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The effect of glucocorticoids on Thrombospondin-1, Osteocalcin and the Thrombospondin-1:Osteocalcin ratio in humans

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Abstract

Objective: Thrombospondin-1 (TSP1), a matricellular protein, and Osteocalcin (OCN), a non-collagenous protein secreted by osteoblasts, are known to be up- and down-regulated respectively by glucocorticoids. The aim of this study was to determine if a ratio between TSP1:OCN was altered by changes in glucocorticoid activity in humans.

Design: Prospective observational study.

Setting: Tertiary university hospital in Queensland, Australia.

Patients and Measurements: Patients with Cushing's syndrome (CS, n=19), asthma or giant cell arteritis on chronic prednisolone treatment (PRED, n=13), adrenal insufficiency (AI, n=16) and healthy volunteers (HV, n=20). Plasma TSP1 and serum total OCN were measured by immunoassay at 0800h, 1200h and 1600h in patients with CS, patients with AI taking replacement glucocorticoids, HV before and after 4 mg dexamethasone, and PRED patients pre-dose at 0800h and 4 hours post-dose at 1200h.

Results: Plasma TSP1 in CS was higher ($P<0{\cdot}0001$) and serum OCN was lower ($P<0{\cdot}0001$) than HV. The TSP1:OCN ratio in HV increased significantly after 4 mg dexamethasone ($P<0{\cdot}0001$) and in AI after taking their hydrocortisone replacement therapy ($P<0{\cdot}001$). PRED patients had a higher TSP1:OCN ratio compared to HV at both 0800h and 1200h (both $P<0{\cdot}001$), but no significant change occurred from pre- to post-dose. A TSP1:OCN ratio of >73 at 0800h differentiated CS from HV with a sensitivity of 95% and a specificity of 100%.

Conclusions: The TSP1:OCN ratio is elevated in patients on prednisolone and in patients with CS compared to healthy volunteers. It may be a useful biomarker of total body glucocorticoid activity in humans.

Introduction

Glucocorticoids are widely prescribed medications for a diverse range of health problems and are used in many medical sub-specialties.[1] When used in pharmacological doses of more than 5 mg per day of prednisolone or equivalent, glucocorticoids are effective anti-inflammatory

and immunosuppressive drugs, but their use is associated with several adverse effects.[2] Endogenous glucocorticoid excess, as seen in Cushing's syndrome (CS), is characterized by pathologically elevated cortisol secretion, absence or blunting of the serum cortisol diurnal rhythm and lack of glucocorticoid suppression.[3] Exogenous and endogenous glucocorticoid excess can result in serious side effects such as weight gain, diabetes, profound muscle weakness, osteoporosis, marked bruising, prominent striae, cataracts and hypertension.[2, 4] Side effects usually correlate with longer use and higher dosage of glucocorticoids[5] and there is a dose-dependent increase in mortality for patients taking glucocorticoids[6] as well as for patients with CS.[7]

Glucocorticoids are also used for physiological replacement in states of cortisol deficiency such as hypopituitarism and primary adrenal insufficiency. In this setting, the therapeutic aim is to mimic normal daily cortisol production, but a major issue in replacement therapy for adrenal insufficiency is that traditional doses have been excessive.[8] Currently there is no established biomarker of glucocorticoid activity in clinical use.

Thrombospondin-1 (TSP1) is a matricellular protein, first discovered in platelets.[9] It is widely expressed in diverse tissues including endothelial cells, monocytes /macrophages and adipocytes and is readily measurable in humans.[10] Previously our group showed that plasma TSP1 increased both in healthy volunteers after a single dose of dexamethasone and in patients with adrenal insufficiency after an increase in their maintenance hydrocortisone and furthermore was elevated in patients with CS, compared to healthy volunteers.[11]

Osteocalcin (OCN) is a vitamin K-dependent, non-collagenous protein synthesized by osteoblasts and considered a marker of bone formation.[12] Glucocorticoid-treated patients have decreased OCN levels, reflecting diminished osteoblastic activity.[13] Furthermore, data suggest that OCN is involved in pathogenesis of glucocorticoid-induced dysmetabolism,[14] and indicate reduced serum OCN in patients with CS.[15]

Neither TSP1 nor OCN alone are sufficiently sensitive or specific to be clinically useful biomarkers of glucocorticoid activity. However, the use of a ratio between an up-regulated and down-regulated glucocorticoid-responsive protein potentially amplifies the observed changes and might improve performance both as a diagnostic test for glucocorticoid excess and for use in therapeutic drug monitoring for patients on exogenous glucocorticoids. This

study therefore aimed to test the hypothesis that the TSP1:OCN ratio was altered in people with acute and chronic states of increased glucocorticoid activity such as CS and patients taking exogenous glucocorticoids, compared to healthy individuals.

Material and Methods

Study design and setting

This prospective study was performed at the Princess Alexandra Hospital, Brisbane, Australia. The Metro South Human Research Ethics Committee, Brisbane approved the study protocol and written informed consent was obtained from all participants.

Participants

In total, we included 19 patients with Cushing's syndrome (CS), 13 patients on chronic supra-physiological prednisolone treatment (PRED), 16 patients with adrenal insufficiency (AI) and 20 healthy volunteers (HV). In all patients and HV vital signs, comorbidities, current medication, and symptoms related to glucocorticoid excess were assessed.

Healthy volunteers

Twenty HV, ten male and ten female, were studied. On day one, venous blood was sampled at 0800h, 1200h and 1600h in a non-fasting state. On day two, separated from day one by at least 48h, each participant took 4 mg of dexamethasone orally at midnight, followed by repeat venous sampling at 0800h, 1200h and 1600h the following day.

Patients with Cushing's syndrome

Nineteen patients with CS were enrolled in the study, of whom 15 (79%) had Cushing's disease (adrenocorticotrophic hormone (ACTH) secreting pituitary adenoma) and 4 (21%) had ACTH-independent hypercortisolism of adrenal origin. All patients with CS had clinical features of cortisol excess, including at least 2 out of: characteristic weight distribution (18/19), thinning of the skin (16/19), easy bruising (14/19) and proximal myopathy (13/19). They were diagnosed with CS according to the Endocrine Society Clinical Practice Guidelines[16] with at least two abnormal diagnostic tests (elevated 24h urinary free cortisol, elevated late night salivary cortisol or failure to suppress serum cortisol to 50 nmol/L after 1 mg dexamethasone), in combination with a normal or elevated plasma ACTH in the case of Cushing's disease[16] or suppressed ACTH in adrenal Cushing's. Three participants had

recurrence or persistence following previous trans-sphenoidal resection of an ACTH-secreting pituitary adenoma and had a clinically mild phenotype at the time of testing. One patient had cyclical Cushing's disease, but was tested during an "on" phase. There was a range of biochemical severity, with urinary free cortisol (UFC) excretion varying from 0.7-22.7 times the upper limit of the reference range (ULN) - median UFC 2.8x ULN (IQR 1.8-4.5). In all patients, venous blood was sampled across the day at 0800h, 1200h and 1600h in a non-fasting state for serum cortisol, plasma ACTH, plasma TSP1 and serum OCN, after the diagnosis of CS was confirmed but prior to the institution of any specific therapy. Of the 15 patients with Cushing's disease, 11 later had histological confirmation of an ACTH-secreting pituitary adenoma, 2 had negative histology but biochemical and clinical remission post-operatively, 1 was deemed medically unsuitable for surgery and 1 (the patient with cyclical Cushing's) declined follow-up prior to any intervention. All patients with adrenal Cushing's underwent successful unilateral adrenalectomy.

Patients on chronic prednisolone treatment

In total, 13 patients either with asthma (n=8, 62%) or giant cell arteritis (n=5, 38%) on chronic prednisolone treatment (PRED) were included. All patients were on oral prednisolone or equivalent therapy of ≥7·5 mg per day for a minimum of three months with stable dosage in the preceding four weeks. Blood sampling occurred before taking their usual prednisolone dose at 0800h and 4 hours after at 1200h.

Patients with adrenal insufficiency

Out of 16 patients with AI, 12 had primary adrenal insufficiency (75%) and 4 had secondary adrenal insufficiency (25%). Serial venous blood samples were drawn across the day at 0800h, 1200h and 1600h while patients received their routine glucocorticoid replacement (either hydrocortisone or cortisone acetate) at 0800h, 1200h and 1600h for those on a thrice daily regimen (n=10, 63%), or 0800h and 1400h for those on a twice daily regimen (n=6, 37%).

Blood sampling and laboratory tests

Blood samples were immediately centrifuged and stored at -80°C. Plasma TSP1 was determined in a batch analysis using our previously published ELISA.[17] Reanalysing assay precision after the purchase of a new TSP1 antibody, the inter-assay co-efficient of variation (CV) was 9% and the intra-assay CV was 3%. Half-life of TSP1 has been reported to be 9

hours.[10] Serum total OCN was measured with a commercial ELISA (MicroVue Osteocalcin EIA kit 8002, Quidel, San Diego, CA, USA), as per manufacturer's instructions. The limit of the detection was 0·5 ng/mL and co-efficient of variation (CV) was 6·5% and 10·5% within and between assays respectively at 18·5 ng/mL. Serum cortisol was measured by Beckman DxI800 immunoassay method (Beckman Coulter, Brea, CA, USA) CV 8.1% at 124 nmol/L, 5·1% at 627 nmol/L.

Statistical analysis

Categorical variables are expressed as frequency (percentage), Gaussian continuous variables as mean ± standard deviation (SD), and non-Gaussian continuous variables as median and (interquartile range, IQR). Most data sets did not satisfy parametric assumptions and were therefore log transformed prior to analysis with two-way repeated measures analysis of variance (ANOVA). In HV, two-way repeated measures ANOVA was performed on the log transformed data to assess the effect of treatment (dexamethasone) and time. Two-way repeated measures ANOVA on log-transformed data was also used to assess the effect of participant group and time when comparing patients with CS, AI and PRED to HV. Data are depicted graphically as box and whisker plots (median, interquartile range, range). Serum cortisol was normally distributed among HV and CS, so the untransformed cortisol data were used for the two-way repeated measures ANOVA.

Intergroup comparisons were made applying the chi-square test, Mann-Whitney U test or Kruskal-Wallis test, as appropriate. Paired data were analysed using the paired t test or non-parametric equivalent (Wilcoxon signed rank test). To quantitate TSP1, OCN and TSP1:OCN ratio accuracy in distinguishing between HV and CS, we performed receiver operator characteristic (ROC) curve analysis, and report area under the ROC curve (AUC) and its 95% CI. Analyses were performed using Graph Pad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Testing was two-tailed; $P<0·05$ was considered statistically significant.

Results

Baseline characteristics

Baseline characteristics of the study population are provided in Table 1.

Median age of HV (female n=10, male n=10) was 26 years (IQR 23-30). Healthy volunteers had a median blood systolic pressure of 122 mmHg (IQR 114-136) and a body mass index (BMI) of 23.8 kg/m2 (IQR 22.7-25.2). The median age of patients with CS was 46 years

(IQR 41-55), and 17 participants (89%) were female. Median systolic blood pressure was 141 mmHg (IQR 132-152) and median BMI was 32.2 kg/m2 (IQR 28.6-36.8). Seven patients (38%) had a manifest diabetes mellitus, 14 patients (74%) had hypertension and three patients (16%) had treatment-requiring osteoporosis. Eight PRED patients had asthma (62%) and five (38%) were diagnosed with giant cell arteritis. Patients were taking a median dose of 14 mg prednisolone (IQR 10-20) and median duration of prednisolone treatment was 24 months (IQR 5-60). Median systolic blood pressure was 132 mmHg (IQR 125-142) and median BMI was 30.6 kg/m2 (IQR 29.7-34.4). Three patients (23%) had diabetes mellitus, nine (69%) had hypertension and eight (62%) had osteoporosis. Median age for patients with AI was 53 years (IQR 39-65) and 11 (69%) were female. The median hydrocortisone replacement dose was 20 mg/day (IQR 18-22) and 12 patients (75%, those with primary AI) were on fludrocortisone therapy – median dose 0.1 mg/day, (IQR 0.04-0.16). Median systolic blood pressure was 119 mmHg (IQR 114-128) and median BMI was 26.1 kg/m2 (IQR 24.0-31.9).

Serum cortisol

Diurnal serum cortisol concentrations in HV and CS are depicted in Figure 1, while the individual 0800h cortisol concentrations are outlined in Figure 2. In the HV group, median cortisol levels were 393 (IQR 241-483) nmol/L at 0800h and decreased significantly during the day as expected along the circadian rhythm (P<0·0001). After 4 mg dexamethasone at midnight, serum cortisol and plasma ACTH were suppressed throughout the sampling period, while median serum cortisol was elevated in the CS at 0800h: 511 (IQR 412-554) nmol/L and showed only a slight decline during the day (P=0·012). Patients with CS had higher cortisol levels compared to HV at all three time points (P<0·0001, Figure 1). In the PRED patients, 0800h serum cortisol was low due to hypothalamic-pituitary-adrenal axis suppression, and very low in the patients with AI. Using Kruskal Wallis one-way ANOVA, 0800h cortisol was significantly lower in both AI and PRED groups compared to the HV group (P<0·0001, Figure 2).

TSP1 and OCN in healthy volunteers before and after dexamethasone

On day one at 0800h, median plasma TSP1 was 271 (IQR 237-336) ng/mL, median serum OCN was 11·0 (IQR 7·5-13·7) ng/mL and the TSP1:OCN ratio was 24·0 (IQR 17·8-46·0), Table 2. Two-way ANOVA on log transformed data demonstrated that all parameters remained unchanged during the day (P=0·80, P=0·79 and P=0·99 respectively). The increase

in plasma TSP1 after dexamethasone in this group has been previously reported.[11] Following dexamethasone, OCN levels were lower compared to the control day (all time points P<0·0001, Figure 3A). Median OCN levels after dexamethasone were 6·8 (5·0-8·8) ng/mL at 0800h and decreased further during the day (P=0·0006). Accordingly, median TSP1:OCN ratio was lowest at 0800h: 53·3 (IQR 28·7-85·7) and increased during the study day (P=0·02). The TSP1:OCN ratio in HV was significantly higher after 4 mg dexamethasone than the control day at all time points (main effect of treatment: P<0·0001, Figure 3B).

TSP1 and OCN in patients with Cushing's syndrome

Plasma TSP1 was significantly higher in CS compared to HV at all three time points (0800h: P=0·0002, 1200h and 1600h: P<0·0001, Table 2). At 0800h a plasma TSP1 >389 ng/mL identified patients with CS with a sensitivity of 79% and a specificity of 85% (AUC 0·84 (95% CI 0·70-0·97). At 1200h and 1600h the diagnostic accuracy of plasma TSP1 for CS remained similar. Serum OCN was significantly lower in patients with CS compared to HV (0800h: P<0·0001, 1200h: P=0·0009, 1600h: P<0·0001, Table 2). ROC analysis showed the best sensitivity and specificity for the diagnosis of Cushing's syndrome with a single OCN measurement at 0800h: (serum OCN <6·5 ng/mL, AUC 0·95 (95% CI 0·88-1·0), sensitivity: 84%, specificity: 90%). The TSP1:OCN ratio was significantly higher in patients with Cushing's syndrome compared to HV at all time points (P<0·0001), see Table 2 and Figure 4A.

TSP1 and OCN in patients with adrenal insufficiency

Plasma TSP1 levels in patients with AI did not change during the day (overall P=0·097). Compared to HV, TSP1 levels were higher only at 1200h (P=0·02, Table 2). Serum OCN levels were highest at 0800h: 13·4 (IQR 9·8-15·9) ng/mL and decreased during the day – 1200h: 10·4 (IQR 7·7-14·5) ng/mL, 1600h: 8·9 (IQR 6·5-12·1) ng/mL, P=0·005. At all time points, OCN levels were similar to HV, but were higher than PRED or CS groups (Table 2). The TSP1:OCN ratio in patients with AI was lowest at 0800h: 28·2 (IQR 23·2-38·8) and increased across the day after taking the usual replacement therapy 1200h: 32·9 (IQR 26·9-48·2), 1600h: 43·5 (IQR 29·4-58·3), (P=0·0009). The TSP1:OCN ratio in AI was similar to HV at 0800h and 1200h. However, at 1600h the TSP1:OCN ratio was higher in AI compared to HV (P=0·027), see Figure 4B.

TSP1 and OCN in patients on chronic prednisolone treatment

Plasma TSP1 in PRED patients was similar before and four hours after taking their medication (P=0·27). There was no difference in TSP1 compared to HV at 0800h and 1200h (P=0·62, and P=0·15), see Table 2. On the contrary, OCN was significantly lower in PRED patients compared to HV at 0800h (P<0·001, Table 2) and 1200h (P<0·01), but there was no significant change from pre- to post-dose (P=0·44). The TSP1:OCN ratio in PRED patients was similar at 0800h: 59·2 (IQR 38·8-91·9) compared to 1200h: 49·3 (IQR 36·4-134·6), P=0·74. PRED patients had significantly higher TSP1:OCN ratio compared to HV at both time points (both P<0·001, Table 2 and Figure 4C). Furthermore, the pre-dose TSP1:OCN ratio was lower in the PRED group compared to CS at 0800h (P<0·01), However, after receiving the dose of prednisolone, the ratio was similar for both patient groups at 1200h (Table 2 and Figure 4C).

Diagnostic Performance of the TSP1:OCN ratio in Cushing's syndrome

The best diagnostic accuracy for the TSP1:OCN ratio was at 0800h, which is outlined in Figure 5. A TSP1:OCN ratio of >73 distinguished patients with CS from HV with a sensitivity of 95% and a specificity of 100% – AUC 0·997 (95% CI 0·99-1·0), P<0·0001. The TSP1:OCN ratio at 0800h did not correlate with the 24h urinary free cortisol, expressed as fold change from the upper limit of the reference range (P=0.32).

Effect of inverting the ratio

The investigators chose to express the numerically higher value of the upregulated protein as the numerator and the numerically lower value of the downregulated protein as the denominator, leading to an increase in the TSP1:OCN ratio when glucocorticoid activity rose. Inverting the ratio to express it as OCN:TSP1 resulted in numeric values in the range of 0.01-0.1 which fell in response to increased glucocorticoid exposure. There was no difference in the statistical significance of the measure regardless of which parameter was the numerator or denominator.

Discussion

This prospective observational study demonstrates that the TSP1:OCN ratio increases in response to both acute and chronic glucocorticoid exposure in humans. Firstly, the TSP1:OCN ratio increased in HV after a single dose of dexamethasone, and patients on chronic supra-physiological prednisolone treatment had a higher TSP1:OCN ratio compared to HV. Secondly, the TSP1:OCN ratio was elevated in patients with CS compared to HV with

high sensitivity and specificity, reflecting elevated endogenous cortisol secretion. Thirdly, the TSP1:OCN ratio increased during the day in patients with AI receiving their daily glucocorticoid supplementation, indicating the effect of replacement therapy.

Several studies have shown that both TSP1 and OCN are affected by glucocorticoids in humans.[11, 13, 15] Glucocorticoids inhibit OCN gene expression in osteoblasts via a negative glucocorticoid response element (GRE) in the OCN promoter,[18] but the precise molecular mechanism of the TSP1 induction has not been elucidated. The current study suggests that a ratio between TSP1 and OCN reflects total body glucocorticoid activity in both an acute and chronic setting. Furthermore, in patients with CS, the TSP1:OCN ratio might be useful as a novel diagnostic marker. Establishing a diagnosis of CS can be challenging. The sensitivity and specificity of the various diagnostic tests varies depending on several patient factors. False-positive elevation of 24-hour urinary free cortisol may be seen in patients with high fluid intake and any physiological or pathological condition that increases cortisol production.[16] False-negative results of urine cortisol may occur in patients with moderate to severe renal impairment.[19] In obese subjects and in women taking the oral contraceptive pill, up to 50% false positive results have been reported with the 1 mg dexamethasone suppression test.[16, 20] The diagnostic accuracy of the late-night salivary cortisol in CS is similar to that of 24-hour urinary free cortisol and the 1 mg dexamethasone test, with several studies reporting a sensitivity between 92-100% and a specificity between 93-100%.[21] Nevertheless, falsely elevated results can occur in patients with blunted circadian rhythm, i.e. depressive illness, shift workers and critically ill patients.[22] Furthermore, a recent study including elderly men reported that 20% of all participants and 40% of diabetic hypertensive subjects had at least one falsely elevated late-night salivary cortisol.[23] While we acknowledge that a larger pool of patients both with and without CS needs to be studied, our results show the TSP1:OCN ratio from a single morning blood draw, shows potential as a screening test.

A reliable biomarker for the glucocorticoid action would also be useful in patients with AI, as sometimes replacement doses can be excessive.[8] A higher mortality rate has been noted in patients with AI[24] – possible explanations include supra-physiological maintenance doses[25] and a poor diurnal glucocorticoid exposure-time profile.[26] Our results showed that the TSP1:OCN ratio increased during the day after taking their usual replacement therapy, and was significantly elevated compared to HV at 1600h, possibly reflecting higher than ideal afternoon dosing in these patients. Once-daily dual release hydrocortisone, an alternative

replacement therapy to twice or thrice daily immediate release hydrocortisone, provides a more circadian-based serum cortisol profile, improving weight, blood pressure and glucose metabolism.[27] We hypothesise that the afternoon TSP1:OCN ratio in patients on once-daily dual release hydrocortisone would be lower than those on conventional replacement therapy. Of note, the patients with AI had very low morning cortisol but a normal morning TSP1:OCN ratio, while those on PRED had a low morning cortisol but an elevated TSP1:OCN ratio. This may indicate that these patients on conventional replacement largely maintain normal tissue glucocorticoid activity overnight or that the measure is not as sensitive to detect low compared to high glucocorticoid activity, while patients on supraphysiological prednisolone maintain elevated glucocorticoid activity over the 24h period.

The question arises whether the TSP1:OCN ratio reflects recent or longer term glucocorticoid exposure (akin to the relationship between HbA1c and glucose). Given our findings, we suggest that the relationship between the TSP1:OCN ratio and glucocorticoids is similar to that between IGF1 and growth hormone (GH). IGF1 is a more sensitive marker of GH excess than GH deficiency and is a measure of integrated GH exposure rather than a reflection of the concurrent GH concentration.

The following limitations of our study are acknowledged. TSP1 and OCN concentrations may be affected by factors other than glucocorticoids. It is known that TSP1 is elevated in patients with diabetes, particularly in patients with both diabetes and cardiovascular disease.[28] However, data on the relationship between TSP1 and cardiovascular disease are conflicting and more research is warranted.[29] Our earlier study did not show any association between plasma TSP1 and age or BMI among HV, which needs to be confirmed with a larger sample size.[11] Platelet activation markedly increases TSP1 levels, which are approximately 100-fold higher in serum than plasma,[17] so careful sample collection is required to avoid this. The precision of the immunoassays for TSP1 and OCN may result in errors which are minimised using batch analysis nullifying a higher inter-assay CV. However, this is largely a technical issue which we believe could be overcome (changing the TSP1 antibody for example more than halved the previous inter-assay CV), noting that immunoassays remain in widespread clinical use in the broad field of Endocrinology. It is also noted that using a ratio between two independent variables potentially has the effect of compounding error, reducing diagnostic precision.

OCN levels can be affected by several conditions. Previous studies showed that OCN is affected by androgens and oestrogens.[30] Oestrogen replacement therapy which counteracts the biochemical changes caused by increased bone turnover associated with menopause can affect OCN levels.[31] However, in our CS patients we found no difference in OCN levels in postmenopausal women compared to premenopausal women (data not shown). Furthermore, several studies have explored the role of OCN in the regulation of glucose metabolism in humans and have reported associations of reduced OCN levels with hyperglycaemia, insulin resistance, type 2 diabetes and obesity.[32-34] Our HV had a lower BMI compared to patients with CS and the rate of metabolic syndrome was higher in our patients with CS which might have affected our results. Further, osteoporosis and especially anti-resorptive agents might have influenced our results, as OCN is a marker of bone formation. Our results remained similar excluding patients with known osteoporosis (n = 3 for Cushing's patients and n= 6 for PRED patients). However, due to the rather small patients group this findings have to be interpreted with caution and larger studies are needed to draw a definitive conclusion.

Lastly, we acknowledge that the sample size in our study is small and further studies of larger cohorts, especially including more control subjects with conditions such as obesity, hypertension and type 2 diabetes are clearly needed to establish a reference range for non-Cushing's patients.

In summary, the TSP1:OCN ratio reflects glucocorticoid activity in several different patient cohorts. The TSP1:OCN ratio increased in HV after a single dose of dexamethasone and rose during the day in patients with AI receiving their usual daily glucocorticoid supplementation. Furthermore, the TSP1:OCN ratio was high in patients on chronic prednisolone treatment and in patients with CS. We propose that the TSP1:OCN ratio is a promising glucocorticoid biomarker, which may provide adjuvant information to the standard diagnostic tests for Cushing's syndrome and potential therapeutic drug monitoring in patients on glucocorticoids.

Declaration of interest:

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Legends for Figures:

Figure 1

Mean ± standard error of the mean (SEM) diurnal serum cortisol in healthy volunteers (n=20) under resting conditions (○) and after 4mg of dexamethasone orally administered at midnight (●) compared to patients with Cushing's syndrome (■, n=19). *** P<0·001 and **** P<0·0001 compared to healthy volunteers.

Figure 2

Serum cortisol at 0800h in healthy volunteers (n=20) under resting conditions (○), patients with Cushing's syndrome (■, n=19), patients on chronic supra-physiological prednisolone therapy (▼, n=13) and adrenal insufficiency on replacement hydrocortisone or cortisone acetate (▲, n=16). The 0800h cortisol represents a pre-dose concentration in both groups receiving glucocorticoids. * P<0·05 vs HV and AI, ** P<0·01 vs HV and CS, *** P<0·0001 compared to HV and CS.

Figure 3

A – Serum osteocalcin (OCN) in healthy volunteers (n=20) under resting conditions (○) and after 4 mg dexamethasone orally administered at midnight (●). *** P<0·001 and **** P<0·0001 compared to control day.

B – Thrombospondin-1 to osteocalcin (TSP1:OCN) ratio in healthy volunteers under resting conditions (○) and after 4 mg dexamethasone orally administered at midnight (●). *** P<0·001 and **** P<0·0001 compared to control day.

Figure 4

A – Thrombospondin-1 to osteocalcin (TSP1:OCN) ratio in patients with Cushing's syndrome (n=19, ■) and healthy volunteers (n=20, □). ****P<0·0001 compared to healthy volunteers.

B – Thrombospondin-1 to osteocalcin (TSP1:OCN) ratio in patients with adrenal insufficiency (n=16, ■) and healthy volunteers (n=20, □). *P<0·05 compared to healthy volunteers.

C – Thrombospondin-1 to osteocalcin (TSP1:OCN) ratio in patients with Cushing's syndrome (n=19, ■), patients on chronic supra-physiological prednisolone treatment (n=13, ■) and healthy volunteers (n=20, □). The 0800h sample in the patients on prednisolone was drawn pre-dose and the 1200h sample was drawn 4h post-dose. *** P<0·001 and **** P<0·0001 compared to healthy volunteers. †† P<0·01 compared to Cushing's syndrome.

Figure 5

Diagnostic performance of the TSP1:OCN ratio in Cushing's syndrome at 0800h. The dashed line indicates a TSP1:OCN ratio of 73, where both are expressed in ng/mL. ROC curve (inset) AUC 0·997 (95% CI 0·99-1·0), P<0·0001. Sensitivity 95%, Specificity 100%.

Table 1: Baseline Characteristics

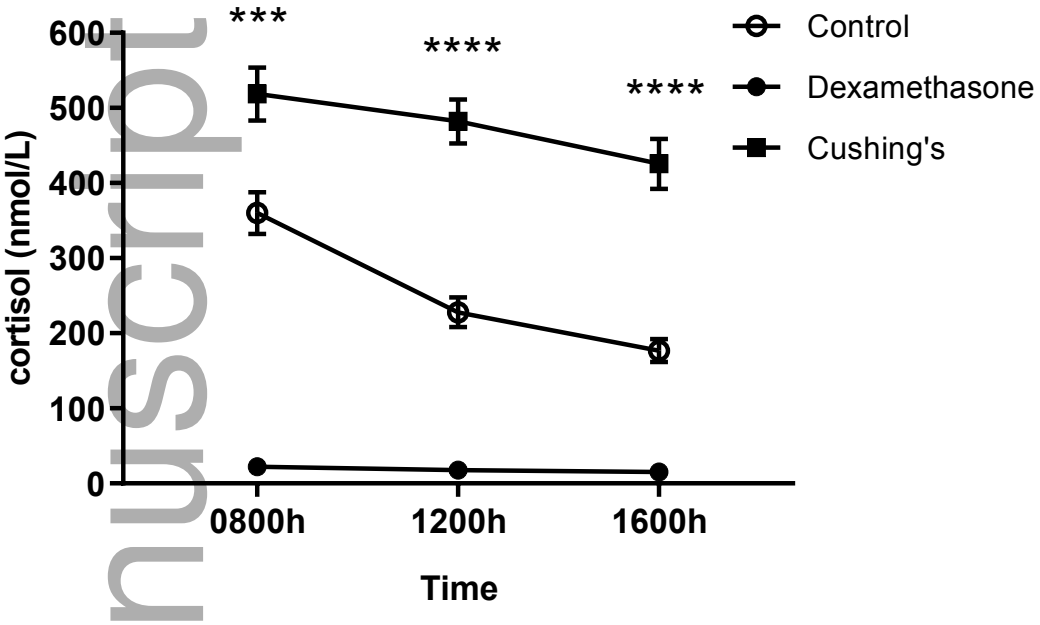
Characteristics	**Healthy volunteers (n = 20)**	**Adrenal insufficiency (n = 16)**	**Cushing's patients (n = 19)**	**Patients on chronic prednisolone (n = 13)**
Median (IQR) age, *y*	26 (23-30)	53 (39-65)	46 (41-55)	66 (58-72)
Female, *n (%)*	10 (50)	11 (69)	17 (89)	4 (31)
Median (IQR) prednisolone dose, *mg*				14 (10-20)
Median (IQR) duration of prednisolone treatment, *months*				24 (5-60)
Median (IQR) hydrocortisone dose, *mg*		20 (18-22)		
Median (IQR) vital signs				
Systolic BP, *mmHg*	122 (114-136)	119 (114-128)	141 (132-152)	132 (125-142)
Diastolic BP, *mmHg*	79 (71-82)	78 (71-82)	85 (81-97)	81 (76-86)
Body mass index, kg/m^2	23·8 (22·7-25·2)	26·1 (24·0-31·9)	32·2 (28·6-36·8)	30·7 (29·9-35·0)
Clinical variables				
Diabetes mellitus *n (%)*	0 (0)	3 (15)	7 (38)	3 (23)
Hypertension *n (%)*	0 (0)	3 (15)	14 (74)	9 (69)
Osteoporosis *n (%)*	0 (0)	1 (5)	3 (16)	6 (46)

Table 2

Median (IQR) plasma thrombospondin-1 (TSP1), serum osteocalcin (OCN) and TSP1:OCN ratio in healthy volunteers (HV), and patients with Cushing's syndrome (CS), adrenal insufficiency on physiological glucocorticoid replacement (AI) and patients on supra-physiological doses of prednisolone (PRED).

Condition	Healthy Volunteers (n=20)	Cushing's syndrome (n=19)	P- Value HV vs. CS	Adrenal insufficiency (n=16)	P- Value HV vs AI	Prednisolone (n=13)	P- Value HV vs PRED
Plasma TSP1 (ng/mL) 0800h	271 (237-336)	467 (399-630)	0.0002	374 (326-402)	ns	274 (249-388)	ns
Plasma TSP1 (ng/mL) 1200h	278 (193-357)	507 (386-576)	< 0.0001	356 (318-398)	0.02	324 (254-424)	ns
Plasma TSP1 (ng/mL) 1600h	294 (219-376)	510 (383-673)	< 0.0001	378·(340-402)	ns	N/A	
Serum OCN (ng/mL) 0800h	11·0 (7·5-13·7)	4·1 (3·3-5·9)	< 0.001	13·4 (9·8-15·9)	ns	5·9 (3·5-7·4)	< 0.001
Serum OCN (ng/mL) 1200h	10·2 (7·9-14·0)	6·5 (3·6-8·3)	0.0009	10·4 (7·7-14·5)	ns	5·9 (3·2-10·1)	< 0.01
Serum OCN (ng/mL) 1600h	11·4 (8·4-14·3)	6·5 (3·6-8·3)	<0.0001	8·9 (6·5-12·1)	ns	N/A	
TSP1:OCN ratio 0800h	24·0 (17·8-46·0)	91·0 (83·2-157·3)	< 0.0001	28·2 (23·2-38·8)	ns	59·2 (38·8-91·9)	< 0.001
TSP1:OCN ratio 1200h	25·5 (18·7-47·1)	87·7 (64·8-131·7)	< 0.0001	32·9 (26·9-48·2)	ns	49·3 (36·4-134·6)	< 0.001
TSP1:OCN ratio 1600h	25·4 (20·5-34·5)	102·1 (71·9-160·5)	<0.0001	43·5 (29·4-58·3)	0.027	N/A	

Figure 1:



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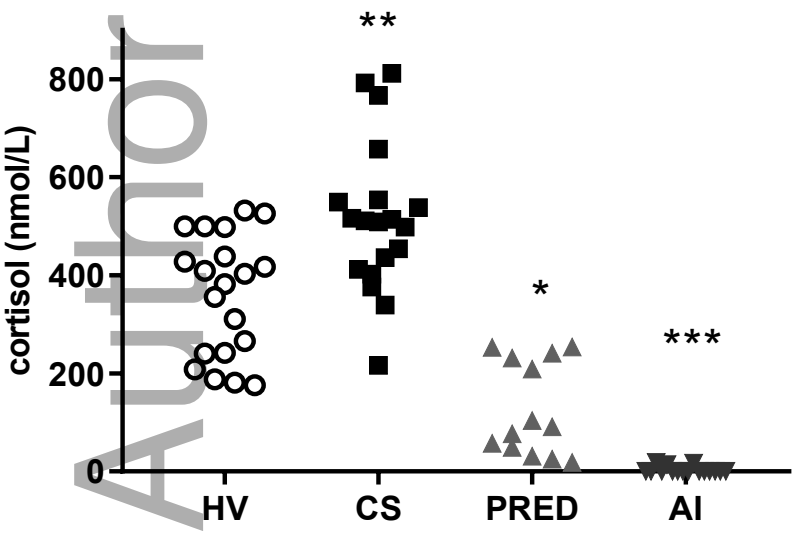


Figure 3

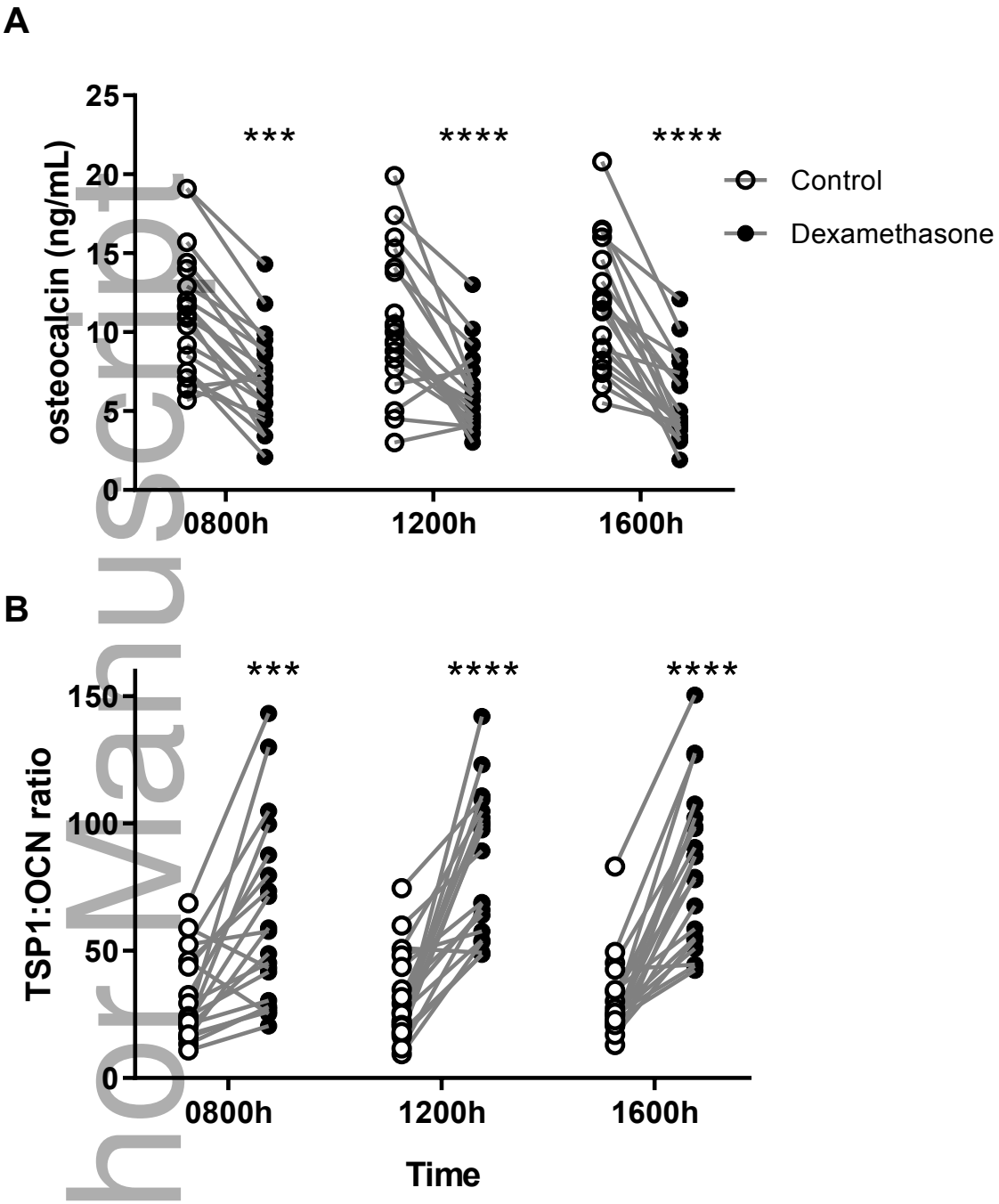


Figure 4

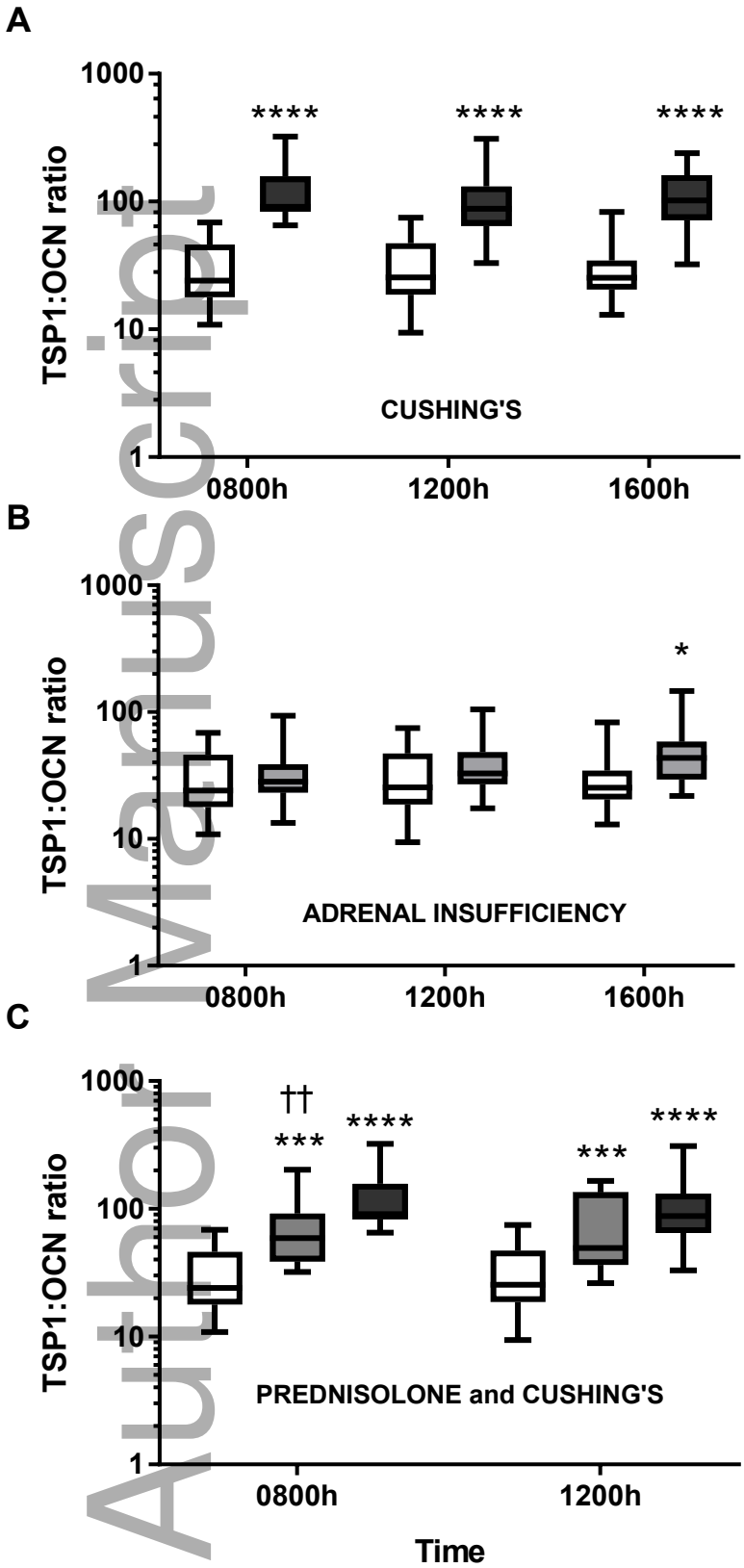
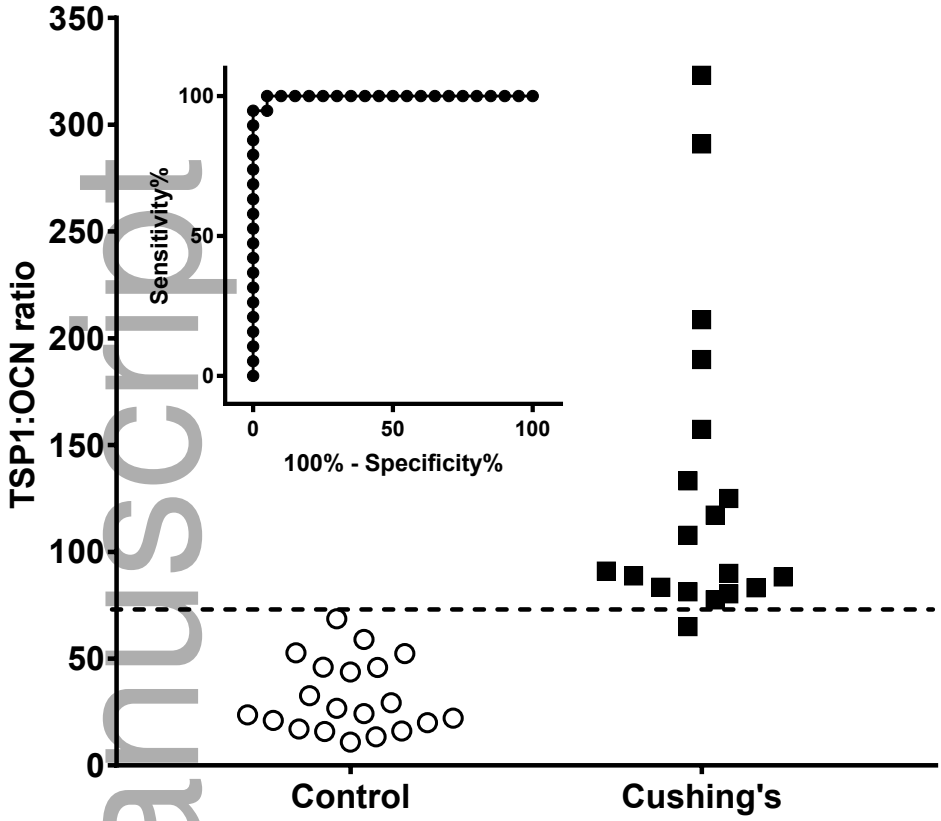


Figure 5



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