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## Enhanced oxidative stress and platelet activation in patients with Cushing's syndrome

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### Summary

#### Objective

Cushing Syndrome (CS) is implicated by increased cardiovascular risk (CVR) leading to increased morbidity and mortality. Oxidative stress (OS) and platelet activation (PA) are associated with increased CVR. However, scarce data of OS in CS exist. Our objective was to determine the oxidant–antioxidant balance in CS.

#### Design

Fourteen patients with CS at diagnosis and fourteen healthy subjects (NS) were evaluated OS by measuring plasma 15-F<sub>2t</sub>-IsoP, PA by thromboxane B<sub>2</sub> levels (TXB<sub>2</sub>), and antioxidant reserve measuring total antioxidant capacity (TAC) and serum vitamin E.

#### Results

15-F<sub>2t</sub>-IsoP and TXB<sub>2</sub> levels were significantly higher ( $P < 0.01$ ) in CS, while vitamin E levels were higher in NS ( $P < 0.03$ ). 15-F<sub>2t</sub>-IsoP levels were significantly higher ( $P < 0.01$ ) in complicated vs not-complicated CS and NS and significantly higher ( $P < 0.03$ ) in CS not-complicated vs NS. TXB<sub>2</sub> levels were significantly reduced ( $P < 0.03$ ) in NS vs complicated and not-complicated CS. A negative correlation between Vitamin E and UFC was observed in CS ( $P < 0.05$   $r = -0.497$ ). TXB<sub>2</sub> correlated with glucose, HbA1c and T-score ( $P < 0.05$   $r = 0.512$ ,  $P < 0.03$   $r = 0.527$  and  $P < 0.01$   $r = 0.783$ , respectively) and HDL ( $P < 0.01$   $r = -0.651$ ). 15-F<sub>2t</sub>-IsoP correlated with triglycerides, HbA1c and diastolic pressure ( $P < 0.01$   $r = 0.650$ ,  $P < 0.03$   $r = 0.571$  and  $P < 0.05$   $r = 0.498$ , respectively) and HDL ( $P < 0.03$   $r = -0.594$ ).

#### Conclusions

This study emphasizes the major role of OS in CS. As our findings demonstrated that enhanced OS and PA take place in this rare metabolic disorder which is associated with increased CVR, it could be suggested that these biochemical alterations can further contribute in the pathogenesis of atherosclerosis, increased CVR and mortality in CS.

#### Introduction

Cushing's syndrome (CS), the clinical condition that refers to the manifestations induced by chronic exposition to glucocorticoid (GC) excess, may result from various causes. It is a complex, rare endocrine condition with potential serious complications if untreated or inadequately managed, associated with high morbidity and mortality rates, being the cardiovascular complications among the predominant causes.[1] In patients with untreated CS, the cardiovascular mortality is four to five times higher compared with the general population.[2] However, with modern-day treatments, the mortality rate after successful cortisol normalization does not differ significantly from the general population,[2] although in patients with cured CS, an increased prevalence of atherosclerosis even several years after remission of hypercortisolism seems to persist.[3]

The oxidative stress (OS) is involved in the pathogenesis of atherosclerosis and cardiovascular diseases (CVD).[4, 5] OS is the loss of the normal homeostatic balance between reactive oxygen species (ROS) and

antioxidant defences, resulting in excess production of ROS which are detrimental and toxic to cells and tissues causing membrane lipid peroxidation and consequently lipid and DNA damage leading to apoptosis and protein impairment.[6, 7]

On the other hand, data about the effects of OS and CS are scanty. Previous studies *in vitro* have reported that the acute administration of GC is able to inhibit the production of ROS.[8] On the contrary, there is evidence that the endogenous chronic hypercortisolism does not inhibit the OS and the protective effects of GC are suppressed by pro-oxidative processes or insufficient antioxidant systems.[9] These findings are further supported by studies indicating significant higher levels of malondialdehyde (MDA), an indicator of OS, in patients with overt CS compared with cured CS and healthy subjects.[10] In addition, animal studies showed that chronic GC or ACTH treatment increases the plasma F<sub>2</sub>-isoprostane (F<sub>2</sub>-IsoP) concentration, a marker of lipid peroxidation, suggesting an increased production of ROS and a further derangement in the oxidant–antioxidant balance.[11]

The quantification of F<sub>2</sub>-IsoPs, particularly the 15-F<sub>2t</sub>-Isoprostane (15-F<sub>2t</sub>-IsoP), represents a highly precise and accurate index of OS.[4-6, 12] It has been demonstrated that its levels are increased in metabolic disorders such as diabetes mellitus (DM), atherosclerosis, hypercholesterolaemia, obesity and in several other diseases.[13] Moreover, 15-F<sub>2t</sub>-IsoP is found to induce platelet activation (PA) via thromboxane A<sub>2</sub> (TXA<sub>2</sub>) receptors and has vasoconstrictive properties.[14]

It is known that TXA<sub>2</sub>, an eicosanoid hormone, causes platelet release, activation and accumulation and exerts potent vasoconstrictive effects.[15] It is rapidly degraded into its inactive form, thromboxane B<sub>2</sub> (TXB<sub>2</sub>), which represents a reliable indicator of TXA<sub>2</sub> biosynthesis.[15] Previous studies have demonstrated that TXA<sub>2</sub> and 15-F<sub>2t</sub>-IsoP are involved in the initiation and progression of atherosclerosis and, in addition, it was found to correlate with different metabolic pathologies such as DM, obesity, hypertension and hypercholesterolaemia.[14, 16, 17] These correlations could be ascribed to the already known interaction between OS and PA.[14]

As mentioned, in different pathological conditions, there is an altered balance between ROS and antioxidant production. It is known that living organisms developed complex antioxidant systems to counteract ROS including enzymes such as catalase and glutathione peroxidase; macromolecules such as albumin and ferritin; and an array of small molecules, including vitamin C, vitamin E (VitE) and reduced glutathione. In several diseases such as DM, obesity and CVD, there is a loss of antioxidant reserve of which VitE represents a significant marker, being one of the most important antioxidants.[18-20] In fact, it scavenges lipid peroxy radicals, resulting in the blockade of lipid peroxidation and is also capable to inhibit platelet aggregation.[4, 21, 22]

As OS occurs when there is an imbalance between ROS and antioxidant capacity, additional information may be revealed by the evaluation of the total antioxidant capacity (TAC) which represents a measure of the antioxidant cumulative effects. In fact, what it does measure is low molecular antioxidants, phytochemicals, antioxidant minerals and some other important antioxidant such as  $\alpha$ -lipoic acid.[4] It has been shown that TAC levels were reduced in hypertension, CVD and DM.[23, 24] Finally, there are data showing a correlation between F<sub>2</sub>-IsoPs and TAC in DM.[25]

Based on these premises, to clarify and determine the oxidant–antioxidant balance in patients with active CS, we assessed OS by measuring plasma levels of 15-F<sub>2t</sub>-IsoP and TXB<sub>2</sub> as a noninvasive index of PA and the antioxidant reserve evaluating TAC and serum vitamin E levels.

## **Subjects and methods**

### **Subjects**

Fourteen patients with hypercortisolism (three men, 11 women, age mean  $\pm$  SEM:  $48.33 \pm 3.89$  years, BMI  $28.54 \pm 4.31$  kg/m<sup>2</sup>) were included in this pilot study at the time of the diagnosis (group CS). The causes of hypercortisolism were pituitary adenoma (Cushing's disease) in 10 patients, adrenal cortex adenoma in three patients and an ectopic production of ACTH in one patient. The patients were characterized by typical clinical features of Cushing's syndrome, and the diagnosis of Cushing's syndrome was made according to international criteria, including high daily urinary free cortisol (UFC), absent cortisol suppression after low-dose dexamethasone test [ $>1.8$   $\mu$ g/dl (50 nm)] and lack of the cortisol circadian rhythm [midnight cortisol  $> 7.5$   $\mu$ g/dl (207 nm)];[26] the diagnosis of pituitary or adrenal cortex adenoma was verified by histological examination. From this group, seven patients (50%) had DM, four of them (57.1%) were treated with oral antidiabetic drug(s) or were on diet only, and three (42.8%) were on insulin treatment. In addition, eight patients (57.1%) were hypercholesterolaemic, five of them (62.5%) were treated with cholesterol-lowering drug, and the rest of the group was on cholesterol-lowering diet (37.5%). Furthermore, nine patients (64.2%) suffered from arterial hypertension and were treated with antihypertensive drugs. None of the patients had submitted to a major cardiovascular event. Finally, seven patients (50%) suffered from osteoporosis, and in five of them (71.4%) were identified vertebral fractures by vertebral morphometry. Finally, two patients (14.2%) had a recent history of lower extremity deep venous thrombosis, one of them complicated by pulmonary thromboembolism, treated with anticoagulation therapy, and, furthermore, three patients (21.4%) presented fibrinogen levels as also inflammation markers (C-reactive protein, CRP; and erythrocyte sedimentation rate, ESR) slightly above the upper limit of the normal range.

We have further subdivided our patient group in those with complications (DM, hypercholesterolaemia, arterial hypertension, osteoporosis, coagulation disorders) (CS compl, nine patients, two men, seven women) and in those without any complications (CS not compl, five patients, one man, four women). There was not any significant difference in age between the two patient groups.

Fourteen healthy subjects similar for sex, age and BMI (three men, 11 women, age mean  $\pm$  SEM  $43.09 \pm 1.0$  years, BMI  $26.04 \pm 0.92$  kg/m<sup>2</sup>) without any metabolic, cardiovascular and/or bone complications, free of any medical treatment served as controls (group NS). Smokers or ex-recent smokers were excluded from our study population.

The study protocol had been approved by an independent, local ethics committee, and written informed consent was obtained from all subjects.

### **Study design**

Anthropometric (weight, height, BMI and waist circumference, WC) and the following laboratory parameters were measured in patients and controls: 15-F<sub>2t</sub>-IsoP, TXB<sub>2</sub>, TAC, VitE, fasting glucose, glycosylated haemoglobin (HbA1c), total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides (TG), ACTH, cortisol, INR, PT, PTT, fibrinogen, CRP and ESR levels. Blood pressure was measured in the right arm, with any subject in a relaxed sitting position, using a mercury sphygmomanometer placed at heart level, and was calculated on average by three measurements, according to international criteria.[27]

Blood samples were taken in the morning after a 12-h overnight fast, between 07:30 and 08:00 am. UFC was also assayed in the patients. Routine serum determinations were performed by standard laboratory methods: glucose (mg/dl) total cholesterol, HDL and triglycerides (mg/dl) by enzymatic colorimetric method (Roche Diagnostic GmbH, Mannheim, Germany); low density lipoprotein (LDL) cholesterol was calculated as [(total cholesterol)/(HDL + (triglycerides/5))]. According to the World Health Organization (WHO) criteria, obesity was considered as body mass index (BMI)  $> 30$  kg/m<sup>2</sup> and overweight as BMI  $> 25$  kg/m<sup>2</sup>,[28] DM as fasting glucose levels  $\geq 126$  mg/dl (7.0 mm), HbA1c  $\geq 6.5\%$  or 2-h plasma glucose  $\geq 200$  mg/dl (11.1 mm) during an OGTT in two consecutive determinations,[29] dyslipidaemia according to the National Cholesterol

Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) criteria.[30] Hypertension was defined as systolic blood pressure (SBP) >140 mmHg or diastolic blood pressure (DBP) >90 mmHg according to WHO/ISH 2003.[27] In patients treated with antihypertensive drugs, pretreatment blood pressure values were considered for the diagnosis of hypertension. Osteoporosis was defined as a *T*-score < -2.5 at the hip or spine in postmenopausal women and in men over 50 years.[31] A *Z*-score of -2.0 or lower was defined as 'below the expected range for age', while a *Z*-score above -2.0 was 'within the expected range for age' for women before menopause and in men younger than 50 years of age.[32]

### **Hormone measurements**

Blood samples were centrifuged immediately after collection, and plasma and serum samples were frozen at -20 °C until assay. Plasma ACTH levels (pg/ml) were measured in duplicate by an immunoradiometric assay (IRMA CTK; DiaSorin, Vercelli, Italy). Serum cortisol levels (µg/l) were measured in duplicate by a RIA (Immunotech, Marseilles, France). UFC (µg/day) was evaluated by CMIA (chemiluminescent microparticle-based immunoassay) automated on Architect i2000 platform (Abbott Diagnostics, Abbott Park, IL, USA).

Plasma 15-F<sub>2t</sub>-IsoP levels (ng/ml) were measured with an enzyme immunoassay (EIA) method using a standard commercial kit (DRG Internationa Inc., Springfield, NJ, USA). Plasma TXB<sub>2</sub> levels (ng/ml) were also performed by an EIA method using a standard commercial kit (DRG Internationa Inc., Springfield, NJ, USA). Plasma TAC levels (nmol/µl) were determined using a colorimetric method (Biovision Inc., Milpitas, CA, USA). Serum VitE (µg/ml) was determined with reverse-phase high-performance liquid chromatography (Chromsystems, Gräfelfing, Germany).

### **Statistical analysis**

Oxidative parameters are expressed as mean, standard error of the mean (SEM) and relative 95% confidence interval (95% CI) of either absolute values. Variations of TXB<sub>2</sub>, 15-F<sub>2t</sub>-IsoP, VitE and TAC between the NS group, CS group, CS complicated and CS not-complicated at each time point were compared by means of nonparametric Mann–Whitney test. Correlations of TXB<sub>2</sub>, 15-F<sub>2t</sub>-IsoP, VitE and TAC between themselves, UFC and the metabolic parameters were analysed with the Spearman's test. Differences with a *P*-value <0.05 were considered statistically significant. SPSS (Statistical Package for the Social Science) version 15.0 was used for the analysis.

### **Results**

The demographic, clinical and hormonal data of the study population are expressed in the Table 1.

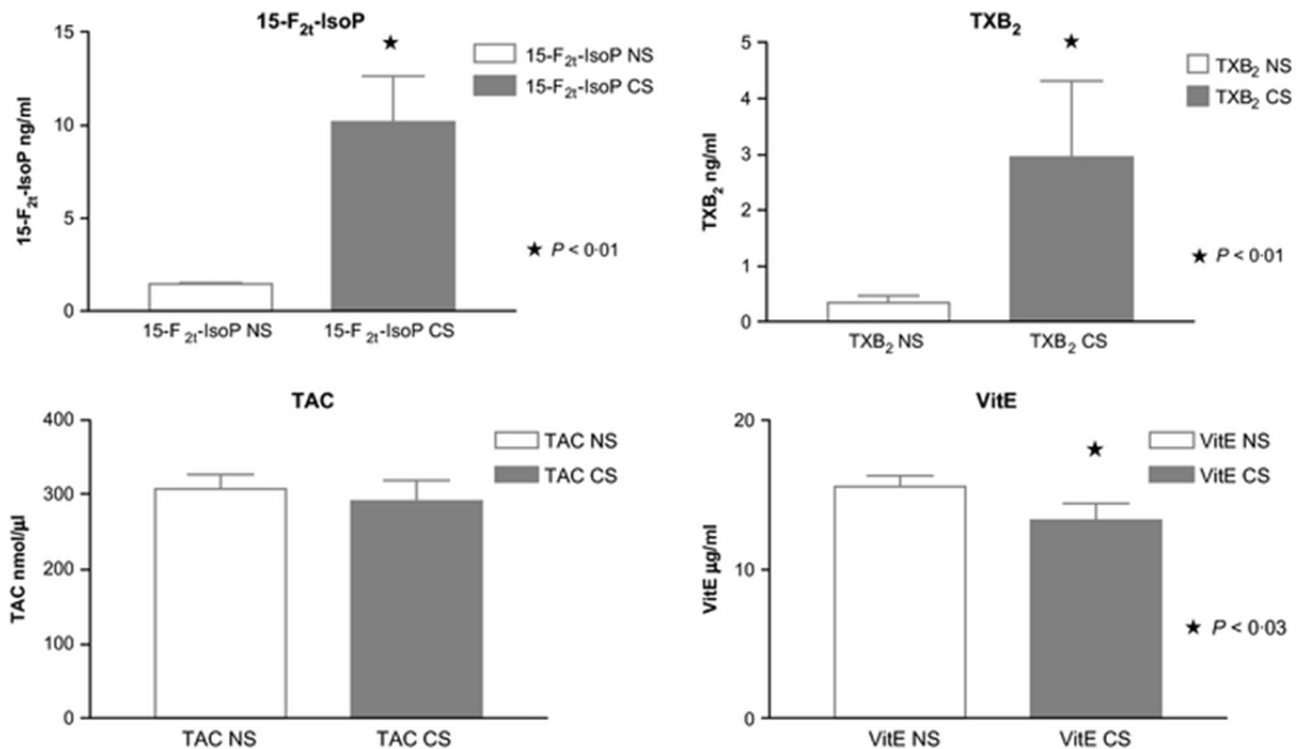
**Table 1.** Demographic, hormonal and clinical data of the patients

Gender (M/F)	3/11
Age (years)	48.33 ± 3.89
BMI (kg/m <sup>2</sup> )	28.54 ± 4.31
WC (cm)	98.55 ± 3.88
UFC (10–90 µg/24 h)	321.73 ± 90.38
1 mg overnight dexamethasone suppression test (DST, <1.8 µg/dl = 50 nm)	14.40 ± 2.71
Fasting glucose (mg/dl; 1 mg/dl = 0.0555 mm)	91.91 ± 11.36
HbA1c (%)	6.35 ± 0.7
Total cholesterol (mg/dl; 1 mg/dl = 0.0259 nm)	225.55 ± 15.89
LDL cholesterol (mg/dl; 1 mg/dl = 0.0259 nm)	130.93 ± 10.67
Non-HDL (mg/dl; 1 mg/dl = 0.0259 nm)	262.00 ± 25.00
HDL cholesterol (mg/dl; 1 mg/dl = 0.0259 nm)	53.82 ± 4.62
Triglycerides (mg/dl; 1 mg/dl = 0.0113 nm)	166.82 ± 47.56
SBP (mmHg)	136.82 ± 5.73
DSP (mmHg)	85.91 ± 3.49
15-F <sub>2t</sub> -IsoP (ng/ml)	10.20 ± 2.43
TXB <sub>2</sub> (ng/ml)	2.96 ± 1.36
TAC (nmol/µl)	290.49 ± 28.97
Vitamin E (µg/ml)	13.30 ± 1.10

**15-F<sub>2t</sub>-IsoP, TXB<sub>2</sub>, VitE and TAC differences between the NS and CS**

15-F<sub>2t</sub>-IsoP and TXB<sub>2</sub> levels were significantly higher ( $P < 0.01$ ) in CS with respect to the control group (Fig. 1), and VitE was higher in NS with respect to CS ( $P < 0.03$ ) (Fig. 1), while no differences were recorded for TAC levels between the two groups (Fig. 1).

**Figure 1.**

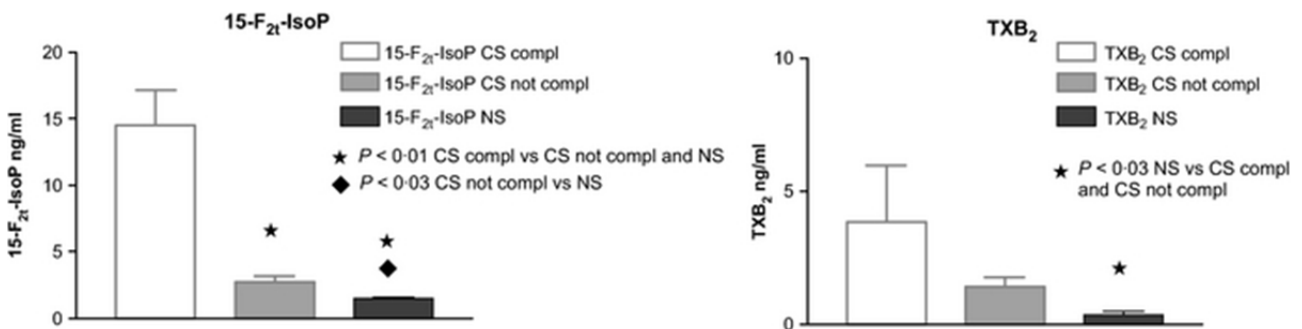


15-F<sub>2t</sub>-IsoP, TXB<sub>2</sub>, TAC and VitE levels in normal subjects (NS) and in patients affected by Cushing's Syndrome (CS).

### 15-F<sub>2t</sub>-IsoP, TXB<sub>2</sub>, VitE and TAC differences between NS, complicated and not-complicated CS

15-F<sub>2t</sub>-IsoP levels were significantly higher ( $P < 0.01$ ) in complicated with respect to not-complicated CS and to NS, and its levels were also significantly higher ( $P < 0.03$ ) in CS not-complicated with respect to NS (Fig. 2); TXB<sub>2</sub> levels were significantly reduced ( $P < 0.03$ ) in NS vs complicated and not-complicated CS and demonstrated higher levels in complicated CS with respect to not-complicated patients without reaching statistical significance (Fig. 2). No differences were observed between NS, complicated and not-complicated CS for VitE and TAC (data not shown).

**Figure 2.**

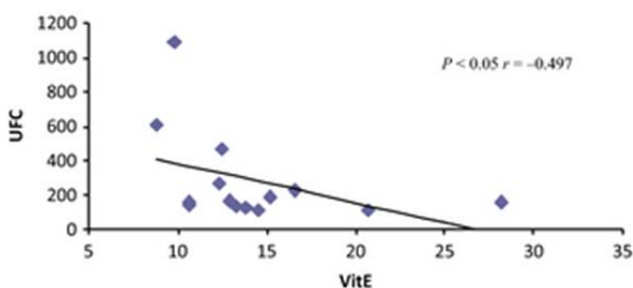


15-F<sub>2t</sub>-IsoP and TXB<sub>2</sub> levels in patients affected by Cushing's Syndrome complicated (CS compl) and not-complicated (CS not compl).

### 15-F<sub>2t</sub>-IsoP, TXB<sub>2</sub>, VitE and TAC correlations

VitE levels were negatively correlated with UFC levels ( $P < 0.05$ ,  $r = -0.497$ ) (Fig. 3). No other significant correlation was detected for 15-F<sub>2t</sub>-IsoP, TXB<sub>2</sub> and TAC with UFC and for 15-F<sub>2t</sub>-IsoP, TXB<sub>2</sub>, VitE and TAC between themselves (data not shown).

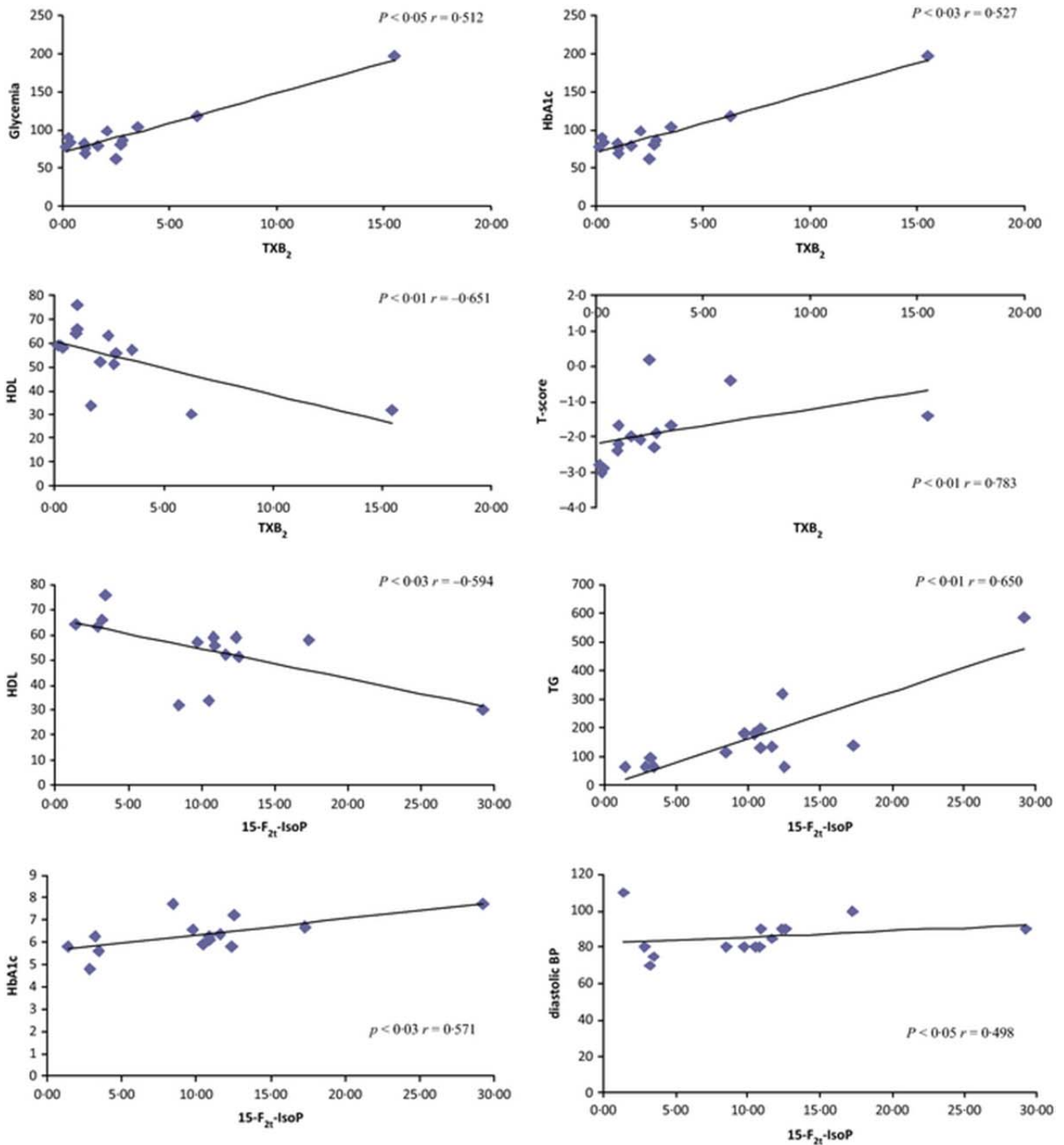
**Figure 3.**



Correlation between VitE and urinary free cortisol (UFC).

Concerning the metabolic parameters, the following significant correlations were observed: TXB<sub>2</sub> levels were positively correlated with glucose, HbA1c and T-score ( $P < 0.05$   $r = 0.512$ ,  $P < 0.03$   $r = 0.527$  and  $P < 0.01$   $r = 0.783$ , respectively) and negatively with HDL ( $P < 0.01$   $r = -0.651$ ) (Fig. 4). 15-F<sub>2t</sub>-IsoP levels were positively correlated with TG, HbA1c and diastolic blood pressure ( $P < 0.01$   $r = 0.650$ ,  $P < 0.03$   $r = 0.571$  and  $P < 0.05$   $r = 0.498$ , respectively) and negatively with HDL ( $P < 0.03$   $r = -0.594$ ) (Fig. 4). No significant correlations were detected for VitE and TAC with the metabolic parameters (data not shown).

Figure 4.



Correlations between TXB<sub>2</sub> and 15-F<sub>2t</sub>-IsoP and metabolic parameters.

## Discussion

In our present study, we evaluated the effects of chronic hypercortisolism on the oxidative stress and antioxidant reserve and their interactions. To our knowledge, this is the first study that evaluated the 15-F<sub>2t</sub>-IsoP levels, a highly precise marker of OS, in patients with overt CS and its correlation with other parameters of the oxidant–antioxidant balance.

We have demonstrated that plasma 15-F<sub>2t</sub>-IsoP and TXB<sub>2</sub> levels, which are reliable markers of the oxidative stress and platelet activation, respectively, [5-7, 13, 16] are significantly increased in patients affected by CS.



In contrast, vitamin E levels, an important marker of the antioxidant status, are significantly lower in CS compared with control group. Furthermore, it has been shown a trend towards a reduction regarding TAC levels in CS.

In previous studies, it has been shown, measuring MDA levels, that high oxidative stress occurs in CS. However, it should be pointed out that increase in MDA levels is a poorly specific marker, as many different aldehydes are formed during the lipid peroxidation process and an overexpression of its level could be happened. In contrast, 15-F<sub>2t</sub>-IsoP is considered as the best available and most sensitive biomarker of the oxidative injury; moreover, isoprostane could represent an independent risk marker of CHD.[4, 5]

Our results showed higher levels of 15-F<sub>2t</sub>-IsoP in patients affected by CS with metabolic, cardiovascular and bone complications compared with patients affected by CS but without secondary complications and, overall, in patients with CS compared with and normal subjects. Similar findings were also noticed for the TXB<sub>2</sub> levels. These findings seem to be very interesting. Alterations in oxidative stress parameters have also been observed in patients with metabolic disorders without chronic hypercortisolism,[14, 16, 17] thus indicating a clear association between oxidative derangement and metabolic abnormalities. Based on our data, it can be hypothesized that chronic increased cortisol concentrations induce a significant derangement in pro-oxidative markers, particularly in susceptible patients who, in turn, develop secondary metabolic complications; alternatively, the occurrence of metabolic alterations in these patients can act significantly and synergically with hypercortisolism to worsen the oxidative processes. To this regard, an important limitation of our study is the absence of a control group of non-CS patients matched for metabolic risk factors other than cortisol status: indeed, only the comparison with these patients could demonstrate if chronic hypercortisolism '*per se*' is an independent source of oxidative stress. Moreover, we cannot exclude the possibility that CS patients without complications are simply displaying early attributes of metabolic syndrome driven by the CS, being at a different '*phase*' in the natural history of the condition. In this case, the possibility of an epiphenomenon is strong.

On the other hand, we did not observe proportional modifications regarding the antioxidant parameters. A possible explanation could be that at the time of the CS diagnosis, perturbations of the antioxidant defences, and particularly of the total antioxidants effects represented by TAC levels, were not evident, probably, due to an adequate intake of protective antioxidants effective opposing the increased production of ROS.

Another interesting result of our study was the inverse correlation among UFC and Vitamin E levels. This datum reinforces our concept about the depletion of this antioxidant in patients with endogenous hypercortisolism at diagnosis compared with healthy subjects matched for sex, age and BMI. On the other hand, we did not observe any correlation between UFC and the marker of oxidative stress. This may possibly happen because Vitamin E seems to be an early marker of the oxidant-antioxidant status even in the presence of mild hypercortisolism, although more data are needed to confirm this hypothesis.

Some other important findings of the present study were the correlations between the metabolic parameters and the markers under study. In fact, it has been found a significant positive correlation between glycaemic indices and isoprostane and thromboxane levels. Furthermore, it has been shown an inverse correlation between the 15-F<sub>2t</sub>-IsoP, TXB<sub>2</sub> and HDL levels. A significant association between 15-F<sub>2t</sub>-IsoP and DBP has also been noticed; however, as the gradient of the best-fit line is low, it seems that there is not a strong biological significance. Although there are different studies which had observed these correlations in patients with primary metabolic disorders,[14, 16, 17] it should be mentioned that there are no literature data comparing these parameters in patients affected by CS and secondary metabolic complications.

Different studies have reported that obesity is positively correlated with plasma 15-F<sub>2t</sub>-IsoP and TXB<sub>2</sub> levels and negatively correlated with plasma Vitamin E and TAC levels.[33, 34] Our statistical analysis had not

revealed any significant correlation, probably because of the lower mean BMI in our population as the majority of patients were not obese and of the different body composition and fat distribution of the patients between the various studies.[35] Furthermore, we did not find any significant correlation between isoprostane levels and sex, gender or age; this result is in agreement with some previous studies, although controversial data have been reported.[6, 24, 31, 34] It should not be ignored the low male to female ratio in our study group.

Previous studies have demonstrated a correlation between  $15\text{-F}_{2t}\text{-IsoP}$  and  $\text{TXB}_2$  levels in various metabolic disorders, but these data have not been confirmed in patients affected by CS with secondary metabolic alterations.[15, 17, 18] This finding is not easy to be explained, but some hypotheses can be drawn: a lack of correlation due to the low number of subjects enrolled; alternatively, it can simply reflect the short time follow-up.

Besides the need to compare patients with CS with a group of non-CS patients matched for metabolic abnormalities, it would be also interesting to verify whether the homeostatic balance of the oxidant–antioxidant status in patients with Cushing's syndrome after disease remission could be reversed, or further derangement may occur during disease recurrence, and to verify the involvement of possible persistent metabolic abnormalities on oxidant damage in cured patients. This could have a clinical relevance as the oxidative biomarkers could be considered themselves as cardiovascular risk factors.

In conclusion, this study shows an enhanced oxidative injury and platelet aggregation in patients affected by Cushing's syndrome. As this rare metabolic disorder is associated with an increased cardiovascular risk, it could be suggested that these biochemical alterations can further contribute to the pathogenesis of atherosclerosis, increased cardiovascular risk and mortality in Cushing's syndrome.

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