over 100. However, with the growing interest and more research groups working in this area, the methods used in long-term corticosteroid analyses increased, as well. Overall, the methods used are very comparable, with some variations in procedures among laboratories. As mentioned in Chapter 1, enzyme-linked immunosorbent assay (ELISA), luminescence immunoassay (LIA), radioimmunoassay (RIA), or liquid chromatograph–mass spectrometry (LC–MS/MS) have all been used for cortisol quantification [10, 11, 47-50]. In the beginning, immunoassays were the most popular, as they are technically straightforward and easy to implement. However, immunoassays come with drawbacks such as cross-reactivity and the limitation to single component measurement per assay. More advanced methods, based on mass spectrometry, have been long available, but were costly and unsuited for high-throughput routine laboratory measurements. During the last years, however, mass spectrometry has overcome these limitations and has

become the preferred method for steroid analysis in high-quality clinical research due to its superior specificity and sensitivity.

With the growing interest in long-term cortisol analyses, comparability and standardization of methods between laboratories seemed paramount, as there can be significant interassay variability among commercially available immunoassays, not to mention the extraction protocols in the laboratories themselves. This need was acknowledged by several laboratories, who subsequently worked together to examine interlaboratory differences. Four different laboratories divided hair samples of the same persons and animals and analyzed them using their different methods, comparing four immunoassay methods and two LC–MS/MS methods. The results showed that when all laboratories analyze a common batch of hair, each laboratory's immunoassay long-term cortisol levels were highly positive correlated with the results of their LC–MS/MS methods (with r^2-values ranging between .88 and .98) [51]. The absolute values of the long-term cortisol determined by each group differed for the immunoassays, however, the correlations between the different assays were rather strong. In addition, long-term cortisol determined by the two LC–MS/MS methods produced virtually identical results ($r^2 = 0.98$). Therefore, the development of the LC–MS/MS method and the ongoing transition in different laboratories from immunoassays to LC–MS/MS allows for the better comparability between studies. Furthermore, the high sensitivity and the addition of other adrenal and sex steroids broaden the scope of clinical use of hair analysis to a variety of psychopathological and somatic diseases.

Despite the many developments regarding the method of long-term corticosteroid analysis in hair, there are still open questions and limitations. One uncertainty is the way in which cortisol is incorporated in the hair shaft, and where this happens. This is important as this will provide information about possible external factors that can influence the concentration of long-term corticosteroids. The hair shaft consists of three parts; the medulla, the cortex, and the cuticle. At present, it is assumed that incorporation happens via diffusion from the capillary system directly into the hair root. Other possible incorporation mechanisms are the incorporation via sebum or sweat, however, an *in vivo* examination revealed that long-term cortisol levels were not acutely altered by interventions that induced significant sweating, and that long-term cortisol levels were unrelated to sweat cortisol levels or to individuals' sweating rate [52], rendering this possibility unlikely.

Another issue of importance in long-term cortisol research is the hair growth rate, especially when creating timelines. Generally, it is assumed that hair grows one centimetre per month, with a range of 0.6 – 1.4 cm/month [53, 54]. It has been shown that hair growth rate differs between ethnicities. Asian hair is reported to grow the fastest (~ .48 mm/day, therefore 1.44 cm/month), whereas African hair is reported to grow the slowest (~ .26 mm/day, therefore .78 cm/month), and Caucasian hair to lie in between

(~ 40 mm/day, therefore 1.2 cm/month) [55, 56]. As intraindividual long-term cortisol levels are relatively stable over months to years [57], a different growth rate should not influence the results when assessing one value (of e.g. three months, i.e. three cm) as general indicator of the long-term cortisol value. However, it does matter when creating hair timelines and should therefore be considered in the analysis. One limitation of long-term cortisol levels is obvious when it comes to persons who are bald or suffer from substantial hair loss at the scalp. These individuals cannot be included in studies on long-term cortisol. Hair loss can be associated with psychological stress [58, 59], with hormonal imbalances [60], and with psychiatric disorders such as anorexia nervosa [61] or trichotillomania. Only if hair loss is that substantial that no hair is available at the posterior vertex of the scalp, short-term cortisol level assessment remains the main method to investigate cortisol values.

Regarding the other cortisol assessments available, a short overview has already been given in the Introduction (Chapter 1).

Short-term measurements offer information on cortisol levels in a time range of seconds up to one day. One technique is the point-measurement, in which cortisol levels at one or more specific time points are measured in saliva or serum. This provides information on acutely circulating cortisol levels and requires minimal effort, however, they are highly fluctuating during the day. Short-term measurements also offer the possibility to assess cortisol day curves, in which several samples of saliva or serum are taken during the day. This method provides information about the circadian rhythm and mean cortisol levels during the day. Disadvantages for the participant include intense sampling, in case of serum sampling also invasive sampling that requires admission. The CAR, part of the day curve, is assessed by collecting samples of serum or saliva in the first hour after awakening and informs about basal activity as well as the response to awakening. While it is not as intensive as a day curve assessment, it is highly dependent on the time of awakening. In case of saliva sampling, the compliance of patients is crucial, in case of serum sampling, again an admission is required. Urinary free cortisol requires the sampling of 24 hours of urine. It reflects the total cortisol production in one day and is non-invasive; however, it also depends on participants' compliance and is cumbersome for the participant. A further rather new method is the assessment of cortisol from finger nails [62]. Fingernail cortisol levels were shown to moderately correlate with hair cortisol and salivary cortisol [63]. Fingernail cortisol offers the possibility to retrospectively assess long-term cortisol (as does hair cortisol), however, not much is known about the nail growth rate and influencing factors (both for growth rate as for cortisol content). It has been hypothesized that four to five months are required for nails to fully extend from the mail matrix [63] and that nail growth rate increases with temperature and declines with age [64], however, nothing is known about effects of for example nail polish or nail-biting habits. One further difficulty arises regarding the creation of timelines. It has

been stated that hormones are integrated into nails via passive diffusion from capillaries to the nail matrix and that they become incorporated into keratin during nail formation [65]. However, other studies show that nail growth only occurs to 80% in the matrix, whereas the remaining 20% are formed during the progressive growth in the nail bed [66]. Therefore, for now, it is not known whether timelines are an option in fingernail cortisol analysis.

Table 1 provides a summary of these different methods.

Table 1. Methods to measure cortisol levels

Test	Sample	Information	Advantages	Disadvantages
Point-Measurement	Saliva or serum	Cortisol level at one specific time point	Minimal effort, gives detailed current information	Highly fluctuating during the day In serum: invasive
Cortisol awakening rise	Saliva or serum	Cortisol levels' basal activity and response to awakening	Provides detailed information In saliva: patients can collect it at home	Highly dependent on time of awakening In serum: invasive, admission required In saliva: compliance of patient required
Cortisol day curve	Saliva or serum	Mean cortisol levels during the day and circadian rhythm	Provides information of day rhythm and mean cortisol levels during the day	Intensive for patients In serum: invasive, admission required
Urinary free corisol	Urine	Total cortisol production in one day	Information on total production	Cumbersome for patient; compliance required
Hair cortisol	Hair	Mean cortisol in one or more months	Informs on mean cortisol levels for months to years Non-invasive, easy to collect Potential to create historical timelines	Hair length ≥1 cm required, some issues not resolved
Nail cortisol	Finger nail	Mean cortisol in several months	Informs on mean cortisol levels for months Non-invasive, easy to collect	New method, Historical timelines not possible many issues not resolved

Summary of short-term and long-term cortisol measurement methods.

A recent study investigated the comparability of methods regarding salivary cortisol, urine cortisol, and hair cortisol, and measured all three matrices for one month (3x daily salivary cortisol, 1x weekly urine cortisol, 1x hair cortisol) [67]. The authors found that long-term cortisol was associated with the daily integrated salivary cortisol levels but not with the integrated CAR, the integrated diurnal slope, or integrated urinary free cortisol. They concluded that long-term cortisol levels of one month measured in hair provide a reliable estimate of a long-term integrated free cortisol production that cor-

responds with one-month integrated daily salivary cortisol production measured in the same period. This supports the notion that long-term cortisol, as measured in hair, is a valid method to assess cortisol if one is interested in long-term values that can be assessed retrospectively and with minimal disadvantages for the participant. However, long-tem cortisol does not provide information about HPA axis dynamics.

8.3   Clinical Implications and Future Perspectives

The clinical applicability of long-term corticosteroid research seems to increase with the fine-tuning of the method. In general, analysis of cortisol in scalp hair has provided researchers with a readily applicable tool to measure cortisol exposure over extended periods of time. Studies by our group and by others have indicated that long-term corticosteroid analysis may provide a new tool to investigate the chronic HPA axis activity in clinical practice. One of the unique advantages is the creation of timelines in hair. The clinical applicability has been reviewed and discussed by our group [68]. For both psychopathological and somatic health factors, Wester and van Rossum concluded that long-term cortisol measurements can be used to evaluate disturbances of the HPA axis, to diagnose specific diseases (such as cyclic hypercortisolism) and to evaluate (hydrocortisone) treatment. They state that potentially, long-term cortisol analyses could form a future marker for a cardiovascular risk profile and may serve as a treatment evaluation target.

For now, the question regarding the potential of long-term cortisol analysis as a new biomarker is still not answered. At this moment, it seems that a distinction in the potential is to be made for mental health and somatic health. For mental health, long-term cortisol analyses have shown only small effect sizes regarding healthy controls and patient groups [38]. Furthermore, as the alterations in cortisol values are only subtle, one cannot use the long-term cortisol values to discriminate between patients and controls. Therefore, at present time, it seems that long-term cortisol analysis is a valuable research tool for psychiatric and psychological studies; however, clinical applications are not possible at the moment. It may be different for somatic health. As previous research has shown, long-term cortisol analysis has high sensitivity and specificity in the diagnosis of both cyclic and non-cyclic CD [69], with percentages comparable to standard salivary and urinary cortisol. It therefore seems that clinical applicability is well justified for somatic health.

Regarding future applications, there are several directions that are worthy to be pursued. One direction is the application of timelines also in mental health. Here, one possibility is the assessment of long-term corticosteroid levels before and after an (stressful) event to investigate whether and how the levels change. In the case of long-term corticosteroid analysis, hair strands can be divided into different segments, allowing the participating individuals to serve as their own controls. First studies using

this method have been published. One study investigated children's long-term cortisol levels before and after school entry [70] and found that levels were higher after school entry. Other studies examined the influence of traumatic events on long-term cortisol levels while using the individuals' levels before the event as baseline level [71, 72].

The next important step in long-term cortisol research to further establish this claim is the investigation of interventions. These interventions can be divided into a) interventions aiming at symptom reduction and in parallel studying hypothetical changes in long-term cortisol levels, or b) interventions aiming at lowering or increasing long-term cortisol levels and in parallel studying hypothetical changes in symptoms. First intervention studies have been published. In a retrospective study, our research group showed that in patients with (cyclic) Cushing's Disease, the treatment trajectory can be observed in hair [69]; thereby demonstrating applicability for this sort of research. Until now, three studies have been published on patients attending a stress management program, and on long-term cortisol levels at the start and the end of the training. In the first study, participants at a smoking cessation intervention showed decreased long-term cortisol levels after a mindfulness training and smoking abstinence [73]. In the second study, patients with structural heart disease participated at a mindfulness training, however, the effect of the mindfulness training on long-term cortisol levels was not significant [74]. In the third study, participants attended a stress management program and had lower long-term cortisol levels in the last session than in the first session [75]. Taken together, the first studies seem promising that long-term cortisol analysis might be a novel biomarker for the evaluation of interventions. Another area that is of interest for future research is the individual sensitivity to cortisol. It has been hypothesized that a person's vulnerability to stress is essential in the development of psychopathology [76]. One factor that determines the individual vulnerability is the genetic sensitivity to cortisol. The genetic sensitivity is expressed by two steroid receptors; the fast-acting, high-sensitive mineralocorticoid receptor (MR) and the slow(er)-acting and less-sensitive glucocorticoid receptor (GR). Genetic polymorphisms of MR and GR account for variations in receptor expression and sensitivity [77]. Several single nucleotide polymorphisms (SNPs) have been detected to have specific effects on MR and GR function. For the MR (NR3C2) gene, these are −2G/C and I180V. Carriers of -2G/C respond with higher cortisol levels to psychosocial stress [78], show enhanced optimism and a lower risk for depression in females [79]. Carriers of I180V present with a higher prevalence of depression [80, 81]. For the GR (NR3C1) gene, research has mainly focused on four polymorphisms that have been associated with distinct differences in glucocorticoid sensitivity, as assessed with the dexamethasone suppression tests (DST), and/or changes in glucocorticoid receptor transactivation or transrepression activity. Two of these polymorphisms have been associated with a profile consistent with a more resistant response to glucocorticoids (ER22/23EK [82-85], 9β [86, 87]), whereas the other two polymorphisms (N363S [88, 89],

BclI [90, 91]) seem indicative of a more sensitive profile. So far, no studies have been published in which long-term cortisol levels and glucocorticoid receptor sensitivity have been combined. Integration of these two factors may provide exciting opportunities for clinical research, as high long-term cortisol levels against a background of a hypersensitive glucocorticoid receptor may synergistically produce stronger adverse health effects compared to participants with a more resistant glucocorticoid receptor. Therefore, we propose that future research combining these two aspects adds to a more complete picture of an individual's cortisol profile.

8.4   Concluding Remarks

In this thesis, we have shown that elevated long-term cortisol levels play a role in several stress-related diseases and disorders. A graphic overview is illustrated in Figure 1.
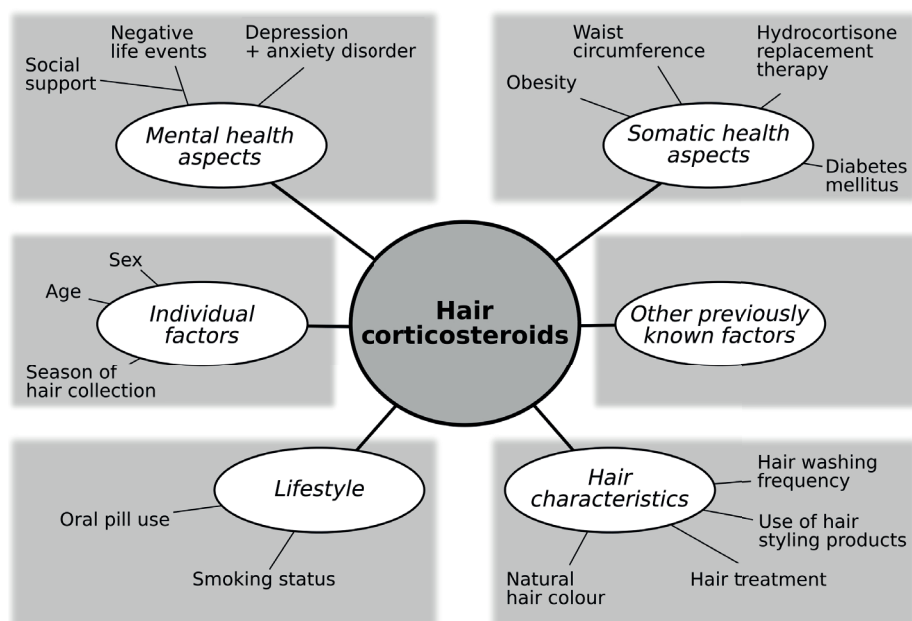

Figure 1. *An overview of the significant associations shown in this thesis.*

More research is required to gain further insight into the underlying factors. The combination of endocrine, genetic and psychological paradigms is a prerequisite to an integrated approach that aims to understand etiology and mechanisms of HPA axis dysregulation. Long-term cortisol research has repeatedly been used for this aim, with promising results. In the long run, this integrated approach will eventually help to predict the response to one or another pharmacological or psychotherapeutic treatment, and thus, to design personalized tailored interventions.

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09 Summary and Samenvatting

SUMMARY

Cortisol, the main glucocorticoid in humans, is commonly known as "the stress hormone". This thesis examined associations between long-term cortisol as measured in scalp hair and different aspects of stress-related diseases and disorders. Research was conducted in two areas; associations between long-term cortisol and the mind, and associations between long-term cortisol and the body.

Chapter 1 provides a general introduction to cortisol and the HPA-axis. The regulatory mechanisms of the HPA-axis are summarized, and different kinds of dysregulations are explained. Related, different methods to measure cortisol as product of the HPA-axis activity are described. The term "stress" as well as its effect is introduced, and an overview of studies on long-term cortisol and chronic stress as well as stress-related disorders is given. Chapter 1 ends with the aims of the thesis.

In **Chapter 2**, we used data of 760 participants without current psychopathology of the Netherlands Study of Depression and Anxiety (NESDA) and examined which sociodemographic, health and lifestyle, and hair (treatment) characteristics affect long-term cortisol and cortisone levels measured in scalp hair. We evaluated the following characteristics in our analyses: sociodemographic factors: sex, age, educational level (years of attained education), and ancestry (North-European or not North-European); health and lifestyle factors: alcohol consumption, smoking behaviour, physical activity levels, waist and hip circumference, presence of diabetes mellitus, hypertension, other chronic diseases, and in women only: use of oral contraceptives, and pregnancy; hair (treatment) characteristics: natural hair colour, dyeing of hair, bleaching of hair, perming of hair, hair washing frequency, use of any products except for shampoo on the scalp on the day of sample collection, day of sample collection (indicative for season). For long-term cortisol, age, diabetes mellitus, and use of oral contraceptives (in women) were the strongest determinants. For long-term cortisone, age and hair washing frequency were the strongest determinants. Therefore, these variables should be considered when examining long-term corticosteroid levels.

In **Chapter 3**, we compared long-term corticosteroid levels of 245 persons without psychopathology with those of 655 persons with a remitted depressive and/or anxiety disorder and with those of 266 persons with a current depressive and/or anxiety disorder. Participants with a current comorbid depressive and anxiety disorder presented with significantly higher long-term cortisol. Long-term cortisol was not different in patients with a remitted disorder or with a current single diagnosis. Additional analyses revealed that the severity of current symptoms was associated with higher long-term cortisol levels. The intake of SSRIs was also associated with higher long-term cortisol. Long-term cortisone was associated with the presence of social phobia, but not with other current or remitted diagnoses. Interestingly, the significant results were found in participants

with a current diagnosis, whereas remitted persons did not show altered long-term corticosteroid levels. This poses a possible answer to the question whether a dysregulated HPA-axis in mentally ill patients is a state or trait phenomenon. A stronger association with active cortisol rather than inactivated cortisol (cortisone), as found in our study, may indicate that the alterations in long-term cortisol associated with psychopathological characteristics seem to be a state rather than a trait. This would imply that during the episode of a disorder, the HPA-axis is dysregulated, but that the cortisol levels return to normal once the patient remits. This could be supported by the results that remitted patients showed long-term corticosteroid levels comparable to healthy controls.

In **Chapter 4**, we examined the association between long-term cortisol of 71 patients with bipolar disorder and the number of stressful life events, the amount of social support, and mood in the three months prior to the hair assessment and between long-term cortisol and mood in the subsequent three months. The total number of life events was not associated with long-term cortisol; however, the number of *negative* life events was associated with increased long-term cortisol. Social support showed an inverse association with long-term cortisol in patients reporting negative life events. Long-term cortisol and mood were neither associated in the three months prior to hair sampling nor in the subsequent three months. This study indicates that patients who experienced recent negative life events have increased long-term cortisol levels, which seem to be attenuated by social support.

In **Chapter 5**, we compared the long-term cortisol levels of 47 obese patients with those of 41 overweight and of 87 normal-weight participants, and found that obese participants had higher long-term cortisol levels than overweight and normal weight participants. Between overweight and normal weight participants, no difference in long-term cortisol was detected. It may be that the association between increased weight and long-term cortisol only reveals itself in a more extreme phenotype of adiposity. It may also indicate that chronically elevated cortisol leads to obesity, although causality cannot be concluded from this study.

In **Chapter 6**, we compared long-term cortisol levels of 132 patients with primary or secondary adrenal insufficiency on hydrocortisone replacement therapy with those of 42 patient controls with a pituitary disease without adrenal insufficiency and of 195 healthy controls. We demonstrated that long-term cortisol levels were higher in patients with primary or secondary adrenal insufficiency on hydrocortisone replacement therapy than in patient controls and healthy controls. We further showed that the hydrocortisone dose correlated with long-term cortisol. Additional analyses revealed that male patients with adrenal insufficiency on hydrocortisone replacement had higher long-term cortisol levels than female patients, and that patients with adrenal insufficiency had higher body mass index (BMI) than healthy controls. The association between long-term cortisol and

BMI could suggest a mild overtreatment that may lead to adverse anthropometric side effects, especially in males.

Chapter 7 describes the association between long-term cortisol, hydrocortisone intake, and quality of life (QoL) in 120 patients with stable adrenal insufficiency. Patients reported impairments in 14 of 15 QoL subscales. More impairment in physical aspects of QoL correlated with higher long-term cortisol and higher daily hydrocortisone intake, an effect that was more pronounced in female patients. These results show that patients with AI report several impairments in QoL which are associated with hydrocortisone intake, and to a lesser extent reflected by chronic systemic cortisol exposure as measured by hair cortisol. This suggests that QoL impairments in patients with AI are not per se the effect of overtreatment with hydrocortisone.

Finally, in **Chapter 8**, the implications of the main findings are discussed, methodological considerations are reviewed, and suggestions for future research directions are provided.

SAMENVATTING

Cortisol, het belangrijkste glucocorticoïdhormoon bij de mens, staat ook wel bekend als "het stresshormoon". In dit proefschrift werden de verbanden onderzocht tussen lange-termijn cortisol, zoals gemeten in hoofdhaar, en verschillende aspecten van stressgerelateerde ziekten en -stoornissen. Het onderzoek werd uitgevoerd op twee gebieden; associaties tussen lange-termijn cortisol en de psyche, en associaties tussen de lange-termijn cortisol en het lichaam.

In **Hoofdstuk 1** wordt een algemene inleiding gegeven op het gebied van cortisol en de hypothalamus-hypofyse-bijnier (HPA)-as. De wijzen waarop de HPA-as wordt gereguleerd zijn hierin samengevat, en verschillende manieren waarop deze regulatie kan zijn verstoord. Verder worden verscheidene methoden beschreven waarop cortisol, het product van de HPA-as, kan worden gemeten. De term "stress" en de effecten hiervan worden ingeleid, en een overzicht van studies op het gebied van lange-termijn cortisol, chronische stress en stressgerelateerde stoornissen wordt gegeven. Hoofdstuk 1 eindigt met de doelstellingen van dit proefschrift.

In **Hoofdstuk 2** onderzochten wij gegevens van 760 deelnemers zonder recente psychische stoornissen van de Nederlandse studie naar Depressie en Angst (NESDA), en onderzochten bij hen welke sociodemografische, gezondheids- en leefstijlaspecten, en eigenschappen van hoofdhaar van invloed zijn op lange-termijn corticosteroïd spiegels gemeten in hoofdhaar. Wij onderzochten in onze analyses de volgende eigenschappen: sociodemografische factoren: geslacht, leeftijd, opleidingsniveau (gevolgd onderwijs in jaren), en afkomst (Noord-Europese of niet Noord-Europese); gezondheid en leefstijl: alcoholconsumptie, roken, lichamelijke activiteit, taille- en heupomtrek, de aanwezigheid van diabetes mellitus, hypertensie, andere chronische aandoeningen, en alleen bij vrouwen het gebruik van orale anticonceptiva en zwangerschap; eigenschappen van hoofdhaar: natuurlijke haarkleur, verven, blonderen en permanenten van het haar, frequentie van haarwassen, gebruik van haar(styling)producten (met uitzondering van shampoo) op de dag van de haarverzameling, en de dag van de haarverzameling (als indicatie voor het seizoen). Leeftijd, diabetes mellitus en het gebruik van orale anticonceptiva (bij vrouwen) waren de sterkste invloeden op het lange-termijn cortisol. Leeftijd en frequentie van haarwassen waren de sterkste invloeden op het lange-termijn cortison. Daarom zou met deze variabelen rekening moeten worden gehouden bij de analyse van lange-termijn corticosteroïdwaarden.

In **Hoofdstuk 3** vergeleken we de lange-termijn corticosteroïdwaarden van 245 personen zonder psychische stoornissen met die van 655 mensen met een depressieve en/of angststoornis in het verleden, en 266 mensen met een actuele depressieve en/of angststoornis. Deelnemers met zowel een actuele depressie als angststoornis hadden een significant hoger lange-termijn cortisol dan andere deelnemers. Het lange-termijn

cortisol verschilde niet tussen patiënten met een aandoening in het verleden, of met een huidige diagnose van een enkele stoornis. Uit aanvullende analyses bleek dat de ernst van de huidige depressive en/of angstige symptomen geassocieerd was met een hoger lange-termijn cortisol. De inname van SSRI's was ook geassocieerd met een hoger lange-termijn cortisol. Lange-termijn cortison was geassocieerd met de diagnose van een sociale fobie, maar niet met andere actuele of vroegere diagnoses. Wat opvalt, is dat de significante resultaten alleen werden gevonden in de deelnemers met een actuele stoornis, terwijl personen met een diagnose in het verleden geen verandering in lange-termijn corticosteroïdwaarden vertoonden. Deze resultaten geven een mogelijk antwoord op de vraag of een ontregelde HPA-as bij patiënten met een psychopatho-logische diagnose een "state" of "trait" fenomeen is. Een sterkere associatie met actief cortisol, in plaats van geïnactiveerd cortisol (cortison), zoals in onze studie, zou kunnen aangeven dat de veranderingen op lange-termijn cortisol geassocieerd met psychopa-thologische kenmerken meer wijzen in de richting van een "state" dan een "trait". Dit impliceert dat de HPA-as ontregeld is tijdens een actuele episode van stoornis, maar dat de cortisolwaarden weer normaliseren zodra de patiënt is hersteld. Dit wordt verder ondersteund door de bevinding dat patienten met een diagnose in het verleden verge-lijkbare lange-termijn corticosteroïd waarden hebben met gezonde controles.

In **Hoofdstuk 4** hebben we bij 71 patiënten met een bipolaire stoornis de as-sociaties onderzocht tussen lange-termijn cortisol waarden en het aantal stressvolle levensgebeurtenissen, de hoevelheid sociale steun, de stemming in de drie maanden voorafgaand aan de haar verzameling, en de stemming in de drie maanden volgend op de haarverzameling. Het totale aantal levensgebeurtenissen hield geen verband met lange-termijn cortisolwaarden; het aantal *negatieve* gebeurtenissen was echter wel ge-associeerd met een verhoogd lange-termijn cortisol. Sociale steun toonde een negatief verband met lange-termijn cortisol bij patiënten die negatieve levensgebeurtenissen hadden meegemaakt. Lange-termijn cortisol waarden waren niet geassocieerd met stemming in de drie maanden voorafgaand aan de haar verzameling, noch in de drie daaropvolgende maanden.

De resultaten van deze studie laten zien dat patiënten die recente negatieve levens-gebeurtenissen hebben meegemaakt hogere lange-termijn cortisol levels hebben, wat echter lijkt te worden verminderd door een toename in sociale steun.

In **Hoofdstuk 5** vergeleken we de lange-termijn cortisolwaarden van 47 obese patien-ten, met die van 41 mensen met overgewicht en 87 mensen met normaal gewicht. Wij vonden dat obese mensen hogere lange-termijn cortisol waarden hebben dan mensen met overgewicht en normaal gewicht. Tussen mensen met overgewicht en een normaal gewicht werd geen verschil in lange-termijn cortisol gedetecteerd. Het zou kunnen zijn dat de associatie tussen hoger gewicht en lange-termijn cortisol waarden zich pas bij een ernstig overgewicht (obesitas) openbaart. Het zou tevens kunnen betekenen dat

chronisch verhoogde cortisol tot obesitas leidt, maar een oorzakelijk verband kan uit deze studie niet geconcludeerd worden.

In **Hoofdstuk 6** vergeleken we de lange-termijn cortisolwaarden van 132 patiënten met primaire of secundaire bijnierschorsinsufficiëntie die werden behandeld met hydrocortisonsubstitutietherapie, met die van 42 controlepatiënten die een hypofyse-aandoening zonder bijnierschorsinsufficiëntie, en 195 gezonde controles. We lieten zien dat lange-termijn cortisol waarden hoger waren bij patiënten met primaire of secundaire bijnierschorsinsufficiëntie onder hydrocortisonsubstitutietherapie dan controlepatiënten en gezonde controles. Daarnaast correleerde de dosis hydrocortison met lange-termijn cortisol. Uit aanvullende analyses bleek dat mannelijke patiënten met bijnierschorsinsufficiëntie onder hydrocortisonsubstitutietherapie een hoger lange-termijn cortisol hadden dan vrouwelijke patiënten. Patiënten met bijnierschorsin-sufficiëntie hadden tevens een hogere body mass index (BMI) dan gezonde controles. Het verband tussen lange-termijn cortisol en BMI zou een milde overbehandeling kunnen suggereren, die zou kunnen leiden tot nadelige bijwerkingen op het gebied op lichaamssamenstelling, met name bij mannen.

Hoofdstuk 7 beschrijft het verband tussen lange-termijn cortisol, het gebruik van hydrocortison, en kwaliteit van leven (*quality of life*, QoL) bij 120 patiënten met stabiele bijnierschorsinsufficiëntie. Patiënten meldden afname in 14 van de 15 QoL subschalen. Een slechtere score op de fysieke aspecten van QoL correleerde met een hoger lange-termijn cortisol, en een hogere dagelijkse inname van hydrocortison, een effect dat sterker was bij vrouwelijke patiënten. Deze resultaten tonen aan dat patiënten met bijnierinsufficiëntie meerdere beperkingen in levenskwaliteit rapporteren, welke geas-socieerd zijn met hydrocortison inname, en in mindere mate worden gereflecteerd door een chronische systemische blootstelling aan cortisol zoals gemeten met haarcortisol. Dit suggereert dat de verminderde kwaliteit van leven bij patiënten met bijnierinsuf-ficiëntie niet enkel het effect van overbehandeling met hydrocortison is.

Ten slotte worden in **Hoofdstuk 8** de implicaties van de belangrijkste bevindingen besproken, methodologische beschouwingen worden samengevat, en worden aanbe-velingen voor toekomstig onderzoek gegeven.

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