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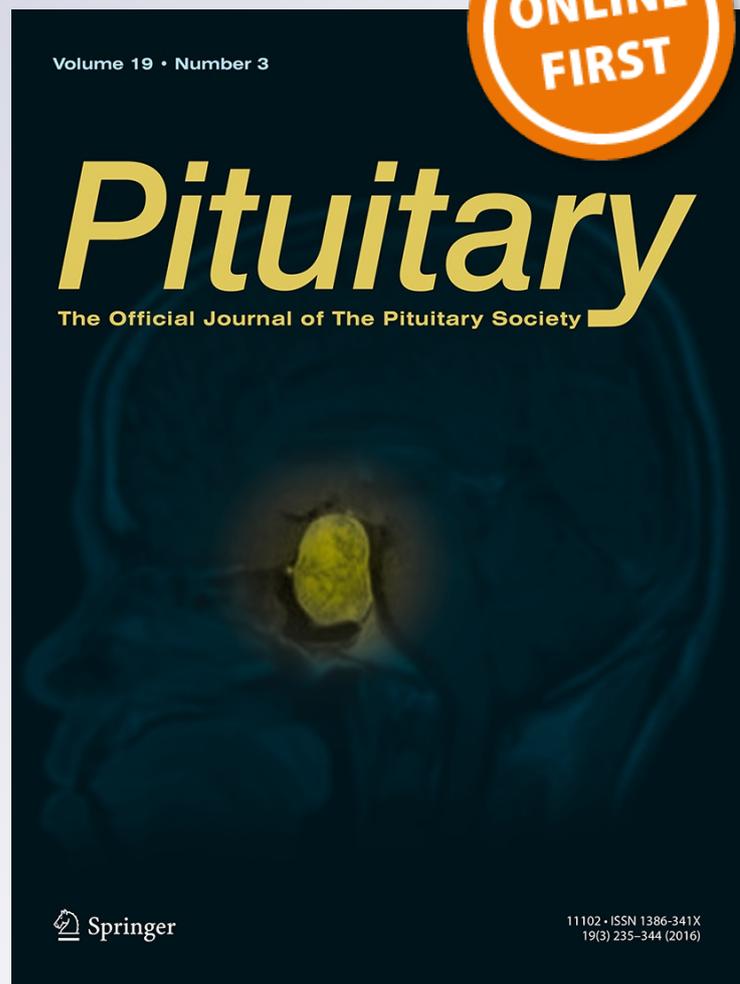B. Morales-Romero, W. Jiménez,
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Volume 19 · Number 3

Pituitary
The Official Journal of The Pituitary Society

ONLINE FIRST

Springer

11102 · ISSN 1386-341X
19(3) 235–344 (2016)

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Accuracy of immunoassay and mass spectrometry urinary free cortisol in the diagnosis of Cushing's syndrome

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Abstract

Purpose Urinary free cortisol (UFC) determination by highly specific methods as mass spectrometry instead of commercially available antibody-based immunoassays is increasingly recommended. However, clinical comparisons of both analytical approaches in the screening of Cushing's syndrome (CS) are not available. The aim of this study was to evaluate the diagnostic value of mass spectrometry versus immunoassay measurements of 24 h-UFC in the screening of CS.

Methods Cross-sectional study of 33 histologically confirmed CS patients: 25 Cushing's disease, 5 adrenal CS and 3 ectopic CS; 92 non-CS patients; and 35 healthy controls. UFC by immunoassay (UFCxIA) and mass spectrometry (UFCxMS), urinary free cortisone (UFCo) and UFC:UFCo ratio were measured, together with creatinine-corrected values. Sensitivity, specificity, AUC and Landis and Koch concordance index were determined.

Results AUC for UFCxIA and UFCxMS were 0.77 (CI 0.68–0.87) and 0.77 (CI 0.67–0.87) respectively, with a kappa coefficient 0.60 and strong Landis and Koch concordance index. The best calculated cutoff values were 359 nmol/24 h for UFCxIA (78 % sensitivity, 62 % specificity) and 258.1 nmol/24 h for UCFxMS (53 % sensitivity, 86 % specificity). The upper limit of UFCxIA and UCFxMS reference ranges were 344.7 and 169.5 nmol/24 h respectively. Sensitivity and specificity for CS diagnosis at these cutpoints were 84 and 56 % for UFCxIA and 81 and 54 % for UFCxMS.

Conclusions According to our data, both methods present a very similar diagnostic value. However, results suggest that lower cutoff points for mass spectrometry may be necessary in order to improve clinical sensitivity.

Keywords Cushing's syndrome · Urinary free cortisol · Mass spectrometry · Immunoassay

M. Careaga, F. A. Hanzu, I. Halperin and G. Casals have contributed equally to this work.

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Introduction

The chronic state of hypercortisolism is associated with significant morbidity that severely impairs quality of life [1, 2], so that early detection of mild cases should be attempted. The diagnostic workup of a patient with a possible Cushing's syndrome (CS) can be challenging for several reasons. First, the spectrum of clinical presentation of CS is broad, and mild cases may be difficult to detect on the basis of clinical symptoms. Second, all screening tests of endogenous hypercortisolism have limitations related with their sensitivity and/or specificity, and results may be modified by several confounding factors. After exclusion

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of exogenous glucocorticoid exposure, three first line screening tests recommended for the diagnosis of CS interrogate different aspects of the pathophysiology of this challenging disease. These are: (1) failure to achieve the nadir of the cortisol circadian rhythm-evaluated by late night salivary cortisol; (2) loss of sensitivity to glucocorticoid negative feedback—tested by low dose dexamethasone suppression testing; and (3) increased production of cortisol—assessed by 24 h urinary free cortisol (UFC) measurement. All these tests have strengths and weaknesses [3–5].

Measurement of 24-h UFC excretion has the advantage of being unaffected by either short-term fluctuations in cortisol or by varying plasma protein binding capacities. 24-h UFC is thought to reflect overall daily cortisol production and is easy to collect in large study populations if compared to serial blood sampling. However, several factors such as the urine volume, renal function, sex, age, smoking, alcohol consumption, exercise frequency, stressfull events and body max index (BMI) can modify results [6]. The methodology for determining UFC is another source of variability. In fact, it has been reported that commercially available antibody-based immunoassays widely used for measurement of UFC levels are subject to considerable inaccuracy mainly due to cross-reactivity with cortisol metabolites [7, 8]. In contrast, mass spectrometry is considered the most specific method to determine true UFC since cross-reactivity with cortisol metabolites is nonexisting [3, 5]. In this regard, mass spectrometric measurements are increasingly recommended [3, 9]. However, there is a lack of direct comparative studies between mass spectrometry and immunoassay measurements evaluating the clinical impact of this methodological change.

The aim of the current study was to evaluate the diagnostic value of mass spectrometry vs immunoassay measurements of UFC in the screening of CS.

Subjects and methods

Patients

Cross-sectional study was performed in all patients with medical conditions suggestive of hypercortisolism, referred for diagnosis to the Endocrinology and Nutrition department of the Hospital Clínic of Barcelona between January 2011 and January 2015, that presented UFC > 276 nmol/24 h (>100 μg/24 h) measured by immunoassay. These included:

1. Thirty-three patients with CS with age at diagnosis 47.2 ± 14.5 years. The diagnosis of CS was based on Endocrine Society and European Society of Endocrinology (ESE) criteria [3]. All the subjects had final histological confirmation of CS: Twenty-five had Cushing's disease (CD), five adrenal CS and three ectopic CS.

2. Ninety-two non-CS patients with medical conditions suggestive of CS (age 50.3 ± 16.8 years) including adrenal incidentaloma, pituitary adenoma or men with osteoporosis.

3. Additionally, a third group of thirty-five healthy subjects (age 47.5 ± 14.7 years) was recruited from the Hospital Clinic staff. None showed signs or symptoms of hypercortisolism nor had a history of adrenal incidentaloma or severe and/or chronic illness. None were taking exogenous glucocorticoids or drugs that could interfere with the hypothalamic–pituitary–adrenal axis.

The diagnosis of endogenous CS was established according to the guidelines of both the ESE and the Endocrine Society, by repeated elevated levels of urinary free cortisol by immunoassay (our routine method), loss of circadian rhythm (elevated free night salivary cortisol) and the lack of suppression of cortisol secretion after dexamethasone. Localization of the cortisol secreting tumour was determined by ACTH level, cortisol suppression test with 8 mg dexamethasone, imaging tests and inferior petrosal sinus sampling (IPSS) when necessary [3].

The following parameters were specifically assessed in each of these three groups: body mass index (BMI), urinary free cortisol by immunoassay (UFCxIA), urinary free cortisol by mass spectrometry (UCFxMS), urinary free cortisone (UFCo), cortisol/cortisone ratio (UFC:UFCo) and creatinine-corrected UFCxIA, UFCxMS and UFCo.

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 gave informed written consent.

Laboratory measurements

Blood samples were collected at 8 a.m., after overnight fast. Main biochemical and hormonal parameters were measured in serum with standard methods in the Biochemistry and Molecular Genetics Department of our hospital as previously described [10]. Plasma ACTH levels were measured in duplicate using an immunoradiometric assay in accordance with the manufacturer's instructions (^{125}I ACTH IRMA, DiaSorin, Stillwater, MN, USA). The limit of detection was 1.5 pg/ml and within-assay and between-run precision were <6 %.

UFC immunoassay measurements were performed following the routine methods employed in the laboratory of our hospital using the standard chemiluminometric immunoassay (LIAISON, Diasorin, Italy) after a previous extraction of

urine with dichloromethane. The inter-assay coefficient of variation (CV) at 312 nmol/L (n = 20) was 7.4 %.

UCF and UFCo mass spectrometry measurements were performed by gas chromatography–mass spectrometry (GC–MS) as previously described [11]. Cortisol-d4 and cortisone-d7 (Sigma Aldrich) were used as internal standards. In brief, after sample extraction and derivatization, final extracts were injected on a Shimadzu GCMS QP2010 Ultra instrument. Steroids were separated in a Sapines-5MS capillary column (30 m × 0.25 mm internal diameter × 0.25 m film thickness) from Teknokroma with helium as a carrier gas. Mass detector was operated in selected ion monitoring (SIM) mode. A full validation of the assay including linearity, intra-assay and inter-assay imprecision and bias, was performed and confirmed the assay to be robust for routine clinical purposes. Calibration curves for UFC and UFCo were linear over the concentration range 3–5500 nmol/L. The inter-assay CV for UFC at 127 noml/L (n = 20) was 4.7 %. The inter-assay CV for UFCo at 28 nmol/L (n = 20) was 7.0 %. The lowest limit of quantification, defined as the lowest concentration measurable in urine with CV 20 %, was 5 nmol/L. The recoveries of added cortisol and cortisone in urine ranged from 99–104 and 96–103 %, respectively. In addition, the accuracy of cortisol measurements was 97–102 % when evaluated with the human serum European Reference Material ERM-DA 192.

Statistical analysis

Continuous data are shown as mean and SD or median and range. Comparisons between groups were performed using *T* test or one-way ANOVA followed by Bonferroni when assuming equal variances or Games-Howell when assuming different variances. Categorical variables were compared using chi- square test or Fisher's exact test when the cell count was <5. Upper limit of the reference ranges were obtained as mean ± 2DS. Sensitivity and specificity were calculated at different cut-off levels to carry out receiver operating curve (ROC) analyses. Kappa coefficient and Landis and Koch concordance index between both measurements techniques were determined. Correlations between metabolic parameters and hormonal parameters across the study population, were assessed using the Person method. Statistical analysis was performed using the SPSS 20 software package. The significance level was set at *p* value <0.05 for all the tests.

Results

The clinical characteristics of healthy controls, non-CS patients and CS patients are presented in Table 1. There were no differences in age, BMI and gender among the three groups, while both patients with confirmed CS and those with suspected CS presented more comorbidities like type 2 diabetes (T2D), hypertension and obesity than healthy controls. Patients with CS had higher levels of UFCxIA, UFCxMS, UFCo and UFC:UFCo than non-CS patients.

Table 2 presents the upper limits of the reference ranges obtained in the group of healthy individuals. At these cutoffs, sensitivity and specificity for the diagnosis of CS were calculated for UFCxIA, UFCxMS, UFCo and their creatinine corrected values. It is noteworthy that the upper limit of the reference range for UFCxIA (344.7 nmol/24 h) was almost twice than that for UFCxMS (169.5 nmol/24 h).

ROC curves were created to establish the optimal threshold values for each test and its diagnostic efficiency for CS (Fig. 1). The AUC for UFCxIA and UFCxMS was respectively 0.77 (CI 0.68–0.87) and 0.77 (CI 0.67–0.87), with a kappa coefficient of 0.60 and strong Landis and Koch concordance index (Fig. 1; Table 2). The best calculated cutoff value for UFCxIA according to ROC-curve analysis was 359 nmol/24 h (78 % sensitivity, 62 % specificity) whereas the best calculated cutoff value for UCFxMS according to ROC-curve analysis was 258 nmol/24 h (53 % sensitivity, 86 % specificity).

UFC creatinine corrected values consistently presented a higher AUC in comparison with 24-h values (Fig. 1). In contrast, UFCo and UFC/UFCo ratio presented lower AUC than UFC (Fig. 1). UFC and UFCo levels were higher in CS patients in comparison with the non-CS group. Within the CS group, the highest values of UFC, UFCo and UFC/UFCo ratio were found in the ectopic CS patients (Fig. 2).

Correlations

Both urinary free cortisol measured by immunoassay and by mass spectrometry were correlated with ACTH, glucose and HDL-c (UFCxIA = ACTH r: 0.390 *p* < 0.001; Glucose r: 0.282 *p* = 0.002; HDL-c r: 0.301 *p* = 0.009) (UFCxMS = ACTH r: 0.384 *p* < 0.001; Glucose r: 0.248 *p* = 0.007; HDL-c r: 0.313 *p* = 0.006). The ratio cortisol: cortisone was correlated with glucose r: 0.198 *p* = 0.040, HDL-c r: 0.266 *p* = 0.029 and total cholesterol r: 0.245 *p* = 0.026. UFCo was correlated with ACTH r: 0.462 *p* < 0.001, Glucose r: 0.221 *p* = 0.018, HDL-c r: 286 *p* = 0.013, total cholesterol r: 0.185 *p* = 0.049.

Discussion

Determination of UFC levels using highly specific analytical methods as mass spectrometry instead of commercially available antibody-based immunoassays is increasingly

Table 1 Baseline characteristics of the study population

Parameters	CS (n = 33)	Non-CS (n = 92)	Healthy controls (n = 35)
Age (years)	47.2 ± 14.5	50.3 ± 16.8	47.5 ± 14.7
Sex (M/F)	14/19	44/48	17/18
T2D (%)	7 (4.5)	10 (6.4)	0 (0)
HTA (%)	21 (13.4)	37 (23.6)	0 (0)
OB (%)	10 (6.4)	20 (15)	1 (0.8)
DLP (%)	10 (6.4)	23 (14.9)	1 (0.8)
BMI (kg/m^2)	27.9 ± 5.6	28.6 ± 6.8	23.9 ± 5.4
UFCxIA (nmol/24 h)	1734.9 ± 4140*	384.2 ± 207.5	189.6 ± 86.9
UFCxMS (nmol/24 h)	918.8 ± 2350.6*	167.5 ± 91.6	94.6 ± 38.9
UFCo (nmol/24 h)	553.4 ± 630.5*	270.2 ± 159.0	134.8 ± 66.4
UFCxIA (nmol/g creat)	1406.4 ± 2941.1**	302.2 ± 209.5	180.2 ± 67.6
UFCxMS/Cr (nmol/g creat)	742.2 ± 1650.5**	129.9 ± 83.4	90.3 ± 38.4
UFCo (nmol/g creat)	470.1 ± 516.5*	214.3 ± 157.6	130.9 ± 72.5
UFC:UFCo ratio	1.1 ± 0.8*	0.7 ± 0.7	0.8 ± 0.6
ACTH (pg/ml)	54.3 ± 70.3	36.8 ± 41.7	–
Glucose (mmol/l)	5.6 ± 1.8	5.5 ± 1.5	4.7 ± 0.3
TC (mmol/l)	4.9 ± 0.8	4.7 ± 0.9	4.8 ± 0.4
HDL-c (mmol/l)	1.8 ± 0.8*	1.4 ± 0.6	1.6 ± 0.2
LDL-c (mmol/l)	2.7 ± 0.7	2.7 ± 0.9	2.9 ± 0.5

CS Cushing's syndrome, *T2D* type 2 diabetes, *HTA* hypertension, *OB* obesity, *DLP* dyslipidemia, *BMI* body mass index, *UFCxIA* urinary free cortisol by immunoassay, *UFCxMS* urinary free cortisol by mass spectrometry, *UFCo* urinary free cortisone, *UFC:UFCo* cortisol:cortisone ratio, *TC* total cholesterol, *HDL-c* high density lipoprotein cholesterol, *LDL-c* low density lipoprotein cholesterol

* $p < 0.05$ CS versus non-CS; ** $p < 0.001$ CS versus non-CS

Table 2 AUC, sensitivity (SE) and specificity (SP) for the diagnosis of Cushing's syndrome

	AUC	SE (%)	SP (%)
UFCxIA > 344.7 nmol/24hs	0.77	84	56
UFCxIA > 304.4 nmol/g Cr	0.79	81	69
UFCxMS > 169.5 nmol/24 h	0.77	81	54
UFCxMS > 167.5 nmol/g Cr	0.79	67	79
UFCo > 235.7 nmol/24 h	0.69	67	53
UFCo > 242.4 nmol/g Cr	0.67	54	74
UFC:UFCo > 1.32	0.69	30	96

N = 125, included all patients evaluated for hypercortisolism. Cut-offs are based on the upper reference range of the healthy control group

AUC area under the curve, *UFCxIA* urinary free cortisol by immunoassay, *UFCxMS* urinary free cortisol by mass spectrometry, *UFCo* urinary free cortisone, *UFC:UFCo* cortisol:cortisone ratio

recommended [3, 7, 12]. However, there is no evidence of what clinical significance this change may represent in the screening for CS due to the lack of direct clinical comparisons between both analytical approaches. In our study, reference values were obtained in the same group of healthy controls by both immunoassay and mass spectrometry, generating comparable cut points based on UFC levels above the standard range recommended in the Endocrine Society Clinical Practice Guideline [3]. If we assume that mild forms of CS should be detected, a highly sensitive screening test with low risk of false negative results should be favored, even if this implies lower specificity. Therefore, when analytical methods are assessed it is adequate to include patients with both mild and severe hypercortisolism in the study population, as in our work, because this reflects better the diagnostic problems found in the clinical setting.

In our study, the cutpoint (upper limit of reference range) obtained for UFCxIA was approximately twice of that obtained for UFCxMS (344.7 vs 169.5 nmol/24 h) which is in agreement with previous studies reporting a high analytical bias in immunoassay measurements [8], and confirming the low analytical capability of UFC immunoassays to detect true cortisol. This low analytical specificity has led to recommendations against their use in clinical diagnostic [13]. At present, clinical guidelines encourage the use of more specific methods, such as mass spectrometry, for determination of steroids. In addition to the superior specificity of mass spectrometry, other factors such as overcoming low agreement among immunoassays together with the possibility of simultaneous determination
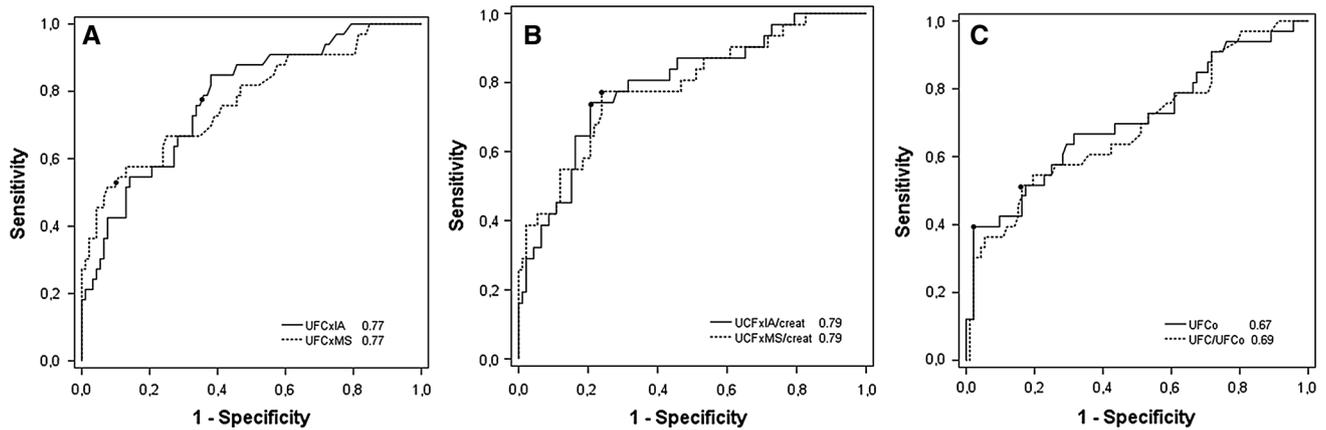
Fig. 1 ROC curves for UFCxIA and UFCxMS (**a**), UFCo and UFC: UFCo (**b**) and creatinine-corrected UFCxIA and UCFxMS (**c**). Calculated optimal cutoffs indicated on the ROC curves were: 359 nmol/24 h (UFCxIA) and 258 nmol/24 h (UFCxMS) (**a**); 196 nmol/24 h (UFCo) and 0.97 (UFC:UFCo) (**b**); 129 nmol/g crea (UFCxIA) and 55 nmol/g crea (UFCxMS)
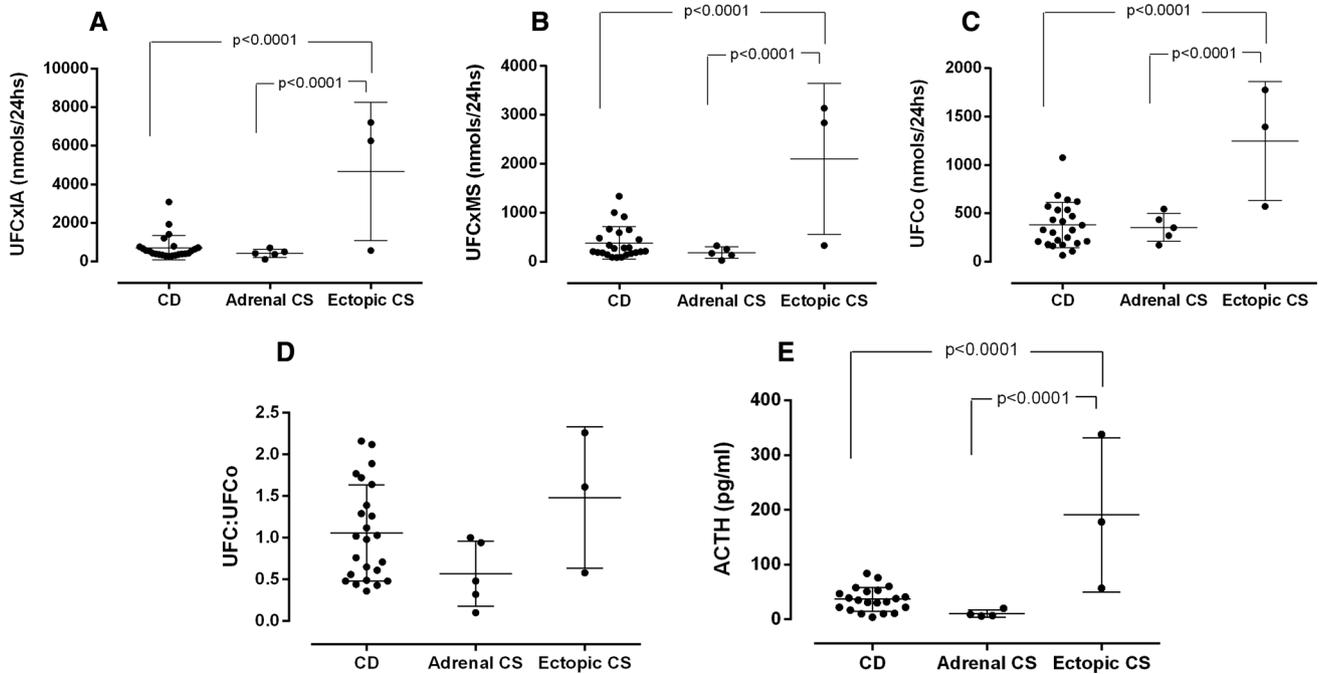

Fig. 2 UFCxIA (**a**), UFCxMS (**b**), UFCo (**c**), UFC:UFCo ratio (**d**) and ACTH (**e**) differences between CS patients according to etiology. *CD* Cushing's disease, *CS* Cushing's syndrome

of several other metabolites including exogenous steroids have favoured its gradual incorporation in many clinical applications of steroid hormones analysis. The evaluation of the clinical impact of these methodological changes is mandatory in order to make proper decisions.

In the last years, routine UFC measurement by GC–MS was performed in our center in all patients with clinical conditions suggestive of CS and UFC higher than 276 nmol/24 h as measured by immunoassay. The diagnostic value of UFC by both immunoassay and mass spectrometry were compared. At the recommended cutpoints (high standard range), sensitivity was lower for mass spectrometry measurements in comparison with immunoassay (81 vs 84 %), suggesting that mass spectrometry cutoffs based on references ranges should be lowered. This possibility has been recently anticipated [5]. In this regard, the higher sensitivity of immunoassay measurements could be explained by a greater presence of metabolites that cross-react with the immunoassay in the urine of CS patients. In fact, it has also been suggested that this cross-reactivity, far from being spurious, may better reflect overall glucocorticoid production [13–15].

It is important to note that, in our study, determination of UFC by immunoassay was performed after solvent extraction. In fact, immunoassays used to measure UFC are generally based on the serum cortisol assay methods and, therefore, prone to significant interferences with metabolites that are present in much higher concentrations in urine than in serum [3, 8]. For this reason prior extraction of urine is considered essential to ensure better specificity [13].

In our study, UFC mass spectrometry measurements were performed by gas chromatography mass spectrometry (GC–MS). Both GC–MS and LC–MS/MS (liquid chromatography tandem mass spectrometry) are considered "gold" standard for UFC analysis, with an excellent agreement between the two methods [8]. In comparison with GC–MS, LC–MS/MS is usually less time-consuming than GC–MS, it does not generally require a derivatization step and allows high-throughput. In contrast, GC–MS usually presents a better chromatographic resolution and allows comprehensive steroid profiling and novel steroid metabolome discovery [16].

Areas under the ROC curves were equal in both UFC methods. Specifically, the AUC in both methods was 0.77, with a coefficient kappa 0.60, and a strong Landis and Koch concordance index. A detailed analysis of the ROC curves pointed out that, at the same sensitivity, low levels of UFC are more specific when measured by immunoassay; on the contrary, high levels of UFC, at the same specificity, are less sensitive when measured by immunoassay. This suggests that UFC immunoassay measurements may be more useful for CS screening and that UFC mass spectrometry measurements may be more valuable for excluding CS. Optimal cutoff points based on ROC curves were also in the same direction.

Both UFC and late night salivary cortisol are reported to be highly accurate in identifying patients with CS [17]. Late night salivary cortisol has an excellent diagnostic performance and is recognized as a robust, convenient test for screening and diagnosis of Cushing's syndrome with a sensitivity and specificity higher than 90 % [18]. In a recent study [19], LC–MS/MS measurements of UFC presented also very high diagnostic performance (AUROC 0.98). In contrast, our study with GC–MS presents lower diagnostic accuracy (AUROC 0.77). This difference may be in part explained by the fact that patients with low UFC levels measured by immunoassay (UFC < 276 nmol/24 h or <100 μg/24 h) were not included in our study. If they had been included, AUROC of UFC by both immunoassay and GC–MS would have been most probably improved, since none CS with UFC < 276 nmol/24 h was diagnosed during the study period.

In our laboratory, we routinely measure creatinine levels in all urine samples in order to help assessing the completeness of the 24 h collection or discarding overcollection. Interestingly, in our study UFC creatinine-corrected values presented higher areas under the ROC curves for the diagnosis of CS than non-creatinine-corrected values (0.79 vs 0.77 for UFCxIA and 0.79 vs 0.77 for UFCxMS). This suggests that, in addition to assess sample adequacy, 24-h creatinine measurements may add clinical significance to UFC results. In contrast, although significant differences were found in UFCo and UFC:UFCo ratio between CS and non-CS patients, these parameters had no additional value compared to UFC. These data are in concordance with the findings of Ceccato et al. [14].

In our study, ectopic CS patients presented higher UFC, UFCo and UFC:UFCo ratio in concordance with previous findings of literature. The UFC:UFCo ratio has been reported to be a possible marker of this type of CS [14]. Higher UFC:UFCo ratios in CS patients suggest a saturation of the 11β-HSD2 enzyme. As the group of Ceccato, we divided our population into two according to UFC:UFCo values higher or lower than the upper limit of the reference range. The group with the higher ratio also showed more elevated UFC levels, so also we believe that only high cortisol levels are able to saturate 11β-HSD2 activity leading to an excess of cortisol that could explain the typical co-morbidities in ectopic CS [14, 19–22].

This study has limitations. Although ours is a third-level referral centre for CS, the number of patients studied was relatively low; first of all, CS is a rare disease and the incidence is low; in the second place, we use stringent inclusion criteria that enabled us to accurately analyze the sensitivity and specificity of UFC. In addition, our study evaluated only one of the several immunoassays available for UFC measurements. Taking in consideration that cortisol metabolites may present different amount of cross-reactivity in different immunoassays, the same results may not be applicable to all immunoassays. However, this cross-reactivity is common to all immunoassays, suggesting that results with others immunoassays should be in the same direction as ours.

In summary, in the present study we compared for the first time, immunoassay and mass spectrometry UFC measurements for the screening of CS. Our results showed that the diagnostic value of both methods was very similar. However, results suggest that it may be necessary to lower the cutoff points for mass spectrometry below the upper limit of the standard range to maintain clinical sensitivity.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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