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

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Chapter 3

Salivary markers of stress system activation and social withdrawal in humans

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Abstract

Social withdrawal is an early and common feature of psychiatric disorders. Hypothalamic-pituitary-adrenal (HPA)-axis activation through increased salivary cortisol (sC) and sympathetic activation through increased salivary alpha-amylase (sAA) may play a role. We aimed to study whether the link between increased sC and sAA on the one hand and depression on the other hand is mediated by social withdrawal. In this cross-sectional, observational study, sC and sAA measures were measured in seven saliva samples in 843 participants (231 psychiatric patients and 612 healthy controls). Social withdrawal was assessed through the Brief Symptom Inventory (BSI)-, the Short Form 36-, and the Dutch Dimensional Assessment of Personality Pathology social withdrawal subscales, and analyzed using linear regression and mediation analyses. On average, participants were 44.0 years old (SD=12.8; 64.1% female). Basal and diurnal sAA were unrelated to any social withdrawal scale and depression. Certain sC measures were positively associated with the BSI social withdrawal subscale (i.e., area under the curve with respect to the increase, beta=0.082, p=0.02; evening sC value: beta=0.110, p=0.003; and mean sC value: beta=0.097; p=0.01). We found limited support for statistical mediation by social withdrawal (measured using a composite social withdrawal score) on the relationship between evening sC and depression. Thus, although we found no support for a role of basal and diurnal sAA in social withdrawal, HPA-axis activation may partly aggravate social withdrawal in depressive disorders.

1. Introduction

Social withdrawal, or withdrawal from family, friends, and colleagues, is a common feature of several psychiatric disorders, such as major depressive disorder (Saris et al., 2017; Bora & Berk, 2016; Kupferberg, Bicks, & Hasler, 2016), anxiety disorders (Saris et al., 2017) , and schizophrenia (Galderisi, Mucci, Buchanan, & Arango, 2018), and is also one of the earliest signs of these disorders (e.g. Cross, Scott, & Hickie, 2017; Nelis et al., 2011). In line with the National Institute of Mental Health Research Domain Criteria (RDoC) project aimed at identifying new ways of classifying psychiatric disorders based on dimensions of (neuro-)biological measures and observable behavior, social withdrawal has been posited to be related to a more stable endophenotype that is more closely connected to biological pathways than psychiatric disorders are (Porcelli et al., 2018; van der Wee et al., 2018). Increased social withdrawal can lead to poor social functioning and social isolation, and can in turn cause feelings of loneliness. Loneliness prevalence of people reporting frequent feelings of loneliness in European countries has been found to range from approximately 5-7% in the West and North to approximately 11% in the East, with an overall European average of 7.9% (d'Hombres, Schnepf, Barjakovà, & Teizeira, 2018). Constructs such as social withdrawal, social isolation, poor social functioning, and loneliness partially overlap. However, for ease of interpretation, social withdrawal will be used as an umbrella term throughout this paper.

Several psychiatric disorders, such as MDD, have been associated with heightened inflammation (Najjar et al., 2013). Inflammatory activity has been found to enhance amygdala activity (a region involved in social withdrawal in animals and associated with social avoidance in humans) to socially threatening images, implicating amygdala activity involvement in sickness-induced social withdrawal (Inagaki et al., 2012). Brain-derived neurotrophic factor (BDNF), dopamine and their receptors likely play important roles, as is supported by experimental studies in rodents investigating the biology of social withdrawal (Campi et al., 2014; Greenberg et al., 2014).

Whereas low levels of social withdrawal have been found to be positively related to longevity, physical-, psychological-, and emotional well-being (Holt-Lunstad, Smith, & Layton, 2010; Uchino, 2006), high social withdrawal has been associated with severe detrimental health outcomes, such as depression (Cacioppo, Hawkley, & Thisted, 2010; Cacioppo, Hughes, Waite, Hawkley, & Thisted, 2006), adverse coronary condition rates (Holt-Lunstad, Smith, Baker, Harris, & Stephenson, 2015; Patterson & Veenstra, 2010; Sorkin, Rook, & Lu, 2002), alcoholism (Qualter et al., 2015), increased mortality rates (Holt-Lunstad et al., 2015; Patterson & Veenstra,

2010; Sorkin, Rook, & Lu., 2002), increased suicidality (Conroy & Smith, 1983; Peck, 1983), and Alzheimer's disease (Wilson et al., 2007). Additionally, associations between social withdrawal and alterations in hypothalamic-pituitary-adrenocortical (HPA) axis activity have been found (Adam, Hawkley, Kudielka, & Cacioppo, 2006; Steptoe, Owen, Kunz- Ebrecht, & Brydon, 2004). Thus, it seems that the dimension of social withdrawal may aid in linking overlapping biological underpinnings across several conditions (van der Wee et al., 2018; Porcelli et al., 2018). The identification of dimensional behavioral phenotypes across disorders may help to deepen our understanding of the biology involved and complements the approach to incorporate dimensional measures as in the DSM-5 system.

Social withdrawal has often been associated with HPA-axis activation (Adam, Hawkley, Kudielka, & Cacioppo, 2006; Arnetz, Theorell, Levi, Kallner, & Eneroth, 1983; Doane & Adam, 2010; Grant, Hamer, & Steptoe, 2009; Hawkley, Cole, Capitanio, Norman, & Cacioppo, 2012; Pressman et al., 2005), although certain findings were not consistent with this (Cacioppo et al., 2002; Steptoe et al., 2004). Activation of the HPA-axis leads to increased circulatingconcentrations of free cortisol in the body. St udies investigating basal cortisol levels in psychiatric disorders have rendered equivocal results. Many studies have found evidence of hypersecretion of cortisol in depressed and anxious patients (Gil et al., 2008; Goodyer, Herbert, Tamplin, & Altham, 2000; Lenze et al., 2011), although long-term stress may lead to a downregulation or exhaustion of the HPA-axis, and less hypercortisolemia (Wardenaar et al., 2011). Extensive literature suggests that hypercortisolemia may be a biological risk factor for depression, and a diminished negative feedback in depressed patients compared to healthy controls after a low-dose dexamethasone-suppression test has been well established (DST; Goodyer et al., 2000; Pruessner et al., 2003a; Stetler & Miller, 2005; Vreeburg et al., 2009, 2010; Wardenaar et al., 2011). Evidence for a temporal relationship starting with social withdrawal and subsequent depression has also been found (Cacioppo, Hawkley, & Thisted, 2010; Engeland et al., 2006).

In contrast to data on sC, salivary alpha amylase (sAA) is a relatively new candidate marker of autonomic nervous system (ANS) functioning and reactivity. The ANS consists of two systems, the parasympathetic nervous system (PNS), that is responsible for the body's rest and digest response, and the sympathetic nervous system (SNS), responsible for the fight or flight response. sAA is secreted in response to neurotransmitter stimulation from salivary glands that are innervated by sympathetic as well as parasympathetic nerves (Vester & Garrett, 1987). Studies have provided evidence that stress increases SNS activity and decreases PNS activity, leading to an increase in epinephrine and norepinephrine levels and a decrease in the

level of acetylcholine. This can lead to an increase in the level of pro-inflammatory cytokines (e.g. TNF-alpha, IL-1, IL-6) and interferons, and decrease the level of anti-inflammatory cytokines (e.g. IL-10), possibly initiating a state of enhancing low-grade inflammation, which in turn may induce indoleamine 2,3-dioxygenase activity, increase the kynurenine/tryptophan ratio. This can lead to an imbalance between neurotoxic and neuroprotective kynurenine metabolites. Ultimately, this can lead to neurodegenerative changes of the brain, that could leave the brain more susceptible to, for example, depression (Won & Kim, 2016). Associations have also been reported between inflammatory cytokines, the kynurenine pathway, and the neurotoxicity of the metabolites in the pathway in depression (Hazari & Bhad, 2015; Jo, Zhang, Emrich, & Dietrich, 2015).

Recently, a study conducted by our group within the same sample population found higher levels of sAA at awakening in MDD patients in comparison to both healthy controls and patients with other common psychiatric disorders (i.e. predominately anxiety and somatoform disorders; Bauduin et al., 2018). Another study found elevated afternoon sAA levels in patients with generalized social anxiety disorder (van Veen et al., 2008). This indicates that elevations of sAA within disorders seem to be time-point specific, suggesting the importance of diurnal patterns. Importantly, these sAA elevations in MDD and social anxiety also suggest a potential role of sAA in social withdrawal. Moreover, a recent study found that social withdrawal was related to heightened sympathetic nervous system activation in response to social stress (Duijndam, Karreman, Johan, & Kupper, 2020). Furthermore, increased social withdrawal has been found to be a mediating variable in the relationship between salivary cortisol (sC) and depression (Tse & Bond, 2004). No studies have explored the interrelationships between sAA, social withdrawal, and depression to date.

In this study, we examine the relationship between sC and sAA with social withdrawal using salivary samples collected in a naturalistic setting. We used a mixed sample of psychiatric out-patients and controls to analyze associations along the dimension of social withdrawal. Moreover, we explored whether the putative relationship between biological stress system activation and depression is mediated through social withdrawal. Based on previous literature, we hypothesized that high sC and sAA levels would be positively associated with social withdrawal. Furthermore, we expected that social withdrawal would mediate the relationship with both salivary markers of stress system activation and depression.

2. Methods

2.1 Participants

The population recruited for this study came from the Routine Outcome Monitoring (ROM) and NormQuest cohorts. The ROM participants were ambulatory outpatients that participated in an extensive ROM interview between 2007 and 2011 as part of routine patient care. ROM is restricted to patients referred for treatment of mood, anxiety, and somatoform disorders. In order to partake in the ROM, participants must have sufficient command of the Dutch language. Detailed ROM protocol information has previously been published elsewhere (de Beurs et al., 2011). NormQuest participants were recruited from the general population via the registration at medical practices as a reference group for the ROM patients (Schulte-van Maaren et al., 2013). These participants were stratified for age, gender, and urbanization in order to reflect the ROM patient population as accurately as possible within this naturalistic study design. According to the Mini International Psychiatric Interview (MINI-Plus; van Vliet & de Beurs, 2007), more than 90% of the NormQuest population were not diagnosed with a psychiatric disorder. Exclusion criteria for the NormQuest controls were: (i) treatment in a secondary psychiatric care center in the last six months and/or dependence on drugs or alcohol; ii) a hearing impairment or limited cognitive abilities (i.e. aphasia, severe dyslexia or dementia); iii) illiteracy or insufficient mastery of the Dutch language, and; iv) a terminal disease.

A total of 949 participants consisting of ROM participants with either a (comorbid) mood, anxiety, or somatoform (MAS-) diagnosis and healthy controls collected saliva samples for our Mood, Anxiety, and Somatoform disorders and HPA-axis biobank (MASHbank), using a protocol that was similar to the protocol used in the Netherlands Study of Depression and Anxiety (NESDA, Vreeburg et al., 2010). The protocol was approved by the LUMC Ethical Review Board, participants gave written consent, and the study was carried out in accordance with the principles of the declaration of Helsinki. After explanation of the protocol, informed consent was obtained from all of the participants. Sample forms and ROM interviews without a recorded date were excluded (n = 44). Participants that returned samples more than 60 days after the ROM interview were also excluded (n = 23), as well as NormQuest participants using psychotropic medication, patients whose medication use was not specified, and all participants lacking demographic information (n = 26, n = 7, and n = 6 respectively). Thus, our final sample consisted of 843 (88.8%) of 949 participants.

2.2 Saliva sampling

Participants were given detailed written instructions in which they were asked to collect saliva samples on a regular working day using Salivettes® (Sarstedt, Germany) at awakening, after 30, 45, and 60 minutes (in order to be able to calculate the area under the curve with respect to the ground (AUCg; which measures total cortisol output), and the area under the curve with respect to the increase (AUCi, which measures change in cortisol over repeated measures; Khoury et al., 2015). Participants were also asked to collect saliva samples at 10:00 PM and 11:00 PM, as earlier studies have found elevated evening cortisol in MDD patients (Young et al., 1994; Vreebrug et al., 2009; Bauduin et al., 2018), and at awakening the following day (to determine the effect of a dexamethasone suppression test (DST) on sC levels), which is in accordance with the NESDA protocol for the MASHbank. Participants were therefore asked to ingest 0.5 mg of dexamethasone after collecting the 11:00 PM sample on day 1.

Participants were asked to record the sampling times on a sampling form and store the samples in the refrigerator. When all samples were collected, they were returned by regular postal mail after which the Salivettes were centrifuged, the saliva aliquoted, and subsequently 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 immunoanalyzer, Roche Diagnostics, Basel, Switzerland), as previously described in van Aken et al. (2003). The functional detection limit was 2.0 nmol/l and the intra- and inter-assay 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 alpha-amylase 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. Intra assay coefficient of variation ranged from 0.7% to 2.7% for the combined predilution step and the analysis across the entire study period. Inter assay coefficient of variation was lower than 5% throughout this study.

2.4 Data cleaning

Both sC and sAA samples 3 SD higher than the mean were excluded (sC n = 66 of the 5901 samples (1%); sAA n = 114 of the 5901 samples (< 2%)), as these values exceed the range that is realistic for sC and sAA samples and are likely to be caused by measurement issues (e.g., bleeding gums or gingivitis). However, for the sake of completeness we also ran the analyses using all of the original values, which resulted in largely comparable findings. Missing values were then imputed using linear regression for cases remaining in the dataset (n = 170; 2.9% values for sC and n = 129; 2.2% values for sAA).

2.5 Psychometric rating scales for social withdrawal

Currently, there is no broadly accepted gold-standard with which to measure social withdrawal. In this study, three subscales were used in order to measure social withdrawal. The first is a subscale is part of the Brief Symptom Inventory (BSI; Derogatis & Melisaratos, 1983). The BSI is a 53-item self- report inventory which uses a 5-point Likert scale (range 0-4) and evaluates the psychological symptom status of individuals on 9 primary symptom dimensions. It has been found to be a valid and reliable measurement instrument (Aroian & Patsdaughter, 1989; Croog et al., 1986; Derogatis & Melisaratos, 1983). Regarding our focus on social withdrawal, we used the Dutch version of the BSI social withdrawal subscale consisting of five items. These items are related to the withdrawn lifestyle that is commonly experienced in patients with schizophrenia or depression. A higher score on this subscale indicates a higher level of social withdrawal. An example of an item on this subscale is “Feeling lonely even when you are with people”. Cronbach’s alpha of this subscale in this study is 0.782 (N = 843).

The second subscale used to measure social withdrawal is part of the Short Form 36 (SF-36; Ware & Sherbourne, 1992). The SF-36 is a 36 item self-report survey used to determine an individual’s general health status over eight domains. The SF-36 is a valid and reliable measurement instrument (Razavi & Gandek, 1998). In this study we used the scores given on the social functioning subscale of this survey, which consists of two items. One of the two items in this subscale is “During the past 4

weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?". Cronbach's alpha of this subscale is 0.872 (N = 843).

The third social withdrawal subscale is part of the Dutch Dimensional Assessment of Personality Pathology – Short Form (DAPP-SF; van Kampen, de Beurs, & Andrea, 2008). The DAPP-SF comprises 136 of the original 290 items of the Dimensional Assessment of Personality Pathology – Basic Questionnaire (DAPP-BQ; Livesley & Jackson, 2001). Scores range from 1 (very unlike me) to 5 (very like me). The DAPP-SF has been found to be both a valid and reliable questionnaire (de Beurs, Rinne, van Kampen, Verheul, & Andrea, 2009). The DAPP- SF social avoidance subscale in this questionnaire consists of 6 items geared towards establishing whether one has a low level of affiliation, is fearful of interpersonal hurt, has defective social skills, has a desire for improved affiliative relationships, and for the level of social apprehensiveness. A translation of one of the items on the subscale is "At parties or meetings I tend to avoid people". Cronbach's alpha for this subscale in this study is 0.897 (n = 420).

A composite social withdrawal scale was created using the items from the BSI, SF36, and DAPP social withdrawal subscales. This scale was created by computing the standardized z-scores of each subscale and averaging these scores into one composite. Subscales missing <=1 observation were included in the composition of the social withdrawal composite scale. This scale was used for means of visual interpretation of sC and sAA values at each time point (see Figures 1A and B), and as the scale reflecting social withdrawal in the statistical mediation analysis. Cronbach's alpha for this subscale in this study is 0.780 (n = 420).

2.6 Psychometric depression rating scales

The Montgomery-Åsberg Depression Rating Scale (MADRS; Montgomery & Asberg, 1979) is a 10-item observer-rated instrument assessing the range and severity of depressive symptoms. Items are rated on a 7-point Likert scale anchored at 4 points (i.e. 0 indicating the no abnormality, 6 indicating severe abnormality). Summed total scores lie between 0 and 60. The MADRS has a good internal consistency and reliability (Goekoop & Zwinderman, 1994; Montgomery & Asberg, 1979). The time frame for the MADRS encompasses the previous seven days.

2.7 Statistical analysis

As published in a previous paper by our group (Bauduin et al, 2018), sC and sAA were

positively skewed and therefore naturally loge transformed before analyses. Baseline characteristics were compared across the mixed group consisting of patients with mood-, anxiety-, and/or somatoform diagnoses and healthy controls using analysis of variance for continuous variables and chi-squared tests for categorical variables. These variables were summarized using means, percentages, and standard deviations. Associations between the three social withdrawal subscales and the seven sC and sAA samples were determined by means of Pearson's correlations.
The measures computed for both the sAA and sC samples were the AUCg, the AUCi, mean evening sAA levels, and mean sAA levels at awakening. The relationship between sAA AUCg levels, AUCi levels, mean evening sAA levels, and sAA levels at awakening with the BSI, the SF-36, and the DAPP social withdrawal subscales were determined by means of both unadjusted and adjusted linear regression. In the adjusted model, NE ancestry, age (continuous), and alcohol use (dichotomous) were included. These are variables that earlier studies have reported may influence sAA concentrations (Perry et al., Rigaud et al., 2015; Veen et al., 2012). Additionally, we adjusted for gender and educational level (no/low education versus middle/high education).

The relationship between sC AUCg levels, AUCi levels, mean evening sC levels, and sC levels at awakening after the DST with the BSI, the SF-36, and the DAPP social withdrawal subscales were determined by means of both unadjusted and adjusted linear regression. The model was adjusted for 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 (as a continuous variable), hours of sleep (<6 hours of sleep, >6 hours of sleep), weekday versus weekend, and alcohol use the day of testing in adherence, where possible, to the expert consensus guidelines (Stalder et al., 2016).

In order to investigate whether social withdrawal is a mediating variable in the relationship between sC and sAA elevations and increased depression severity as measured on the MADRS, a statistical mediation analysis was run using the Preacher and Hayes mediation method (Preacher & Hayes, 2008). Potential mediation was determined by directly testing the significance of the indirect effect of the independent variable (IV) on the dependent variable (DV) through the mediating variable (M; the c'-path). This is quantified as the product of the effects of the IV on M (a -path) and the effect of M of DV (b-path). Analyses were performed using a multiple mediator model with a bootstrapping approach whereby the point estimate

of the indirect effect was deduced by means of 5000 estimates of the a x b path. 95% confidence intervals (CI) of the empirical distribution were estimated using cut-offs for the 2.5% highest and lowest scores. Mediating effects were considered to be significant when the CI did not include zero. A two-sided p-value <0.05 was considered statistically significant. To determine effect sizes, the completely standardized indirect effect was determined using the following formula: abcs = ßMXßYM (Preacher & Kelley, 2011). IBM SPSS Statistics for Windows version 24 (IBM Corp. Armonk, N.Y., USA) was used for data-analysis.

3. Results

The mean age of the sample was 44.0 years (64.1% female; N = 843). MADRS scores means were 2.00 (IQR 0-7), as this variable is positively skewed. Further sample characteristics can be found in Table 1. A strong correlation van found between the SF36 and the BSI social withdrawal subscales ($r = 0.64$; $p < 0.001$), the BSI and the DAPP-SF social withdrawal subscales ($r = 0.60$; $p < 0.001$), and the SF36 and the DAPP social withdrawal subscales ($r = 0.45$; $p < 0.001$).

Pearson's correlations between the seven sC measurements indicated that all measurements were significantly positively associated with one another ranging from 0.286 through 0.775. Pearson's correlations between the seven sAA measurements indicated that these were also positively associated with one another ranging from 0.480 through 0.811. sC and sAA were often only weakly correlated with correlation coefficients ranging between -0.069 and 0.209.

Table 1. Socio-demographic variables for the population sample (MAS-disorder patients and healthy controls)

Variables	N = 843
Sociodemographic characteristics:	
Gender, female (%)	540 (64.1)
Age in years, mean (SD)	44.00 (12.8)
Higher education (%)	599 (71.1)
North European ancestry (%)	715 (84.8)
Alcohol use on sampling days (Yes) %	247 (29.3)*
Sampling Factors:	
Time of awakening (mean, SD)	7:01 (0:58)
Hours of sleep (less than 6 hours) %	168 (19.9)*
Workday (%)	577 (68.4)*
Light Season (April-September)	373 (44.2)
Clinical Characteristics:	
MADRS total score (median, SD)	1.10 (1.10)
BSI social withdrawal subscale (median, SD)	0.00 (0.53)
SF-36 social functioning subscale (median, SD)	1.50 (0.93)
DAPP-SF social avoidance (N = 420; median, SD)	1.83 (0.97)
Composite subscale (mean, SD)	0.00 (1.00)
Assessment characteristics:	
Time interval between assessment and saliva sampling (median, SD)	7.0 (10.00)
Medication use n (%):	
Using any psychotropic medication	129 (15.3)
TCAs	8 (0.9)
SSRIs	55 (6.5)
Benzodiazepines	30 (3.6)
Stimulants	1 (0.1)
Antipsychotics	8 (0.9)
Stabilizers	8 (0.9)
Other ADs	19 (2.3)

*:Alcohol use n = 16 (<2%) missing; hours of sleep n=9 (%) missing; workday n = 4 missing ; MADRS n = 1 missing, SF-36 n = 19; BSI n = 5; composite subscale z-scores 72 missing.

Associations between salivary alpha amylase and social withdrawal subscales
Associations between AUCg, mean sAA levels at awakening, and diurnal sAA with the SF36 social withdrawal subscale were found to be significant ($p = 0.01$, $p = 0.01$, and $p = 0.005$ respectively). However, none of these associations remained significant after adjustment for
covariates (see Table 2). Age was found to be the covariate of most influence in the adjusted analyses ($p < 0.01$). None of the other covariates were found to be significant. A visual
representation of sAA values per time point can be found in Figure 1A. The sample is categorized in quartiles (i.e. the adjusted scores on the composite subscale divided into four groups of equal size) for visual purposes only in order to facilitate interpretability. In this Figure the moment of ingestion of the dexamethasone has been included ('oral dexamethasone'). We have denoted this in both Figures 1A and 1B for continuity purposes, as sAA and sC values were derived from the same saliva sample, although dexamethasone does not influence sAA levels (as can be seen in Figure 1A).

Associations between salivary cortisol and social withdrawal subscales
Significant associations between sC AUCg, AUCi, evening sC, diurnal sC and the BSI social withdrawal subscale were found in unadjusted models, before adjusting for covariates (see Table 3). After multivariate adjustment only associations between AUCi, evening sC, diurnal sC, and the BSI social withdrawal subscale remained significant ($p = 0.02$; $p = 0.003$; $p = 0.01$ respectively). Evening sC was found to be significantly associated with the SF36 social withdrawal subscale, however this association did not remain significant after adjustment for covariates. The DAPP social withdrawal subscale consisting of 420 observations (thus approximately half of the number of observations recorded on the SF36 and BSI social withdrawal subscales) was not significantly associated with sC at any of the time points (see Figure 1B for visual representation of sC levels per time point). The covariates of largest influence in all of the analyses were working on the day of saliva collection and sleeping less than six hours before the day of saliva collection ($p < 0.001$ respectively).

Table 2. Associations between salivary alpha-amyase (sAA) and social withdrawal subscales.

		N	BSI Social withdrawal	N	SF36 Social Functioning	N	DAPP social avoidance
AUCg (U/ml)	Crude	763	0.048 (*p*=0.18)	749	**0.033 (*p*=0.01)**	445	0.052 (*p*=0.28)
	Adjusted	749	0.029 (*p*=0.43)	735	0.021 (*p*=0.11)	433	0.044 (*p*=0.37)
AUCi (U/ml)	Crude	763	-0.013(*p*=0.72)	749	<-0.001 (*p* =0.56)	445	0.033 (*p* =0.49)
	Adjusted	749	0.005 (*p* =0.90)	735	<0.001 (*p* =0.63)	433	0.043 (*p* =0.39)
Mean T1 and T7 (nmol/ml),	Crude	767	0.054 (*p*=0.14)	753	**0.092 (*p*=0.01)**	449	0.013 (*p*=0.79)
at awakening	Adjusted	741	0.083(*p* =0.07)	739	0.049 (*p* =0.18)	437	<0.001 (*p*=0.998)
Diurnal sAA	Crude	726	0.051 (p=0.17)	712	**0.104 (p=0.005)**	420	0.043 (p=0.38)
	Adjusted	715	0.034 (p=0.37)	701	*0.071 (p=0.057)*	410	0.028 (p=0.58)

*Adjusted for gender, age, Northern European ancestry, and alcohol use.
**Values presented in Table are standardized beta-coefficients using linear regression models.

Table 3. Associations between salivary cortisol (sC) and social withdrawal subscales.

		N	BSI Social withdrawal	N	SF36 Social Functioning	N	DAPP social avoidance
AUCg (U/ml)	Crude	769	**0.076 (*p*=0.03)**	755	0.014 (*p*=0.70)	451	0.013 (*p*=0.78)
	Adjusted*	749	0.067 (*p*=0.06)	735	-0.007 (*p*=0.85)	436	0.016 (*p*=0.74)
AUCi (U/ml)	Crude	769	**0.079 (*p*=0.03)**	755	0.043 (*p*=0.24)	451	0.028 (*p*=0.55)
	Adjusted*	749	**0.082 (*p*=0.02)**	735	0.057 (*p*=0.11)	436	0.038 (p=0.44)
Evening sC	Crude	765	**0.147 (*p* < 0.001)**	749	**0.113 (*p* = 0.002)**	442	0.034 (*p* = 0.48)
(U/ml)	Adjusted*	747	**0.110 (*p* = 0.003)**	731	0.050 (*p* = 0.17)	428	0.027 (p = 0.59)
sC after DST(U/ml),	Crude	752	0.027 (*p*=0.45)	739	0.019 (*p*= 0.61)	448	-0.068 (*p* = 0.15)
at awakening	Adjusted*	720	0.007 (p=0.86)	709	-0.015 (*p*=0.69)	425	-0.069 (*p*=0.16)
Diurnal sC	Crude	726	**0.112 (p=0.002)**	712	0.064 (p=0.09)	420	0.009 (p=0.86)
	Adjusted*	710	**0.097 (p=0.01)**	696	0.020 (p=0.58)	407	0.010 (p=0.84)

*Adjusted for gender, age, Northern European ancestry, education level, season, time of awakening, hours of sleep, weekday versus weekend, and alcohol use.
**Values presented in table 1 are standardized beta-coefficients using linear regression models.

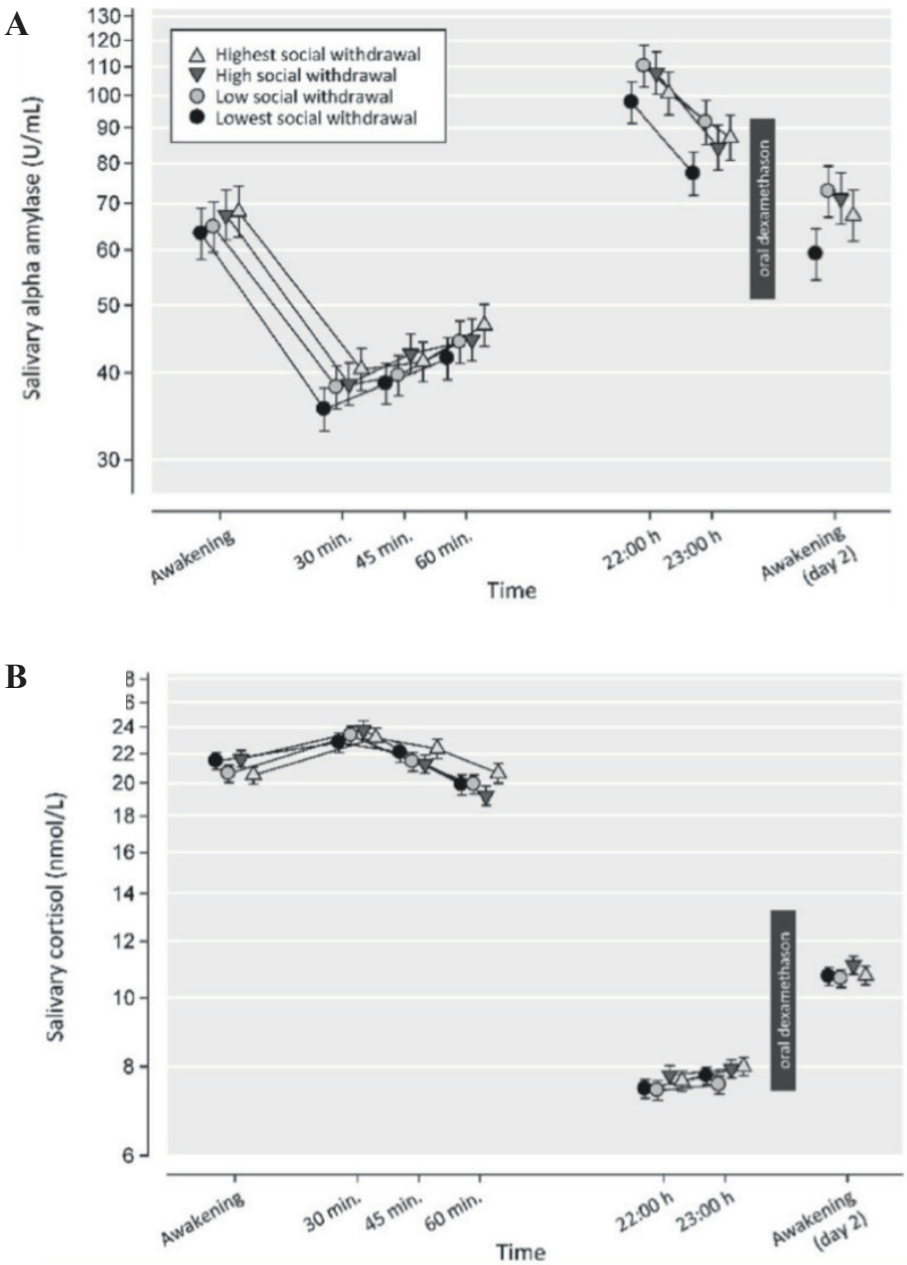

Figure 1. (A) A visual representation of sAA levels according to the composite SW scale at each time point. (B) A visual representation of sC levels according to the composite SW scale at each time point.

Mediation analyses

Using Preacher and Hayes' mediation model (2008), a statistical mediation analysis was run to determine whether social withdrawal is a mediating variable in the relationship between sAA and sC elevations on the one hand and increased depression severity as recorded on the MADRS on the other hand (see Figure 2). After adjustment for covariates, social withdrawal, which was measured using the composite scale comprised of all the three standardized subscales, was not found to have a mediating effect in the relationship between sAA and MADRS scores at any of the time points. Social withdrawal was only found to have a significant mediating effect in the relationship between evening sC and MADRS scores (see Mediating effects (M) in Table 4 and Figure 2). The effect size for this significant association was calculated using the Completely Standardized Indirect Effect (abcs = 0.06, 95% CI: 0.012-0.111), indicating a small effect size.

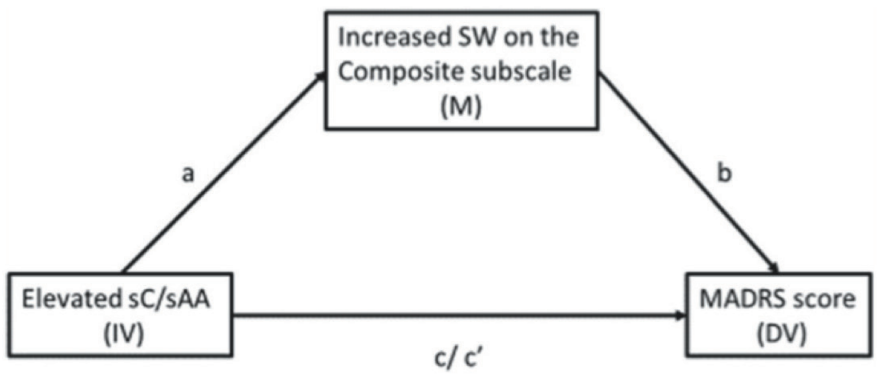

Figure 2. Model of elevated sC/sAA, increased SW as scored on the composite scale, and increased MADRS scores suggesting that increased SW is the intermediate factor between elevated sC/sAA secretion and increased depression scores.

Table 4. Potentioal mediating effects of the social withdrawal (SW) composite on the relationship between sAA and sC levels (IV) and MADRS score (DV).

	Model	n	Effect of IV on M (a)	Effect of M on DV (b)	Direct effect of IV on DV (c')	Mediating effect (a x b; 95% CI) (M)	Total effect of IV on DV (c)
sAA indicators (IV):							
AUC_g	Crude	764	0.0728*	-0.0196	-0.0196	**0.0518 (0.0032; 0.0974)**	0.0322
	Adjusted	750	0.0478	-0.0259	-0.0259	0.0338 (-0.0150; 0.0790)	0.0079
AUC_i	Crude	764	-0.0124	-0.0157	-0.0157	-0.0088 (-0.0585; 0.0387)	-0.0245
	Adjusted	750	0.0147	-0.0056	-0.0056	0.103 (-0.0434;0.0611)	-0.0047
Mean T1 and T7	Crude	768	0.0716*	0.0004	0.0004	**0.0509 (0.0072; 0.0914)**	0.0514
at awakening	Adjusted	754	0.0424	-0.0073	-0.0076	0.0312 (-0.0168; 0.0761)	0.0235
Mean sAA	Crude	727	0.0793*	-0.0267	-0.0267	**0.0565 (0.0066; 0.1046)**	0.0298
	Adjusted	716	0.0560	-0.0354	-0.0354	0.0396 (-0.0094; 0.0882)	0.0042
sC indicators (IV):							
AUC_g	Crude	770	0.0425	0.0551*	0.0551*	0.0300 (-0.0207; 0.0826)	0.851*
	Adjusted	750	0.0263	0.0537	0.0537*	0.0181 (-0.0309; 0.0695)	0.0718*
AUC_i	Crude	770	0.0033*	0.0049	0.0049	0.0445 (-0.0152; 0.1058)	0.0494
	Adjusted	750	0.0669	-0.0055	-0.0055	0.0462 (-0.0152; 0.1073)	0.0407
Evening SC	Crude	727	0.1341***	0.0683***	0.0683*	**0.0943 (0.0423; 0.1507)**	0.1626***
	Adjusted	711	0.0941*	0.0665*	0.0665*	**0.0644 (0.0118; 0.1165)**	0.1309***
sC after DST	Crude	686	0.0309	0.0845**	0.0845**	0.0217 (-0.0300; 0.0729)	0.1062**
at awakening	Adjusted	666	0.0003	0.0904**	0.0904**	0.0002 (-0.0513; 0.0519)	0.0407*
Mean sC	Crude	727	0.0846*	0.0832**	0.0832**	**0.0596 (0.0076; 0.1119)**	0.1428***
	Adjusted	711	0.0591	0.0857**	0.0857**	0.0405 (-0.0107; 0.0919)	0.1262**

MADRS, Montgomery-Åsberg Depression rating scale; BSI, Brief Symptom Inventory; Composite subscale, a composite of the BSI social withdrawal, the SF-36 social functioning, and the Dimensional Assessment of Personality Pathology – Short Form (DAPP-SF) social avoidance subscales. Mediation analyses according to Preacher & Hayes: IV denotes the Independent variables, M denotes mediating variable, DV denotes dependent variable, a denotes effect of IV on M, b denotes effect of M on DV, c' denotes direct effect, a x b denotes indirect mediating effect, c denotes total effect.

sAA adjusted for gender, age, Northern European ancestry, and alcohol use. sC adjusted for gender, age, Northern European ancestry, education level, season, hours of sleep, weekday versus weekend, and alcohol use.

*** *p-value* < .001; ** *p-value* < .01; * *p*-value < .05.

4. Discussion

The current study examined the association between sAA and sC with three social withdrawal subscales in a naturalistic sample of psychiatric out-patients and healthy controls. Although no significant associations were found between sAA and social withdrawal subscales, significant associations were found between sC AUCi, evening sC, and diurnal sC samples with social withdrawal, but only as measured on the BSI social withdrawal subscale. Furthermore, social withdrawal was not found to be a mediating factor in the relationship between sC and sAA on the one hand and the severity of depressive symptoms on the other hand. Therefore, our findings do not support the idea that increased basal and diurnal sAA are associated with social withdrawal, but suggest that HPA axis activation may be implicated in this relationship.

Recent studies have found an association between sAA, an enzyme that is increasingly secreted under the stimulation of the ANS, and certain psychiatric disorders at specific time points. More specifically, we found elevated levels of naturalistic sAA at awakening in patients with a current major depressive disorder (MDD) diagnosis in comparison to patients with (comorbid) anxiety and somatoform disorders and healthy controls, in a previous analysis of the current sample (Bauduin et al., 2018). Also, a study with gSAD patients and healthy controls found elevated sAA levels in a naturalistic patient sample (van Veen et al., 2008). These findings gave rise to the idea that elevations in sAA may be disease and time-point specific reflections of ANS activation. As more targeted research has been geared towards the understanding of social withdrawal as a common endophenotype across various psychiatric disorders and research has indicated that there may be an association between HPA-axis activation and social withdrawal, we hypothesized a role of the ANS in the relationship with social withdrawal as well. However, no significant associations were found between sAA and any of the social withdrawal subscales indicating that sAA (as measured in this study) is unlikely to be an important biomarker of social withdrawal. We also did not find evidence for a mediating role of social withdrawal in the relationship between sAA and depression severity. In sum, the findings suggest that ANS activation as assessed using the basal and diurnal sAA samples collected in this study is unlikely to be involved in the etiology of social withdrawal.

Ample research has found that the hypersecretion of the stress hormone cortisol secreted under activation of the HPA-axis is associated with a variety of psychiatric disorders (Gil et al., 2008; Goodyer et al., 2000; Lenze et al., 2011). Our findings

offer further support for this as the statistical mediation analysis replicated these findings (i.e. the effect of the independent variable on the dependent variable), and also found this positive association between the sC AUCg, evening sC, sC after the DST, and mean sC levels and the MADRS scores, increasing the validity with regard to our sAA findings. Previous studies investigating the association between social withdrawal and HPA-axis activity have found equivocal results, echoing findings from animal studies (Hawkley et al., 2012; Cacioppo, Cacioppo, Capitanio, & Cole, 2015), the majority of which have found a positive association between sC and social withdrawal (Adam et al., 2006; Arnetz, Theorell, Levi, Kallner, & Eneroth, 1983; Doane & Adam, 2010; Grant, Hamer, & Steptoe, 2009; Hawkley, Cole, Capitanio, Norman, & Cacioppo, 2012; Pressman et al., 2005; Steptoe et al., 2004). This was also partially the case with regard to the current study. We found significant positive associations between sC markers and the BSI social withdrawal subscale, but not between sC and the other social withdrawal subscales.

Regarding social withdrawal as a putative mediating variable between sC and depression, an earlier statistical mediation analysis found that social withdrawal mediates the association between sC and depression as measured on the Beck Depression Inventory (Tse & Bond, 2004). However, this study did not adjust for numerous influential covariates (Stalder et al., 2016; i.e. the model was adjusted only for gender, age, and cortisol concentration). In our study we found similar results, namely that social withdrawal mediated part of the relationship between evening and mean sC samples and depression, although this effect only remained significant with regard to the evening sC samples after covariate adjustment with a very small effect size. It is possible that we were unable to find the mediating effect of social withdrawal in the unadjusted analyses with the other sC samples due to the lack of specificity of the composite scale or the limited number of scores in the higher range of the MADRS. It may likewise be the case that we were unable to replicate the results from the previous study in all of our sC samples because we used a different and sophisticated mediation method (Preacher & Hayes, 2008). A previous study conducted by our group using the same saliva samples as in the current study found elevated evening sC, and not morning sC levels, in depressed subjects. This is likely to have influenced the significant mediation effect we found. Although other studies have found similar results (Young et al, 1994; Vreeburg et al., 2009), the most likely possibility is that social withdrawal is only a minor mediating factor in the association between sC and depression, as it is plausible that the significant but relatively small statistical mediation effect found in the earlier paper would be attenuated if all influential covariates would have been accounted for.

There were a number of limitations in this study. The first is the cross-sectional nature of the study, which does not allow for causal inferences. Second, the subscales that were used to measure social withdrawal may not have been sensitive enough in their ability to measure social withdrawal without enough precision. Also, the DAPP-SF social withdrawal subscale, the psychometric tool with the strongest face validity to measure social withdrawal, had some missing observations in comparison to the other two social withdrawal subscales. Third, changes in subjects' body position were not considered, as standing to sitting and vice versa can induce rapid changes in sAA enzyme activity (Bosch, Veerman, de Geus, & Proctor, 2010). Fourth, although participants were given strict instructions to refrain from eating, drinking, smoking, or brushing teeth 15 minutes prior to sampling, it should be noted that due to the ambulatory and naturalistic setting of this study it is more difficult to monitor compliance than in a systematic clinical trial. However, a previous study using the same saliva samples as the current study replicated the well-established diurnal curve of sC, suggesting adequate validity (Bauduin et al., 2018). A further potential limitation may be that we were unable to adjust for psychotropic medication use as there were no participants who used psychotropic medication in the healthy control group. Also, although we adjusted our analyses for several potential confounders, there may be residual confounding (e.g. menstrual cycle, gingivitis). Furthermore, only a small group of participants suffered from moderate to severe depression (i.e. n = 61 (7%) with MADRS score >19). Therefore, our findings cannot be extrapolated to patients with more severe depressive psychopathologies or patients with other psychiatric disorders. Moreover, selection bias may also be the case, as highly socially withdrawn patients may not have volunteered to participate in the study. Finally, we did not record the postage day of the saliva samples and were therefore unable to correct for possible warming effects during postal delivery. However, if this were to have an effect on the samples, it is likely to have added random error, leading to findings approaching the null hypothesis.

Strengths of this study include the dimensional approach of the construct social withdrawal as an endophenotype across psychiatric disorders using three subscales. Furthermore, we were able to include a reasonably large naturalistic sample of consecutive ROM out-patients with MAS- disorders alongside a large sample of healthy controls that were specifically recruited as a reference group for the ROM participants. Moreover, saliva samples were obtained over the course of the day using a non-invasive sampling method, which is suitable for clinical use (Nater & Rohleder, 2009).

Future research investigating social withdrawal should use dedicated psychometric assessment tools to measure social withdrawal, for example the World Health Organization Disability Assessment Schedule 2 social withdrawal scale (Üstün et al., 2010), and should also differentiate between state and trait characteristics of social withdrawal (Doane & Adam, 2010). Furthermore, the association between social withdrawal and stress-related psychiatric disorders should preferably use prospective longitudinal designs, and include a wider severity range of psychopathology. Also, although previous research has found an association between sC and social withdrawal, the effect sizes in these studies were generally rather small and these studies often did not adjust for a number of important covariates (i.e. Stalder et al., 2016). Therefore, it may be prudent to also further explore associations that have previously been found to be promising biological markers for social withdrawal, namely the cardiovascular system (Steptoe et al., 2004; Grant, Hamer, & Steptoe., 2009), and other stress systems that involve inflammation and immune activation (i.e. acute-phase responses and natural killer cell counts; Steptoe et al., 2004), as heightened inflammatory activity seems to be involved in social withdrawal, as part of sickness behavior (Inagaki et al., 2012). Furthermore, in light of findings from experimental studies, possible gender differences in the relationship between SNS activation and social withdrawal should also be further explored (Campi et al., 2014; Greenberg et al., 2014).

In conclusion, this study found evidence for the idea that a chronic HPA-axis activation may stimulate social withdrawal, however that basal and diurnal sAA levels are less likely to be involved.

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Appendix

BSI social withdrawal subscale items (items 3, 14, 34, 44, and 53 on the BSI):

- The idea that someone else can control your thoughts
- Feeling lonely even when you are with people
- The idea that you should be punished for your sins
- Never feeling close to another person
- The idea that something is wrong with your mind

SF-36 social functioning subscale items (items 20 and 32 on the SF-36):

- During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups? (Answers range between 1 (not at all) and 5 (extremely).
- During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)? (Answers range between 1 (all of the time) and 5 (none of the time).

DAPP-SF social avoidance subscale items (items 7, 15, 27, 114, 127, and 128 on the DAPP): Subtraits of the DAPP-SF social avoidance subscale are low affiliation, fearful of interpersonal hurt, defective social skills, desire for improved affiliative relationships, social apprehensiveness.

- Ik voel me niet erg zeker van mezelf in het gezelschap van anderen
- Op feestjes of bijeenkomsten neig ik ernaar om mensen te ontlopen
- In iedere groep maak ik me zorgen dat ze me buiten zullen sluiten of zullen afwijzen
- Mensen maken me zenuwachtig
- Ik weet nooit hoe ik me moet gedragen als er andere mensen bij zijn
- Ik zou willen dat ik gezelliger met andere mensen kon omgaan

Free translation to English:

- I am not very sure of myself in the company of others
- At parties or meetings I tend to avoid people
- In every group I am concerned that they will shut me out or reject me
- People make me nervous
- I never know how to behave when other people are present
- I wish I could interact with other people more socially

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