The diagnostic performance of urinary free cortisol is better than the cortisol:cortisone ratio in detecting *de novo* Cushing's syndrome: the use of a LC–MS/MS method in routine clinical practice

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Abstract

Objective: The Endocrine Society Clinical Guidelines recommend measuring 24-h urinary free cortisol (UFF) levels using a highly accurate method as one of the first-line screening tests for the diagnosis of Cushing's Syndrome (CS). We evaluated the performance of UFF, urinary free cortisone (UFE), and the UFF:UFE ratio, measured using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method.

Subjects and methods: The LC–MS/MS was used to analyze UFF and UFE levels in 43 surgically confirmed CS patients: 26 with Cushing's disease (CD, 16 *de novo* and ten recurrences), 11 with adrenal CS and six with ectopic CS; 22 CD patients in remission; 14 eu-cortisolemic CD patients receiving medical therapy; 60 non-CS patients; and 70 healthy controls. Sensitivity and specificity were determined in the combined groups of non-CS patients, healthy controls, and CD in remission.

Results: UFF > 170 nmol/24 h showed 98.7% specificity and 100% sensitivity for *de novo* CS, while sensitivity was 80% for recurrent CD patients, who were characterized by lower UFF levels. The UFF:UFE and UFF + UFE showed lower sensitivity and specificity than UFF. Ectopic CS patients had the highest UFF and UFF:UFE levels, which were normal in the CD remission patients and in those receiving medical therapy.

Conclusions: Our data suggest high diagnostic performance of UFF excretion measured using LC–MS/MS, in detecting *de novo* CS. UFF:UFE and UFF + UFE assessments are not useful in the first step of CS diagnosis, although high levels were found to be indicative of ectopic CS.

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Introduction

Cushing's syndrome (CS) is a rare disease with patients often presenting with a less than clear clinical picture and at times only isolated signs, although many of its typical symptoms are common in the general population (1, 2).

When efficient diagnostic and screening strategies are used in specific populations (diabetes mellitus and osteoporosis), a significantly higher number of CS diagnoses are made (3, 4).

The Endocrine Society Clinical Practice Guidelines (1) recommends initially testing for CS using one test with high diagnostic accuracy: 24-h urinary free cortisol (UFF) excretion, late night salivary cortisol (LNSC), or 1 mg dexamethasone suppression test (DST). It has been seen that many of the commercially available antibody-based immunoassays for measurement of UFF levels can give falsely high cortisol values due to cross-reactivity with its metabolites (5). While liquid chromatography–tandem mass spectrometry (LC–MS/MS) is considered an accurate method to determine UFF levels (1), it is not widely used in clinical practice (5, 6, 7).

The balance between cortisol and cortisone (E) levels is predominantly regulated by 11- β -hydroxysteroid dehydrogenase (11- β -HSD) types 1 and 2 (5, 8). In the event of excessive cortisol secretion, as occurs in CS, 11- β -HSD2 can be saturated, causing an increase in the UFF:UFE, which has been described in ectopic CS (9); the UFF+UFE sum is not, instead, influenced by 11- β -HSD 2.

The aim of this study was to determine the accuracy of UFF levels, measured using LC–MS/MS in distinguishing CS from non-CS, and to examine the additional diagnostic value of the UFF:UFE ratio for making a differential diagnosis of CS.

Subjects and methods

Patients

Two hundred and nine patients referred to the Endocrinology Unit of the University of Padova Medical Center between December 2011 and December 2013 were assessed. These included:

- i) Forty-three patients with CS (age at diagnosis 48 ± 18 years). CS suspicion was based on the lack of cortisol suppression <50 nmol/l after 1 mg DST and LNSC levels >5.24 ng/ml (10) in at least two samples. All the subjects had final histological confirmation of CS: 26 had Cushing's disease (CD, 16 *de novo* and ten recurrences after a minimum of 12 months of remission), 11 with adrenal CS (six adenoma and one carcinoma), and six with ectopic CS (two pancreatic and four bronchial neuroendocrine tumors with positive adrenocorticotrophic hormone (ACTH) immunostaining). The following parameters were assessed in each of these patients: body weight (kg), waist circumference (normal: <94 cm in males and <80 cm in females), BMI (kg/m^2), potassium levels (normal range 3.4–4.5 mEq/l), blood pressure (hypertension was defined as systolic blood pressure ≥ 130 mmHg and/or diastolic ≥ 85 mmHg or on antihypertensive treatment), lipid profile (dyslipidemia was diagnosed when plasma triglycerides ≥ 1.7 mmol/l or HDL <1 mmol/l in males and <1.3 mmol/l in females, or in patients receiving treatment), diabetic mellitus (fasting glucose ≥ 5.5 mmol/l or patients being treated for diabetes). Patients with three or more of the following parameters were considered as being affected with the metabolic syndrome: elevated waist circumference, increased triglycerides, reduced HDL levels, hypertension, and diabetic mellitus (11).
- ii) Twenty-two patients (age 50 ± 15 years) in remission from CD (median follow-up 52 months, range 14–96). Patients were considered in remission after a minimum of 6 months following pituitary surgery and when at least two of the following criteria were met: i) need for postoperative corticosteroid replacement therapy; ii) low serum cortisol levels early after surgery (<50 nmol/l, low/normal morning serum cortisol level for at least 6 months after pituitary surgery; and iii) significant changes in clinical features (12). None of the patients received glucocorticoid substitutive therapy for at least 1 month before urine collection; all had normal LNSC and cortisol levels <50 nmol/l after 1 mg DST (both were assessed every 12 months during the follow-up).
- iii) Fourteen CD patients (age 52 ± 14 years) receiving medical therapy and showing normal LNSC levels and cortisol suppression <50 nmol/l after 1 mg-DST: eight patients were being treated with ketoconazole (two with 200 mg/daily, four with 400 mg/daily, and two with 600 mg/daily, duration of therapy 16–54 months), six patients were receiving both ketoconazole (400–600 mg/daily) as well as cabergoline (3.5 mg/weekly, duration of therapy 16–54 months), five were receiving pasireotide (600 μg b.i.d., for 10 months) and one temozolamide (400 mg daily for 5 days every 28 days, the patient underwent 18 cycles of therapy).
- iv) Sixty subjects with medical conditions suggestive of hypercortisolism (age 45 ± 16 years), cortisol <50 nmol/l after 1 mg DST, and normal LNSC (at baseline and after 12 months).
- v) Seventy healthy subjects (age 47 ± 21 years) recruited from the staff of the Endocrinology Unit and the Laboratory Medicine Department of the University of Padova Medical Centre. All underwent a comprehensive clinical examination and detailed medical

histories were taken. None showed signs or symptoms of hypercortisolism, none had a history of adrenal incidentaloma or severe and/or chronic illness (in particular of an endocrine origin). None were taking exogenous glucocorticoids or drugs that could interfere with the hypothalamic–pituitary–adrenal axis.

The study was carried out in accordance with the guidelines in the Declaration of Helsinki, the Local Ethics Committee approved the protocol, and all patients/subjects gave informed consent.

Urinary glucocorticoid analysis

For 24-h urine collections, the patients were instructed to discard the first morning urine void and to collect all urine for the next 24 h, so that the morning urine void on the second day was the final collection. The sample was kept refrigerated from collection time until it was analyzed: a 10 ml aliquot sample was taken and centrifuged at 3000 *g* for 10 min at room temperature. Then 2 μ l formic acid and 50 μ l deuterated internal standard solution (d_4 -cortisol and d_7 -cortisone) were added to 500 μ l of urine supernatant or calibrators. The solution was vortexed for 30 s and centrifuged at 16 000 *g* for 5 min at room temperature. This was followed by 20 μ l supernatant added to 200 μ l 0.1% formic acid water solution and placed in the autosampler of the LC–MS/MS. UFF and UFE levels were measured, as is routine in our center, using an Agilent HPLC series 1200 triple quadrupole mass spectrometer, Agilent 6430, equipped with an electrospray ionization source in a positive ionization mode (Agilent Technologies, Palo Alto, CA, USA). The on-line cleanup/enrichment was carried out using a cartridge Zorbax Extend-C18 2.1 $\times$ 12.5 mm, 5 μ m particle size, and the HPLC separation by a 4.6 $\times$ 50 mm, 1.8 μ m particle size, analytical column Zorbax Eclipse XDB-C18 (Agilent Technologies). Quantitative analysis was carried out in the multiple reaction monitoring mode. The method was linear up to 625 and 1125 nmol/l, with a lower quantification limit of 5 and 6 nmol/l for UFF and UFE respectively. Within-run and between-run coefficients of variation (CV) were <5 and 6% for UFF and 6 and 8% for UFE respectively. The mean recoveries were 106% for UFF and 104% for UFE. The intra-individual variability of UFF was determined by calculating the CV ($CV = s.d./mean \times 100$).

All the patients provided two to five complete 24-h urine collections, the average values from all of the

collections were used in the final analysis. The healthy controls provided one sample.

The reference range used in our department, determined using the LC–MS/MS method described above, is for UFF 16–170 nmol/24 h, UFE 41–364 nmol/24 h, and UFF:UFE 0.14–1.09 (13); these values are comparable with others obtained using LC–MS/MS methods (5, 6, 14).

Data and statistical analysis

UFF, UFE, UFF:UFE, and UFF+UFE excretion in the different CS groups was compared. The *de novo* CS group was defined as: *de novo* CD+adrenal CS+ectopic CS. The rate of false-positive test results in the combined groups of non-CS patients, healthy controls, and CD remission patients (considering the last as a group in which recurrence was possible) was determined. Continuous data are shown as mean and s.d. or median and range. We compared the groups using the Mann–Whitney *U*-test for non-parametrical data; categorical variables were compared using the χ^2 test or Fisher's exact test when the cell count was <5. Sensitivity and specificity were calculated at different cut-off levels to carry out receiver operating curve (ROC) analyses. Linear regression was used to examine the relationship between UFF or UFF:UFE and age. Statistical analysis was performed using the SPSS 17 Software package (SPSS, Inc.). The significance level was set at *P* value <0.05 for all the tests.

Results

UFF excretion

UFF levels were higher in the CS patients with respect to all the other groups, and the highest values were found in the ectopic CS patients and in the single patient with adrenal carcinoma (Table 1 and Fig. 1). The CV ranged between 4 and 114% (median 39%).

Sensitivity and specificity of UFF >170 nmol/24 h were respectively 95.3 and 98.7% for the CS patients (Table 2), with positive and negative predictive values of 95.3 and 98.5%. The best calculated cut-off value according to ROC-curve analysis was 197 nmol/24 h (sensitivity 95.3% and specificity 100%). When only the 33 *de novo* CS patients were considered, the sensitivity and specificity of UFF >170 nmol/24 h were 100 and 98.7% respectively with positive and negative predictive values 94.3 and 100% respectively; according to ROC analyses, UFF >197 nmol/24 h showed 100% sensitivity and specificity for *de novo* CS. Two of the ten recurrent CD

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Table 1 UFF, UFE, and UFF:UFE median levels (range) in nmol/24 h in selected groups of patients.

Group		M/F	UFF	UFE	UFF:UFE
CS (n=43)	CD (n=26)	9/17	398 (27–3344) ^{a,b,c,d}	448 (58–1606) ^{a,b,c,d}	1.05 (0.45–2.26) ^{a,b,c,d}
	CD <i>de novo</i> (n=16)	7/9	548 (281–3344) ^{a,b,c,d,e}	576 (286–1606) ^{a,b,c,d,e}	1.23 (0.47–2.26) ^{a,b,c,d}
	CD recurrence (n=10)	2/8	289 (27–948) ^{a,b,c,d}	306 (58–659) ^{a,b,c,d}	1.00 (0.45–1.91) ^{a,b,c,d}
	Adrenal CS (n=11)	1/10	808 (208–10360) ^{a,b}	693 (192–1325) ^{a,b}	1.80 (0.70–12.95) ^{a,b}
	Ectopic CS (n=6)	1/5	5992 (421–29120) ^{a,b,f}	884 (365–1840) ^{a,b,f}	4.92 (1.15–26.47) ^{a,b,f}
CS <i>de novo</i> (n=33)	CD <i>de novo</i> (n=16)	9/24	808 (208–29120) ^{a,b}	619 (192–1840) ^{a,b}	1.52 (0.47–26.47) ^{a,b}
	Adrenal CS (n=11)				
	Ectopic CS (n=6)				
	CD on medical therapy (n=14)	4/10	64 (27–168)	148 (13–464)	0.33 (0.20–0.62)
Controls (n=152)	CD in remission (n=22)	5/17	58 (15–151)	151 (19–375)	0.41 (0.28–0.86)
	Non-CS (n=60)	12/48	51 (10–180)	131 (26–426)	0.41 (0.16–1.66)
	Healthy controls (n=70)	20/50	58 (16–186)	155 (36–463)	0.38 (0.14–1.19)

M, male; F, female; UFF, urinary free cortisol; UFE, urinary free cortisone.

^a $P < 0.001$ vs healthy subjects.

^b $P < 0.001$ vs non-CS.

^c $P < 0.001$ vs CD in medical therapy.

^d $P < 0.001$ vs CD in remission.

^e $P < 0.05$ vs CD recurrence.

^f $P < 0.001$ vs CD.

patients had normal UFF levels, resulting in an 80% sensitivity. There were no false-negative UFF results in the patients with adrenal or ectopic CS.

Those CS patients with UFF < 1.5 times the upper normal limit (< 255 nmol/l) were considered as being affected with mild hypercortisolism. None of the ectopic CS patients did, but one out of 11 of the adrenal CS (9%) and four out of 26 (15%) of the CD patients had a mild form and all were with recurrent CD. While UFF levels were higher in the *de novo* than in the recurrent CD patients ($P < 0.05$, Table 1), the frequency of mild hypercorticism was higher in the latter (four out of ten, 40%) with respect to the former (0 out of 16, $P < 0.05$).

UFF:UFE ratio

The diagnostic performance of UFE was worse than that of UFF (Table 2); the ROC-curve based cut-off (281 nmol/24 h) showed a higher accuracy in the detection of CS with 86% sensitivity and 93.4% specificity. Seven out of ten of the recurrent CD patients had a normal UFE excretion, while all of the ectopic CS patients had increased levels.

The optimal cut-off for UFF:UFE according to ROC-curve analysis was 0.73 (sensitivity 86% and specificity 92.8%). When a threshold of 1.09 (upper reference limit (15)) was used, the UFF:UFE ratio showed a lower degree

Figure 1

Scatter plot of UFF (a), UFE (b), and UFF:UFE (c) in the different populations: Cushing's syndrome (n=43), CD receiving medical therapy (n=14), CD in remission (n=22), non-CS (n=60), and healthy controls (n=70). Full black line indicates ROC-calculated

threshold values in the *de novo* CS and non-CS patients. Open circle, *de novo* CD (n=16); open triangle, recurrent CD (n=10); open square, adrenal-CS (n=11); and cross, ectopic CS (n=6); filled circle, control groups described above.

Table 2 Sensitivity (SE, %), specificity (SP, %), and AUC for UFF, UFE, and UFF:UFE.

	CS			de novo CS		
	AUC	SE	SP	AUC	SE	SP
UFF > 170 nmol/24 h	0.976	95.3	98.7	1.00	100.0	98.7
UFE > 364 nmol/24 h	0.941	69.8	96.7	0.979	81.8	96.7
UFF:UFE > 1.09	0.940	58.1	96.7	0.953	66.7	96.7

AUC, area under the curve.

of accuracy in the detection of CS (Table 2). Ectopic CS patients had the highest UFF:UFE values; the ROC generated threshold of UFF:UFE > 1.51 showed 83.3% sensitivity and 73.1% specificity (AUC 0.885) in distinguishing ectopic from pituitary ACTH secretion. The UFF:UFE ratio was normal in the two recurrent CD patients who had normal UFF and in all of the eu-cortisolemic CD patients (Table 1). The diagnostic accuracy of the UFF + UFE sum was similar to that of the UFF:UFE ratio. It was higher in the CS with respect to the non-CS patients and the healthy controls, it was higher in the ectopic CS with respect to the CD patients, and it showed higher sensitivity (93%) and specificity (94.7%) in detection of CS (UFF + UFE > 432 nmol/24 h, AUC 0.971). It had only a slightly lower diagnostic accuracy in distinguishing between ectopic CS and CD (sensitivity 83.3% and specificity 69.2% if UFF + UFE > 1264 nmol/24 h, AUC 0.833).

The patients with an UFF:UFE > 1.09 showed higher UFF levels than did those with the ratio < 1.09 (for CS median UFF 1115 vs 308 nmol/24 h, $P < 0.001$ and for CD median 630 vs 305 nmol/24 h, $P < 0.01$); there were no differences in these groups with regard to body weight, BMI, prevalence of diabetes, hypertension, dyslipidemia, or metabolic syndrome. CS patients with UFF:UFE > 1.09 had lower waist circumference (median 94 vs 108 cm, $P < 0.01$) and lower potassium levels (median 3.7 vs 3.9 mEq/l, $P < 0.05$) as well as a higher rate of hypokalemia. Seven, in fact, out of 25 (one CD, one adrenal, and five ectopic CS) patients with UFF:UFE > 1.09 had serum potassium levels < 3.4 mEq/l (median 2.4, range 1.8–2.8 mEq/l) and a median UFF:UFE of 5.21 (range 1.15–26.47), while none of the patients with UFF:UFE ratio < 1.09 had hypokalemia ($P < 0.05$). UFF:UFE levels were normal (in seven out of ten) or only slightly elevated in the recurrent CD patients.

Sex and age difference

No gender differences linked with UFF or UFF:UFE values were found in any of the groups. Considering those

patients with ectopic CS or adrenal carcinoma as malignant CS, UFF and UFF:UFE levels decreased with age in the non-malignant CS (UFF $y = -32.958x + 2347.5$, $r^2 = 0.2373$, $P < 0.01$ and UFF:UFE $y = -0.0175x + 2.1395$, $r^2 = 0.1341$, $P < 0.05$). In the *de novo* non-malignant CS patients (to avoid bias linked with lower UFF levels in recurrent CD) UFF and UFF:UFE levels decreased with age: UFF $y = -44.059x + 3111.2$, $r^2 = 0.3672$, $P < 0.01$ and UFF:UFE $y = -0.0247x + 2.6218$, $r^2 = 0.2423$, $P < 0.05$; (Fig. 2).

UFF levels decreased with age ($y = -1.2243x + 135.38$, $r^2 = 0.2071$, $P < 0.05$) to a greater degree in the CD remission patients with respect to the healthy subjects ($r^2 = 0.108$, $P < 0.01$); no age-related variation was found in UFF or in the UFF:UFE ratio in the CD patients receiving medical therapy or in the non-CS subjects.

Discussion

When evaluating patients for the diagnosis of CS, the Endocrine Society recommends determination of UFF levels

Figure 2

Logistic regression in age-related decreasing UFF levels and UFF:UFE ratio in the non-malignant *de novo* Cushing's syndrome patients. Open circles represent patient's measurements.

using highly accurate methods instead of commercially available antibody-based immunoassays (1, 5, 15). In this study, UFF levels, routinely determined in our centre using the LC–MS/MS method, were compared among healthy subjects, CS patients, and non-CS patients presenting clinical features suggestive of hypercortisolism but with normal LNSC and cortisol <50 nmol/l after 1 mg DST. The diagnostic value of the UFF:UFE ratio was also assessed (5, 9, 16).

This is the first work, to our knowledge, assessing healthy controls and a series of consecutive surgically confirmed CS patients as well as patients in whom the diagnosis of CS was excluded, with the intent of analyzing the sensitivity and specificity of UFF values measured using the LC–MS/MS method.

UFF values obtained using the LC–MS/MS method did not produce any false-negative test results in the *de novo* CS patients, without mild hypercortisolism: the reference ranges for UFF used in a laboratory are of utmost importance in view of their crucial role in cases of suspected hypercortisolism. In accordance with (17) report, we found, moreover, a high inpatient variability in two or more samples collected for evaluation of UFF levels.

In our center, a patient is considered as having recurrent CD when, after a stable clinical/biochemical remission, he/she shows signs of worsening health status and hypercortisolism confirmed by elevated LNSC levels and lack of cortisol suppression to 1 mg DST (we routinely used the most sensitive cut-off even in this case). Confirming previously described data regarding LNSC (10, 18, 19), higher UFF excretion was found in the *de novo* with respect to the recurrent CD patients, and a higher rate of mild hypercortisolism was found in the latter. We also found normal UFF excretion in two out of ten recurrent CD patients. One study in the literature, in fact, reported that elevated UFF levels appear later with respect to other tests in recurrent CD patients (20). During the routine follow-up of these patients, early diagnosis of recurrence (based on pathological results of two other screening tests) can be made even in the event that UFF levels are normal or only slightly elevated, due to lower sensitivity and specificity of UFF in this particular clinical setting. It is, nevertheless, important to recognize recurrence at an early stage when hypercortisolism is still mild. Endocrinologists should thus carry out at least two screening tests rather than two urine collections during the post-operative follow-up of patients who have surgical remission of CD.

UFF:UFE and UFF+UFE have no additional value with respect to UFF in cases of recurrent CD. In accordance with (16) data, we found no differences in the UFF:UFE

levels in the healthy controls and CD patients receiving medical therapy.

Just as was reported with regard to LNSC levels, ectopic CS patients had higher UFF, UFF+UFE sum, and UFF:UFE ratios than did the CD patients (10). The UFF:UFE ratio has been reported to be a marker of this type of CS (8) and a higher UFF:UFE ratio was indeed found in the ectopic CS patients studied here. Further studies are needed to establish whether the ratio can be considered as a biomarker for ectopic ACTH, which is of occult origin in 20% of ACTH-dependent cases (21).

Higher UFF:UFE ratios in CS patients suggest that there is 11- β -HSD2 saturation, which is typical of CS (5, 8, 16). If we divide our CS into two groups whose UFF:UFE ratios are higher or lower with respect to the local upper reference limit of 1.09, the group with the higher ratio showed elevated UFF levels. It is probable that only high cortisol levels are able to saturate 11- β -HSD2 activity leading to an excess of cortisol production that could explain the typical co-morbidities in CS. In addition, the patients with UFF:UFE ratio >1.09 had low potassium levels and a high rate of hypokalemia, confirming that excess cortisol saturates 11- β -HSD2 and leads to cortisol-related mineralocorticoid activity.

We observed a linear regression in age-related decreasing levels of UFF and UFF:UFE in the non-malignant *de novo* CS, a finding which needs to be examined by future studies assessing large CS populations (Fig. 2).

This study has some limitations. Although ours is a third-level referral centre for CS, the number of patients studied was relatively low. Stringent inclusion criteria (i.e. the histological confirmation of CS or both normal LNSC and the lower cut-off after 1 mg DST to define non-CS) were, nevertheless, used to enable us to accurately analyze the sensitivity and specificity of UFF. Studies using larger numbers of patients with discordant test results are warranted to verify the diagnostic accuracy of UFF measured using the LC–MS/MS method and to establish the UFF:UFE ratio as a biomarker of ectopic CS.

In conclusion, in view of its accuracy, UFF levels measured using the LC–MS/MS method can be considered a useful element during the routine clinical assessment of patients with suspected hypercortisolism. Measurement of UFF levels was found to be reliable in detection of *de novo* CS. Its sensitivity in diagnosing recurrent CD was instead limited, probably due to higher levels of mild hypercortisolism rather than to methodological issues. The UFF:UFE ratio does not appear to be useful in the first step of CS diagnosis, but it may be important in forming the differential diagnosis of ACTH-dependent hypercortisolism.

Declaration of interest

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

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