Chapter 9

General discussion
This thesis contains several studies on the relationship between salivary cortisol measures – as indicators of activity of the hypothalamus-pituitary-adrenal (HPA) axis - and depressive and anxiety disorders. For all these studies, we used data from the Netherlands Study of Depression and Anxiety (NESDA), which recruited 2981 persons in the age of 18 through 65 years. In this Chapter, the main findings will be reviewed, methodological considerations and clinical implications will be discussed and suggestions for future research directions will be given.

**SUMMARY OF MAIN FINDINGS**

Sociodemographic, health and sampling factors and salivary cortisol levels

Using data from 491 controls without psychopathology from the Netherlands Study of Depression and Anxiety (NESDA), we examined the influences of sociodemographics, health factors and sampling factors on salivary cortisol measures (Chapter 2). We observed that sex, smoking, physical activity, and season of saliva collection were the most consistent determinants of salivary cortisol indicators. Women showed an increased cortisol awakening response (CAR), flatter diurnal slope, and more cortisol suppression after dexamethasone. Smoking was associated with higher morning and evening cortisol and less cortisol suppression. Physically active persons had higher morning cortisol levels, a steeper diurnal slope, and showed more cortisol suppression. Saliva collection in months with relatively less day light was associated with higher morning and evening cortisol, flatter diurnal slope and less cortisol suppression after dexamethasone ingestion. In addition, older age was related to higher evening and post-dexamethasone cortisol levels, and cardiovascular disease to lower 1-hour awakening cortisol and a lower CAR. Finally, several sampling factors were significant determinants of especially 1-hour awakening cortisol and diurnal slope: early time of awakening and less sleep were associated with a higher CAR and flatter diurnal slope, and working on the sampling day with higher overall morning cortisol and a steeper diurnal slope. No significant consistent associations were found for: education, weekday, daily alcohol intake, body mass index, pain, diabetes, allergy or lung diseases or other chronic diseases in the main analyses. However, more days in pain was associated with a blunted CAR, steeper cortisol decline and more cortisol suppression after dexamethasone intake in additional analyses excluding all persons with chronic diseases or medication use. Therefore, these sociodemographics (sex, age), health factors (smoking, physical activity, cardiovascular disease) and sampling factors (sleep duration, season, awakening time and working on the sampling day) should be considered as covariates when examining salivary cortisol measures.
Table 1  Associations between salivary cortisol indicators and depressive and anxiety disorders, course and somatic consequences

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<th>Cortisol awakening curve</th>
<th>Basal level</th>
<th>DST</th>
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<td>AUCg</td>
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<td><strong>Psychopathology</strong></td>
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<td>- Remitted MDD</td>
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<td>- Current MDD</td>
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<td>ns</td>
<td>T5+</td>
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<td>- Remitted anxiety disorder</td>
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<td>+1</td>
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<tr>
<td>- Current anxiety disorder</td>
<td>ns</td>
<td>PDA+</td>
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<td>- Comorbidity a</td>
<td>+1</td>
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<td><strong>Psycho-active medication a</strong></td>
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<td>- Use of TCA</td>
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<td>- SSRI</td>
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<td>- Other antidepressants</td>
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<td><strong>Familiarity b</strong></td>
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<td>- Diagnosed parental history</td>
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<td>- Self-reported parental history</td>
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<td><strong>Psychological traits b</strong></td>
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<td>- Hopelessness reactivity</td>
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<td>- Other traits</td>
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<td><strong>Chronic course (vs remission) a</strong></td>
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<td><strong>Metabolic syndrome</strong></td>
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<td>- Waist circumference</td>
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<td>- Systolic blood pressure</td>
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<td>- Glucose, triglycerides, HDL</td>
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**Abbreviations**: DST=dexamethasone suppression test, AUCg/i=area under the curve with respect to the ground/increase, MDD=Major depressive disorder, T=trend, T5=cortisol at 22h00, T7=cortisol after dexamethasone at the next morning, PDA=panic disorder with agoraphobia, TCA=tricyclic antidepressant, SSRI=selective serotonin reuptake inhibitor, HDL=high density lipoprotein
+ = positively, - = negatively, ns = not significantly associated

a Analyses were conducted among persons with psychopathology
b Analyses were conducted among persons free of psychopathology

**Salivary cortisol levels in depressive and anxiety disorders**

In **Chapter 3**, we compared salivary cortisol levels of 308 persons without psychopathology with 579 persons with remitted major depressive disorder (MDD) and 701 persons with current MDD. Remitted as well as current MDD was associated with a modestly but significantly increased cortisol awakening curve as compared to persons without lifetime psychiatric disorders (see Table 1). Second, although not confirmed with 23h00 data, significantly higher 22h00 evening cortisol levels were found for the current MDD group. However, MDD groups did not show more non-suppression after 0.5 mg dexamethasone ingestion, current MDD even seemed to be associated with more suppression. Additional analyses on several depression characteristics revealed no significant associations for most characteristics (e.g. chronicity, symptom severity, childhood trauma), except for a higher cortisol awakening response among depressed subjects with comorbid anxiety. In addition, the use of psycho-active medication was generally associated with decreased cortisol levels and less cortisol suppression after dexamethasone (Table 1). These results imply that HPA axis activity as measured through salivary cortisol levels is significantly – although modestly - increased in both remitted as well as current depressed outpatients. The latter finding suggests that either
the dysregulation was present before the onset of the disorder, perhaps reflecting a biological vulnerability or it is a result of a depressive episode, a ‘scar’-effect.

In Chapter 4, we compared salivary cortisol levels of persons with a remitted \( n = 311 \) or current \( n = 774 \) anxiety disorder (generalized anxiety disorder, social phobia and/or panic disorders with or without agoraphobia) to 342 persons without psychopathology. After adjustment for relevant covariates, we observed a higher cortisol awakening response for persons with a current anxiety disorder. The cortisol awakening curve for remitted anxiety held the intermediate position between current anxiety and controls. Additional analyses on type of disorder and presence of MDD revealed that persons with panic disorder with agoraphobia and persons with a comorbid MDD exhibited higher cortisol awakening responses as compared to persons with other anxiety disorders and no MDD (Table 1). No differences were found in evening cortisol or cortisol suppression after dexamethasone ingestion between persons with anxiety disorders and controls (Table 1).

The cortisol awakening curve as a trait

To further examine state versus trait effects and analyze whether a higher cortisol awakening curve could already be observed in unaffected persons at high risk of depressive or anxiety disorders, we conducted a study on parental history of depressive and anxiety disorders (Chapter 5). Parental history was ascertained by self-report \( n = 114 \) as well as Composite International Diagnostic Interview (CIDI) diagnoses (in a subgroup: \( n = 74 \)). Results showed that CIDI-diagnosed parental history was associated with a higher cortisol awakening curve as compared to 180 persons without parental history, independent of neuroticism, childhood trauma and life events (Table 1). However, self-reported parental history was not associated with a higher cortisol awakening curve. Furthermore, the cortisol awakening curve was similar to that of 1262 patients with MDD or panic disorder with agoraphobia. These results suggest that an increased cortisol awakening curve might represent a trait and a biological vulnerability for the development of depressive and/or anxiety disorders. A reason why we did not find differences between self-reported parental history and controls could be that the diagnosed parental history group contained more severe psychopathology with possibly more familial loading.

In Chapter 6, we examined whether psychological traits (associated with psychopathology), such as personality and cognitive reactivity, were associated with the cortisol awakening curve, to further investigate whether the increased cortisol awakening curve in persons without psychopathology represents a trait rather than a state effect. This was done in a sample consisting of 381 persons without a lifetime psychiatric history. No traits were significantly associated with the cortisol awakening response, except for a positive association
with hopelessness (Table 1). Higher scores on hopelessness – a trait associated with depression and suicidality – were associated with a higher cortisol awakening response.

Consequences: course of depressive and anxiety disorders and the metabolic syndrome

Chapter 7 describes a longitudinal study on the association between salivary cortisol measures at baseline and the course of psychopathology in 837 patients with depressive and/or anxiety disorders. We observed that compared to persons experiencing remission during the two-year follow up, persons with a lower CAR were at a higher risk of developing a chronic course. Evening cortisol and cortisol suppression after dexamethasone intake were not associated with a chronic course (Table 1). The association appeared to be similar across baseline disorders (anxiety disorder only, depressive disorder only or comorbid disorders). A lower CAR could be the result of a longer underlying activation of the HPA axis due to chronic or severe stress exposure, finally resulting in exhaustion of the system resulting in down-regulation of receptors of the HPA axis.

In Chapter 8, the association between salivary cortisol measures and the metabolic syndrome was examined in 1883 persons. In addition, measures of the autonomic nervous system (ANS) were related to the metabolic syndrome as well as salivary cortisol measures. Components of the metabolic syndrome included: waist circumference, triglyceride levels, high-density lipoprotein levels, systolic blood pressure and glucose levels, and were also separately analyzed. Results showed that ANS measures were strongly associated with (components of) the metabolic syndrome, whereas salivary cortisol measures were not (Table 1). Furthermore, ANS measures and HPA axis measures were not correlated. These results indicate that salivary cortisol levels are not associated with cardiovascular risk as measured through the metabolic syndrome and that basal HPA axis activity is independent of ANS activity.

In short, the results presented in this dissertation show that salivary cortisol levels, although influenced by many factors are on average slightly higher in persons with psychopathology. More specifically, the cortisol awakening curve is modestly but significantly higher in persons with a remitted or current MDD or a current panic disorder with agoraphobia. Furthermore, evidence was gathered that this higher cortisol awakening curve is more of a trait than a state characteristic, since it is also present in unaffected persons with parental history of depression or anxiety, and with hopelessness reactivity. When exploring consequences, we found that the cortisol awakening response at baseline was inversely associated with the risk of a chronic course over two years, but was not associated with the metabolic syndrome.
GENERAL IMPLICATIONS

Especially the CAR – but not other cortisol indicators - was associated with psychopathology. We included three HPA axis indicators because they each represent a different aspect of HPA axis activity. The CAR reflects the increase upon awakening and is regarded to be a distinct feature of the HPA axis. The CAR is often measured with two formula's according to Pruessner. The area under the curve to the ground (AUCg) is an indication of the total secretion during the first hour, the area under the curve to the increase (AUCi) is more related to the sensitivity of the system, emphasizing changes of time and is calculated as AUCg minus the awakening samples times one hour. It has been recommended to measure both the AUCg as well as the AUCi, since these measure different aspects of the cortisol awakening response. Hellhammer et al. reported that the AUCg is more related to traits and AUCi to momentary states such as stress load. Therefore, it seems that the AUCg reflects underlying hypo- or hypoactivity of the HPA axis and is more trait-related and the AUCi reflects the CAR, a distinct phenomenon, more related to situational factors. Evening levels indicate basal cortisol levels, since cortisol levels are generally low before night time. The dexamethasone suppression test (DST) is used to measure the negative feedback system. The low dose of dexamethasone (0.5 mg) was used to suppress cortisol levels, but not completely, allowing the detection of subtle differences in feedback function.

By far, the most associations were found between psychopathology (or other investigated variables) and the cortisol awakening curve and not with evening cortisol or cortisol suppression after dexamethasone intake (Table 1). In prior studies on cortisol levels during the day, associations are often strongest for morning cortisol (e.g. 10-12). Possible reasons include methodological issues (see also Methodological considerations), or that the CAR is truly the most important characteristic in the relation with depressive and anxiety disorders in outpatients, as compared to evening cortisol and the DST. Possibly, the CAR measurement was affected by less measurement error compared to evening cortisol and DST, maybe because the CAR is based on more sampling points. In addition, some levels in the evening are possibly close to the detection limit of the cortisol assay where generally measurement error is higher. The AUCg and evening cortisol might both be reflective of basal activity, however, AUCg might be a better indicator, or peak levels are more affected than basal levels. Possibly, the pulsatile nature of cortisol release is better picked up by morning cortisol levels. Indeed, the correlation between morning cortisol levels with ACTH levels is high. Also, awakening might be regarded as a natural stressor and cortisol differences tend to be more pronounced after exposure to a stressor. Another explanation could be that morning cortisol is reported to be under genetic influences, while evening levels are not and that we investigated mainly trait markers, which could therefore be more related to morning cortisol. The results could indicate that basal levels as measured in the evening and the negative
feedback system as measured with the DST are not as much affected in depressed or anxious outpatients, while the CAR is. Previous studies examining the CAR, evening cortisol and the DST together in relation with depressive and anxiety disorders are scarce. The DST, could be only associated with severe, psychotic depression as compared to the moderately severe depression and anxiety disorders found in outpatients. It appears that the CAR is more sensitive to moderate degrees of depression than traditional measures such as the DST.

**HPA axis and psychopathology: more trait than state characteristic**

We observed a higher CAR in persons with remitted as well as current MDD and current panic disorder with agoraphobia (**Chapters 3 and 4**). Therefore, the higher cortisol awakening curve might represent a trait indicator for MDD and possibly panic disorder with agoraphobia, present before the onset of these disorders, or it might represent a 'scar'-effect. Given the cross-sectional nature of the design of these studies, the causal directions are unclear. However, in order to obtain an indication of whether the elevated cortisol awakening curve could be present before onset of these disorders, we examined persons at risk for depression and anxiety disorders. We investigated whether it was associated with parental history of depression or panic disorder and psychological traits in unaffected persons. We found an elevated cortisol awakening curve in unaffected persons with diagnosed parental history of depression or panic disorder and in unaffected persons with high scores on hopelessness reactivity. Furthermore, previous studies also reported a higher CAR in persons recovered from depression or in unaffected persons with parental history. In addition, a recent study reported that a higher CAR was predictive of the onset of depression in adolescents. Therefore, an elevated cortisol awakening curve could reflect a trait, more specifically, a biological vulnerability for depression (and possibly panic disorder). In accordance with the study by Hellhammer, this trait effect was mainly reflected by the AUCg and not the AUCi. However, presence of panic disorder with agoraphobia was only associated with the CAR as measured with the AUCi, which could reflect a higher stress load or arousal in these patients, more related to situational factors. For instance, Thorn et al. reported a positive association between arousal scores and the AUCi and not the AUCg, although this was in persons without psychopathology. Since the group of persons with only remitted panic disorder or with parental history of panic disorder was too small, we could not sufficiently examine whether the higher CAR in patients with current panic disorder with agoraphobia reflects a trait or a state marker.

Possible mechanisms underlying the biological vulnerability as reflected by an increased cortisol awakening curve, include genetic and environmental factors. Heritability of depressive and anxiety disorders is around 30-40%. Hopelessness is for 30% genetically determined.
In addition, the cortisol awakening curve is under genetic control while the other diurnal measures are not\(^{12,22,23}\). In a twin study, Wust et al. reported relatively high heritability for the AUCg and AUCi (48% and 40%, respectively)\(^{12}\). Kupper et al. reported considerable genetic influence for the awakening sample and 30 min post-awakening (34 and 32%), but no influence on the CAR\(^{23}\). Bartels et al. reported heritability of 22-24% for T1 and 56-59% for the sample 60 min post-awakening in children\(^{22}\). Possibly, the AUCg is more genetically determined than the AUCi. The serotonin transporter gene (5-HTTLPR) could mediate these effects, since it has been associated with depression\(^{24}\), hopelessness\(^{25}\) and the CAR\(^{26}\). This genetic vulnerability could also be mediated through polymorphisms of the mineralocorticoid (MR) and glucocorticoid (GR) receptors\(^{27}\) or receptors involved in corticotrophin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) hormone signalling, such as the CRH receptor\(^{28}\).

Childhood trauma, life events and daily stressors could further play a role. We did adjust for childhood trauma and life events, and these did not impact on our results. This suggests that these factors are not likely to be of much importance. However, we did not assess daily stressors or other adversities, which could still be related to the development of depression and anxiety\(^{29,30}\), and might lead to hopelessness owing to ineffective coping styles. In addition, a differential home environment could also be of influence, e.g., less structure provided by parents and chronic family stress, which may have lead to cortisol differences\(^{31,32}\). Furthermore, exposure to parental depression might have induced stress among the children, since remitted or current depression are associated with personality deviances and role function problems of the parents\(^{33,34}\). Interactions between genetic and environmental factors could also be important. Bet et al. reported an interaction between GR genes and childhood adversity, which was associated with depressive symptoms as well as cortisol levels\(^{35}\).

This thesis provided little evidence for state effects on cortisol levels. For instance, no effects of characteristics, such as daily hassles and symptom severity were observed as measured in our studies (Chapter 3 and 4). We did find effects of sampling factors on the CAR, such as awakening time, season and sleep (Chapter 2). However, it is unclear whether these reflect state or trait effects, since these could also reflect traits such as chronotype\(^{36}\) and seasonal variation in mood (seasonality)\(^{18}\). Then again, we did not measure current stress at the day of sampling, which could have had an effect on cortisol levels. Previous studies on state effects showed mixed results. For example, Thorn et al. reported arousal scores to be associated with AUCi and not the AUCg, especially in persons with trait seasonality. Surprisingly, state stress was not associated with AUCg or AUCi in their study\(^{18}\). Polk et al. also studied state versus trait effects on the CAR. They reported no relation with state negative or positive affect, whereas trait negative affect was positively associated with CAR in men only\(^{37}\). Hellhammer et al. reported larger effects of situational...
factors on the AUCi than on the AUCg. Adam et al. examined the influences of day-to-day experiences on the CAR in older adults. They reported that lower wake-up levels were predictive of fatigue and physical symptoms later that day, and that feelings of loneliness or sadness on the prior day were associated with an increased CAR the next day\textsuperscript{38}. These state effects could be different for patients as compared to controls. Peeters et al. showed that in contrast to healthy controls, depressed outpatients exhibited no increase in cortisol after negative events\textsuperscript{39}. Taken together, these findings suggest that there are some day-to-day associations of variations in emotions with morning cortisol levels.

We did found some – although minor - effects of psycho-active medication on cortisol levels. A lower CAR was observed in persons using tricyclic antidepressants (TCAs). In addition, TCA use and selective serotonin reuptake inhibitor (SSRI) use were associated with less suppression after dexamethasone (\textit{Chapter 3}). Antidepressants could have a direct effect on the HPA axis through up-regulation of the GR and MR leading to normalization of the HPA axis\textsuperscript{40}. However, it is also possible that there is an underlying pathophysiology involving more severe and/or more chronic depressive episodes, resulting in the use of TCA as well as a lower CAR.

\textbf{Previous literature on cortisol levels in depressed or anxious outpatients: inconsistencies}

Studies analyzing the CAR in depressed outpatients have reported a blunted\textsuperscript{41} or an increased CAR\textsuperscript{42, 43} in persons with depression or depressive symptoms compared to controls. Studies in depressed outpatients using daytime cortisol levels (other than the CAR) also showed mixed results, although mostly showing no difference compared to controls\textsuperscript{39, 44-48}. An often mentioned possible explanation is that subtypes of depression explain these inconsistencies. However, we did not find differences in the CAR between persons with atypical versus melancholic depression or other differences in characteristics, such as chronicity, suicide attempts, depression severity or childhood trauma. Possible explanations for inconsistencies in the previous literature – based on this thesis - are: inclusion of remitted MDD in the control group resulting in no or smaller effects, differences in proportion of comorbidity with anxiety, use of TCAs, inclusion of panic disorder with agoraphobia or a large proportion of parental history of depression in controls and differences in inclusions of covariates, such as smoking status and awakening time. In addition, since we found a blunted CAR in patients developing a chronic course, possibly differences in inclusion of chronic depressives contribute to the heterogeneity of results.

Only few studies examined the CAR in relation with anxiety disorders. One report found a higher CAR in generalized anxiety disorder compared to controls in an older cohort\textsuperscript{49}, no difference in CAR was found between patients with social phobia and control participants\textsuperscript{50}, and there are no previous reports on the CAR in panic disorder. Literature on daytime
cortisol levels in anxiety disorders other than the CAR proved to be inconsistent, especially for panic disorder, reporting elevated\textsuperscript{51-53} as well as normal\textsuperscript{54, 55} basal cortisol levels. When examining cortisol levels in anxiety disorders the following factors should be taken into account; comorbidity with remitted or current MDD, agoraphobia in panic disorder, use of TCA, parental history of depression or panic disorder in controls and important covariates.

A low CAR was associated with chronic psychiatric course. When examining the 2-year chronic course in patients with current depressive and/or anxiety disorders, we observed a lower CAR for persons with a chronic course over two years (\textit{Table 1, Chapter 7}). Lower cortisol levels have previously been found in depression in the elderly, especially in persons with chronic and recurrent depressive episodes\textsuperscript{47, 56} and/or physical frailty\textsuperscript{57}. The development of a chronic course is possibly due to chronic stress, accompanied by long-term activation of the HPA axis and resulting in exhaustion of the system leading to a lower cortisol levels\textsuperscript{58, 59}. Miller et al. conducted a meta-analysis on chronic stress and HPA axis activity and reported that morning cortisol is especially lowered when time has passed since the occurrence of the stressor and the stressor is no longer present, and when the stressor concerns an uncontrollable threat to the physical self, or traumatic stressor\textsuperscript{60}.

Exposure to chronic stress could also be reflected in the CAR, since several studies have reported a relationship between chronic or severe stress exposure and a blunted CAR\textsuperscript{61, 62}. Although, we adjusted for symptom duration and childhood trauma, we did not actually assess chronic stress and we did not capture lifetime exposure to stress, or specific aspects of a stressor. Therefore, chronic stress or high allosteric load could play a role in explaining our result. A possible mechanism for hypocortisolism after chronic stress is down-regulation of the CRH receptors in the pituitary, following a longer period of stress-induced hypothalamic CRH secretion, resulting in lower ACTH and cortisol levels\textsuperscript{59}. Alternatively, reduced biosynthesis or depletion of CRH, ACTH and/or cortisol or increased sensitivity of the HPA axis to negative feedback could play a role in hypocortisolism\textsuperscript{58, 59}. The latter could not be confirmed in our study, since we did not find a significant association between cortisol suppression after dexamethasone ingestion and the risk of a chronic course.

It is unclear whether the same mechanisms apply to the CAR, since this is a distinct feature of the HPA axis and its regulatory processes are not yet understood. However, the hippocampus could play a role in explaining the results, since a reduced hippocampus has been associated with a blunted CAR\textsuperscript{10, 63}. In addition, a smaller hippocampus is found in chronically depressed patients\textsuperscript{64}. Possibly, chronic stress or symptom duration leads initially to HPA axis hyperactivity which may damage the hippocampus and leads to down-regulation of receptors of the HPA axis, resulting in lower cortisol levels. Although a reduced hippocampus may lead to hypercortisolism in general, by decreased inhibition of the HPA axis, the integrity
of the hippocampus appears to be a requisite for the CAR\textsuperscript{10, 65, 66}. The CAR has been hypothesized to be associated with "an activation of prospective memory representations at awakening enabling individual’s orientation about the self in time, and space as well as anticipation of upcoming demands", with an important role for the hippocampus\textsuperscript{65}.

Other underlying chronic disorders, such as posttraumatic stress syndrome (PTSD) and chronic fatigue syndrome could also account for the results, since these disorders have been associated with a lower CAR\textsuperscript{11, 67}. Alternatively, these may share a common pathophysiology with chronic depression. Finally, a blunted CAR could also be the result of non-compliance with the time protocol (see \textit{Methodological considerations}).

\textbf{Cortisol levels were not strongly associated with somatic outcomes}

In this thesis, no evidence has been provided for a relationship between salivary cortisol measures and somatic diseases, such as the metabolic syndrome (\textbf{Chapter 8}), except for an association between cardiovascular disease and a lower CAR in controls (\textbf{Chapter 2}). This could imply that the cortisol levels (within a normal range) are not related to cardiovascular risk, measured through the metabolic syndrome. Two prior studies also reported no association between the CAR and the metabolic syndrome or its components in 65-year old men\textsuperscript{68} and elderly women\textsuperscript{69}, while a recent study reported a positive association for the metabolic syndrome, in women and not in men, aged 45-70 years\textsuperscript{70}. Most studies on the CAR and cardiovascular risk have focused on visceral obesity. A higher\textsuperscript{71-73} as well as a lower\textsuperscript{74} CAR have been associated with measures of abdominal obesity. We did find a significantly lower CAR in persons with cardiovascular disease free from psychopathology and a trend towards a lower CAR for a larger waist circumference. However, our sample was perhaps too young to detect these chronic somatic consequences. Central obesity is one of the main components of MetSyn and might precede other metabolic dysregulations\textsuperscript{75}. In older samples, the association between cortisol levels and cardiovascular risk is more consistent\textsuperscript{76}. Important work has been done by Rosmond and Björntop, who reported that in men, a reduced variation in the diurnal cortisol pattern (including low morning cortisol) was associated with metabolic dysregulations and predicted higher risk of cardiovascular events after five years\textsuperscript{77}. A low CAR as a result of long-term dysregulation or chronic stress might be a risk factor for metabolic abnormalities, possibly due to a decrease in inhibition of inflammation leading to more inflammation, which could result in somatic consequences, such as cardiovascular disease.
METHODOLOGICAL CONSIDERATIONS
Assessment of HPA axis activity

Measuring cortisol in saliva samples has many advantages. The sampling method can be performed at home, under normal conditions, and the collection of saliva is stress-free and non-invasive. This allows for a good indication of cortisol levels during day-to-day life, with minimal influence of the sampling method on the HPA axis. Cortisol as measured in saliva reflects the unbound biologically active form. Correlations of free and total cortisol are high, especially when saturation with cortisol binding globulin is low. It is easily possible to obtain measurements directly at awakening and multiple measurements during the day, to obtain an indication of the diurnal rhythm of cortisol levels, and it can easily be used in large-scale studies. Advantages of a large sample are inclusion of important covariates and increasing the generalizability of the results. Adjustment for covariates is important since many factors (external as well as internal) influence the HPA axis, of which we included most of the known influential factors. Often the number of saliva samples in large-scale studies is limited due to high costs. The minimum number of samples is one wake up sample, one sample 30-45 min after awakening and one bedtime sample. Additional samples, as we used, allow for a better definition of the diurnal cortisol curve. Unlike most large studies, we also included a salivary low dose DST. The response rate for saliva collection in our study was 74% and comparable to the mean response rate of 77% (range 25-93%) found for large-scale studies. We feel this is a high response, considering that most other large-scale studies were population-based and we included many persons with psychopathology, and considering that these respondents underwent an extensive examination. Persons who provided sufficient saliva samples differed from the remainder of the NESDA sample on some characteristics, i.e. responders were generally older, more educated, less often smokers, less often currently depressed or anxious (Chapters 2-8). No differences were observed in sex, body mass index, physical activity, metabolic syndrome, or chronic course. This could have resulted in an under-representation of persons with depressive or anxiety disorders in our studies. Possibly, effects would become stronger when we were able to include these respondents in our cortisol studies.

There are no generally accepted reference values for salivary cortisol levels. Even though the determinants were largely in accordance with previous reports, the 1-hour awakening cortisol in our study was lower compared to most other studies. A mean increase from awakening to 30 min later of 9.3 nmol/l (± 3.1 nmol/l), range 3.9-15.0 nmol/l, has been reported for 12 studies in the review by Clow et al. Therefore, the increase of 4.0 nmol/l (34.2%) found in our study was at the lower end of the range. The evening cortisol levels in our studies were comparable to levels found in other studies. Concerning the DST, non-suppression rates were difficult to compare to other studies, since several different methods
exist. Our suppression ratio of around 2.5 (=60% suppression) was in between the 54% suppression observed in 93 psychiatric outpatients and 74% in 33 controls. Most studies use rather arbitrary cut off values. Possible explanations for differences in cortisol values between studies are non-compliance (discussed below), differences in cortisol assays, and heterogeneity of the samples.

One of the most important issues when measuring salivary cortisol, is compliance with the sampling protocol. This is especially true for the CAR, since it is dependent on awakening and therefore it is crucial to take the first sample immediately after awakening. When there is a delay in sampling, the peak of cortisol levels may be missed, which could lead to negative CARs. This could explain why 26% of persons did not show a rise in cortisol level within the first hour after awakening. Kudielka et al. reported that when electronically monitoring sampling times, non-compliance resulted in a lower CAR. Electronically monitoring sampling is a way to check timing. However, it does not monitor time of awakening. Kupper et al. did monitor awakening through electrocardiograms and motility measurements in 700 participants. They reported that 11% showed a negative CAR, which was explained by later awakening than reported in most persons (n=77/700). However, Dockray et al. reported that even when awakening is closely monitored, still 15% of participants exhibit a negative CAR. In addition, reports suggest that the concordance between reported and actual awakening times is high. Future studies are warranted to evaluate whether a negative response is an artifact or represents a feature of the HPA axis. It remains to be determined, however, whether compliance is different across groups. Also for the DST, compliance with dexamethasone ingestion is essential. To examine the compliance, we measured dexamethasone levels with a radioimmunoassay using the anti-dexamethasone antibody from IgG Corporation Nashville, TN, functional detection limit is 0.4 nmol/l and reported cross-reactivity for cortisol is 0.04%.

Among 47 respondents with a T1/T7 ratio lower than 1.5 (indicative of non-suppression) who reported dexamethasone ingestion, we found detectable dexamethasone levels (>0.4 nmol/l) in the T7 saliva samples among 90%, indicating that non-compliance with dexamethasone ingestion is not likely to be frequent.

As we mentioned before, we did not measure current stress or state effects on the day of saliva sampling. We did instruct respondents to obtain the samples on a representative day without unusual amounts of stress. However, state effects (such as variations in mood, sleep) could still have played a role.

Furthermore, when the CAR differs between groups, this is an strong indication that there are differences in HPA axis activity. On the other hand, when there are no differences in the CAR, it could still be that HPA axis function is altered. For instance, the sensitivity of the adrenal gland could be affected, which may lead to higher levels of CRH and ACTH to maintain the same level of cortisol. Since we did not measure CRH and ACTH, future
research is needed to elucidate whether these hormones are affected in disorders for which we found no alterations in CAR, such as social phobia. Also, since cortisol as measured in saliva reflects the unbound free cortisol, it could still be that there are differences in total cortisol.

Finally, there are some remarks on the reliability of the measurement of saliva samples to be made. Although the intra-individual stability is reported to be reasonable for the cortisol awakening curve (AUCi correlations: 0.32-0.43, AUCg correlations: 0.39-0.67), the reliability significantly increases when measured on two days for the AUCg, or six days for the AUCi. However, to minimize costs and burden of participants, most large studies measure salivary cortisol on one day. Possibly, this might (partly) be compensated by the large sample size. Indeed, we observed several associations that were reported before in smaller-scale studies, which may support that we can compensate sampling on one day through a large sample size. Furthermore, measuring a complete CAR after dexamethasone could have increased the reliability of the DST. Concerning evening cortisol, possibly, a measure of diurnal rhythm across the day would have been more informative, including afternoon samples. However, it has been reported that diurnal slopes based on the wake up sample and the bedtime sample measured on two days, correlate 0.94 with slopes based on 6-7 samples per day on two days.

Assessment of psychopathology

DSM-IV based CIDI diagnoses were available for depressive and anxiety disorders. The CIDI is a standardized interview conducted by specially trained staff. It has a high inter-rater reliability, high test-retest reliability, and high validity for depressive and anxiety disorders. Depressive and anxiety disorders were studied in tandem, which is important since these disorders show high comorbidity and could have overlapping pathophysiologies. A distinction was made between remitted and current disorder to examine state versus trait effects and also persons at risk of depression or anxiety disorders due to diagnosis of the parents were included in the study. Also, multiple well established measures of psychological characteristics were ascertained. The longitudinal design of the study allowed us to examine whether baseline cortisol levels predict the course of depressive and anxiety disorders. Several depressive and anxiety disorders were diagnosed in the study, however, we did not diagnose PTSD. Although overt PTSD was an exclusion criterion, it could still be that persons with PTSD were included. Possibly, this could have influenced our results. The influence could be rather limited, since a recent meta-analysis by Meeuwisse et al. showed no significant differences in cortisol levels between persons with and without PTSD, except in certain subgroups.
CLINICAL IMPLICATIONS

The CAR as a diagnostic tool

In order to evaluate the clinical relevance of the cortisol awakening curve, we would like to speculate on the role of the CAR as a diagnostic tool, biomarker or research tool. The aim of a diagnostic test is to discriminate between persons with and without the disease. However, the effect size of an increased cortisol awakening curve in MDD is not large enough to distinguish persons with and without MDD. There is too much overlap between cases and controls. In addition, it is possibly a trait marker therefore not suitable as diagnostic tool or biomarker, which need to measure a state. Furthermore, a higher CAR was also associated with panic disorders with agoraphobia and possibly with other disorders. A low specificity and sensitivity are therefore expected. Second, a diagnostic test needs to present precise, accurate and reproducible information. This criterion is also not sufficiently met for the CAR, since it is influenced by many factors, is not that easy to measure (it needs to be measured on 2-6 days) and is highly dependent on compliance. These factors will lead to a decreased reliability. Finally, the clinical relevance of the CAR remains to be determined. In this thesis, it was not associated with diagnoses or characteristics that could not be ascertained by psychiatric evaluation, except perhaps the risk of a chronic course. Taken together, the CAR does not fulfill the criteria for a diagnostic test for depression.

The CAR as a research tool

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes (major causal pathway) or pharmacological responses to a therapeutic intervention. The CAR is not a very objective measurement as it is influenced by many factors, highly dependent on awakening, which is difficult to control, and compliance. The CAR reflects HPA axis activity; the AUCg reflects the basal activity and the AUCi the response to awakening. A higher or lower CAR has been associated with disease, such as MDD, panic disorder with agoraphobia and chronic fatigue syndrome. HPA axis hyperactivity is believed to be a major causal pathway for MDD. There is some indication that antidepressants normalize HPA axis dysregulation. We also observed a lower CAR in TCA users. However, a recent meta-analysis reports no significant differences in cortisol pre- and post-treatment. The utility of cortisol as an outcome measure may be limited to subgroups of patients. Therefore, it is too early to say whether the CAR fulfills criteria for a biomarker. Possibly, the utilization of cortisol measures as biomarker can improve by using multiple measurements of cortisol level and using the change in cortisol levels as a biomarker. Alternatively, the Dexamethasone/CRH test is sensitive in measuring treatment response, although this was tested in inpatients only.
A biomarker is not a substitute for clinical judgement, however, it could be important for the development of new antidepressants or anxiolytics.

Since we (and others) found evidence that an increased cortisol awakening curve represents a trait, this curve could be used as an endophenotype in genetic analyses. An endophenotype is a psychiatric term, used for a measurable internal component along the pathway between disease and genotype\textsuperscript{109}. An endophenotype should be associated with the disease, be heritable, state-independent, co-segregate with illness within families and should be found in non-affected family members at a higher rate than in the general population\textsuperscript{109}. We found some evidence that a higher cortisol awakening curve is associated with MDD and panic disorder with agoraphobia, parental history of MDD or panic and that it is state-independent. However, effects are small and especially the heritability needs to be further investigated.

Although it has no apparent clinical use (yet) as a diagnostic tool or biomarker, it is a valuable tool to be used in research on understanding the pathophysiology of stress-related disorders, such as depressive and anxiety disorders. Salivary cortisol could be causally connected to depressive and/or anxiety disorders and could therefore be used as a biomarker or endophenotype in research. Promising results have been presented in this thesis and other studies\textsuperscript{65} for the relevance of the CAR in (especially mental) disorders. The usefulness of the CAR as a research tool has also a potential clinical benefit, since it improves our knowledge of the pathophysiology of stress-related disorders and could therefore eventually lead to development of treatment or prevention strategies.

**Medication and prevention**

Although medication with a purpose to normalize cortisol levels is being developed, indication of these medicines in outpatients with depression or anxiety disorders is too premature. Blockage of the GR has been observed to be efficacious in depression, but only in the most severe and psychotic type\textsuperscript{110}. This thesis does not provide evidence for a severely dysregulated HPA axis in outpatients with depressive and anxiety disorders. Whether we observed clinically relevant differences in the CAR remains to be examined, since the effect sizes were small to medium. Of the disorders, lifetime MDD and current PDA seem the most promising candidates. Our results also implicate that current antidepressants already normalize HPA axis function, however, this needs to be examined in experimental studies. Finally, a lower CAR was associated with a chronic course, when this is replicated in more studies, this finding could lead to a more intensive treatment of persons exhibiting a low CAR in order to prevent a chronic course.
FUTURE RESEARCH DIRECTIONS
This thesis aimed to provide a further insight into the possible link between salivary cortisol levels and depression and anxiety disorders. This could give rise to more studies examining the relevance of these measures, in particular the CAR. To determine the generalizability of our results to extended samples, additional studies examining depressed inpatients or patients with bipolar disorder, and larger samples with specific anxiety disorders (e.g. PTSD) can be informative. In addition, to further examine whether the elevated cortisol awakening curve represents a biological vulnerability for depressive and anxiety disorders, longitudinal studies including genetic markers (e.g. MR and GR polymorphisms) and additional environmental factors (e.g. childhood experiences) are recommended. Also, additional large-scale longitudinal studies are required to confirm our findings for the role of HPA axis activity in especially the course of depressive and anxiety disorders. These studies should further unravel the involvement of potential additional genetic factors and chronic stress. Finally, more research is needed to examine the role of functional and structural brain changes associated with low versus high HPA axis activity in order to learn more about the actual underlying brain mechanisms that are in place. Especially (functional) neuroimaging of hippocampus, amygdala and prefrontal cortex could be informative since these brain structures are considered of prime importance in HPA axis regulation and/or mood regulation.

CONCLUSION
This thesis aimed to provide more insight into the role of the HPA axis in depressive and anxiety disorders in outpatients, taking into account important characteristics in a large sample. Taken together, the results of this thesis show increased morning cortisol levels in remitted and current MDD and current panic disorder. In contrast, a blunted CAR was associated with a chronic course over two years. So, the HPA axis indicator associated with presence of depression and anxiety disorders was completely different from the one predicting course of depression and anxiety disorders. These appear to reflect two distinct pathways. First, a higher cortisol awakening curve (AUCg) was found to reflect a biological vulnerability marker for depression and panic disorder. Second, among patients with depression and anxiety disorders, a lower CAR (AUCi) – possibly reflecting a high allosteric load during life – was found to predict a chronic course trajectory.
Reference


