Stressed-out stress systems: dysregulated stress-systems in the pathophysiology of stress-related disorders

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Chapter 2

Elevated salivary alpha-amylase levels at awakening in patients with depression

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Abstract

Introduction

Specific Major Depressive Disorder (MDD) biomarkers could help improve our understanding of MDD pathophysiology and aid in the refinement of current MDD criteria. While salivary cortisol (SC) can differentiate between healthy controls and patients with psychiatric disorders, salivary alpha amylase (sAA), may be a putative candidate biomarker for MDD specifically.

Methods

In a naturalistic cohort of consecutive out-patients and healthy controls, sAA and SC were determined in 833 participants (97 MDD patients, 142 patients with other mood, anxiety, and/or somatoform (MAS-) disorders, and 594 healthy controls). Samples were collected at 7 different time points (at awakening, after 30, 45, and 60 min, at 10:00 p.m., at 11:00 p.m., and at awakening on day 2).

Results

The mean age of the sample was 43.8 years (SD=12.9; 63.9% female). Concerning sAA, MDD patients had higher sAA levels upon awakening on two consecutive days ($p=0.04$, $p=0.01$ respectively), as well as a higher area under the curve with respect to the increase (AUCi; $p=0.04$) in comparison to both controls and the other MAS-disorders group. Regarding SC, mean levels of evening SC were elevated in MDD patients ($p=0.049$) in comparison to both controls and the other MAS-disorders group. SC values on day 2 after ingestion of dexamethasone were elevated in both MDD patients and the other MAS-disorders group ($p=0.04$, $p=0.047$ respectively).

Conclusions

sAA at awakening and not cortisol differentiates MDD from other psychiatric disorders in outpatients. This suggests that sAA may be a valuable candidate biomarker specifically for MDD.

1. Background

Major depressive disorder (MDD) is one of the most common psychiatric disorders and the leading cause of disability worldwide (WHO, 2017). Despite extensive research, no specific biomarkers for MDD have been identified to date (Fried and Nesse, 2015). Although the mean level of salivary cortisol (SC; a hormone reflecting hypothalamus-pituitary-adrenal (HPA) axis activity) has often been found to be elevated in patients with mood, anxiety, and somatic disorders (MAS-disorders), this possible biomarker lacks the potential to distinguish among these disorders (Rief et al., 1998; Hellhammer et al., 2009). Recent studies suggest that salivary alpha amylase (sAA), a newer candidate biomarker that involves the autonomic nervous system (ANS) axis, may differentiate MDD from other MAS-disorders more specifically than SC (van Veen et al., 2008; Ishitobi et al., 2010; Booij et al., 2015).

sAA is an enzyme that is secreted under autonomic regulation in the oral cavity and accounts for 40–50% of salivary protein content (Rohleder et al., 2004; Schumacher et al., 2013). Studies with healthy controls have demonstrated that sAA presents a distinct diurnal cycle opposing that of SC (Nater et al., 2007; Rohleder et al., 2004), with lower levels in the morning and higher levels in the early evening. The enzymatic activity and quantity of sAA have been found to vary among individuals in relation to certain environmental factors, such as stress levels and circadian rhythms (Granger et al., 2007; Chatterton et al., 1996). Elevations of sAA levels are indicative of increased autonomic activity and have been found to occur in response to neurotransmitter (i.e. norepinephrine) stimulation (Garrett, 1999; Nater et al., 2005). Studies with healthy controls have shown that sAA is highly sensitive to acute stress-related changes, with increasing levels during psychosocial stress tasks (i.e. Trier Social Stress Test, Kirschbaum et al., 1993; Nater et al., 2005, 2006; Rohleder et al., 2004). Within the MAS-patient population, interventional studies using psychosocial stress tasks in the afternoon (between 12:00 and 5:00 p.m.) have provided evidence that sAA levels in patients with anxiety disorders and MDD increase more than those of healthy controls (Tamura et al., 2013; Tanaka et al., 2012a, 2012b).

To our knowledge, five previous observational studies on sAA have been conducted in patients with mood and anxiety disorders: two in a laboratory setting and three in a naturalistic setting. The laboratory studies found low baseline sAA levels in MDD patients in the morning (Cubala and Landowski, 2014), and elevated sAA levels in the afternoon (Ishitobi et al., 2010) in comparison to healthy controls. Two naturalistic studies were conducted with MDD patients and found higher sAA levels in the morning (no time specified), afternoon (six hours later), and early evening (six hours

after the afternoon sample) in comparison to healthy controls (Booij et al., 2015), as well as elevated levels of sAA in MDD patients using tricyclic antidepressants (but not SSRIs) in the late evening (between 10:00 and 11:00 p.m; Veen et al., 2013). In a naturalistic study with generalized anxiety disorder (gSAD) patients, elevated sAA levels were found in the area under the curve with respect to the ground (AUCg) on day 1 and at 4:00 p.m. on day 2 in comparison to healthy controls (van Veen et al., 2008). In sum, based on the small amount of naturalistic observational research carried out to date, it seems morning, afternoon, and early evening sAA levels may differentiate between MDD and healthy controls, whereas late evening sAA levels are less successful in doing so. Furthermore, gSAD patients seem to have elevated AUCg and afternoon sAA values in comparison to healthy controls. However, no studies have investigated sAA levels in patients with MAS-disorders at awakening to date.

In this study, we investigated awakening and diurnal levels of basal sAA and SC in patients with MDD, other MAS-disorders, and healthy controls. Our main aim was to determine whether sAA may differentiate MDD from other MAS-disorders at certain time points. Due to the naturalistic nature of our study, SC levels were determined to compare our samples to the distinct diurnal pattern of SC found in earlier studies. Replicating these SC diurnal patterns indicate adherence to the sampling protocol to a certain extent, and thus increase the validity of the saliva samples collected. We hypothesized that MDD patients would have the same diurnal sAA patterns as those found in healthy controls. However, based on the existing literature, we expected that the morning levels of sAA would be higher in the MDD patient group than in the other MAS-disorders patient group and the healthy controls group. Furthermore, we expected to find an elevation of sAA in the AUCg in both the MDD and other MAS-disorders patient groups in comparison to healthy controls. Finally, as previous studies have found the differences in SC levels to be most pronounced after a low-dose dexamethasone-suppression test (DST; Pruessner et al., 2003a; Stetler and Miller, 2005; Vreeburg et al., 2009, 2010; Wardenaar et al., 2011), we expected both the MDD group and other MAS-disorders group to have higher SC levels in comparison to the healthy controls upon the DST.

2. Methods

2.1. Participants

Participants were recruited from two different sources: The Routine Outcome Monitoring (ROM) and NormQuest cohorts. Patients who were recruited participated in ROM interviews between 2007 and 2011. A detailed description of the ROM protocol has previously been published elsewhere (De Beurs et al., 2011).

These were ambulatory patients who were referred to one of three Riverduinen Mental Health Clinic locations (GGZ Leiden RijnVeste, GGZ Lisse, or GGZ Katwijk mental health clinics), or the Department of Psychiatry in the Leiden University Medical Center (LUMC). These patients all underwent extensive ROM interviews as part of routine patient care and the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM IV) diagnoses were assessed by means of the Mini International Neuropsychiatric Interview (MINI-Plus; van Vliet and De Beurs, 2007). Healthy controls were recruited from the NormQuest cohort, a study that was initiated to obtain a reference group for the ROM patients (Schulte-van Maaren et al., 2013). NormQuest participants were recruited from the general population via medical practitioners. The NormQuest sample was stratified for gender, age, and urbanization to reflect the ROM patient group. The majority of the complete Norm-Quest sample (> 90%) did not have a psychiatric diagnosis according to the Mini International Neuropsychiatric Interview (MINI-Plus; van Vliet and De Beurs, 2007).

All of the recruited participants were asked to collect seven saliva samples on a regular working day for our biobank for Mood, Anxiety, and Somatoform disorders and the HPA-axis (MASHbank). In line with the protocol used in the Netherlands Study of Depression and Anxiety (NESDA, Vreeburg et al., 2010), participants were instructed to refrain from eating, drinking, smoking, or brushing teeth within the preceding 15 min of sampling. The protocol was approved by the LUMC Ethical Review Board. Of the 949 saliva samples collected from the patient and reference groups, 44 forms were returned without a date or lacked a ROM interview date. Cases that sampled>=60 days after the ROM interview were also removed from the dataset (n=23) as were cases missing Brief Symptom Inventory (BSI; Derogatis, 1975) total scores (n=15). Furthermore, we excluded NormQuest controls using psychotropic medication (n=26), participants from all groups whose medication use was unknown (n=7), as well as all participants lacking demographic information (n=6), resulting in a total of 39 excluded subjects. The final sample (N=828) was then categorized into three groups, namely 594 controls, 95 MDD patients, and 139 “other MASdisorders” patients with predominately current anxiety and/or somatoform disorders (43 patients in this group either did not receive a diagnosis on the MINI-Plus or were diagnosed with other psychiatric disorders such as remitted MDD with comorbid alcohol and/or drug abuse).

2.2. Saliva sampling

To determine the diurnal patterns, seven samples were obtained by means of Salivettes® (Sarstedt, Germany). A Salivette is a cotton wad in a plastic tube.

Participants were asked to remove the wad from the tube, chew on it gently for at least 2 min, place the wad back into the tube, and store it in the refrigerator. The instructions stated to collect morning saliva prior to breakfast at four time points: at awakening (T1), after 30 min (T2), after 45 min (T3), and after 60 min (T4). The fifth (T5) and sixth (T6) samples were to be collected at 10:00 p.m. and 11:00 p.m. respectively. After the sixth sample, participants were asked to prepare for a DST by ingesting 0.5 mg of dexamethasone. At awakening the next morning, participants were asked to collect the final saliva sample (DST/ T7). At each time point, participants were instructed to write down the exact time that saliva was collected on the sampling form. After return by mail, Salivettes were centrifuged, and the saliva was aliquoted and stored at −80 °C.

2.3. Laboratory analysis of salivary cortisol and sAA

Cortisol analysis was performed by the Cortisol (gen 1) competitive electrochemiluminescence immunoassay (ECLIA, Modular E170 immunoanalyser, Roche Diagnostics, Basel, Switzerland), as described in van Aken et al. (2003). The functional detection limit was 2.0 nmol/l and the intra- and interassay variability coefficients in the measuring range were less than 10%. Assays were repeated if cortisol levels were very high (> 80 nmol/l) or very low (< 1 nmol/l). All very high samples remained high and the mean of both values was used. In 80% of the very low samples, the repeated cortisol value was within the normal range and used for analysis, otherwise the mean was used. Three cortisol indicators were used: 1-hour awakening cortisol; evening cortisol; and DST.

After overnight thawing of the saliva at 4 °C, samples for alphaamylase analysis were diluted 50-fold with a Hamilton Microlab 500 B/ C dilutor in physiological saline solution (Versylene® Fresenius, Cat. Nr. B230551). Analyses were performed using a kinetic colorimetric assay for total amylase activity (Cat Nr. 03183742, Roche Diagnostics, Mannheim, Germany) on a routine clinical chemistry analyzer. The total amylase assay is standardized to the IFCC reference measurement procedure (Lorentz, 1998), guaranteeing worldwide comparability of the data. Amylase activities are measured and expressed in IU/L at 37 °C. Within-run imprecision for the control pool ranged from 0.7% to 2.7% for the combined predilution step and the analysis across the entire study period. Between-run analytical imprecision was lower than 5% throughout this study.

2.4. Data cleaning

SC and sAA samples with values higher than 3 SD from the mean were denoted

as missing (SC n=66; sAA n=114) as they exceed the realistic range for SC and sAA and are likely to be the result of measurement issues (e.g. gingivitis or bleeding gums). Missing data was imputed for cases that remained in the dataset by means of linear regression for both missing SC values (n=170; 2.9%), and for sAA values (n=129; 2.2%). Participants missing>=2 morning saliva samples and/or>=1 evening saliva sample were excluded (cortisol n=27; sAA n=20). Participants that did not note a sampling time and those that sampled outside of the sampling time ranges (for details of sampling times, see Appendix) were also excluded (morning SC and sAA n=29; evening SC and sAA n=34). Participants that did not ingest the 0.5 mg of dexamethasone (n=87) were excluded from the SC DST analyses. No cases were removed from the sAA samples in this regard, as no negative feedback effect is to be expected upon DST.

2.5. Saliva sample indicators

Three sAA indicators were used: the AUCg, and with respect to the increase (AUCi), and evening sAA levels. The AUCg and the AUCi were calculated in accordance with Pruessner's et al. formulas (2003b), and evening sAA levels were calculated using the T5 and T6 samples (T5+T6/2). Five salivary cortisol indicators were used: two measures reflecting the cortisol awakening response (CAR), the evening cortisol value, DST, and the cortisol suppression ratio (CSR). The CAR measures the increase in cortisol over four fixed time points within the first hour after awakening (e.g. T1-T4; Fries et al., 2009). These morning cortisol values allow for the calculation of the AUCg, and AUCi, which are used to reflect the dynamics of the CAR (Pruessner et al., 2003b; Fekedulegn et al., 2007). The low-dose DST assesses the (dis-)inhibition of the HPAaxis (Carroll et al., 1976), and the CSR indicates the cortisol suppression ratio (T1/T7).

2.6. Other measurements and covariates

The BSI (Derogatis, 1975), is a 53-item self-report questionnaire that was designed as a rapid screening method for symptoms of psychological disorders. In this study, the BSI total score was used as an index of general psychopathology. The total score is the mean score of all of the 53 items, whereby each item can range between 0–4 (a higher score indicates more general psychopathology). The BSI has been found to be both a reliable and valid measurement instrument (Croog et al., 1986; Conoley and Kramer, 1989).

Certain specific factors have been found to influence sAA levels and should be taken into account as potential confounders in epidemiological studies. There is evidence that carbohydrate intake increases sAA response in individuals whose ancestors

consumed starch-rich diets. Thus, those of Southern European ancestry experience a larger increase in sAA levels in comparison to those of Northern European ancestry (Perry et al., 2007; Rigaud et al., 2015). Furthermore, a study conducted in a sample of healthy participants (N=487), found that sAA levels are influenced by age and alcohol use (Veen et al., 2012). In addition to ancestry status, age was entered as a continuous variable and alcohol use the day of testing as a dichotomous variable. Additionally, we adjusted for gender and education level (no/low education versus middle/high education). Analysis of covariance (ANCOVA) was used to adjust for these sociodemographic factors.

Regarding SC, the following covariates were entered into the model: gender, age, Northern European (NE) ancestry, education level (to adjust for social economic status; no/low education versus middle/high education), season (more daylight hours versus less daylight hours), time of awakening (continuous), hours of sleep (< 6 h of sleep,>6 h of sleep),weekday versus weekend, and alcohol use the day of testing in adherence, where possible, to the expert consensus guidelines denoted by Stalder et al. (2016) to yield adjusted means.

2.7. Statistical analysis

Both the cortisol values and the sAA values showed positively skewed distributions and were therefore naturally loge transformed before analyses. Back-transformed geometric means and 95% confidence intervals (CI) of the mean are presented in the tables. The baseline characteristics of the three groups (MDD, other MAS-disorders, and healthy controls) were compared by means of $\chi 2$–tests for categorical variables and analyses of variance statistics (ANOVA) for continuous variables. These variables were expressed by means, percentages, and standard deviations (see Table 1 for further details). A twosided p-value<0.05 was considered statistically significant. No adjustments were made for multiple comparisons. IBM SPSS Statistics for Windows version 23 (IBM Corp. Armonk, N.Y., USA) was used for dataanalysis.

3. Results

3.1. Demographic characteristics

Characteristics of the three groups, (i) MDD patients (N=95, of whom 6 patients were diagnosed with dysthymia), (ii) patients with other MAS-disorders (N=139), and (iii) healthy controls (N=594), are presented in Table 1. The mean age of this sample was 43.8 years (SD=12.9), of which 63.9% was female. Gender distribution was similar amongst the three groups. MDD patients and patients with the other MAS-disorders

were more often less educated, less often of Northern European ancestry, less likely to have a partner, less likely to have a job, more likely to smoke, sleep fewer hours at night, and collect samples more often during the weekend in comparison to healthy controls. Use of alcohol on sampling days, sampling season, and time of awakening did not differ significantly between the groups. MDD patients had higher mean BSI total scores in comparison to both of the other groups. Of the patients in the MDD group, 34 patients had a comorbid anxiety disorder (35.1%), 8 patients had a comorbid somatic disorder (8.2%), and 11 patients had both disorders (11.3%). Regarding the time-interval between the interview and saliva sampling, patients in the other MAS-disorders group had the most days between the interview and saliva sample return, followed by the MDD group. The median time between the ROM interview and the sampling was 7.0 days (25th-75th percentile: 3–14). Regarding medication use, patients in the MDD group used more medication than those in the other MAS-disorders group (see Table 1).

3.2. Salivary alpha-amylase (sAA)

Comparisons of sAA levels between groups are presented in Table 2. sAA levels were found to be significantly elevated in the MDD group at T1, AUCi, and T7 ($p= 0.01$; $p= 0.04$; $p < 0.001$), in comparison to the sAA levels in healthy controls (see Fig. 1A). After multivariate adjustment (i.e. adjusted for gender, age, Northern European ancestry, education level, and alcohol use), these sAA levels remained significantly elevated in comparison to the control group (T1: $p= 0.04$ AUCi: $p= 0.04$; T7: $p= 0.01$ respectively). sAA levels in the other MAS-disorder group were lower than those of healthy controls at all time points, although none of these differences were statistically significant. These differences remained statistically insignificant after adjusting for confounders (see Table 2). Pearson correlations between mean sAA levels at awakening were found to be significantly associated with BSI depression subscale scores ($p=0.015$), indicating that there is a relationship between sAA levels and depression symptom severity. Finally, sAA levels were able to differentiate between the MDD group and the other MAS-disorders group at these sampling points after multivariate adjustment (T1: $p= 0.005$; AUCi: $p= 0.009$; T7: $p= 0.01$).

Table 1. Comparison of demographic and clinical characteristics between controls, patients with MDD, and patients with other MAS-disorders.

Variables	Controls (n = 594)	MDD[a] (n = 95)	Other[a] (n = 139)	p-value[c]
Sociodemographic characteristics:				
Female (%)	64.1	66.3	61.2	0.70
Age in years (mean, SD)	42.8 ± 12.5	47.2 ± 13.0	45.4 ± 13.6	0.50
Higher educational (%)	75.6	57.9	66.2	< 0.001
Northern European ancestry (%)	86.9	74.7	81.3	0.005
Marital status with partner (%)	73.7	55.8	67.6	0.001
Employment status (%):				
Employed (full-/part-time)	83.3	38.9	54.0	< 0.001
Unemployed/Sick leave	5.4	38.9	21.6	
Retired/stay-at-home parent	11.3	22.1	24.5	
Smokers n (%)[d]	80 (13.7)	27 (30.3)	29 (21.5)	< 0.001
Alcohol use on sampling days (%)				
Yes	69.3	79.8	71.6	0.13
No	30.7	20.2	28.4	
Sampling Factors:				
Time of awaking (mean, SD) [d]	6:57 ± 0:58	7:10 ± 1:03	7:08 ± 0:52	0.36
Hours of sleep (%)[d]				
< 6 hours	16.1	36.3	26.1	< 0.001
> 6hours	83.2	61.5	72.5	
Workday (%)[d]	77.0	44.0	54.8	< 0.001
Light season (April-September)	46.3	41.1	47.5	0.58
Clinical characteristics n (%):				
current comorbid anxiety disorder	–	35 (36.8)	6 (4.3%)	
current comorbid somatic disorder	–	8 (8.4)	6 (4.3%)	
current comorbid anxiety and somatic disorder	–	10 (10.5)	6 (4.3%)	
BSI total score (mean, SD)	0.18 ± 0.19	1.37 ± 0.67	0.59 ± 0.47	< 0.001
Assessment characteristics:				
Time interval between assessment and saliva sampling (days, SD)	9.2 ± 9.1	10.6 ± 11.5	12.4 ± 12.7	0.03
Medication use n (%)[b]:				
Using any psychotropic medication	–	49 (51.6)	31 (22.3)	< 0 .001
TCAs	–	5 (5.3)	3 (2.2)	< 0.001
SSRIs	–	25 (26.3)	14 (10.1)	< 0.001
Benzodiazepines	–	15 (15.8)	11 (7.9)	< 0.001
Stimulants	–	0 (0.0)	0 (0.0)	–
Antipsychotics	–	5 (5.3)	3 (2.2)	< 0.001
Stabilizers	–	3 (3.2)	3 (2.2)	< 0.001
Other ADs	–	16 (16.8)	3 (2.2)	< 0.001

[a] MDD denotes the patient group with current Major Depressive Disorder and/or current Dysthymia (n = 89 and n = 6 respectively). Other denotes the patient group with remitted mood disorders and/or other (comorbid) MAS-disorders.

[b] TCA denotes Tricyclic Antidepressant; SSRI denotes Selective Serotonin Reuptake Inhibitor; Other AD denotes other Antidepressants.

[c] P-values were achieved by using χ^2-tests for categorical variables and analyses of variance statistics (ANOVA) for continuous variables.

[d] smoking information was missing for 2.2% of the sample; alcohol intake information was missing for 3.4% of the sample; Time of awakening was missing for 2.4% of the sample; hours of sleep information was missing for 1.0% of the sample; workday information was missing for 2.2% of the sample.

Table 2. Comparison of morning and evening salivary alpha amylase (sAA) variables between controls and MDD patients and controls and other MAS-disorders patients.

Variables	n	Controls	n	MDD patients	p-value	n	Other MAS-disorders	p-value
Crude values								
Morning sAA variables								
T1 (U/ml) at awakening	554	63.8 (57.8; 70.4)	83	90.9 (71.8; 115.1)	**0.01**	129	55.2 (45.3; 67.4)	0.23
T2 (U/ml) 30' after awakening	554	37.3 (34.4; 40.6)	83	46.0 (37.6; 56.2)	0.07	129	34.9 (30.3; 40.3)	0.48
T3 (U/ml) 45' after awakening	554	40.1 (37.1; 43.5)	83	43.8 (36.3; 52.9)	0.43	129	36.5 (31.6; 42.2)	0.30
T4 (U/ml) 60' after awakening	554	44.4 (41.0; 48.0)	83	48.5 (38.0; 62.0)	0.44	129	40.2 (34.4; 47.0)	0.28
AUC_g (U/ml)	554	44.4 (41.2; 47.9)	83	54.3 (45.3; 64.9)	0.06	129	40.4 (35.5; 46.0)	0.21
AUC_i (U/ml) [a]	554	−38.3 (-44.4; -32.2)	83	−60.0 (-80.3; -39.7)	**0.04**	129	−30.9 (-41.3; -34.2)	0.28
Evening sAA variables								
T5 (U/ml) at 10 PM	553	101.7 (94.0; 109.9)	85	108.5 (86.4; 136.3)	0.56	124	92.6 (79.2; 108.1)	0.31
T6 (U/ml) at 11 PM	553	79.3 (72.9; 86.4)	85	98.2 (79.0; 122.1)	0.07	124	75.5 (63.3; 89.9)	0.62
Evening cortisol (U/ml)	553	89.8 (83.1; 97.1)	85	103.2 (83.2; 128.0)	0.21	124	83.6 (71.9; 97.2)	0.43
T7 (U/ml), at awakening	549	63.7 (57.6; 70.5)	83	97.7 (79.7; 119.7)	**< 0.001**	127	61.8 (50.2; 76.1)	0.80
Adjusted values [b]								
Morning sAA variables								
T1 (U/ml) at awakening	542	66.1 (60.1; 72.7)	82	86.9 (67.9; 111.4)	**0.04**	128	55.4 (45.5; 67.4)	0.11
T2 (U/ml) 30' after awakening	542	38.3 (35.4; 41.5)	82	43.9 (35.7; 54.0)	0.23	128	34.6 (29.3; 40.7)	0.27
T3 (U/ml) 45' after awakening	542	41.6 (38.5; 44.8)	82	41.9 (34.3; 51.0)	0.95	128	36.1 (30.9; 42.2)	0.11
T4 (U/ml) 60' after awakening	542	45.2 (41.7; 49.0)	82	46.4 (37.7; 57.2)	0.81	128	40.0 (33.9; 47.1)	0.19
AUC_g (U/ml)	542	45.8 (42.6; 49.2)	82	51.8 (43.0; 62.4)	0.22	128	40.0 (34.6; 46.4)	0.11
AUC_i (U/ml) [a]	542	−39.5 (-45.6; -33.4)	82	−57.1 (-72.9; -41.3)	**0.04**	128	−30.5 (-43.0; -17.9)	0.20
Evening sAA variables								
T5 (U/ml) at 10 PM	543	103.5 (95.6; 111.9)	85	107.0 (87.6; 130.9)	0.75	123	92.9 (78.8; 109.4)	0.25
T6 (U/ml) at 11 PM	543	81.5 (74.8; 88.7)	85	94.1 (75.8; 117.0)	0.23	123	74.9 (62.7; 89.5)	0.41
Evening sAA (U/ml)	543	91.8 (85.0; 99.2)	85	100.4 (82.4; 122.4)	0.41	123	83.4 (70.9; 98.1)	0.30
T7 (U/ml) at awakening	537	66.0 (59.9; 72.8)	82	92.7 (72.0; 119.4)	**0.01**	126	61.0 (49.8; 74.6)	0.48

Table shows sample sizes, back-transformed geometric means and 95% confidence intervals (CI) of the mean.

[a] These geometric means were not back-transformed as the variable was normally distributed.

[b] Adjusted for gender, age, Northern European ancestry, education level, and alcohol use.

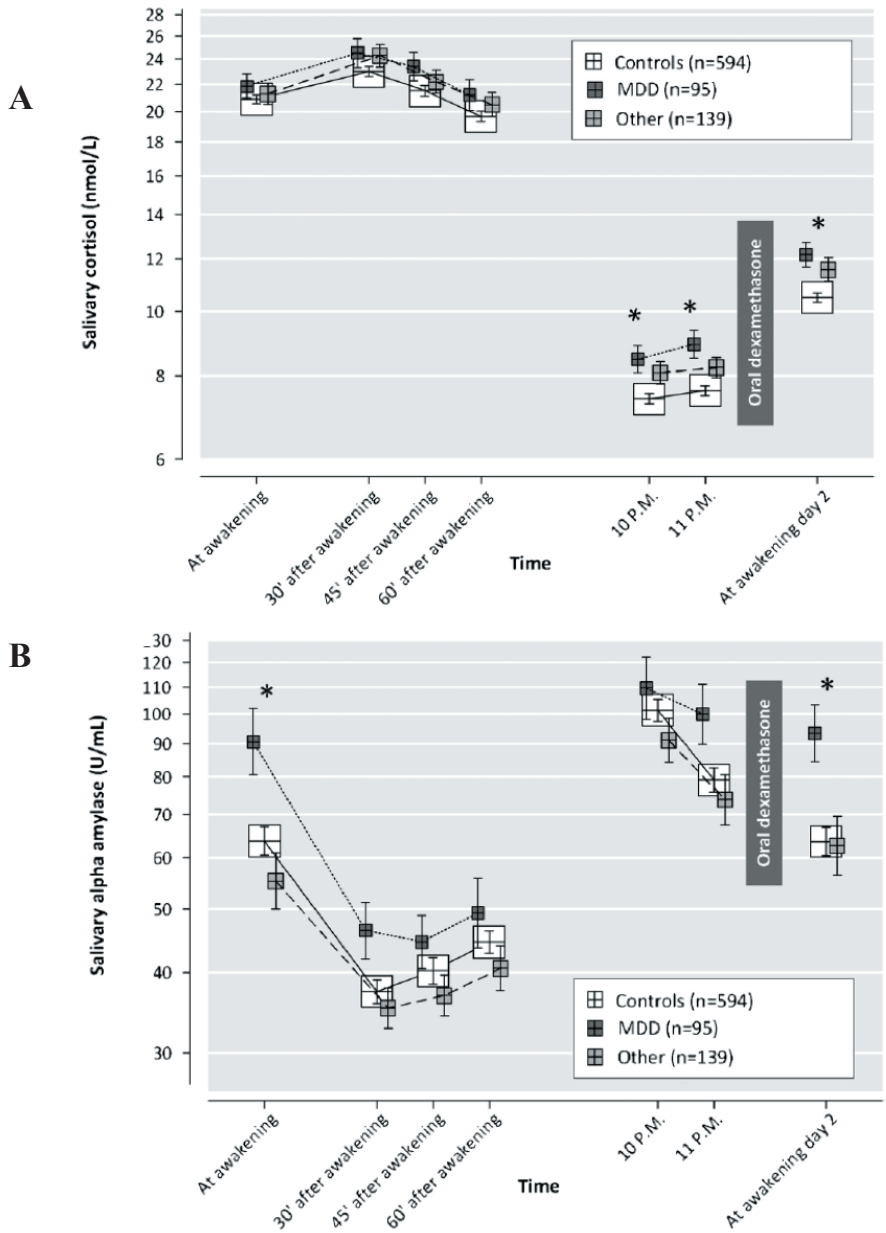

Figure 1. Unadjusted mean values of salivary alpha amylase [A] and salivary cortisol [B] levels according to psychiatric diagnoses presented in Table 1. Figure A and B show awakening, diurnal, and awakening day 2 sAA and SC levels (± S.E.M.) in MDD patients, patients with other MAS-disorders, and healthy controls. Error bars represent standard errors. * = $p < 0.05$.

3.3. Salivary cortisol

Comparisons of SC levels between groups are presented in Table 3. We found no significant differences with regard to the CAR between the three groups in the unadjusted model. Comparisons of unadjusted evening SC levels between the three groups showed that patients in both the MDD group and other MAS-disorders group presented significantly higher T5 ($p= 0.004$; $p= 0.02$), T6 ($p < 0.001$; $p= 0.03$), and mean evening cortisol levels ($p < 0.001$; $p= 0.01$). DST levels were also elevated in both patient groups ($p= 0.001$; $p= 0.02$ respectively) in comparison to the control group (see Table 3 and Fig. 1B). Patients in the MDD group also had a significantly elevated CSR in comparison to the other MAS-disorders group and healthy controls ($p= 0.03$). After multivariate adjustment (i.e. adjusted for gender, age, Northern European ancestry, education level, season, time of awakening, hours of sleep, weekday versus weekend, and alcohol use), SC levels at T6 and mean evening cortisol levels remained significantly higher in the MDD group (T6: $p= 0.04$; mean evening cortisol: $p= 0.049$) in comparison to the other MAS-disorders group and the healthy controls group.

DST remained significantly elevated in both the current MDD group and the other MAS-disorders group in comparison to the control group ($p= 0.04$ and $p= 0.047$; see adjusted values in Table 3). There were no significant differences in SC levels between the MDD group and the other MAS-disorders group at any of the sampling times.

Table 3. Comparison of morning and evening cortisol variables between controls and MDD patients and controls and other MAS-disorders patients.

Variables	n	Controls	n	MDD/DYS	p–value	n	Other MAS-disorders	p–value
Crude values								
Morning cortisol variables								
T1 (nmol/L) at awakening	555	20.9 (20.3; 21.5)	84	21.8 (20.0; 23.7)	0.33	132	21.3 (19.8; 22.9)	0.61
T2 (nmol/L) 30' after awakening	555	22.8 (22.1; 23.6)	84	24.1 (22.0; 26.5)	0.24	132	24.4 (22.5; 26.3)	0.09
T3 (nmol/L) 45' after awakening	555	21.4 (20.7; 22.1)	84	23.4 (21.2; 25.7)	0.07	132	22.4 (20.7; 24.2)	0.27
T4 (nmol/L) 60' after awakening	555	19.5 (18.9; 20.2)	84	20.9 (18.9; 23.1)	0.17	132	20.6 (19.0; 22.4)	0.20
AUC_g (nmol/L/h)	555	21.5 (21.0; 22.1)	84	22.9 (21.2; 24.7)	0.11	132	22.6 (21.1; 24.2)	0.20
AUC_i (nmol/L/h)[a]	555	1.0 (0.4; 1.6)	84	1.7 (-0.5; 3.8)	0.42	132	1.8 (0.6; 3.0)	0.22
Evening cortisol and DST variables								
T5 (nmol/L) at 10 PM	555	7.4 (7.1; 7.6)	87	8.5 (7.7; 9.3)	**0.004**	125	8.1 (7.5; 8.8)	**0.02**
T6 (nmol/L) at 11 PM	555	7.6 (7.3; 7.8)	87	9.0 (8.1; 9.9)	**< 0.001**	125	8.3 (7.7; 8.9)	**0.03**
Evening cortisol (nmol/L)	555	7.5 (7.3; 7.7)	87	8.7 (8.0; 9.5)	**< 0.001**	125	8.2 (7.6; 8.8)	**0.01**
DST (nmol/L), at awakening	522	10.5 (10.2; 10.8)	85	12.2 (11.2; 13.2)	**0.001**	125	11.6 (10.7; 12.6)	**0.02**
CSR (nmol/L)	566	3.8 (3.7; 3.9)	86	3.6 (3.5; 3.8)	**0.03**	135	3.7 (3.5; 3.9)	0.18
Adjusted values[b]								
Morning cortisol variables								
T1 (nmol/L) at awakening	540	21.1 (20.4; 21.7)	82	21.3 (19.6; 23.2)	0.79	129	20.9 (19.6; 22.3)	0.82
T2 (nmol/L) 30' after awakening	540	29.9 (22.1; 23.7)	82	23.9 (21.8; 26.2)	0.50	129	24.5 (22.8; 26.3)	0.14
T3 (nmol/L) 45' after awakening	540	21.4 (20.6; 22.2)	82	23.3 (21.2; 25.6)	0.10	129	22.6 (21.0; 24.3)	0.19
T4 (nmol/L) 60' after awakening	540	19.5 (18.8; 20.2)	82	21.1 (19.2; 23.2)	0.13	129	21.0 (19.5; 22.6)	0.07
AUC_g (nmol/L/h)	540	21.6 (21.0; 22.3)	82	22.7 (21.0; 24.5)	0.26	129	22.6 (21.3; 24.0)	0.20
AUC_i (nmol/L/h)[a]	540	0.9 (0.3; 1.5)	82	1.9 (0.3; 3.5)	0.27	129	2.2 (1.0; 3.5)	0.06
Evening cortisol and DST variables								
T5 (nmol/L) at 10 PM	540	7.5 (7.2; 7.8)	86	8.1 (7.4; 8.9)	0.12	123	8.0 (7.4; 8.6)	0.13
T6 (nmol/L) at 11 PM	540	7.7 (7.5; 8.0)	86	8.5 (7.8; 9.3)	**0.04**	123	8.1 (7.6; 8.7)	0.22
Evening cortisol (nmol/L)	540	7.6 (7.4; 7.8)	86	8.3 (7.7; 9.0)	**0.049**	123	8.0 (7.5; 8.6)	0.14
DST (nmol/L) at awakening	504	10.6 (10.2; 10.9)	78	11.7 (10.7; 12.7)	**0.04**	118	11.4 (10.7; 12.2)	**0.047**
CSR (nmol/L)	551	3.8 (3.7; 3.9)	84	3.6 (3.4; 3.8)	0.07	132	3.7 (3.5; 3.8)	0.18

Table shows sample sizes, back-transformed geometric means and 95% confidence intervals (CI) of the mean.

[a] These geometric means were not back-transformed as the variable was normally distributed.

[b] Adjusted for gender, age, Northern European ancestry, education level, season, time of awakening, hours of sleep, weekday versus weekend, and alcohol use.

4. Discussion

The current study examined the characteristics of diurnal sAA and SC in a naturalistic sample of psychiatric out-patients and healthy controls. We found consistent elevated sAA levels at awakening over two days in MDD patients in comparison to healthy controls and patients with other MAS-disorders, indicating increased early morning sympathetic nervous system activation in this patient group. Evening SC at 11.00 p.m. and mean evening SC levels were found to be significantly increased in MDD patients. Elevated levels of SC after dexamethasone ingestion on day two were found in both the MDD patient group and in the other MAS-disorder group. Our findings suggest that sAA levels at awakening may be a putative new candidate biomarker for MDD specifically.

Research has indicated that the parotid glands contribute most to sAA, reflecting ANS, and in particular sympathetic nervous system (SNS) activation via synergistic sympathetic-parasympathetic interactions (Bosch et al., 2011). Increases of sAA in MAS-patients have been recorded in response to both psychological and physical stress (Schumacher et al., 2013), suggesting that sAA may be a promising marker of SNS dysregulations in MAS-disorders in general. However, previous studies investigating sAA levels in the MAS-disorder patient population have been few, whereof none have determined the diurnal profile of sAA (Tamura et al., 2013; Tanaka et al., 2012a, 2012b; van Veen et al., 2008; Cubala and Landowski, 2014; Booij et al., 2015; Ishitobi et al., 2010; Veen et al., 2013). Moreover, the study designs employed have been heterogeneous. For example, sample sizes ranged from 30 (Booij et al., 2015) to 1683 participants (Veen et al., 2013), sampling settings differed (i.e. naturalistic setting versus laboratory setting), as did sampling times (morning, afternoon, early evening, late evening), numbers of samples collected (1–90), and patient populations (MDD, remitted MDD, gSAD patients, panic disorder, and healthy controls).

Our study is the first to investigate sAA levels at awakening in a larger sample of MDD patients and patients with other MAS-disorders. This, along with the multiple sampling points in the morning and in the evening, makes it possible to extend our current knowledge regarding naturalistic sAA levels in patients with MAS-disorders. To date, three previously conducted naturalistic studies investigating sAA levels in MDD patients found elevated sAA levels in MDD patients in comparison to healthy controls, which is partially in line with the results of this study. Specifically, significantly elevated morning, afternoon, and early evening sAA levels were found

in MDD patients in comparison to healthy controls (Booij et al., 2015), significantly elevated afternoon sAA levels in MDD patients in comparison to healthy controls (Ishitobi et al., 2010), and significantly elevated levels of late evening sAA in MDD patients using tricyclic antidepressants in comparison to MDD patients not using this medication and healthy controls (Veen et al., 2013). We were unable to replicate these results with regard to potential differences in sAA levels between participants using TCAs and those who were antidepressant medication free, as our sample size consisted of only 8 participants using TCAs (n=5 in the MDD group and n=3 in the other MAS disorders group). A sensitivity analysis excluding these participants did not alter our results significantly. We were able to replicate the absence of differences in sAA levels between MDD patients and healthy controls in the late evening (Veen et al., 2013). One study found significantly lower morning sAA levels in MDD patients in comparison to healthy controls (Cubala and Landowski, 2014). We did not find any significant differences between the groups in morning sAA levels, with the exception of the novel finding of differences between the groups directly after awakening.

With regard to SC, we did not find any differences in morning SC levels between MDD patients, patients with other MAS-disorders, and healthy controls. This is not surprising as study findings have shown a high level of variability regarding CAR activity. On the one hand, studies have found similar morning SC concentrations in depressed patients and healthy controls (e.g. Strickland et al., 2002), as was the case in the current study. On the other hand, studies have also found a blunted cortisol response in patients with MDD in comparison to healthy controls (Burke et al., 2005; Stetler and Miller, 2005). Again, other studies have found an increased CAR for both AUCg and AUCi variables in MDD patients in comparison to healthy controls (e.g. Bhagwagar et al., 2005; Pruessner et al., 2003a; Vreeburg et al., 2009; Wardenaar et al., 2011). Our study did find significantly elevated levels of evening cortisol in both the MDD patient group as well as other MAS-disorders patient groups in comparison to healthy controls before adjustment. This difference remained significant for the MDD group after adjusting for influential covariates (Stalder et al., 2016). These results support the findings from two earlier studies conducted with MDD patients and healthy controls in which evening cortisol levels were found to be (partially) increased in depressed subjects (Young et al., 1994; Vreeburg et al., 2009). We also found that SC levels post-dexamethasone intake remained elevated in both the MDD patient group and other MAS-disorders group after adjustment for covariates. These findings provide further evidence for differences in HPA stress-axis regulation among patients with MAS-disorders and healthy controls, however again highlight

the inability of SC to clearly distinguish between MAS disorders.

Our findings are relevant to the ongoing search for possible biomarkers differentiating between MAS-disorders as it seems that basal sAA levels at awakening may distinguish MDD patients from patients with other MAS-disorders. The identification of a putative biomarker that might distinguish MDD from other psychiatric disorders has both scientific and clinical implications in that it may improve our understanding of MDD pathophysiology, may function as an aid in predicting or interpreting response to treatment, and may help in improving current MDD criteria.

We believe that a strength of our study was that we included a MDD and other MAS-disorders, therefore likely reflecting the general MAS-disorder patient population to a reasonable extent and thus increasing the external validity of the findings. Secondly, we were also able to compare our clinical sample to a large sample of healthy controls that were specifically recruited as a reference group for the ROM participants, from which the patient sample in this study was derived. Thirdly, the two saliva samples collected directly after awakening on day 1 and day 2 allowed us to verify our initial finding of sAA differentiating between MDD patients and patients with other MAS-disorders and healthy controls by means of our day 2 sample. Finally, we collected saliva samples using a non-invasive and inexpensive sampling method, which is suitable for clinical use (Nater and Rohleder, 2009).

Our study has a number of potential limitations that should be acknowledged. Firstly, due to the cross-sectional nature of this study we cannot determine the directionality of the relationship between sAA and MDD. However, findings of a prior study within remitted MDD patients (Bagley et al., 2011) seem to suggest that increased sAA levels reflect a state of current depression rather than a vulnerability factor. Secondly, in an ambulatory and naturalistic setting, compliance is more difficult to monitor than in a systematic clinical trial. Although strict instructions were given to the participants regarding protocol adherence (i.e. to refrain from eating, drinking, smoking, or brushing teeth within the preceding 15 min of sampling alongside the importance of adhering to the sampling time frames), we did not ask our participants to refrain from other factors such as the consuming of antioxidants and napping before sampling, as the potential bias through these factors had not yet been well established at the time of data collection. However, these factors are unlikely to influence sAA and SC concentrations at awakening. Additionally, and in accordance with the Salivette® instructions for use manual, participants were instructed to gently chew on the swab for at least two minutes.

This may have affected the levels of sAA recorded as chewing has been found to increase salivary flow rate and in turn sAA concentrations (Bosch et al., 2011). As we did not measure salivary flow rate we were unable to take this into account. Passive drool methods for saliva sampling (DeCaro, 2008) may have yielded more accurate values, although outcomes with regard to whether or not salivary flow rate is independent of stress-induced sAA increase have been inconsistent and evidence has been found supporting both theories (Rohleder et al., 2006; Bosch et al., 2003; Beltzer et al., 2010). Therefore, salivary flow rates should be taken into account in future studies. It should be noted that we instructed our participants to keep the swab in their mouths for two minutes, and research has indicated decreases in sAA concentration and output within the first several minutes of chewing-induced secretion (Proctor and Carpenter, 2001). Moreover, an objective verification method of participants' awakening time (e.g. by means of wrist actigraphy), as well as electronic monitoring devices may have provided more accuracy in the cortisol assessments after awakening (e.g. Broderick et al., 2004; Stalder et al., 2016). However, this was not feasible in the current study, and may therefore have led to some (random) measurement error. A possible explanation for not finding any significant group differences in morning SC parameters could be due to errors in timing. However, a sensitivity analysis excluding samples with less strict timeframes (Stalder et al., 2016) was conducted and the AUCg and AUCi findings remained non-significant. Furthermore, although the samples were collected in a naturalistic setting, we found the distinct diurnal patterns of sAA and SC found in earlier studies (e.g. Nater et al., 2007; Rohleder et al., 2004; Kirschbaum and Hellhammer, 1989), supporting the validity of our results. A further potential limitation may be that we were unable to adequately adjust for psychotropic medication use in our analyses as there were no participants who used psychotropic medication in the healthy control group. Also, information with regard to (childhood and adulthood) trauma exposure was lacking for a large portion of the sample and we could therefore not take this into account. However, although several studies have found that trauma-exposed individuals experience an increase in cortisol levels (e.g. Pico-Alfonso et al., 2004; Klaassens et al., 2010a, 2010b), other studies did not find this effect (e.g. de Kloet et al., 2007; Rohleder et al., 2004; Gill et al., 2008). Moreover, a meta-analysis found that neither adulthood trauma nor PTSD were associated with differences in HPA-axis functioning (Klaassens et al., 2012). Still, this meta-analysis did not look into the effect of childhood trauma exposure on HPA-axis functioning. It is therefore of importance to include information on trauma exposure in future studies to further explore the possible relationship between trauma and HPA-axis and ANS-axis activation. A further limitation is that we did not record the day that

saliva samples were posted by participants and were therefore unable to correct for possible warming effects during postal delivery. However, if this were to have an effect it is likely to have added random measurement error, and thus result in findings approaching the null hypothesis. Finally, collecting samples at more time points over a number of days would have reflected the diurnal patterns of SC and sAA more accurately and further increased the reliability of the SC and sAA levels measured at each time point (Hellhammer et al., 2007).

In conclusion, our findings support earlier findings that sAA levels are increased in patients with current MDD and offer further evidence towards the recently emerging hypothesis that sAA may be a potential biomarker for MDD. Although this study indicates that sAA levels are higher at awakening only in patients with MDD, there is a possibility that these levels are also higher in patients with other psychiatric disorders. Further research is necessary in this regard. Future studies should replicate the elevated awakening sAA levels found in MDD patients in comparison to other MAS-disorders using larger sample sizes to further substantiate the validity of these findings. Furthermore, creating expert consensus guidelines with regard to assessing sAA, as has been done for the CAR (Stalder et al., 2016), may be supportive in obtaining consistent data in future studies. Also, samples should be collected at more time points throughout the day (i.e. during the course of the afternoon and the early evening), and ideally also over more days (Hellhammer et al., 2007).

Appendix

Sampling time frames morning:
Time between T1 and T2< =60 and>=15 min, time between T2 and T3< =45 min and>=5 min, time between T3 and T4< =45and>=5 min, and time between T1 and T4< =90 and>=44 min.

Sampling time frames evening:
Time between T5 and T6< =120 min and>=0 min.

References

1. Bagley, S.L., Weaver, T.L., Buchanan, T.W., 2011. Sex differences in physiological and affective responses to stress in remitted depression, 2018. *Physiol. Behav. 104*(2), 180–186.
2. Beltzer, E.K., Fortunato, C.K., Guaderrama, M.M., Peckins, M.K., Garramone, B.M., Granger, D.A., 2010. Salivary flow and alpha-amylase: collection technique, duration, and oral fluid type. *Physiol. Behav. 101*(2), 289–296.
3. Bhagwagar, Z., Hafizi, S., Cowen, P.J., 2005. Increased salivary cortisol after waking in depression. *Psychopharmacology 182*(1), 54–57.
4. Booij, S.H., Bos, E.H., Bouwmans, M.E., van Faassen, M., carrKema, I.P., Oldehinkel, A.J., de Jonge, P., 2015. Cortisol and α-amylase secretion patterns between and within depressed and non-depressed individuals. *PloS one 10*(7) e0131002.
5. Bosch, J.A., de Geus, E.J., Veerman, E.C., Hoogstraten, J., Amerongen, A.V.N., 2003. Innate secretory immunity in response to laboratory stressors that evoke distinct patterns of cardiac autonomic activity. *Psychosom. Med. 65*(2), 245–258.
6. Bosch, J.A., Veerman, E.C., De Geus, E.J., Proctor, G.B., 2011. α-amylase as a reliable and convenient measure of sympathetic activity: don't start salivating just yet! *Psychoneuroendocrinology 36*, 449–453.
7. Broderick, J.E., Arnold, D., Kudielka, B.M., Kirschbaum, C., 2004. Salivary cortisol sampling compliance: comparison of patients and healthy volunteers. *Psychoneuroendocrinology 29*(5), 636–650.
8. Burke, H.M., Davis, M.C., Otte, C., Mohr, D.C., 2005. Depression and cortisol responses to psychological stress: a meta-analysis. *Psychoneuroendocrinology 30*(9), 846–856.
9. Carroll, B.J., Curtis, G.C., Mendels, J., 1976. Neuroendocrine regulation in depression: I. Limbic system-adrenocortical dysfunction. *Arch. Gen. Psychiatry 33*(9), 1039–1044.
10. Chatterton, R.T., Vogelsong, K.M., Lu, Y.C., Ellman, A.B., Hudgens, G.A., 1996. Salivary α-amylase as a measure of endogenous adrenergic activity. Clin. *Physiol. Funct. Imaging 16*(4), 433–448.
11. Conoley, J.C.E., Kramer, J.J., 1989. *The tenth mental measurements yearbook*. Buros Inst. Mental Meas.
12. Croog, S.H., Levine, S., Testa, M.A., Brown, B., Bulpitt, C.J., Jenkins, C.D., Williams, G.H., 1986. The effects of antihypertensive therapy on the quality of life. *N. Engl. J. Med. 314*(26), 1657–1664.
13. Cubala, W.J., Landowski, J., 2014. Low baseline salivary alpha-amylase in drug-naïve patients with short-illness-duration first episode major depressive disorder. *J. Affect. Disord. 157*, 14–17.
14. De Beurs, E.D., den Hollander-Gijsman, M.E., van Rood, Y.R., Van der Wee, N.J., Giltay, E.J., van Noorden, M.S., Zitman, F.G., 2011. Routine outcome monitoring in the Netherlands: practical experiences with a web-based strategy for the assessment of treatment outcome in clinical practice. Clin. Psychol. *Psychother. 18*(1), 1–12.
15. De Kloet, C.S., Vermetten, E., Heijnen, C.J., Geuze, E., Lentjes, E.G.W.M., Westenberg, H.G.M., 2007. Enhanced cortisol suppression in response to dexamethasone administration in traumatized veterans with and without posttraumatic stress disorder. *Psychoneuroendocrinology 32*(3), 215–226.
16. DeCaro, J.A., 2008. Methodological considerations in the use of salivary α-amylase as a stress marker in field research. *Am. J. Hum. Biol. 20*(5), 617–619.
17. Derogatis, W.G., 1975. Brief Symptom Inventory. Clinical Psychometric Research, Baltimore. Fekedulegn, D.B., Andrew, M.E., Burchfiel, C.M., Violanti, J.M., Hartley, T.A., Charles, L.E., Miller, D.B., 2007. Area under the curve and other summary indicators of repeated waking cortisol measurements. *Psychosom. Med. 69*(7), 651–659.

18. Fried, E.I., Nesse, R.M., 2015. Depression sum-scores don't add up: why analyzing specific depression symptoms is essential. *BMC Med. 13*(1), 72.
19. Fries, E., Dettenborn, L., Kirschbaum, C., 2009.). The cortisol awakening response (CAR): facts and future directions. Int. J. *Psychophysiol. 72*(1), 67–73.
20. Garrett, J. R., Ekström, J., & Anderson, L. C. (1999). Effects of autonomic nerve stimulations on salivary parenchyma and protein secretion. *Neural mechanisms of salivary gland secretion, 11*, 59-79.
21. Gill, J., Vythilingam, M., Page, G.G., 2008. Low cortisol, high DHEA, and high levels of stimulated TNF-α, and IL-6 in women with PTSD. *J. Trauma Stress 21*(6), 530–539.
22. Granger, D.A., Kivlighan, K.T., El-SHEIKH, M.O.N.A., Gordis, E.B., Stroud, L.R., 2007. Salivary α-amylase in biobehavioral research. *Ann. N.Y. Acad. Sci. 109*8(1), 122–144.
23. Hellhammer, J., Fries, E., Schweisthal, O.W., Schlotz, W., Stone, A.A., Hagemann, D., 2007. Several daily measurements are necessary to reliably assess the cortisol rise after awakening: state-and trait components. *Psychoneuroendocrinology 32*(1), 80–86.
24. Hellhammer, D.H., Wüst, S., Kudielka, B.M., 2009. Salivary cortisol as a biomarker in stress research. *Psychoneuroendocrinology 34*(2), 163–171.
25. Ishitobi, Y., Akiyoshi, J., Tanaka, Y., Ando, T., Okamoto, S., Kanehisa, M., Kawano, A., 2010. Elevated salivary α-amylase and cortisol levels in unremitted and remitted depressed patients. Int. J. *Psychiatry Clin. Pract. 14*(4), 268–273.
26. Kirschbaum, C., Hellhammer, D.H., 1989. Salivary cortisol in psychobiological research: an overview. *Neuropsychobiology 22*(3), 150–169.
27. Kirschbaum, C., Pirke, K.M., Hellhammer, D.H., 1993. The 'Trier social stress test'–a tool for investigating psychobiological stress responses in a laboratory setting. *Neuropsychobiology 28*(1-2), 76–81.
28. Klaassens, E.R., Giltay, E.J., van Veen, T., Veen, G., Zitman, F.G., 2010a. Trauma exposure in relation to basal salivary cortisol and the hormone response to the dexamethasone/ CRH test in male railway employees without lifetime psychopathology. *Psychoneuroendocrinology 35*(6), 878–886.
29. Klaassens, E.R., van Veen, T., Giltay, E.J., Rinne, T., van Pelt, J., Zitman, F.G., 2010b. Trauma exposure and hypothalamic–pituitary–adrenal axis functioning in mentally healthy Dutch peacekeeping veterans, 10–25 years after deployment. *J. Trauma Stress 23*(1), 124–131.
30. Klaassens, E.R., Giltay, E.J., Cuijpers, P., van Veen, T., Zitman, F.G., 2012. Adulthood trauma and HPA-axis functioning in healthy subjects and PTSD patients: a metaanalysis. *Psychoneuroendocrinology 37*(3), 317–331.
31. Lorentz, K., 1998. Approved recommendation on IFCC methods for the measurement of catalytic concentration of enzymes part 9. IFCC method for α-amylase (1, 4-α-DGlucan 4-Glucanohydrolase, EC 3.2. 1.1). *Clin. Chem. Lab. Med. 36*(3), 185–203.
32. Nater, U.M., Rohleder, N., 2009. Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: current state of research. *Psychoneuroendocrinology 34*(4), 486–496.
33. Nater, U.M., Rohleder, N., Gaab, J., Berger, S., Jud, A., Kirschbaum, C., Ehlert, U., 2005. Human salivary alpha-amylase reactivity in a psychosocial stress paradigm. *Int. J. Psychophysiol. 55*(3), 333–342.
34. Nater, U.M., La Marca, R., Florin, L., Moses, A., Langhans, W., Koller, M.M., Ehlert, U., 2006. Stress-induced changes in human salivary alpha-amylase activity—associations with adrenergic activity. *Psychoneuroendocrinology 31*(1), 49–58.
35. Nater, U.M., Rohleder, N., Schlotz, W., Ehlert, U., Kirschbaum, C., 2007. Determinants of the diurnal course of salivary alpha-amylase. *Psychoneuroendocrinology 32*(4), 392–401.

36. Perry, G.H., Dominy, N.J., Claw, K.G., Lee, A.S., Fiegler, H., Redon, R., Carter, N.P., 2007. Diet and the evolution of human amylase gene copy number variation. *Nat. Genet. 39*(10), 1256.
37. Pico-Alfonso, M.A., Garcia-Linares, M.I., Celda-Navarro, N., Herbert, J., Martinez, M., 2004. Changes in cortisol and dehydroepiandrosterone in women victims of physical and psychological intimate partner violence. *Biol. Psychiatry 56*(4), 233–240.
38. Proctor, G.B., Carpenter, G.H., 2001. Chewing stimulates secretion of human salivary secretory immunoglobulin *A. J. Dent. Res. 80*(3), 909–913.
39. Pruessner, M., Hellhammer, D.H., Pruessner, J.C., Lupien, S.J., 2003a. Self-reported depressive symptoms and stress levels in healthy young men: associations with the cortisol response to awakening. *Psychosom. Med. 65*(1), 92–99.
40. Pruessner, J.C., Kirschbaum, C., Meinlschmid, G., Hellhammer, D.H., 2003b. Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. *Psychoneuroendocrinology 28*(7), 916–931.
41. Rief, W., Shaw, R., Fichter, M.M., 1998. Elevated levels of psychophysiological arousal and cortisol in patients with somatization syndrome. Psychosom. Med. 60(2), 198–203.
42. Rigaud, S., d'Errico, F., Vanhaeren, M., 2015. Ornaments reveal resistance of North European cultures to the spread of farming. *PloS one 10*(4), e0121166.
43. Rohleder, N., Nater, U.M., Wolf, J.M., Ehlert, U., Kirschbaum, C., 2004. Psychosocial stress-induced activation of salivary alpha-amylase: an indicator of sympathetic activity? *Ann. N.Y. Acad. Sci. 1032* (1), 258–263.
44. Rohleder, N., Wolf, J.M., Maldonado, E.F., Kirschbaum, C., 2006. The psychosocial stressinduced increase in salivary alpha-amylase is independent of saliva flow rate. *Psychophysiology 43*(6), 645–652.
45. Schulte-van Maaren, Y.W., Carlier, I.V., Giltay, E.J., van Noorden, M.S., de Waal, M.W., van der Wee, N.J., Zitman, F.G., 2013. Reference values for mental health assessment instruments: objectives and methods of the Leiden Routine outcome monitoring study. *J. Eval. Clin. Pract. 19*(2), 342–350.
46. Schumacher, S., Kirschbaum, C., Fydrich, T., Ströhle, A., 2013. Is salivary alpha-amylase an indicator of autonomic nervous system dysregulations in mental disorders?—A review of preliminary findings and the interactions with cortisol. *Psychoneuroendocrinology 38*(6), 729–743.
47. Stalder, T., Kirschbaum, C., Kudielka, B.M., Adam, E.K., Pruessner, J.C., Wüst, S., Miller, R., 2016. Assessment of the cortisol awakening response: expert consensus guidelines. *Psychoneuroendocrinology 63*, 414–432.
48. Stetler, C., Miller, G.E., 2005. Blunted cortisol response to awakening in mild to moderate depression: regulatory influences of sleep patterns and social contacts. *J. Abnorm. Psychol. 114*(4), 697.
49. Strickland, P.L., Deakin, J.W., Percival, C., Dixon, J., Gater, R.A., Goldberg, D.P., 2002. Bio-social origins of depression in the community. *Br. J. Psychiatry 180*(2), 168–173.
50. Tamura, A., Maruyama, Y., Ishitobi, Y., Kawano, A., Ando, T., Ikeda, R., Ninomiya, T., 2013. Salivary alpha-amylase and cortisol responsiveness following electrical stimulation stress in patients with the generalized type of social anxiety disorder. *Pharmacopsychiatry 46*(07), 225–260.
51. Tanaka, Y., Ishitobi, Y., Maruyama, Y., Kawano, A., Ando, T., Imanaga, J., Tsuru, J., 2012a. Salivary alpha-amylase and cortisol responsiveness following electrical stimulation stress in panic disorder patients. *Neurosci. Res. 73*(1), 80–84.
52. Tanaka, Y., Ishitobi, Y., Maruyama, Y., Kawano, A., Ando, T., Okamoto, S., Hanada, H., 2012b. Salivary alpha-amylase and cortisol responsiveness following electrical stimulation stress in major depressive disorder patients. Prog. Neuro-Psychopharmacol. *Biol. Psychiatry 36*(2), 220–224.
53. van Aken, M.O., Romijn, J.A., Miltenburg, J.A., Lentjes, E.G., 2003. Automated measurement of cortisol, 1408 1409, salivary cortisol. *Clin. Chem. 49*(8), 1408–1409.

54. van Veen, J.F., Van Vliet, I.M., DeRijk, R.H., Van Pelt, J., Mertens, B., Zitman, F.G., 2008. Elevated alpha-amylase but not cortisol in generalized social anxiety disorder. *Psychoneuroendocrinology 33*(10), 1313–1321.
55. van Vliet, I.M., De Beurs, E., 2007. Het Mini Internationaal Neuropsychiatrisch Interview (Mini). Een kort gestructureerd diagnostisch psychiatrisch Interview voor DSM-IV en ICD-10-stoornissen [The Mini International Neuropsychiatric Interview (Mini). A short structured diagnostic psychiatric Interview for DSM-IV and ICD-10 disorders]. T*ijdschrift voor Psychiatrie 49*(6), 393–397.
56. Veen, G., Giltay, E.J., Vreeburg, S.A., Licht, C.M., Cobbaert, C.M., Zitman, F.G., Penninx, B.W., 2012. Determinants of salivary evening alpha-amylase in a large sample free of psychopathology. *Int. J. Psychophysiol. 84*(1), 33–38.
57. Veen, G., Giltay, E.J., Licht, C.M., Vreeburg, S.A., Cobbaert, C.M., Penninx, B.W., Zitman, F.G., 2013. Evening salivary alpha-amylase, major depressive disorder, and antidepressant use in the Netherlands study of depression and anxiety (NESDA). *Psychiatry Res. 208*(1), 41–46.
58. Vreeburg, S.A., Hoogendijk, W.J., van Pelt, J., DeRijk, R.H., Verhagen, J.C., van Dyck, R., Penninx, B.W., 2009. Major depressive disorder and hypothalamic-pituitary-adrenal axis activity: results from a large cohort study. *Arch. Gen. Psychiatry 66*(6), 617–626.
59. Vreeburg, S.A., Zitman, F.G., van Pelt, J., DeRijk, R.H., Verhagen, J.C., van Dyck, R., Penninx, B.W., 2010. Salivary cortisol levels in persons with and without different anxiety disorders. *Psychosom. Med. 72*(4), 340–347.
60. Wardenaar, K.J., Vreeburg, S.A., van Veen, T., Giltay, E.J., Veen, G., Penninx, B.W., Zitman, F.G., 2011. Dimensions of depression and anxiety and the hypothalamo-pituitary-adrenal axis. *Biol. Psychiatry 69*(4), 366–373.
61. World Health Organization, 2017. Depression [Fact Sheet]. Retrieved from. http://www.who.int/mediacentre/factsheets/fs369/en/.
62. Young, E.A., Haskett, R.F., Grunhaus, L., Pande, A., Weinberg, V.M., Watson, S.J., Akil, H., 1994. Increased evening activation of the hypothalamic-pituitary-adrenal axis in depressed patients. Arch. *Gen. Psychiatry 51*(9), 701–707.