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**Cushing's Disease: a model for stress-related  
neuropsychiatric disorders?  
New evidences from MRI volumetric data**

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## Riassunto

La malattia di Cushing (Mdc) è frequentemente associata a disturbi psichiatrici e alterazioni neuronali. Una delle più antiche evidenze della psichiatria biologica dimostra che più del 50% dei soggetti con depressione melanconica non rispondono al test di soppressione al desametasone e presentano caratteristiche cliniche, in parte, simili al Morbo di Cushing (Pseudo-Cushing). La formazione ippocampale, il sistema limbico, la corteccia prefrontale e il nucleo caudato sono le strutture più coinvolte nel danno mediato da glucocorticoidi nella depressione maggiore. La Mdc rappresenta un ottimo modello per studiare il ruolo del cortisolo nel determinismo delle alterazioni strutturali del sistema nervoso centrale ben documentate in diversi disturbi psichici, in particolare in corso di depressione maggiore. Sono stati studiati 20 pazienti affetti da Mdc e venti controlli sani. Le misurazioni volumetriche sono state condotte lungo l'asse longitudinale dell'ippocampo separandone le strutture del corpo, della testa e della coda, per l'amigdala, il caudato, il cingolo anteriore e la corteccia prefrontale subgenuale. I volumi studiati sono stati correlati agli indici biochimici di funzionamento dell'asse ipotalamo-ipofisi-surrene. Il confronto fra i volumi ha evidenziato una riduzione della testa dell'ippocampo di sinistra. I livelli del cortisolo post test di soppressione al desametasone correlavano negativamente con il volume cerebrale globale e i volumi dei nuclei caudati bilateralmente, rivelando una plasticità di tali strutture in relazione ai livelli circolanti di cortisolo.

## Summary

Cushing's disease (CD) frequently causes Major Depression and neural damages. These patients experience high levels of endogenous cortisol, the naturally occurring GC. More than 50% melancholic patients do not suppress HPA axis with a single dose of Dexamethasone and have "pseudo-Cushing" features. Hippocampal formation, limbic system, prefrontal cortex and caudate nucleus seem to be the most implicated structure in the supposed glucocorticoid neuronal damage in Major Depression. These observations underline why CD provides a unique opportunity to study the potential role of cortisol in the well documented structural alterations of CNS in humans. Twenty CD patients and matched controls were examined. MRI measurements were performed separately for the hippocampal head, body and tail, amygdala, caudate and prefrontal cortex areas. We studied the relationship of various brain structure's volumes with the degree of HPA hyperactivity. Group comparison showed that patients had left hippocampal atrophy, mainly involving the hippocampal head. Cortisol levels after DST were negatively correlated with whole brain volume and caudate volume bilaterally, revealing a neuroplastic modulation of these structures in dependence with cortisol hyperproduction.

**Keywords:** Cushing's Disease; Depression; Cortisol; DST; Hippocampus; Caudate Nucleus

# 1 Introduction

Endogenous Cushing's Syndrome (CS) results from chronic exposure to excess glucocorticoids produced by the adrenal cortex. It may be caused by excess ACTH production (80–85%), usually by a pituitary corticotroph adenoma [Cushing's disease (CD)], less frequently by an extrapituitary tumor (ectopic ACTH syndrome), or very rarely by a tumor secreting CRH (ectopic CRH syndrome). CS can also be ACTH-independent (15–20%) when it results from excess secretion of cortisol by unilateral adrenocortical tumors, either benign or malignant, or by bilateral adrenal hyperplasia or dysplasia.

Major depression is the most common comorbid disorder of patients suffering from Cushing's Disease (CD). Depending on the series, 50–80% of patients with CD meet Diagnostic and Statistical Manual of Mental Disorders IV criteria for major depression. A minority of patients have other psychopathological manifestations including mania, anxiety, and cognitive dysfunction (1). If psychotic symptoms occur, they are likely to be a complication of mania or severe depression. Suicidal tendency also has been reported in patients with CD (2). The presence of depressive symptoms can be an early manifestation of CD and correlates with the severity of the clinical presentation (1). In contrast to adults, hypercortisolemic children suffering from Cushing's Disease have been reported to exhibit obsessive-compulsive behavior (3).

One of the first discovery in biological psychiatry was that more than 50% of melancholic patients did not suppress surrenal cortisol production after Dexamethasone Suppression Test (DST) (4). Those with a DST non suppression also had a high normal to exaggerated response to metyrapone similar to that observed in CD

(5). An analysis of over 150 studies reported that 43% of persons with major depressive disorder (MDD), 67% with psychotic depression, 41% with mania, and 78% with mixed mania have DST non-suppression (6) (7). This non-suppression is the consequence of an hyperactivity of HPA during acute episodes of depression and its persistence after antidepressant treatment is predictive of a non-response to treatment and chronicity. Such an hyperactivity has been interpreted by many authors (8) on the basis of their clinical data, as an hypothalamic Corticotropin Releasing Hormone (CRH) hypersecretion that has a direct effect on the pituitary and adrenal activity and rhythms, causing an hyperactivity of the pituitary and adrenal gland with hypercortisolism and loss of circadian rhythms, and an activating effect on extra-hypothalamic CNS structures such as the Locus Coeruleus-Ne system, which mediates some of the behavioural and sympathetic action of CRH (9). Clinical data suggest that normalisation of the CRH activity is obtained by AD treatment and that clinical non-responses are seen when such normalisation doesn't occur. CRH antagonists have also shown to have antidepressant action both in animal and humans (10).

On the basis of these observations patients with spontaneous CD can be considered a powerful model in which investigate the consequences of hypercortisolemia on brain structure and functions in humans (11). Recent studies have utilized brain imaging to examine the association of elevated cortisol with cerebral volumes in CD. Two structures were found to be altered during CD: the hippocampus and the caudate nucleus. Hippocampal formation shows a considerable atrophy in CD patients (11) (12) and also caudate nucleus seems to be sensible to cortisol modifications (13) (14).

Moreover neuropsychiatric symptoms in Cushing's syndrome patients are directly correlated with circulating cortisol levels (15) (16). As was the case with individuals administered exogenous glucocorticoids, patients with Cushing's syndrome demonstrated a pattern of cognitive disturbance that is consistent with hippocampal dysfunction (involving episodic but not semantic memory) (17) (18).

These patients experienced high levels of endogenous cortisol, the naturally occurring GC, for periods of months to years.

The hippocampus as a target of glucocorticoid (GC) activity is an area of increasing scientific and clinical interest (19) (20). This is due to several related factors. First, the hippocampus expresses high levels of receptors for the stress-responsive adrenal-glucocorticoids and an elevated HPA axis is one of the hallmark neuroendocrine markers of depression. Second, the hippocampus plays a significant role in negative feedback regulation of the HPA axis, which controls glucocorticoid release and is often disinhibited in many stress-related disorders. Third elevated glucocorticoid can lead to atrophy or loss of hippocampal neurons, which in turn can lead to further loss of the feedback inhibition of the HPA axis provided by this structure (21). Animal studies in rodents and primates indicate that chronic exposure to elevated glucocorticoid concentrations or chronic stress results in dendritic atrophy in hippocampal CA3 neurons (22) and can result in hippocampal pyramidal cell loss (23). In addition, as shown in animals, glucocorticoids increase the rate of age-dependent cell loss in the hippocampus (24). The hippocampus is one of several limbic brain structures implicated in the pathophysiology and treatment of mood disorders. Preclinical and clinical studies demonstrate that stress and depression lead to

reductions of the total volume of this structure and atrophy and loss of neurons in the adult hippocampus. One of the cellular mechanisms that could account for alterations of hippocampal structure as well as function is the regulation of adult neurogenesis. Stress exerts a profound effect on neurogenesis, leading to a rapid and prolonged decrease in the rate of cell proliferation in the adult hippocampus. In contrast, chronic antidepressant treatment up-regulates hippocampal neurogenesis, and could thereby block or reverse the atrophy and damage caused by stress. Recent studies also demonstrate that neurogenesis is required for the actions of antidepressants in behavioral models of depression (21).

Reduced hippocampal volume has been reported in several psychiatric disorders, including depression, posttraumatic stress disorder (PTSD) (25) and schizophrenia (26). The importance of the hippocampus in the pathophysiology of major depressive disorder (MDD) is supported by a substantial body of evidence from clinical studies. Two meta-analytic studies confirmed that the hippocampus is consistently reduced in patients with major depression (27) (28).

In humans, a possible association of the caudate nucleus with mood is suggested by a small literature from disparate study populations, particularly major depressive disorder (MDD) and obsessive-compulsive disorder. Studies using structural MRI have compared caudate volume in patients with MDD to normal subjects. Reduced caudate volume in MDD has been found in some but not all studies, so this is not yet conclusive (29). Two studies using functional imaging in MDD before and after treatment demonstrated pre-treatment differences from normal subjects as well as changes after treatment unilaterally in right striatum (30) (31). A recent

review of functional neuroimaging findings enforce the role of caudate nuclei in mood relevant neurocircuitry (32).

The literature supports a neuroendocrinological model for major depression as an hypercortisolemic-stress related disorder where hippocampus may be the most vulnerable and neuroplastic structure of the brain at the same time (19) (33). These observations underline why CD provides a unique opportunity to study the potential role of cortisol in the well documented structural alterations of CNS in humans.

In this study we sought to examine in CD patients the volumes of hippocampus, caudate and other brain structures (amygdala, anterior cingulate, SGPFc and whole Brain Volume) and to evaluate the relationships between brain volumes, psychometric variables and biochemical data with the purpose to better understand the link between hypercortisolemia and potential volumetric changes in mood-relevant neurocircuitry.

## 2 Methods and materials

### 2.1 Subjects

We limited the study group to patients with CD, the most common form of spontaneous Cushing Syndrome (CS). CD results from hypersecretion of pituitary ACTH. Although other etiologic types of CS, such as adrenal adenomas, also exhibit elevated cortisol, they differ in potentially confounding variables, such as the suppression of pituitary ACTH. The study included 20 patients with CD. Of the 20 participants, 15 were women and 5 were men, approximating the gender ratio seen in CD. Patients were admitted to the Neurosurgery Department of Padua University for diagnostic studies to confirm the presence of CD and before the surgical treatment for microadenoma.

All patients provided written informed consent. Mean age ( $\pm 1$  SD) for patients at the time of diagnosis was  $35,9 \pm 12,2$  years.

All patients met standard clinical and biochemical diagnostic criteria for CD. Clinical criteria included a disease-compatible history and physical findings (e.g., truncal obesity, skin and muscle atrophy, and "moon facies"). Biochemical criteria included high basal cortisol production (increased cortisol secretion rates, 24-hour urinary free cortisol and mean total plasma cortisol levels), high plasma ACTH levels, lack of normal cortisol circadian rhythm, lack of normal suppression with a low dose (2 mg). All patients received an MRI at the time of diagnosis to determine the presence of a pituitary tumor and to obtain volumetric measurements of the brain structures of interest.



The control group consisted of healthy controls carefully matched for sex, age and education.

A structured interview (M.I.N.I. Mini International Neuropsychiatric Interview) (34) was administered to rule out the presence of current or past psychiatric illness for CD patients and controls. Further exclusion criteria for healthy controls were first-degree relatives with a psychiatric disorder, any relevant medical disorders as well as history of alcohol/substance abuse. Participants had no clinically significant brain pathology, as determined by the neuroradiologist.

## 2.2 Biochemical measurements

### *Dexamethasone suppression tests (DST)*

The DST are used to differentiate CS patients from those who do not have CS (35) (36). The overnight DST consists of the oral intake of 2 mg dexamethasone between 23:00 and 24:00 h, followed by measurement of fasting plasma cortisol between 08:00 and 09:00 h the following morning. The specificity of the test is, however, limited, due to potential misclassification of patients with increased CBG, acute and chronic illness, or pseudo-CS. Occasionally, otherwise healthy individuals fail to suppress cortisol to this level also (35). For any DST, interfering conditions causing an apparent lack of suppression include: decreased dexamethasone absorption, drugs enhancing hepatic dexamethasone metabolism (barbiturates, phenytoin, carbamazepine, rifampicin, meprobamate, aminoglutethimide, methaqualone), increased concentration of CBG (estrogen treatment, pregnancy) and pseudo-Cushing states. (35) (37) (38) (39).

### *Twenty-four-hour urinary free cortisol (UFC)*

The 24-h urinary cortisol gives an integrated index of the free (unbound) cortisol that circulated in the blood during this period of time. In contrast to plasma cortisol levels, which measure total cortisol, unbound and bound, it is not affected by factors that influence corticosteroid-binding globulin (CBG) levels (36) (39). Due to the possibility of intermittent hypercortisolism, if the index of suspicion is high and the first result is normal, up to three 24-h urine collections should be performed. If cortisol excretion results are normal in three collections, then CS is highly unlikely, providing that renal function is normal. Mild CS is also unlikely although the alteration of the other tests can define a mild CS. Urinary creatinine may also be measured to verify the adequacy of the urine collection. If glomerular filtration rate is less than 30 ml/min, the urinary cortisol excretion is decreased and may thus be normal despite the presence of excessive cortisol production. In children, the urinary cortisol excretion should be corrected for body surface area/1.72 m<sup>2</sup>. Measurement of urinary cortisol by immunoassays (RIA, immunometric assays) is influenced by various metabolites of cortisol and some synthetic glucocorticoids, whereas measurements using HPLC allow the separation of various urinary glucocorticoids and metabolites. HPLC has a high sensitivity and specificity, but occasionally interfering substances, such as carbamazepin and digoxin, can also coelute with cortisol and produce false elevations of the UFC (40) (41). The recent introduction of mass spectrometry combined with gas chromatography or HPLC may overcome these problems; however, these techniques are more expensive, are not widely available, and have not yet been validated extensively. UFC values can be extremely variable in CS. UFC values 4-fold

greater than the upper limit of normal are very rare, except in CS, and therefore can be considered diagnostic for this condition. Milder elevations of urinary cortisol can be found in conditions such as chronic anxiety, depression, and alcoholism, all of which are also known as pseudo-Cushing states (35) (39), and in normal pregnant women. Urinary cortisol may not identify subclinical or preclinical CS in which hypercortisolism is still mild, and for this reason and the others cited above, it cannot be considered as a universal single screening test for the detection of CS.

#### *ACTH measurement.*

ACTH is rapidly degraded by plasma proteases. To prevent this, the assays for the determination of plasma ACTH levels require collection of blood into a prechilled EDTA tube, placement in an ice water bath, and rapid delivery to the laboratory for refrigerated centrifugation. Only assays such as the two-site immunoradiometric assays, which can reliably detect values less than 10 pg/ml (2 pmol/liter), should be used. ACTH concentrations below the level of detection or below 10 pg/ml (2 pmol/liter) at 0900 h with concomitant increased production of cortisol suggest an ACTH-independent cause of CS. However, ACTH levels may not be fully suppressed in some patients with adrenal CS and intermittent or concomitantly relatively low secretion of cortisol. Plasma ACTH values greater than 20 pg/ml (4 pmol/liter) suggest an ACTH-dependent cause. For values between 10 and 20 pg/ml (2–4 pmol/liter), a CRH stimulation test is indicated, with measurement of plasma ACTH. ACTH levels tend to be higher in ectopic ACTH-secreting CS than in CD; however, the overlap in ACTH values is such that ACTH values alone rarely distinguish between the two conditions (35) (42).

## 2.3 Psychometric tests and clinical variables

Patients' symptoms were assessed with the 17-item HDRS (43), Beck Depression Inventory (BDI) (44), State Trate Anxiety Inventory (45), Hamilton Anxiety Rating Scale (46) and Clinical Global Impression scale-severity (CGI) (47) on the day before of MRI examination to analyze relationships between volumetric data and concurrent symptom state and severity.

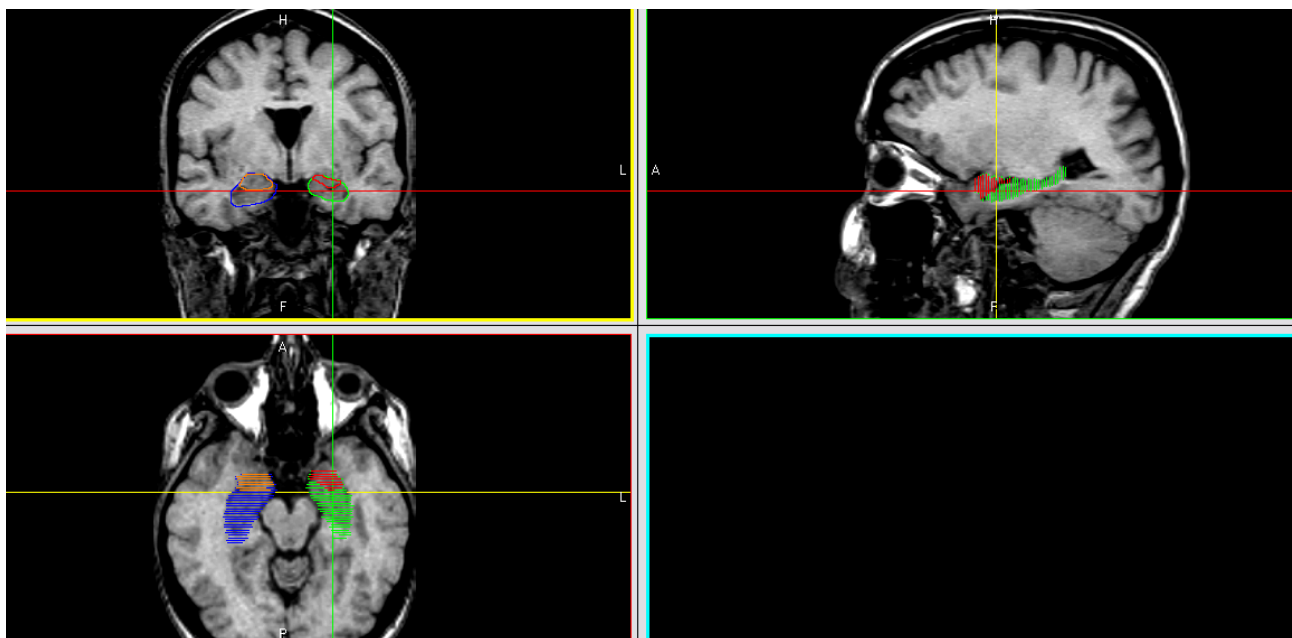
## 2.4 Image Acquisition

Magnetic resonance imaging acquisition was done in a 1. T Picker. A sagittal scout series was conducted to confirm image quality and patients' head position and to find a midline sagittal image. A T1-weighted sagittal scout image was acquired to obtain a graphic prescription of the coronal and axial images. Three-dimensional (3D) gradient echo imaging (spoiled gradient recalled acquisition) was conducted in the sagittal plane (time to repetition = 25 msec, time to echo = 5 msec, nutation angle = 40°, field of view = 24 cm, slice thickness = 1.5 mm, number of excitations = 1, matrix size = 256 × 192) to obtain 124 images covering the entire brain. To rule out any neuroradiological abnormalities, we obtained a T2 and proton density image in the axial plane.

## 2.5 Image Analysis

An Acer PC workstation running the semiautomated software DCM View for Windows (Padova Ricerche, Italy) was used to perform the anatomical measurements. All VOIs were initially manually traced in the coronal view and subsequently edited in sagittal and axial

views. This procedure allowed to perform corrections in the tracing process, with an optimization of the volume measurements. In fact some anatomical details are better identifiable in the sagittal or axial plane. (Fig.1)



*Fig. 1:* The Regions of interest (Roi's) of hippocampus and amygdala in coronal, sagittal and axial projections

All volumetric measurements were performed, with subjects' identities and diagnoses masked, by an expert neuroradiologist (P.A.). The interrater reliabilities (intraclass correlation coefficients: ICC) were calculated for two evaluators (P.A. and T.T.) who independently traced each region of interest in 10 scans. The reliabilities were  $r = 0.97$  (ICC) (Whole Brain volume, WBV),  $r = 0.97$  (ICC) (left hippocampus),  $r = 0.96$  (ICC) (right hippocampus),  $r = 0.92$  (ICC) (left hippocampal tail),  $r = 0.89$  (ICC) (left hippocampal body),  $r = 0.97$  (ICC) (left hippocampal head),  $r = 0.96$  (ICC) (right hippocampal tail),  $r = 0.88$  (ICC) (right hippocampal body)  $r = 0.95$  (ICC) (right hippocampal head),  $r = 0.98$  (ICC) (left caudate nucleus), and  $r = 0.90$  (ICC) (right caudate nucleus),  $r = 0.95$  (ICC) (left amygdala), and  $r = 0.91$

(ICC) (right amygdala),  $r = 0.97$  (ICC) (left anterior cingulate),  $r = 0.95$  (ICC) (right anterior cingulate),  $r=0.89$  (ICC) (left SGPF) and  $r=0.93$  (ICC) (right SGPF).

The volumes of these brain structures were expressed in cubic centimeters.

## 2.6 Anatomical Landmarks

Hippocampal and amygdalar volumes were estimated using a manual tracing technique and defined anatomic criteria (48). The segmentation of hippocampal head, tail and body was realized by the neuroradiologist (P.A.), who is expert in hippocampal neuroanatomy, with the following method.

In order to trace the Hippocampal Tail (HT), the most anterior slice of the HT we used was the first slice where the Sylvius aqueduct was clearly seen in full profile. The right and left HT transitions did not always appear on the same slice, as a result of minor differences in head position during the acquisition or due to anatomical differences. The most posterior sections of the HT is represented from the slice in which the grey matter of hippocampus at the level of the lateral ventricle is no longer visible. The hippocampal body (HB) was easier to outline: the first slice after the one where the cerebral pedunculi are clearly recognizable represents the front margin of the body and last slice, that precedes the slice in which the Sylvius aqueduct is well visible, represents the posterior margin of the body. The most posterior slice of the Hippocampal Head (HH) was the first slice where the cerebral pedunculi are clearly visible. Rostrally, the first slice we use is the one in which the hippocampus begins to show a characteristic triangular shape from the overlying

amygdala and in which the body of the caudate and the third ventricle become visible.(Fig. 2)

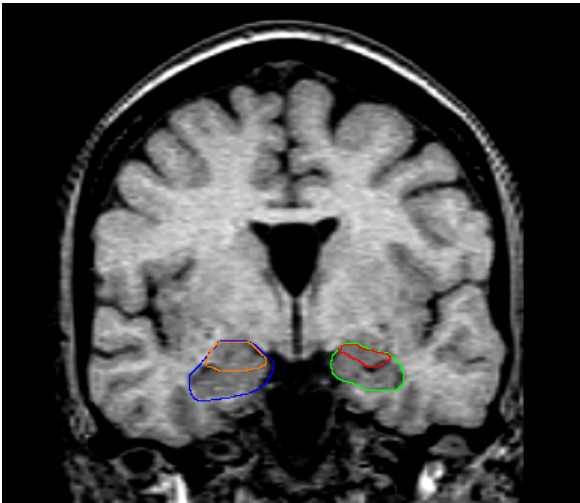


Fig. 2: Manual delimitation of right and left hippocampi and amygdale

Tracing of the caudate started anteriorly when the caudate was visible for the first time and continued posteriorly until the tail of the caudate could no longer be reliably discerned. This generally occurred a few slices before the point at which the tail curved ventrally to border the atrium of the lateral ventricles. The lateral ventricles and internal capsule were the respective medial and lateral borders. (Fig. 3)

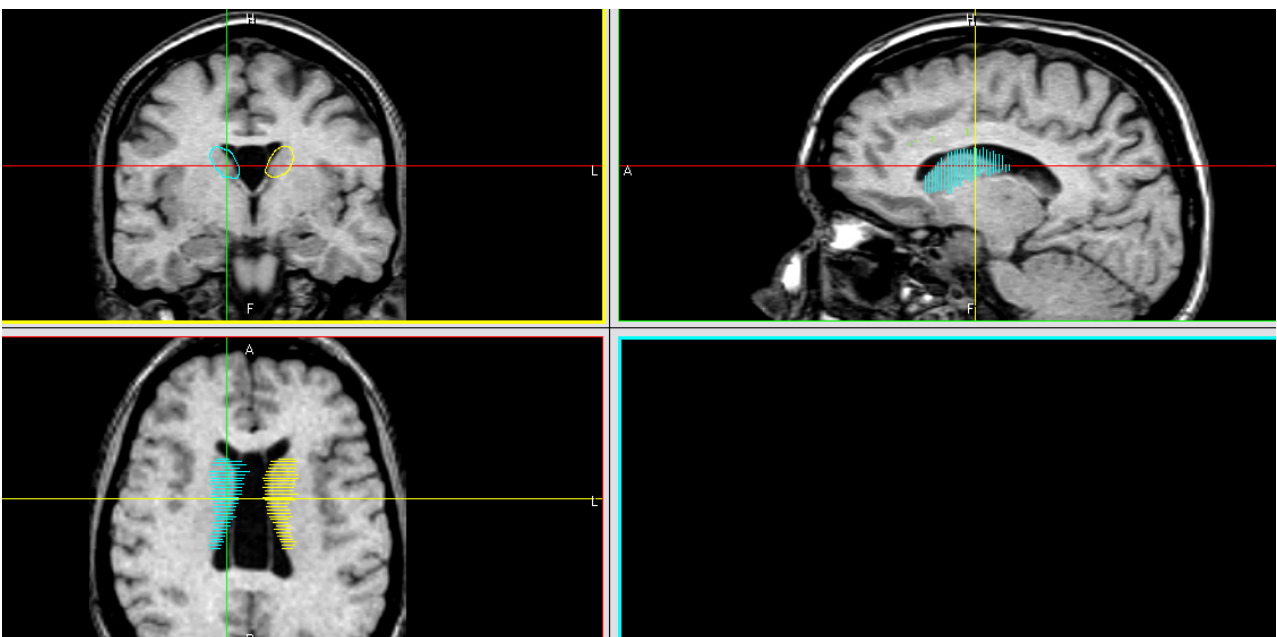
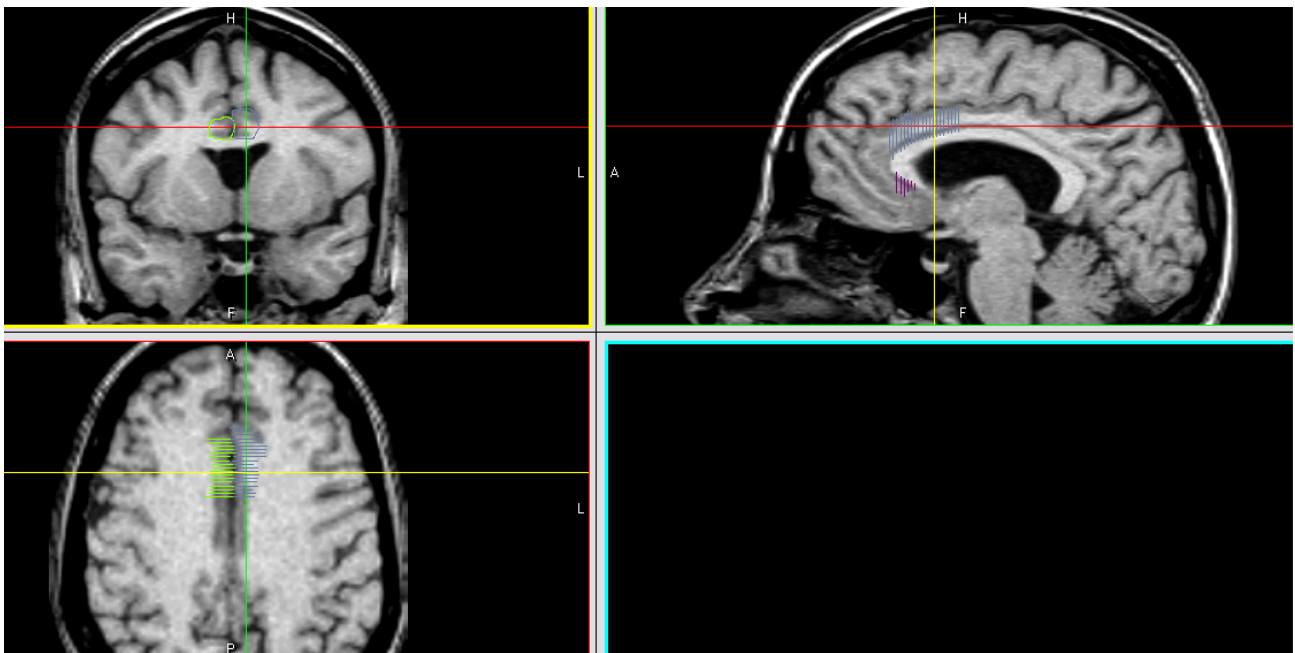


Fig. 3: The Roi's of caudate nuclei in coronal, sagittal and axial projections

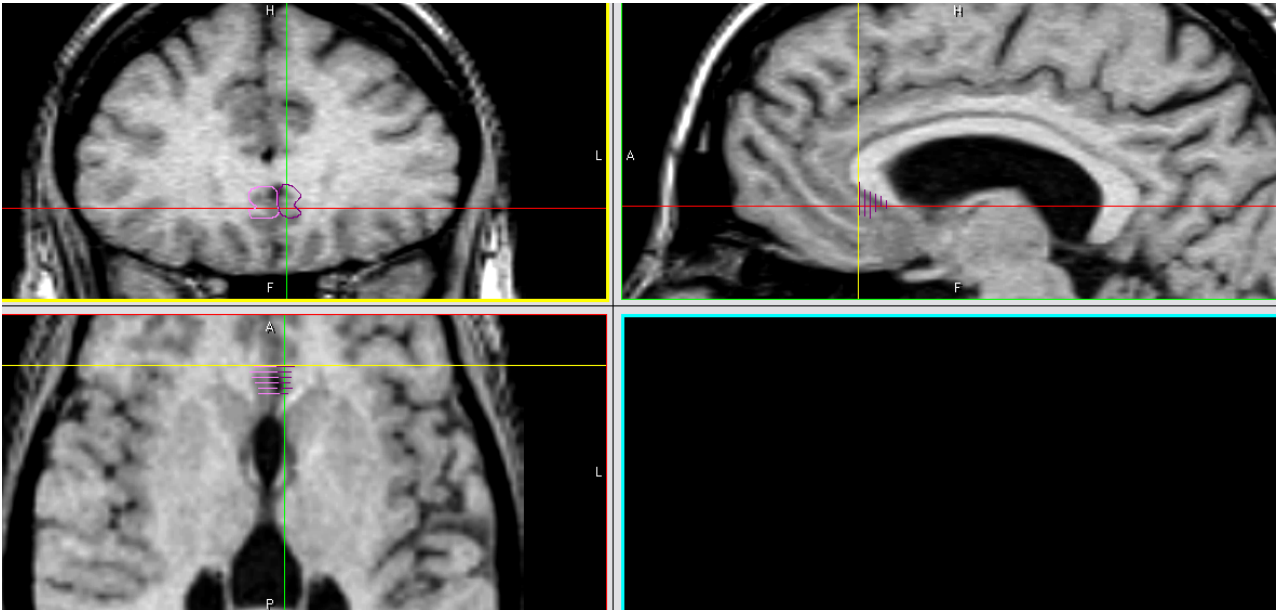
Right and left subregions of the ACC were traced separately in the coronal plane. The tracing was performed on coronal slices. Briefly, for the anterior cingulate, the tracing began at two slices anterior to the most anterior slice where the genu of the corpus callosum was visible, with the cingulate sulcus as the upper limit and the callosal sulcus as the lower limit defining the cingulate gyrus. The tracing progressed caudally on all slices until the slice where the anterior commissure was most apparent was reached. The anterior commissure marked the posterior limit of the anterior cingulate. The tracings at left and right side were done separately. (Fig. 4)



*Fig. 4:* The Roi's of anterior cingulate in coronal, sagittal and axial projections

Right and left SGpFC were defined as all gray matter in the first full gyrus inferior to the corpus callosum with the anterior boundary defined by the first coronal plane, which intersects the anterior portion of the corpus callosum, and the posterior boundary defined as the last slice before the internal capsule is first visualized (49) (50). (Fig. 5)





*Fig. 5: The Roi's of SGPF in coronal, sagittal and axial projections*  
 For measuring the whole Brain Volume (WBV) total cerebral gray and white matter (including temporal lobes, the optic chiasm, the pituitary but excluding the cerebellum and CSF) were included.

## 2.7 Statistical Analysis

T tests for nonpaired samples were used to compare group differences in WBV, left and right hippocampi, caudate, amygdalar, cingulated and SGPF volumes. The .05 level of significance with two-sided testing was used to reject the null hypotheses.

Volumes were then normalized using the following formula (51) for volume correction: standardized volume = absolute volume / whole Brain Volume of the same subject X 1000 cm<sup>3</sup>.

The Pearson correlation coefficient with level of significance set at 0.05 and two-tailed p values was used to determine the significance of correlations among and between MRI measures, psychometric (HDRS, BDI, HARS, STAI and CGI scores) and biochemical data.

All data are reported as unadjusted means ± SD. SPSS 11.5 for windows (SPSS, Chicago, Illinois) was used for all analyses.

# 3 Results

## 3.1 Study population

The study included 20 patients with CD. Of the 20 participants, 15 were women and 5 were men, approximating the gender ratio seen in CD. The same number of healthy controls carefully matched for sex, age and education were enrolled into the study. Patients did not differ from healthy comparison subjects in sex, age and years of education (Table 1).

*Table 1.* Characteristics of the study population

	<b>CD patients</b>	<b>Control subjects</b>
Number of subjects	20	20
Females	15	15
Males	5	5
Age (years)	35,98(±12,2)	32,9 (±10)
Education (years)	13,5 (±4)	15,2 (±3)

Continuous variables are displayed as mean values ± STD

The study group was comprised of a consecutive series of 20 subjects (15 female, 5 male) with CD (which results from hypersecretion of pituitary ACTH). This gender ratio approximates that usually seen in CD. At the time of evaluation, no subject had an acute medical condition requiring treatment, and none had clouding of consciousness. Patients were treated for their benign pituitary microadenomas, all confined to the sella turcica, with transsphenoidal pituitary surgery, which avoids any invasion of brain. Immediately following surgery, all patients exhibited the

expected complete suppression of ACTH and cortisol secretion, demonstrating the success of the surgical treatment. Replacement therapy was adjusted at regular intervals to insure that cortisol concentration remained normal until recovery of spontaneous hypothalamic-pituitary-adrenal axis function.

### 3.2 Psychometric Data

Five patients did not complete the psychometric evaluation. Considering the cut-offs reported in the cited literature the mean scores of psychometric scales have no pathological value (table 2)

*Table 2.* Clinical psychometric characteristics of the population

	<b>CD patients (N=15)</b>
HDRS	5,7 ( $\pm$ 5,8)
BDI	11 ( $\pm$ 8)
HARS	9,4 ( $\pm$ 7,4)
STAI-X	39,1 ( $\pm$ 10,1)
STAI-Y	42,1 ( $\pm$ 10,4)
CGI-S	2 ( $\pm$ 1,3)

Continuous variables are displayed as mean values  $\pm$  STD. HDRS: Hamilton Depression Rating Scale, BDI: Beck Depression Inventory, HARS: Hamilton Anxiety Rating Scale, STAI-X\_Y: State Trait Anxiety Inventory, CGI: Clinical Global Impression.

### 3.3 Biochemical Data

A complete biochemical study, comprehending 24h-UFC (Urinary Free Cortisol on 24 hours), DST (Cortisol concentrations after Dexametasone Suppression Text) and ACTH test, was available for all 20 CD patients but we decided to limit the analysis to the biochemical findings obtained the days before the MRI acquisition otherwise the correlation could not be reliable. For 12 patients the UFC was done the day before the MRI scan, for 9 the ACTH test and for 10 the DST.

### 3.4 Volumetric data

#### 3.4.1 WBV

Whole Brain volume (WBV) was similar in patients and controls.

#### 3.4.2 Absolute hippocampal volume

Patients displayed only a trend toward significance ( $p=0,05$ ) for reduced absolute left hippocampal volume and no significant difference for absolute right hippocampus.

The segmentation between hippocampal head (HH), body (HB) and tail (HT) revealed marked differences between patients and control subjects for left ( $p=0,004$ ) and right HH ( $p=0,02$ ).

#### 3.4.3 Hippocampal volumes after normalization for whole Brain volume (WBV)

Normalization through division by whole brain volume (52) is frequently used for comparison of hippocampal volume measurements ("WBV-corrected volumes").

Normalization process confirmed the significant differences for left hippocampal head WBV corrected (HH) ( $p=0,01$ ) and showed a trend for right HH WBV corrected ( $p=0,06$ ).

### 3.4.4 Absolute caudate volume

We did not find significant differences in absolute caudate volumes.

### 3.4.5 Caudate volumes after normalization for whole Brain Volume (WBV)

We did not find significant differences in caudate volumes after normalization for whole Brain Volume (WBV)

### 3.4.6 Absolute and WBV-corrected Amygdalar, Anterior Cingulate and SGPFC volume

We did not find significant differences in absolute amygdala, anterior cingulate cortex (ACC) and subgenual prefrontal cortex (SGPFC) volumes. As expected, we did not observe significant differences for Amygdala, ACC and SGPFC, after correction for WBV.

*Table 3.* Comparison of hippocampal, caudate, amygdalar, Anterior Cingulate Cortex (ACC) and SubGenual Prefrontal Cortex (SGPFC) volumes between depressed patients and controls

Volumes (Cm <sup>3</sup> )	Cushing Disease Patients (n=20)	Control subjects (n=20)	<i>INV.T</i> value	<i>P</i> Value
Whole brain volume (WBV)	925,22 (±88,4)	975,95(±116,6)	1,33	0,19
Absolute right hippocampal volume	3,96 (±0,89)	4,35 (±0,71)	1,51	0,13
Right hippocampal volume(WBV-corr)	4,31 (±0,99)	4,48 (±0,71)	0,64	0,52
Absolute Right Hippocampal Head	1,01 (±0,48)	1,45(±0,71)	2,26	0,02
Right Hippocampal Head (WBV-corr)	1,10 (±0,52)	1,47 (±0,73)	1,83	0,07
Absolute Right Hippocampal Body	1,85 (±0,33)	1,72 (±0,39)	1,06	0,29
Right Hippocampal Body (WBV-corr)	2,01 (±0,35)	1,79 (±0,46)	1,63	0,11
Absolute Right Hippocampal Tail	1,11 (±0,33)	1,18 (±0,26)	0,77	0,44
Right Hippocampal Tail (WBV-corr)	1,20 (±0,37)	1,22 (±0,30)	0,19	0,84

Absolute left hippocampal volume	3,93 ( $\pm 0,84$ )	4,43 ( $\pm 0,73$ )	2,01	0,05
Left hippocampal volume (WBV-corr)	4,27 ( $\pm 0,93$ )	4,54 ( $\pm 0,52$ )	1,11	0,27
Absolute Left Hippocampal Head	0,91 ( $\pm 0,43$ )	1,36 ( $\pm 0,52$ )	3,03	0,004
Left Hippocampal Head (WBV-corr)	0,98 ( $\pm 0,46$ )	1,39 ( $\pm 0,48$ )	2,69	0,01
Absolute Left Hippocampal Body	1,83 ( $\pm 0,33$ )	1,73 ( $\pm 0,40$ )	0,81	0,41
Left Hippocampal Body (WBV-corr)	1,99 ( $\pm 0,37$ )	1,79 ( $\pm 0,43$ )	1,57	0,12
Absolute Left Hippocampal Tail	1,19 ( $\pm 0,37$ )	1,33 ( $\pm 0,36$ )	1,19	0,24
Left Hippocampal Tail (WBV-corr)	1,30 ( $\pm 0,42$ )	1,36 ( $\pm 0,29$ )	0,53	0,60
Absolute right caudate volume	3,14 ( $\pm 0,69$ )	2,77 ( $\pm 0,96$ )	1,39	0,18
right caudate volume (WBV-corr)	3,41 ( $\pm 0,73$ )	2,87 ( $\pm 1,05$ )	1,86	0,08
Absolute left caudate volume	2,82 ( $\pm 0,55$ )	2,60 ( $\pm 1,01$ )	0,89	0,38
left caudate volume (WBV-corr)	3,08 ( $\pm 0,67$ )	2,70 ( $\pm 1,09$ )	1,35	0,18
Absolute right amygdalar volume	1,52 ( $\pm 0,39$ )	1,46 ( $\pm 0,41$ )	0,51	0,61
right amygdalar volume (WBV-corr)	1,65 ( $\pm 0,39$ )	1,52 ( $\pm 0,48$ )	0,96	0,34
Absolute left amygdalar volume	1,61 ( $\pm 0,37$ )	1,56 ( $\pm 0,45$ )	0,41	0,68
left amygdalar volume (WBV-corr)	1,75 ( $\pm 0,39$ )	1,62 ( $\pm 0,52$ )	0,84	0,40
Absolute Right Anterior Cingulate Cort	2,17 ( $\pm 0,76$ )	2,45 ( $\pm 0,47$ )	1,41	0,17
Right Ant Cingulate Cort (WBV-corr)	2,33 ( $\pm 0,75$ )	2,54 ( $\pm 0,49$ )	1,01	0,32
Absolute Left Anter Cingulate Cortex	1,62 ( $\pm 0,44$ )	1,83 ( $\pm 0,49$ )	1,39	0,17
Left Anter Cingulate Cort (WBV-corr)	1,76 ( $\pm 0,47$ )	1,91 ( $\pm 0,60$ )	0,91	0,36

Tissue volumes are displayed as mean values  $\pm$  STD in cubic centimeters (cm<sup>3</sup>). Absolute measurements are the uncorrected volumes. WBV-corr = corrected for differences in brain volumes.

## 3.5 Correlations

### 3.5.1 correlation between age, education and volume measurements

WBV negatively correlated with age in the whole sample (patients and controls). Hippocampal volumes positively correlated in the whole sample (patients and controls considered together) with education level.

### 3.5.2 Correlation between HDRS, BDI, HARS, STAI X-Y, CGI and volume measurements

We did not observe a significant correlation between the mentioned clinical variables and the studied regions volumes.

### 3.5.3 Correlation between UFC, DST, ACTH test and volume measurements

No significant correlations were seen between UFC concentrations and all the studied structures.

No significant correlations were seen between DST cortisol concentrations and all absolute hippocampal measurements, also considering the segmentation between hippocampal head, body and tail.

DST cortisol concentration was negatively correlated with absolute right caudate and absolute left caudate ( $r = -0.80$ ,  $P < 0.02$ ;  $r = -0.11$ ,  $P < 0.02$ ).

DST cortisol concentration was negatively correlated with whole Brain Volume ( $r = -0.82$ ,  $P < 0.02$ ).

ACTH measures did not correlate with all the studied volumes.

## 4 Discussion

In the present study, volume of the head of the hippocampus was smaller in patients with CD compared with healthy subjects. We have assessed the possible differences in sensitivity of the hippocampus along its longitudinal axis (head, body and tail) in Cushing's patients. Damage was mainly located in the head of the hippocampus, and the tail and the body were not significantly affected. To our knowledge, this is the first study to investigate limbic and prefrontal structures and caudate nuclei in Cushing Disease and identified a focal vulnerability, linked to rostral portions of hippocampus to endogenous glucocorticoid neurotoxicity.

It may be that the hippocampal head is especially sensitive to biochemical/neuroendocrinological alterations in CD. This sensitivity could be attributed to a different head to tail gradient of excitatory-inhibitory cell content: in fact there is a greater excitatory cell density and lower inhibitory cell density in the anterior hippocampus compared to the posterior one (53).

The head of the hippocampus contains a large proportion of the CA1 and CA1' fields of the cornu ammonis (54). Moreover the head of the hippocampus is a densely vascular structure (55) and consists of CA1 neurons that are particularly sensitive to stress and glucocorticoids (56). The role of adrenal steroids in the structural neuronal remodeling is well known and it reflects many interactions with neurochemical systems in the hippocampus, including serotonin,  $\gamma$ -aminobutyric acid, and excitatory amino acids (57) (58) (59). Probably the most important interactions are



those with excitatory amino acids such as glutamate. Excitatory amino acids released by the mossy fiber pathway play a key role in the structural remodeling of the hippocampus, and regulation of glutamate release by adrenal steroids may play an important role (58) (60) (61). Glutamate excito-toxicity is one putative mechanism affecting the neuronal plasticity in CD. Elevated glucocorticoids increase the release and extra-cellular concentrations of excitatory amino acids, and may set off a cascade leading to changes in architecture, though not necessarily cell death.

The hippocampal head seems to be more vulnerable to cortisol mediated neuronal damage in CD. Some considerations about the role of this substructure are appropriated:

the head or the anterior part of the hippocampus has reciprocal connections with the prefrontal cortex (62) (63), and it is important in the encoding of associative memories (64). In fact patients with Cushing's syndrome demonstrate a pattern of cognitive disturbance that is consistent with hippocampal dysfunction (involving episodic but not semantic memory) (17) (18).

Preclinical studies suggest that lesions in the hippocampal formation early in development result in behavioral abnormalities consistent with frontal lobe dysfunction in adulthood (65), while removal of an abnormal hippocampus improves prefrontal cortical function (66). Decreased volume of the anterior hippocampus has been correlated with lower scores on tests of executive function in schizophrenia (67), while larger volume of the anterior hippocampus has been positively correlated with prefrontal cortical activation (68), suggesting that a smaller anterior hippocampus predicts dysfunction of the prefrontal cortex.

we did not find caudate nuclei volume reductions in our sample. The present study did not evidenced structural abnormalities in the prefrontal cortex although literature clearly shows cognitive alterations in CD patients linked with prefrontal functions; it is possible that structural abnormalities in the CA1 neurons and dentate gyrus in the head of the hippocampus could lead to secondary functional abnormalities in the prefrontal cortex, in absence of structural abnormalities.

Some considerations about the relationships between hippocampal structure and functions, HPA axis are needed in interpreting data.

First hippocampus play an important role in HPA axis regulation: the hippocampus is a primary CNS target for corticosteroids (69). Thus, injury to the hippocampus by corticosteroids could lead to elevated levels of corticotropin releasing hormone (CRH), adrenocorticotropin (ACTH), and cortisol. However, hippocampal interactions with the HPA axis are complex and may be mediated, at least in part, by the paraventricular nucleus (PVN). HPA axis inhibition by the hippocampus appears to be mediated by negative feedback from circulating glucocorticoids. Destruction of the dorsal hippocampus attenuates the ability of dexamethasone to suppress the stress response. Loss of the normal glucocorticoid fast feedback, mediated, in part, by the hippocampus, has been reported in humans with depression and animals in chronic stress paradigms (70) (71) (72).

Therefore, elevated levels of cortisol secondary to hippocampal damage could produce further injury to the hippocampus itself, and consequently, even greater increases in cortisol levels due to

impaired feedback mechanisms to suppress cortisol release, a concept termed the "glucocorticoid cascade hypothesis" (73).

In our patients sample may occur this process and our evidences confirm the peculiar vulnerability of the hippocampus to hypercortisolemia.

Surprisingly we did not find negative correlations between volumes and UFC levels, as other studies did (11) (12), conversely post DST cortisol levels correlated negatively with WBV and caudate volumes bilaterally. While other studies demonstrated a sensible reduction in cerebral volume we did not reveal significant differences in WBV between patients and controls, but interestingly brain volume seems to be vulnerable to DST cortisol concentrations. This evidence must be considered in the interpretation of this study. Literature better supports the hippocampal sensitivity to circulating cortisol, but our data do not confirm this hypothesis, based upon the evidence of the elevated presence of GC receptors on hippocampal neurons. On the other hand, whole brain is markedly bigger than hippocampus and GC receptors are distributed in other brain regions. This result reveal the global neurotoxicity in CD of cortisol on the brain. Some, but not all of the several structural volumetric studies in patients with primary depressive disorder showed decreased caudate volume compared to normal subjects. Functional imaging studies during major depressive episodes demonstrated reduced functional activity in the basal ganglia, particularly caudate (74). Functional imaging studies have examined this region both prior and subsequent to the treatment of MDD. In a positron emission tomography study, patients prior to treatment showed higher glucose metabolism in several brain regions, including the right dorsal caudate head, compared to normal subjects. After treatment,

only activity in the right caudate decreased significantly (30). Even in healthy controls, as well as patients with depression, fMRI studies have shown caudate changes after behavioral induction of temporary (75). Research on obsessive-compulsive disorder has also suggested functional involvement of the right caudate. Decreased rCBF in the right caudate of obsessive-compulsive disorder patients, but not patients with panic disorder or healthy subjects, has been demonstrated (76). Ventral striatum, including ventral parts of the caudate nucleus, has been implicated in mood in animal studies. The ventral striatum has connections to the limbic system, receiving fibers from hippocampus. In humans, subjects in early-stage intense romantic love viewing photographs of the loved person showed significant activation in right but not left postero-dorsal caudate body and medial caudate nucleus (77). Possible biochemical mechanisms underlying striatal changes in depression and CD may include glutamate. In Huntington's disease, glutamate excito-toxicity is thought to be primarily responsible for the death of striatal neurons. Glutamate excito-toxicity is one likely mechanism affecting the brain in CD. Elevated glucocorticoids increase the release and extra-cellular concentrations of excitatory amino acids, and may set off a cascade leading to changes in architecture, though not necessarily cell death.

The findings reported here of correlation between caudate volumes and DST provide another line of evidence in humans that the striatum, particularly the caudate, is a major component in human brain circuits involved in mood regulation. Caudate sensitivity to cortisol concentrations suggests that effects on caudate may be a mechanism by which the less substantial but frequent elevations of

cortisol or alterations in its diurnal rhythm in psychiatric disorders could affect mood regulation.

We conclude that neural damage, with consequent loss of brain volume is limited to hippocampal head, while brain volume "in toto" seems to be sensible to the marked cortisol variation observed in CD.

Signs of hippocampal atrophy should be specifically evaluated in course of CD and major depression, and further studies are needed to better understand the role of HPA axis in regulating neuromodulation and neurotoxicity and mood.

## 5 Limits of the investigation

This study is limited in several aspects, which should be taken into consideration when interpreting its results. First, we could not precisely register an important variable as the “duration of the illness”. In our opinion considering the simple evidence of the first diagnosis as the begin of the illness is too simplistic and not reliable, when the literature, and our experience with these patients, clearly shows that diagnosis is difficult and often occurs many years after the first clinical signs. Consequently our sample may be too heterogeneous: we collected young patients with a probably short illness duration together with adults with more than twenty years of CD. This variability may represent an important confound relatively to the HPA axis and the reliability of the correlations we found. In fact, in our opinion, the volume measurements and/or the results of biochemical investigations may change in dependence to the duration of the illness. First neurotoxicity is a reversible process for a limited time, secondly in young patients with recent onset is possible that hippocampus is able to exercise a structural and functional compensatory role, while in older patients the hippocampal damage becomes irreversible and the loss of the inhibition on HPA axis contributes to worsen the damage.

Secondly the number of patients in which biochemical measurements were completed was limited and more definitive conclusions will be possible with a larger sample.

The third limit of this investigation concerns the meaning of the strength correlations we found between DST cortisol levels and the volumes we studied. DST is used in clinical neuroendocrinological

practice as an instrument for determine a differential diagnosis; it is utilize to specify if an illness is present or not, but it is not usually employed for clarifying the level and the gravity of the illness CD. Generally the UFC levels are preferred. The evidence we found establish a close correlation between cortisol levels after DST and volume measurements. Further studies are needed for clarify if DST is reasonably available with this purpose.

Another limit of our investigation is represented by the peculiar clinical severity of the patients we studied. In fact all CD patients, who participated in this investigation, were recruited in the Neurosurgery department close before the surgical treatment. This sample can not be considered completely representative of all CD patients, but only of those who did not respond to the usual not surgical medications. The HPA axis alterations we measured may be influenced by the severity of the clinical status, the mean long illness duration and the medications.

## 6 validity of the model and The Clinical Relevance

Do our results confirm the theory that Cushing Disease is a powerful model for stress related neuropsychiatric disorders as some cited authors sustaine?

The principal result of this study is the detection of hippocampal head atrophy in CD patients while all the other cerebral structure we studied are not damaged. In this prespective we confirmed the vulnerability to Glucocorticoids effects on hippocampal formation with the original evidence of the selective atrophy of the head of the hippocampus.

Some considerations about the conceptualization of “stress” in medicine are worthwhile to better analyze if CD may be considered a close paradigm of stress related psychiatric disorders: the brain is the key organ of the response to stress because it determines what is threatening and, therefore, potentially stressful, as well as the physiological and behavioral responses which can be either adaptive or damaging. Stress is commonly defined as a state of real or perceived threat to homeostasis. Maintenance of homeostasis in the presence of aversive stimuli (stressors) requires activation of a complex range of responses collectively known as the stress response. Activation of the stress response initiates a number of behavioral and physiological changes that improve an individual’s chance of survival when faced with homeostatic challenges. Behavioral effects of the stress response include increased awareness, improved cognition, euphoria, and enhanced analgesia. Physiological adaptations



initiated by activation of this system include increased cardiovascular tone, respiratory rate, and intermediate metabolism, along with inhibition of general vegetative functions such as feeding, digestion, growth, reproduction, and immunity. Due to the wide array of physiologic and potentially pathogenic effects of the stress response, a number of neuronal and endocrine systems function to tightly regulate this adaptive process.

Moreover stress involves two-way communication between the brain and the cardiovascular, immune, and other systems via neural and endocrine mechanisms. Beyond the "flight-or-fight" response to acute stress, there are events in daily life that produce a type of chronic stress and lead over time to wear and tear on the body ("allostatic load"). Yet, hormones associated with stress protect the body in the short-run and promote adaptation ("allostasis"). The brain is a target of stress, and the hippocampus was the first brain region, besides the hypothalamus, to be recognized as a target of glucocorticoids. Stress and stress hormones produce both adaptive and maladaptive effects on this brain region throughout the life course. Early life events influence life-long patterns of emotionality and stress responsiveness and alter the rate of brain and body aging. The hippocampus, amygdala, and prefrontal cortex undergo stress-induced structural remodeling, which alters behavioral and physiological responses.

These considerations clarify the strength link between the construct "stress" and cortisol action on hippocampus and help to confirm the appropriateness to investigate CD patients in biological psychiatry research.

Further consideration about neuroendocrinological differences in HPA hyperactivation in major depression and CD are needed.

Hypercortisolemia and ACTH overproduction are similar in CD and depression, but production of CRH is profoundly suppressed in CD and stimulated in major depression. This evidence may partially explain the radical differences in the pathophysiology of these two distinct disorders and confirms the need of better investigate the role of hippocampal formation in major depression, when considering the well demonstrated role of hippocampus in regulation of CRH activity.

Another substantial difference regards the volumetric findings in CD and depressive disorders and the potential role of cortisol neurotoxicity. Despite hypercortisolemia is more severe in CD, literature reports that depressive patients show more frequently structural alteration than CD patients. This evidence leads at least two further hypothesis: firstly cortisol-induced damage is not the only neurotoxic mechanism that occurs in course of major depression; secondly depressed patients may present structural abnormalities prior the onset of the disease, as a trait vulnerability marker.

Moreover the utility of a clinical model is also based upon the evidence of a practical/clinical relevance of its results.

Our data contribute to enforce the clinical utility of brain imaging and biochemical data in neuropsychiatric disorders for example in differentiating between depression with or without GC hypersecretion.

Much more work is needed, given that there are now only a handful of human studies, in carrying out prospective studies of stress-related (i.e. major depression and PTSD), and in determining whether hippocampal atrophy, as a reversible process, may be used as a diagnostic target and a marker in the monitoring in the course of the illness and/or in the treatments responsiveness.

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