Chapter 6

Salivary cortisol measures in major depression: comparison of inpatients and outpatients

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Abstract

Hypothalamic-pituitary-adrenal (HPA) axis dysregulation has been associated with major depressive disorder (MDD) and its severity, but results have been inconsistent. We compared patients with MDD from three different treatment settings (a primary care outpatient, a secondary care outpatient and one inpatient sample), with regard to several salivary cortisol measures.

The outpatient samples were drawn from the ongoing Netherlands Study of Depression and Anxiety (NESDA; 141 primary care and 199 secondary care outpatients with MDD), whereas the MDD inpatient sample (n =47) was recruited from five Dutch mental health hospitals. Cortisol levels in 7 saliva samples yielded the cortisol awakening response (CAR), evening cortisol, and cortisol levels after a 0.5-mg dexamethasone ingestion (dexamethasone suppression test (DST)). These were compared using analysis of covariance after adjustment of covariates.

The MDD patients had a mean age of 43.1 years (SD=11.6), and 37.5% were male. Inpatients, when compared to outpatients, showed higher cortisol levels at awakening (*P*=.01), higher evening cortisol (*P*<.001) and higher cortisol levels after dexamethasone ingestion (*P*<.001). In addition, inpatients with the highest severity scores (n=20) showed lower cortisol levels in the CAR (*P*= .01) and more non-suppression in the DST (*P*=.04) as compared to those with lower severity scores (n=27).

Inpatients showed higher cortisol levels at awakening, higher evening cortisol levels and higher cortisol levels after dexamethasone ingestion, indicating hypersecretion of cortisol.

Introduction

Dysregulation of the hypothalamic-pituary-adrenal axis (HPA-axis) is thought to play a central role in the pathophysiology of major depressive disorder (MDD).1;2 Cortisol is physiologically produced in reaction to stress, but in case of chronic inappropriate secretion it may induce unwanted adverse effects such as appetite and mood changes,1 as well as metabolic alterations such as insulin resistance, obesity, and hyperlipidemia.3

Cortisol levels reflect HPA-axis activity and can be measured in serum, 24 hour urine and saliva samples. Salivary cortisol reflects the active unbound fraction of cortisol and is considered to be the less stressful and invasive way of sampling.4 Chronically increased cortisol levels have frequently been found in MDD patients.5-9 The more extreme hypercortisolism found in Cushing's disease, is often accompanied by MDD and unfavourable cardiovascular outcomes.[10;11] Some studies suggested that chronic or more severe MDD may be accompanied by exhaustion of the HPA axis, as non-linear associations existed with cortisol levels.[12-14]

The collection of serial saliva samples enables the assessment of several cortisol measures, such as the cortisol awakening rise (CAR), area under the curve (AUC) with respect to the ground (AUC_g) and to the increase (AUC_i), evening cortisol and the dexamethasone suppression test (DST). The CAR is the natural increase of cortisol levels within 60 minutes after awakening,[15] which has frequently found to be increased among depressed subjects.[9;16] A blunted response, however, has been reported as well.[17;18] The following two measures reflect the dynamics of the CAR: the AUC_g and the AUC_i. The AUC_g is an estimate of the total cortisol secretion during the first hour after awakening and is related to the individual's total cortisol secretion throughout the day.[19] The AUC_i is a more dynamic HPA-axis measure that reflects cortisol reactivity.[19;20] Decreased CAR dynamics, objectively expressed by a decreased AUC_i, might reflect exhaustion of the HPA-axis and have been associated with more severe and/or chronic forms of depression.[12-14] Evening cortisol levels have been less extensively studied but were also found to be increased in depressed patients.[9] The dexamethasone suppression test (DST) depends on the negative feedback of the HPA axis,[21] and has repeatedly showed (more) non-suppression in patients with severe MDD.[8;9;22]

Most studies compared outpatient with psychopathology versus healthy controls. Studies on inpatients are far less numerous.[17;23-25] More severe symptomology and MDD with psychotic features have been associated with both higher basal cortisol levels and (more) DST non-suppression.[25-27] Also a blunted CAR was found in inpatients with MDD,[17;23;28] although it is unclear whether the severity of depression played a role. A meta-analysis[29] showed that hospitalization is associated with lower cortisol of a larger effect size when comparing depressed versus non-depressed groups, which was independent of symptom severity.

The Dutch mental care system follows a stepped care model.[29;30] This implies that increased treatment intensity, based on increased severity, can be offered in a more

specialized or advanced treatment setting. Therefore, in order to examine the effect of both depression severity and hospitalisation on cortisol, we systematically compared cortisol measures (i.e., morning, evening and DST cortisol measures) in MDD patients across three treatment settings (i.e., a primary care, a secondary outpatient and an inpatient sample). Based on the literature discussed above we expect inpatients to show more disturbances in different cortisol measures.

Methods

Participants

This study used three samples: two outpatient samples and one inpatient sample. Both outpatient samples consisted of participants of the Netherlands Study of Depression and Anxiety (NESDA), a Dutch longitudinal cohort study among 2981 individuals aged 18 through 65 years. Recruitment took place from September 2004 through February 2007. The detailed description of the NESDA baseline assessments has been reported elsewhere.[31] The first outpatient sample was drawn from primary mental health care outpatients, through 65 general practitioners (GPs). The second outpatient sample was drawn from secondary mental health care centers, where subjects have access to only after referral by a GP.

The inpatient sample (n=80) was recruited from five mental health hospitals, all part of GGZ Rivierduinen, a mental health care organization in the Netherlands, from January 2007 through December 2009. The detailed description of the recruitment process and inclusion criteria have been described elsewhere.[32] After inclusion, the interview, medical exam and blood draw were performed at the admission ward by a singular, by NESDA trained, researcher (FL). The study protocol was approved by the ethical review board of all participating centers and all participating subjects provided written informed consent.

In order to systematically compare the samples, only outpatients with a current MDD (1-month recency), were selected. Diagnosis of current depressive disorder was assessed by means of the composite international diagnostic interview (CIDI, WHO version 2.1), which classifies diagnoses according to the Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM-IV) criteria (American Psychiatric Association, 2001). Subsequently, we excluded a total of 3 pregnant and 2 breastfeeding women, and 42 subjects on corticosteroid treatment, leaving an initial sample of 525 outpatients and 78 inpatients. Further selection was made based on cortisol sampling participation (i.e. at least three valid morning cortisol samples had to be available) leaving 141 primary care outpatients, 199 secondary care outpatients and 47 inpatients as the definitive study sample.

Sociodemographic, clinical and sampling characteristics

Sociodemographic factors included sex, age, mean attained years of education and ancestry (Northern European or not). Sampling factors comprised awakening time and month with more daylight, according to earlier identified relevant factors.[33] General health characteristics

included smoking status (current/former/never), a current diagnosis of alcohol dependence and the presence of diabetes mellitus and/or cardiovascular disease (CVD). Because impairment of concentration and the anhedonic state of inpatients, we were frequently unable to assess detailed depression severity scales in this group. The number of CIDI items (CIDI-score), nine in total, reflecting the nine depression criteria according to the DSM-IV criteria, was therefore chosen as a proxy for depression severity. Other than the outpatient samples, the inpatients were additionally screened for the presence of psychotic features relying on the clinical experience of both the investigator (FL) and the treating psychiatrists. Medication use within the past month was registered by observation of drug containers brought in, and coded according to the Anatomical Therapeutic Chemical Classification System (ATC),[34] with special attention to the use of antidepressant and antipsychotic medication. Antidepressants were categorized into: selective serotonin-reuptake inhibitors (SSRIs [N06AB]), tricyclic antidepressants (TCAs [N06AA]) and 'other antidepressants' (which only consisted of serotonin and norepinephrine reuptake inhibitors (SNRIs) and mirtazapine [N06AX]).

Salivary cortisol measures

During the baseline interview, participating subjects were asked to collect saliva samples, after which they received instructions. For the outpatient samples this was done at their homes. Detailed description of outpatients saliva collection has previously been published.[33] For inpatients the samples were collected at the admission ward, when needed with the assistance of the nursing staff. Saliva samples were obtained using cotton Salivettes (arrested AG and Co, Nümbrecht, Germany) at 7 time points. Instructions prohibited eating, smoking, drinking and teeth brushing within 15 minutes before sampling.

The CAR includes 4 morning sampling points: at awakening (T1), at 30 (T2), 45 (T3) and 60 (T4) minutes afterwards. The two evening values were collected at 10 PM (T5) and 11 PM (T6). Dexamethasone suppression was measured by cortisol sampling the next morning at awakening (T7) after ingestion of 0.5 mg of dexamethasone the previous day (directly after the saliva sample was taken at 11:00 PM; i.e. T6). Saliva samples were stored in the refrigerators of the participants or in those of the admission ward, and returned by regular mail. After receipt, Salivettes were centrifuged at 2000*g* for 10 minutes, aliquoted, and stored at −80°C. Cortisol analysis was performed by competitive electrochemiluminescence immunoassay (E170; Roche, Basel, Switzerland) as described by van Aken et al.[35] The functional detection limit was 0.07 µg/dL (to convert to nanomoles per liter, multiply by 27.588) and the intra-assay and inter-assay variability coefficients in the measuring range were less than 10%. Data cleaning excluded values greater than 2SD above the mean. In line with an earlier publication of our group,[33] five cortisol indicators were used: two measures reflecting the CAR, the evening cortisol level, cortisol suppression after dexamethasone ingestion (DST), and the cortisol suppression ratio (CSR).

Cortisol awakening response (CAR)

We calculated the area under the curve (AUC) with respect to the ground (AUC_g) and with respect to the increase (AUC_i) based on the formulas by Pruessner et al.[19] For AUC analyses, at least 3 samples had to be available. For those with 1 missing cortisol value (i.e. 28 primary care outpatients, 26 secondary care outpatients, and 2 inpatients), the missing values were imputed using linear regression analyses including information on the 3 remaining cortisol levels, sex, age and smoking status.

Evening Cortisol

Data cleaning implied that T5 and T6 values greater than 2 SD above the mean were excluded (i.e., 10 and 6 respectively). This was only the case in outpatients. Evening cortisol was calculated as the mean of T5 and T6 cortisol levels, as the correlation coefficient between both values was rather strong (r = 0.89)

Dexamethasone Suppression Test (DST) and cortisol suppression ratio

Data cleaning implied that 15 values needed to be excluded because of values greater than 2 SD above the mean. In addition to the DST, the cortisol suppression ratio was calculated by dividing the cortisol value at awakening on the first day (T1) by the cortisol value at wakening the next day (T7) after ingestion of 0.5 mg dexamethasone the evening before. In addition, to indicate non-suppressors more clearly, we also used a dichotomized indicator of the T1/T7 ratio, with a ratio below 0.16354 (representing 1 SD below the mean) denoting non-suppression.

Statistical analyses

All cortisol values showed near normal distributions, except for the cortisol suppression ratio, which was log transformed before analyses.

Sociodemographic characteristics, sampling factors, health and clinical characteristics between inpatients and the two outpatient samples were compared by Chi-square test for categorical variables and by analyses of variance (ANOVA) for other variables, and described using percentages and means and standard deviations (SD) for categorical and quantitative variables respectively.

Analyses of covariance (ANCOVA) were conducted to analyse differences in CAR (with AUC_i and AUC_g), evening cortisol levels (cortisol level at 10 PM (T5), at 11 PM (T6) and the mean of both levels), cortisol suppression after dexamethasone ingestion, the DST (cortisol at T7) and the cortisol suppression ratio (log ratio of T1/T7), adjusting for demographic and sampling factors, and general health characteristics (i.e. sex, age, education, Northern European ancestry, awakening time, month with more daylight, smoking, alcohol dependence, diabetes, and CVD).

Additional multivariate linear regression analyses were conducted to assess the association between cortisol values and several clinical characteristics, adjusting for relevant covariates.

Variables	Primary care outpatients (n=141)	Secondary care outpatients (n=199)	Inpatients (n=47)	P-value
Sociodemographic characteristics				
Male sex (%)	34.8	36.2	51.1	.12
Age in (years) (mean, SD)	46.1 (11.4)	40.2 (10.9)	43.8 (11.2)	< .001
Educational level (years) (mean, SD)	11.9 (3.2)	11.8 (3.3)	10.7 (3.6)	.06
Northern European ancestry (%)	90.8	95.0	80.9	< .01
Sampling factors				
Time of awakening (mean, SD)	7:24 (1:13)	7:38 (1:12)	6:53 (1:05)	.001
Sampling in month with more daylight (%)	55.3	58.3	46.8	.36
General health characteristics (%)				
Smoking status				
Current	36.2	39.7	40.4	
Former	39.0	30.2	19.1	.09
Never	24.8	30.2	40.4	
Alcohol dependence	20.6	21.1	21.3	.99
Diabetes	5.0	5.0	4.3	.98
CVD	8.5	4.5	2.1	.16
Clinical characteristics				
CIDI-score (mean, SD)	6.9 (1.2)	7.4 (1.2)	8.1 (0.9)	< .001
Highest severity (9 CIDI items; %)	10.6	21.1	42.6	< .001
Psychotic features (interview; %)	0.0	0.0	12.8	< .001
Medication use (%)				
SSRI	17.0	37.7	40.4	< .001
TCA	0.7	5.0	19.1	< .001
Other antidepressant	4.3	13.1	31.9	< .001
Antipsychotics	0.0	5.0	19.1	< .001

Table 1. Comparison of demographic, health and clinical characteristics between primary care and secondary care outpatients and inpatients with current MDD.
Means with standard deviations (SD) or percentages are given, when appropriate. Abbreviations: MDD, major depression disorder; CVD, cardiovascular disease; CIDI, composite international diagnostic interview; SSRI, selective serotonin-reuptake inhibitor; TCA, tricyclic antidepressant.

As the most significant disturbances in cortisol levels were found for the most severely affected samples [25-27] we examined the impact of depression severity in more detail with in the inpatient group. This group was dichotomized based on the median of the CIDI-score of the inpatients: one with a CIDI-score of 9 and one with 8 or less. Using analyses of covariance we compared both inpatient subgroups with regard to all cortisol variables. As symptom severity is conceptually a continuous variable, additional regression analyses were performed to examine the effect of the CIDI count severity measure as a continuous parameter.

Variables	n	Primary care outpatients	n	Secondary care outpatients	n	Inpatients	P-value
Crude values							
- T1 (nmol/L), at awakening	141	17.38 (0.58)[a]	199	16.29 (0.48)[a]	47	19.51 (1.00)[b]	.01
- T2 (nmol/L), 30´ after awakening	141	21.61 (0.81)	199	20.13 (0.68)	47	21.26 (1.41)	.36
- T3 (nmol/L), 45´ after awakening	141	20.11 (0.82)	199	19.03 (0.69)	47	20.23 (1.42)	.53
- T4 (nmol/L), 60´ after awakening	141	17.72 (0.82)	199	17.16 (0.69)	47	19.14 (1.41)	.47
- AUC_g (nmol/L/h)	141	19.69 (0.65)	199	18.53 (0.55)	47	20.30 (1.13)	.22
- AUC_i (nmol/L/h)	141	2.31 (0.52)	199	2.23 (0.44)	47	0.79 (0.90)	.31
Adjusted values							
- T1 (nmol/L), at awakening	141	17.21 (0.58)[a]	199	16.23 (0.49)[a]	46	20.37 (1.04)[b]	.002
- T2 (nmol/L), 30´ after awakening	141	21.19 (0.82)	199	20.36 (0.69)	46	21.79 (1.46)	.59
- T3 (nmol/L), 45´ after awakening	141	19.92 (0.82)	199	19.14 (0.69)	46	20.52 (1.45)	.62
- T4 (nmol/L), 60´ after awakening	141	17.74 (0.82)	199	17.13 (0.68)	46	19.36 (1.45)	.39
- AUC_g (nmol/L/h)	141	19.45 (0.66)	199	18.62 (0.55)	46	20.81 (1.16)	.22
- AUC_i (nmol/L/h)	141	2.23 (0.53)	199	2.39 (0.44)	46	0.45 (0.93)	.17

Table 2. Comparison of morning cortisol variables between primary care and secondary care outpatients and inpatients with current MDD.
Means with standard errors (SE) are given.
Adjusted means are calculated by analysis of covariance adjusted for sex, age, education, Northern European ancestry, awakening time, month with more daylight, smoking, alcohol dependence, diabetes and CVD.
AUC_g denotes the area under the curve with respect to the ground.
AUC_i denotes the area under the curve with respect to the increase.
[a,b]: Values in the same row with different superscript letters denote statistical significance, $P < 0.05$ (post hoc Sidak test).

Variables	n	Primary care outpatients	n	Secondary care outpatients	n	Inpatients	P-value
Crude values							
- T5 (nmol/L), at 10 PM	139	5.93 (0.35)	192	5.40 (0.30)[a]	47	7.32 (0.61)[b]	.02
- T6 (nmol/L), at 11 PM	138	5.41 (0.31)[a]	197	5.32 (0.26)[a]	47	7.84 (0.53)[b]	< .001
- Evening cortisol	140	5.70 (0.29)[a]	198	5.39 (0.24)[a]	47	7.58 (0.50)[b]	< .001
- DST (nmol/L), at awakening	140	7.23 (0.33)[a]	190	7.12 (0.28)[a]	43	9.83 (0.59)[b]	< .001
- Cortisol suppression ratio	140	0.39 (0.02)	190	0.38 (0.02)	43	0.31 (0.03)	.07
Adjusted values							
- T5 (nmol/L), at 10 PM	139	5.83 (0.34)	192	5.47 (0.29)[a]	46	7.23 (0.59)[b]	.03
- T6 (nmol/L), at 11 PM	138	5.44 (0.30)[a]	197	5.30 (0.25)[a]	46	7.78 (0.5)[b]	< .001
- Evening cortisol	140	5.65 (0.27)[a]	198	5.42 (0.23)[a]	46	7.52 (0.47)[b]	<.001
- DST (nmol/L), at awakening	140	7.12 (0.33)[a]	190	7.22 (0.28)[a]	42	9.92 (0.61)[b]	< .001
- Cortisol suppression ratio	140	0.40 (0.02)	192	0.37 (0.02)	42	0.32 (0.03)	.12
- Non-suppression	140	10.7	190	12.1	42	20.9	.27

Table 3. Comparison of evening and Dexamethasone suppression test (DST) cortisol variables between primary care and secondary care outpatients and inpatients with MDD.

Means with standard errors (SE) are given.

Evening cortisol levels denote the mean of T5 and T6 cortisol levels.

DST denotes Dexamethasone suppression test (T7 cortisol level after dexamethasone ingestion).

Cortisol suppression ratio is calculated as natural log of T1 cortisol divided by T7 cortisol after DST.

Adjusted means are calculated by analysis of covariance adjusted for sex, age, education, Northern European ancestry, awakening time, month with more daylight, smoking, alcohol dependence, diabetes and CVD.

[a,b]: Values in the same row with different superscript letters denote statistical significance, $P < 0.05$ (post hoc Sidak test).

All tests were two-tailed with $P < .05$ denoting statistically significance. Statistical analyses were done with SPSS version 20.0 statistical software (SPSS Inc., Chicago, USA).

Results

Sociodemographic and sampling factors, general health and clinical characteristics

Characteristics across treatment settings are presented in Table 1. The mean age of all subjects was 43.1 years (SD=11.6), and 37.5% was male. Primary care outpatients were significantly older than the others two groups, while secondary care outpatients more often had Northern ancestry. Inpatients woke up at an earlier time, showed higher depression severity levels, more psychotic features and used more psychotropic drugs.

With regard to saliva sampling the groups did not differ significantly in response rate. Of the eligible patients with MDD, 70.2% of the primary care outpatients, 63.8% of the secondary care outpatients and 58.8% of the inpatients completed all 4 morning saliva samples (P = .08). Among inpatients, cortisol sampling participants did significantly differ from non-participants with regard to the mean CIDI-score (8.7 for non-participants *vs.* 8.1 for participants; p=.004) and the prevalence of highest severity (96.8% of the non-participants had a CIDI-score of 9 vs. 72.3% of the participants, p=.005). Outpatient cortisol sampling participants were more often from Northern European ancestry (93.5% vs. 86.3%, p=.004), were more often former (32.9% vs.18.1%, p=<.001), or current smoker (39.1% vs. 58.3%, p=<.001), and had less often a CIDI-score of 9 (27.2% vs. 35.8%, p=.01), although the overall mean CIDI-score components was of borderline significance (p=.06).

Cortisol awakening response (CAR)

Comparisons between inpatients and outpatients with regard to morning cortisol levels (CAR) are presented in Table 2. Inpatients presented only higher mean T1 cortisol levels compared to primary and secondary care outpatients in both crude and adjusted models.

Evening cortisol and DST

Comparisons between inpatients and outpatients with regard to T5, T6 and mean evening cortisol levels and DST are presented in Table 3. Inpatients showed significantly higher levels in all evening cortisol and DST variables in adjusted analyses. The cortisol suppression ratio did not differ across treatment settings (Table 3), nor did the percentage of non-suppression (primary care outpatients 10.7%, secondary care outpatients 12.1% and inpatients 20.9%, P= .20) even after adjustment. The adjusted values between treatment settings of all cortisol measures are shown in Figure 1.

Based on earlier findings on the association between psychotic depression and higher DST levels,[27;36] we additionally compared inpatients with and without psychotic features with regard to morning (AUC_g, AUC_i), evening and cortisol levels after dexamethasone ingestion. Other than expected, we found no differences between inpatients with and without psychotic

depression (results not shown), but this could be due to the small number of inpatients suffering from psychotic depression (n=6).

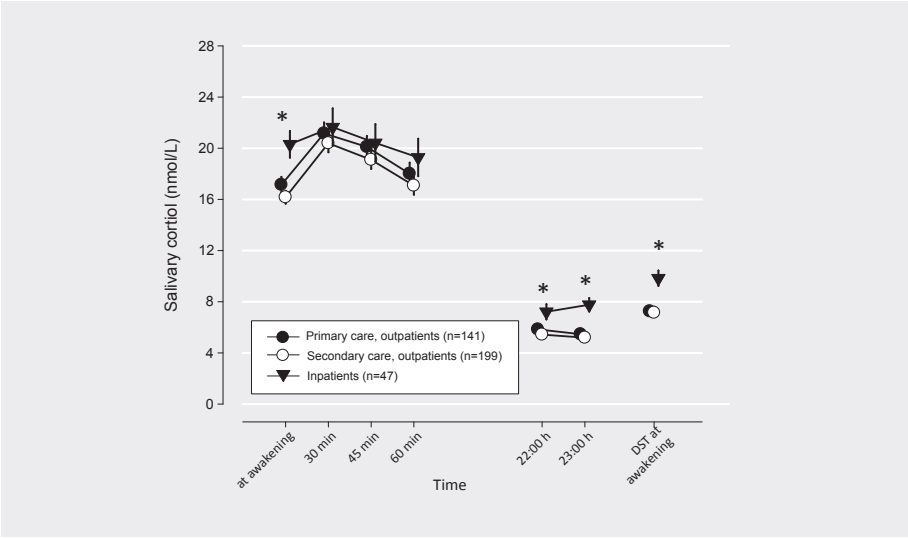


Figure 1. Adjusted salivary cortisol levels in the morning, evening and after a Dexamethasone suppression test (DST) in primary care and secondary care outpatients and inpatients with MDD.
Cortisol levels were adjusted for sex, age, education, Northern European ancestry, awakening time, month with more daylight, smoking, alcohol dependence, diabetes and CVD. Mean values are given, with error bars representing standard errors. * P-value < .05.

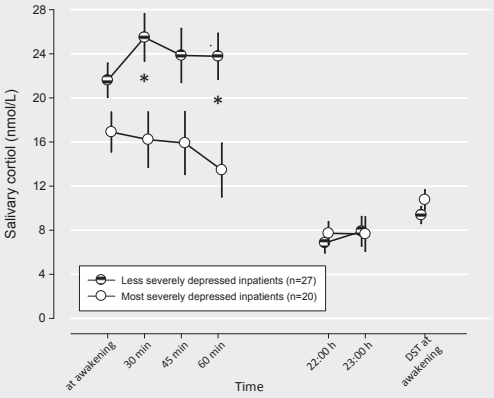
Additional analyses

Additional multivariate linear regression analyses to investigate whether the differences across the three patient groups might be explained by clinical characteristics showed a small effect of the CIDI-score on the AUC_g (β= - 0.10, *P*= .03, in fully adjusted models) indicating that higher CIDI-scores were associated with a smaller AUC_g, although the AUC_g values did not differ across treatment settings. Further, we found a small negative effect of TCA use on AUC_i (β= - 0.10, *P*= .03, in fully adjusted models). The characteristic 'treatment setting' had a consistent effect on evening and DST cortisol values in adjusted analyses (β_{T5} = 0.14, *P* < .001; β_{T6} = 0.25, *P* < .001; β_{T7} = 0.20, *P* < .001; β_{DST} = – 0.10, *P* = .04). Further, in fully adjusted models, we found a medication effect of TCA (β= 0.14, *P*= < .01) and antipsychotics (β = 0.14, *P* < .01) on DST.

Next, we compared the two inpatient subgroup based on the CIDI severity using analyses of covariance. Similarly, these results showed lower T2, T4 and AUC_g values among the most severely depressed (CIDI-score = 9; n=20) compared to those with lower severity (CIDI-score

Variables	n	Less severely depressed inpatients	n	Most severely depressed inpatients	P-value
Morning cortisol variables					
- T1 (nmol/L), at awakening	27	21.59 (1.55)	20	16.90 (1.80)	.07
- T2 (nmol/L), 30´ after awakening	27	25.48 (2.14)	20	16.22 (2.49)	.01
- T3 (nmol/L), 45´ after awakening	27	23.85 (2.44)	20	15.90 (2.84)	.06
- T4 (nmol/L), 60´ after awakening	27	23.77 (2.08)	20	13.46 (2.42)	.01
- AUC_g (nmol/L/h)	27	23.89 (1.86)	20	15.97 (2.16)	.01
- AUC_i (nmol/L/h)	27	2.96 (1.06)	20	-0.94 (1.23)	.07
Evening cortisol and DST variables					
- T5 (nmol/L), at 10 PM	27	6.85 (0.90)	20	7.72 (1.05)	.56
- T6 (nmol/L), at 11 PM	27	7.90 (1.33)	20	7.65 (1.55)	.91
- Evening cortisol level (nmol/L)	27	7.37 (0.91)	20	7.68 (1.06)	.84
- DST (nmol/L), at awakening	27	9.37 (0.76)	20	10.76 (0.90)	.28
- Cortisol suppression ratio	27	0.36 (0.04)	20	0.22 (0.05)	.04
- Non-suppression (%)	25	12.0	18	33.3	.051

Supplementary Table. Comparison of cortisol variables between less severely depressed inpatients versus most severely depressed inpatients.
Means with standard errors (SE) are given.
Less severely depressed inpatients had a CIDI-score of 5 through 8 MDD symptoms, while most severely depressed inpatients had a CIDI-score of 9 symptoms.
Adjusted means are calculated by analysis of covariance adjusted for sex, age, education, Northern European ancestry, awakening time, month with more daylight, smoking, alcohol dependence, diabetes and CVD.



Supplementary Figure. Adjusted salivary cortisol levels in the morning, evening and after a Dexamethasone suppression test (DST) in less severely depressed inpatients (CIDI count 5 through 8) versus the most severely depressed inpatients (CIDI-score = 9).
Cortisol levels were adjusted for sex, age, education, Northern European ancestry, awakening time, month with more daylight, smoking, alcohol dependence, diabetes and CVD. Mean values are given, with error bars representing standard errors. * P-value < .05.

<9; n=27) In sum, the most severe affected group showed lower cortisol levels in T2, T4, AUC_g (Supplementary Table and Figure). For evening and DST levels, the "most severe" subgroup showed lower cortisol suppression ratio (P = .04) and slightly more non-suppression (P = .051).

Discussion

In this study we compared primary and secondary care outpatients with inpatients, all with a current MDD, with regard to several cortisol measures. Our main finding was that inpatients showed higher morning cortisol levels at awakening (T1), higher evening cortisol levels and higher cortisol levels after dexamethasone ingestion (DST levels). Many studies have examined the association between depression and cortisol levels. A large meta-analysis quantitatively summarized the literature comparing hypothalamic-pituitary-adrenal (HPA) axis function between depressed and non-depressed individuals,[29] indicating that HPA-axis over-activity is found in depression, especially in older inpatients, independently from depression severity. Still, studies examining cortisol measures among depressed inpatients include primarily case-control studies.[8;37-39] Studies specifically comparing inpatients and outpatients are even scarcer,[21;25;26] but the three we are aware of indicate that inpatients present higher morning cortisol levels[26] and more non suppression[21;25] compared to outpatients. To our knowledge, our study is the first to systematically compare subjects from three different treatment settings, all suffering from a current major depression, with regard to HPA-axis activity, taking into consideration morning, evening and DST cortisol measures.

Many previous studies described higher morning cortisol levels among depressive subjects, both among inpatients when compared to outpatients or controls,[8;26;39] and among outpatients and community-based studies.[5;9;40] In case of inpatients, increased morning cortisol, a reflection of increased HPA-axis activity, might be caused by on the one hand more severe psychopathology and on the other hand the hospitalization per-se.[26;29] The higher T1-levels found among our inpatient sample are a visible part of higher CAR levels and reflect increased HPA-axis activity. Sleeping disturbances are very common among patients with MDD,[41;42] and the effects of sleep(disturbances) on the HPA-axis are well reported.[43] In addition, brief awakenings increase the time a subject is awake,[43] causing the onset of the CAR to happen earlier in time. It cannot be ruled out that inpatients, more prone to frequent nightly awakenings and often prematurely awake, had their CAR before starting the morning saliva sampling procedure. The measured T1 might in fact be the real T4 and the last visible trace of the extinguishing CAR. However, conflicting results have been described as well.[38]

Evening cortisol levels are usually low. The relatively few studies investigating the association between evening cortisol levels and MDD, found higher evening cortisol levels in the most affected group, both in studies comprising inpatients,[44] and in those including outpatients.[9] These results are in line with our findings. The higher levels might be attributable to HPA-axis over-activity,[45] hospitalization,[26] sleeping disturbances,[43] or the

result of postponing ones normal bedtime.[46]

The most consistent findings have been described for DST, where quite uniformly more non-suppression has been reported in inpatients than outpatients.[8;21;22;47] We found inpatients to have higher cortisol levels after dexamethasone ingestion (DST) and comparable cortisol suppression ratios compared to outpatients, although in the crude models there was a trend towards significance (*P*= .07). Our additional analyses showed that the inpatients with the highest CIDI-scores showed lower values for the cortisol suppression ratio (*P*= .04) and for the percentage of non-suppression (*P*= .05). Strictly speaking, we cannot state that inpatients showed more non-suppression compared to outpatients. However, the finding that the most severely affected inpatients showed lower cortisol levels after dexamethasone ingestion and had more non-suppressors, is consistent with the literature, where studies comparing inpatients to healthy controls showed more non-suppression in the more severely affected group.[8;21;47] The fact that inpatients showed higher cortisol levels in the DST, but no significant difference in the cortisol suppression ratio or the percentage of non-suppression compared to outpatients, may be a consequence of low statistical power, as the "most severe" inpatients group, being the smallest in sample size, was most likely the one responsible for potential differences between inpatients and outpatients.

Depression severity may be of importance,[21] a previous study of our group did not find more non-suppression among currently depressed subjects nor found an association with depression severity.[9] In addition, other than expected[27;36] we did not find DST differences when comparing inpatients with and without psychotic features, while depressive disorders with psychotic features are considered to be the more severe forms of depression. Some studies suggest that characteristics other than severity may play a role, such as a biological vulnerability as an explanation for increased cortisol levels in depression.[48] There is evidence that different depressive subtypes differ not only in their symptom presentation, but also in their biological correlates.[49] 'Treatment setting' might be a better discriminative variable when comparing different 'severity' samples as it bundles different characteristics such as comorbidity, psychotic features, the presence of specific symptoms and medication use.

In our additional analyses comparing in the highest CIDI-score with the other inpatients, we found less elevated cortisol levels (T2, T4, AUC_g, cortisol suppression ratio) in the most severely depressed group (results shown in supplementary Table and Figure). This is further supported by our finding that higher CIDI-scores were inversely associated with AUC_g levels suggesting a blunted CAR.[17] Previous findings[12-14] are in line with the idea that the HPA-axis may be prone to exhaustion during a prolonged and more severe episode of MDD. These results fit the hypothesis that up to a certain "turn-over-point", inpatients show higher cortisol levels. Once this certain level has passed, the HPA-axis is exhausted and inpatients present impaired HPA-axis dynamics (expressed by a blunted CAR and lower AUC_g and AUC_i levels). Although we did not find significant differences in AUC_i, the finding that TCA use, more present among inpatients, was associated with lower AUC_i levels, supports the

idea of impaired HPA-dynamics in a severely affected group. Taking together the above with the finding that inpatients have higher cortisol levels after dexamethasone ingestion, our results might indicate that a significant dysregulation of the HPA-axis and its negative feedback system is confined to severe forms of depression as found in inpatients. This idea is supported by the fact that earlier studies finding DST non-suppression were conducted among more severely inpatients with melancholic or psychotic features.[26;27] Of course, if sleep disturbances are in fact more prominent among the most severely affected group, early awakening might (additionally) contribute to the lower morning cortisol levels, as the CAR might already have been passed and therefore extinguished.

This study presents some limitations. First, we were not able to examine whether the origin of our findings lie in a different biological vulnerability or in a 'scar' effect due to longer existing illness and/or medication use. Second, CIDI-score might be considered as a crude proxy for severity of depression, missing the characteristic to discriminate between different depression subtypes, such as melancholic or atypical depression. In addition, as a consequence of the required minimum of 5 DSM-IV criteria to fulfill the criteria for MDD, all included subjects had a minimum CIDI-score of 5. Consequently, the range of our severity measure was rather limited. Third, the sample of 47 inpatients was rather small, although it is one of the larger inpatient samples studied so far in this field of research. As 77% of eligible inpatients agreed to participate, the 23% drop-outs may have introduced selection bias as the more severely depressed patients may have been less likely to participate. The study presents several strengths as well. To our knowledge, this is the first study to systematically compare cortisol measures across three different treatment settings, all including patients with a current MDD. Further, we examined several cortisol measures reflecting not only basal cortisol levels, but also the dynamics of the HPA-axis. In order to examine the origin of the found differences, future longitudinal studies, with larger inpatient samples, are warranted, taking into consideration more extensive examination of non-suppression. The use of actigraphy along with cortisol sampling is recommended to better monitor and understand the role of awakening and sleep disturbances. Further, saliva cortisol measurements could be supplemented by relatively new methods such as cortisol measurements in hair.50 And, more research is warranted to examine whether there is in fact a "turn-over-point" and to assess which factors contribute to a proper definition of "severity".

Conclusions

This study showed that inpatients present elements of HPA-axis over-activation as expressed by a higher cortisol awakening level (T1), higher evening cortisol levels and higher cortisol levels after dexamethasone ingestion. In addition, the most severely depressed subjects in the inpatients group showed signs of HPA-axis exhaustion, expressed by a blunted CAR.

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