Plasma steroid profiles in subclinical compared to overt adrenal Cushing's syndrome

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Abstract: CONTEXT Diagnosis of subclinical adrenal hypercortisolism is based on several tests of the hypothalamic-pituitary-adrenal axis to establish mild alterations of cortisol secretion and dysregulated cortisol physiology. OBJECTIVE This study assessed whether plasma steroid profiles might assist diagnosis of subclinical Cushing's syndrome (SC). DESIGN Retrospective cross-sectional study. SETTING Two tertiary medical centers. PATIENTS Two hundred and eight patients were tested for hypercortisolism among whom disease was excluded in 152 and confirmed in 21 with overt clinical Cushing's syndrome due to adrenal tumors (AC) compared to 35 with SC. Another 277 age- and gender-matched hypertensive and normotensive volunteers were included for reference. MAIN OUTCOME MEASURES Panel of 15 plasma steroids measured by mass spectrometry with classification by discriminant analysis. RESULTS Patients with SC showed lower (P<0.05) plasma concentrations of dehydroepiandrosterone and dehydroepiandrosterone-sulfate than subjects without SC. The largest increases (P<0.001) in plasma steroids among patients with SC were observed for 11-deoxycortisol and 11-deoxycorticosterone. Nevertheless, concentrations of 11-deoxycorticosterone, 11-deoxycortisol and pregnenolone in patients with AC were higher (P<0.05) than in those with SC. Patients with SC or AC could be distinguished from subjects without disease using the above combination of steroids as precisely as with use of measurements of serum cortisol after dexamethasone. The steroid combination provided superior diagnostic performance compared to each of the other routine biochemical tests. CONCLUSIONS Distinct plasma steroid profiles in patients with SC may provide a simple and reliable screening method for establishing the diagnosis.

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Context: Diagnosis of subclinical adrenal hypercortisolism is based on several tests of the hypothalamic-pituitary-adrenal axis to establish mild alterations of cortisol secretion and dysregulated cortisol physiology.

Objective: This study assessed whether plasma steroid profiles might assist diagnosis of subclinical Cushing's syndrome (SC).

Design: Retrospective cross-sectional study.

Setting: Two tertiary medical centers.

Patients: Two hundred and eight patients were tested for hypercortisolism among whom disease was excluded in 152 and confirmed in 21 with overt clinical Cushing's syndrome due to adrenal tumors (AC) compared to 35 with SC. Another 277 age- and gender-matched hypertensive and normotensive volunteers were included for reference.

Main Outcome Measures: Panel of 15 plasma steroids measured by mass spectrometry with classification by discriminant analysis.

Results: Patients with SC showed lower ($P<0.05$) plasma concentrations of dehydroepiandrosterone and dehydroepiandrosterone-sulfate than subjects without SC. The largest increases ($P<0.001$) in plasma steroids among patients with SC were observed for 11-deoxycortisol and 11-deoxycorticosterone. Nevertheless, concentrations of 11-deoxycorticosterone, 11-deoxycortisol and pregnenolone in patients with AC were higher ($P<0.05$) than in those with SC. Patients with SC or AC could be distinguished from subjects without disease using the above combination of steroids as precisely as with use of measurements of serum cortisol after dexamethasone. The steroid combination provided superior diagnostic performance compared to each of the other routine biochemical tests.

Conclusions: Distinct plasma steroid profiles in patients with SC may provide a simple and reliable screening method for establishing the diagnosis.

Our data suggest that the multistep biochemical testing for diagnosis of subclinical Cushing's syndrome could be simplified by single plasma multisteroid measurements. .

Introduction

Up to 50% of patients with adrenocortical adenomas detected incidentally (adrenal incidentalomas) may have hypercortisolism [1]. In most such cases the classical clinical features of Cushing's syndrome are absent. Such conditions have been described as 'subclinical Cushing's syndrome ' [1], 'subclinical autonomous glucocorticoid hypersecretion' or subclinical hypercortisolism [2]. In this article we use the term 'subclinical Cushing's syndrome' (SC) when referring to this entity.

   Dysregulated cortisol physiology with mild elevations of cortisol secretion is most often recognized during evaluation of adrenal incidentalomas to exclude hypercortisolism [2 3]. Insulin resistance, hypertension, obesity, dyslipidemia, impaired glucose tolerance and diabetes mellitus are frequently associated with both subclinical Cushing's syndrome and overt Cushing's syndrome, thereby contributing to cardiovascular complications and high mortality [4]. Vertebral fractures may also be features of otherwise asymptomatic cortisol excess in osteoporotic patients with SC [5].

   The aforementioned considerations and findings of a 45% prevalence of SC among patients with unilateral adrenal masses with size over 2.5 cm and CT-imaging attenuation below one Hounsfield unit indicate the potential importance of recognizing SC [2]. Nevertheless, a consistent consensus on the criteria used to diagnose SC has yet to be reached. Clinical, radiological and hormonal characteristics of this pathological condition all require consideration. Absence of clinical signs and symptoms related to cortisol hypersecretion in the presence of hypercortisolism provides the generally accepted criteria for SC but is weakened by reliance on recognition of clinical clues.

   Findings of a serum cortisol above 1.8 µg/dl after the dexamethasone suppression test (DST) combined with adrenocorticotrophin (ACTH) concentrations below 10 pg/ml provide one of several criteria for establishing dysregulated cortisol secretion in SC [6]. Elevated 24-h urinary outputs of free cortisol (UFC) have been reported in some studies but are not reliable alone for diagnosis of either overt Cushing's syndrome or SC [2 7]. Measurement of late-night salivary cortisol has provided one method to demonstrate loss of diurnal rhythm of

cortisol secretion in SC patients; however, with cut-off points of the late-night and midnight salivary cortisol ranging from 0.13 µg/dl to 0.55 µg/dl [8] use of this test alone is also unreliable for diagnosis of either AC or SC. Overall, diagnosis of SC remains difficult and requires a positive DST and at least another HPA axis aberration [9].

Use of other adrenal steroids apart from cortisol for diagnosis of SC has not been widely investigated. Altered plasma concentrations of several steroids in patients with adrenocortical adenomas and SC, as measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), have suggested that steroid profiling may provide a useful tool for diagnosis of SC [10]. This has been further supported by an LC-MS/MS steroid profiling study establishing a panel of steroids that can be used to identify patients with Cushing's syndrome and discriminate those with and without ACTH-dependent subtypes [11]. Other studies have indicated that urinary steroid profiling may be useful for diagnosis of overt clinical Cushing's syndrome [12 13].

Using panels of steroids for diagnosing disorders of adrenal steroidogenesis has a more than two decades history, particularly with gas chromatography-mass spectrometry methods applied to congenital adrenal hyperplasia and related disorders of sexual development [14 15]. Use of steroid profiles in adrenal cortical disorders has more recently gained traction with methods employing LC-MS/MS that also take advantage of advances in computational mathematics. Through such advances there is potential for a paradigm shift from unidimensional to multidimensional diagnostic approaches utilizing basic multivariate discriminant and principal components analyses to more sophisticated machine learning methods [11 16 17].

Based on the above promising leads and advances we used LC-MS/MS to analyze the plasma steroid profiles of patients with adrenocortical adenomas associated with SC compared to profiles of patients in whom Cushing's syndrome was excluded (EX) and patients with overt adrenal Cushing's syndrome (AC). The analysis took advantage of well characterized reference intervals in a series of 525 hypertensive and normotensive volunteers, all relevant data available by open access [19]. The aim was to establish from this panel a selection of steroids that could serve as a single test alternative to routine tests for discriminating patients with SC from AC and patients without disease.

Methods

Recruitment of Patients

Patients were enrolled in this bi-centric cross-sectional study from outpatients referred to the Departments of Endocrinology at the Ludwig-Maximillians-Universität München and the University Hospital Dresden. Patients were tested for hypercortisolism due to findings of adrenal masses detected by computed tomography or magnetic resonance imaging as well as for investigation of secondary hypertension. No comparison was made between patients having bilateral adrenal masses with those having unilateral lesions. The test was also performed to screen for Cushing's syndrome in overweight and obese subjects. Hormonal evaluations were also performed to rule out pheochromocytoma and primary hyperaldosteronism. The final study population was restricted to those with either confirmation or exclusion of disease according to current guidelines [18]. Patients with ACTH-dependent Cushing's syndrome were excluded from the analysis.

Cortisol values (fasting serum cortisol >1.8 µg/dl) after an overnight dexamethasone suppression test (DST), 24h urinary free cortisol (upper cut-offs 75.4 µg/24h) (UFC), midnight salivary cortisol (SFC) (>1.5 ng/ml) as well as basal serum cortisol (upper cut-offs 240 µg/l) and ACTH concentrations (<10 pg/ml) were performed to confirm presence of AC or SC. An abnormal dexamethasone suppression test (DST) as one initial screening test in combination with at least one other abnormal test of the hypothalamic-pituitary-adrenal axis was advocated for diagnosis of SC. Overt AC was thereby confirmed in 21 patients. Subjects with no relevant clinical features of Cushing's syndrome were classified as SC (n=35) if they had biochemical evidence of hypercortisolism or as disease excluded (EX) if they showed no biochemical evidence of hypercortisolism (n=152).

Reference population

The reference control group was matched as closely as possible to combined patient groups according to age and sex. A total of 277 normotensive and hypertensive volunteers were thereby selected out of 525 subjects (all at Dresden) recruited initially for establishing specific reference intervals for each of the steroids of the plasma panel [19]. The reference population was included for comparisons to patients in whom hypercortisolism was excluded and to aid correction of impacts of differences in age and sex among the three different patient groups (Table 1). All subjects provided written informed consent under protocols approved by the local ethics committee at each center.

Steroid profiling

The 15 adrenal steroid panel including aldosterone, cortisol, 11-deoxycortisol, 21-deoxycortisol, corticosterone, 11-deoxycorticosterone, aldosterone, 18-oxocortisol, 18-hydroxycortisol, cortisone, progesterone, 17-hydroxyprogesterone, pregnenolone, androstenedione, dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S) was assayed by LC-MS/MS as described elsewhere [20].

Cortisol after DST, UFC, SFC and basal plasma cortisol and ACTH were analyzed within the routine clinical laboratories at both centers.

Blood sampling was performed in the morning between 08:00 to 10:00 A.M after an overnight fast. Samples were collected into blood tubes containing lithium heparin or ethylendiaminetetraacetic acid and stored at -80°C until analyses of steroid profiles.

Statistical analyses

Statistical analyses were carried out utilizing the JMP statistic software package (SAS Institute Inc., Cary, NC). The Wilcoxon and Steel Dwass all-pairs tests were used for non-parametric comparisons of demographic and routine biochemical data involving multiple groups. For parametric multivariate analyses, all data were logarithmically transformed before analyses. Corrections for age and sex in comparisons that involved the reference population involved multivariate calculations of least square means using age and sex as covariates with final display of data derived by the exponents of logarithmically transformed data to calculate geometric means and respective plus and minus standard errors. For least square means multivariate comparisons, significance of differences were assessed using the Tukey HSD test. For other multivariate analyses (e.g., discriminant analyses) data were normalized according to age- and gender-specific upper cut-offs of reference intervals as established elsewhere [19].

For distinguishing patients with and without SC, receiver-operating characteristic (ROC) curves were constructed by logistic regression. Selection of a minimal panel of the most useful steroids for diagnosis was established using stepwise regression. Discriminant analysis with stepwise variable selection was further employed to assess the use of plasma steroid combinations for distinguishing patients with SC from AC and EX groups. Details concerning the discriminant platform of the JMP statistics software package are available on-line (https://www.jmp.com/support/help/14-2/disciminant-analysis.shtml). Results from steroid profiling were compared to those derived from routine measurements of UFC, SFC, plasma ACTH and plasma cortisol before and after DST. Descriptions of underlying mathematical and statistical concepts, associated methodological details and additional statistical analyses are available in the on-line supplemental file [41].

Results

Patient characteristics and routine test results

Patients with SC were older at presentation compared to other groups while those with AC showed a higher proportion of females compared to other groups (Table 1). Plasma cortisol concentrations after DST in SC patients were as expected higher (P<0.05) than in the EX group but lower (P<0.005) compared to those of AC patients. Midnight salivary free cortisol as well as UFC revealed higher (P<0.05) values in AC und SC patients than in the EX group. Basal plasma ACTH concentrations were lower (P<0.01) in SC patients than in the EX group but higher (P<0.0005) compared to AC patients. Cortisol concentrations after DST showed minimal overlap between AC or SC patients and the EX group, in keeping with use of this test as part of the gold standard for classification.

Steroid profiles

Significances of differences among groups were assessed by models with multivariate analyses to correct for differences in sex and age (Table 2). Among the 15 steroids of the panel, 11-deoxycortisol and 11-deoxycorticosterone were consistently increased (P<0.05) in both groups of patients with AC and SC compared to both the reference and EX groups (Figure 1). Plasma concentrations of cortisol were increased (P<0.05) in AC and SC groups above the reference but not the EX group. Plasma aldosterone showed no differences between groups, whereas 18-oxocortisol and 18-hydroxycortisol in patients with SC and AC and the EX group were higher (P<0.05) than concentrations of the reference group.

Plasma concentrations of DHEA and DHEA-S were the only two steroids that were consistently lower (P<0.05) in patients with AC and SC than both reference and EX groups, with additionally lower concentrations of both these two steroids in AC than SC (Figure 1). Androstenedione was lower in AC than the EX and reference groups. Corticosterone was higher (P<0.05) in SC compared to the EX group. There were no differences in plasma concentrations of 21-deoxycortisol, cortisone and 17-hydroxyprogesterone among the four groups, whereas pregnenolone showed unusually lower (P<0.05) concentrations in EX and SC groups compared to reference as well as SC compared to AC. Progesterone concentrations were lower (P<0.05) in SC and AC groups compared to reference.

Diagnostic test performance

A combination of 14 steroids was established using a stepwise analysis to provide optimal diagnostic performance for distinguishing patients with and without SC. With the full profile,

the analysis indicated 6 steroids (11-deoxycortisol; 11-deoxycorticosterone, DHEA-S, DHEA, androstenedione and progesterone) that provided the highest discriminatory power with respective F-ratios of 41.7, 35.6, 34.8, 28.6, 9.4 and 8.7. With omission of steroids to obtain different steroid combinations 11-deoxycortisol always maintained the highest rank. With discriminant analysis this combination provided a misclassification rate of only 14.4%. With exclusion of 18-oxo-cortisol, the combination of the remaining 14 steroids provided a lower misclassification rate of 4.8%. Using this combination, areas under ROC curves showed higher (P<0.01) diagnostic performance of the steroid panel compared to salivary free cortisol, basal ACTH, basal serum cortisol, and UFC (Figure 2, Table 3). The area under the ROC curve for the steroid panel did not differ from the area under the ROC curve for the DST.

Classification of Patients with SC, AC and NS

Routine measurements of UFC, midnight salivary free cortisol, basal plasma ACTH and serum cortisol combined with the DST provided optimal discrimination of the three groups of patients with areas under ROC curves of 1.000, 0.9930 and 0.9941 for AC, EX and SC respectively (Figure 3). Eighty-three percent of all patients with SC and 100% of patients with AC were correctly classified by the combination of all five routine measurements. Four percent of patients of the EX group were incorrectly classified using the combination of routine tests.

With use of the steroid panel 97% of patients with SC were correctly classified, whereas up to 5% of patients with AC and of the EX group were misclassified (Figure 3). Areas under the ROC curve of the steroid panel ranged from 0.9962, 0.9904 and 0.9901 for AC, EX and SC respectively, similar to those observed for the combination of routine tests (Figure 3). Although both methods could completely distinguish patients with SC from AC, identification of patients among the SC group suffered fewer false negatives with use of the steroid profile than with the routine test combination.

Discussion

The main findings of our study are two-fold. First, we show for the first time that the measurement of multiple steroids from a single baseline plasma sample is able to identify patients with SC with high accuracy and distinguish them from normal controls and subjects in who disease was excluded. Second, we demonstrate that patients with SC, although heterogeneous in terms of standard diagnostic tests and glucocorticoid output, have a distinct steroid fingerprint, which separates them from patients with AC in discriminant analysis. Our data extend those of our previous publications addressing similar diagnostic questions in primary aldosteronism [21-23]. All together these data suggest that in the future cumbersome multistep biochemical testing for diagnosis of adrenal pathologies could be radically simplified by single plasma multisteroid measurements.

Numerous previous studies have shown that no single test to establish hypercortisolism has 100% sensitivity or perfect accuracy resulting in patients incorrectly labeled with SC due to false-positive results [24 25]. Lack of specificity of conventional tests can have far-reaching consequences, as for example in patients with pronounced metabolic syndrome who might become candidates for unilateral adrenalectomy when presenting with false-positive tests for autonomous cortisol secretion [14]. Inadequate diagnostic sensitivity also remains a

pressing problem. Missing the correct diagnosis in a mildly symptomatic patient with a true cortisol hypersecreting tumor can delay adequate treatment with potential for an adverse outcome.

Nocturnal salivary cortisol, an early hallmark of hypercortisolism, has been extensively shown in some studies to offer poor sensitivity for SC [1 9 22]. Our data confirm in a large data set a poor discrimination of patients with SC compared to those with AC using salivary free cortisol measurements. Elevations in UFC have been widely used to predict the chronic manifestation of subtle cortisol excess despite the drawbacks due to common sampling errors and problems with cross-reactivity to other steroids in immunoassays. Of note, patients with SC in our study had similar mean levels of UFC as patients without Cushing syndrome. However, UFC provided reasonable separation of AC patients from other groups. In summary, the ROC curves of the routine tests for diagnosis of SC in our study revealed lower diagnostic effectiveness of these methods compared to the selected steroid combination.

Interestingly, of the various tests examined for differentiating patients with and without SC all performed poorly except for the DST. This can be in part explained by the strong emphasis clinicians involved in our study likely placed on the DST, which according to the recently published European guideline [14] classifies autonomous cortisol secretion according to this test. There has been disagreement on the best cut-off for cortisol after DST. A recent study revealed that the optimal value could even be lower than the commonly used value of 1.8 µg/dl (50 nmol/l) [2 26]. The response of cortisol after DST in patients who do not exhibit signs and symptoms specific for AC have to be analyzed together with other results from routine tests. Indeed, some of the patients with positive results of the DST were eventually categorized as NS since there was no evidence of abnormal cortisol excess in other hormonal parameters, making this a source of uncertainty [24]. The more common threshold value of 1.8 µg/dl was used in our study to show that the level of cortisol after the DST remains the most sensitive assay alongside the combination of steroids to subgroup patients with subtle autonomous cortisol hypersecretion.

More recently, the discussion about cut-offs for correct classification of hormonal excess has moved towards prediction of long-term outcomes in SC. It is a well-established concept that hypercortisolism can lead to associated comorbidities, such as metabolic, musculoskeletal and cardiovascular diseases [2], similar to patients with overt Cushing's syndrome. A retrospective study analysed outcome, using the classification of patients with adrenal incidentalomas as non-secreting or as stable SC according to the cortisol levels after DST and in another cohort with increasing cortisol levels on follow-up. This arbitrary classification was able to identify patients with increased cardiovascular events and mortality rates [27].The data were confirmed by two quite similar studies [28 29]. Future studies will need to show whether a classification based on steroid finger printing will make it possible to predict long term outcome in patients with SH.

A number of studies have reiterated the advantages of LC-MS/MS over immunoassays for measurements of steroids [10 11 15]. Here we additionally show that plasma 11-deoxycortisol, 11-deoxycorticosterone, DHEA, DHEA-S and corticosterone can reliably distinguish patients with and without SC. Circulating levels of DHEA-S have been found in the high normal range in patients with ACTH-dependent CS, whereas both AC and SC are characterized by decreased circulating basal concentrations of DHEA-S and DHEA [30-32]. The inverse association of DHEA-S and cortisol excess has been revealed in several studies

to be exclusively associated with adrenocortical adenoma [33] [11]. Indeed our data also confirm lowered DHEA and DHEA-S in patients with AC and SC, supporting previous suggestions that ACTH is a major determinant of their secretion [34 35].

Low levels of progesterone in both SC and AC patients in our study can be explained by two mechanisms. Continuously elevated cortisol levels inhibit the pituitary-gonadal axis leading to anovulatory cycles, prohibiting the action of progesterone in the uterus as well as the secretion of GnRH from the thalamus [36]. This effect likely contributes to low progesterone in our mainly female premenopausal cohort with florid AC. In the gender-balanced, predominantly postmenopausal SC cohort, precursor substrate flow characteristics, enhanced by partially suppressed plasma ACTH, could result in the pattern of low plasma pregnenolone and progesterone concentrations. Indeed, chronic ACTH excess has been shown to influence intraadrenal utilization of delta5-pregnene leading to elevation of pregnenolone [37 38], which is very much in line with our findings.

Our study has several limitations, one of which involved the higher age of the SC than other cohorts and the female predominance of the AC cohort. Since the latter is an established observation in AC [39] and since adrenal incidentalomas that prompt consideration of hypercortisolism are predominantly found in the elderly [2 40], neither of these issues are easily addressed by matched populations. Rather we addressed these potential confounders by use of models and normalizations that took advantage of data from a larger reference population. It nevertheless remains possible that even with these corrections age, sex and menstrual phase differences may have contributed to differing patterns of steroids among AC and SC groups. Another limitation was that the diagnosis and exclusion of hypercortisolism was based on routine tests that are not infallible. Indeed findings that some steroids showed differences between reference and EX groups and that the EX group showed levels of many steroids intermediate between reference and SC and AC groups raises the possibility of milder forms of adrenal cortical dysfunction within the EX group that were not identified by routine tests. Finally, it would have been useful to assess outcomes after adrenalectomy. However, the indication of adrenalectomy for patients with SC is often not achieved.

In conclusion, this study establishes a method to identify patients with SC using LC-MS/MS measurements of a panel of adrenal steroids. Performance of the steroid profile was more advanced than salivary and urinary free cortisol and showed similar accuracy to use of routine test combinations that included the DST. Thus, the plasma steroid panel could serve as a single test alternative to screen for SC or to confirm the findings on DST. Versatility of a single steroid profiling method for detecting other disorders of adrenal steroidogenesis provides another advantage for use of the method in the routine laboratory.

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Disclosure Summary:
The authors declare that they have no financial relationships that could be broadly relevant to the work.
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The authors declare that they have no conflict of interest relevant to this article.

References

1. Debono M, Newell-Price J. Subclinical hypercortisolism in adrenal incidentaloma. *Curr Opin Endocrinol Diabetes Obes* .2015;22(3):185-92.
2. Di Dalmazi G, Pasquali R, Beuschlein F, Reincke M. Subclinical hypercortisolism: a state, a syndrome, or a disease? *Eur J Endocrinol* .2015;173(4):M61-71.
3. Nieman LK. Update on subclinical Cushing's syndrome. *Curr Opin Endocrinol Diabetes Obes* .2015;22(3):180-4.
4. Akaza I, Yoshimoto T, Iwashima F, Nakayama C, Doi M, Izumiyama H, Hirata Y. Clinical outcome of subclinical Cushing's syndrome after surgical and conservative treatment. *Hypertens Res* .2011;34(10):1111-5
5. Chiodini I, Vainicher CE, Morelli V, Palmieri S, Cairoli E, Salcuni AS, Copetti M, Scillitani A. MECHANISMS IN ENDOCRINOLOGY: Endogenous subclinical hypercortisolism and bone: a clinical review. *Eur J Endocrinol*.2016;175(6):R265-r82.
6. Di Dalmazi G, Pasquali R. Adrenal adenomas, subclinical hypercortisolism, and cardiovascular outcomes. *Curr Opin Endocrinol Diabetes Obes* .2015;22(3):163-8.
7. Chiodini I. Clinical review: Diagnosis and treatment of subclinical hypercortisolism. *J Clin Endocrinol Metab*.2011;96(5):1223-36.
8. Doi M, Sekizawa N, Tani Y, Tsuchiya K, Kouyama R, Tateno T, Izumiyama H, Yoshimoto T, Hirata Y. Late-night salivary cortisol as a screening test for the diagnosis of Cushing's syndrome in Japan. *Endocr J*. 2008;55(1):121-6
9. Gagliardi L, Torpy DJ. Subclinical Cushing's syndrome in adrenal incidentaloma: a common problem or an artefact of current diagnostic testing? *Clin Endocrinol (Oxf)*.2010;72(2):277-8.
10. Di Dalmazi G, Fanelli F, Mezzullo M, Casadio E, Rinaldi E, Garelli S, Giampalma E, Mosconi C, Golfieri R, Vicennati V, Pagotto U, Pasquali R. Steroid Profiling by LC-MS/MS in Nonsecreting and Subclinical Cortisol-Secreting Adrenocortical Adenomas. *J Clin Endocrinol Metab*.2015;100(9):3529-38.

11. Eisenhofer G, Masjkur J, Peitzsch M, Di Dalmazi G, Bidlingmaier M, Gruber M, Fazel J, Osswald A, Beuschlein F, Reincke M. Plasma Steroid Metabolome Profiling for Diagnosis and Subtyping Patients with Cushing Syndrome. Clin Chem. 2018;64(3):586-96.

12. Kotlowska A, Puzyn T, Sworczak K, Stepnowski P, Szefer P. Metabolomic Biomarkers in Urine of Cushing's Syndrome Patients. *Int J Mol Sci*.2017;18(2).

13. Kikuchi E, Yanaihara H, Nakashima J, Homma K, Ohigashi T, Asakura H, Tachibana M, Shibata H, Saruta T, Murai M. Urinary steroid profile in adrenocortical tumors. *Biomed Pharmacother*.2000;54 Suppl 1:194s-97s.

14. Shackleton C. Clinical steroid mass spectrometry: a 45-year history culminating in HPLC-MS/MS becoming an essential tool for patient diagnosis. *J Steroid Biochem Mol Biol*.2010;121(3-5):481-90.

15. Wooding KM, Auchus RJ. Mass spectrometry theory and application to adrenal diseases. *Mol Cell Endocrinol* .2013;371(1-2):201-7.

16. Wilkes EH, Rumsby G, Woodward GM. Using Machine Learning to Aid the Interpretation of Urine Steroid Profiles. *Clin Chem*. 2018;64(11):1586-95.

17. Arlt W, Biehl M, Taylor AE, Hahner S, Libe R, Hughes BA, Schneider P, Smith DJ, Stiekema H, Krone N, Porfiri E, Opocher G, Bertherat J, Mantero F, Allolio B, Terzolo M, Nightingale P, Shackleton CH, Bertagna X, Fassnacht M, Stewart PM. Urine steroid metabolomics as a biomarker tool for detecting malignancy in adrenal tumors. *J Clin Endocrinol Metab*.2011;96(12):3775-84.

18. Fassnacht M, Arlt W, Bancos I, Dralle H, Newell-Price J, Sahdev A, Tabarin A, Terzolo M, Tsagarakis S, Dekkers OM. Management of adrenal incidentalomas: European Society of Endocrinology Clinical Practice Guideline in collaboration with the European Network for the Study of Adrenal Tumors. *Eur J Endocrinol*.2016;175(2):G1-G34.

19. Eisenhofer G, Peitzsch M, Kaden D, Langton K, Pamporaki C, Masjkur J, Tsatsaronis G, Mangelis A, Williams TA, Reincke M, Lenders JWM, Bornstein SR. Reference intervals for plasma concentrations of adrenal steroids measured by LC-MS/MS: Impact of gender, age, oral contraceptives, body mass index and blood pressure status. *Clin Chim Acta*.2017;470:115-24.

20. Peitzsch M, Dekkers T, Haase M, Sweep FC, Quack I, Antoch G, Siegert G, Lenders JW, Deinum J, Willenberg HS, Eisenhoher G. An LC-MS/MS method for steroid profiling during adrenal venous sampling for investigation of primary aldosteronism. *J Steroid Biochem Mol Biol*. 2015;145:75-84.

21. Meyer LS, Wang X, Susnik E, Burrello J, Burrello A, Castellano I, Eisenhofer G, Fallo F, Kline GA, Knosel T, Kocjan T, Lenders JWM, Mulatero P, Naruse M, Nishikawa T, Peitzsch M, Rump LC, Beuschlein F, Hahner S, Gomez-Sanchez CE, Reincke M, Williams TA. Immunohistopathology and Steroid Profiles Associated With Biochemical Outcomes After Adrenalectomy for Unilateral Primary Aldosteronism. *Hypertension*. 2018; 72(3):650-657.

22. Eisenhofer G, Dekkers T, Peitzsch M, Dietz AS, Bidlingmaier M, Treitl M, Williams TA, Bornstein SR, Haase M, Rump LC, Willenberg HS, Beuschlein F, Deinum J, Lenders JW, Reincke M. Mass Spectrometry-Based Adrenal and Peripheral Venous Steroid Profiling for Subtyping Primary Aldosteronism. *Clin Chem* 2016;62(3):514-24.

23. Williams TA, Peitzsch M, Dietz AS, Dekkers T, Bidlingmaier M, Riester A, Treitl M, Rhayem Y, Beuschlein F, Lenders JW, Deinum J, Eisenhofer G, Reincke M. Genotype-

Specific Steroid Profiles Associated With Aldosterone-Producing Adenomas. *Hypertension*. 2016;67(1):139-45.

24. Stewart PM. Is subclinical Cushing's syndrome an entity or a statistical fallout from diagnostic testing? Consensus surrounding the diagnosis is required before optimal treatment can be defined. *J Clin Endocrinol Metab*. 2010;95(6):2618-20.

25. Nunes ML, Vattaut S, Corcuff JB, Rault A, Loiseau H, Gatta B, Valli N, Letenneur L, Tabarin A. Late-night salivary cortisol for diagnosis of overt and subclinical Cushing's syndrome in hospitalized and ambulatory patients. *J Clin Endocrinol Metab*.2009;94(2):456-62.

26. Friedman TC. An update on the overnight dexamethasone suppression test for the diagnosis of Cushing's syndrome: limitations in patients with mild and/or episodic hypercortisolism. *Exp Clin Endocrinol Diabetes.* 2006;114(7):356-60.

27. Di Dalmazi G, Vicennati V, Garelli S, Casadio E, Rinaldi E, Giampalma E, Mosconi C, Golfieri R, Paccapelo A, Pagotto U, Pasquali R. Cardiovascular events and mortality in patients with adrenal incidentalomas that are either non-secreting or associated with intermediate phenotype or subclinical Cushing's syndrome: a 15-year retrospective study. *Lancet Diabetes Endocrinol*.2014;2(5):396-405.

28. Morelli V, Reimondo G, Giordano R, Della Casa S, Policola S, Palmieri S, Salcuni AS, Dolci A, Mendola M, Arosio M, Ambrosi B, Scillitani A, Ghigo E, Beck-Peccoz P, Terzolo M, Chiodini I. Long-term follow-up in adrenal incidentalomas: an Italian multicenter study. *J Clin Endocrinol Metab*.2014;99(3):827-34.

29. Debono M, Bradburn M, Bull M, Harrison B, Ross RJ, Newell-Price J. Cortisol as a marker for increased mortality in patients with incidental adrenocortical adenomas. *J Clin Endocrinol Metab*.2014;99(12):4462-70.

30. Boizel R, de Peretti E, Cathiard AM, Halimi S, Bost M, Berthezene F, Saez JM. Pattern of plasma levels of cortisol, dehydroepiandrosterone and pregnenolone sulphate in normal subjects and in patients with homozygous familial hypercholesterolaemia during ACTH infusion. *Clin Endocrinol (Oxf)*. 1986;25(4):363-71

31. El Asmar N, Rajpal A, Selman WR, Arafah BM. The Value of Perioperative Levels of ACTH, DHEA, and DHEA-S and Tumor Size in Predicting Recurrence of Cushing Disease. *J Clin Endocrinol Metab*.2018;103(2):477-85.

32. Francucci CM, Caudarella R, Rilli S, Fiscaletti P, Ceccoli L, Boscaro M. Adrenal incidentaloma: effects on bone metabolism. *J Endocrinol Invest*.2008;31(7 Suppl):48-52

33. Yener S, Yilmaz H, Demir T, Secil M, Comlekci A. DHEAS for the prediction of subclinical Cushing's syndrome: perplexing or advantageous? *Endocrine*. 2015;48(2):669-76.

34. Morio H, Terano T, Yamamoto K, Tomizuka T, Oeda T, Saito Y, Tamura Y, Sasano H. Serum levels of dehydroepiandrosterone sulfate in patients with asymptomatic cortisol producing adrenal adenoma: comparison with adrenal Cushing's syndrome and non-functional adrenal tumor. *Endocr J*.1996;43(4):387-96.

35. Yamaji T, Ishibashi M, Sekihara H, Itabashi A, Yanaihara T. Serum dehydroepiandrosterone sulfate in Cushing's syndrome. *J Clin Endocrinol Metab*.1984;59(6):1164-8.

36. Lee SM, Hahm JR, Jung TS, Jung JH, Kang MY, Kim SJ, Chung SI. A case of Cushing's syndrome presenting as endometrial hyperplasia. *Korean J Intern Med*. 2008;23(1):49-52.

37. Bermudez JA, Lipsett MB. Early adrenal response to ACTH; Plasma concentrations of pregnenolone, 17-hydroxypregnenolone, progesterone, and 17-hydroxyprogesterone. *J Clin Endocrinol Metab*.1972;34(1):241-3.

38. McKenna TJ, Miller RB, Liddle GW. Plasma pregnenolone and 17-OH-pregnenolone in patients with adrenal tumors, ACTH excess, or idiopathic hirsutism. *J Clin Endocrinol Metab* .1977;44(2):231-6.

39. Pecori Giraldi F, Moro M, Cavagnini F, Study Group on the Hypothalamo-Pituitary-Adrenal Axis of the Italian Society of E. Gender-related differences in the presentation and course of Cushing's disease. *J Clin Endocrinol Metab* .2003;88(4):1554-8.

40. Haan RR, Visser JBR, Pons E, Feelders RA, Kaymak U, Hunink MGM, Visser JJ. Patient-specific workup of adrenal incidentalomas. *Eur J Radiol Open.* 2017;4:108-14.

41. Masjkur J, Gruber M, Peitzsch M, Kaden D, Di Dalmazi G, Bidlingmaier M, Zopp S, Langton K, Fazel J, Beuschlein F, Bornstein SR, Reincke M, Eisenhofer G. Data from: Plasma steroid profiles in subclinical compared to overt adrenal Cushing's syndrome. *OpARA - Open Access Repository and Archive 2019.* Deposited 28.03.2019. https://opara.zih.tu-dresden.de/xmlui/handle/123456789/1389

Figure 1. Results of steroids of the 15-steroid panel in patients with ACTH-independent Cushing's syndrome (AC and SH) and in patients in whom the disease was excluded compared to that of the reference population. Values of all steroids are shown as least square means corrected for age and sex and determined from exponents of logarithmically-transformed data (i.e geometric means) with similarly determined positive and negative standard errors. All figures depict plasma steroid concentration in ng/mL. *P<0.05, different from reference; †P<0.05 different from excluded; §P<0.05, different from adrenal Cushing.

Figure 2. Results of model comparisons of the steroid profile and routine diagnostic test. ROC curves are shown altogether in one panel for the 5 routine screening tests and a selection of 14 steroids of the steroid panel, which exhibit optimal discrimination of patients with ACTH-independent Cushing's syndrome to patients in whom the disease was excluded. Prediction accuracy of each group was measured as area under the curve (AUC) in related table. The groups are distinguished by different color lines.

Figure 3. Results of discriminant analyses for use of routine diagnostic tests (A,B,C) compared to 14 steroids of the steroid panel (D,E,F) that provided optimal discrimination of the 3 patient groups (adrenal Cushing ▲ , subclinical hypercortisolism ●, excluded ◆). Two-dimensional canonical plots are shown in panels B and E; ROC curves with areas under curves are shown in panels A and D; whereas predicted versus actual groupings according to discriminant analyses are shown in panels C and F. Routine diagnostic tests included measurements of salivary and urinary free cortisol, DST and basal plasma cortisol, and basal plasma ACTH. Steroids for the selected steroid profile included 11-deoxycortisol, 11-deoxycorticosterone, DHEA, DHEA-S, androstenedione, aldosterone, cortisol, corticosterone, cortisone, 18-hydroxycortisol, 21-deoxycortisol, 17-hydroxyprogesterone, progesterone and pregnenolone. For the analyses of routine clinical tests, complete results for all 5 tests were only available in 169 of the 208 patients.

Table 1. Characteristic of data and routine biochemical test results for patients screened for Cushing's syndrome according to diagnosis

Group of Subjects	Demographics			Biochemistry				
	N	Gender	Age	Cortisol				ACTH
				DX Test (μg/dL)$^\phi$	Salivary (ng/mL)$^\phi$	Serum (ng/mL)$^\phi$	Urinary free (μg/24hr)$^\phi$	(pg/mL)$^\phi$
Reference	277	99/178	47 (19-81)					
Excluded	152	58/94	48 (16-80)	1.1 (0.5-4.8)	1.1 (0.1-14.9)	84 (47-286)	62 (4-191)	17 (1-244)
Subclinical Cushing	35	17/18	65* § (45-80)	3.6 *§ (1.8-20.6)	2.4* (0.2-9)	74§* (4.7-257)	63§ (5-145)	12§* (1-40)
Adrenal Cushing	21	3/18	46 (17-72)	14* (2.7-23.9)	3.9* (0.3-11.8)	156* (21-271)	599* (82-195)	4* (2-35)

$^\phi$ Data for age and routine biochemistry are shown as median and ranges. ACTH, adrenocorticotropin. DX, dexamethasone ; * p<0.05, different from excluded; § p<0.05, different from adrenal Cushing; ¶ p<0.05, different from reference

**Table 2.** Effect of age and gender in Plasma concentration of adrenal steroids in all groups of population

| Steroid	Model Effect p-value		
	Grouping	Age	Gender
11-Deoxycortisol	<.0001*	0.7802	<.0001*
11-Deoxycorticosterone	<.0001*	0.2416	0.8601
DHEA	<.0001*	<.0001*	0.1527
DHEA-SO4	<.0001*	<.0001*	<.0001*
Aldosterone	0.2236	0.0058*	0.7109
Androstenedione	0.0012*	<.0001*	0.0098*
18-Hydroxycortisol	<.0001*	0.1432	<.0001*
Pregnenolone	<.0001*	<.0001*	0.1417
Cortisol	0.0001*	<.0001*	0.9772
18-oxocortisol	<.0001*	0.0206*	0.0037*
Cortisone	0.1724	<.0001*	0.0044*
21-Deoxycortisol	0.2211	0.4671	0.0152
Corticosterone	0.0147*	<.0001*	0.5445
Progesterone	0.0178*	<.0001*	<.0001*
17-Hydroxyprogesterone	0.4040	<.0001*	<.0001*

*Significantly involved effect

Table 3. Probability models of steroid profile compared to all five routine diagnostic tests

| Probability (Subclinical Cushing) Predictor	Vs. Probability (Subclinical Cushing) Predictor	Probability >Chi2
Steroid Profile	DST Serum Cortisol	0.4053
Steroid Profile	Saliva Free Cortisol	<.0001*
Steroid Profile	Basal Plasma Cortisol	<.0001*
Steroid Profile	Basal Plasma ACTH	<.0001*
Steroid Profile	Urinary Free Cortisol	<.0001*
DST Serum Cortisol	Saliva Free Cortisol	<.0001*
DST Serum Cortisol	Basal Plasma Cortisol	<.0001*
DST Serum Cortisol	Basal Plasma ACTH	<.0001*
DST Serum Cortisol	Urinary Free Cortisol	<.0001*
Saliva Free Cortisol	Basal Plasma Cortisol	0.3550
Saliva Free Cortisol	Basal Plasma ACTH	0.0344*
Saliva Free Cortisol	Urinary Free Cortisol	0.6491
Basal Plasma Cortisol	Basal Plasma ACTH	0.1534
Basal Plasma Cortisol	Urinary Free Cortisol	0.6828
Basal Plasma ACTH	Urinary Free Cortisol	0.1631

*Significantly distinguished
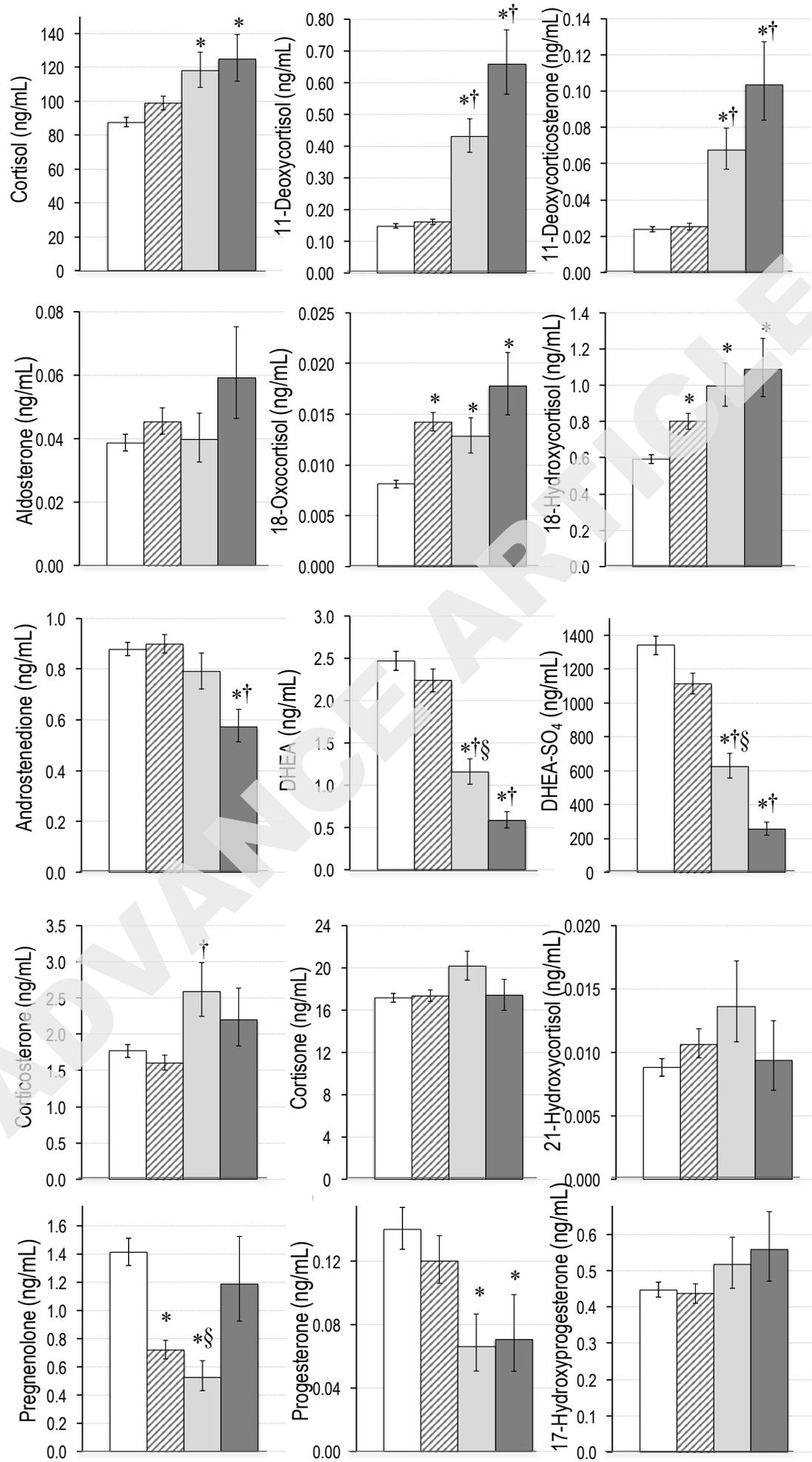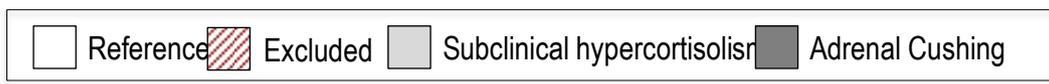
Predictor	AUC
Probability (Subclinical Cushing)-Steroid Profile	0.9577
Probability (Subclinical Cushing)-DST Serum Cortisol	0.9421
Probability (Subclinical Cushing)-Saliva Free Cortisol	0.7143
Probability (Subclinical Cushing)-Basal Plasma Cortisol	0.6441
Probability (Subclinical Cushing)-Basal Plasma ACTH	0.5683
Probability (Subclinical Cushing)-Urinary Free Cortisol	0.6810
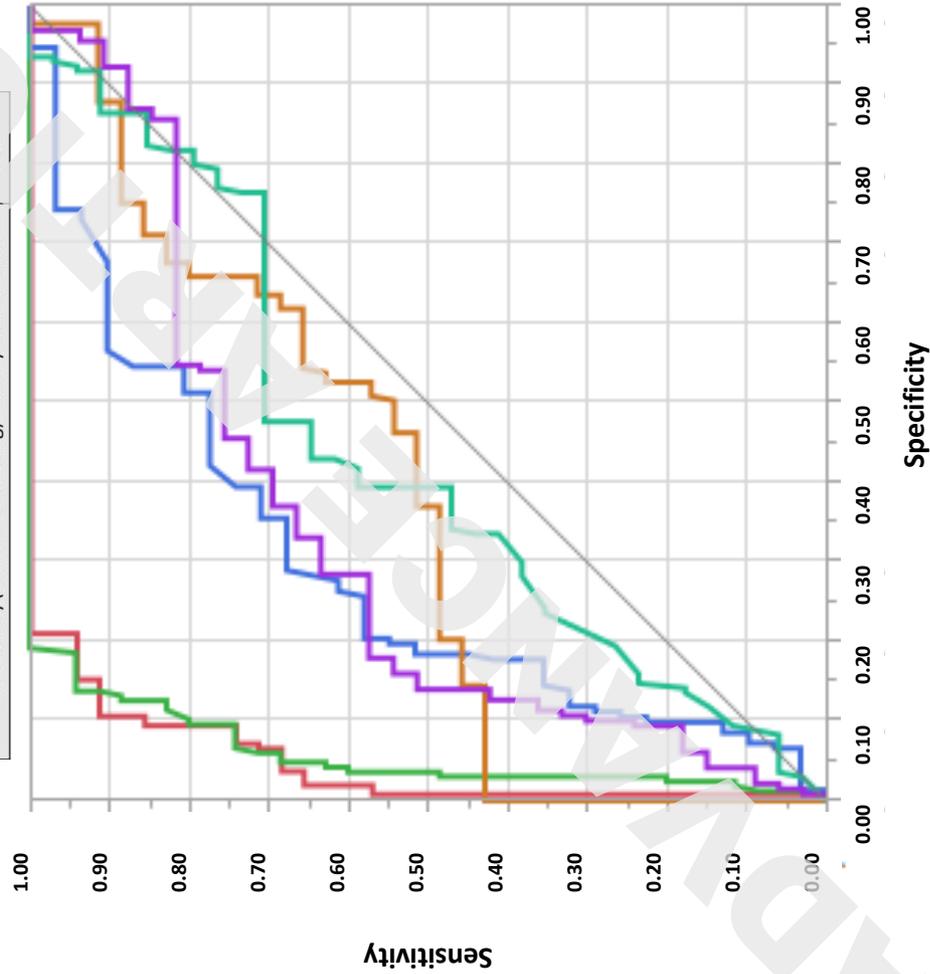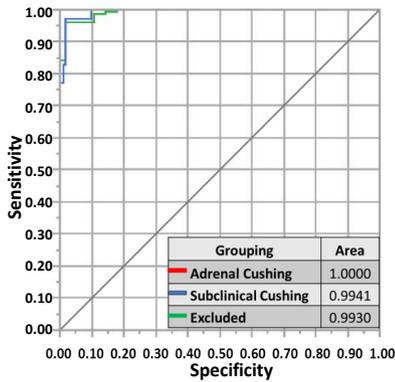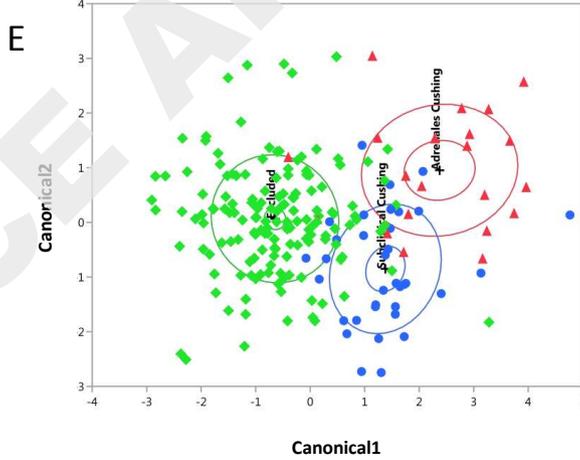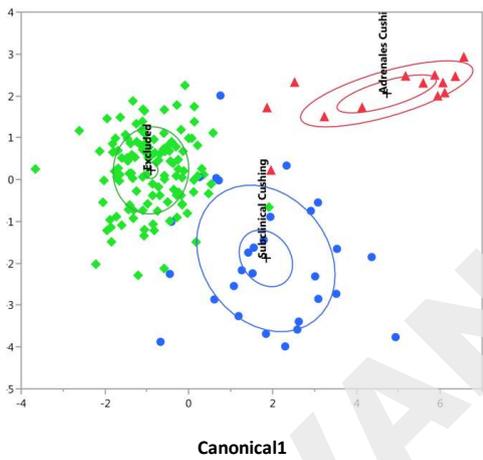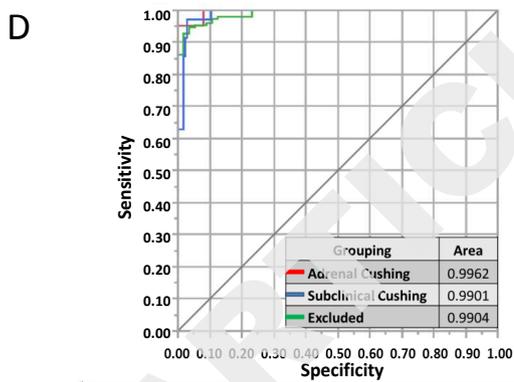
Routine Test

Grouping	Area
Adrenal Cushing	1.0000
Subclinical Cushing	0.9941
Excluded	0.9930

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	13	0	0	100%
Subclinical Cushing	0	24	5	83%
Excluded	2	3	122	96%

Steroid Profile

D

Grouping	Area
Adrenal Cushing	0.9962
Subclinical Cushing	0.9901
Excluded	0.9904

E

F

Actual Grouping	Predicted Grouping			
	Adrenal Cushing	Subclinical Cushing	Excluded	
Adrenal Cushing	20	0	1	95%
Subclinical Cushing	0	34	1	97%
Excluded	3	5	144	95%