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ORIGINAL ARTICLE

STEROID PROFILING IN THE DIFFERENTIAL DIAGNOSIS OF CUSHING'S SYNDROME AND DIAGNOSIS OF MACS

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Abstract. Background: *Cushing's syndrome (CS) is the consequence of the exposure of tissues to extremely high levels of glucocorticoids. Early diagnosis and treatment are the mainstay of optimizing patient outcomes and improving their quality of life. In the recent years steroid profiling by LC-MS sheds more light on the diagnosis of CS.* ***Materials and methods:*** *This was a retrospective cross-sectional study.* ***Objective:*** *To investigate serum steroid precursor differences between different etiological forms of CS and to suggest a steroid panel for the diagnosis of MACS in patients with adrenal incidentalomas.* ***Results:*** *Our studied patients with CD had significantly lower levels of 11-deoxycorticosterone (p = 0.047) and 17 OH progesterone (p = 0.024) compared to those with adrenal forms of CS. In out cohort of patients with adrenal incidentalomas, those with MACS had significantly lower levels of androgens (DHEA, p = 0.001) and cortisone (p = 0.015) and higher levels of 11-deoxycortisol (p = 0.039) compared to the patients with non-secreting adenomas (NSA).* ***Conclusion:*** *Introducing LC-MS based steroid profiling would be very helpful in the diagnostic process of patients with CS.*

Key words: *Cushing's syndrome, adrenal incidentalomas, steroid profiling, mild autonomous cortisol secretion*

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INTRODUCTION

Cushing's syndrome (CS) is the consequence of the exposure of tissues to extremely high levels of glucocorticoids. Left untreated it has detrimental effects on the body leading to extreme morbidity and mortality rates corresponding to the duration and severity of hypercortisolism [1]. Early diagnosis and treatment are the mainstay of optimizing patient outcomes and improving their quality of life [2]. The diagnostic process however usually require a multi-step approach, including baseline and dynamic hormone tests and imaging studies and every part of it has its subtleties [3]. While the diagnosis can be unmistakable in overt forms, in milder cases it remains a challenge as there is no clinical feature that is absolutely predictive and discriminatory [4]. The screening tests recommended by the Endocrine society clinical practice guideline have several pitfalls [5]. The tests for differential diagnosis have to be precisely performed and need the right interpretation. And the imaging studies can even make the diagnostic process more complicated in cases where the pituitary

adenoma is so small that it is hard to be visualized (50 % of cases) [6].

Mild autonomous cortisol secretion (MACS) is a biochemical phenomenon used to describe abnormal cortisol secretion (defined as serum cortisol levels post dexamethasone > 50 nmol/L (> 1.8 μg/dL) in patients who don't have the classical clinical signs and symptoms of hypercortisolism [7]. Nevertheless, they have increased rates of hypercortisolism-associated comorbidities and cardiovascular risk factors [8]. That's why the identification of these patients seems of great importance for their appropriate management. MACS is diagnosed in up to 48% of patients with adrenal incidentaloma referred to endocrine units for hormonal evaluation [8].

Undoubtedly introducing new methods into the diagnostic process of patients with CS would be very helpful both for the distinguishing between the ACTH-dependent and ACTH-independent forms of CS and for the diagnosis of the milder cases, especially asymptomatic like those with MACS.

In the recent years steroid profiling by LC-MS is getting more and more into the routine practice. It is the preferred method for steroid analysis as the cross-reactivity of the conventional immunoassays is avoided. Furthermore, the simultaneous measurement of multiple analytes belonging to the three lines of the steroidogenesis opens a new horizon in the investigation of adrenocortical diseases [9-11]. As for the diagnosis of hypercortisolism a single analysis with the well selected steroid panel may have a power equal to or even better than that of the first and second line tests taken together [12].

OBJECTIVE

To investigate serum steroid precursor differences between different etiological forms of CS and to suggest a steroid panel for the diagnosis of MACS in patients with adrenal incidentalomas.

MATERIALS AND METHODS

Subjects

It was a retrospective cross-sectional study. We enrolled all the adult patients referred to the Hypothalamus-Pituitary-Adrenal Diseases Clinic (Sofia, Bulgaria) between January 2022 and June 2023 for the following 2 reasons:

1. Hormonal evaluation of an adrenal incidentaloma (n = 256)

2. Patients with symptoms suspicious for Cushing's syndrome (n = 135)

We excluded those with a history of steroid intake during the last year and on medications, interfering with steroidogenesis. For the patients referred for adrenal incidentaloma we excluded those with formations with malignant characteristics, known oncological and infiltrative diseases and also patients with pheochromocytoma and primary aldosteronism. For the final analysis we also excluded patients with adrenal incidentaloma aged > 65 years because of the recent findings that in older patients the clinical relevance of MACS is decreasing [13] in patients without signs and symptoms of overt Cushing's syndrome, a post-dexamethasone cortisol level above 50 nmol/L (>1.8 μg/dL). Finally, 92 patients were enrolled – 68 with adrenal incidentalomas (35 with non-secreting adenomas (NSA) and 33 with MACS) and 24 with CS (10 with CD and 14 with adrenal form of CS).

All the participants in the study signed an informed consent. The study was approved by the local Ethics Committee.

Hormonal evaluation with the routine tests

For the patients who were referred for testing for CS, hypercortisolism was confirmed by the first line screening tests – elevated urinary free cortisol (UFC), impaired circadian cortisol rhythm with increased midnight serum and salivary cortisol levels, lack of suppression after 1 mg DXM suppression test and 2 mg/2 days DXM suppression test. For differential diagnosis we used ACTH, 8 mg/2 days DXM suppression test, CRH test and DDAVP test.

For the patients without clinical signs and symptoms typical for CS, who were referred for hormonal evaluation of an adrenal incidentaloma 1 mg DXM suppression test was used to identify those with MACS. We used the cut-off of 50 nmol/l (1.8 μg/dL) according to the current guidelines [7].

Serum and urinary free cortisol (UFC, nmol/24 h) were measured by highly sensitive and specific RIA (Immunotech, Beckman Coulter Co., France) with intra-assay and inter-assay coefficients of variations ≤ 5.8% and 9.2%, respectively and analytical sensitivity of 5 nM.

For the determination of salivary cortisol automated highly sensitive competitive electrochemiluminescence immunoassay (ECLIA) using Elecsys Cortisol reagent kit (Roche) was used, which is in our routine practice since 2006 [14]. Plasma ACTH was determined by highly-sensitive, specific IRMA method (ACTH Thermo Scientific BRAHMS, Germany) with analytical sensitivity 0.26 pmol/l and functional sensitivity, measured by 20%th intertest variation coefficient – 0.52 pmol/l.

Serum steroid measurements

For the steroid profiling blood samples were taken in the morning from all the patients. Serum was extracted, stored at -80ºC until being analyzed by liquid chromatography mass spectrometry (LC-MS). The panel included 14 steroids: aldosterone (0.01-0.45), 11-deoxycorticosterone (< 54), corticosterone (1.65-40.51), cortisol (134-644), 11-deoxycortisol (0.13-2.58), cortisone (28.9-91.0), 21-deoxycortisol (< 0.445), progesterone (0.04-0.7 for men and 0.07-55 for women), 17-hydroxyprogesterone (0.87-6.24), androstenedione (1.53-8.28 for men and 1.06-7.72 for women), dehydroepiandrosterone (DHEA) (2.5-46.7 for men and 1.7-38.3 for women), testosterone (7.6 37.2 for men and 0.31-2.29 for women), dihydrotestosterone (0.5-2.7 for men and 0.2-1.6 for women), estradiol (0.04-0.2 for men and 0.14-2.7 for women).

Subtyping of the patients

According to the lab results (the routine first and second-line tests) and the clinical signs and symptoms we divided the patients into 4 groups: 1) Cushing's disease (CD); 2) ACTH-independent form of CS 3) adrenal adenoma with mild autonomous cortisol secretion (MACS) – defined as serum cortisol after 1 mg dexamethasone suppression test > 50 nmol/l in patients without clinical signs of overt CS; 4) control group – patients with non-secreting adrenal adenomas (NSA).

Statistical analysis

Data calculations and analyses were performed using SPSS 23.0 (IBM, Armonk, NY, USA) and GraphPad Prism 8.0.1 (GraphPad Software, San Diego, California USA). Categorical variables are presented with numbers and percentages. Kolmogorov-Smirnov test was used to assess the distribution of continuous variables. Median and interquartile range are used to describe variables with non-normal distribution. Non-parametric methods were used to evaluate differences between groups – Mann-Whitney test when comparing two groups and Kruskal-Wallis test for three groups. Statistical significance was considered when $p < 0.05$.

RESULTS

Demographic, clinical and biochemical characteristics of studied patients (n = 92), divided into four groups according to the lab results (the routine first and second-line tests) and the clinical signs and symptoms, are shown in Table 1.

Females were the predominating sex in all the subgroups.

The youngest group was that of patients with CS, followed by the NSA, adrenal CS and the oldest group was that of patients with MACS.

In our cohort of patients with adrenal incidentalomas (n = 68) MACS was diagnosed in 49% of the patients (n = 33).

No significant differences in body mass index were observed.

Patients with the highest levels of UFC and the highest percentage of cortisol-associated comorbidities were those with CD, followed by adrenal CS. Patients with MACS and NSA showed comparable results.

Patients with MACS had significantly higher levels of 11-deoxycortisol and lower levels of DHEA and cortisone compared to those with NSA.

Table 1. Demographic, clinical and biochemical characteristics of studied patients (n = 92) divided into four groups

	CD	Adrenal form of CS	MACS	NSA
Number	10	14	33	35
Gender				
Males, n (%)	2 (20)	3 (21,4)	8 (24)	11 (31,4)
Females, n (%)	8 (80)	11 (78,6)	25 (76)	24 (68,6)
Age, years	41,5 (32-56)	51,5 (37,5-63)	59 (46-62)	46 (44-64)
Cortisol after 1 mg DXM nmol/l	799 (382-881)	349,3 (210,2-753,4)	86,75 (62,2-144,3)	26.2 (21-33,3)
UFC nmol/24h* (38-275)	813 (414-1487)	399,7 (151-648)	122,6 (67,6-177,5)	123,1 (64,2-168,2)
ACTH pmol/L * (2.2-12.2)	17,45 (9,25-31,05)	1.0 (1,0-1,3)	4,9 (2,6-7,0)	7,95 (4,85-11,3)
BMI kg/m^2	27 (25-39)	30,4 (24,8-33)	29 (24,5-34)	30,8 (26-34)
Hypertension, n (%)	9 (90)	9 (64,3)	22 (66,6)	25 (71,4)
Carbohydrate Abnormalities				
Diabetes, n (%)	5 (50)	4 (28,6)	6 (18,2)	6 (17,1)
Prediabetes, n (%)	3 (30)	2 (14,3)	5 (15,2)	5 (14,3)
Dyslipidemia, n (%)	8 (80)	10 (71,4)	18 (54,5)	22 (62,9)

Table 2. Steroid Profiling by LC-MS in patients with adrenal incidentalomas – MACS and NSA

	NSA N = 35	MACS N = 33	p
Aldosterone, nmol/l	0,14 (0,07-0,24)	0,13 (0,08-0,3)	0,413
Cortisol, nmol/l	407,9 (279,5-513)	453,3 (261,8-551)	0,496
Corticosterone, nmol/l	10,65 (7,1-20,1)	14,82 (5,11-21,33)	0,811
11-deoxycortisol, nmol/l	1,12 (1,6-1,81)	1,89 (0,83-2,84)	0,039
21-deoxycortisol, nmol/l	0,2 (0,2-0,2)	0,2 (0,2-0,2)	0,037
Cortisone, nmol/l	65,13 (55,7-71,5)	56,68 (42,6-65,8)	0,015
Dihydroepiandrosterone, nmol/l	9,5 (6,0-17,6)	5,01 (3,95-7,38)	0,001
11-deoxycorticosterone, nmol/l	0,14 (0,14-0,21)	0,19 (0,14-0,36)	0,081
17-OH-progesterone, nmol/l	2,4 (0,86-3,49)	1,42 (1,91-3,94)	0,519

Table 3. Steroid Profiling by LC-MS in patients with ACTH-dependent and ACTH-independent CS

	CD N = 10	Adrenal CS N = 14	p
Aldosterone, nmol/l	0,07 (0,07-0,11)	0,08 (0,07-0,14)	0,346
Cortisol, nmol/l	564,9 (312,8-623,33)	443,3 (372,8-602,6)	0,682
Corticosterone, nmol/l	13,4 (4,68-15,99)	14,85 (9,35-39,13)	0,242
11-deoxycortisol, nmol/l	1,41 (0,7-1,89)	2,12 (1,15-3,35)	0,065
21-deoxycortisol, nmol/l	0,2 (0,2-0,2)	0,2 (0,2-0,2)	0,222
Cortisone, nmol/l	51,62 (42,3-76,5)	58,68 (51,1-67,1)	0,598
Dihydroepiandrosterone, nmol/l	5,5 (3,29-15,77)	3,95 (3,22-6,19)	0,241
11-deoxycorticosterone, nmol/l	0,13 (0,08-0,14)	0,17 (0,13-0,46)	0,047
17-OH-progesterone, nmol/l	1,15 (0,82-1,31)	1,63 (1,18-2,34)	0,024

Patients with adrenal forms of CS had significantly higher levels of 11-deoxycorticosterone and 17-OH-progesterone. DHEA and cortisol were higher in patients with CD, while cortisone and 11-deoxycortisol were higher in patients with adrenal CS, however they didn't reach statistical significance.

For most of the patients in all the groups 21-deoxycortisol values were below the sensitivity levels of the LC-MS assay we used.

We compared each group of patients with the three others separately. The steroids that had statistical significant differences are shown in figure 1. The groups between which we found significant differences are marked with an asterisk.

For the analysis of the sex steroids we divided the patients by sex and included only menopausal women. We combined the 2 forms of CS in 1 group. The results are shown in table 4.

Testosterone was significantly lower in men with CS compared to men with NSA and MACS (p = 0.003).

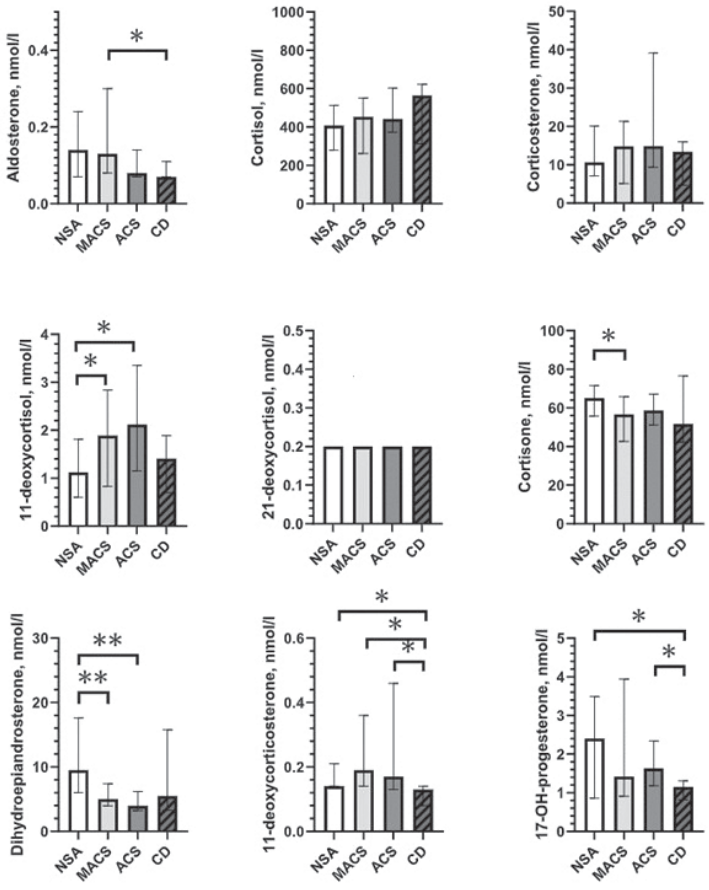

Fig. 1. Steroids with statistically significant differences between the groups

Legend: The * shows the groups between which statistically significant differences are found.

Table 4. Analysis of the sex steroids

	NSA (20/11)	MACS (22/8)	CS (18/5)	p
Estradiol, nmol/l				
Women	0,08 (0,08-0,22)	0,08 (0,08-0,08)	0,09 (0,08-0,36)	0,269
Men	0,11 (0,09-0,16)	0,10 (0,08-0,18)	0,1 (0,08-0,11)	0,577
Testosterone, nmol/l				
Women	1,15 (0,84-1,45)	0,76 (0,54-1,15)	0,66 (0,44-1,6)	0,208
Men	14,09 (11,63-17,33)	14,46 (12,96-17,81)	7,15 (0,81-7,35)	0,003
Dihydrotestosterone, nmol/l				
Women	0,2 (0,2-0,44)	0,2 (0,2-0,2)	0,2 (0,2-0,2)	0,213
Men	1,24 (0,82-1,54)	0,98 (0,80-1,86)	0,52 (0,2-0,83)	0,074
Androstendione, nmol/l				
Women	2,55 (1,87-4,05)	1,88 (1,06-3,3)	3,21 (1,46-5,25)	0,672
Men	2,72 (2,37-3,48)	2,85 (1,96-3,41)	3,41 (1,38-4,88)	0,866
Progesterone, nmol/l				
Women	0,58 (0,58-2,0)	0,58 (0,58-0,58)	0,58 (0,58-0,58)	0,116
Men	0,58 (0,58-0,58)	0,58 (0,36-0,58)	0,58 (0,58-0,58)	0,812

DISCUSSION

The aim of our study was to emphasize the importance of LC-MS multisteroid profiling in the routine clinical practice by providing evidence of a differential steroid profile among 1) patients with different etiological forms of CS and 2) patients with adrenal incidentalomas with MACS, i.e. subtle forms of hypercortisolism who can be missed with the routine screening tests.

Differential steroid profile among patients with different etiological forms of CS

Studies investigating steroidogenesis in patients with hypercortisolism have proven that these patients have some specific alterations in the steroid precursors that may be sufficient both to set the diagnosis and to distinguish the etiological form. Attempts to find the steroid panel which to classify CS patients by etiology date back many years and still go on [15-18]. Despite the improvement in the methods used for steroid detection, there is still no consensus pointing out the distinctive steroid fingerprints in the different forms of CS.

Our studied patients with CD had significantly lower levels of 11-deoxycorticosterone (p = 0.047) and 17-OH-progesterone (p = 0.024). DHEA and cortisol were higher in patients with CD, while cortisone and 11-deoxycortisol were higher in patients with adrenal CS, however they didn't reach statistical significance. For most of the patients in all the groups 21-deoxycortisol values were below the sensitivity levels of the LC-MS assay we used. Previous studies proved its positive correlation with the ACTH values – higher in CD and lower in ACTH-independent CS [12]. The same team pointed out 10-steroid panel with a good accuracy in subtyping patients with CS and found positive correlation between the levels of ACTH and aldosterone [12]. They also combined the steroid profiling with 1 mg dexamethasone suppression test and found out that the discriminative power of these tests taken together had the same effectiveness as the combination of all the routine screening tests [12].

Distinguished steroids in the differential diagnosis of CS are the adrenal androgens. As adrenal androgens are stimulated mainly by ACTH and suppressed in cases of suppressed ACTH (ACTH-independent forms of CS) respectively it can be hypothesized that they can be used as reliable markers to distinguish the two main forms of CS. DHEAS has a historical significance in the differential diagnosis of patients with hypercortisolism. Yamaji et al. measured serum DHEAS levels in patients with adrenal and pituitary CS and in healthy controls and found that it was significantly higher in CD than in healthy individuals and significantly lower in adrenal CS compared to the group of healthy controls [15]. Results of other studies also supported the diagnostic value of the adrenal androgens (DHEA and DHEA-S) [11, 19]. Gao et al. highlighted the advantages of DHEA-S compared to ACTH – longer half-life and stable levels throughout the day. The team suggested the combination of DHEA-S, DHEA and androstendione as an additional test in the differential diagnosis, especially in cases of falsely elevated ACTH [19]. Ciftchi et al. even defined a cut-off value for DHEA-S as an etiology marker – 20% of the reference interval [20]. The lack of significant difference in DHEA values between our groups with ACTH-dependent and ACTH-independent forms of CS may be due to the small number of patients.

Differential steroid profile among patients with MACS and NSA

MACS belongs to the group of ACTH-independent forms of CS. That's why in the steroid profiles of patients with MACS the changes are similar to those of patients with adrenal overt CS, though milder abnormalities are observed [21]. Furthermore, the lack of clear clinical presentation in these patients makes the diagnosis even harder. And yet, these patients are of increased risk of hypercortisolism-associated comorbidities and special measures are necessary [8].

In out cohort of patients with adrenal incidentalomas, those with MACS had significantly lower levels of androgens (DHEA, p = 0.001) and cortisone (p = 0.015) and higher levels of 11-deoxycortisol (p = 0.039). Cortisol, corticosterone and 11-deoxycorticosterone were higher and 17-OH-progesterone was lower in patients with MACS than those with NSA, however they didn't reach statistical significance.

Similar results published Di Dalmazi et al. and they also found correlations between levels of cortisol and DHEA and components of the metabolic syndrome (waist circumference) [22]. In another study aiming to identify steroid metabolomics typical for patients with CS cortisol, 11-deoxycortisol, 21-deoxycortisol, corticosterone were determined as the most specific steroids for identifying patients with hypercortisolism [12]. They defined 11-deoxycortisol as the most powerful predictor of CS with a correlation between the levels of the steroid and the severity of hypercortisolism [12]. Berke et al. explored the utility of steroid profiling in patients with adrenal incidentalomas and found that patients with autonomous cortisol secretion were characterized with lower levels of DHEA and DHEAS and higher levels of 11-deoxycortisol and 11-deoxycorticosterone than those with NSA [23].

CONCLUSION

Undoubtedly introducing LC-MS based steroid profiling would be very helpful into the diagnostic process of patients with CS both for the distinguishing between the ACTH-dependent and ACTH-independent forms of CS and for the diagnosis of the milder cases, especially MACS.

Limitations of the study are the small number of patients and the retrospective nature of the study.

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