Title: AFFECTIVE ALTERATIONS IN PATIENTS WITH CUSHING'S SYNDROME ARE ASSOCIATED WITH DECREASED BDNF AND CORTISONE LEVELS

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Abstract

Objective: Affective alterations and poorer quality of life often persist in patients with Cushing's syndrome (CS) after correction of hypercortisolism. Brain-derived neurotrophic factor (BDNF) is involved in the regulation of the hypothalamic-pituitary-adrenal axis (HPA) and is highly expressed in brain areas controlling mood and response to stress. Our aims were to assess affective alterations after long-term remission of CS and evaluate whether they are associated with serum BDNF levels, and salivary cortisol (SalF) and/or cortisone (SalE) concentrations.

Subjects and methods: Thirty six CS patients (32 females/4 males; mean age (±SD), 48.8±11.8 years) in remission for a median of 72 months, and 36 gender-, age-, and BMI-matched controls were included. No patients were on hydrocortisone replacement. Beck Depression Inventory II (BDI-II), Center for Epidemiological Studies Depression Scale (CES-D), Positive Affect Negative Affect Scale (PANAS), State Trait Anxiety Inventory (STAI), Perceived Stress Scale (PSS) and EuroQoL and CushingQol questionnaires were completed to evaluate anxiety, depressive symptoms, affect, stress perception and quality of life (QoL) respectively. Salivary cortisol was collected twice a day (at 8 a.m. and 11 p.m.) on two consecutive days and measured using liquid chromatography/tandem mass spectrometry (LC/TMS). BDNF was measured in plasma using an ELISA assay.

Results: CS patients showed worse scores in all questionnaires than controls: STAI ($p<0.001$), BDI ($p<0.001$), CES-D ($p<0.001$), PANAS ($p<0.01$), PSS ($p<0.01$) and

EuroQoL (p<0.01). A decrease in BDNF values was observed in CS as compared with controls (p=0.038), and low BDNF levels were associated with more anxiety (r=-0.247, p=0.037), depressive symptoms (r=-0.249, p=0.035), stress (r=-0.277, p=0.019) and affective balance (r=0.243, p=0.04). No significant differences in salivary cortisol were observed between CS patients and controls, but morning salivary cortisone concentrations were inversely associated with trait anxiety (r=-0.377, p=0.040) and depressed affect (r=-0.392, p=0.032) in CS patients, but not in controls. Delay to diagnosis was associated with depressive symptoms (BDI-II: r=0.398, p=0.036 and CES-D: r=0.449, p=0.017) and CushingQoL scoring (r=-0.460, p<0.01).

Conclusions: Low BDNF levels are associated with affective alterations in "cured" CS patients. Assessment of SalE may be a marker of affective status in these patients.

Keywords: Cushing's syndrome, anxiety, stress, depression, BDNF, salivary cortisol, salivary cortisone

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INTRODUCTION

Cushing's syndrome (CS) is a rare endocrine disorder caused by chronic exposure to cortisol excess [1]. Patients with CS show poor neuropsychological status, including depression, anxiety and mania, which also affect health-related-quality of life (QoL) [2-6]. Recent evidence indicates that some of the behavioral dysfunctions observed in the active phase of CS do not fully revert after correction of hypercortisolism [7-15]. In particular, an increased prevalence of affective alterations, such as anxiety, emotional lability and apathy, has been described in patients with Cushing's disease (CD) at an average of 11 years of remission, as compared not only with healthy subjects, but also with patients who had been treated for non-functioning pituitary macroadenomas [16]. As a matter of fact, recent magnetic resonance (MR) imaging studies showed structural and functional brain damage in CS patients long-term after remission, mainly at those neural structures, rich in glucocorticoid receptors (GR), which are involved in stress reactivity and emotional processing [17-20]. These findings suggest that previous over-exposure to cortisol may cause irreversible effects on brain, thus leading to sustained psychological symptomatology after eucortisolism is reached [21]. Of note, Dorn et al. reported an inverse association between persistent psychopathology and morning cortisol values in CS patients in long-term remission, indicating that a sustained derangement of the hypothalamic-pituitary-adrenal axis after restoration of eucortisolism may increase the individual vulnerability to stressors [8].

Brain-derived neurotrophic factor (BDNF), a member of the nerve growth factor (NGF) family, is a prominent mediator of neuronal differentiation, survival, and plasticity, and is highly expressed in the hippocampus and prefrontal cortex, which constitute a neural circuitry controlling mood and response to stress [22]. Decreased BDNF expression in these regions has been described in several models of rodents exposed to chronic stress, and has been associated with depression-like behavior [23]. In humans, serum BDNF levels are lower in stress-induced affective disorders, including major depression disease (MDD), compared with healthy controls, and are increased by antidepressant treatment [22]. Noteworthy, BDNF is involved in the regulation of the hypothalamic-pituitary-adrenal axis (HPA), in that the intracerebroventricular administration of this neurotrophin enhances the expression of corticotrophin releasing factor (CRF) in the hypothalamus of adult rats, leading to elevation of plasma corticosterone levels [24].

Assessment of salivary cortisol (SalF), a surrogate for free serum cortisol (SerF), is routinely used as a biomarker of psychological stress and depressive symptoms [25], and has also been advocated in clinical practice as an accurate test to diagnose CS [26] or ascertain postoperative outcome in CD patients [27]. Yet, because 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) converts cortisol (SalF) to cortisone (SalE) in the parotid gland, the latter is the most abundant glucocorticoid in saliva and, therefore, may be a source of interference in the most frequently used immunoassays [28]. Indeed, it has been recently demonstrated that salivary cortisone (SalE) is highly correlated to SerF concentrations under both basal and stimulated conditions, suggesting that it could be more reliable than SalF in assessing the biological activity of cortisol [29]. To the best of our knowledge no data have been published thus far on SalE in CS patients in long-term remission.

We hypothesized that 1) BDNF is decreased in “cured” CS and modulates mood and stress perception in these patients; 2) morning and late-night SalE concentrations are associated with impaired emotional status in CS patients in long-term remission.

Thus, the aim of this study was to examine the relationship between BDNF levels, depressive and anxiety symptoms, impairment of stress perception and emotional status, as well as poor HRQoL, in patients treated for CS. In addition we aimed at determining if there was an association between SalF and/or SalE, as assessed by a highly sensitive and specific liquid chromatography/tandem mass spectrometry (LC/TMS) method, and depressive symptoms as well as BDNF values in our patients.

SUBJECTS

We studied 36 patients with Cushing’s syndrome in remission [32 females and 4 males; mean age (±SD), 48.83±11.8 years; 20 females (63%) were premenopausal] who were included successively in our study while attending the endocrine clinic at our hospital.

They were matched for age, gender and body mass index (BMI) with 36 patients who were recruited through advertisement at the blood donor center of our institution (Table 1).

The diagnosis of CS was made after clinical, biochemical and radiological evaluation, based on internationally agreed guidelines. All patients had abnormal values on at least two of the following tests: elevated UFC, late night salivary or serum cortisol, 1-mg overnight dexamethasone suppression test (ODST) or 48-h 2-mg/day low-dose dexamethasone suppression test (LDDST).

Twenty-five patients had Cushing's disease (CD) due to a microadenoma (n=33) or a macroadenoma (n=3). The remaining 11 patients had an adrenal adenoma. All the CD patients underwent transsphenoidal surgery (TSS) from 12 to 288 months (median: 78 months) previously, and 6 of them (24%) also received radiotherapy from 18 to 96 months (median: 54 months), after unsuccessful surgery (n=2) or relapse (n=4). All the patients diagnosed with an adrenal adenoma underwent adrenalectomy from 12 to 312 months (median: 54 months) previously.

Twenty patients (56%) received preoperative treatment with steroidogenesis inhibitors in order to control clinical symptoms of hypercortisolism from 1 to 22 months (median: 4.6 months) prior to surgery.

Delay to diagnosis was defined as the time elapsed from onset of symptoms, as referred by patients, and final diagnosis of CS.

CS was considered in remission if either adrenal insufficiency was demonstrated (basal morning cortisol < 100 nmol/l [<4μg/dl] and/or undetectable 24-h free urinary cortisol) or morning cortisol suppression (<50 nmol/l, <1.8 μg/dl) after 1 mg dexamethasone overnight was observed. Thirty patients (83%) had received hydrocortisone (HC) replacement for a median of 9 months (range: 3-60 months) after surgery. At the time of study entry, no patient was on HC, for a median of 94 months (range: 5-204 months). Duration of remission was defined as the time elapsed from diagnostic confirmation of remission to study entry.

At study entry, three patients (8%) had growth hormone deficiency (GHD) on replacement with recombinant human GH; five women (14%) were gonadotropin-deficient on estrogen/progesterone hormone replacement, and five (14%) were hypothyroid, three due to TSH deficiency and two due to primary hypothyroidism (all on L-thyroxine replacement).

Exclusion criteria were: active disease, patients with ascertained adrenal insufficiency (i.e. morning cortisol values below 5 μg/dl or stimulated cortisol levels below 18 μg/dl) [30] and/or taking replacement therapy with hydrocortisone, diabetes mellitus, diagnosis of severe depression or other psychiatric condition, and patients taking more than one psychoactive drug.

During the study visit, patients and controls underwent a fasting blood drawing for the measurement of BDNF. They were also given four commercially available cotton devices (Salivette, Sarstedt, Numbrecht, Germany) for collection of morning (between 0730 and 0800 h) and late-night (2330 and 2400 h) saliva samples on two consecutive days. Subjects were instructed to refrain from brushing their teeth, smoking, eating or drinking other than water for at least 60 minutes before sampling. They were also instructed to store the Salivettes, after saliva sampling, at 4°C in a home refrigerator and send back by mail within 7 days. The cortisol diurnal rhythm (CDR) was then calculated. A normal CDR was defined by late-night salivary cortisol value of less than 75% of the morning value [31].

All subjects gave full informed consent and the study was approved by the local ethics committee.

ASSAYS

Salivary cortisol was measured by liquid-chromatography-tandem mass spectrometry (LC/TMS). Salivary cortisol (SalF) and salivary cortisone (SalE) were measured with a LC-MS/MS assay with lower limits of quantitation 0.39 nmol/liter (SalF) and 0.78 nmol/liter (SalE); intra-assay CVs of less than 9.3% and less than 7.9%; and interassay CVs of less than 9.7% and less than 10.3% at 1.8–52.2 nmol/liter of SalF and 3.6–96 nmol/liter of SalE, respectively [28-29]. The ratio SalF/SalE was calculated as a marker of 11β-HSD2 activity.

Brain-derived neurotrophic factor (BDNF) in plasma was performed by a Quantikine® ELISA (R&D Systems Europe LTD, UK). The limit of detection was less than 20 pg/mL. Inter-assay variation was 7.6 to 11.3% and intra-assay variation 3.8 to 6.2%.

MEASUREMENTS OF AFFECTIVE STATE

State-Trait Anxiety Inventory

State-Trait Anxiety Inventory (STAI) is a self-reported measure that includes two subscales to evaluate two types of anxiety: state anxiety (anxiety related to current events) and trait anxiety (anxiety as a personal characteristic). Each subscale has 20 questions with a four point scale ranging from 0-3. The total score for each subscale can range from 0-60. Higher scores indicate higher levels of anxiety [32].

Beck Depression Inventory-II

Beck Depression Inventory-II (BDI-II) is a self-reported measure of the severity of depressive symptoms. It has 21 items with a four point scale ranging from 0-3. The total score is the sum of each item-rating and can range from 0-63. Higher scores indicate more severe depressive symptoms. The BDI-II can be separated into affective and somatic dimensions [33-34].

Center for Epidemiological Studies Depression Scale

Center for Epidemiological Studies Depression Scale (CES-D) includes 20 items measuring subject frequency rate of depressive symptoms on a four-point scale ranging from 0-3. CES-D can be separated into four subscales assessing: 1) depressed affect; 2) vegetative depression; 3) loss of well-being; and 4) interpersonal relationships. Higher scores indicate more frequency of depressive symptoms [35].

Positive and Negative Affective Schedule

Positive and Negative Affective Schedule (PANAS) is a self-reported measure of positive and negative emotions in the last week. PANAS consists on 20 words (10 reflect positive affect and 10 negative affect) which are rated from 1 to 5. Higher score indicates more intensity in the emotion. The affective balance (total score) is calculated as the difference between positive affect and negative affect [36].

Perceived Stress Scale

Perceived Stress Scale (PSS) measures the perception of situations appraised as unpredictable or uncontrollable over the last month and includes 14 items with a 5-point response scale. A higher score indicates a higher level of perceived stress [37].

MEASUREMENTS OF HEALTH-RELATED QUALITY OF LIFE (QoL)

EuroQoL

EuroQoL is a generic questionnaire of QoL that is divided in 2 parts. EuroQoL-5D profile evaluates five health dimensions (mobility, self-care, usual activities, pain/discomfort, and anxiety/depression) with a single item with three possible answers (having no problems, having some or moderate problems, being unable to do/having extreme problems).

EuroQoL-VAS is a visual analogue scale that evaluates the self-perception of current health [38], ranging from 0 (worst possible) to 100 (best possible QoL).

CushingQoL

CushingQoL is a disease-generated questionnaire, which consists of 12 items referring to problems relevant to CS patients. Each item has five categories of response related to frequency and degree of agreement with the sentence. The total score is the sum of each item-rating (range 12 to 60) and higher scores indicate better QoL [6,14].

STATISTICAL ANALYSIS

Data were analyzed using SPSS 21.0 statistical package for Windows (SPSS Inc., Chicago, IL, USA) with a level of significance of $p<0.05$. All quantitative data are expressed as mean±SD (Gaussian distribution) or as median (range) (non-Gaussian distribution). Data distribution was analyzed by the Kolmogorov-Smirnov test. Comparisons between the two groups were performed using Student's t-test and Mann-Whitney's U. Correlations among variables were studied using Pearson's correlation coefficient for parametric measures and Spearman's rho for non-parametric measures. Furthermore, stepwise multiple linear regression analyses were performed in order to assess potential predictors of CushingQoL scoring.

Results

HORMONE PARAMETERS

No differences in either SalF or SalE were observed between CS patients and healthy controls. Cortisol variability measured by CDR and ratio SalF/SalE were also similar in CS and controls (See Table 1). BDNF values were significantly lower in CS patients than controls (433.5±278.8 pg/mL vs. 602.8±391.1 pg/mL, $p=0.038$) (Figure 1).

AFFECTIVE STATE AND QoL

CS patients showed both higher state and trait anxiety scores than controls (state, 22.9±13.4 vs. 12.2±8.1; trait, 25.8±13.7 vs. 14.9±8.5; $p<0.001$ for both comparisons) (Table 2). Total score of BDI-II was higher in CS patients than controls, indicating more depressive symptoms (11.3±10.2 vs. 3.4±2.9, $p<0.001$). CS patients scored worse on affective and somatic subscales of BDI-II than controls (affective, 6.7±1.3 vs. 1.7±0.3; somatic, 4.6±0.5 vs. 1.7±0.3; $p<0.001$ for both comparisons) (Table 2). Twenty-four CS patients (67%) showed minimal symptoms, 4 (11%) mild symptoms, 5 (14%) showed moderate symptoms and three (8%) severe symptoms of depression.

CS patients also reported greater total depressive symptoms than controls using the CES-D (16±13.2 vs. 6.5±6.4; $p<0.001$). CS patients scored higher on subscales of depressed affect, vegetative depression and loss of well-being compared to healthy controls (depressed affect, 4.6±0.9 vs. 1.4±0.4; vegetative depression, 5.3±0.9 vs.

2±0.4; loss of well-being, 5.5±0.6 vs. 2.9±0.5; p<0.01 for all comparisons). No differences in the subscale of interpersonal relationships were found between CS and controls. On PANAS, the affective balance score was lower in CS patients than healthy controls (7.5±2.4 vs. 19.3±1.8; p<0.001). CS patients scored higher on negative affect and lower on positive affect than controls, as assessed by PANAS (negative affect, 18.6±9.4 vs. 13.6±3.7; positive affect, 26±8.7 vs. 32.9±9.2; p<0.01 for both comparisons) (Table 2).

Total score of PSS was higher in CS patients than healthy control (24.9±9.8 vs. 17.6±8; p<0.01), indicating greater stress perception (Table 2).

CS patients showed poorer QoL than healthy controls, as assessed by EuroQoL-VAS (70.9±19.8 vs. 84.5±14.5; p<0.01). Cushing QoL in CS patients was 40±9.9.

RELATIONSHIP BETWEEN HORMONE VALUES, AFFECTIVE STATE AND QoL

BDNF levels were negatively correlated with the trait anxiety score of STAI (r=-0.247, p=0.037), the affective dimension score of BDI-II (r=-0.249, p=0.035), and the perception of stress (r=-0.277, p=0.019) measured by PSS. Moreover, BDNF was correlated with the affective balance and positive affect of PANAS (affective balance, r=0.243, p=0.04; positive affect, r=0.295, p=0.012). These associations remained significant after correcting for age.

There was an inverse correlation between level of morning salivary cortisone and scores on trait anxiety of STAI (r=-0.377, p=0.040) and depressed affect of CES-D (r=-0.392, p=0.032) (Figure 2). Midnight SalF/ SalE ratio was negatively correlated with BDNF levels (r=-0.401, p=0.035) in CS patients but not in controls.

On the other hand, delay to diagnosis was related to the total score of BDI-II and total score of CES-D (BDI-II: r=0.398, p=0.036 and CES-D: r=0.449, p=0.017), two measurements of depressive symptoms, and CushingQoL scores (r=-0.460, p<0.01) (Figure 3). These correlations lost significance after correction for age. We did not find any correlations between duration of remission and both affective state and QoL measurements.

When CushingQoL scoring was entered as a dependent variable and age, delay to diagnosis, total score of BDI-II as independent variables in a forward stepwise regression model, total score of BDI-II was the only predictor of CushingQoL (β=-0.647; p<0.001) in CS patients.

Discussion

We have shown that CS patients in long-term remission have low levels of circulating BDNF, which are associated with more anxiety trait, depression and perceived stress, and less affective balance and positive affect, regardless of age and SalF/SalE concentrations. These findings support the hypothesis that BDNF plays an important role in the modulation of affective state and stress perception, even long-term after correction of hypercortisolism. In addition, we have shown that SalE levels early in the morning are inversely associated with anxiety trait and depressed affect.

BDNF is an important regulator of the HPA axis adaptive response to stress [39]. In animal models, central infusion of BDNF activates the HPA axis, while chronic restraint stress down-regulates glucocorticoid receptor expression in the brain, alter BDNF function, and induces anxiety- and depression-like behaviors in rats [40]. The expression of BDNF is reduced in animal models of depression at the level of the hippocampus and prefrontal cortex, both being important brain regions involved in stress responsivity and emotional functioning [41]. Because circulating BDNF concentrations decrease in mood disorders and increase after successful treatment in humans, it is a well-established biomarker of depression [22]. We have shown an association between low BDNF and impaired mood and stress perception in CS patients but not in matched controls.

BDNF levels have not been studied in patients with active CS thus far, but *in vivo* experiments have demonstrated a negative association between chronic exposure to corticosterone and BDNF expression in the brain regions regulating the response to stress [39-40]. BDNF expression increases in the hippocampus of adrenalectomized rats and returns to normal after treatment with dexamethasone [42]. Moreover, continuous administration of corticosteroids decreases BDNF mRNA in the frontal cortex of rats, which is associated with atrophy of the limbic structures [40]. Of note, frontal cortical thining has been previously described in CS patients after long-term remission [19]. Consistently, we have shown a negative association between BDNF and midnight SalF/ SalE ratio in our CS patients only. Thus, the observed decrease in BDNF may be one of the persisting abnormalities in "cured" CS patients after the achievement of eucortisolism, and may reflect irreversible structural and/or functional changes in neural circuitries as a consequence of previous exposure to cortisol excess.

Another important finding of our study is that morning SalE is inversely related to depressed affect and anxiety trait in "cured" Cushing's syndrome only, in contrast with SalF, which was not associated with any psychological dimensions in the groups analyzed. Recent evidence suggests that SalE may reflect more accurately fluctuations in SerF than SalF and, therefore, may be useful to measure the biological activity of cortisol in several conditions associated with HPA axis dysregulation, including CS and mood disorders [28]. Whereas SalF assessment is clinically used in both endocrine and psychiatric conditions as a diagnostic marker of hypercortisolism [26] and psychological stress [25] respectively, data on SalE in these contexts are scanty. To the best of our knowledge, this is the first time that SalE has been measured in patients who suffered CS, and is related to residual morbidity.

Although levels of SalE were similar between CS patients and controls, we documented an inverse relationship between morning SalE and measures of mood dysfunction. HPA hypoactivity has been described in patients with atypical depression as well as several stress-related states showing an heterogenous spectrum of depressive symptoms, such as chronic fatigue syndrome and fibromyalgia [43]. Dorn et al. found that atypical depression was the most prevalent psychopathology in CS patients one year after remission, and lower morning baseline concentrations of cortisol were related to higher numbers of psychological symptoms, including anxiety, depression and psychoticism [3]. This finding led the authors to speculate that derangement of the HPA axis, namely low CRH production or relative glucocorticoid deficiency, may persist long-term despite correction of hypercortisolism and contribute to the maintanence of affective disorders in remitted CS [3, 44]. We cannot support this hypothesis, because we did not extensively evaluate the HPA axis responsiveness under stimulated conditions. In addition, although we cannot exclude that some of our patients had relative glucocorticoid deficiency at study entry, hydrocortisone replacement was withdrawn in all of them, after recovery of the HPA axis was confirmed by an ACTH stimulation test [45].

Although our CS patients showed clear depressive symptomatology and scored worse than controls on several scales assessing mood and affect, they did not meet the criteria for clinical depression, in line with the results of prior studies [11, 16, 46]. This indicates that patients previously exposed to cortisol excess maintain an increased vulnerability to stress, leading to a longstanding “subtle”, but nevertheless clinically relevant, psychopathology. The pathogenesis of this is probably multifactorial, not only including dysregulated HPA axis (Heim), but also coping strategies and personality traits [16], impaired decision making [19], previous stress induced by suffering a chronic disease [5], and structural/functional alterations of brain regions controlling emotional functioning and stress modulation [17].

In our study, delay to diagnosis was related with depressive symptoms and QoL. Recent evidence suggests that early diagnosis and treatment are clinical predictors of QoL after achieving remission of CD [47]. However, we have found that depressive symptomatology is the main negative predictor of QoL rather than delay to diagnosis. Thus, our data suggest that affective alterations, mainly depressive symptoms, might play a pivotal role in affecting patients' wellbeing and daily living after long-term remission, in line with findings observed in active patients [48]. Therefore, screening for subtle affective alterations should be included in the regular follow-up of CS patients.

This study has several limitations. The small sample size, an unavoidable drawback of the studies on rare diseases, may also account for the lack of significance in some of the analyses performed. Another limitation is its crosssectional design, which prevented us from inferring causalty from these data. In addition, because BDNF plays a role in the regulation of energy homeostasis and food intake, circulating BDNF levels in serum could be influenced by other factors, including body weight and nutritional habits [49].

In conclusion, altered affectivity, mainly anxiety, depressive symptoms, negative affect and stress, persist long-term in patients previously treated for CS, and SalE concentrations might be a potential marker of the affective status in these patients. Low levels of BDNF could be involved in the pathophysiology of the affective alterations observed in patients with CS in remission.

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Table 1. Clinical characteristics of CS patients and healthy controls.

	CS patients (n=36)	Healthy controls (n=36)	*P value*
Gender (female/male)	32/4	32/4	p=0.99
Age (years)	48.8±11.8	48.8±11.7	p=0.99
Body mass index (BMI, kg/m^2)	27±4.7	26.1±5.7	p=0.46
Origin of CS:		-	
- Pituitary adenoma	25 (69%)		
- Adrenal adenoma	11 (31%)		
Transsphenoidal surgery	25 (69%)	-	
Radiotherapy	6 (24%)	-	
Previous medical treatment with steroidogenesis inhibitors	20 (56%)	-	
Delay to diagnosis (months)	24 (7-132)	-	
Duration of remission (months	72 (3-312)	-	
Gonadotropin deficiency	3 (8%)		
Growth hormone deficiency	5 (14%)		
Hypothyroidism	5 (14%)		
Morning salivary cortisol (nmol/L)	5.8±3.9	6.3±4.1	p=0.63
Late-night salivary cortisol (nmol/L)	1.6±1.1	1.2±0.9	p=0.16
Morning salivary cortisone (nmol/L)	16.5±9.9	17.1±9.2	p=0.81
Late-night salivary cortisone (nmol/L)	5.9±6	4.4±3.2	p=0.23
Cortisol diurnal rhythm (CDR) (%)	51.8±11.3	55.5±18.3	p=0.87
Morning SalF/ SalE ratio	0.39±0.19	0.37±0.2	p=0.65
Midnight SalF/ SalE ratio	0.38±0.31	0.32±0.23	p=0.45

All values are expressed as mean (±SD); nonparametric values are expressed as median (range)

Table 2. Affective state in CS patients and healthy controls.

STAI, State-Trait Anxiety Inventory; BDI-II, Beck Depression Inventory-II; CESD, Center for Epidemiological Studies Depression Scale; PANAS, Positive and Negative Affectice Schedule; PSS, Perceived Stress Scale.

Questionnaires	**CS patients (n=36)**	**Healthy controls (n=36)**	**p value**
STAI			
Anxiety state	22.9±13.4	12.2±8.1	p<0.001
Anxiety trait	25.8±13.7	14.9±8.5	p<0.001
BDI-II			
Total score	11.3±10.2	3.4±2.9	p<0.001
Affective dimension	6.7±1.3	1.7±0.3	p<0.001
Somatic dimension	4.6±0.5	1.7±0.3	p<0.001
CES-D			
Total score	16±13.2	6.5±6.4	p<0.001
Depressed affect	4.6±0.9	1.4±0.4	p=0.001
Vegetative depression	5.3±0.9	2±0.4	p=0.001
Loss of well-being	5.5±0.6	2.9±0.5	p=0.002
Interpersonal relationship	0.56±1.2	0.25±0.5	p=0.185
PANAS			
Affective balance (or total score)	7.5±2.4	19.3±1.8	p<0.001
Positive affect	26±8.7	32.9±9.2	p=0.002
Negative affect	18.6±9.4	13.6±3.7	p=0.005
PSS			
Total score	24.94±9.8	17.56±8	p=0.001

Figure 1.

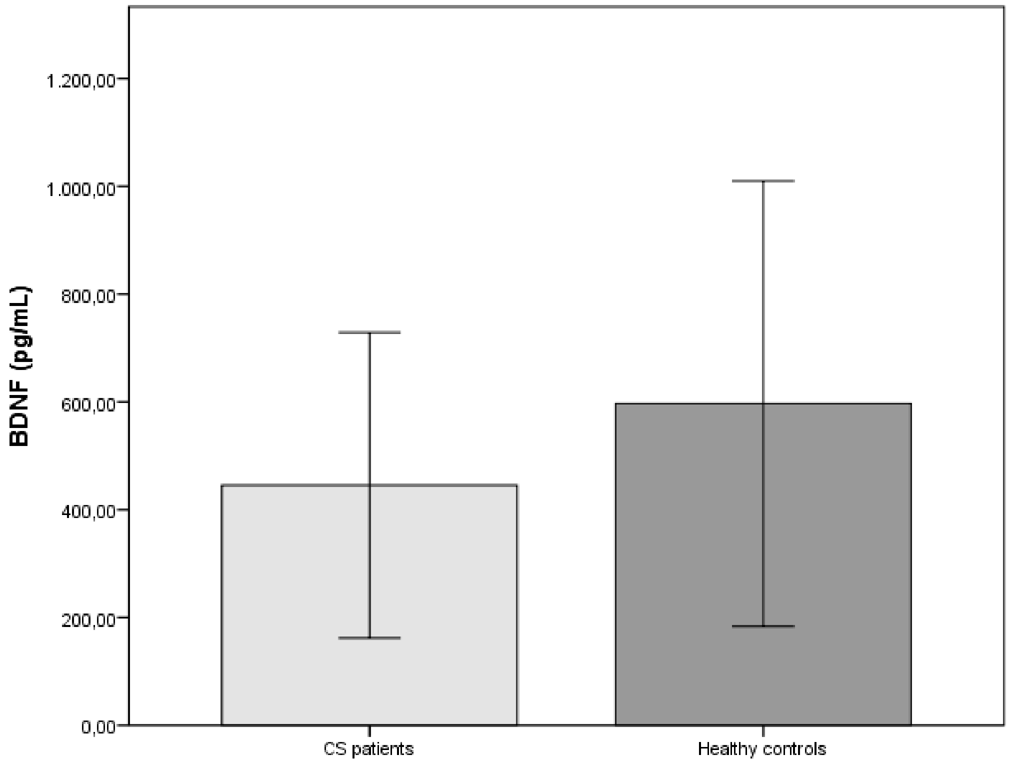
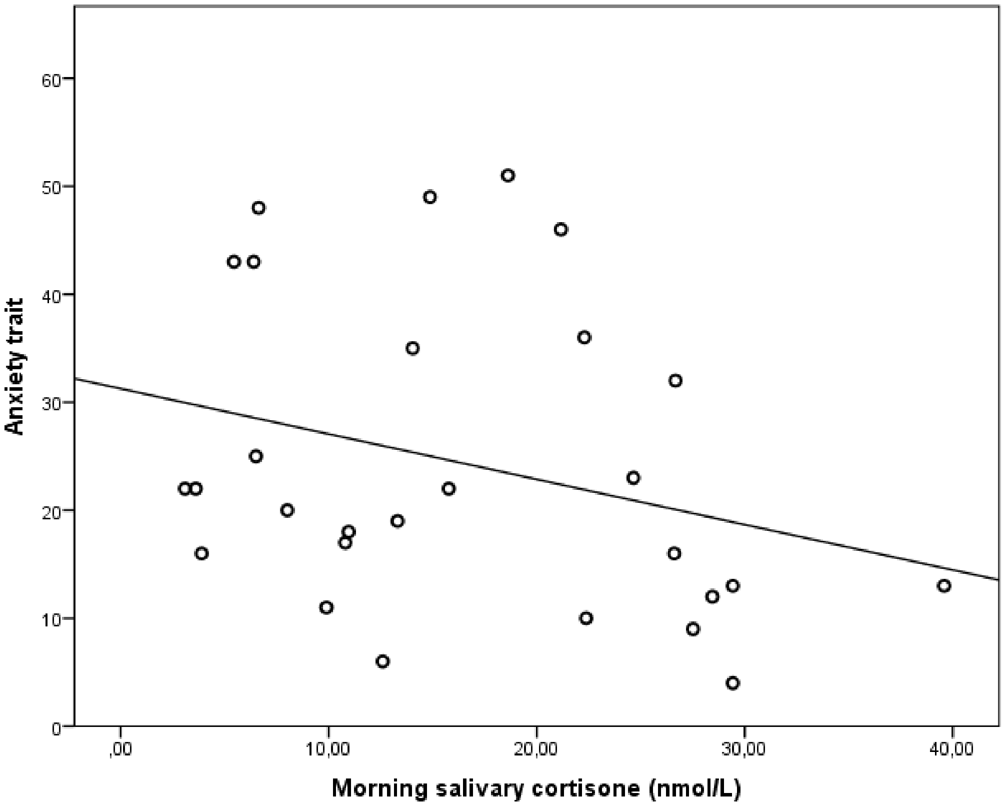

BDNF (pg/mL)
1.200,00
1.000,00
800,00
600,00
400,00
200,00
0,00
CS patients
Healthy controls

Figure 2.


Anxiety trait
0
10
20
30
40
50
60
,00
10,00
20,00
30,00
40,00
Morning salivary cortisone (nmol/L)

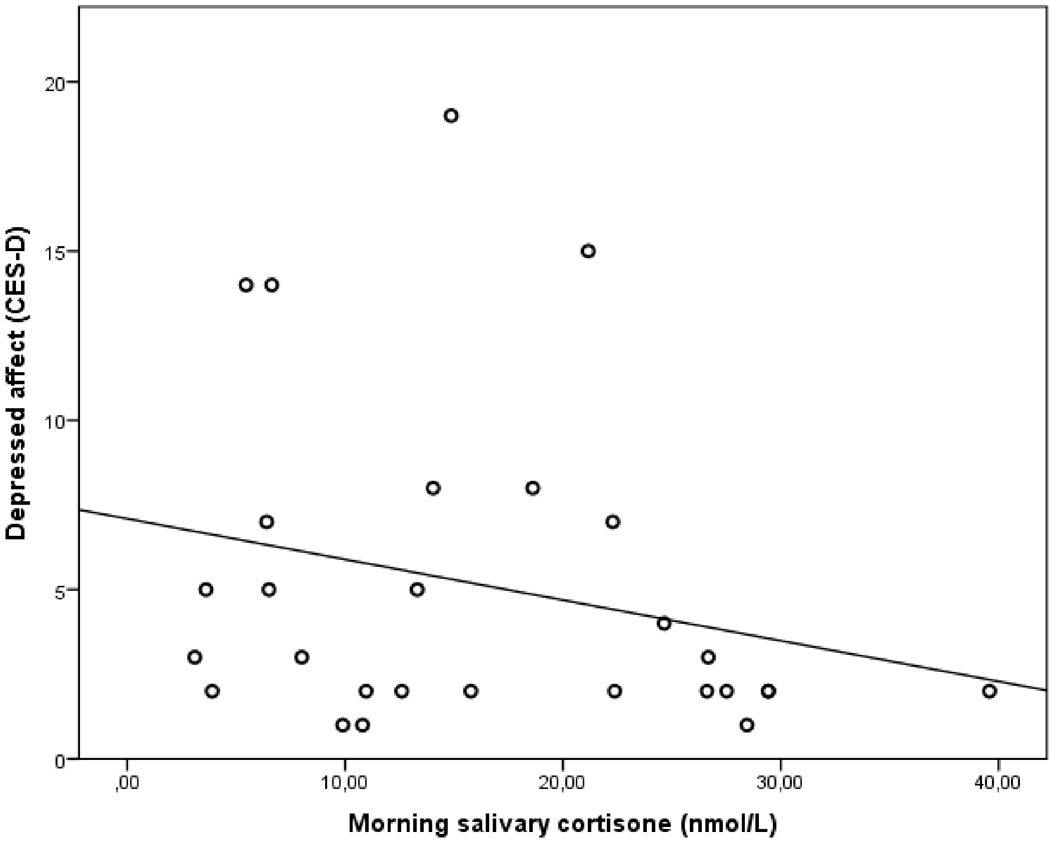
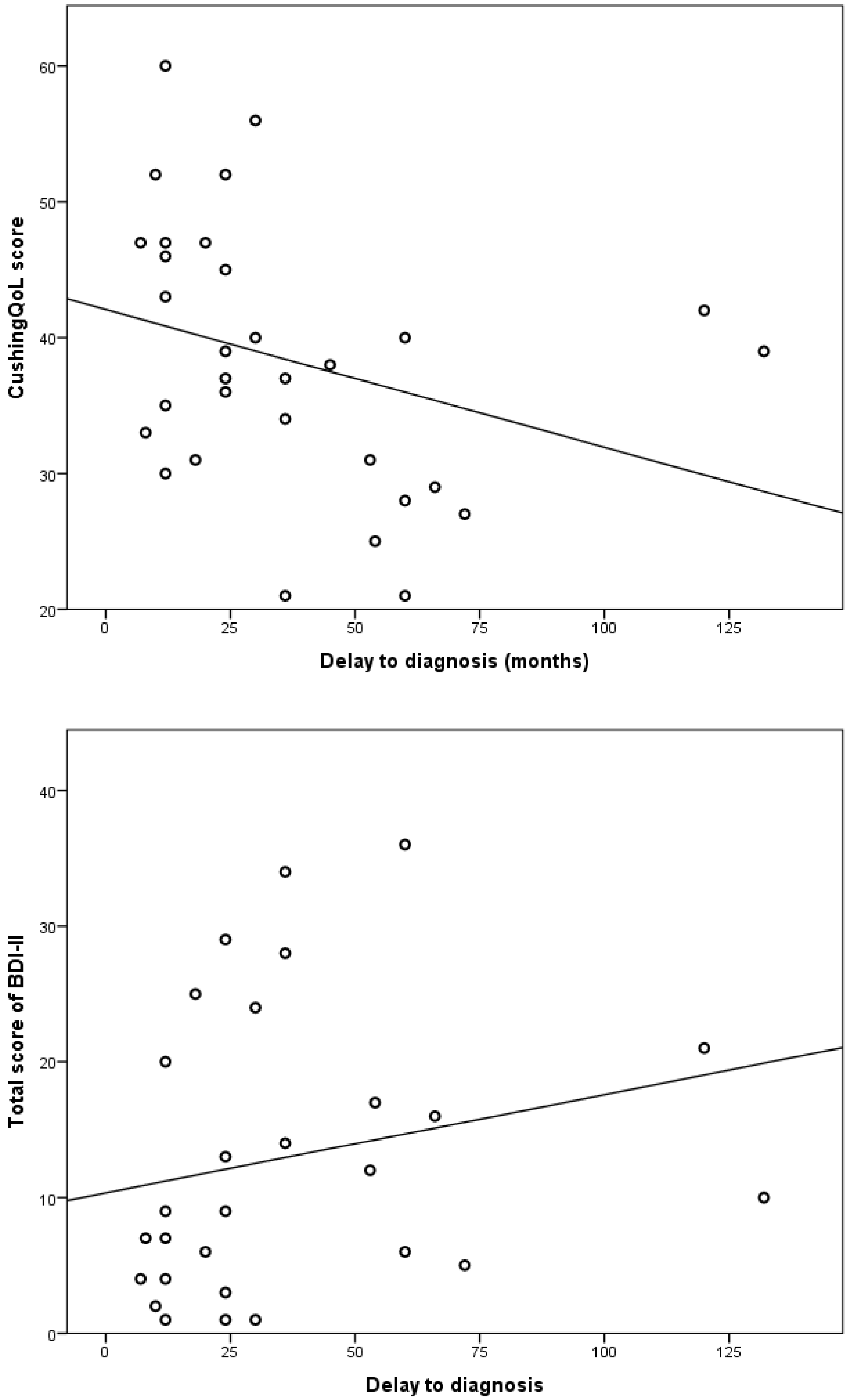

Depressed affect (CES-D)
0
5
10
15
20
,00
10,00
20,00
30,00
40,00
Morning salivary cortisone (nmol/L)

Figure 3.


CushingQoL score
60
50
40
30
20
0
25
50
75
100
125
Delay to diagnosis (months)
Total score of BDI-II
40
30
20
10
0
0
25
50
75
100
125
Delay to diagnosis

1