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Chapter 1

Introduction and thesis outline

Introduction

All organisms must maintain a complex and dynamic equilibrium, or homeostasis, which is constantly challenged by internal and external forces termed stressors. Stress occurs when homeostasis is threatened or perceived to be so; homeostasis is reestablished by various physiological and behavioral adaptive responses. During evolution the organism has developed a stress-system to deal with factors which may disturb homeostasis, of which the Hypothalamus-Pituitary-Adrenal (HPA) axis is an important part. Neuroendocrine hormones, such as cortisol, play a major role in the maintenance of basal homeostasis as responses to threat, and are involved in the pathogenesis of diseases characterized by dyshomeostasis.[1;2]

The stress-system needs to have a far-reaching power over the metabolism of the body, because in times of crisis the body should be able to acutely redirect metabolism to enable a fight, flight or freeze reaction. Especially in humans, along with the development of the stress system, psychological stress factors became more important, such as fear anticipation. It is generally assumed that the organism did not develop a new stress system to cope with these psychological stressors, but used the already working stress-system, again with the HPA axis as an important part.

If the stress-system plays an important role in psychological stress, it is to be expected that depression and anxiety disorders are accompanied by dysfunctions of this system.[3-5] This hypothesis has been tested, mainly in research using cortisol concentrations in blood or saliva as marker for the function of the hypothalamus-pituitary-adrenal (HPA) axis. Thus far, the results were inconsistent: hypercortisolism, hypocortisolism and normal cortisol levels were found.[6-14]

Before one can conclude that thus the HPA axis and the stress-system are not involved in depression and anxiety disorders, several other explanations should be considered. Cortisol may not be the right marker for the stress-system in general or the HPA axis in particular. As cortisol has such a central place in the stress-system this is not very probable. Besides, the problem is not that abnormal cortisol levels are absent in patients with depressive and anxiety disorders: Abnormalities have been found repeatedly. The problem is that the abnormalities were inconsistent. Therefore, another possibility should be considered, i.e., that the phenotype or clinical picture is described insufficiently. This is less improbable than it may seem: the validity of the most used diagnostic classification system, the DSM-IV, has been questioned frequently. To date, the DSM has focused solely on face or clinical validity, the assertion that the diagnoses correspond to clinicians' subjective views of a disorder. This is a weak form of validity only requiring consensus among clinical experts. One common form of validity is expressed by sensitivity and specificity, which are both low for DSM-IV diagnoses due to the extensive comorbidity, the high within-category

heterogeneity, and the overlap of DSM-IV diagnoses by sharing criteria. Ideally, the validity of a diagnosis is determined by the correlation between the diagnosis and another criterion of the disease, for instance a biological parameter, which is considered as 'gold standard'. As discussed above, such a gold standard has not been found.

In this thesis we explore whether a dimensional approach to describe the clinical picture is a better way to disentangle the relationship between phenotype and HPA axis functioning than the DSM-IV categories. In addition to and as a consequence of that starting point, not the clinical picture (diagnosis or dimension) was central in our studies, but cortisol levels. In other words, we investigated per dimension to what extent differences in scores on that dimension correlated with cortisol levels and not which cortisol levels were found in major depression and which in anxiety disorders.

In depression and anxiety also abnormalities in metabolic and immune parameters have been found, but again the results were inconsistent. To date, none of the metabolic and immune markers were sufficiently specific to contribute to the diagnosis of major depression.[15] Therefore, we also investigated the relation of the cortisol levels with those biological markers, hoping to find a more consistent picture.

In conclusion, we moved from an approach that puts psychiatric diagnosis in the center to an approach that puts the HPA axis in the center (see figure 1). In the following part of this introducing chapter, background information and an outline of this thesis are presented.

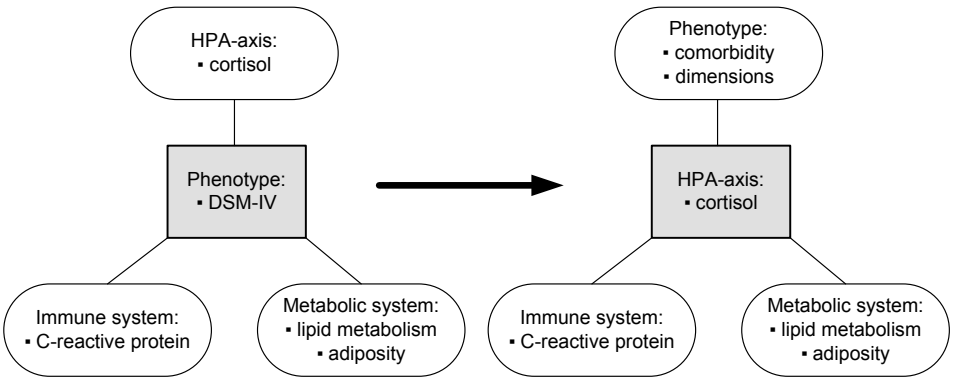

Figure 1. *Structure of thesis*
Associations between HPA axis function, phenotypic characteristics, metabolic and immune factors in depression and anxiety disorders. Associations might be bidirectional and may also exist between 'nodes', but are left out in the picture for the purpose of clarity.

Major depressive disorder and anxiety disorders

Depression and anxiety disorders are invalidating affective disorders accompanied by diminished functioning or well-being and increased mortality. Major depressive disorder (MDD) is characterized by a depressed mood and/or the loss of interest and pleasure in nearly all activities. In addition to these essential features, alterations in appetite, sleep disturbances, psychomotor changes, fatigue and decreased energy, feelings of worthlessness, cognitive problems (e.g. inability to make decisions), and thoughts of death are considered to be characteristics symptoms. For a diagnosis according to DSM-IV (APA, 1994), one of the essential features together with at least four additional symptoms have to be present most of the day, nearly every day, for at least two weeks. MDD is a common psychiatric disorder, with a 12 month prevalence of 5.8% in the Netherlands.[16]

Anxiety disorders are characterized by an excessive feeling of overwhelming anxiety, irrational fear and avoidance behaviour. The anxiety is often accompanied by physical symptoms such as sweating, cardiac disturbances, diarrhea or dizziness. Anxiety disorders include panic disorder, social anxiety disorder, post-traumatic stress disorder (PTSS), obsessive-compulsive disorder, generalized anxiety disorder, and specific phobia. Each anxiety disorder has specific symptoms, but all the symptoms cluster around excessive, irrational fear and threat. The 12 month prevalence of all anxiety disorders is 12.4% in the Netherlands.[16] Depressive and anxiety disorders are considered as stress-related disorders, marked by a dysfunction of the HPA axis.[5]

Stress

Stress and stress vulnerability are assumed to play major etiological roles in depression and anxiety disorders. The acute stress response is reflected in the rapid activation of the sympathetic nervous system, which leads to the release of epinephrine and norepinephrine. The sympathic pathways (epinephrine) elevate heart rate, blood pressure, respiration, glucose synthesis and cognitive arousal/attention. Simultaneously, the parasympathic pathways (e.g., norepinephrine and other catecholamines) are activated leading to a decrease in food intake, sleep and sexual drive. As part of the stress response, the hypothalamus releases corticotrophin-releasing hormone (CRH) inducing the release of adrenocorticotropic hormone (ACTH) from the pituitary gland, which stimulates the adrenal cortex to produce and secrete corticosteroids. This leads to elevated circulating levels of corticosteroids, in man mainly cortisol (figure 2). The acute stress response is adaptive to homeostasis. In the long term, chronic or repeating stress may impair physiological functions, such as growth, reproduction, metabolism, and immunocompetence, and imposes an increased risk for depression and anxiety disorders.[17-19]

The underlying risk for the development of depression and anxiety disorders can be conceptualized as an accumulation of daily hassles, lifestyle, and major life events that interact with the genetic constitution and predisposing early life experiences.[5] The relationship between life stress and depressive disorders is well established.[17] Post (1992) asserted that the nature of the relationship between stressful life events and depression changes as function of the longitudinal course of the disease.[20] Post's basic premise is that the first episode of a depressive disorder is more likely to be preceded by major psychological stressors than subsequent episodes. At the basis of this premise are two distinct models that offer potential mechanisms of this empirical observation: behavioral stress sensitization and electrophysiological kindling. Stress sensitization is observed by the fact that less and less life events are needed to elicit depression across the course of the disorder. Kindling is the observation that after an initial sensitization to stressors, recurrences of depression occur autonomously, in the absence of stressors.[21] In a recent meta-analysis, evidence was provided that first onsets of depression were more likely than recurrences to be preceded by severe life events, supporting Post's view.[22]

HPA axis

The HPA axis is an important neuroendocrine system involved in stress coping. HPA axis activity is predominantly studied in this thesis by measuring one of its final products: cortisol, the main stress hormone in humans. In reaction to both physical and psychological stress, CRH is released from the paraventricular nucleus in the hypothalamus. As a consequence, a neuroendocrinological cascade is initiated, with CRH stimulating the release of ACTH from the pituitary gland. In turn, ACTH binds to receptors in the outer cortex of the adrenal glands, resulting in the secretion of the steroid hormone cortisol (Figure 2).

Plasma cortisol release is tightly regulated by negative feedback at the level of the pituitary, hypothalamus and hippocampus. Cortisol acts through binding to mineralocorticoid (MR) and glucocorticoid receptors (GR). Low levels of cortisol are sufficient to occupy the high-affinity MR, a receptor involved in the maintenance of homeostasis, which exerts tonic inhibition of the HPA axis. During stress, when cortisol levels are high, also the low-affinity GR are occupied. The GR mainly acts to prevent overshoot of primary defense reactions and shuts of the HPA axis. The balance between MR and GR modes is thought to be essential for cell homeostasis, mental performance, and health. This 'yin-yang' concept in stress regulation is fundamental for genomic strategies to understand the mechanistic underpinning of corticosteroid-induced stress-related disorders such as depression and anxiety disorders.[5;23]

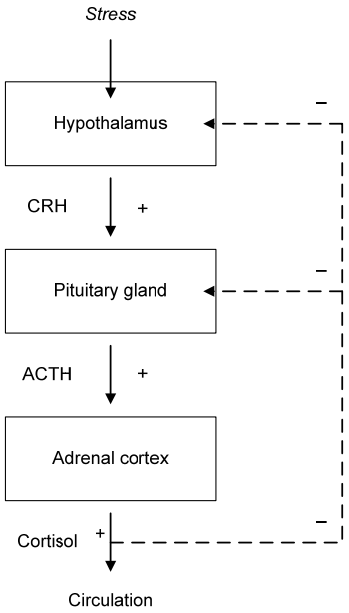

Figure 2. *Hypothalamic-pituitary-adrenal axis*

Cortisol release shows a clear diurnal rhythm. Cortisol levels peak about half an hour after awakening, with a 50% to 100% increase in cortisol levels compared to the levels during the rest of the day. The lowest level are found around midnight (Figure 3).[24] The main effect of cortisol is an increase of blood glucose levels via glucogenesis (glucose synthesis) and glycogenolysis (glucose release from storage). During the acute phase of the stress response the release of cortisol result in a replenishment of depleted energy levels. Later it provides energy for the long-term demands.

The integrity of the HPA axis can be evaluated using a variety of paradigms in basal and challenge conditions. As indicator of basal HPA axis function the saliva cortisol day curve is assessed in many studies. In addition, several neuroendocrine challenge tests have been developed to study HPA axis activation. A formerly frequently used test is the dexamethasone suppression test (DST), which examines whether negative feedback processes can be inhibited by the oral administration of dexamethasone (DEX) (usually 0.5 to 1.0 mg). DEX pretreatment normally results in a suppression of pituitary adrenocorticotropic hormone (ACTH) release and thus reduces secretion of cortisol from the adrenals. However, if DEX is given to hypercortisolemic individuals, cortisol suppression may be incomplete, resulting in less DEX suppression (DST-nonsuppression).

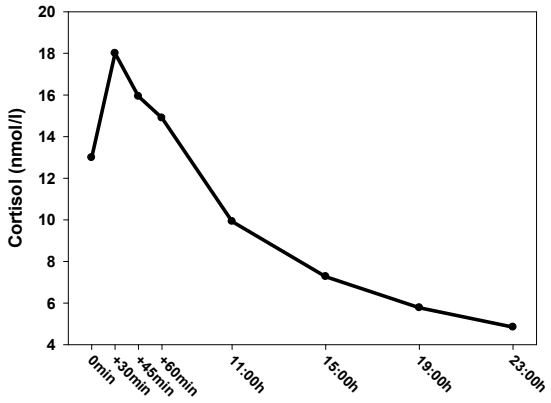

Figure 3. *One example of a saliva cortisol day curve*

In the mid nineties the DEX/CRH test was introduced, to examine HPA activity under the condition of suppressed glucocorticoid feedback. The application of the DEX/CRH test requires individuals to take 1.5 mg DEX orally at 23:00h on the night before the test day. On the day of the test itself, 100 micrograms human CRH are administered at 15:00h intravenously as a bolus, and blood samples for the determination of plasma cortisol and ACTH are drawn every 15 min from 15:00h (pre CRH) to 16:45h. Excessive ACTH and cortisol responses are indicative of a disturbed negative-feedback regulation and an overactive HPA system as is frequently seen in stressed and/or depressed individuals.[25] The DEX/CRH test has been reported to be more sensitive (above 80%) than the DST (about 20-50%) in differentiating MDD patients from healthy controls and it has therefore been argued that the DEX/CRH test unveils subtle HPA axis disturbance not detected by the DST.[6;26]

HPA axis dysfunctions in depression and anxiety disorders

Previous studies show inconsistent and contradictory findings regarding HPA dysfunctions in patients with depression and anxiety disorders. Hyperactivity of the HPA axis is a frequent finding in MDD.[9;13] Approximately 50-60% of patients with MDD show higher baseline ACTH and cortisol levels and diminished negative feedback, resulting in an escape from DEX suppression in the DST. After challenge with CRH under pretreatment with DEX, depressed patients show increased ACTH and cortisol responses to CRH.[6;25] However, minor or no alterations of the HPA system were found in dysthymic and chronically depressed patients.[8;10] Studies in outpatients and community populations have also provided limited evidence of HPA axis dysfunctions in depression.[27-30] Furthermore, in older depressive patients, associations were found with hypercortisolism as well as with hypocortisolism,

indicating the presence of a non-linear, U-shaped association between depression and cortisol.[31;32]

Studies in primary anxiety disorder patients have revealed less robust HPA axis dysregulations. The majority of the studies suggest that basal cortisol and ACTH concentrations are unaltered.[9] In panic disorders patients, elevations of basal cortisol and rates of nonsuppression in DST are reported that are slightly elevated compared to normal subjects, but much lower than those observed in depressive patients. Furthermore, higher HPA axis responsiveness was found in panic disorder patients compared with healthy controls following injection with CRH, and to CRH following DEX pretreatment.[7;11] Patients with social anxiety disorder did not differ from controls in basal 24-h urinary and salivary cortisol levels [14;33;34] and in response to DST.[14;34] However, in response to the Trier Social Stress Test, patients with social anxiety disorder had a significantly larger cortisol response than controls.[35] Patients suffering from PTSD mainly show lower baseline cortisol levels, increased CRH concentrations, increased sensitivity to the suppressive effects of DEX, and blunted ACTH response to CRH stimulation test. These findings indicate enhanced negative feedback capacity and an increased sensitivity of glucocorticoid receptors in the HPA system.[36] Elevated cortisol levels were also found in PTSD studies, specifically in patients with comorbid depression.[37]

Phenotype

The phenotype refers to the observable characteristics or symptoms of an individual. In psychiatry, different phenotypic approaches are used, but the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV classification system is most commonly used. A major problem of this categorical approach to phenotyping is the high comorbidity and low specificity due to the huge overlap in symptoms between depression and anxiety disorder diagnoses.

Psychiatric comorbidity

One explanation for the variability and inconsistencies in results of studies on dysfunctions of the HPA axis in patients with depression and anxiety disorders is comorbidity. A large epidemiological survey in the United States showed a lifetime prevalence rate for comorbid depression and anxiety disorders of 41%.[38;39] Feinstein first introduced the term comorbidity in the medical literature in 1970 (Feinstein, 1970). Comorbidity refers to two or more distinct co-occurring psychiatric disorders in an individual patient. Comorbidity of depression and anxiety disorders is widely understood to be associated with increased severity, persistence, and functional impairment.[40] There might be several possible explanations for the high frequency of comorbidity between anxiety disorders and (often temporally secondary) depressive

disorders, e.g., anxiety disorders could be a causal risk for depressive disorders, or anxiety and depressive disorders could be both the consequence of a common underlying factor. The last assumption is strengthened by the evidence of a shared genetic vulnerability for anxiety and depressive disorders.[41] As a consequence, the use of categorical DSM IV axis I diagnoses to search for specific underlying neuroendocrine dysfunctions might have limited potential, because depression and anxiety disorders may share some etiological factors.

A small number of studies on HPA axis function included a separate group of patients with comorbid depression and anxiety disorders, in addition to a group of patients who suffered from only the pure disorder (usually the depressive disorder). In the DST, patients with comorbid panic disorder and depression show higher rates of nonsuppression than those with pure panic disorder; the rates of the comorbid group were comparable to the rates seen in pure depression.[42;43] Patients with mixed anxiety and depressive disorder do also show DST nonsuppression rates similar to those seen in depression.[44] Mixed findings were found for PTSD with comorbid depression. Some studies showed low baseline cortisol and enhanced negative feedback to DEX,[45;46] whereas others showed increased baseline cortisol.[37] When using a psychological challenge test, such as the Trier Social Stress Test, ACTH was significantly higher in depressed patients compared to controls, and cortisol showed a trend in the same direction. However, this increase was completely due to those depressed patients who also had a comorbid anxiety disorder. The pure depressed patients did not show an increase.[47]

To summarize, studies on psychiatric comorbidity and the HPA axis, mostly found that depression is more robustly linked to HPA axis dysregulation than anxiety is. Depression might thus 'dominate' the neuroendocrine picture when disorders are comorbid.[30] However, when using a psychological challenge test, the presence of an anxiety disorder seems to modify the effect of the presence of depression on cortisol levels. As far as we know, no studies were done on the influence of psychiatric comorbidity on the responsivity to the more recently developed DEX/CRH test in patients with depression and anxiety disorders.

Categories versus dimensions

One way of handling the problem of comorbidity in psychiatric research might be by looking for alternative approaches to phenotyping. Although the categorical DSM-IV diagnoses have resulted in a significant improvement in worldwide communication among clinicians and make outcome of research worldwide comparable,[48] one of the disadvantages is the use of a threshold level of symptoms in deciding whether a diagnosis is present or not. Furthermore, there is a high amount of overlap in

symptoms between diagnoses. A problem related to the overlap is the already discussed high prevalence of comorbidity.

Assessing dimensions might be an alternative phenotypic approach to categorical DSM-IV diagnosing. A dimensional approach has several advantages. Firstly, dimensions replace categorical comorbidity by providing patient-specific diagnostic profiles. Secondly, dimensions might be better suited to help us understand relationships with biological, anatomical and genetic factors, because genetic transmission of psychopathology may operate at the level of individual dimensions or symptoms rather than at diagnostic or syndromal levels.[49] Lastly, dimensions provide quantitative scores, with which a more adequate description of symptom severity is possible, the sensitivity and statistical power is increased and, of course, the dichotomy of categorical diagnoses is avoided.

Several dimensional models have been proposed for assessing depression and anxiety disorders, such as the approach-withdrawal model, the valence-arousal model, and the tripartite model.[50] All of these models posit that depression and anxiety share a common distress dimension, whereas other dimensions discriminate these disorders.

A. Clark and Watson's tripartite model is designed to handle the high comorbidity rates of depression and anxiety disorders by taking into account overlapping as well as distinct features of anxiety and depression [51]. The model posits two broad factors of temperament, namely positive affect and negative affect. Positive affect includes traits such as enthusiasm, excitement seeking, gregariousness, and energy. Negative affect includes emotions such as sadness, guilt, hostility, uneasiness, fear, and self-dissatisfaction. The third dimension of the tripartite model is autonomic arousal. Its symptoms are physiological and include symptoms such as dizziness, shortness of breath, racing heart, and shaky hands. Low positive affect (also called the 'anhedonic depression' dimension) is thought to be rather specific for depression, whereas autonomic arousal (also called the 'anxious arousal' dimension) is rather specific for anxiety, as is seen in panic disorder. High negative affect (also called the 'general distress' dimension) is a non-specific factor that relates to both depression and anxiety, and is seen as a measure of severity of psychopathology.
B. The approach-withdrawal system of Davidson and colleagues posits two separate systems of motivation and emotion: an approach and a withdrawal system. While the tripartite model is proposed as part of a larger biobehavioral system, the core of this model is an affective system. The approach system is viewed as being responsible for the generation of positive affect, which is elicited when one moves towards an incentive, reward or positive stimulus. Activation of the withdrawal system is also hypothesized to

elicit arousal. The withdrawal system is purported to be responsible for the generation of certain aspects of negative affect, such as 'fear' or 'disgust' that one experiences while in close proximity to an aversive stimulus. It is suggested that depression can be seen as an underactivation of the approach system and/or an overactivation of the withdrawal system. An overactivation of the withdrawal system is also proposed as being related to anxiety leading to inhibiting behavior and increase of arousal when confronted with an aversive stimulus.[50;52]

C. The valence-arousal model was introduced by Heller and colleagues, and is an elaboration of the approach-withdrawal model. This model characterizes depression as Davidson does (i.e., decreased approach behavior and subsequent lower positive affect), but distinguished two subtypes of anxiety disorders, one associated with a dimension of anxious apprehension (e.g., obsessive compulsive disorder and generalized anxiety disorder) and another associated with a dimension of anxious arousal (e.g., panic disorder).[50;53] This is somewhat consistent with the tripartite model that suggests that arousal is specific to certain anxiety disorders (like panic disorder) and that there may be other components that are unique to other anxiety disorders. The tripartite model, however, subsumes anxious apprehension under a general negative affect factor. Thus, a key distinction between the tripartite and Heller model is that anxious apprehension is viewed as factor separate from negative affect.

The three models show a large conceptual overlap in their definition of the dimensions, e.g., all models differentiate between a positive and negative affect factor. However, the models slightly differ in the way that anxiety is taken into account as one or more separate dimensions. The approach-withdrawal system and the valence-arousal model are frequently studied in relation to neural substrates by using the Positive and Negative Affects Scales (PANAS).[54] One advantage of the tripartite model is the availability of a validated questionaire that assesses positive and negative affect, as well as anxious arousal, the so called Mood and Anxiety Symptom Questionnaire (MASQ).[55-57]

The metabolic system

Metabolic homeostasis is a crucial parameter of the adaptive stress response, since the activation of the HPA axis exerts potent transient effects on most of the metabolic pathways. Stressful conditions induce the rise of circulating cortisol, subsequently followed by increases of gluconeogenesis in the liver, lipolysis and protein degradation at multiple tissues (e.g., muscle, bone, skin). Consequently, most of the accessible stores of glucose, lipids, and amino acids are mobilized in order to be used as substrates that will supply the required energy to cope with the imposed stressor and

restore the internal milieu. The activated HPA axis antagonizes reproductive, growth and thyroid axis in order to temporally suspend every energy consuming process which at the moment is not essential for survival. The transient nature of the adaptive response renders its antagonized effects temporally beneficial for survival, rather that damaging.[58] In contrast, chronic stress lead to detrimental metabolic complications as described beneath. Figure 4 depicts the interactions between cortisol and parameters of the metabolic system.

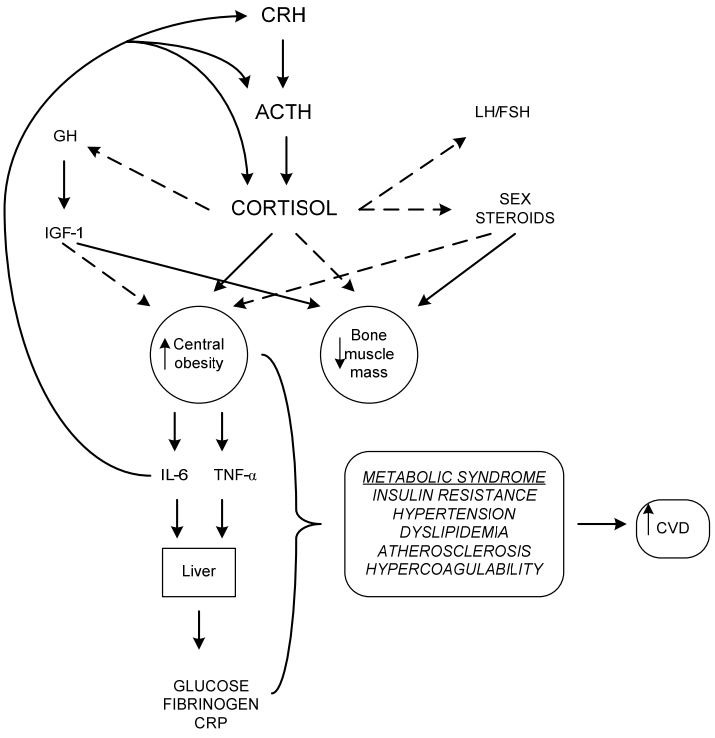

Figure 4. *Interactions between cortisol and metabolic parameters*
+ denotes stimulation, and - denotes inhibition.

Depression, anxiety and cardiovascular disease

Patients with depression and anxiety disorders have a two to fourfold increased risk of developing cardiovascular disease (CVD).[59-61] Several plausible mechanisms may explain the link between depression (and possibly also anxiety) and CVD. Pathophysiological alterations caused by depression and anxiety have been described, including impairment of platelet functions[62;63] and a decreased heart rate variability as a consequence of an imbalance in the autonomic tone.[64;65] Furthermore, immune

activation has been implicated in the pathogenesis of atherosclerosis and consequent CVD.[66] Unhealthy lifestyles, such as smoking, low physical activity, and poor dietary habits, are well-known cardiac risk factors and have been found to be more common among depressed than nondepressed persons.[67-69] Lastly, the link between depression and CVD may be caused by pharmacotherapeutic treatment. Antidepressants, in particular tricyclic antidepressants, may have a cardiotoxic effect.[70-72] Additionally, depression has been hypothesized to be associated with the, so called, metabolic syndrome.[73] The metabolic syndrome is described as a clustering of risk factors associated with CVD and diabetes. It includes at least three of the following conditions: abdominal obesity, high triglyceride levels, low high density lipoprotein (HDL) cholesterol, high blood pressure and high fasting glucose.[74]

Cortisol and lipid metabolism

Cortisol has important effects on the lipid metabolism and body composition. Cortisol activates lipoprotein lipase, the gatekeeper of lipid accumulation in adipocytes. Furthermore, cortisol in the presence of insulin inhibits the lipid mobilizing system. As a consequence, free fatty acids increase, and dyslipidaemia develops with elevated serum levels of total cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides, and decreased serum levels of HDL cholesterol. In the long term, cortisol excess also leads to an increase in visceral adiposity.[75] Visceral adiposity refers to the distribution of fat around the abdomen ('apple-shaped'), which is associated with an increased risk of CVD. The metabolic effects of cortisol are clearly demonstrated by the effects of synthetic glucocorticoids during anti-inflammatory and immunosuppressive therapy[76] and in Cushing's disease.[77;78] The typical side effects of long-term exposure to high levels of cortisol or synthetic glucocorticoids are elevated serum levels of LDL cholesterol and triglycerides, lower HDL cholesterol, and elevated body-mass index (BMI) and waist-to-hip ratio (WHR).[79;80;77;78] The few studies addressing these associations in patients with depression and anxiety disorders showed contradictory results. In a large cross-sectional survey of elderly depressed patients, high 24h urinary cortisol levels were associated with the metabolic syndrome, which includes high triglycerides and low HDL cholesterol.[73] However, in another study higher salivary cortisol levels (measured at three time points during the day) were associated with lower LDL cholesterol levels in 41 overweight depressed patients (BMI > 25 kg/m^2), but not in 37 patients of normal weight.[81] Up to now, the association between lipids and cortisol levels in patients with anxiety disorders has not been studied. Investigating different aspects of HPA axis function (e.g., overall cortisol release, responsivity of the stress system) in relation to lipid metabolism might contribute to further disentanglement of this relationship.

The immune system

Besides associations between HPA axis and lipid metabolism, there is also an interaction between the HPA axis and the innate and adaptive immune system. Proinflammatory cytokines, i.e., tumor necrosis factor α (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6), activate the HPA axis, leading to an increase in plasma cortisol levels. Cortisol, on its turn, inhibits the release of proinflammatory cytokines (Figure 5). One aspect of the innate inflammatory process is the acute phase response, with C-reactive protein (CRP) as a key pro-inflammatory marker.

Acute phase response

CRP is a proinflammatory acute-phase reactant, predominantly produced in the liver. The release of CRP is regulated by an inflammatory cascade of reactions, which involve, among others, proinflammatory cytokines.[82;83] The main biological function of CRP is its ability to recognize pathogens and damaged cells of the host and to mediate their elimination by recruiting the complement system, which subsequently activates and attracts phagocytic cells.[83] Due to its capability to bind to and modulate the function of mononuclear phagocytes, a process that is called opsonisation, CRP induces the release of the proinflammatory cytokines IL-1, IL-6, and TNF-α by these cells[84] and, therefore, might indirectly stimulate cortisol release. Cortisol acts synergistically with IL-6 to enhance the release of CRP.[85] On the other hand, cortisol is a potent endogenous anti-inflammatory agent with immunosuppressive effects. It has a strong capacity to suppress immune cell functions, particularly during the early development of the inflammatory response. It significantly decreases the production of cytokines and other mediators of inflammation (e.g., platelet activating factor, nitric oxide, prostanoids). However, not much is known yet about the direct pathways from CRP to cortisol release. We assume that a bidirectional relationship between CRP and cortisol plays an important role in maintaining the physiological homeostasis during the adaptive response to noxious stressors.[86] Figure 5 depicts the Interactions between cortisol and parameters of the immune system).

Thesis outline

The main purpose of the present thesis is to investigate the associations between the HPA axis and phenotypic, metabolic and immune factors in patients with depression and anxiety disorders who were free of psychotropic medication. In the first two studies the HPA axis is used as a way to redefine the phenotype of patients suffering from depression and anxiety symptoms. The last two studies are on interactions between the HPA axis and the metabolic and immune system, focusing on, respectively, lipid metabolism and the acute-phase response. For all of this, we

assessed parameters involved in baseline HPA axis regulatory processes and used a neuroendocrine challenge design, the DEX/CRH test. Furthermore, we collected indices of lipid metabolism, adiposity, acute phase response and we determined the frequency of six well-characterized CRP polymorphisms. The study population consists of outpatients with depressive and/or anxiety disorders and healthy controls. By means of these studies, we hope to reach a better understanding of the correlates and determinants of the complex HPA system in depression and anxiety disorders.

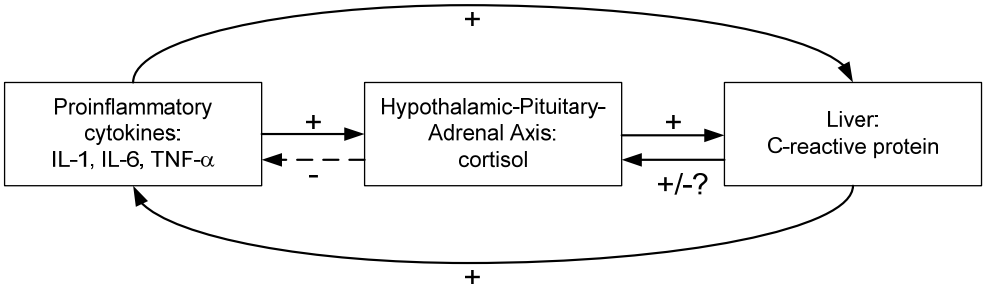

Figure 5. *Interactions between proinflammatory cytokines interleukin-1 (IL-1), tumor necrosis factor a (TNF-a), interleukin-6 (IL-6) and cortisol and C-reactive protein (CRP)*
+ denotes stimulation, and - denotes inhibition.

As a preface to the empirical studies of this thesis, we wrote an introductory article on the need for alternative ways of phenotyping of mood, anxiety and somatoform disorders in biological research **(chapter 2)**.

In the first empirical study, we hypothesized that psychiatric comorbidity might be an explaining factor for the heterogeneous outcome of the DEX/CRH studies in patients with depression. The attention for psychiatric comorbidity, although it is a frequently occurring phenomenon, is remarkably limited. Furthermore, comorbidity was often not addressed as explaining factor for the broad range in cortisol and ACTH values within and between DEX/CRH studies. We investigated whether psychiatric comorbidity affects the responsivity to the DEX/CRH test in patients with depression, who were free of psychotropic medication **(chapter 3)**.

In the second empirical study a dimensional model was used in the search for underlying HPA axis dysfunctions of the clinical phenotype. For this, we choose the tripartite model of anxiety and depression, because it is broadly accepted in adult psychiatry[87-89] and because a validated questionaire exists to assess the dimensions is available.[55] Continuous psychological dimensions selected for their predictiveness of HPA-dysfunctions were proposed to be the advantageous way in reaching an

understanding of the biological causations in depression and anxiety, and may be complementary to DSM-IV diagnoses when doing neuroendocrine research **(chapter 4)**.

Next, we investigated the interaction between the HPA axis and lipid metabolism in depression and anxiety disorders. The effects of cortisol on lipid metabolism (and adiposity) make HPA axis dysfunctions one of the possible mediator of the association between depression and anxiety disorders and CVD.[73] We studied two aspects of the HPA axis function (i.e., basal cortisol release over the day, and circadian cortisol variability as indicator of the responsivity of the stress system) in relation to lipid metabolism and adiposity **(chapter 5)**.

In the last study, associations were explored between CRP haplotypes with plasma CRP levels and basal salivary cortisol in a genetic association study. Six well-characterized CRP polymorphisms that are known to influence plasma CRP levels were used in order to explore the relationship between CRP levels and salivary cortisol levels over the day **(chapter 6)**.

In **chapter 7** the results of the different studies and the interface between them will be discussed. The final part of this chapter includes some future perspectives.

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Chapter 2

Need for alternative ways of phenotyping of mood, anxiety, and somatoform disorders in biological research

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Abstract

Variation in psychiatric symptomatology is continuous and does not coalesce into fairly well defined categorical DSM-IV clusters. As a consequence, DSM-IV fails to meaningfully integrate information generated by neuroendocrine research. Continuous psychological dimensions selected for their predictiveness with respect to endophenotypes, as biological intermediate factors, are proposed to be the best way in reaching an understanding of the causations in mood, anxiety and somatoform disorders.

Introduction

Nowadays, psychopathology is mostly described in terms of diagnostic categories according to the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV). An important advantage of this system is that it yields reliable diagnoses, especially with respect to classical psychiatric disorders like depression and panic disorder, which are subsumed under axis I in the DSM-IV. However, the validity is open to debate. Firstly, the majority of patients shows a complex presentation of a wide range of psychiatric symptoms, often leading to more than one axis I diagnoses, simultaneously. Therefore, the face validity of the categorical approach of the DSM-IV has been questioned. Secondly, in general, each DSM-IV diagnosis requires the presence of a minimum number of symptoms out of a list of symptoms characterizing the disorder. However, the threshold level is mostly chosen arbitrarily, but above the mean number of symptoms found in the general population. As a consequence, the DSM-IV excludes a large group of persons with below-threshold psychopathology. Thirdly, as a diagnosis does not require the presence of all symptoms listed for the diagnosis, patients with the same DSM-IV disorder may differ greatly with respect to their symptoms. For example, two depressive patients may suffer from opposite symptoms, e.g. hyposomnia versus hypersomnia. By using DSM-IV classification, this clinical heterogeneity is not specified or adequately described. Fourthly, no close relationship between the DSM-IV axis I diagnoses and biological markers has been found. For instance, notwithstanding the indications that stress plays an important role in the development of mood, anxiety and somatoform (MAS) disorders, only in about half of the patients hypothalamic-pituitary-adrenal (HPA) axis dysregulations are found. Furthermore, often opposite findings are found within one diagnostic entity, e.g. hyper- and hypocortisolism in respectively melancholic and atypical depression.[1;2]

Does this imply that we look at the wrong biological markers or do we make the wrong groupings of the phenotype? In this article we explore the latter possibility and propose the need for alternative ways of phenotyping of MAS disorders in biological research.

Phenotype: Diagnosing MAS disorders

In 1990, Van Praag proposed a new diagnostic approach, named functionalization and verticalization. Functionalization comprises converting categorical diagnoses into the psychic dysfunctions underlying the psychopathological symptoms. This enables the verticalization, by which is meant connecting the psychic dysfunction with the underlying neurobiological substratum. To do so, a sequential analysis is required, i.e.

determination of the sequence of appearance of symptoms, because it is hypothesized that the first symptoms, called front runners by Van Praag, carry a primary character with respect to neuroendocrine dysfunctions. Examples are the associations between serotonergic dysfunctions and disturbances in anxiety, aggression regulation and impulse control, and between dopaminergic dysfunctions and disturbances in motoricity.[3-5]

Unfortunately, for many types of psychic dysfunctions the front runners are unknown or difficult to determine. Instead of the front runners, the dimensions underlying the psychic dysfunctions may also be an appropriate link between psychopathology and neuroendocrine dysfunctions. Dimensional models, in contrast to functionalization and verticalization, do not require a sequentional analysis of psychic dysfunctions, because it is hypothesized that for each patient assessment on all dimensions that cover the psychopathology is sufficient for meaningfull integration with the information generated by neuroendocrine research. Several dimensional models have been proposed for assessing mood and anxiety disorders, such as the tripartite model, approach-withdrawal model, and valence-arousal model. All these models posit that mood and anxiety disorders share a common distress dimension, but they also can be distinguished from each other by particular characteristics.[6] A shortcoming of these models is that they still use the DSM-IV classification as frame of reference by proposing dimensions with assumed predictiveness for DSM-IV diagnoses instead of looking for dimensions with a high concordance with biological markers, the so called endophenotypes. The development of a new dimensional model, independent of DSM-IV diagnoses, and external validated with endophenotypes, is needed.

Endophenotype: The crucial link in between

An endophenotype is a biological marker of a phenotype closer to relevant gene action than the phenotype itself. Endophenotypes should be continuously quantifiable and predict disorders probabilistically. In the case of psychopathology, endophenotypes may be neurophysiological, biochemical, endocrinological, neuroanatomical, cognitive, or neuropsychological in nature. As MAS disorders are linked to stress, it is hypothesized that dysfunction of one of the important stress systems, the HPA-axis, is an endophenotype of these disorders.

Indeed some indications have been found that HPA-axis dysfunction is an endophenotype of MAS disorders diagnosed according to the DSM-IV. About half of the patients with a major depressive disorder show a hyperactivity of the HPA-axis. Studies of anxiety disorders revealed less robust HPA-axis dysregulations. Some, but not all patients with posttraumatic stress disorders, show hypocortisolism.

Hypocortisolims has been reported in 20-25% of patients with somatoform disorders.[7-9] Given the questionable validity of diagnoses based on the DSM-IV, no large correlations between diagnoses of this type and biological markers are to be expected.

A few studies have examined HPA-axis activity in relation to psychic dysfunctions, instead of DSM-IV classification. Hyperactivity of HPA-axis is considered to play an important role for individual symptoms, such as enhanced anxiety, decreased responsiveness to the environment, decreased diurnal variation, disturbed sleep, altered psychomotor functions, decreased appetite and libido, and impaired cognition. Reduced HPA-axis activity, mediated by an enhanced negative feedback, is associated with symptoms, such as hypersomnia, hyperphagia, lethargy, and fatigue.[10-12]

The relationship between dimensional models and HPA-axis activity has, so far known, never been studied.

A model to study dimensions of mood, anxiety and somatisation and HPA-axis functioning

We propose that the development of a dimensional model that covers the symptomatology of all three MAS disorders is needed to reach more insight in its biological substrate. By using psychological questionnaires that assess a broad spectrum of symptoms, one can look for underlying dimensions that adequately and precisely describe MAS psychopathology. Dimensions don't need to have predictive value for separate DSM-IV diagnoses, but should be externally validated with biological markers, such as HPA-axis function. Basal HPA-axis activity can be measured by assessment of the cortisol diurnal pattern. HPA-axis reactivity can be examined with challenge tests like the combined dexamethasone/corticotrophin-releasing hormone (CRH) challenge test, which proved to be a sensitive measure (above 80%) in differentiating depressive patients from healthy controls.[13] It is used to examine HPA reactivity under the condition of suppressed glucocorticoid feedback as a reflection of the sensibility and responsivity of the pituitary. We hypothesize that combining these phenotypic and endophenotypic data will lead to more clarity about psychopathological processes in MAS disorders.

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Chapter 3

The influence of psychiatric comorbidity on the dexamethasone/CRH test in major depression

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Abstract

Objective: The outcome of the dexamethasone/corticotropin-releasing-hormone (DEX/CRH) test in depressed patients is heterogeneous. The present study investigated whether comorbidity of anxiety or somatoform disorders might be an explaining factor for this finding.

Methods: The DEX/CRH test was administered in 36 pure major depressive outpatients, 18 major depressive outpatients with a comorbid anxiety and/or somatoform disorder, and 43 healthy controls. Patients were free of psychotropic medication. Group differences in responsivity to the DEX/CRH test were analysed.

Results: Depressive patients with comorbidity showed a significant lower cortisol response compared to pure depressive patients (p=.04) and controls (p=.003). Group differences between MDD patients with and without comorbidity in cortisol responses disappeared after adjustment for post-DEX cortisol concentrations (p=.34).

Conclusions: An enhanced suppression of cortisol to 1.5 mg DEX is present in a subgroup of depressed patients with psychiatric comorbidity. Distinct hypothalamic-pituitary-adrenal (HPA) axis dysfunctions are revealed when comorbidity is taken into account.

Introduction

Investigating the function of the hypothalamic-pituitary-adrenal (HPA) axis, hyperresponsiveness to the dexamethasone/corticotropin-releasing-hormone test (DEX/CRH test) is a frequent finding in patients with major depressive disorder (MDD).[1-8] Yet, some studies found no change[9-12] or even hyporesponsiveness [13]. Actually, cortisol responses to the DEX/CRH test showed a broad range within and between studies. For instance, mean peak plasma cortisol values in depressed patients ranged from about 150 nmol/l[13] to about 300 nmol/l.[14]

There are several explanations for these heterogeneous findings. Firstly, depression severity of the patients differed widely between studies, ranging from a mean HDRS-score of 17[10] to about 28.[3;6] Secondly, patients on medication were often included, whereas antidepressive medication may affect HPA-axis function. One study found that antidepressants restored HPA-axis function, and that this preceded symptom resolution,[7] although this was not found in another study.[15] Thirdly, heterogeneous findings may also be explained by the inclusion of patients with bipolar depression in some studies.[4;6;8]

We hypothesize that psychiatric comorbidity might be an explaining factor for the heterogeneous outcome of the DEX/CRH studies. The attention for psychiatric comorbidity, although it is a frequently occurring phenomenon, is remarkably limited.[10;16;17] Most studies did not report on comorbid psychiatric disorders,[4;8] while another study mentioned comorbidity, but did not address it in the statistical analyses.[12] In the present study comorbidity was limited to anxiety and somatoform disorders, because they frequently co-occur with major depression.

In the present study we investigated whether psychiatric comorbidity and severity of psychopathology affect the responsivity to the DEX/CRH test in 50 patients with MDD, who were free of psychotropic medication. Forty-three healthy controls were included as a reference group.

Methods

Participants

Fifty patients suffering from MDD were recruited from the outpatient department of the mental health center Rivierduinen in Leiden, the Netherlands. All patients met the criteria of a MDD according to the DSM-IV criteria in the Dutch version of the Mini International Neuropsychiatric Interview Plus 5.0.0-R (MINI-Plus).[18;19] Comorbidity, assessed with MINI-Plus, was allowed for anxiety disorders (i.e., panic disorder, social anxiety disorder, obsessive compulsive disorder, generalized anxiety disorder or posttraumatic stress disorders) and undifferentiated somatoform disorders, but other

axis I disorders were a reason for exclusion. The MINI-Plus was administered by trained interviewers. Patients were divided into two groups, one containing 32 depressed patients without comorbidity (MDD/pure group) and 18 depressed patients with a comorbid anxiety and/or somatoform disorder (MDD/com group). Forty-three healthy controls were recruited by advertisement in local news papers asking for healthy persons willing to participate in a study on the biological stress system in relation to depression and anxiety. Around 55 persons responded to the advertisements. Controls were included if they had no present or lifetime history of any axis I psychiatric disorder as assessed by the MINI-Plus and neither reported any traumatic experience in history. Patient groups and control group were comparable for age ($F_{2,88}$= 1.7; p=0.20) and gender ($\chi^2_{2,88}$= 1.1; p=0.57). Exclusion criteria for patients and controls were a history of neurological or endocrine diseases or other serious unstable medical conditions. Furthermore, patients and controls with substance or alcohol abuse, as well as pregnant or breast feeding women and premenopausal women with ovarectomy were excluded. None of the controls used any psychotropic medication. Patients using psychotropic medication with exception of a low dose of a benzodiazepine (equivalent to 30 mg oxazepam daily) were also excluded. If psychotropic medication was used within the last 14 days (for fluoxetine six weeks) patients were excluded. Patients were included on admission before the start of (pharmaco)therapy, or during treatment when medication was tapered in case of clinical ineffectiveness before the switch to another medication. Additionally, patients and controls using corticosteroids, antidiabetics, estrogens, (anti)thyroid hormone, or herbal medication (e.g., Valerian, St. Johns Wort) were excluded. All participants had a routine physical examination and laboratory blood tests. Prior to participation, written informed consent was obtained from all participants. The study was approved by the local ethics committee of the Leiden University Medical Center.

Dexamethasone/CRH test

The DEX/CRH test was performed according to the protocol developed by Heuser (Heuser et al., 1994). Participants took one tablet of DEX of 1.5 mg orally at 23:00h on the evening before neuroendocrine testing. They attended the research unit of the hospital the following day at 12:30h and had a light lunch upon arrival. At 13:45h a cannule was placed in the antecubital fossa. Participants were fasting throughout the experiment, remained semi-supine and non-sleeping. Human CRH (100 µg) (Ferring Hoofddorp, The Netherlands) was administered via the cannule at 15:02h. Blood samples for assessment of cortisol and adrenocorticotropic hormone (ACTH) were collected at 15:00h (pre-CRH), 15:30h, 15:45h, 16:00h, 16:15h, 16:30h and 16:45h.

Blood samples were obtained in EDTA tubes (Sarstedt) on ice and were stored directly at -70 ^{o}C before assessment. The determination of cortisol in plasma was

performed with a competitive electrochemiluminescence immunoassay (ECLIA) using a Modular Analytics E170 immunoassay analyzer from Roche Diagnostics (Mannheim, Germany). The detection limit for cortisol was 2.0 nmol/l and the intra- and inter-assay variability coefficients in the measuring range were less than 10%. The determination of ACTH was performed with a solid-phase, two-site sequential chemiluminescent immunometric assay using an Immulite 2500 immunoassay analyzer from DPC (Los Angeles, USA). The detection limit for ACTH was 1.1 pmol/l and the total precision in the measuring range was about 5%.

Measure of severity of psychopathology

Patients completed the Dutch translation of the Brief Symptom Inventory (BSI), a shortened version of the Symptom Checklist (SCL-90), that is used to measure psychological complaints or symptoms.[20] The total BSI-score generates an overall measure of psychopathological symptom severity. Internal consistency of the BSI is very good (Cronbachs α=0.96), and validity is sufficient.[20]

The Short Comprehensive Psychopathology Rating Scale (sCPRS)[21] is a semi-structured diagnostic interview, developed to assess psychiatric symptoms and most often used for screening purposes. Two indices have been derived from the shortened version (25 items) of the CPRS, the Brief Anxiety Scale (BAS)[22] to measure anxiety severity and the Montgomery Åsberg Depression Rating Scale (MADRS)[23] to measure depression severity.

Statistical analyses

Group differences for clinical characteristics were assessed by independent t-tests and one-way analysis of variance (ANOVA) for continuous variables, and χ^2-tests for categorical variables.

As indices of DEX suppression we used the 15:00h plasma concentrations of cortisol and ACTH after oral DEX intake, but before CRH administration, reported in analyses as BASELINE. For determination of the hormonal responses to the additional CRH administration in DEX-pretreated participants, the total area under the curve (AUC) was calculated with the trapezoid formula.[24] Oneway ANOVA was used to investigate group differences in BASELINE and AUC measures. Groupwise comparisons were adjusted for multiple testing using least significant difference (LSD) correction. Log-transformed values of cortisol and ACTH measures were used, because data were positively skewed. Analyses were adjusted for age, gender, and smoking status.

Additional exploration of the data was undertaken to investigate the impact of severity of psychopathology on the responsivity to the DEX/CRH test in patients. The total BSI-score was used as global index of psychiatric disease severity.

Furthermore, the influence of post-DEX suppression of cortisol on the responsivity to the additional CRH administration was examined. One-way ANOVA was repeated with AUC as dependent variable and the BSI total-score and BASELINE added, respectively, as covariates. Analyses were performed using Statistical Package or the Social Science version 16.0 (SPSS 16.0). Analyses were two-tailed with the level of significance set at .05.

Table 1. Clinical characteristics of MMD/pure group, MDD/com group, and control group

	MDD/pure group (n=32)	**MDD/com group (n=18)**	**Control group (n=41)**	**Test, p-value**
Age	33.8 ± 11.2	28.4 ± 8.2	33.9 ± 12.3	$F_{2,88}$=1.7, p=0.20
Gender (% females)	18 (56.3%)	11 (61.1%)	28 (68.3%)	$\chi^2_{2,88}$=1.1, p=0.57
Smoking (% smokers)	18 (56.3%)	10 (55.6%)	4 (9.8%)	$\chi^2_{2,88}$=21.1, p<0.01
BMI (kg/m²	24.4 ± 4.1	25.8 ± 9.1	24.4 ± 3.8	$F_{2,88}$=0.46, p=0.64
Age of onset MDD	23.9 ± 11.3	21.5 ± 7.1		$t_{1,48}$=0.82, p=0.42
Recurrent MDD (% patients)	23 (71.7%)	10 (55.6%)		$\chi^2_{1,48}$=1.4, p=0.24
⇒ Panic disorder		4		
⇒ Social anxiety disorder		5		
⇒ Obessive-compulsive disorder		1		
⇒ Post-traumatic stress disorder		6		
⇒ Undifferentiated somatoform disorder		3		
Total BSI score	1.5 ± .64	1.7 ± .53		$t_{1,48}$=1.1, p=0.30
MADRS score	23.1 ± 6.8	24.1 ± 6.2		$t_{1,48}$=0.49, p=0.63
BAS score	15.0 ± 5.5	18.6 ± 6.2		$t_{1,48}$=2.1, p=0.047

Data are presented as means ± standard deviation (SD) or n (percentage).
BAS = Brief Anxiety Scale; BMI = body-mass index; BSI = Brief Symptom Inventory; MADRS = Montgomery Åsberg Depression Rating Scale.

Results

Clinical characteristics

Clinical characteristics are presented in table 1. Patient groups and controls did not differ significantly for gender, age, and body-mass index (BMI). Significant group differences were found for smoking status, with patients smoking more frequently compared to controls. Patient groups did not differ in age of onset of MDD or recurrence of episodes of MDD. Furthermore, patient groups did not differ for the total BSI-scores, and MADRS, but the MDD/com group showed a significantly higher score on the BAS compared with the MDD/pure group.

Cortisol and ACTH responses to the DEX/CRH test

Figure 1 shows the cortisol and ACTH responses to the DEX/CRH test. One-way ANOVA revealed a non-significant group difference for $BASELINE_{cortisol}$ ($F_{2,88}$=2.3, p=0.11). Post-hoc tests, adjusted for multiple comparisons, showed a lower cortisol concentration at 15:00h for the MDD/com group as compared to the MDD/pure group (p=0.04). Regarding the cortisol response to the additional CRH administration, one-way ANOVA revealed a significant main effect of group for $AUC_{cortisol}$ ($F_{2,88}$=5.0, p=0.01). In post-hoc tests, the MDD/com group showed a significantly lower $AUC_{cortisol}$ as compared to the MDD/pure group (p=0.03) and controls (p=0.002). The MDD/pure group and controls did not differ in their cortisol response to the DEX/CRH test (p=0.32).

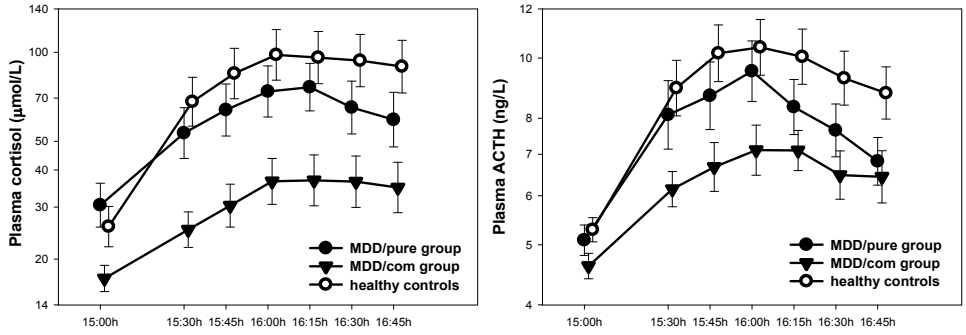

Figure 1. *Cortisol and ACTH response to the DEX/CRH test*
Data are graphical presented as means with bars representing SE; patients are subdivided according to DSM-IV categories. DEX 1.5 mg was orally taken at 23:00h on the evening before. CRH 100 µg was administered at 15:02h. Cortisol and ACTH levels are depicted on a logarithmic scale.

No group differences were found for $BASELINE_{ACTH}$ ($F_{2,88}$=1.4, p=0.25). Regarding the ACTH response to the additional CRH administration, one-way ANOVA revealed

a trend towards significance for the main effect of group for AUC_{ACTH} ($F_{2,88}$=2.8, p=0.07). Post hoc tests revealed that this trend could be attributed to a lower ACTH response for the MDD/com group as compared to controls (p=0.02), whereas no group differences in AUC_{ACTH} were found for the other contrasts.

Subsequently, all analyses were adjusted for the effect of age, gender, and smoking status. None of the main findings were significantly affected by these covariates (data not shown).

Comparisons between the MDD/pure group and MDD/com group

Additional analyses were performed on the differences in cortisol and ACTH response to the DEX/CRH test between the MDD/pure group and MDD/com group. One-way ANOVA was performed with, subsequentially, $BASELINE_{cortisol}$ and $AUC_{cortisol}$ as dependent variables and with age, gender, and smoking status added as covariates (model 1). None of these covariates showed to be significantly associated with $BASELINE_{cortisol}$ or $AUC_{cortisol}$ and group differences remained significant or changed to a trend towards significance with lower cortisol levels in the MDD/com group compared to the MDD/pure group (respectively, p=0.06, p=0.03). In a next step, the BSI total-score was added as a covariate to the analysis, to investigate whether severity of psychopathology could explain the differences in cortisol responsivity to CRH administration (model 2). The BSI total-score was not significantly associated with $BASELINE_{cortisol}$ and $AUC_{cortisol}$, and the main group effect remained significant or changed to a trend towards significance (respectively, p=0.06, p=0.02). The same findings were found after adjustment for either the MADRS or BAS (data not shown). In a last step (model 3) we repeated the analysis with $AUC_{cortisol}$ and adjusted for $BASELINE_{cortisol}$ to examine whether the differences in post-DEX suppression of cortisol at 15:00h (pre-CRH) could explain the differences in cortisol responsivity to CRH administration (model 3). $BASELINE_{cortisol}$ was significantly associated with the $AUC_{cortisol}$ (p<0.001) and the main group effect was no longer significant (p=0.24) (table 2).

Same analyses were done with, subsequentially, $BASELINE_{ACTH}$ and AUC_{ACTH} as dependent variables. No significant group differences were found between the MDD/pure group and the MDD/com group for all models (table 2).

In sensitivity analyses, patients with PTSD and patients with somatoform disorders were omitted to explore whether the lower cortisol responses to the DEX/CRH test could be ascribed to the somatoform disorders or post-traumatic stress disorder patients within the MDD/com group. After omitting patients with PTSD (n=6), group differences remained significant for $BASELINE_{cortisol}$ and $AUC_{cortisol}$ (respectively, $F_{1,42}$=5.6, p=0.02, $F=_{1,42}$=4.2, p=0.046). When $AUC_{cortisol}$ was adjusted for $BASELINE_{cortisol}$ (model 3), the main group effect was no longer significant

($F_{1,38}$=0.18, p=0.68). In reanalyses when excluding patients with somatoform disorders (n=3), group differences remained also significant for $BASELINE_{cortisol}$ and $AUC_{cortisol}$ (respectively, $F_{1,46}$=4.3, p=0.04, $F=_{1,46}$=5.1, p=0.03). When $AUC_{cortisol}$ was adjusted for $BASELINE_{cortisol}$ (model 3), the main group effect was again no longer significant ($F_{1,42}$=1.1, p=0.31).

Table 2. BASELINE and AUC of cortisol and ACTH as indices of the responsivity to the DEX/CRH test in MDD/pure group, MDD/com group

	MDD/pure group (n=32)	**MDD/com group (n=18)**	**Test, p-value**
$BASELINE_{cortisol}$ (nmol/L)[a]	30.4 (22.8 – 40.7)	17.2 (11.7 - 25.4)	$F_{1,48}$=5.6, p=0.02
• Model 1	29.4 (22.0 – 39.4)	18.3 (12.3 – 27.1)	$F_{1,45}$=3.7, p=0.06
• Model 2	29.6 (22.0 – 39.8)	18.1 (12.1 – 26.9)	$F_{1,44}$=3.9, p=0.06
$AUC_{cortisol}$ (nmol/L/h)[a]	110.5 (79.2 – 154.3)	57.5 (36.8 – 89.6)	$F_{1,48}$=6.4, p=0.02
• Model 1	107.4 (77.0 – 149.9)	60.5 (38.6 – 94.7)	$F_{1,45}$=4.8, p=0.03
• Model 2	108.8 (77.9 – 152.1)	59.1 (37.6 – 92.8)	$F_{1,44}$=5.4, p=0.02
• Model 3	94.3 (73.1 – 121.7)	76.2 (53.9 – 107.8)	$F_{1,44}$=1.4, p=0.24
$BASELINE_{ACTH}$ (ng/L)[a]	5.1 (4.6 – 5.6)	4.6 (4.0 – 5.3)	$F_{1,48}$=1.3, p=0.25
• Model 1	5.1 (4.6 – 5.6)	4.7 (4.1 – 5.4)	$F_{1,45}$=.87, p=0.36
• Model 2	5.1 (4.6 – 5.6)	4.6 (4.0 – 5.3)	$F_{1,44}$=1.5, p=0.22
AUC_{ACTH} (ng/L/h)[a]	14.1 (11.8 – 16.9)	11.3 (8.9 – 14.3)	$F_{1,48}$=2.3, p=0.14
• Model 1	13.9 (11.6 – 16.7)	11.6 (9.1 – 14.8)	$F_{1,45}$=1.5, p=0.24
• Model 2	14.0 (11.7 – 16.8)	11.5 (9.0 – 14.6)	$F_{1,44}$=1.8, p=0.19
• Model 3	13.6 (11.6 – 16.0)	12.1 (9.7 – 15.1)	$F_{1,44}$=.74, p=0.39

Data are presented as estimated means with 95% Confidence Interval (CI) within groups.
P-values of one-way ANOVAs are presented.
Model 1: adjusted for age, gender, and smoking (yes/no); Model 2: adjusted for age, gender, smoking, and overall severity (BSI total-score); Model 3: adjusted for age, gender, smoking and BASELINE.
[a] log- transformed values were used in statistical analyses because data were positively skewed.

Discussion

In the present study, we investigated whether psychiatric comorbidity and severity of psychopathology affect the responsivity to the DEX/CRH test in depressed patients who were free of psychotropic medication. Our results indicate that comorbidity in depressed patients leads to an enhanced post-DEX suppression of cortisol, and

consequently to a lower cortisol response to CRH administration. After adjustment for post-DEX cortisol concentrations, no difference in cortisol response to the DEX/CRH test was found between depressed patient with and without psychiatric comorbidity. Severity of psychopathology was not associated with the responsivity to the DEX/CRH test.

Our finding of lower cortisol responses to the DEX/CRH in depressed patients with comorbidity as compared to controls is in line with findings of a previous study[13]. In this study, depressive female patients on long-term sick leave showed attenuated cortisol responses to the DEX/CRH test. However, differences in post-DEX cortisol concentrations were not examined in their study and it remained unclear whether comorbidity might have been a contributing factor. In most previous studies, a higher percentage of non-suppression of post-DEX cortisol before CRH administration was found in depressive patients.[1;2;5;7;8;25;26] In some studies using the DEX suppression test also an enhanced post-DEX suppression of cortisol levels was found. Specifically in MDD patients with a history of traumatic life events and/or patients with post-traumatic stress disorder.[27-29] Our finding of an enhanced DEX suppression of cortisol when using the DEX/CRH test in depressed patients with psychiatric comorbidity is therefore, to the best of our knowledge, an important new finding, which needs to be replicated. It seems unlikely that any individual psychiatric comorbid disorder explains our finding, as previous studies on pure anxiety disorders have reported no enhanced post-DEX cortisol suppressive effects.[3;7;30] Sensitivity analyses also indicated that individual psychiatric comorbid disorders, specifically somatoform disorders and PTSD, did not influence our findings. Furthermore, it is unlikely that clinical variables, such as age of onset of MDD or recurrence of episodes of MDD, could explain our finding of the enhanced DEX suppression, because patient groups did not significantly differ on these clinical aspects. Lastly, the use of psychotropic medication could not have influenced the DEX suppression, because we included medication-free patients with a mild to moderate depression (mean MADRS score of 23.5) on admission before the start of (pharmaco)therapy, or during treatment when medication was tapered in case of clinical ineffectiveness before the switch to other medication.

One of the mechanisms that could explain the enhanced suppression to DEX is enhanced central glucocorticoid receptor sensitivity. DEX, a synthetic glucocorticoid, mimics the negative feedback effect of cortisol; it binds with high affinity to the glucocorticoid receptor in the pituitary gland, inhibiting peripheral release of cortisol. The extent to which cortisol release is inhibited after DEX intake indicates central feedback sensitivity.[31;32] The lower cortisol concentrations after DEX intake in our sample of MDD patients with comorbidity indicate increased negative feedback sensitivity in this group of depressed patients.

The influence of comorbidity on HPA-axis regulation in depressed patients has rarely been studied. Some studies used a psychological paradigm, the Trier Social Stress Test (TSST), to investigate the HPA-axis. Purely depressed patients showed no differences in cortisol and ACTH response compared to controls for the TSST. In case of comorbid anxiety disorders an exaggerated ACTH response was found.[33;34] The TSST examines the psychological stress response using a psychological stimulus, while the DEX/CRH test examines the neuroendocrine stress response using a pharmacological stimulus. This basal difference makes it hard to compare their results.

Some methodological limitations on this study should be mentioned. Firstly, the sample size was relatively small, particularly of the MDD/com group. Secondly, we found relatively high cortisol and ACTH values in our control group with a mean peak cortisol value of 212 nmol. In previous studies mean peak cortisol values in healthy controls ranged from about 70 nmol/l[3;12] to about 220 nmol/l.[13] Our relative high cortisol values might be related to a bias in the recruitment of the controls, e.g., mostly non-working participants responded to our advertisements, which might be a distinct population within society. Nevertheless, this has no implications for the presented differences between the MDD/pure group and MDD/com group on HPA-axis dysfunctions. Thirdly, we did not collect further clinical data that might have confounded or mediated the associations, like the duration of the current MDD episode or comorbid disorder, the presence of a family history of psychiatric disorders, and specific symptoms such as sleep disturbances. Other clinical variables that were assessed (i.e., age of onset and recurrence of MDD) did not significantly differ between patient groups. Lastly, we did not consider effects of genotype, personality traits, and early life trauma, all of which might impact the DEX/CRH test results.

We found that an enhanced suppression of cortisol to 1.5 mg DEX is present in a subgroup of depressed patients with comorbid anxiety and/or somatoform disorders. However, no differences in cortisol responsivity to CRH administration were found after adjustment for the post-DEX cortisol concentration, suggesting that a stronger negative feedback to DEX explains the difference.

Our finding of an enhanced DEX suppression of cortisol when using the DEX/CRH test in depressed patients with psychiatric comorbidity is of importance, because it underlines that comorbidity should be taken into account when interpreting the results of studies on the HPA-axis functioning in depressive patients. Replication of this study is needed to validate our results. Furthermore, it remains to be elucidated why psychiatric comorbidity in patients with MDD leads to an enhanced DEX-suppression of cortisol.

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Chapter 4

Basal cortisol levels in relation to dimensions and DSM-IV categories of depression and anxiety

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Abstract

Objective: The Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV classification may fail to adequately distinguish neuroendocrine factors involved in the etiology of depressive and anxiety disorders. Continuous phenotypic dimensions may correlate better with underlying neuroendocrine dysregulations.

Methods: We compared the categorical DSM-IV diagnoses with a dimensional approach in the same group of outpatients with depressive (n=36), anxiety (n=18), and comorbid depressive and anxiety (n=19) disorders, who were free of psychotropic medication, and in 36 healthy controls. The Mood and Anxiety Symptom Questionnaire (MASQ) was used to measure the three dimensions of the tripartite model, i.e., anhedonic depression, anxious arousal, and general distress. The salivary cortisol awakening response (CAR) (0, 30, 45, and 60 min after awakening), and diurnal cortisol decline (11:00h, 15:00h, 19:00h, and 23:00h) were analysed for linear and non-linear associations.

Results: The CAR showed statistically significant non-linear relationships with two MASQ dimensions, i.e., anhedonic depression and general distress, but no differences between DSM-IV categories. The diurnal cortisol decline was linearly related to the MASQ dimensions anhedonic depression and general distress and significantly higher $AUC_{diurnal}$ levels and a steeper slope were found in depressive patients compared to controls using DSM-IV categories.

Conclusion: The present study shows that linear and non-linear associations with salivary cortisol are detected when using phenotypic dimensions and may be complementary to phenotypic DSM-IV categories when doing neuroendocrine research.

Introduction

Stress and stress vulnerability have been hypothesized to play an etiological role in depressive and anxiety disorders, marked by a dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis.[1-3] Previous studies show inconsistent and contradictory findings regarding these HPA dysfunctions in relation to depression and anxiety. Elevated plasma cortisol levels were found in patients with depressive disorder,[4;5] but decreased or no alterations of plasma cortisol levels were found in chronic depressed patients and in a subgroup of atypical depressed patients with an early onset and/or chronic form of depression.[6;7] Studies in outpatients and community populations have provided limited evidence of HPA axis dysfunctions in depression.[8-10] Furthermore, in older depressive patients, associations were found with hypercortisolism as well as with hypocortisolism, indicating the possibility of a non-linear, U-shaped association between depression and cortisol.[11;12] Most studies on primary anxiety disorders suggest that basal cortisol and ACTH concentrations are unaltered. In challenge conditions, higher as well as lower HPA axis responsiveness was found in patients with anxiety disorders as compared to healthy controls.[4;6;7;13-17]

The lack of consistent findings regarding the HPA axis dysfunction in patients with depressive and anxiety disorders could be explained, at least in part, by the limited specificity of the categorical DSM-IV diagnoses. This may be due to the overlap of DSM-IV diagnoses by sharing criteria. Furthermore, the presence of non-linear associations between cortisol measures and psychopathology might have contributed to the inconsistent findings.[11;12]

A dimensional approach may be an alternative in the search for underlying biological dysfunctions of the clinical phenotype. For the present study, we chose the tripartite model of anxiety and depression, because it is broadly accepted in adult psychiatry.[18-20] Clark and Watson's tripartite model is designed to handle the high comorbidity rates of depressive and anxiety disorders[21] through taking account of both overlapping and distinct features of anxiety and depression. Low positive affect (also called the 'anhedonic depression' dimension) is thought to be specific for depression, whereas somatic arousal (also called the 'anxious arousal' dimension) is more specific for anxiety. High negative affect (also called the 'general distress' dimension) is a non-specific factor that relates to both depression and anxiety, and is seen as a measure of severity of psychopathology.

Phenotypic dimensional and categorical approaches can be externally validated by using indices of HPA axis function. Sequential assessment of saliva cortisol is frequently used to assess the diurnal cortisol which represents the activity of the HPA axis under basal conditions. Previous studies indicate that the CAR is under a distinct

regulatory influence apart from the decline of cortisol over the day, and, therefore, should be assessed and analyzed separately.[22-24]

We hypothesize that phenotypic tripartite dimensions may reveal underlying associations with salivary cortisol in addition to phenotypic DSM-IV categories, because the continuum of dimensional scores includes an indication of severity. Moreover, comorbidity is no longer an issue when using a dimensional approach. In the present study, we compared the categorical DSM-IV diagnoses with the dimensional tripartite model of anxiety and depression as correlates of measures of HPA axis function under basal conditions. We used sequential assessments of salivary cortisol over the day, to derive HPA axis measures of the CAR and the diurnal decline.

Methods

Subjects

Thirty-six patients suffering from depressive disorders without comorbidity, 18 patients with anxiety disorders without comorbidity, and 19 patients with comorbid depressive and anxiety disorders were recruited from the outpatient department of the Rivierduinen mental health center in Leiden, the Netherlands. Patients had to meet the criteria of a major depressive disorder and/or an anxiety disorder (i.e., panic disorder, social anxiety disorder, obsessive compulsive disorder, generalized anxiety disorder or post-traumatic stress disorder) according to the DSM-IV criteria confirmed by the Dutch version of the Mini International Neuropsychiatric Interview Plus 5.0.0-R (MINI-Plus).[25;26] Patients with any other axis I disorders were excluded. Forty-three healthy controls were recruited by advertisement. The MINI-Plus was also used in healthy controls to rule out current axis I psychopathology. The MINI-Plus was administered by trained interviewers. Exclusion criteria were a history of neurological or endocrine diseases or other serious unstable medical conditions. Furthermore, pregnant or breast feeding women and premenopausal women with ovarectomy, and subjects using psychotropic medication with exception of a low dose of a benzodiazepine (equivalent to 30 mg oxazepam daily) were excluded. All subjects had to be free of psychotropic medication for at least 2 weeks (fluoxetine for six weeks), except for a low dose of benzodiazepine,. Additionally, subjects using corticosteroids, antidiabetics, estrogens, thyroid hormone, or herbal medication (e.g., Valerian, St. Johns Wort) were excluded. All subjects had a routine physical examination and laboratory blood tests in order to detect and exclude subjects with severe physical comorbidity. Prior to participation, written informed consent was obtained from all subjects. The study was approved by the ethics committee of the Leiden University Medical Center.

Psychological assessments

The Dutch version of the Mini International Neuropsychiatric Interview Plus 5.0.0-R (MINI-Plus)[25;26] was used for psychiatric diagnosing according to DSM-IV criteria.

Subjects completed the Depressive and Anxiety Symptoms Questionnaire (MASQ).[27;28] The MASQ, based on the tripartite model of anxiety and depression, was developed to assess symptoms of depression and anxiety over the previous week. The MASQ consists of 90 items, divided into 5 dimensions: 1) anhedonic depression; 2) anxious arousal; 3) general distress depression; 4) general distress anxiety, and 5) general distress mixed. All items are presented with a 5-point rating scale ranging from 'not at all' (1) to 'very much' (5). Total scores were calculated by summing all items of a particular dimension. The last three dimensions were clustered into one non-specific 'general distress' dimension. In this study the Dutch validated version of the MASQ was used.[29] The internal consistency of the three MASQ dimensions is excellent (all Cronbach's $a \geq .88$).[29]

Saliva cortisol sampling

Face-to-face as well as written instructions concerning saliva sampling prohibited eating, smoking, drinking tea or coffee or brushing teeth within 15 minutes before sampling. Furthermore, no dental work 24 hours prior to sampling was allowed. Saliva samples were obtained at home using salivettes (Sarstedt, Germany) at eight time-points covering the cortisol awakening response (CAR) and the diurnal decline in cortisol. The CAR includes four sampling points; at awakening (T1) and 30 (T2), 45 (T3) and 60 (T4) minutes after awakening. Four additional samples were taken to assess the diurnal decline in cortisol at 11:00h (T5), 15:00h (T6), 19:00h (T7), and 23:00h (T8). Subjects were asked to register the times they actually sampled in order to be able to do a limited check whether participants sampled at the required times. The cortisol curve of a single day is determined by situational factors and, to a smaller extent, by trait factors.[30] Therefore, to reduce measurement error and the effects of day-to-day variation, subjects were asked to provide saliva samples on two consecutive non-working days. Non-working days were chosen for both patients and controls, because hardly any patient was working due to his or her illness. Samples were stored at 4 °C until bringing them to the clinic, within one week after collection. After receipt, salivettes were centrifuged at 2000g for ten minutes, aliquoted and stored at -20 °C. Cortisol analysis was performed by competitive electrochemiluminescence immunoassay (Roche, Switzerland), as described in Van Aken et al. [31]. The lower detection limit was <0.5 nmol/l. The intra-assay coefficients of variance were lower than 7%, the inter-assay coefficients of variance were lower than 8%, except for the very low range. Per sampling point, physiologically unlikely high values were excluded from further analyses (1.3% of the data). We choose a cut-off score of about two

standard deviations above the mean, which equals 50 nmol/l. The rationale for assuming physiologically unlikely high values is that little wounds in the oral cavity might lead to contamination of saliva with blood, resulting in the faulty high cortisol levels in saliva. Saliva cortisol levels are reported as nmol/l and showed a normal distribution. The two cortisol values obtained at the time points on the 2 days were significantly correlated indicating moderate to good intra-individual stability over time (r between 0.43 and 0.58, all $P \leq 0.01$). Therefore, mean cortisol values for each time point were computed for each subject and used in the analyses. If a sample was missing, then the value of the other day was used (4.6% of the data points).

Subjects were asked to report on the following potential confounding factors: cigarette smoking, alcohol and coffee consumption, medication and illicit drug use, vigorous exercise, time of awakening, time of falling asleep the night before, estimation of hours of sleep during the night, the most stressful event of the day, and typicality of the day. Body weight and height were measured to calculate the BMI (kg/m^2).

Statistical analyses

Group differences for clinical characteristics were assessed by independent t-tests and one-way ANOVAs for continuous variables and χ^2-tests for categorical variables. Separate analyses were done for the CAR and diurnal cortisol decline. As markers of overall cortisol concentrations, the total area under the curve of the CAR (AUC_{CAR}) and diurnal decline ($AUC_{diurnal}$) were calculated using the trapezoid formula.[32] In addition, measures of dynamic change were calculated: the awakening rise (max value of T2/T3 minus T1), and the slope of the diurnal decline by estimating a simple linear regression model for each subject by regressing the cortisol values at time of collection from T5 to T8.

In addition to the DSM-IV classification, the same group of patients was also categorized in tertiles for each MASQ dimension (i.e., upper, middle, lower). One-way ANOVA was used for the analysis of group differences in the measures of the CAR and diurnal decline. DSM-IV categories and tertiles of the MASQ dimensions were entered in subsequent analyses as grouping factor. Trend analyses with polynomial contrasts were used when the overall group effect for the phenotypic MASQ dimensions was significant, to examine whether this effect could be explained by either a linear or non-linear (quadratic) effect.

All analyses were adjusted for age, gender, body-mass-index (BMI), the use of oral contraceptives, and awakening time (only for AUC_{CAR}). Smoking was not included as covariate, because there were only 4 smokers in the control group (i.e., 9.3%). In addition, analyses were repeated after excluding subjects with post-traumatic stress disorder (PTSD), because of contradictory findings found in previous studies.[33]

Analyses were performed using Statistical Package or the Social Science version 16.0 (SPSS 16.0). *P*-value < 0.05 was considered statically significant.

Table 1. Clinical characteristics according to DSM-IV patient groups and controls

	Depressive disorder (n = 36)	**Anxiety disorder (n = 18)**	**Comorbid disorder (n=19)**	**Controls (n = 43)**
Age	35.7 ± 12.9	30.3 ± 8.4	28.8 ± 8.7	33.2 ± 12.4
Gender (% women)	25 (69.4%)	14 (77.8%)	9 (47.4%)	29 (67.4%)
Smoking (% smokers)[a]	21 (58.3%)	8 (50.0%)	10 (52.6%)	4 (9.3%)
Contraceptives (% female users)[a]	13/25 (52.0%)	12/14 (85.7%)	5/9 (55.6%)	10/29 (34.5%)
Body-mass-index (kg/m^2)	24.4 ± 4.3	22.8 ± 3.5	26.0 ± 8.7	24.4 ± 3.8
Awakening time day 1 (h:mm)	7:12 ± 0:54	7:41 ± 1:09	7:39 ± 1:11	7:27 ± 0:43
Awakening time day 2 (h:mm)	7:44 ± 1:10	8:08 ± 1:11	8:07 ± 1:26	7:30 ± 0:57
Panic disorder	-	6	4	-
Sociale anxiety disorder	-	9	9	-
Obsessive compulsive disorder	-	1	1	-
Generalized anxiety disorder	-	1	0	-
Post-traumatic stress disorder	-	5	5	-
MASQ anhedonic depression[a,b]	87.8 ± 11.0	69.2 ± 12.1	81.1 ± 12.7	44.0 ± 12.4
MASQ anxious arousal[a,c]	33.5 ± 13.0	30.0 ± 14.3	30.0 ± 10.0	18.6 ± 1.9
MASQ general distress[a,b]	37.8 ± 9.2	30.6 ± 9.7	34.1 ± 8.1	16.8 ± 3.3

Data are presented as means and standard deviation (SD) or percentage within subgroups. Some patients with pure anxiety disorders had more than one anxiety disorder, so the sum does not add up to the total.

[a] significant difference between DSM-IV groups compared with controls; [b] significant difference between DSM-IV groups mutually; [c] log-transformed values were used in statistical analyses because of positively skewed distributions.

Results

Clinical data

Clinical characteristics are presented in table 1. Patient groups and controls did not differ statistically significantly for gender (χ^2=4.30, *P*=0.23), age (*F*(3,112)=1.80, *P*=0.15), BMI (*F*(3,112)=1.21, *P*=0.31) and time of awakening (day1: *F*(3,112)=1.47, *P*=0.23; dag2: *F*(3,112)=2.02, *P*=0.12) and the awakening times were significantly intercorrelated (*r*=.67, *P*<0.001). However, patients were more likely to smoke (χ^2=24.52, *P*<0.001) and, for the female subjects, did more often use oral contraceptives (χ^2=9.40, *P*=0.03) as compared to controls.

As expected, patient groups scored significantly higher on all MASQ dimensions compared to controls (all *P*<0.01). Significant differences between patient groups were found in scores for anhedonic depression (*F*(2,70)=15.13, *P*<0.001) and for general distress (*F*(2,70)=4.04, *P*=0.02), but not for anxious arousal (*F*(2,70)=0.91, *P*=0.41). Patients with a depressive or comorbid disorder scored higher on anhedonic depression and general distress compared to patients with an anxiety disorder.

When divided in tertiles per MASQ dimension, the three patient groups did not differ significantly in age, BMI, smoking and the use of oral contraceptives (data not shown). A significant difference was found for gender between the tertiles of anxious arousal: females were equally distributed across the tertiles, but males were predominantly found in the lower tertile (χ^2=7.89, *P*=0.02).

Salivary cortisol in the CAR

Figure 1 shows the CAR for the different approaches to phenotyping. One-way ANOVA using the DSM-IV categories as a group factor, revealed neither significant differences in AUC_{CAR} nor in awakening rise (Table 2).

Next, we explored whether MASQ dimensions were associated with the CAR. Controls were included in the analyses as a separate group. Significant group differences in AUC_{CAR} were found for anhedonic depression and general distress (trend), but not for anxious arousal (Table 2). Trend analyses with polynomial contrasts were performed to explore whether this group effect could be explained either by a linear or a non-linear association between groups and cortisol measures. For anhedonic depression and general distress, non-linear associations with the AUC_{CAR} were found. Higher cortisol concentrations were found in patients of the middle tertile as compared to the lower and upper tertiles, as well as to controls, indicating an inverted U-shape (Figure 3). Trend analyses were repeated while adjusting for the presence of a depressive and/or anxiety disorder to exclude the possibility for non-linear associations to be ascribed to a disproportional distribution of the DSM-IV categories across the tertiles. However, non-linear associations

remained significant after multivariable adjustment. With regard to the awakening rise, only a trend toward significance was found for all three dimensions, i.e., anhedonic depression, anxious arousal, and general distress (Table 2). In addition, analyses were repeated after excluding subjects with post-traumatic stress disorder (PTSD), because of contradictory findings found in previous studies [33]. Again, our main findings did not change (data not shown).

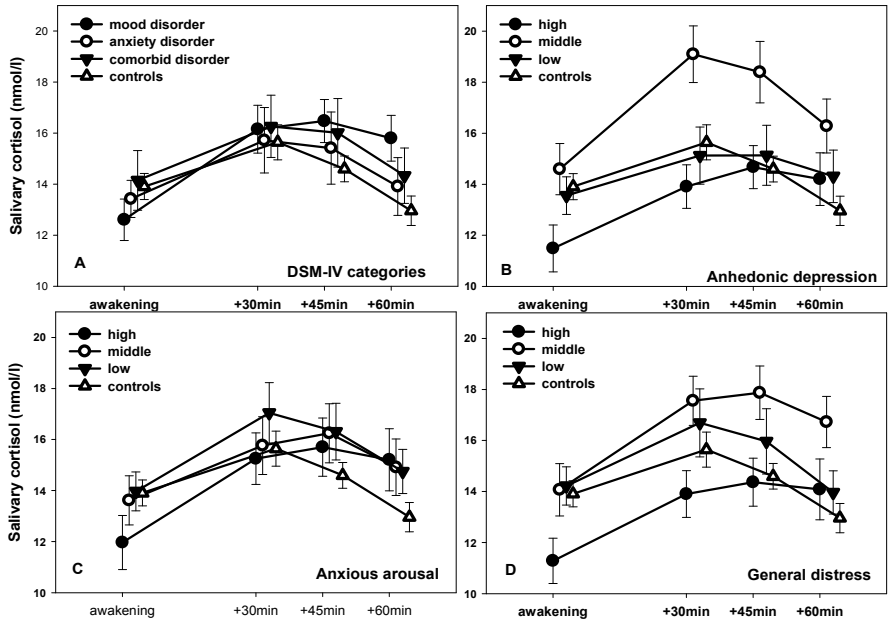

Figure 1. *Cortisol awakening response*
Graphical representation of means with bars representing the standard error (SE); patients are subdivided into DSM-IV categories (A), and for each MASQ dimension into tertiles (upper, middle, lower) for anhedonic depression (B), anxious arousal (C), and general distress (D).

Diurnal decline in salivary cortisol

Figure 2 shows the diurnal decline for the different approaches to phenotyping. One-way ANOVA using the DSM-IV categories as group factor revealed significant differences in $AUC_{diurnal}$ and the slope of the diurnal decline (Table 2). Simple contrast tests, comparing each DSM-IV category with controls, showed significantly higher $AUC_{diurnal}$ levels and a steeper slope in depressive patients compared to controls (t=1.9, P=<0.001; t=-0.74, P=0.01, respectively).

Next, we explored whether MASQ dimensions were associated with the CAR. Controls were included in the analyses as a separate group. Significant group differences in the $AUC_{diurnal}$ were found for anhedonic depression, anxious arousal

(trend), and general distress (Table 2). Trend analyses with polynomial contrasts were performed to explore whether this group effect could be explained either by a linear or a non-linear association between groups and cortisol measures. A linear trend was found for anhedonic depression and general distress. Higher dimensional scores were related to higher cortisol concentrations during the diurnal cortisol decline. No clear linear or non-linear trend could be detected for anxious arousal (Figure 3). With regard to the slope of the diurnal decline, no significant group differences were found for all dimensions, i.e., anhedonic depression, anxious arousal, and general distress (Table 2). Analyses were repeated after excluding subjects with post-traumatic stress disorder (PTSD). Our main findings did not change (data not shown).

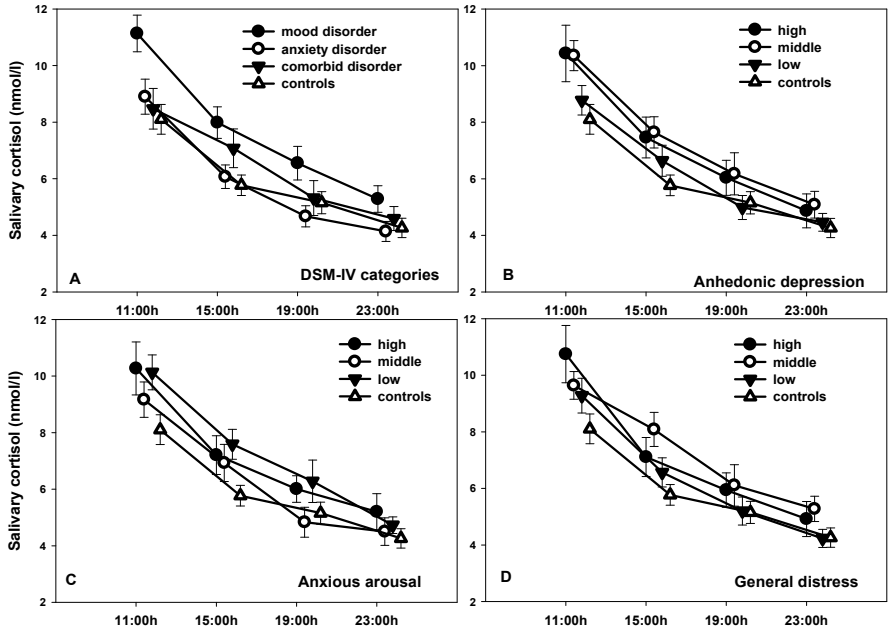

Figure 2. *Diurnal decline in salivary cortisol*
Graphical representation of means with bars representing the standard error (SE); patients are subdivided into DSM-IV categories (A), and for each MASQ dimension into tertiles (upper, middle, lower) of anhedonic depression (B), anxious arousal (C), and general distress (D).

Table 2. Cortisol measures accordingly to DSM-IV categories and MASQ tertiles in controls and patients with anxiety and/or depressive disorders

	n	**AUC_{CAR}[a]**	**Rise[a]**	**$AUC_{diurnal}$[b]**	**Slope[b]**
Controls	45	14.6 ± 3.2	2.5 ± 4.4	68.4 ± 21.4	-1.2 ± 1.2
DSM-IV diagnoses:					
• **Depressive disorder**	36	15.3 ± 4.5	5.0 ± 4.3	91.0 ± 33.4	-1.9 ± 1.3
• **Anxiety disorder**	18	14.8 ± 4.3	3.1 ± 5.8	69.0 ± 19.3	-1.6 ± 0.6
• **Comorbid disorder**	19	15.4 ± 4.8	2.9 ± 4.7	75.7 ± 27.8	-1.3 ± 0.9
Group effect		*F*(3,112)=0.61 *P*=0.61	*F*(3,112)=2.3 *P*=0.09	*F*(3,112)=5.4 *P*=0.002	*F*(3,112)=2.7 *P*=0.045
Anhedonic depression:					
• **Lower tertile**	23	14.6 ± 4.1	2.6 ± 5.6	73.0 ± 23.1	-1.5 ± 0.6
• **Middle tertile**	25	17.4 ± 4.8	5.3 ± 5.3	86.2 ± 27.0	-1.7 ± 1.2
• **Upper tertile**	25	13.5 ± 3.9	4.0 ± 3.5	84.6 ± 38.1	-1.8 ± 1.3
Group effect		*F*(3,112)=4.2 *P*=0.01	*F*(3,112)=2.6 *P*=0.06	*F*(3,112)=2.9 *P*=0.04	*F*(3,112)=1.9 *P*=0.13
Anxious arousal:					
• **Lower tertile**	23	15.8 ± 4.6	3.7 ± 5.4	85.2 ± 30.5	-1.8 ± 1.0
• **Middle tertile**	25	15.2 ± 4.4	3.5 ± 5.0	74.4 ± 26.3	-1.6 ± 1.1
• **Upper tertile**	25	14.5 ± 4.7	4.7 ± 4.3	83.8 ± 33.7	-1.6 ± 1.1
Group effect		*F*(3,112)=1.6 *P*=0.20	*F*(3,112)=2.2 *P*=0.09	*F*(3,112)=2.2 *P*=0.09	*F*(3,112)=1.6 *P*=0.18
General distress:					
• **Lower tertile**	23	15.6 ± 4.9	3.2 ± 5.4	74.1 ± 23.1	-1.7 ± 0.9
• **Middle tertile**	25	16.7 ± 4.1	4.6 ± 5.6	86.6 ± 30.4	-1.5 ± 0.9
• **Upper tertile**	25	13.4 ± 4.1	4.1 ± 3.5	83.5 ± 36.1	-1.9 ± 1.4
Group effect		*F*(3,112)=2.5 *P*=0.07	*F*(3,112)=2.4 *P*=0.08	*F*(3,112)=3.4 *P*=0.02	*F*(3,112)=1.9 *P*=0.14

Data are presented as means and standard deviation (SD) within subgroups; patients are subdivided into DSM-IV categories, and for each MASQ dimension into tertiles (lower, middle, upper). Test results of one-way ANOVAs are presented.

[a] *Adjusted for age, gender, BMI, use of oral contraceptive, and awakening time.*

[b] *Adjusted for age, gender, BMI, and use of oral contraceptive.*

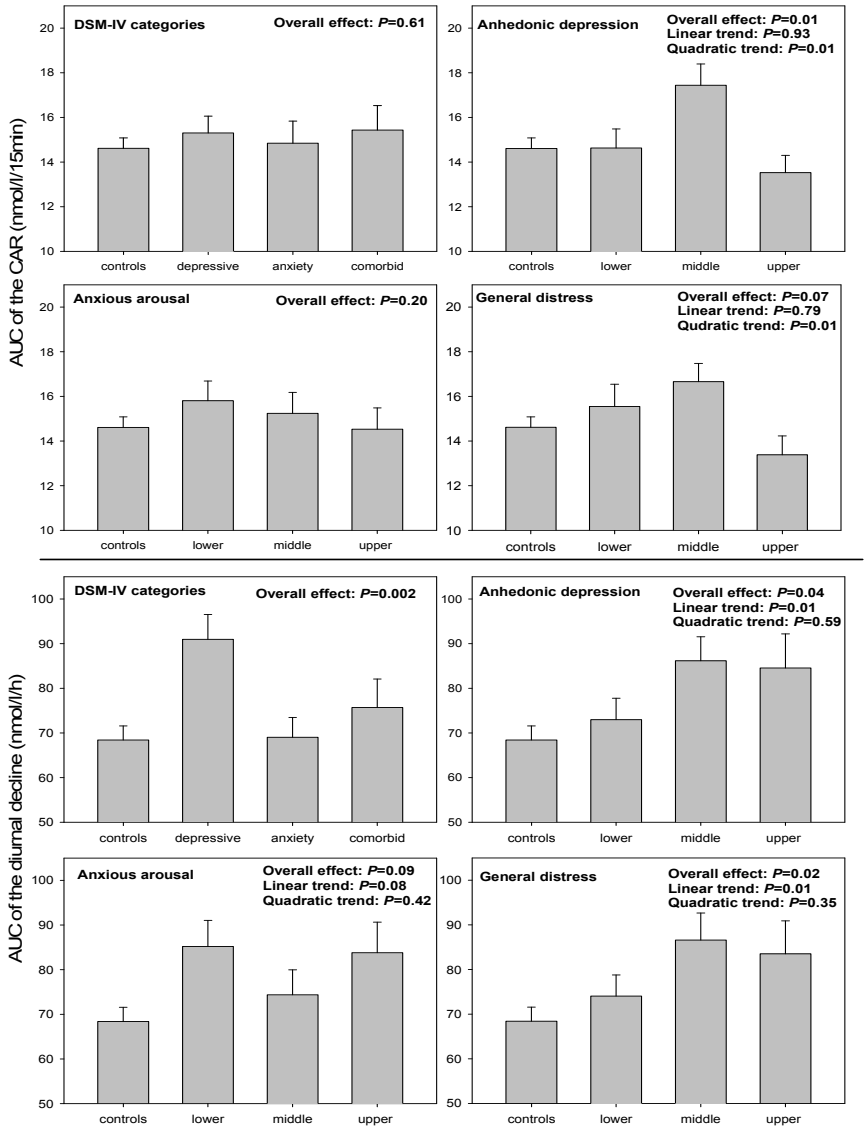

Figure 3. *AUC_{CAR} and $AUC_{diurnal}$ of patients with depression and anxiety and controls*
Graphical representation of means with bars representing the standard error (SE); patients are subdivided in DSM-IV categories (depressive, anxiety, comorbid disorder) and for each MASQ dimension into tertiles (lower, middle, upper). P-values of one-way ANOVAs and post hoc trend analyses are presented, adjusted for age, gender, BMI, use of oral contraceptive, and awakening time (only for AUC_{CAR}).

Discussion

In the present study, we showed that MASQ dimensions are related to the CAR in a non-linear pattern, and to the diurnal cortisol decline in a linear pattern. All patients with depressive and/or anxiety disorders were free of psychotropic medication.

Regarding the CAR, cortisol concentrations showed a nonlinear relationship with the anhedonic depression and general distress dimension of the MASQ, indicating an inverted U-shaped association. This finding would be missed when investigating only phenotypic DSM-IV categories. In contrast, the phenotypic DSM-IV categories of depressive and anxiety disorders did not show measurable differences in cortisol concentrations during the CAR. Our finding of non-linear associations between phenotypic dimensions and cortisol concentrations after awakening is in line with previous studies in which depressive symptoms were associated with both hyperactivity and hypoactivity of the HPA axis.[11;12;34] However, these previous studies were performed in elderly subjects and did not specifically focus on the CAR. In a recent study, a U-shaped association between a dimensional phenotypical approach and basal cortisol levels was found. Similar to our findings, cortisol profiles in seniors showed that in the extreme 'highest' and 'lowest' well-being groups, lower cortisol levels were found during the first hour after awakening [35]. Although the present cross-sectional study design precludes statements on causation, our finding of an inverted U-shaped association between cortisol concentrations in the first hour after awakening and MASQ dimensions might suggest that depression and anxiety initially may lead to enhanced HPA axis activity, resulting in higher morning cortisol levels. Disease progression and increasing severity may eventually lead to a down-regulation of the HPA axis, resulting in lower morning cortisol levels.[36;37] Previous studies provided evidence that an enhanced HPA axis function adapts to chronic and/or severe stress by subsequent down-regulation, resulting in a blunted CAR.[34;38] Also in a recent meta-analysis it was shown that timing of the stressor is a critical element in explaining the variability in HPA axis function. Hormonal activity is elevated, resulting in elevated cortisol output at the stressor onset, but as time passes, this activity diminishes, and cortisol secretion rebounds to below normal.[39]

Regarding the diurnal cortisol decline, cortisol showed a linear relationship with the anhedonic depression and general distress dimensions of the MASQ, indicating that increasing dimensional scores were associated with higher cortisol concentrations. When using a phenotypic categorical approach, higher cortisol concentrations and a steeper slope were found in patients with a DSM-IV depressive disorder compared to controls. The linear association between two MASQ dimensions and cortisol concentrations during the diurnal decline confirms previous findings in which severity of symptoms was related to higher cortisol concentrations.[40] The finding of higher

cortisol concentrations in depressive patients is also in line with previous studies that showed elevated basal cortisol levels in depressive patients during the day. A diminished negative feedback or a higher hypothalamic drive might be responsible for these higher levels.[41-43] With regard to the slope, previous studies found flattened circadian slopes (e.g., smaller slopes) in depressive patients, which seems to contrast with our finding of steeper slopes.[42] This might be due to differences in calculation of the slope. Most studies used the time span between awakening and the evening and excluded the awakening response. We approached the CAR and diurnal decline as separate entities of the cortisol day curve, based on the support of distinct regulatory influences to be involved, and calculated the slope of the diurnal decline from 11:00h to 23:00h.[23]

The non-linear associations during CAR versus the linear associations during the diurnal decline support the idea that the cortisol release during the CAR and during the diurnal decline are under distinct regulatory influences resulting in different kinds of associations (i.e., linear and non-linear) between MASQ dimensions and cortisol concentrations.[22-24] On the other hand, our study may have been underpowered to detect non-linear associations during the diurnal cortisol decline.

In our study, no cortisol measure was able to discriminate between categories of the anxious arousal dimension. This might indicate that the other dimensions, e.g., anhedonic depression and general distress, are more closely related to HPA axis dysfunction than arousal dimensions. This is in line with previous studies on anxiety disorders are characterized by arousal symptoms showing that basal cortisol was unaltered as compared to controls.[4;6;7;13-17] Otherwise, arousal measures, compared to affective measures, might be more subject to temporary fluctuations depending on momentary stressors. Therefore trait arousal may be difficult to assess through a questionnaire.

Some methodological limitations of this study should be mentioned. Firstly, sample sizes were modest, especially of the pure anxiety group and comorbid group. This might have limited the power to detect potential group differences and other associations. Secondly, the pure anxiety group and comorbid group were heterogeneous, because patients with different anxiety disorders were included. However, due to the small number of subjects per anxiety disorder, allocation according to diagnosis was not possible. This heterogeneity might also have limited the power to detect potential group differences. Thirdly, around 37% of the control group and 33% in the patient group showed no rise in cortisol after awakening. At least for healthy controls, this percentage is somewhat higher than previous reported percentages of 25%, which implies the possibility of non-adherence to the study protocol.[44;45] Fourthly, previous studies indicate that in psychiatric in-patients with more severe forms of psychopathology stronger differences in HPA axis measures

were found compared to controls.[46-49] We included outpatients and this may have reduced the sensitivity to detect DSM-IV group differences in HPA axis function. Fifthly, the use of benzodiazepines could have influenced our outcome. However, only 4 patients used a low dose of benzodiazepines (maximum of an equivalent to 30 mg oxazepam daily). Sixthly, we did not collect clinical data on the duration of the current episode, age of onset, number of episodes, and presence of psychotic features of the depressive and/or anxiety disorder. The hypothesis that an enhanced HPA axis reactivity in the short term is followed by down-regulation of the HPA axis in the long term should be explored further in longitudinal studies. Lastly, we did not consider effects of genotype, personality traits, menstrual cycle, sleep-wake rhythm, and early life trauma, all of which might impact HPA axis function.[50]

We conclude, firstly, that anhedonic depression and general distress showed non-linear associations with cortisol concentrations during the CAR in patients with depressive and anxiety disorders who were free of psychotropic medication. Secondly, linear associations were found between these same MASQ dimensions and cortisol concentrations during the diurnal decline. Thirdly, higher cortisol concentrations and a steeper slope were found in patients with a DSM-IV depressive disorder compared to controls. Although, replication of this study is needed, the present study shows that linear and non-linear associations with salivary cortisol are detected when using phenotypic dimensions and may be complementary to phenotypic DSM-IV categories when doing neuroendocrine research.

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Chapter 5

Salivary cortisol, serum lipids and adiposity in patients with depressive and anxiety disorders

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Abstract

Objective: Depressive and anxiety disorders are associated with an increased risk of cardiovascular disease (CVD). Chronic stress induces hypothalamus-pituitary-adrenal (HPA)-axis perturbations, which might subsequently induce atherogenic lipoprotein profiles and adiposity. The aim of the present study was to investigate the relationship between basal saliva cortisol levels and serum lipids and adiposity in psychiatric patients.

Methods: Eight salivary cortisol samples (awakening, 30, 45, and 60 min after awakening, 11:00h, 15:00h, 19:00h, and 23:00h) on two consecutive days in medication-free outpatients with depressive and/or anxiety disorders (n=72) and of healthy controls (n=42) were used to derive two measures of HPA-axis function: basal cortisol concentrations (i.e., area-under-the-curve ($AUC_{cortisol}$)) and circadian cortisol variability ($variability_{cortisol}$). Index z-scores were calculated for dyslipidaemia (from serum triglycerides, inverse high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol) and adiposity (from body-mass index and waist-to-hip ratio).

Results: Regression analyses were conducted to determine the contribution of $AUC_{cortisol}$ and $variability_{cortisol}$ in explaining the variance of respectively the lipid- and adiposity-index. Patients showed a higher mean $AUC_{cortisol}$ compared to healthy controls ($t=2.7$; $p=.01$). Both cortisol parameters were independently associated with dyslipidaemia in patients after adjustment for age, alcohol use, and smoking habits ($\beta=.31$, $p=.02$; and $\beta=-.29$, $p=.02$, respectively), but not in controls. Cortisol measures were not associated with adiposity in either group.

Conclusions: We conclude that elevated basal cortisol concentrations and lower circadian cortisol variability were independently associated with a less favourable lipoprotein profile in patients with depressive and/or anxiety disorders. These data lend support to the hypothesis that the relationship between affective disorders and CVD is partly mediated by HPA-axis perturbations.

Introduction

Several studies have shown that depression is associated with an increased morbidity and mortality from cardiovascular disease (CVD).[1-3] There is growing evidence that the same holds for anxiety disorders.[4-6] In a part of the patients with depressive and anxiety disorders, dysfunctions of the hypothalamic-pituitary-adrenal (HPA) axis are observed. The most frequently reported finding is hypercortisolemia under basal conditions[7;8] Another dysfunction that has been found repeatedly is a lower circadian cortisol variability compared to healthy controls.[9;10] As cortisol influences many metabolic processes, these HPA-axis dysfunctions are plausible mediators of the association between depressive and anxiety disorders on the one side and CVD on the other.[11]

Cortisol is a stress hormone that has effects not only on blood pressure, glucose metabolism, and the immune system, but also on lipoprotein metabolism and body composition. It stimulates lipolysis and decreases the activity of lipoprotein lipase, partly through its effects on insulin sensitivity. As a consequence of hypercortisolism, serum levels of total cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides increase, and serum levels of high-density lipoprotein (HDL) cholesterol decrease. In the long term, this dyslipidaemia due to a cortisol excess leads to an increase in central adiposity,[12-14] an elevated body-mass index (BMI) and a higher waist-to-hip ratio (WHR).[15;16;17;18] These metabolic effects of cortisol are clearly shown in studies on the effects of synthetic glucocorticoids during anti-inflammatory and immunosuppressive therapy[19] and on Cushing's disease.[17;18] In addition, in healthy participants a lower circadian cortisol variability was associated with detrimental metabolic effects, i.e., high triglycerides, high LDL cholesterol, low HDL cholesterol, high BMI, and high WHR.[20-23]

Studies on HPA-axis dysfunctions in patients with depressive disorders are scarce and show contradictory results. In a large cross-sectional survey of elderly depressed patients, high 24h urinary cortisol levels were associated with the metabolic syndrome, including high triglycerides and low HDL cholesterol.[11] However, in another study higher salivary cortisol levels (measured at three time points during the day) were associated with lower LDL cholesterol levels in 41 overweight depressed patients (BMI > 25 kg/m^2), but not in 37 patients of normal weight.[24] Anxiety disorders were associated with dyslipidaemia,[25;26] but to the best of our knowledge, no studies were done on the associations between lipids and cortisol levels in anxiety disorders.

We studied two aspects of the HPA-axis function (i.e., basal cortisol release over the day, and circadian cortisol variability as an indicator of the responsivity of the stress system). Both HPA-axis functions are known to be disturbed in patients with depressive and/or anxiety disorders and are hypothesized to be related to a

detrimental lipid metabolism and adiposity. As far as we know, no previous study focused on both aspects of HPA-axis function and their relationship with parameters of dyslipidaemia and adiposity in patients with depressive and anxiety disorders. As psychotropic medication is a potential confounder that may affect body weight, serum lipids and lipoproteins levels[27;28] as well as cortisol levels, we included only patients free of such medication.

Methods

Participants

Seventy two patients suffering from a depressive and/or anxiety disorder were recruited from the outpatient department of the mental health center Rivierduinen in Leiden, the Netherlands. The Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV diagnoses were confirmed by trained interviewers using the Dutch version of the Mini International Neuropsychiatric Interview Plus 5.0.0-R (MINI-Plus).[29;30] Patients with any other axis I disorder were excluded. Axis I psychopathology was also ruled out by the MINI-plus in 42 healthy controls recruited by advertisement. Exclusion criteria were a history of neurological or endocrine diseases or other serious medical conditions. Furthermore, participants with substance or alcohol abuse, as well as pregnant or breast feeding women and premenopausal women with ovarectomy were excluded. Participants using psychotropic medication with exception of a low dose of a benzodiazepine (equivalent to 30 mg oxazepam daily) were also excluded. If psychotropic medication was used within the last 14 days (for fluoxetine six weeks) participants were excluded. Additionally, participants using corticosteroids, antidiabetics, estrogens, thyroid hormone, or herbal medication (e.g., Valerian, St. Johns Wort) were excluded. All participants had a routine physical examination and laboratory blood tests. Prior to participation, written informed consent was obtained from all participants. The study was approved by the ethics committee of the Leiden University Medical Center.

Saliva cortisol measures

Instructions concerning saliva sampling prohibited eating, smoking, drinking tea or coffee or brushing teeth within 15 minutes before sampling. Furthermore, no dental work 24 hours prior to sampling was allowed. Saliva samples were obtained using Salivettes (Sarstedt, Germany) at eight time-points covering the cortisol day curve. Four samples were collected at awakening (T1) and 30 (T2), 45 (T3) and 60 (T4) minutes after awakening. Four additional samples were taken at 11:00h (T5), 15:00h (T6), 19:00h (T7), and 23:00h (T8). The cortisol curve of a single day is determined by situational factors and, to a smaller extent, by trait factors. Therefore, to reduce

measurement error and the effects of day-to-day variation,[31] participants were asked to provide saliva samples on two consecutive non-working days. Non-working days were chosen for both patients and controls, because hardly any patients were working due to their illness. Samples were stored at 4 °C until bringing them to the clinic, within one week after collection. After receipt, salivettes were centrifuged at 2000g for 10 minutes, aliquoted and stored at -20 °C. Cortisol analysis was performed by competitive electrochemiluminescence immunoassay (Roche, Switserland), as described in Van Aken et al..[32] The lower detection limit was <0.50 nmol/l. The intra-assay coefficients of variance were lower than 7%, the inter-assay coefficients of variance were lower than 8%, except for the very low range. Per sampling point, physiologically unlikely high values (defined as > 50 nmol/l) were excluded from further analyses (1.3% of the data). Saliva cortisol levels are reported as nmol/l and showed a normal distribution. The two cortisol values obtained at the time points on the 2 days were significantly correlated, indicating moderate to good intraindividual stability over time (r between .42 and .68, $p \leq .01$). Therefore, mean cortisol values for each time point were computed for each subject and used in the analyses. If a sample was missing, then the value of the other day was used (for 2.9% of the data).

Anthropometric, lipid, and lipoprotein measures

Body weight and height were measured to calculate the BMI (kg/m^2). The waist circumference was measured midway between the lower costal and iliac crest and the hip circumference was measured at the level of the great trochanters, to calculate the WHR.

Venous blood was sampled with standard venipuncture techniques. Non-fasting triglycerides, total cholesterol and HDL cholesterol (HDL-C plus 3rd generation) were measured in serum by automated enzymatic colorimetric methods using a Modular P analyzer (Roche, Switzerland). LDL cholesterol was calculated according to the Friedewald-formula.[33]

Statistical analyses

The area under the cortisol day curve ($AUC_{cortisol}$) was used as an indicator of the total cortisol excretion over the day, calculated using the trapezoid formula [34]. The AUC of the first four samples at awakening (i.e., T1 to T4) was added to the AUC of the last four samples (i.e., T5 tot T8), because the time between the assessment of T4 and T5 varied depending on the time of awakening. For determination of the circadian variability in salivary cortisol ($variability_{cortisol}$), the within-individual variance between all cortisol assessments (8 samples x 2 days) was calculated. $Variability_{cortisol}$ was log-transformed, because of its positively skewed distribution. For illustrative purposes,

figure 1 represents 2 individual participants with, respectively, a high and low circadian cortisol variability.

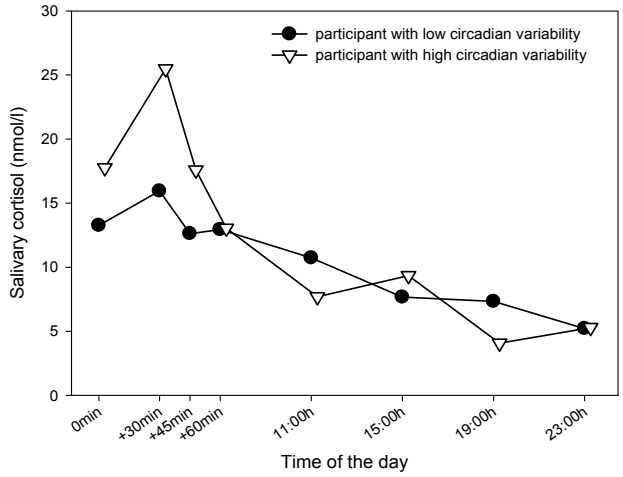

Figure 1. *Circadian cortisol variability*
Graphical representation of salivary cortisol values (means from 2 sampling days) of 2 individual participants with either a high or low circadian cortisol variability. Figure is included for illustrative purposes to demonstrate the concept circadian cortisol variability.

Group differences for clinical characteristics, lipids, lipoproteins, BMI, WHR, and cortisol measures (i.e., $AUC_{cortisol}$ and $variability_{cortisol}$) were assessed by independent t-tests and one-way ANOVAs for continuous variables and χ^2-tests for categorical variables. Next, associations between cortisol measures, lipoprotein profiles, and adiposity were examined in patients and controls. Lipid- and adiposity-indices were calculated for each subject. For the lipid-index, a mean score of the individual z-scores of triglycerides, LDL cholesterol, and inverted HDL cholesterol was calculated, corrected for gender and use of oral contraceptives.[35] For the adiposity-index, a mean score of the individual z-scores of BMI and WHR was calculated, corrected for gender and use of oral contraceptives. Univariate and multivariate linear regression analyses were conducted to determine the individual and independent contribution of $AUC_{cortisol}$ and $variability_{cortisol}$ in explaining the variance of respectively the lipid- and adiposity-index in the patient group and the control group. All regression analyses were adjusted for time of awakening, age, smoking (yes/no), and alcohol consumption (daily-weekly/monthly-none). Analyses were performed using Statistical Package for the Social Science version 16.0 (SPSS 16.0). *P*-value < 0.05 was considered statically significant.

Results

Differences between patients and healthy controls

Clinical characteristics are presented in table 1. Females showed significant higher HDL cholesterol levels (t=3.5, p=.001), and a significant lower WHR (t=-9.0, p<.001) than males. Therefore, lipid- and adiposity-indices were adjusted for gender (and oral contraceptives) in order to take the effects of confounding into account. Patients and controls did not differ statistically significantly for gender, age, BMI, and WHR. However, patients were more likely to smoke (χ^2=22.5, p<.001) and, for the female participants, did more often use oral contraceptives (χ2=6.4, p=.01) as compared to controls. No statistically significant differences between patients and controls were found in serum lipid and lipoprotein levels, but patients showed lower HDL cholesterol levels than controls (t=-3.3, p=.001). Patients showed a higher $AUC_{cortisol}$ values compared to controls (t=2.7; p=.01), but a comparable $variability_{cortisol}$ (t=-.04; p=.97).

Table 1. Clinical characteristics, serum lipid and lipoprotein levels, anthropometric measures, and cortisol measures in 72 patients with depressive and anxiety disorders and 42 healthy controls

	Patients (n=72)	**Controls (n=42)**	**p-value**
Age	32.5 ± 11.3	33.3 ± 12.5	.72
Gender (% females)	47 (65.3%)	28 (66.7%)	.88
Smoking (% smokers)	39 (54.2%)	4 (9.5%)	< .001
Alcohol (% consumers)	38 (52.8%)	29 (69.1%)	.06
Oral contraceptives (% users in females)	30/47 (63.8%)	9/28 (32.1%)	.01
BMI (kg/m^2)	23.9 ± 4.1	24.3 ± 3.7	.63
WHR - males	.94 ± .07	.94 ± .05	.82
- females	.84 ± .05	.83 ± .04	.24
Total cholesterol (mmol/l)	4.80 ± 1.08	4.88 ± 1.11	.73
Triglycerides (mmol/l)	1.52 ± .86	1.41 ± .79	.53
LDL cholesterol (mmol/l)	3.11 ± .93	3.08 ± .99	.87
HDL cholesterol (mmol/l)	1.56 ± .40	1.82 ± .42	.001
$AUC_{cortisol}$ (nmol/l/h)	96.8 ± 32.0	81.8 ± 23.2	.01
$Variability_{cortisol}$ ($nmol^2$)[a]	36.9 ± 27.8	33.4 ± 19.7	.97

Data are presented as means ± standard deviation (SD) or n (percentage within group);

[a] log-transformed values used in t-test, because of positively skewed distributions.

BMI = body-mass index; WHR = waist-to-hip ratio; LDL = low-density lipoprotein; HDL = high-density lipoprotein; AUC = area-under-the-curve. To convert values for cholesterol and triglycerides from mmol/l of to mg/dl, multiply respectively by 38.7 and 88.8.

Associations in the patients

The lipid- and adiposity-indices were significantly correlated (r=.51, p=<.001). The correlation between $AUC_{cortisol}$ and $variability_{cortisol}$ showed a low correlation coefficient (r=.22, p=.07), indicating them to be relatively independent. In univariate regression analyses, $AUC_{cortisol}$ was a significant predictor of the lipid-index. $Variability_{cortisol}$ was not significantly associated with the lipid-index (Table 2). In multivariate models, however, $AUC_{cortisol}$ and $variability_{cortisol}$ were independently associated with the lipid-index, after adjustment for age, smoking status, and alcohol consumption (Table 2). High $AUC_{cortisol}$ and a low $variability_{cortisol}$ were independently predictive for a less favourable lipid/lipoprotein profile. Univariate and multivariate models showed no significant associations between the two cortisol measures and the adiposity-index (Table 2).

Table 2. Univariate and multivariate associations between lipid index, adiposity-index and cortisol measures in patients with depressive and anxiety disorders and in healthy controls

	Patients (n=72)					
	Unadjusted (univariate)		**Unadjusted (multivariate)**		**Adjusted[b] (multivariate)**	
	β	**p**	**β**	**p**	**β**	**p**
Lipid-index						
▪ **$AUC_{cortisol}$**	.24	.048	.28	.02	.31	.02
▪ **Variability[a]**	-.10	.42	-.17	.16	-.29	.02
Adiposity- index						
▪ **$AUC_{cortisol}$**	.09	.47	.05	.71	.09	.51
▪ **Variability[a]**	.20	.10	.19	.13	.08	.54
	Controls (n=42)					
Lipid-index						
▪ **$AUC_{cortisol}$**	.10	.54	.10	.57	.02	.89
▪ **Variability[a]**	.04	.81	.02	.89	.08	.64
Adiposity- index						
▪ **$AUC_{cortisol}$**	.08	.62	.03	.84	-.01	.97
▪ **Variability[a]**	.21	.19	.20	.23	.17	.30

Standardized regression coefficients (β) and p-values are presented;

[a] log-transformed values because of positively skewed distribution;

[b] adjusted for age, smoking, and alcohol consumption;

Lipid-index = mean score of the individual z-scores for triglycerides, LDL cholesterol, and inverse HDL cholesterol, adjusted for gender and use of oral contraceptives;

Adiposity-index = mean score of the individual z-scores for BMI and WHR, adjusted for gender and use of oral contraceptives.

Next, we explored the associations with individual lipids and lipoproteins. BMI was added as predictor to the final model, because of its strong association with lipid and lipoprotein levels. In the final model, $variability_{cortisol}$ was predictive for triglyceride levels and $AUC_{cortisol}$ was predictive for HDL cholesterol. BMI was an independent predictor for almost all serum lipids and lipoproteins (Table 3). When analyses were repeated while adjusting for the presence of depressive disorder (yes/no) and/or anxiety disorder (yes/no), our main findings were not importantly affected (data not shown).

Table 3. Associations between serum lipid levels and cortisol measures in 72 patients with depressive and anxiety disorders

	Unadjusted		**Adjusted[b]**		**Adjusted[c]**	
	β	**p**	**β**	**p**	**β**	**p**
Cholesterol						
$AUC_{cortisol}$	.12	.34	.11	.39	.08	.53
$Variability_{cortisol}$[a]	.07	.60	-.17	.17	-.18	.14
BMI					.22	.06
Triglycerides						
$AUC_{cortisol}$	.24	.06	.18	.19	.12	.33
$Variability_{cortisol}$[a]	-.17	.18	-.26	.05	-.28	.02
BMI					.41	.001
HDL cholesterol						
$AUC_{cortisol}$	-.28	.02	-.31	.02	-.26	.03
$Variability_{cortisol}$[a]	.29	.02	.17	.17	.18	.12
BMI					-.32	.01
LDL cholesterol						
$AUC_{cortisol}$	.18	.15	.20	.15	.16	.23
$Variability_{cortisol}$[a]	-.02	.86	-.21	.11	-.22	.09
BMI					.25	.048

Standardized regression coefficients (β) and p-values are presented;

[a] *logtransformed values because of skewed distribution of data;*

[b] *adjusted for age, gender, use of oral contraceptives, smoking (yes/no) alcohol consumption (daily-weekly/monthly-none), and presence of depressive (yes/no) and/or anxiety disorders (yes/no);*

[c] *additionally adjusted for BMI.*

Associations in healthy controls

The lipid-index and adiposity-indices were significantly correlated (r=.52, p=.001). Again, the correlation between $AUC_{cortisol}$ and $variability_{cortisol}$ was not statistically significant (r=.22, p=.16). Univariate and multivariate analyses showed no significant associations between $AUC_{cortisol}$ and $variability_{cortisol}$ and the lipid-index. Furthermore,

no significant associations were found between $AUC_{cortisol}$ and $variability_{cortisol}$ and the adiposity-index (Table 2).

Discussion

In the present study, higher basal cortisol concentrations were found in patients with depressive and/or anxiety disorders as compared to controls. In patients elevated basal cortisol concentrations and lower circadian cortisol variability were independently associated with a less favourable lipoprotein profile (i.e., higher scores on the lipid-index). However, no associations were found between cortisol measures and indices of adiposity, although the adiposity-index was strongly associated with dyslipidaemia.

The elevated basal cortisol concentrations we found, have been reported frequently for these patient groups.[7;8] Our findings on the association between elevated cortisol levels and dyslipidaemia in our patient group (mean age 32.8 ± 11.7) are in line with a study that showed associations between high 24h urinary cortisol levels and metabolic syndrome (which includes dyslipidaemia) in elderly depressed patients (mean age 74.1± 6.6).[11] This indicates that the positive association between cortisol and dyslipidaemia is not limited to elderly depressed patients. Associations were also found between glucocorticoid administration after organ transplant and hyperlipidaemia.[15;16;36] Furthermore, it was previously shown in healthy subjects that a low circadian cortisol variability, measured in saliva samples, is related to a less favourable lipoprotein profile.[21-23] Such association was confirmed in our patients, but not in controls, maybe because our controls did not show low circadian variability. In summary, the present study supports the hypothesis that elevated basal cortisol concentrations and lower circadian cortisol variability induce dyslipidaemia in patients with depressive and/or anxiety disorders.

In contrast to some previous studies[21;23] we did not find a relationship between cortisol and the adiposity-index. Several factors might contribute to this difference. Firstly, the mean BMI of our participants was about 3 to 4 points lower than in most other studies.[21;23] Secondly, other important factors such as lifestyle behaviours might contribute more to the development of adiposity in patients with depressive and anxiety disorders than HPA-axis perturbations, more specifically changes in dietary intake, sleep and less physical activity.[37] Thirdly, adiposity might be a long term consequence of cortisol excess via dyslipidaemia. Our outpatient group might have been less chronically depressed to be able to assess this effect. Fourthly, individual differences in depressive symptoms (e.g., high versus low appetite) may make it difficult to detect associations between cortisol and indices of adiposity. Fifthly, other neuroendocrine pathways might be involved, including the central sympathic nervous

system, the gonadal and growth hormone axes, as well as leptin levels.[38] Lastly, hypercortisolism seems to be involved in central rather than peripheral adiposity,[38] and we did not directly assess the intra-abdominal fat mass.

Our data support the hypothesis that the higher risk of CVD in patients with affective disorders may partly be explained through the direct effects of HPA-axis perturbations on lipoprotein metabolism. With respect to HPA-axis dysfunctions, an excess of glucocorticoids could contribute to insulin resistance, resulting in increased lipolysis through inhibition of lipoprotein lipase. Increased lipolysis results in increased serum levels of LDL cholesterol, total cholesterol, and triglycerides and decreased serum levels of HDL cholesterol.[39] Little is known about the associations between low circadian cortisol variability in patients with depressive and anxiety disorders and lipid metabolism. It could be hypothesized that a lower cortisol variability marks dysfunctions of the HPA-axis and other endocrine axes that subsequently affect lipid metabolism.[20;21;40]

Some methodological limitations of the present study should be mentioned. Firstly, participants were non-fasting prior to blood sampling for logistical reasons, while food intake is known to increase triglycerides levels. Furthermore, associations between lipid/lipoproteins and cortisol might have been weakened, because blood was collected between participants at different time points during the day (mostly at 9:00h or at 14:00h). Nevertheless, significant associations between the index scores of dyslipidaemia and cortisol measures were found. Secondly, no electronic monitoring of compliance to the sampling protocol was performed.[41;42] Therefore, non-adherence could have affected our data. Thirdly, although we adjusted for several potential confounders, other confounders might have influenced our outcome, such as the duration of the disease, the age of onset, and specific symptoms such as sleep disturbances. Fourthly, the sample size was relatively small. Lastly, the lumping of patients with depressive and anxiety disorders in a single group might have prevented us from finding more clear-cut results. However, in our opinion this is justified because of the extensive co-morbidity and indications for shared psychopathological processes.[43;44] Moreover, we adjusted for the presence of a depressive and/or anxiety disorder and this did not change our results.

We conclude that elevated basal cortisol concentrations and lower circadian cortisol variability were independently associated with a less favourable lipoprotein profile, but not with indices of adiposity in patients with depressive and/or anxiety disorders. These associations were not found in healthy controls. Perturbations of the HPA-axis in patients might partly explain the increased risk of CVD through its effects on lipoprotein metabolism. Our data need replication to confirm our results. Furthermore, prospective studies are needed to determine the impact of chronic psychological stress on the HPA-axis and its metabolic consequences.

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Chapter 6

C-reactive protein polymorphisms are associated with plasma C-reactive protein levels and the cortisol awakening response in basal conditions

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Abstract

Objective: Cortisol effects the acute-phase response, but it is unknown whether C-reactive protein (CRP), a proinflammatory acute-phase reactant, also affects hypothalamus-pituitary-adrenal (HPA) axis activity. In the present study associations were explored between CRP haplotypes with plasma CRP levels and basal salivary cortisol.

Methods: We included 266 physically healthy Caucasian subjects of whom 94 had a psychiatric disorder in a genetic association study. Six tag single nucleotide polymorphisms (SNPs) capturing the common genetic variation of the CRP gene were genotyped (i.e., rs2808628, rs2808630, rs1205, rs1800947, rs1417938, and rs3091244) to yield common CRP haplotypes. Plasma CRP levels, the salivary cortisol awakening response (CAR) (0, 30, 45, and 60 min after awakening), and the diurnal cortisol decline (11:00h, 15:00h, 19:00h, and 23:00h) were assessed.

Results: Rs2808628, rs1205, rs1417938, and rs3091244 showed expected associations with CRP levels and salivary cortisol during the CAR. The 5 most common CRP haplotypes were ordered linearly according to wellcharacterized increasing CRP levels. There was an inverse linear association between CRP haplotypes and cortisol levels during the CAR, but no association with the diurnal cortisol decline.

Conclusion: Genetic variants in the CRP gene are associated not only with higher plasma CRP levels but also with lower salivary cortisol levels after awakening, in basal, non-inflammatory conditions.

Introduction

The immune system and the hypothalamus-pituitary-adrenal (HPA) axis play important roles in maintaining homeostasis by generating adaptive responses to noxious stressors.[1;2] C-reactive protein (CRP), a proinflammatory acute-phase reactant, and cortisol, the main stress hormone of the HPA axis, are both involved. CRP is predominantly produced in the liver and its release is regulated by an inflammatory cascade of reactions, which involve, among others, proinflammatory cytokines.[3;4] Cortisol acts synergistically with the proinflammatory cytokine interleukine (IL)-6 to enhance this effect.[5] The main biological function of CRP is its ability to recognize pathogens and damaged cells of the host and to mediate their elimination by recruiting the complement system, which subsequently activates and attracts phagocytic cells[4] CRP induces the release of proinflammatory cytokines IL-1, IL-6, and TNF-α by these cell.[6] On the other hand, cortisol is a potent endogenous anti-inflammatory agent with immunosuppressive effects. It has a strong capacity to suppress immune cell functions, such as inhibiting the release of proinflammatory cytokines. Not much is known yet about the direct pathway from CRP to cortisol release. However, the bidirectional relationship between CRP and cortisol is assumed to play an important role in maintaining the physiological homeostasis.[2]

Environmental variables and lifestyle behaviours such as dietary intake, smoking, acute and chronic infections, gender, lipid levels, obesity, and blood pressure can contribute to variations in both CRP levels and cortisol levels.[7-9] These variables might also influence the potential cross-sectional relationship between CRP and cortisol. Mendelian randomization refers to the random assortment of genes from parents to offspring that occurs during gamete formation and conception.[10;11] It provides a method to assess, relatively unconfounded, whether CRP – that has an important genetic component of about 35-40%[12] – is causally related to salivary cortisol. Several well-characterized CRP gene polymorphisms are known to be associated with plasma CRP levels, e.g., rs2808628, rs2808630, rs1205, rs1800947, rs1417938, and rs3091244.[12-15]

In the present study we assessed these CRP polymorphisms in a Mendelian randomization design in order to elucidate the relationship between plasma CRP levels and basal salivary cortisol levels over the day under non-inflammatory conditions. We tested the hypothesis that haplotypes of the CRP gene that affect plasma CRP levels, are also associated with basal salivary cortisol levels, especially with cortisol during the awakening response, that shows substantial heritability (40-48%).[16] As indices of activity of the HPA axis, we used sequential assessments of salivary cortisol over the day to derive measures of the cortisol awaking response (CAR) and the diurnal decline.

Methods

Subjects

We included 266 physically healthy subjects, of whom 94 subjects had a depressive and/or anxiety disorder. Data were collected as part of studies on psychiatric and neuroendocrine correlates of the HPA axis.[17;18] Non-psychiatric subjects were recruited by advertisement in local news papers asking for physically and mentally healthy persons willing to participate in a study on the biological stress system in relation to depression and anxiety. Psychiatric subjects were included from the outpatient department of the Rivierduinen mental health center in Leiden, the Netherlands. We allocated non-psychiatric and psychiatric subjects to one experimental group to increase the sample size and statistical power. Exclusion criteria were a history of neurological or endocrine diseases or other serious or unstable medical conditions. Furthermore, subjects with substance or alcohol abuse, as well as pregnant or breast feeding women and premenopausal women with ovariectomy were excluded. Subjects were excluded when using psychotropic medication within the last 14 days, also corticosteroids, antidiabetics, estrogens, thyroid hormone, or herbal medication (e.g., Valerian, St. Johns Wort) were excluded. All subjects had a routine physical examination and laboratory blood tests, excluding a.o. acute infections and chronic inflammatory diseases. Prior to participation, written informed consent was obtained from all subjects. The study was approved by the ethics committee of the Leiden University Medical Center.

Saliva cortisol sampling

Instructions concerning saliva sampling prohibited eating, smoking, drinking tea or coffee or brushing teeth within 15 minutes before sampling. Furthermore, no dental work 24 hours prior to sampling was allowed. Saliva samples were obtained using Salivettes (Starstedt, Germany) at eight time-points covering the cortisol awakening response (CAR) and the diurnal decline in cortisol. The CAR includes four sampling points; at awakening (T1) and 30 (T2), 45 (T3) and 60 (T4) minutes after awakening. Four additional samples were taken to assess the diurnal decline in cortisol at 11:00h (T5), 15:00h (T6), 19:00h (T7), and 23:00h (T8). Subjects registered each time a sample was collected in order to be able to do a limited check whether subjects sampled at the required times. The cortisol curve of a single day is determined by situational factors and, to a smaller extent, by trait factors.[19] Therefore, to reduce measurement error and the effects of day-to-day variation, subjects were asked to provide saliva samples on two consecutive non-working days. Samples were initially stored at 4 °C for a week at most and delivered by the subject to our clinic. After receipt in our clinic, salivettes were centrifuged at 2000g for ten minutes, aliquoted

and stored at -20°C. After receipt, salivettes were centrifuged at 2000g for ten minutes, aliquoted and stored at -20°C. Cortisol analysis was performed by competitive electrochemiluminescence immunoassay (Roche, Switserland), as described in Van Aken et al. [20]. The lower detection limit was 0.50 nmol/l. The intra-assay coefficients of variance were lower than 7%, the inter-assay coefficients of variance were lower than 8%, except for the very low range. Per sampling point, physiologically unlikely high values (i.e., > 50 nmol/l) were excluded from further analyses (1.3% of the data). Saliva cortisol levels are reported as nmol/l and showed a normal distribution. The two cortisol values obtained at the time points on the 2 days were significantly correlated indicating moderate to good intra-individual stability over time (Pearson's r between 0.45 and 0.65, $p \leq 0.001$). Therefore, mean cortisol values for each time point were computed for each subject and used in the analyses. If a sample was missing, then the value of the other day was used (2.9% of the data).

Plasma CRP measurement

Venous blood was sampled with standard venipuncture techniques. CRP levels were measured in plasma by automated enzymatic colorimetric methods using a Modular P analyzer (Roche, Switzerland) with a lower limit of detection being 3 mg/l.

Single nucleotide polymorphism genotyping

Genomic DNA was isolated from the blood samples according to standard procedures. Single nucleotide polymorphisms (SNPs) in the CRP gene were determined the frequency of six well-characterized CRP polymorphisms that showed to be related with plasma CRP levels (Figure 1). I-plex assays were assigned using Assay designer software (Sequenom). Genotyping was performed using the MassArray platform according to the manufacturer's protocols (Sequenom, San Diego, CA). After PCR on 2.5 ng of DNA, a primer tension reaction was performed to introduce mass differences between alleles. After removing salts by adding a resin, ~15 nL of the product was spotted onto a target chip with 384 patches containing matrix. Mass differences were detected using the Brucker Autoflex MALDI-TOF mass spectrometer, and genotypes were assigned real-time user Typer 3.1 software (Sequenom). As quality control, 10% of samples were genotyped in duplicate, and no inconsistencies were observed.

Statistical analyses

The haplotype frequencies were reconstructed using SNPHAP (Clayton, 2002; version 1.3; available online at http://www-gene.cimr.cam.ac.uk/clayton/software/) with an estimation-maximization algorithm. Haplotypes were placed in the order that previously showed to be associated with increasing plasma CRP levels.[15;21] Each of the

CRP polymorphisms was assessed to determine if the observed genotype frequencies were consistent with the expected Hardy-Weinberg proportions by using the χ^2-test. The Linkage Disequilibrium (LD) was assessed using Lewontin's D' statistics.[22]

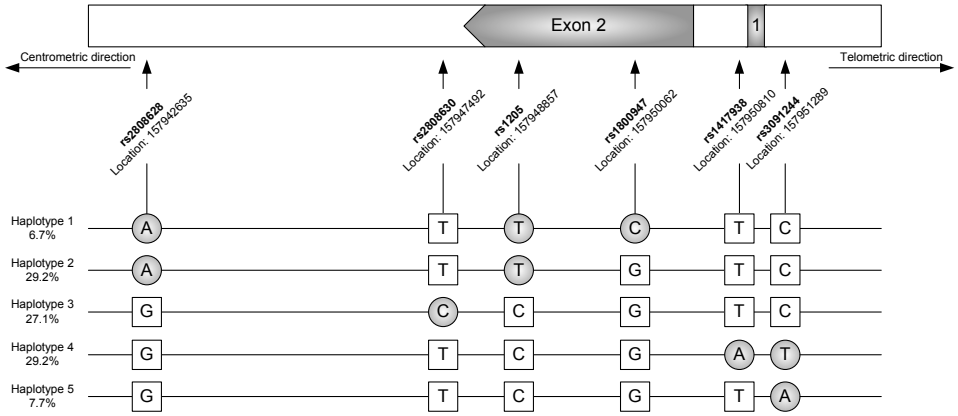

Figure 1. *Schematic overview of the structure of the C-reactive protein (CRP) gene, consisting of 2 exons seperated by a single intron*
The orientation of the gene is marked by arrows, with the gene transcribed from left to right. All single nucleotide polymorphisms (SNPs) were in Hardy-Weinberg equilibrium (HWE; all p's ≥ 0.15). Arrows mark the approximate location on chromosome 1 of the 6 tagSNPs that were in close linkage disequilibrium (D' for all SNP pairs ≥0.96, except for 2 pairs with ≥0.90). The 5 most frequent haplotypes are presented, that are similar to those found in populations from Northern and Western European ancestry. rs3091244 is a tri-allelic SNP located in the promotor region of the CRP gene.

As markers of overall cortisol levels, the total area under the curve of the CAR (AUC_{CAR}) and diurnal decline ($AUC_{diurnal}$) were calculated using trapezoid formula [23]. Furthermore, subjects were dichotomized according to the CRP-level (> 3 mg/l = high risk and ≤ 3 mg/l = low/average risk). The cut points of low/average risk (< 3.0 mg/l), and high risk (> 3.0mg/l) correspond to approximate tertiles of high-sensitivity (hs)-CRP in the adult population.[24] Subjects with > 3.0 mg/l hs-CRP levels are at an increased risk for cardiovascular disease (estimated odds ratio 2.0) and depression (estimated odds ratio 1.6 in men).[24;25]

Binary logistic and linear regression analyses were used to investigate associations between polymorphisms and haplotypes with changes in plasma CRP levels and salivary cortisol levels. Analyses were adjusted for age, gender, smoking status (yes/no), body mass-index (BMI) and group (presence/absence of affective disorder). Non-psychiatric and psychiatric subjects were combined because of the relatively low number of subjects per group to increase statistical power. Analyses were performed

using Statistical Package or the Social Science version 16.0 (SPSS 16.0; SPSS Inc., Chicago, IL, USA). *P*-value < 0.05 was considered statically significant.

Results

Study population

The mean age of the study population was 40.1 ± 13.1 years. The study population consisted of 103 females (38.7%) and 163 males (61.3%) and 38.3% of the subjects were smokers. The mean BMI of the study population was 25.3 ± 4.5 kg/m^2. The genotypes frequencies are listed for each polymorphism in table 1. Rare genotypes (<1%) for rs3091244 and rs1800947 were subsequently collapsed. The genotype frequencies of all polymorphisms were in Hardy-Weinberg equilibrium (*P*s>0.15). All single nucleotide polymorphisms (SNPs) were in Hardy-Weinberg equilibrium (HWE; all p's ≥ 0.15). The six polymorphisms were in close linkage disequilibrium (D' for all SNP pairs ≥0.96, except for 2 pairs with ≥0.90).

Associations with individual CRP genotypes (table 1)

Plasma CRP levels showed the expected differences among subjects with different genotypes of rs2808628, rs1205, rs1417938, and rs3091244. Rs1800947 showed a trend towards significance (p=0.08), but this trend was lost after adjustment for covariates (p=0.15).

The CRP polymorphisms that were related to differences in plasma CRP levels also showed significant differences in cortisol AUC_{CAR} values (i.e., rs1205, rs1417938, rs3091244, and a trend for rs2808628). For the CRP polymorphism rs1205, the CC genotype was associated with lower cortisol AUC_{CAR} values. For the CRP polymorphism rs1417938, the AA genotype was associated with lower cortisol AUC_{CAR} values. For the CRP polymorphism rs3091244, the TT/TA/AA genotype was associated with lower cortisol AUC_{CAR} values. For the CRP polymorphism rs2808628 a trend towards significance was found for the association with lower cortisol AUC_{CAR} values. CRP polymorphisms rs1800947 and rs2808630 did not show differences in cortisol AUC_{CAR} values between the genotypes. No significant associations were found between CRP polymorphisms and cortisol $AUC_{diurnal}$ values.

The CRP levels (as a continuous variable) were not significant correlated with cortisol AUC_{CAR} values and cortisol $AUC_{diurnal}$ values (respectively, Spearman's rho = -0.06, p =0.38, Spearman's rho = -0.09, p =0.16.

Table 1. Plasma CRP levels, AUC_{CAR} values and $AUC_{diurnal}$ values according to genotype frequencies for CRP polymorphisms in 266 subjects

SNP genotype	n	High CRP (%)	AUC_{CAR} (nmol/l/h)	$AUC_{diurnal}$ (nmol/l/h)
Rs2808628				
GG	111	34 (30.6%)	15.2 (14.3 – 16.1)	6.4 (6.0 – 6.9)
GA	113	20 (17.7%)	16.0 (15.0 – 16.9)	6.7 (6.2 – 7.2)
AA	40	7 (17.5%)	16.9 (15.4 – 18.3)	6.6 (5.9 – 7.4)
Missing values	2			
***P*-value for trend**		0.01	0.06	0.57
rs2808630				
TT	140	31 (22.1%)	15.6 (14.8 – 16.5)	6.6 (6.2 – 7.1)
TC	108	28 (25.9%)	16.1 (15.2 – 17.0)	6.4 (5.9 – 6.9)
CC	17	3 (17.7%)	15.3 (12.8 – 17.9)	6.9 (5.6 – 8.2)
Missing values	1			
***P*-value for trend**		0.75	0.76	0.79
rs1205				
CC	110	35 (31.8%)	15.1 (14.1 – 16.0)	6.4 (6.0 – 6.9)
CT	115	21 (18.3%)	16.1 (15.2 – 17.0)	6.6 (6.2 – 7.1)
TT	41	7 (17.0%)	16.8 (15.3 – 18.3)	6.8 (6.1 – 7.6)
Missing values	0			
***P*-value for trend**		0.01	0.03	0.37
rs1800947				
GG	231	59 (25.5%)	15.7 (15.0 – 16.3)	6.5 (6.2 – 6.9)
GC/CC	35	4 (11.4%)	16.6 (15.0 – 18.2)	6.9 (6.1 - 7.7)
Missing values	0			
***P*-value for trend**		0.15	0.29	0.42
rs1417938				
TT	135	25 (18.5%)	16.3 (15.4 – 17.1)	6.7 (6.2 – 7.1)
TA	108	31 (28.7%)	15.5 (14.6 – 16.4)	6.6 (6.1 – 7.1)
AA	21	5 (23.8%)	14.2 (12.1 – 16.2)	5.9 (4.8 – 7.0)
Missing values	2			
***P*-value for trend**		0.04	0.04	0.30
rs3091244				
CC	107	16 (15.0%)	16.5 (15.5 – 17.4)	6.6 (6.1 – 7.1)
CT/CA	97	31 (32.0%)	16.0 (15.1 – 17.0)	7.0 (6.5 – 7.5)
TT/TA/AA	33	8 (24.2%)	14.2 (12.6 – 15.9)	5.9 (5.0 – 6.8)
Missing values	29			
***P*-value for trend**		0.02	0.04	0.57

Percentages of subjects with plasma CRP levels > 3mg/l are presented. Rs3091244 is a triallelic SNP. Geometric estimated means adjusted for age, gender, smoking status (yes/no), BMI, and group (presence/absence of affective disorder) with 95% confidence intervals are presented for AUC_{CAR} (nmol/l/h) and $AUC_{diurnal}$ (nmol/l/h). Test values of binary logistic regression analysis (plasma CRP) linear regression analyses (cortisol measures) were used to test for linear trend.

Associations with CRP haplotypes (table 2)

Five common haplotypes with ≥ 1% frequencies were observed (Figure 1). We numbered haplotypes in our data set according to previous published increasing plasma CRP levels from haplotype 1 to haplotype 5 [15;21] and we confirmed the order in our data set. A statistically significant linear trend, but in reverse direction, was also found for AUC_{CAR} values from haplotype 1 to haplotype 5. No significant associations were found between haplotypes and the cortisol $AUC_{diurnal}$ values (Table 2, Figure 2).

The frequency of haplotype 1 to 5 in the psychiatric subjects was, respectively, 3.2%, 33.9%, 25.3%, 28.0%, 9.7%, and in the non-psychiatric subjects, respectively, 9.0%, 27.0%, 29.9%, 29.7%, and 6.3%. The frequency of the individual haplotypes did not differ significantly between psychiatric and non-psychiatric subjects (linear-by-linear=1.7, p= 0.33), and the effect of group in binary logistic and linear regression analyses with plasma CRP levels and AUC_{CAR} were also not significant (p=0.14 and p=0.40, respectively).

Table 2. Associations between CRP haplotypes with plasma CRP levels and salivary cortisol measures in 266 subjects

Haplotype	**Genotype combination**	**n**	**High CRP (%)**	**AUC_{CAR} (nmol/l/h)**	**$AUC_{diurnal}$ (nmol/l/h)**
1	**ATTCTC**	36 (6.8%)	4 (11.4%)	16.1 (14.5 – 17.7)	6.8 (6.1 – 7.6)
2	**ATTGTC**	153 (28.8%)	31 (21.0%)	16.2 (15.4 – 17.0)	6.3 6.2 – 7.0)
3	**GCCGTC**	140 (26.3%)	34 (24.6%)	15.6 (14.8 – 16.4)	6.6 (6.2 – 7.0)
4	**GTCGAT**	151 (28.4%)	43 (29.1%)	15.0 (14.3 – 15.8)	6.4 (6.0 – 6.8)
5	**GTCGTA**	39 (7.3%)	13 (34.2 %)	15.0 (13.5 – 16.5)	6.6 (5.9 – 7.4)
Other haplotypes		13 (2.4%)			
***P*-value for trend**			0.004	0.03	0.38

Five common haplotypes were constructed from rs2808628, rs2808630, rs1205, rs1800947, rs1417938, and rs3091244. Percentages of subjects with plasma CRP levels > 3 mg/l are presented. Geometric estimated means adjusted for age, gender, smoking status (yes/no), BMI, and group (presence/absence of affective disorder) with 95% confidence intervals are presented for AUC_{CAR} (nmol/l/h) and $AUC_{diurnal}$ (nmol/l/h). Test values of binary logistic regression analysis (plasma CRP) and linear regression analyses (cortisol measures) were used to test for linear trend. Each subject has two haplotypes; total 532 (i.e. 2 x 266).

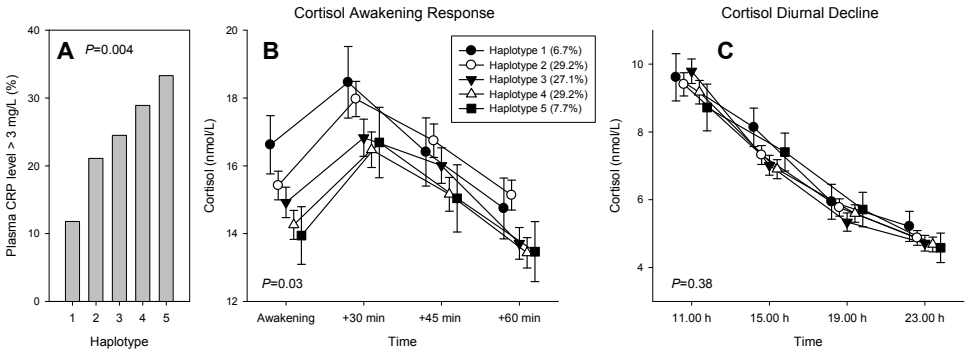

Figure 2. *Crude associations between 5 common CRP haplotypes and plasma CRP levels and salivary cortisol levels across the day in 266 subjects*
Percentages of subjects with plasma CRP levels > 3mg/l are presented (A). Cortisol assessments in the first hour after awakening (B) and during the diurnal decline (C) are presented.

Discussion

The main finding of the present study is that CRP haplotypes were not only associated with differences in plasma CRP levels, but also with differences in salivary cortisol levels during the awakening response. More specific, the present data showed that variants of the CRP gene which were associated with higher CRP levels under basal, non-inflammatory conditions, were also associated with lower cortisol levels after awakening. These findings support the hypothesis of a relationship between CRP and the CAR. To the best of our knowledge, this was the first study that investigated associations between CRP gene polymorphisms and cortisol levels using a Mendelian randomization design.

Our finding of associations between CRP polymorphisms (i.e., rs3091244, rs1417938, rs2808628, rs1205) and plasma CRP levels is consistent with previously published data.[12;15;21] In addition, we showed significant associations between the same CRP polymorphisms and cortisol levels during the CAR. However, we found no associations between CRP polymorphisms and cortisol release during the diurnal decline, suggesting that genetic variation in the CRP gene only affects cortisol levels after awakening under basal, non-inflammatory conditions. The effects on the awakening response may thus be a manifestation of the multiple effects of the CRP gene. This is in accordance with findings of previous studies that evaluated the heritability of cortisol levels. In a twin study of 52 monozygotic (MZ) and 52 dizygotic (DZ) twin pairs, it was concluded that about 40% to 48% of individual differences in cortisol release in the first hour after awakening was attributable to genetic factors,

whereas no genetic influence was found for the remaining diurnal decline.[16] This pattern was replicated in a larger study of 199 MZ and 272 DZ adult twin pairs, in which the cortisol levels at awakening and 30 minutes later showed around 30% heritability, with no significant effects for values recorded later during the day.[26]

Furthermore, we confirmed the clear linear trend in increasing plasma CRP levels from haplotype 1 to haplotype 5,[15;21] but we also found a linear trend, but in inverse direction, between CRP haplotypes and cortisol levels after awakening. The relationship between CRP and cortisol levels has scarcely been investigated. One study showed that the infusion of CRP increases plasma cortisol levels on the short-term.[27] However, CRP infusion leads to an acute increase of CRP to 'pathophysiological levels', which might interact differentially with cortisol release as compared to more longstanding effects of higher CRP levels, which are captured in a Mendelian randomization design. In another study, no significant correlation between CRP levels and cortisol over the day was found in healthy subjects in a cross-sectional study.[28] The association between CRP and cortisol was not the main focus of that study and adjustment for potential confounders was not applied. In general, stressful and proinflammatory conditions, such as acute infections, may increase both CRP and cortisol levels, due to activation of the immune system and HPA axis. But, previous studies showed that in chronic inflammatory conditions, such as rheumatoid arthritis and Sjögren's syndrome,[29-31] cortisol levels were not elevated, despite the presence of chronic high circulating inflammatory cytokine and CRP levels. This phenomenon was referred to as an 'inadequate' low cortisol secretion relative to the chronic inflammatory status.[29;31] It could be hypothesized that a chronic pro-inflammatory state with high CRP levels ultimately results adaptation of the HPA axis, through negative feedback mechanisms, leading to lower cortisol levels. As persistent high cortisol concentrations would predispose to infections,[32] such a cortisol decrease may be adaptive. Our findings are consistent with this hypothesis of a negative feedback of CRP on the HPA axis. We showed that genetically induced higher CRP levels are associated with lower cortisol levels. In other words, CRP effects on cortisol may play a role in maintaining the stress homeostasis. However, in contrast with the direct effects of CRP polymorphisms on CRP levels, the mechanism of effect on cortisol levels likely involves a much more complex network of endogenous interactions that include cytokines.

Some methodological limitations of the present study should be mentioned. Firstly, plasma CRP levels were not analyzed with a high sensitivity assay and the lower limit of detection was 3 mg/l. Nevertheless, the sensitivity was sufficient to confirm the well-known associations between CRP polymorphisms and CRP levels found in previous studies. Secondly, our study may have been underpowered with a relatively small sample size. Data were collected as part of studies on psychiatric and

neuroendocrine correlates of the HPA axis. To increase the statistical power in our analyses, we allocated physically healthy subjects with and without a psychiatric disorder to one experimental group. Although psychiatric and non-psychiatric subjects did not differ in frequency of individual haplotypes, replication in a larger, more uniform and healthy cohort is required. Thirdly, the HPA axis may be involved in the pathology of psychiatric disorders, that some of the subjects were suffering from. However, post-hoc analyses showed no statistical differences between non-psychiatric and psychiatric subjects in cortisol levels neither during the awakening response nor for the diurnal decline. Moreover, there was no interaction with psychopathology for our main effects. Therefore, the effects of CRP polymorphisms on cortisol levels are unlikely due to confounding by psychopathology. Fourthly, although we adjusted for several potential confounders, other confounders that are likely to affect CRP and/or cortisol levels might have influenced our outcome, such as acute (viral) infections. Finally, the design of the study needs to be considered. Our study made use of naturally occurring genetic variation resulting from independent gene assortment, also referred to as Mendelian randomization, as the basis of our analyses of association. Variants of nearby genes in linkage disequilibrium with the CRP polymorphisms may also explain our findings.

We conclude that genetic variants in the CRP gene are associated with differences in plasma CRP and salivary cortisol levels after awakening under basal, non-inflammatory conditions, suggesting that CRP metabolism and cortisol release during the awakening response are closely linked.

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Chapter 7

Summary and discussion

Summary

The objective of this thesis is to extend our knowledge on hypothalamus-pituitary-adrenal (HPA) axis dysfunction in patients with depression and/or anxiety disorders. Nearly all previous studies on this topic start with the psychiatric disorder and study its relationship with the HPA axis function in comparison to healthy controls. These studies showed inconsistent associations between depression and/or anxiety disorders defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV defined and HPA axis dysfunctions. A number of studies show a hyperactive HPA axis.[1-5] However, a normoactive,[2;6;7] and hypoactive[6;8;9] HPA axis were also found in this patient groups. This lack of consistent findings could be explained, at least in part, by the limited specificity of the categorical DSM-IV diagnoses. This may be due to the overlap of DSM-IV diagnoses by sharing criteria. Furthermore, the presence of non-linear associations between cortisol levels and psychopathology might also have contributed to these inconsistent findings.[10;11] In addition, the HPA axis needs to have a far-reaching power over the metabolism of the body, because in times of crisis the body should be able to acutely redirect metabolism to enable a fight, flight or freeze reaction. Therefore, dysfunctions of the HPA axis have to lead to changes in other biological systems. So far as known, not much work is done on this issue. Most studies compare psychiatric patients and controls on differences in function of the biological system under study, but mutual associations between the HPA axis and other biological systems, e.g., the metabolic and immune system, are hardly explored.

In this thesis we have tried to avoid the shortcomings of the DSM-IV classification system by starting with the HPA axis, as key stress system in stress coping. Cortisol, as main end product of the HPA axis in man, exerts diverse effects on a wide variety of physiological systems. We used cortisol concentrations, in diurnal curves as well as assessed in challenge tests, as core parameters by which we attempted to redefine the phenotype of psychopathology. In addition, we studied its relationship with the metabolic and immune system in patients suffering from depression and/or anxiety disorders.

In the first empirical study (**chapter 3**) we investigated the functioning of the HPA axis in patients with depression and comorbid anxiety disorders, depression alone and in healthy controls by means of the dexamethasone (DEX)/corticotropin-releasing hormone (CRH) test. We found an enhanced suppression of cortisol to DEX, with consequentially a lower cortisol response to CRH in the patient group with comorbidity of depression and anxiety disorders compared to the other two groups. We concluded that the degree of HPA axis dysfunctions is influenced by comorbidity. As previous studies did not pay attention to comorbidity, the influence of comorbidity

on HPA axis dysfunctions may partly explain the inconsistencies in the outcomes of previous DEX/CRH studies.

In **chapter 2**, we argue that the study of the relationship between psychiatric symptomatology and an endophenotype like HPA axis functioning should not only take comorbidity into account. As variation in psychiatric symptoms is continuous they do not coalesce into fairly well-defined categorical DSM-IV clusters. Continuous psychological dimensions selected for their predictiveness with respect to endophenotypes, as biological intermediate factors, are proposed to be the best ways in reaching an understanding of the causations in mood, anxiety, and somatoform disorders.

In **chapter 4**, we performed an empirical study on this issue. We investigated whether cortisol concentrations correlate with continuous phenotypic dimensions based on the tripartite model of Watson and Clark. Using these dimensions we found linear as well as non-linear associations with salivary cortisol. These non-linear associations remained unobserved when categorical diagnoses were used. This might be an additional explanation of the inconsistencies found in previous cortisol studies. Therefore, a dimensional approach to phenotyping is a promising new way to study the relations between phenotype and endophenotype in psychiatric disorders.

In **chapter 5**, the relationship between the HPA axis and lipid metabolism and adiposity indices in patients with depression and anxiety disorders was explored. These indices are predictive of the risk of cardiovascular disease (CVD). They are also components of the metabolic syndrome and are often affected in patients with depression and/or anxiety disorders. This study extended previous research by providing evidence that two states of dysfunction of the HPA axis, i.e., elevated basal cortisol levels and lower circadian cortisol variability, were independently related to detrimental lipid metabolism, but not to obesity, in patients with depression and/or anxiety disorders. We propose that the HPA axis, among other factors, influences the metabolic system. These data lend further support to the hypothesis that the relationship between depression and anxiety disorders and CVD is mediated by elevated basal cortisol concentrations and lower circadian cortisol variability.

In the last study **(chapter 6)**, genetic variation of the C-reactive protein (CRP) gene was used as an instrument to investigate associations between plasma CRP and saliva cortisol, by using a Mendelian randomization design, in patients with depression and anxiety disorders. In this study, we showed that genetic variants in the CRP gene were associated not only with higher plasma CRP levels but also with lower salivary cortisol levels after awakening, in basal, non-inflammatory conditions. This suggests that CRP metabolism and cortisol release during the awakening response are closely linked. We proposed a role for the effects of CRP on cortisol in maintaining physiological homeostasis and mediating risks of and protection from diseases, such

as depression and anxiety disorders. Furthermore, this study extended previous research by showing that the Mendelian randomization design might be a fruitful approach studying potential causality of associations between a biomarker and a psychiatric disorder.

General discussion

The results of the studies presented in this thesis extend the literature on HPA axis dysfunction in several ways. Most important, we used a different approach than commonly used in this area of research. Not the clinical picture (diagnosis or dimension) was central, but cortisol levels were used as starting point in our studies. We redefined the phenotype, related to differences in cortisol levels, e.g., hyper- and hypocortisolism. We showed that a dimensional clinical phenotype might be additive to the DSM-IV classification, for understanding underlying HPA axis dysfunctions. Furthermore, we hypothesized that differences in HPA axis activity, e.g. hyper- versus hypoactivity, might be a consequence of a sequence in time in chronic stress conditions. Additionally, we provided evidence that HPA axis dysregulation is associated with the metabolic and immune system in depression and anxiety disorders. In the following parts of the general discussion of this thesis these issues will be discussed in more detail.

HPA axis activity: from hyper- to hypoactivity?

The main conclusion of this thesis is that chronic stress might lead to two states of dysregulation of the HPA axis (**chapter 3 and 4**): either a hyperactive or a hypoactive HPA axis, associated with, respectively hyper- and hypocortisolism. How can these seemingly contradictory effects of chronic stress be explained? Are they distinct entities or part of a sequence in time?

A recent meta-analysis showed that in the first period after the onset of stress cortisol levels are elevated (phase 1: acute and intermediate stress), while later on the levels are below normal (phase 2: chronic stress).[12] A time dependent pattern of this nature is consistent with theories advanced by several researchers on the association between chronicity and hyper- and hypocortisolism.[13-15] Hypercortisolism and hypocortisolism might not be contradictionary, but might simply reflect different time points during the stress process. Unfortunately, our study, as well as most other studies included in the meta-analysis, had a cross-sectional design. But although this design precludes statements on causation and timing, our finding of an inverted U-shaped association between cortisol concentrations in the first hour after awakening and the MASQ dimensions is consistent with the idea that depression and anxiety disorders initially lead to enhanced HPA axis activity, resulting in higher morning

cortisol levels. Increasing severity might eventually lead to an (over)adjustment of the HPA axis by means of (1) the down-regulation of specific receptors on different levels of the axis (hypothalamus, pituitary, adrenals, target cells), (2) reduced biosynthesis or depletion at several levels of the HPA axis (CRF, ACTH, cortisol) and/or (3) increased negative feedback sensitivity to glucocorticoids, all together contributing to lower morning cortisol levels.[14;15] Previous studies provided evidence that an enhanced HPA axis function adapts to chronic and/or severe stress by subsequent down-regulation of receptors, resulting in a blunted CAR.[16;17] Longitudinal studies are better equipped than the studies presented in this thesis to answer questions such as: At what point in time does the HPA axis begin to decline from its peak? When does it drop below a person's baseline? At what point does it reach the asymptote and stop declining? In short, the next wave of studies will need to discern the shape of the time function, and they can only be done using prospective longitudinal designs with repeated assessments of HPA axis functioning.

A theoretical perspective on hyper- and hypocortisolism is also captured by the concept of 'allostatic load' as posited by McEwen.[18] This theory describes an individual's physiological response to stress. Diverse physiological systems accommodate to changing conditions in an effort to achieve stability by change (allostasis), and are thus protective in terms of adaptation. However, over time, this accommodation may produce allostatic load, which refers to the wear and tear that the body experiences due to repeated cycles of allostasis as well as the insufficient turning-on and shutting off of these responses. As so, chronic hyper- or hypoactivity of the stress system may have adverse effects on the organism.

According to McEwen, al least four types of allostatic load can be identified: (1) repeated challenges, (2) failure to habituate with repeated challenges; (3) failure to shut off the response after the challenge is past, and (4) failure to mount an adequate response (see figure 1). The first three types are all associated with a hyperactive HPA axis accompanied by higher cortisol levels, which might lead to manifestations of the metabolic syndrome (insulin resistance, dyslipidaemia, hypertension, hyper-coagulability, and visceral obesity).[19] Type 4 is characterized by a hypoactive HPA axis associated with a lower than needed cortisol, leading to compensatory hyperactivity of the other mediators, e.g. increased levels of cytokines that are normally counter-regulated by cortisol.[19] However, in this theory no explicit hypotheses are formulated on chronological relatedness between type 1 to 3 on one hand and type 4 on the other hand. Presumably, when types 1 to 3 are long lasting, it might eventually develop into a type 4 state of allostatic load.

In summary, homeostatic systems exert their effects in an inverse, U-type dose response relationship. Eustasis is in the middle, optimal range of the curve. Eustasis is the case in healthy persons, but also as intermediate phase switching form a hyper- to

hypofunction of the homeostatic system. Suboptimal effects may be on either side of the curve and can lead to suboptimal adaptation, which may be harmful to the organism in the short or long term. Both hypofunction and hyperfunction of the homeostatic systems have multiple aversive effects. With regard to the HPA axis, a hyper- and hypoactive state, associated with, respectively hyper- and hypocortisolism, refer to both suboptimal sides of the homeostatic stress system.[20] We assume that those states are not distinct entities, but part of a sequence in time leading from a hyperactive (phase 1: acute and intermediate stress) via a normoactive state to a hypoactive state (phase 2: chronic stress) when stress holds on.

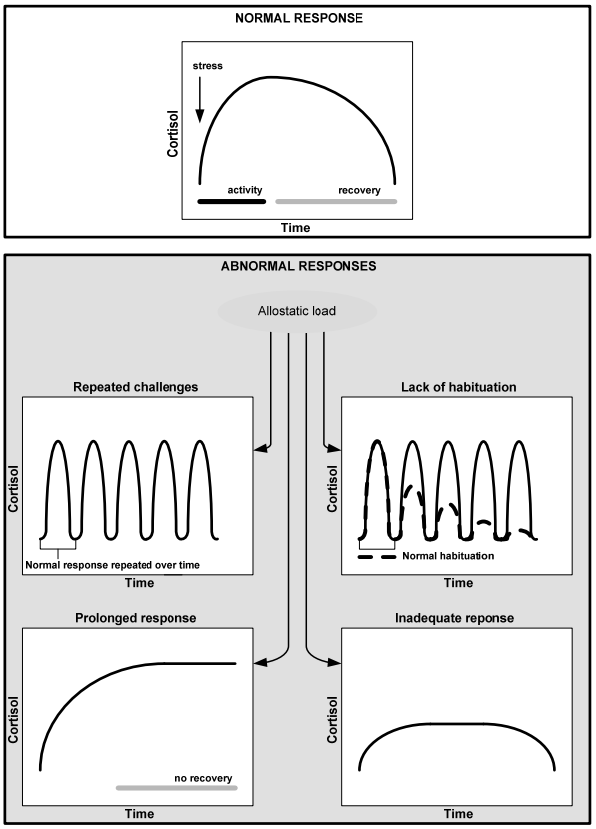

Figure 1. *Four types of allostatic Load The top panel illustrates the normal allostatic response, in which a response is initiated by a stressor, sustained for an appropriate interval, and then turned off. The remaining panels illustrate four conditions that lead to allostatic load: 1) Repeated "hits" from multiple novel stressors; 2) Lack of adaptation; 3) Prolonged response due to delayed shut down; and 4) inadequate response that leads to compensatory hyperactivity of other mediators: e.g., inadequate secretion of cortisol, resulting in increased levels of cytokines that are normally counter-regulated by cortisol..*

In the next paragraphs, we will further discuss the relationships between the two forms of dysregulations of the HPA axis, i.e., hypercortisolism (phase 1) and hypocortisolism (phase 2), and how they relate to clinical phenotypes and parameters of the metabolic and immune system in patients suffering from depression and/or anxiety disorders (see figure 2 for a summary).

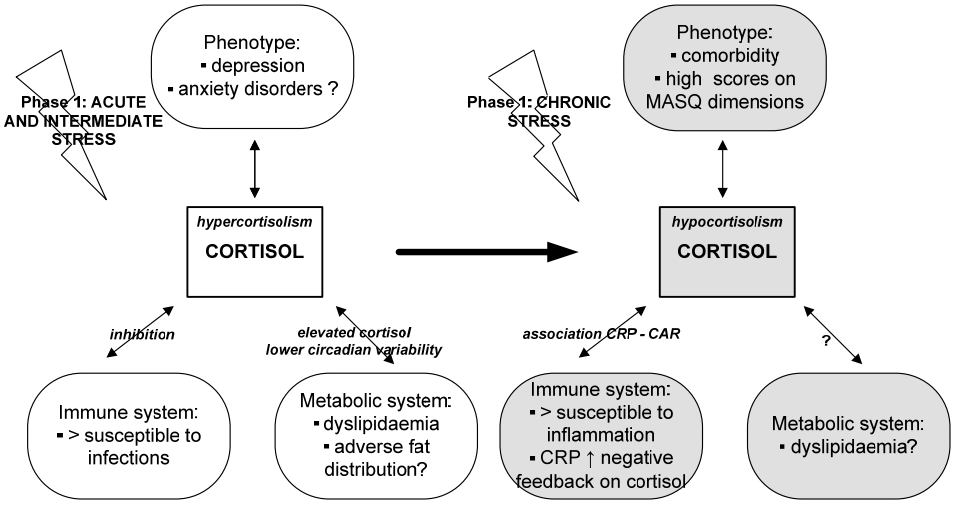

Figure 2. *Schematic summary of this thesis: Associations between, respectively hypercortisolism (right side of figure: phase 1) and hypocortisolism (left side of figure: phase 2) and phenotypic characteristics, metabolic and immune factors in depression and anxiety disorders. Associations relating to a hyperactive hypothalamus-pituitary-adrenal axis are depicted. HPA = hypothalamus-pituitary-adrenal axis; CAR = cortisol awakening response; CRP = C-reactive protein.*

Cortisol and the clinical phenotype

We used cortisol concentrations, by assessing salivary cortisol day curves, as core parameters by which we attempted to redefine the phenotype of psychopathology. We showed that hypercortisolism and hypocortisolism are associated with specific clinical phenotypes. Differences in cortisol levels were found between patients suffering from DSM-IV depressive disorder and controls. In addition dimensional phenotypes provided additional information on linear and nonlinear aspects of cortisol dynamics. The results of our studies on this topic will be discussed in the next two paragraphs.

Hypercortisolism and the clinical phenotype

Higher cortisol concentrations were found in patients with depression according to DSM-IV criteria compared to controls during the diurnal decline, which is in line with previous studies that showed elevated basal cortisol levels in depressive patients during the day.[21-23] A diminished negative feedback or a higher hypothalamic drive might be responsible for these higher levels. As described in chapter 4, we supposed that using the tripartite dimensional model as an alternative approach to phenotyping might provide additional information about HPA axis dysregulations. And indeed, regarding the diurnal cortisol decline, cortisol showed a linear relationship with the anhedonic depression and general distress dimensions of the tripartite model,

indicating that higher cortisol concentrations are associated with higher dimensional scores. The linear association between the two tripartite dimensions and cortisol concentrations during the diurnal decline confirms previous findings in which more severe psychopathology was related to higher cortisol concentrations.[24]

In our study on cortisol and the dimensions of the tripartite model, none of the cortisol measures were associated with the anxious arousal dimension. This might indicate that affective dimensions, e.g., anhedonic depression and general distress, are more closely related to HPA axis dysfunction than arousal dimensions. This is in line with previous studies on anxiety disorders, which are characterized by arousal symptoms, showing that basal cortisol was unaltered as compared to controls.[25-32] Otherwise, arousal measures, compared to affective measures, might be more subject to temporary fluctuations depending on momentary stressors.

In summary, we replicated previous findings of elevated diurnal cortisol levels in patients with a depressive disorder, although this is not a consistent finding in the literature. In addition, we demonstrated linear associations between diurnal cortisol levels and two dimensions of the tripartite model, suggesting that increasing severity (increase in dimensional scores) is initially related to increasing cortisol levels.

Hypocortisolism and the clinical phenotype

We showed hypocortisolism in depressed patients with comorbid anxiety disorders. Lower cortisol concentrations after DEX intake were found in our sample of depressed patients with comorbidity, indicating increased negative feedback sensitivity in this subgroup of depressed patients (**chapter 3**). Increased sensitivity to glucocorticoid negative feedback is one of the most common features of hypocortisolism.[15] As stated before, disease progression and increasing severity might lead to a (over)adjustment, and consequently to a hypoactivity of the HPA axis. This might be the case in depressed patients with comorbid anxiety disorders. Comorbidity of depression and anxiety disorders is widely understood to be associated with increased severity, persistence, chronicity, and functional impairment.[33] The persistence is illustrated by the relatively early age of onset of the pathophysiology in this patient group, who mostly starts with an anxiety disorder later to be accompanied by depression.[34] Furthermore, depressive patients with comorbid anxiety disorders often are characterized by more severe psychopathology.[35]

In addition, in our studies associations were found between hypocortisolism and some dimensional phenotypes. Cortisol concentrations after awakening showed a nonlinear relationship with the anhedonic depression and general distress dimension of the tripartite model, indicating an inverted U-shaped association (**chapter 4**). Our finding of an inverted U-shaped association between cortisol concentrations in the first hour after awakening and tripartite dimensions might suggest that depression and

anxiety initially may lead to enhanced HPA axis activity, resulting in higher morning cortisol levels (phase 1). Disease progression and increasing severity may eventually lead to a down-regulation of the GR receptors, resulting in lower morning cortisol levels (phase 2).[14;15]

In summary, our studies strengthen the hypothesis that ongoing stress and/or an increase in severity of psychopathology leads to a down-regulation of the GR receptors accompanied by an increased negative feedback mechanism and hypocortisolism.

Conclusion

We provided evidence for hypercortisolism during the diurnal decline in depressed patients. Furthermore, we found linear associations between cortisol and severity of psychopathology (e.g., indicated by dimensional scores). Increasing severity is associated with higher cortisol levels during the diurnal decline. On the other hand, we found hypocortisolism in a subgroup of depressed patients with comorbid anxiety disorders. Lower cortisol levels were also found in depressed/anxious patients who score extremely high (upper tertile) on the anhedonic depression and general distress dimensions of the tripartite model. This suggests that a further increase in severity, might initially lead hyperactivity of the HPA axis, but on the longer term switch to a hypoactivity of the HPA axis. The non-linear associations during CAR versus the linear associations during the diurnal decline support the idea that the cortisol release during the CAR and during the diurnal decline are under distinct regulatory influences resulting in different kinds of associations (i.e., linear and non-linear) between tripartite dimensions and cortisol concentrations.[36-38]

Cortisol and the metabolic system

Cortisol, the final hormone effector of the HPA axis, potently affects the overall body metabolism, exerting a broad spectrum of primarily catabolic effects as part of a generalized effort to increase the availability of energy sources when needed in a stress situation. In addition to their direct catabolic actions, cortisol also antagonizes anabolic actions of thyroid hormones, insulin, and sex steroids on their target tissues. This stress-related shift of metabolism from an anabolic to a catabolic state normally reverses upon retraction of the posed stressor. However, chronic stress might dysregulate the HPA axis and consequently the metabolic system.

Hypercortisolism and the metabolic system

In **chapter 5**, we provided evidence that hypercortisolism (and lower circadian cortisol variability) is associated with detrimental lipid metabolism, one of the features of the metabolic syndrome, in patients with depression and/or anxiety disorders.

Hypercortisolism contributes to insulin resistance, resulting in increased lipolysis by inhibition of lipoprotein lipase. Increased lipolysis results in increased serum levels of LDL cholesterol, total cholesterol, and triglycerides and decreased serum levels of HDL cholesterol [39]. Our findings on the association between elevated cortisol levels and dyslipidaemia in our patient group is in line with a study that showed associations between high 24h urinary cortisol levels and metabolic syndrome (which includes dyslipidaemia) in elderly depressed patients with a mean age 74.[40] Our findings suggest that the positive association between cortisol and dyslipidaemia is not limited to elderly depressed patients. In other studies, patients after organ transplantation showed associations between glucocorticoid administration and hyperlipidaemia.[41-43]

Metabolic syndrome is characterized by dyslipidaemia, as well as by visceral obesity, insulin resistance, and hypertension. The metabolic syndrome is a clustering of risk factors for cardiovascular diseases.[44] Epidemiologic studies evaluating the association between depression and metabolic syndrome suggest that a bidirectional relationship exists between these conditions.[45] Results from clinical studies evaluating depression and metabolic syndrome provide a more detailed estimate of the risk as well as mediational factors and consequences associated with these two conditions [45]. However, not all depressed patients show aspects of the metabolic syndrome. We hypothesize that an association between the metabolic syndrome and depression predominantly exists in patients with a hypercortisolemic depression, because chronically elevated cortisol, as explained above, contributes to the most important aspects of the metabolic syndrome, including insulin resistance, visceral obesity, and dyslipidaemia. As far as we know, only one study examined the metabolic syndrome in hypercortisolemic depression. This study suggests a synergistic relationship between depression in elderly, cortisol and metabolic syndrome.[40] No studies were done on metabolic changes in hypocortisolemic depression.

In summary, chronic exposure to stress, leading to HPA hyperactivity, might be potentially damaging because the prolonged glucocorticoid action causes hyperglycemia, dyslipidaemia (increased triglycerides, decreased HDL cholesterol), and hypertension, which all are manifestations of the metabolic syndrome.[46] We provided evidence that hypercortisolism is associated with dyslipidaemia in patients with depression and anxiety disorders. Therefore, hypercortisolism might partly explain the increased risk of CVD through its effects on lipoprotein metabolism.

Hypocortisolism and the metabolic system

Knowledge concerning the metabolic effects of cortisol is primarily based on studies in which varying amounts of cortisol or synthetic glucocorticoids have been given, or on studies of patients with endogeneous hypersecretion, whereas little is known about the metabolic effects of hypocortisolism. In general, patients with Addison's disease

show hypocortisolism and present clinically with weight loss, muscular weakness, and fatigue and a tendency to hypoglycemia, but there is a lack of controlled studies in this field on the nature of the alterations. Therefore, the effects of low cortisol levels on metabolism remain uncertain in these patients. In a recent study, the metabolic effects of acute cortisol withdrawal were investigated on glucose, lipid, and amino acid metabolism in patients with adrenocortical failure. It was shown that cortisol withdrawal increases insulin sensitivity.[47] This finding is in line with previous studies showing that the opposite, insulin resistance, is related to short-term cortisol excess. It is shown that cortisol promotes lipolysis, rendering it possible that increased levels of free fatty acids in the circulation may contribute to the observed insulin resistance.[48] In the study of Christiansen *et al.* no changes were observed in free fatty acids levels or lipid oxidation rates, suggesting that the observed increase in insulin sensitivity is directly related to hypocortisolism, and not to changes in lipid metabolism.[47] However, this study was on the metabolic effects after acute cortisol withdrawal, so not much is known about the relationship between longstanding hypocortisolism and the metabolic system, including lipid metabolism.

Conclusion

A lot of research has been done on the associations between elevated cortisol levels and (lipid) metabolism, but not much is known about the effects of decreased cortisol levels on the metabolic system. We provided evidence that dyslipidaemia, which is one of the characteristics of the metabolic syndrome, is associated with higher cortisol levels in depressed and anxious patients. Our data lend support to the hypothesis that the relationship between depression and CVD is partly mediated by HPA axis perturbations. However, as is shown before, another part of this patient group might be characterized by low cortisol levels. Not much is known yet about associations between hypocortisolism en lipid metabolism. Therefore, more research needs to be done on this topic.

Cortisol and the immune system

The immune system is typically divided into two categories - innate and adaptive - although these distinctions are not mutually exclusive. Innate immunity refers to nonspecific defense mechanisms that come into play immediately or within hours of an antigen's appearance in the body. These mechanisms include physical barriers such as skin, chemicals in the blood, and immune system cells that attack foreign cells in the body. The innate immune response is activated by chemical properties of the antigen. Adaptive immunity refers to antigen-specific immune response. The adaptive immune response is more complex than the innate. The antigen first must be processed and recognized. Once an antigen has been recognized, the adaptive immune

system creates an army of immune cells specifically designed to attack that antigen. Adaptive immunity also includes a "memory" that makes future responses against a specific antigen more efficient.

The immune system is influenced by changes in HPA axis activity There is a tight and reciprocal relationship between the circulating pro-inflammatory cytokines and the HPA axis, an alliance which subserves the preservation of the threatened body homeostasis under conditions of stress and systemic inflammation. The HPA axis has profound inhibitory effects upon the inflammatory response, primarily due to the actions of the glucocorticoids, which inhibit virtually all the mobilized components of the innate and adaptive immune system. This action of corticosteroids is thought to prevent extensive damage to the body potentially inflicted by uncontrolled inflammatory and immune activation.[49;50]

Hypercortisolism and the immune system

Cortisol is the most potent anti-inflammatory hormone in the body. It acts on the immune system by both suppressing and stimulating pro- and anti-inflammatory mediators respectively.[51] While cortisol promotes T helper 2 (Th2) development, for example by enhancing interleukin 4 (IL-4) and interleukin 10 (IL-10) secretion by macrophages,[52] it inhibits inflammatory responses and suppresses the production and release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6).[53] During stress the immune system becomes activated, resulting in a release of cytokines.[20] An important role of cortisol during stress is to suppress the production and activity of pro-inflammatory cytokines, thus restraining the inflammatory reaction and preventing tissue destruction.[18] The inhibitory effect of cortisol on the production of pro-inflammatory cytokines is clearly depicted in the inverse rhythm of cytokine secretion in relation to the plasma cortisol levels that is documented under both basal conditions (circadian rhythm of inhibition) and inflammatory action.[54;55] Accordingly, stress-induced increases in cortisol levels prevent bodily responses from overshooting by its immunosuppressive effects. However, in case of chronic stress associated with hypercortisolism, there is an immunosuppression at virtually every level of the immune and inflammatory responses, including during activation of the innate immune response and in both cellular and humeral acquired immune responses. Therefore, hypercortisolism leads to an enhanced susceptibility to infection.[54;56]

Besides the immunosuppressive effect, cortisol also synergistically stimulates the acute phase response, in combination with IL-6, which includes the production of CRP. CRP increases the ability of blood to coagulate and decreases fibrinolysis and by their pro-atherosis action have a negative effect on longevity.[57] Not much is known about the direct pathway from CRP to cortisol release. We assume a bidirectional

relationship between CRP and cortisol to play a role in maintaining the physiological homeostasis. Therefore, a negative feedback mechanism between CRP and cortisol might be present. Our study on this issue will be discussed in the next paragraph.

Hypocortisolism and the immune system

Hypocortisolism, as is observed in patients suffering from chronic or severe stress-related disorders, may result in an overactivity of the immune system in terms of increased inflammatory responses due to the impaired suppressive effects of low cortisol. This assumption is supported by studies reporting elevated levels of pro-inflammatory cytokines in stress-related disorders characterized by hypocortisolism, e.g., chronic fatigue syndrome and fibromyalgia.[58-60]

In **chapter 6**, we tried to elucidate the relationship between plasma CRP levels and basal salivary cortisol levels over the day under non-inflammatory conditions. We showed that genetic variants in the CRP gene are associated not only with higher plasma CRP levels but also with lower salivary cortisol levels after awakening. In general, stressful and pro-inflammatory conditions, such as acute infections, may increase both CRP and cortisol levels, due to activation of the immune system and HPA axis. But, previous studies showed that in chronic inflammatory conditions, such as rheumatoid arthritis and Sjögren's syndrome,[61-63] cortisol levels were not elevated, despite the presence of chronic high circulating inflammatory cytokine and CRP levels. This phenomenon was referred to as an 'inadequate' low cortisol secretion relative to the chronic inflammatory status.[61;63] It could be hypothesized that a chronic pro-inflammatory state with high CRP levels ultimately results in adaptation of the HPA axis, by negative feedback mechanisms, leading to lower cortisol levels. In other words, CRP effects on cortisol may play a role in maintaining the stress homeostasis. However, in contrast with the direct effects of CRP polymorphisms on CRP levels, the mechanism of the effect on cortisol levels likely involves a much more complex network of endogenous interactions that include, among others, cytokines.

Conclusion

To summarize, the connections between the HPA axis and the immune system provide a finely tuned regulatory system required for health. Chronic hypercortisolism leads to an overall suppression of immune responses and to an enhanced susceptibility to infection, whereas hypocortisolism is associated with an enhanced susceptibility to inflammation.[55] In addition, we provided evidence that genetic variants in the CRP gene that are associated with higher plasma CRP levels, are also associated with lower salivary cortisol levels after awakening, in basal, non-inflammatory conditions, suggesting the presence of a negative feedback mechanism between CRP and cortisol to maintain homeostasis.

Strengths and limitations

A major strength of our studies is that they departed from the level of the stress system where the stress reaction starts, namely the HPA axis. By using this approach, we were able to avoid the problems that are related to the lack of validity of the categorical DSM-IV diagnoses, as described before.

Another advantage of our approach of taking the HPA axis as starting point is that we were able to investigate the nonlinear aspect of the HPA system, which characterizes all homeostatic systems. Suboptimal function (i.e., hyper- versus hypofunction) is observed at both sides of a homeostatic system, e.g. the HPA axis. And indeed, part of the patients with depression and anxiety disorders, are characterized by hypercortisolism (i.e., hyperfunction), and part by hypocortisolism (i.e., hypofunction). Both dysfunctional states are assumed to differ in phenotypic presentation, and in their effects on metabolic and immune parameters, as is shown in our studies.

Furthermore, saliva sampling was used to assess cortisol levels over the day. Cortisol levels measured in saliva provide a valid and reliable correlate of serum or plasma cortisol concentrations. Cortisol in saliva is 100% unbound and biologically active.[64] The available literature clearly suggests that despite lower concentration in saliva, saliva cortisol is even closer correlated with the free cortisol fraction in serum compared to total serum cortisol.[65]

In addition, measurement of cortisol in saliva has many advantages over determination from blood samples. Saliva sampling is non-invasive and therefore not painful (avoiding the risk of stress-induced modulation of cortisol levels), it does not require trained medical personnel and can be repeated frequently. In addition, the collected samples need no special treatment and are stable at room temperature for up to 7 days. These advantages mean that samples can reliably be taken in a normal ambulatory setting, for example the subject's own home, as they go about their routine activities.

An additional strength of our studies is the collection of multiple salivary samples over the day on two consecutive, non-working days, to allow controlling for day to day variations. The cortisol curve of a single day is determined by situational factors and – to a smaller extent – by trait factors. Therefore, to reduce measurement errors and the effects of day-to-day variations,[66] subjects were asked to provide saliva samples during two consecutive non-working days. Non-working days were chosen to reduce confounding, because most patients were non-working due to their illness. Another important advantage is that the subjects of our studies were free of psychotropic medication. There is evidence that this kind of medication might influence HPA axis function.[67]

Last but not least, several biological and genetic markers were assessed (i.e., lipids/lipoproteins, indices of adiposity, acute phase reactants, and CRP polymorphisms), which enabled us to investigate associations of HPA axis dysfunctions with parameters of the metabolic and immune system.

In the last study, genetic variation of the CRP gene was used as an instrument to investigate associations between CRP and cortisol by using the Mendelian randomization design. Instrumental variables, such as genetic variants, can be used to strengthen causal inferences in non-experimental situations. Alleles are generally unrelated to confounding factors, in particular, socioeconomic position and life style factors that distort the interpretations of observational epidemiology. Furthermore, disease processes do not alter genotypes and therefore associations between the genotype and disease outcomes cannot be affected by reverse causality. Unlike randomized controlled trials, Mendelian randomization studies can be conducted in a representative population without the need for exclusion criteria. It is important to note that the aim of Mendelian randomization studies is not to identify functional genes for an outcome, but the approach depends on knowing about the function of the genes before undertaking the study.[68;69]

However, the findings of this thesis also need to be valued in the light of some methodological limitations. As discussed before, a major limitation is the cross-sectional design of our studies, which precludes conclusions about changes in HPA axis dysfunctions over time. Longitudinal studies are needed to answer this aspect of HPA axis function.

Secondly, the sample size was relatively small, which might have lead to a lack of statistical power. Nevertheless, despite the relatively small sample size we were able to detect several significant associations between cortisol and phenotypic, metabolic and immune parameters.

Thirdly, subjects sampled saliva at home without electronic monitoring. Some precautions were taken to avoid non-compliance. For instance, subjects were asked to register the times they actually sampled in order to be able to do a limited check whether subjects sampled at the required times. Furthermore, subjects received the explicit instruction to lay the first salivette for sampling next to their bed, so minimal time is lost between awaking and the first sampling. No evidence was present for differences in compliance between groups (for instance, depressed patients and healthy controls), but some influence of non-compliance might still have been present.

Fourthly, previous studies found larger differences in HPA axis measures in psychiatric in-patients with more severe forms of psychopathology compared to controls. We included outpatients, and not inpatients with severe psychopathology, and this may have reduced the sensitivity to detect HPA axis dysfunctions.

Fifthly, no valid, world-wide used dimensional model of depression and anxiety disorders is yet available. Therefore, for the study in this thesis, we chose the tripartite model of anxiety and depression, because it is the most broadly accepted in adult psychiatry.[70-72] Clark and Watson's tripartite model is designed to handle the high comorbidity rates of depressive and anxiety disorders[73] by taking account of both overlapping and distinct features of anxiety and depression. An additional advantage of the tripartite model is the availability of a validated questionaire that assesses the three dimensions of the model, the so called Mood and Anxiety Symptom Questionnaire (MASQ).[74-76]

Practical advices for future cortisol research

Salivary cortisol has emerged as an easy-to-collect, relatively inexpensive, biological marker of stress. However, circulating cortisol is highly variable and is responsive to a wide range of factors that should be considered when incorporating this measure in research. Two advantages that salivary cortisol has over plasma cortisol are that samples can be collected through relatively noninvasive techniques and that they can be timed without depending on the availability of a laboratory or health care professional. Based on the experiences during the studies presented in this thesis, we provide some advices for salivary cortisol sampling in future research.

1. To control for the effects of circadian and diurnal rhythms, the time of day samples are collected should be standardized. Ideally, samples are collected at fixed time points during the day for all subjects. The number of samples obtained must also be established. More samples provide more information on individual fluctuations. We think 3 à 4 samples within the first hour after awakening are needed to assess the CAR followed by 4 samples during the rest op the day with time intervals of 3-4 h to assess the underlying diurnal profile, e.g., at 11:00h, 15:00h, 19:00h and 23:00h.[77;78]
2. Regarding the waking modus. In a previous study it was shown that CAR did not differ on days were subjects woke up spontaneously or used the alarm clock [77]. We advice, however, to use the spontaneous waking modus in combination with an alarm clock. Sampling after spontaneous awakening should be the primary instruction, because, patients with depression and/or anxiety disorders are often early awakeners and might therefore wake up before the alarm clock is set. In addition, an alarm clock should be set, for instance at 7:00h, because if subjects tend to sleep late, they often doze for some time through which the CAR might be missed.
3. Saliva samples should be collected on more than one day, in order to be able to test the reliability of sampling and/or the interindividual stability. The CAR of a single day is determined to a great extent by situational factors and only for a

small proportion by trait factors, and from two to six days is necessary to achieve reliable trait measures, since state factors bias data from a single day. The total area under the curve (AUC_t) is a good choice for assessing hyper- and hypocortisolism. Sufficient reliability was observed for the AUC_t after 2 days.[66] Therefore, to reduce measurement error and the effects of day-to-day variation, subjects should be asked to provide saliva samples on at least two consecutive days.

4. The materials and techniques used to collect samples may influence the accuracy of testing. A common way of obtaining saliva for analysis involves the use of the commercially available Salivatte (Saarsredt). The Salivette looks like a 2-inch cotton dental roll and is packed in a plastic test-tube-like containers. Subjects are instructed to chew or mouth the Salivette for approximately 30 to 45 seconds. In a recent study it was shown that that salivary cortisol measurement with Salivettes is a reliable prediction method of total and calculated free serum levels. It is a convenient method for saliva collection, handling a laboratory processing.[79] Although tasteless, the Salivette has a consistency that some subjects find unpalatable. Another strategy to assure the collection of adequate samples is to use salivary stimulants, such as flavored drink crystals or sugarless gum. However, salivary stimulants should be used with caution. Although the rate of salivary flow does not affect the cortisol levels, salivary stimulants may alter salivary pH, causing an elevation is the cortisol assay.[80]
5. Samples should be collected either on working days or on non-working days for both the cases and the controls. Anticipation of the working day is associated with an enhanced cortisol response.[81-83] In psychiatric samples, a considerable number of patients are known to be non-working. Therefore, it is preferable in psychiatric studies to sample on non-working days for the sake of comparability between patients and healthy controls.
6. Ingestion of food should be avoided 30 min prior to sampling as it increases the salivary cortisol concentrations.[84;85]
7. To ensure that samples are collected in a consistent, standardized manner, protocols for research teams should be developed, in order to make the outcomes of different studies comparable.
8. Patients treated with antidepressants should be excluded, for it is known that antidepressants influence the HPA axis regulation via up-regulation of MR and GR receptor function and by restoration of the disturbed feedback control.[67]
9. Besides stress, the HPA axis is influenced by many situational factors. In a recent study on confounding factors of salivary cortisol indicators in a large sample without psychopathology it was shown that sociodemographic variables (gender, age), sampling factors (awakening time, working versus non-working day,

sampling month, sleep duration) and health indicators (smoking, physical activity, cardiovascular disease) influence different features of salivary cortisol levels. Smoking had the most consistent effect on cortisol levels. These factors should be considered in psychoneuroendocrinology research and assessed in an accurate and systematic way.[86] In a sample size of about 50-100 subjects, one should match the groups under study on age and gender and adjust for smoking, sampling month and sleep duration. Subject should be asked to abstain from heavy physical exercise on the days of sampling and patients with somatic diseases that influence the HPA axis, e.g. cardiovascular diseases, should be excluded.

10. Finally, prospective studies of cortisol sampling are needed in order to be able to investigate changes in HPA axis function over time, for instance in circumstances of ongoing stress.

Clinical implications

The DSM-IV is a diagnostic system that describes and lists the currently recognized psychiatric disorders and the criteria for diagnosing them. Nearly two decades after the release of the current edition, DSM-IV, the fifth edition of DSM (DSM-V) is planned for publication around 2012. Despite the enormous advantages represented in the DSM-IV, psychiatric diagnosing remains problematic. The principal reason is that our present understanding of brain and behavior, and, therefore, of pathophysiology of mental illnesses is still in a very early stage and etiology-based diagnoses are scarcely possible. For purposes of communication, it is important to maintain the best classification system that we have; at the same time we must guard against reifying provisional diagnoses, thus inhibiting scientific progress. The human brain is the most challenging object of study in the history of human science, and illnesses termed 'psychiatric disorders' represent dysfunctions of the highest integrative functions of the brain including cognition, emotional regulation, and executive function. It is timely to ask whether neuroscience has progressed to a point that the next DSM-version can usefully incorporate information about brain structure and function. Genetic, anatomic and biological markers might enhance diagnostic homogeneity. If, however, the lumping and splitting of symptoms that gave rise to the current classification was in error, then the search of biological correlates of these disorders will not prove to be fruitful. Instead, one should create circumstances in which information from genetics, cognitive neuroscience, brain imaging, and animal studies can contribute to defining the clinical phenotype.[87]

One way in which neuroscience could contribute to the development of better diagnostic criteria without prematurely disrupting the current relatively reliable but imperfect (because of their limited validity) clinical diagnoses is through the creation of experimental diagnostic criteria for research purpose that could shadow the

'official' criteria in the DSM-V. Experimental approaches towards a novel classification of mental disorders could take different forms, depending on the situation: 1) dimensional approaches; 2) the identification of clinical significant symptom clusters for which there are compelling hypotheses about underlying neural circuits; and 3) the abandonment of fine-scale splitting of disorders to yield to larger 'spectrum' disorders, the constituents of which are presumed to share pathophysiological features.[87] Furthermore, as we showed in this thesis, starting at the level were the stress reaction starts, i.e. the HPA axis, might also contribute to a better redefinition of the clinical phenotype.

In summary, we demonstrated that a dimensional approach is able to reveal the nonlinear aspects to HPA axis function. In addition, we propose that differentiation between hyper- and hypoactive functional states of the HPA axis, might contribute to a better redefining of the clinical phenotype and to new insights and developments in pharmacological treatment. Furthermore, we propose it to be valuable to collect information about the state of the metabolic and immune system in order to get an impression of the impact of the stress on the body function and the risk of somatic comorbidity.

Future perspectives

Practicing science is a never ending story; numerous fascinating questions remain to be answered. The studies of this thesis provide some new insight in HPA axis dysfunctions. This thesis describes new observations and elucidated several facets of HPA axis dysfunctions by relating it to phenotypic, metabolic and immune factors that might be involved. However, the puzzle is far from being solved.

We propose that a dimensional approach might be a promising addition to the categorical DSM-IV diagnoses, because the results of the studies presented in this thesis add evidence that dimensions are predictive for HPA axis dysfunction. There are several potential benefits of adding a dimensional approach to the categorical DSM system. The most immediate is that it provides quantitative scores, and therefore including an indication of severity. Furthermore, it provides a consistent methodology of fine-tuned phenotyping across individual patients. Another advantage of a dimensional approach is that quantification may help to increase statistical power in research. Many more subjects are needed to achieve the same power with a categorical diagnosis, than with a dimensional one, under assumption that the underlying psychopathology is continuous. Lastly, a dimensional phenotype is capable to reveal the nonlinear aspect of HPA axis dysfunction. This is an important advantage, as we showed that patients with depression and/or anxiety are no homogenous group neither with respect to underlying HPA axis dysfunctions. Subgroups of

hypercortisolemic and hypocortisolemic patients are present, presumably asking for different kinds of (pharmacological) treatment.

Commitment to a dimensional elaboration for depression and anxiety disorders (and other psychiatric disorders) could also offer new perspectives on the problem of comorbidity, the co-occurrence of two or more distinct disorders. A dimensional approach could replace the awkwardness of categorical comorbidity with patient-specific dimensional profiles.[88] However, there is no consensus as yet about a uniform, validated, dimensional approach. Consensus is necessary for cross-study comparability. Therefore, more work should be done on the refinement of the dimensional phenotyping of depression and anxiety disorders. We propose that a dimensional model should include specific dimensions that reflect the psychopathology of the psychiatric disorders under study, along with two other dimensions: one reflecting overall severity of psychopathology (e.g., the general distress dimension of the tripartite model) and another reflecting chronicity. As motivated in this thesis, severity and chronicity are assumed to determine time-dependent changes in HPA axis dysfunction, i.e., from initial hyperactivity to consequentially hypoactivity. These additional two dimensions are, in our opinion, essential components of the clinical phenotype.

Lastly, we foresee an important role for biomarkers in diagnosing depressive and anxious patients. It is reasonable to think that assessment of cortisol levels in an individual patient might on the longer term guide our choices for pharmacological and psychological treatment.

Concluding remarks

In this thesis, we provide evidence in research on endophenotypes of psychopathology that it is fruitful not to take the clinical picture (diagnosis or dimension) central, but the HPA axis as a core stress underlying psychopathophysiology in stress-related disorders. Homeostatic systems, including the HPA axis, are by nature nonlinear in their function (i.e. dose response effects), with suboptimal states of function at both sides of the curve, e.g. hyper- versus hypofunction. By taken the HPA axis as starting point, we were able redefine the clinical phenotype in relation to both dysfunctional states, i.e., hyper- and hypocortisolism. Furthermore, starting with the stress system enabled us to investigate the effects of HPA axis dysfunction on the metabolic and immune system. Based on the studies presented in this thesis, we hypothesize that the nonlinear aspect of HPA axis function reflects different time points during the stress process, from a hyperactive HPA axis when the stress begins to, eventually, a hypoactive HPA axis when stress holds on. Secondly, we hypothesize that both dysfunctional states of the HPA axis, i.e., hyper- and hypocortisolism, differ in their effects on the metabolic and

immune system. Ultimately, understanding these mechanisms should result in earlier, more specific and therefore more effective interventions, because eventually it is all about improving the well-being of our patients.

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Nederlandse samenvatting

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Depressie en angststoornissen zijn psychiatrische stoornissen die gepaard gaan met een grote lijdensdruk en een sterke afname van de kwaliteit van leven door beperkingen in het functioneren en een hogere sterftekans. Een depressie wordt gekenmerkt door een sombere stemming en/of een afname van het plezier kunnen hebben in dingen. Daarnaast wordt een depressie gekenmerkt door veranderingen in de eetlust, slaapproblemen, vermoeidheid, afname van energie, gevoelens van waardeloosheid, cognitieve problemen (twijfelzucht) en gedachten over de dood. In de loop van een jaar lijdt bijna 6% van de Nederlandse bevolking aan een depressie.

Angststoornissen worden gekenmerkt door overweldigende, irrationele angsten en vermijdingsgedrag. De angst gaat vaak gepaard met lichamelijke symptomen zoals zweten, hartkloppingen, diarree en duizeligheid. Om die angst zoveel mogelijk te voorkomen vermijden mensen met angststoornissen vaak de situaties die angst uitlokten. Tot de angststoornissen behoren de volgende ziekten: paniekstoornis, sociale angststoornis, posttraumatische stressstoornis, obsessieve-compulsieve stoornis, gegeneraliseerde angststoornis en de specifieke fobie. De angststoornissen onderscheiden zich van elkaar door de situaties waarin de angst optreedt. In de loop van een jaar lijdt ruim 12% van de Nederlandse bevolking aan een angststoornis.

Depressie en angststoornissen worden gekenmerkt door een ontregelde reactie op stress. Dit is het gevolg van een leven waarin zorgen routine zijn en ingrijpende levensgebeurtenissen vaker voorkomen dan normaal. Ook een ongezonde leefwijze draagt bij aan het ontstaan van deze ziekten. Niet iedereen die onder zulke omstandigheden leeft krijgt een depressie of angststoornis. Erfelijke eigenschappen en traumatische gebeurtenissen in de eerste levensjaren (misbruik en emotionele verwaarlozing) maken mensen veel 'vatbaarder' voor deze ziekten.

De ontregelde reactie op stress blijkt ook uit de afwijkende werking van de hypothalamus-hypofyse-bijnierschors as (HPA-as), een in het Nederlands ingeburgerde afkorting gebaseerd op de Engelse naam Hypothalamus-Pituitary-Adrenal axis. De HPA-as is heel belangrijk voor de omgang met stress. Stress activeert de HPA-as. Daardoor neemt de afgifte van corticotropin releasing hormone (CRH) in de hypothalamus toe. CRH stimuleert de hypofyse tot afgifte van adrenocorticotroop hormoon (ACTH). ACTH zet op zijn beurt de bijnieren weer aan tot afgifte van cortisol (een belangrijk stresshormoon bij mensen). Cortisol heeft grote invloed op andere biologische systemen, zoals het metabole systeem (houdt zich bezig met de stofwisseling) en het immuunsysteem (bescherming tegen lichaamsvreemde stoffen en ziekten). Het uiteindelijke effect van die invloed is dat we onze reserves zo efficiënt mogelijk inzetten om de stress het hoofd te bieden. Doordat cortisol zijn eigen afgifte remt dooft onder normale omstandigheden de reactie op stress vanzelf uit. Dit wordt

negatieve terugkoppeling genoemd. Heel veel cortisol legt de activiteit van de HPA-as zelfs helemaal stil.

Hoe meer stress iemand heeft, hoe actiever zijn HPA-as is en hoe meer cortisol uit de bijnieren vrijkomt. De concentratie van cortisol kan goed worden gemeten in bloed en speeksel. In het onderzoek beschreven in dit proefschrift deden we dat in het speeksel onder andere omdat dat voor proefpersonen het minst vervelend is.

Hoe de HPA-as op stress reageert, kan worden onderzocht door dit systeem op een kunstmatige manier 'uit te dagen' met een zogenaamde 'challenge'-test. Een voorbeeld is de dexamethason (DEX)-CRH test. Dexamethason is een stof dat op het lichaam ongeveer dezelfde effecten heeft als cortisol. Zo remt het de HPA-as sterk. In de DEX-CRH-test krijgt een proefpersoon 's avonds zoveel dexamethason dat de HPA-as de volgende dag vrijwel niet meer actief is. 's Middags krijgt de proefpersoon CRH ingespoten. Onder normale omstandigheden zou dat de afgifte van cortisol sterk stimuleren. Bij gezonde mensen die zijn 'voorbehandeld' met dexamethason stijgt de afgifte van cortisol echter maar weinig vanwege het remmende effect dat dexamethason nog steeds op de HPA-as heeft. Bij depressieve mensen die de DEX-CRH test ondergaan stijgt de hoeveelheid cortisol in bloed en speeksel wel sterk. Bij hen is iets mis met de negatieve terugkoppeling. Hun HPA-as werkt niet naar behoren

Het doel van dit proefschrift betrof het vergaren van meer kennis over het disfunctioneren van de HPA-as bij patiënten met een depressie en/of angststoornis. Gangbaar in de onderzoeken tot nu toe was om eerst een psychiatrische stoornis als uitgangspunt te kiezen, bijvoorbeeld depressie, en vervolgens de relatie met de HPA-as te bestuderen. Deze onderzoeken laten inconsistente en soms zelfs tegenstrijdige uitkomsten zien. In geval van depressie, zijn vergeleken met gezonde proefpersonen zowel hogere als lagere concentraties cortisol gevonden of geen verschillen. Deze inconsistente uitkomsten kunnen samenhangen met de manier waarop psychiatrische stoornissen worden geclassificeerd. Dat gebeurt tegenwoordig vrijwel altijd met de 'Diagnostic and Statistical Manual of Mental Disorders' (DSM)-IV. Een probleem van dit systeem is dat veel patiënten meer dan één diagnose hebben (een hoge comorbiditeit), terwijl veel klachten een kenmerk zijn van verschillende ziekten (een lage specificiteit) ten gevolge van de grote overlap in symptomen tussen depressie en angststoornissen.

Een alternatieve diagnostische benadering is het gebruik van dimensies (continue meetschaal). Een dimensionele benadering heeft een aantal voordelen. Als eerste vervangen dimensies de categorische comorbiditeit door het genereren van individuele diagnostische profielen op basis van scores per dimensie. Ten tweede kan het gebruik van dimensies ons kunnen helpen om meer inzicht te krijgen in de relaties met biologische en genetische factoren, omdat ze mogelijk sterker gerelateerd zijn aan disfuncties op dit niveau.

In de onderzoeken die beschreven zijn in dit proefschrift hebben we geprobeerd de tekortkomingen van de DSM-IV classificatie te vermijden door onze insteek te kiezen op het niveau van de HPA-as, omdat dit ons centrale systeem is in de omgang met stress. Voor de onderzoeken in dit proefschrift hebben wij cortisol dagcurves gemeten en de cortisol afgifte tijdens een 'challenge' test gemeten en gebruikt als centrale uitkomstmaten voor de HPA-as functie. Het onderzoek is uitgevoerd bij patiënten met een depressie en/of angststoornis en bij gezonde proefpersonen. Met behulp van cortisol hebben we geprobeerd om een alternatieve manier van diagnosticeren van het klinisch beeld te ontwikkelen. Daarnaast hebben we gekeken naar de relatie onderzocht tussen de HPA-as en het metabole en immuunsysteem.

In **hoofdstuk 2** wordt beschreven dat het noodzakelijk is om alternatieve manieren van diagnosticeren ontwikkelen om de biologische verstoringen die ten grondslag liggen aan psychiatrische stoornissen beter te kunnen begrijpen. Als alternatief voor het DSM-IV systeem wordt het gebruik van dimensies voorgesteld. In dit hoofdstuk wordt gesteld dat psychiatrische symptomen continu van aard zijn in tegenstelling tot de categorische DSM-IV diagnoses. Goede psychologische dimensies zijn in staat om afwijkingen in cortisol afgifte te voorspellen. Wij veronderstellen dat dimensies dichter bij biologische verstoringen staan dan DSM-IV diagnoses.

In het **hoofdstuk 3** worden de resultaten beschreven van een onderzoek naar het functioneren van de HPA-as bij depressieve patiënten met en zonder comorbide angststoornis in vergelijking met gezonde proefpersonen. Er werd gebruik gemaakt van de DEX/CRH test. Patiënten met een depressie en een comorbide angststoornis lieten een sterkere onderdrukking van cortisol na DEX-inname zien dan de andere twee onderzoeksgroepen. De conclusie van dit onderzoek was dat comorbiditeit invloed heeft op de HPA-as functie. Voorgaande onderzoeken hielden vaak geen rekening met de invloed van comorbiditeit. Daarom kunnen onze bevindingen deels een verklaring vormen voor de eerdere inconsistente uitkomsten in onderzoeken naar het functioneren van de HPA-as in patiënten met depressie en angststoornissen.

In **hoofdstuk 4** wordt de uitkomst van een onderzoek gepresenteerd naar de relatie tussen cortisolconcentraties en psychologische dimensies bij patiënten met depressieve en/of angststoornissen. Er werd gebruik gemaakt van het tripartiete dimensionele model van Clark en Watson. Zij onderscheiden in hun model 3 dimensies: “general distress” (negatief affect), “anhedonic depression” (gebrek aan positief affect) en “anxious arousal” (lichamelijke angstsymptomen). Wij vonden lineaire en non-lineaire (omgekeerde U-curve) verbanden tussen cortisolconcentraties en dimensies. Bij een lineair verband neemt de biologische verstoring (bijv. cortisolafgifte) toe als de klachten toenemen. Een non-lineair verband (omgekeerde U-curve) betekent dat bij toename klachten de biologische verstoring eerst toeneemt (hogere cortisol afgifte), maar later afneemt (lagere cortisol afgifte). Non-lineaire

verbanden kunnen wel worden aangetoond met dimensies, maar niet met DSM-IV diagnoses. De conclusie van ons onderzoek was dat de omgekeerde U-curve wijst op een omkering van HPA-as activiteit bij aanhoudende/toenemende klachten. Depressieve en angstklachten leiden in eerste instantie leidt tot een hyperactieve HPA-as (hogere cortisolconcentraties). Een verdere toename van klachten (hogere dimensionele scores) kan leiden tot een switch naar een hypoactieve HPA-as (lagere cortisolconcentraties), wat gezien kan worden als een (over)aanpassing van de HPA-as.

In **hoofdstuk 5** hebben we de relatie tussen de HPA-as en het lipidenmetabolisme en overgewicht bestudeerd in patiënten met een depressie en/of angststoornis. Bekend is dat een afwijkend lipidenprofiel en overgewicht risicofactoren voor het ontwikkelen van hart- en vaatziekten zijn. Wij vonden dat twee uitkomstmaten van disfunctie van de HPA-as (verhoogde cortisol afgifte en vlakkere cortisol dagcurve) gerelateerd zijn aan een afwijkend lipidenprofiel, maar niet aan overgewicht. Een verhoogde cortisolafgifte wijst op een hyperactief stresssysteem en een vlakkere cortisolcurve duidt op een gebrek aan flexibiliteit van het stresssysteem. Dit onderzoek ondersteunt de hypothese dat de relatie tussen depressie en/of angststoornissen met hart- en vaatziekten, onder andere, loopt via een verstoorde HPA-as functie.

In **hoofdstuk 6** wordt een onderzoek besproken waarin een andere onderzoeksmethodiek is toegepast, namelijk 'Mendelian Randomization'. Deze methodiek houdt in dat er gebruik wordt gemaakt van genetische verschillen tussen mensen. In dit onderzoek hebben we gekeken naar de relatie tussen C-reactief proteïne (CRP) en speeksel cortisol. CRP is een eiwit dat in de lever wordt geproduceerd als er een ontstekingsproces gaande is in het lichaam. CRP wordt daarom vaak gebruikt wordt als maat voor ontsteking. Cortisol, samen met andere ontstekingsfactoren, stimuleert de afgifte van CRP. Daarnaast heeft cortisol ook een remmend effect op het immuunsysteem. Het samenspel tussen CRP en cortisol wordt verondersteld een belangrijke rol te spelen bij de fysiologische homeostase. Homeostase is het vermogen van de mens om zijn interne milieu constant te houden, ook onder omstandigheden van psychische (bijv. depressie) of lichamelijke (bijv. ontsteking) stress. Dit onderzoek laat zien dat CRP een remmende invloed heeft op cortisolafgifte, wat gezien kan worden als een negatieve terugkoppeling voor de handhaving van de homeostatische balans.

De resultaten die in dit proefschrift beschreven worden vormen een uitbreiding op de reeds bestaande literatuur over de disfunctie van de HPA-as bij depressie en/of angststoornissen. De belangrijkste meerwaarde van dit proefschrift is dat we gekozen hebben voor aan andere benadering dan meestal wordt gekozen in dit onderzoeksveld. Niet het klinische plaatje (de diagnose of het symptoom) stond centraal, maar de

cortisolconcentraties zijn gebruikt als uitgangspunt in onze onderzoeken. Wij hebben het klinische beeld geherdefinieerd op basis van verschillen in cortisolconcentraties. Onze uitkomsten laten zien dat een dimensioneel model toegevoegde waarde heeft voor de DSM-IV classificatie, omdat het in staat is non-lineaire verbanden tussen psychopathologie (uitgedrukt in dimensionele scores) en cortisol te ontdekken. Op basis van deze uitkomst hebben wij de hypothese geformuleerd dat stress is geassocieerd met een hyperactiviteit van de HPA-as, maar op termijn, bij aanhoudende stress, kan de hyperactiviteit overgaan in een hypoactiviteit van de HPA-as. Enige bescheidenheid is hier echter op zijn plaats, omdat onze onderzoeken cross-sectioneel van opzet zijn (eenmalige meting). Hierdoor mogen eigenlijk geen uitspraken gedaan kunnen worden over het verloop in de tijd. Om iets over HPA disfuncties in de loop van de tijd te kunnen zeggen zijn prospectieve onderzoeken nodig, waarbij proefpersonen meerdere malen worden gemeten in de loop van de tijd.

Verder hebben wij bewijs geleverd dat verstoringen van de HPA-as geassocieerd zijn met ontregelingen van het metabole systeem wat zorgt voor een groter risico op cardiovasculaire zieken. Ook hebben we laten zien dat CRP en cortisol elkaar over en weer beïnvloeden wat bijdraagt aan een homeostatisch evenwicht.

Onderzoek doen is een 'never-ending story'; diverse, fascinerende vragen blijven onbeantwoord. Dit proefschrift geeft nieuwe inzichten in de relatie tussen verstoringen van de HPA-as en het klinisch beeld en metabole en immuunfactoren. De puzzel is echter nog lang niet opgelost.

Ons voorstel is om een dimensionele benadering toe te voegen aan de DSM-IV classificatie, omdat dit proefschrift laat zien dat dimensies meer zicht geven op onderliggende biologische verstoringen. Er zijn meerdere voordelen te noemen van het toevoegen van een dimensionele benadering aan het categoriale DSM-IV systeem. De belangrijkste is dat het leidt tot kwantitatieve scores, waardoor ook een ernstmaat is meegenomen in het beschrijven van de psychopathologie. Daarnaast voorziet deze benadering in een nauwkeurige en consistente methodiek om het klachtenpatroon van een individuele patiënt te beschrijven. Een ander voordeel van de dimensionele benadering is dat de kwantificatie bijdraagt aan een vergroting van de statistische kracht ('power') in onderzoek. Je hebt veel meer patiënten nodig om dezelfde statische kracht te bereiken met categoriale diagnoses dan met dimensies, er vanuit gaand dat de onderliggende psychopathologie continu van aard is. Tot slot is een dimensioneel benadering in staat om het non-lineaire aspect van de HPA-as te laten zien. Dit is van belang omdat depressieve en angstpatiënten geen homogene groep vormen, ook niet wat betreft hun HPA-as disfuncties. Subgroepen van patiënten met hypercortisolisme (hoog cortisol) of hypocortisolisme (laag cortisol) zijn aanwezig, waardoor het goed voorstelbaar is dat deze subgroepen ook om verschillende soorten (medicamenteuze) behandelingen vragen. Overgaan tot een dimensionele uitbreiding voor depressie en

angststoornissen (en mogelijk ook andere psychiatrische stoornissen) biedt ook een ander perspectief op het probleem van de comorbiditeit, de gelijktijdige aanwezigheid van twee of meer stoornissen. Een dimensionele benadering vervangt categoriale comorbiditeit door patiëntspecifieke dimensionele profielen.

Er is echter nog geen consensus over een eenduidig, gevalideerd, dimensioneel model. Deze consensus is nodig om de uitkomsten van verschillende onderzoeken met elkaar te kunnen vergelijken. Er moet daarom nog meer werk verricht worden aan het ontwikkelen van een valide dimensioneel model voor van depressie en angststoornissen. Wij pleiten voor een dimensioneel model met specifieke dimensies die het klachtenpatroon van een patiënt goed beschrijven. Daarnaast zijn twee andere dimensies van belang: een dimensie die de ernst van het klachtenpatroon weergeeft, en een dimensie die de duur van de klachten weergeeft. In dit proefschrift wordt de hypothese geformuleerd dat ernst en duur leiden tot opeenvolgende HPA-as disfuncties over de tijd, te weten eerst een hyperactiviteit (hogere cortisol afgifte door bijnierschors) gevolgd door een hypoactiviteit (lagere cortisol afgifte door bijnierschors).

Tot slot, voorspellen we een belangrijke rol voor biomarkers bij het diagnosticeren van depressieve en angstpatiënten. Het is niet ondenkbaar dat de bepaling van cortisolconcentraties in speeksel of bloed in een individuele patiënt in de toekomst richtinggevend zal worden voor de keuze van onze medicamenteuze en/of psychologische behandeling en voor het voorspellen van het ziekteverloop.

Dankwoord

Dankwoord

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Curriculum Vitae

Curriculum Vitae

Gerthe Veen werd op 27 februari 1971 geboren in Nootdorp. In 1988 behaalde zij haar VWO-diploma aan scholengemeenschap Guido de Brès te Amersfoort. Aansluitend heeft zij een jaar psychologie en filosofie vakken gevolgd aan een college in Palos Heights, een voorstad van Chicago, in Verenigde Staten van Amerika. Van 1990 tot 1995 studeerde zij gezondheidswetenschappen, afstudeerrichting geestelijke gezondheidkunde, aan de Universiteit Maastricht. Daarnaast heeft zij van 1992 tot 1998 geneeskunde gestudeerd aan dezelfde universiteit. Na het behalen van haar artsendiploma startte zij haar loopbaan als arts-assistent en onderzoeker bij het Utrechts Medisch Centrum, waar zij werkte tot juni 1999. Daarna heeft zij een jaar gewerkt als arts-assistent bij het Regionaal Psychiatrisch Centrum in Woerden, onderdeel van Altrecht. Vanaf 1 maart 2000 is zij gestart met de opleiding tot psychiater bij GGZ Meerkanten in Ermelo (opleider: Dr. T. Kuipers). In dat kader heeft zij haar stage sociale psychiatrie gedaan bij het Regionaal Psychiatrisch Centrum Zeist, onderdeel van Altrecht (opleider: Drs. M.A. de Pater-Zijlstra). Haar keuzestage heeft zij in de kinderpsychiatrie gedaan bij De Bascule in Amsterdam (Opleider: Prof. Dr. Th. A.H. Doreleijers). Op 1 september 2004 heeft zij haar opleiding tot psychiater afgerond. Aansluitend is zij als psychiater gaan werken bij de ambulante volwassenenzorg van GGZ Leiden en omstreken, onderdeel van Rivierduinen, bij de zorglijn voor stemmings-, angst- , en somatoforme (SAS) stoornissen. Gelijktijdig is zij gestart met een wetenschappelijk onderzoek bij het Leids Universitair Medisch Centrum. Het onderzoek was gericht op het (dis)functioneren van het biologische stresssysteem, de hypothalamus-hypofyse-bijnier as, bij patiënten met een depressie en/of angststoornis. Dit resulteerde in dit proefschrift.

List of publications

List of publications

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