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## **Analysis of steroid profiles by mass spectrometry: a new tool for exploring adrenal tumors?**

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## **Abstract**

The assay of multiple steroids by mass spectrometry coupled with chromatography, combined with data analysis using an artificial intelligence approach, has become more widely accessible in recent years. Multiple applications for this technology exist for the study of adrenocortical tumors. Taking advantage of the capacity of malignant cortical tumor secretion of non-bioactive precursors, it provides an additional diagnostic approach that can point to the nature of a tumor. These encouraging perspectives have been based to date only on pilot retrospective studies. However, this has changed in 2020 with the publication of data from the EURINE-ACT study. This very large prospective European study provided more nuanced evidence for the benefit of combining the measurement of a panel of steroids with essential imaging tools. This study also facilitated our understanding and provided more precise characterisation of autonomous steroid secretion, particularly in the case of subclinical cortisol-secreting adrenocortical adenomas. This article will focus on our current knowledge on the potential utility of mass spectrometry for diagnosis of both the nature of an adrenal tumors and their secretion.

**Key words:** Mass spectrometry, adrenal steroids, machine learning, adrenal tumors, adrenocortical carcinoma, autonomous cortisol secreting adenoma

## **I- Introduction**

The biosynthesis of steroid hormones from cholesterol occurs in the adrenal cortex, the gonads and in some peripheral tissues. In the adrenal cortex, all steroids are synthesised via the actions of enzymes from the steroid dehydrogenase and cytochrome P450 families (Figure 1). In clinical practice, in terms of adrenal tumor pathology, it is the terminal products of steroidogenesis, aldosterone, cortisol and androgens, that are measured. Nevertheless, measurement of hormone precursors and their metabolites can be of use in several clinical situations which will be outlined in this article.

## **II- Principle of mass spectrometry coupled with chromatography**

Steroid hormones and their metabolites are first extracted from a urine or blood sample. The second step consists of separating the steroids by either liquid or gas chromatography. Gas chromatography requires more pre-analytical steps than liquid chromatography, including extraction, evaporation, and chemical derivation steps. After this step, steroids are then ionised, charged and detected after their passage through the quadrupoles (where they are fragmented), according to their mass/charge ratio.

Currently, mass spectrometry coupled with gas chromatography (GC-MS) remains less feasible in clinical practice due to the constraints of the various pre-analytical steps. Conversely, the simplified pre-analytical steps in liquid chromatography, and the commercialization of standardised assay kits that are 'ready to use', has enabled greater use of steroid assays by tandem mass spectrometry coupled with liquid chromatography (LC/MS-MS) in endocrine laboratories.

Compared to older immunoassay techniques, the major analytical advantage of mass spectrometry is its excellent specificity (1). Some learned societies actually advocate relying exclusively on this assay technique, notably in the case of androgen assays (2). Older immunoassay techniques lacked specificity due to cross-reactivity, resulting from the structural similarity of the steroid molecules being measured (Figure 1).

Several clinical situations illustrate this problem of a lack of specificity. An example is the administration of exogenous glucocorticoids which can be the cause of iatrogenic Cushing's syndrome. The assay of prednisone, a glucocorticoid used as an anti-inflammatory drug, can lead to an over-estimation of free urinary cortisol concentration and thus give rise to a false suspicion of endogenous hypercortisolism (3).

The surveillance of patients being treated with steroidogenesis inhibitors, such as metyrapone or osilodrostat, represents another example. These drugs act by blocking the enzymatic activity of 11 beta-hydroxylase, leading to an accumulation of 11-deoxycortisol. Older immunoassay techniques for cortisol could falsely recognise this molecule, which is structurally very similar to cortisol (4) and thus over-estimate the serum cortisol concentration, wrongly suggesting that the drug treatment was ineffective.

The question remains as to whether there is a clinical benefit in exclusively using the LC/MS-MS assay technique despite it being more onerous and more technically demanding. In fact, Oßwald et al. recently compared the assay performance of LC/MS-MS to that of two new immunoassay techniques, ADVIA Centaur (Siemens) and LIAISON (Diasorin), for measuring urinary free cortisol (5). The concentrations of free urinary cortisol, measured by the two immunoassays, were higher than those measured by LC/MS-MS, probably due to detection of particular interfering steroids. However, the concentrations of free urinary cortisol levels found by these two immunoassay methods correlated very well with the levels measured by LC/MS-MS :  $r=0.96$  and  $r=0.99$  respectively ( $p<0.001$ ). Notably, after analysis of ROC curves, the diagnostic performances of these two methods were not significantly different from that of LC/MS-MS for the diagnosis of hypercortisolism. Therefore, an equivalent diagnostic precision to mass spectrometry can be obtained using some currently available immunoassays.

### **III Mass spectrometry and *machine learning***

Aside from analytical considerations, mass spectrometry coupled with chromatography has the capacity to measure a panel of steroids in a single sample. In view of the complexity of analysing

multiple steroids, the potential benefit of machine learning techniques makes sense. The principal is as follows: through artificial intelligence, the machine acquires competency without the need for human input. During the first phase of apprenticeship, the computer produces a probabilistic algorithm based on known data or known observations. In an example based on our topic, the computer would first learn which steroids are associated ‘most probably’ with an adrenal tumor of etiology A, or with a tumor of etiology B.

Next, the computer would ‘indicate a probable diagnosis’, remembering that it is a probabilistic tool. For example, if the panel of steroids for tumor X corresponds with diagnosis A then the tumor is very probably a tumor of etiology A.

There are as many machine learning processes as there are potential clinical applications and biostatistical methods. A recent article has described the application of various algorithms of machine learning that permitted automatic interpretation of urinary steroid profiles in diverse clinical settings (from adrenal enzymatic blockade to secreting adrenocortical adenomas) (6).

#### **IV Diagnosis of adrenal tumor malignancy**

Malignant adrenocortical tumors (*Adrenocortical carcinoma, ACC*) are rare cancers with an annual incidence estimated at between 0.5 and 2 cases per million population (7). Its prognosis remains very poor, particularly for metastatic forms of the disease (8). Complete surgical resection of a localised ACC provides the only possibility for cure. Research on clinical, morphological, and equally, on biochemical signs that could be markers of malignancy in adrenal tumors is thus crucially important. The goal in this situation is to direct a patient to an experienced surgeon without delay (8).

Reports of individual cases and series with limited patient numbers have described elevated concentrations of steroid precursors secreted by ACC when compared to cortical adenomas (9,10,11). One of the hypotheses to explain this is a possible dedifferentiation of malignant adrenal cortical cells with the loss of the full complement of enzymes not permitting complete steroidogenesis to occur. The presence of elevated steroid precursors therefore orients the diagnosis towards an ACC.

In 2011, Arlt et al. published data from a large series of adrenal tumors from the ENSAT network (12). This study retrospectively compared the profiles of 32 steroids, analysed by GC-MS, in 24-hour urine samples from 102 patients presenting with cortical adenomas and 45 patients with ACC. These 32 urinary steroids were present in very different concentrations in the two groups, revealing a secretory biological signature that was significantly associated with ACC.

The ‘*machine learning*’ step (*Generalized Matrix Learning vector Quantization* GMLVQ, in this study), allowed the identification of a panel of 9 steroids that are the most discriminating in terms of diagnosis of ACC. After analysis of ROC curves, this panel was able to diagnose ACC with a sensitivity and specificity of 88%. The steroids that provided the best discrimination were metabolites and precursors of glucocorticoids and androgens. Notably, elevation of THS (tetrahydro-11-deoxycortisol), a metabolite of 11 deoxycortisol, elevation of pregnenediol, a metabolite of 17-OH pregnenolone, as well as elevation of pregnenetriol, a metabolite of 17-OH progesterone, which have been previously mentioned in earlier studies.

The exhaustive analysis of urinary steroid metabolites by mass spectrometry allowed close to 90% of ACC cases to be diagnosed, according to results from this first study. These data confirmed the greater secretion of non-bioactive precursors by ACC in comparison to benign cortical adenomas. However, it is important to take into account the limitations of this first retrospective study, where more than 2/3 of the ACC cases were metastatic, and consequently presented no real diagnostic challenge. Later studies have since reported similar results (13,14).

Two subtle differences need to be kept in mind concerning the recent study by Schweitzer et al., these being that the panel of steroids was extracted from a plasma sample and that analysis was carried out using LC-MS/MS (15). These two methodological considerations make the method more feasible in clinical practice. Furthermore, the study evaluated the performance of a panel of 14 steroids in the diagnosis of tumor type in adrenal tumors. To this end, the authors compared the steroid profiles of 42 patients with ACC to 66 with cortical adenomas. Additionally, the study took into account the sex of the patient in interpretation of results.

The positive predictive values (PPV) of a panel of 6 steroids for diagnosis of ACC was 92 and 96% for males and females respectively, while the negative predictive values (NPV) were respectively 90 and 86%. These first retrospective studies suggest the potential for the use of mass spectrometry combined with *machine learning* for the diagnosis of tumor type in adrenal tumors. Its practical utility in clinical practice remains to be demonstrated in a large prospective series of patients with adrenal tumors where the tumor type is not clearly known.

Very recently, the EURINE-ACT study has provided additional arguments in favor of this biochemical method (16). This prospective, multicenter study, included 2,017 adrenal tumors enrolled over a period of 5 years, illustrating the strength of the European ENSAT network. The study design mirrored the current clinical practice of clinical endocrinologists. The aim of the study was to evaluate the diagnostic performance of a panel of urinary steroids combined with **two other essential tools** for the diagnosis of malignancy in an adrenal tumor; the tumor size and its imaging characteristics (on CT, MRI or FDG-PET). In this study the panel of urinary steroids, measured by LC-MS/MS, was stratified into 3 categories for malignancy risk (low, intermediate or high), based on a *machine learning* algorithm.

This very large study provides epidemiological confirmation of the distribution of adrenal tumor types. In more than 20 endocrine recruitment sites, aside from known neoplasms and excluding pheochromocytomas, more than 90% of tumors were cortical adenomas and only 5% were ACC.

The study first evaluated the performance of imaging techniques in diagnosis of tumor type. In this large series, only 2/98 ACC presented with a tumor size less than 4 cm. The size of adenomas was more variable, though more than 80% of these were smaller than 4 cm. Clearly, ACC are thus large-sized tumors.

In terms of imaging characteristics, all of the cortical adenomas in the series were homogeneous, unlike 68% of the ACC which appeared heterogeneous. Lastly, only two cases of malignant tumors (including one ACC) had a spontaneous density < 20 Hounsfield Units (HU). The combination of size and spontaneous density of the tumor is useful for excluding malignancy: in this series no ACC



presented with a size smaller than 4 cm AND had a spontaneous density of less than 20 HU. These data would thus suggest that the threshold of 10 HU proposed in the 2016 ESE recommendations (17) should be raised to 20 HU, and confirm the data of recent publications. In fact, based on results from a French (Bordeaux) cohort, the combination of these two criteria, that is size > 4 cm and spontaneous density < 20 HU, are associated with a PPV of 98.6% for diagnosis of a benign adenoma (18).

Individually, the positive predictive value for a high risk steroid profile was greater than that for the other two tools (size and suspicious imaging characteristics) for the diagnosis of an ACC, being 34.6% vs 19.7%. In other words, 34.6% of tumors presenting a high risk steroid profile in this series were ACC. Thus, more than 65% of tumors considered as 'high risk', based on their steroid profile, are false positives (i.e. not ACC). This result brings into question the **individual** benefit of using the steroid panel in diagnosis of malignancy of an adrenal tumor. The PPV increases when the diagnostic methods are combined two at a time, being 64.1% when the high risk steroid panel is combined with tumor size > 4 cm, and 65.6% when the high risk steroid panel is combined with suspicious imaging characteristics.

The value is in combining the three diagnostic tools. In this case, when an adrenal tumor is:

- greater than 4 cm in size
  - presents with suspicious imaging characteristics (density, heterogeneity),
  - and presents with a high risk profile for urinary steroid panel
- ... the PPV was 76.4%, meaning that more than ¾ of these tumors were ACC

In Figure 2 we summarise these results and outline the benefit of using the steroid panel data combined with the two imaging parameters (size > 4 cm and suspicious imaging characteristics). In the case of a tumor with a steroid panel considered 'high risk', and also high risk according to imaging criteria, we recommend that immediate surgery is proposed, since the PPV for an ACC is greater than 75%. Conversely, in cases with a low risk profile, a strategy of short term surveillance can be suggested in view of the low probability (<3%) of the tumor being an ACC. If in the above cases the two strategies can be justified, the decision becomes more difficult in the case of tumors classified as

'intermediate risk' where the treatment strategy needs to be discussed by expert teams. This last scenario illustrates well the limits of these probabilistic tools. Mass spectrometry coupled with chromatography appears to be a useful tool for diagnosing the nature of an adrenal tumor. The multiparameter assay has the advantage of showing a biochemical secretory signature of ACC, particularly of hormone precursors. However, the conclusions of the EURINE-ACT study cast doubt on the individual benefit for diagnosing malignancy, while a combined 'triple test' strategy represents an interesting possibility for increasing the PPV for an ACC, which would allow a rapid decision concerning surgery of these tumors to be made.

## V Characterization of secretory phenotype

### a. Autonomous cortisol secreting adenoma (ACSA)

While the diagnosis of a clear cortisol-secreting adenoma poses no real difficulty, autonomic secretion of low cortisol levels is much more difficult to detect using currently available biochemical methods. These autonomous cortisol secreting adenomas (ACSA) by definition do not lead to an overt Cushing's syndrome. However, their secretory autonomy is responsible for a 'braking' effect on the hypothalamic-pituitary-adrenal (HPA) axis of varying intensity.

The overnight dexamethasone suppression test (ODST) is currently the testing method that is recommended by the European Society of Endocrinology (17). In fact, this test shows the degree of **autonomy** of cortical adenoma secretion, but not the level of secretion. A post-suppression test cortisol level of  $<50$  nmol/L excludes secretory autonomy and has an excellent sensitivity in testing for ACSA (17). However, the limitations of the test must be kept in mind, notably the rate of false-positives. Rapid clearance or poor digestive tract absorption of dexamethasone can explain false-positives for the ODST. By using simultaneous measurement of serum cortisol and dexamethasone in a large cohort of patients presenting with an adrenal incidentaloma and in control subjects, Ueland et al. showed that a 'low serum concentration' of dexamethasone was responsible for 12 – 22% of false-positives in patients with an adrenal incidentaloma (19). Patients taking oral estrogens also represent a classic cause of false-positives, due to the increase in hepatic secretion of cortisol binding globulin (CBG), which binds cortisol.

For diagnosis of an ACSA, it is expected that measurement of ACTH will give low plasma concentrations in the morning. However, in a series of 198 adrenal incidentalomas, a discordance was found between the plasma concentration of ACTH at 8:00 am and cortisol levels after ODST in 27% of cases (20). The biochemical methods available for evaluating the HPA axis are thus imperfect. The question can be posed 'is there a benefit of using mass spectrometry in diagnosis of ACSA?'

In a recent series published in 2019, Masjkur et al. compared a panel of plasma steroids measured using LC/MS-MS in 277 control subjects, 152 subjects presenting with non-functional adrenal incidentalomas, 35 patients presenting with ACSA and in 21 subjects with an adrenal adenoma causing overt Cushing's syndrome (21). A post ODST cortisol level of > 50 nmol/L was used to define ACSA (17). The secretory profiles of patients with an ACSA were qualitatively equivalent, though proportionally smaller, to those patients presenting with a cortical adenoma responsible for overt Cushing's syndrome.

Thus, similar to cortical adenomas responsible for overt Cushing's syndrome, ACSA show lower concentrations of DHEA, DHEAS and progesterone, but higher concentrations of pregnenolone, 11-deoxycortisol and 11-deoxycorticosterone compared to normal subjects and to those with non-functional incidentalomas.

Using ROC analyses, the authors compared the diagnostic performance, for identifying ACSA, of a panel of 14 steroids to several classical static biochemical methods: free urinary cortisol concentration, plasma ACTH concentration and serum or salivary cortisol concentration. The diagnostic performance of the steroid panel was better than each of the static biochemical tests ( $p < 0.01$  for comparison of AUC-ROC for the panel against that of each biochemical test) (Figure 3). Overall, the panel of steroids was able to distinguish patients presenting with ACSA from those with non-functional incidentalomas with the same precision as the overnight dexamethasone suppression test. In addition, the combination of routine biochemical methods had performances equivalent to using the steroid profile alone in diagnosing non-functional adenomas, ACSA, and overt Cushing's syndrome. In summary, it is striking that the steroid panel, which requires only a single venous blood sampling, has identical performance to a panel of biochemical tests.

Mass spectrometry could be used to replace the ODST or be used to confirm results of that test. For this reason the use of the steroid panel may be beneficial in those situations, as mentioned above, where the ODST may not always give accurate results. The identification of ACSA is important. Besides the problem of their diagnosis, discussed above, there is the question of their somatic impact in the absence of comorbidities specific to hypercortisolism. Numerous cross-sectional studies have shown an increased prevalence of hypertension or type 2 diabetes in patients presenting with an ACSA when compared to patients with non-functional adenomas (22). In addition, three studies of large independent cohorts, with long-term follow-up, reported a higher incidence of cardiovascular disease in patients with ACSA compared to patients presenting with a non-functional adrenal incidentaloma (23, 24, 25). The study of Di Dalmazi et al., even showed increased mortality from cardiovascular causes in the ACSA group, compared to non-functional incidentalomas (23). Lastly, according to the analysis in the study of Debono et al., the cortisol concentration after a ODST constitutes an independent marker of cardiovascular events (24).

More recently, Morelli et al., carried out a retrospective cross-sectional analysis of 518 patients presenting with an adrenal incidentaloma who were followed for a median duration of 13 years (26). These authors used a sophisticated mathematical model, an *artificial neural network*, which has the goal, in brief, of finding associations and statistical interactions between different parameters. They found that for each increase in plasma cortisol of 1 µg/dL after administration of 1 mg of dexamethasone, the risk of a cardiovascular event increased by 1.3. Certainly, this represents a statistical approach and thus does not confirm causality. However, the hypothesis of a deleterious effect of autonomous cortisol secretion is plausible, independent of cardiovascular comorbidities in these patients.

In addition to the specific pathogenic role of cortisol suggested in ACSA, what is the role of other secreted steroids? Could the use of mass spectrometry possibly improve the phenotyping of these patients, for example by identifying the patients with a higher cardiovascular risk?

Di Damalzi et al. recently reported that patients with ACSA (n=46) presented with basal concentrations of corticosterone and cortisol levels that were higher than in patients with non-

functional adenomas (n=120) (27). Additionally, the subjects with ACSA showed reduced suppression of the concentrations of these steroids after an overnight dexamethasone suppression test. These results raise the hypothesis that there is a degree of autonomous secretion of corticosterone in ACSA.

In the basal situation, the concentrations of cortisol and corticosterone, post 1mg dexamethasone, were significantly associated with the prevalence of severe resistant hypertension (defined by prescription of more than 3 anti-hypertensive drugs). During a median follow-up of 3 years, patients presenting with ACSA had more cardiovascular events and hypertension than patients with non-functional adenomas. By multivariate analysis (taking into account cardiovascular risk factors), the basal concentration of corticosterone secreted by an ACSA was an independent marker of the appearance of these events. An elevation of 1ng/mL in corticosterone concentration was associated with an increased cardiovascular risk of 1.06 (p=0.031).

This very preliminary study suggests a specific role for the secretion of corticosterone, a metabolite precursor of mineralocorticoids, in cardiovascular morbidity in ACSA, likely acting via mineralocorticoid receptors.

Certainly, these results need to be confirmed. However, analysis of the steroid profile of these benign cortical tumors shows a heterogeneity of secretion, and suggests some 'leakage' between the different steroidogenesis pathways.

#### b- Primary hyperaldosteronism

Until recently, primary hyperaldosteronism, seen in Conn's adenoma was considered as uniquely an alteration in mineralocorticoid secretion. However, in 2017, Arlt et al., showed that some cases of Conn's adenoma also present with urinary excretion of cortisol and glucocorticoid metabolites, in similar proportions to steroid excretion in some cases of ACSA (28).

Immunohistochemical and immunofunctional study of these tumors have even shown that some Conn's adenomas excrete more glucocorticoids due to an increased expression of CYP11beta2, a key enzyme in glucocorticoid synthesis! Such co-secretion of cortisol and aldosterone in hyperaldosteronism has been named '*Connshing Syndrome*'. This may represent one of the

pathophysiological pathways explaining the greater prevalence of type 2 diabetes, osteoporosis and metabolic syndrome in patients with primary aldosteronism, compared with those that present with a so-called 'standard' hypertension. Does this, therefore, mean the end of the dichotomy between Conn's and Cushing's syndromes?

## **Conclusion**

The multiparametric approach to measuring steroids using mass spectrometry coupled with *machine learning* is a new tool which is increasingly available thanks to the technological progress in endocrine laboratories. The possibilities for its use in adrenal tumor pathology are extensive.

Belief in the potential of mass spectrometry for the exploration of adrenal tumors follows the same classical path seen for all new scientific tools. The first publications were met with great enthusiasm by the scientific community, both for studying ACC (12) and for primary hyperaldosteronism (28). However, some reservations are in order, as are shown by more recent publication of studies with a higher level of proof [for example the EURINE-ACT study (16)]. Finally, though the theoretical concept is 'solid', the real clinical benefit remains to be demonstrated.

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Figure 1: The 'steroid universe': schematic diagram of adrenal steroidogenesis

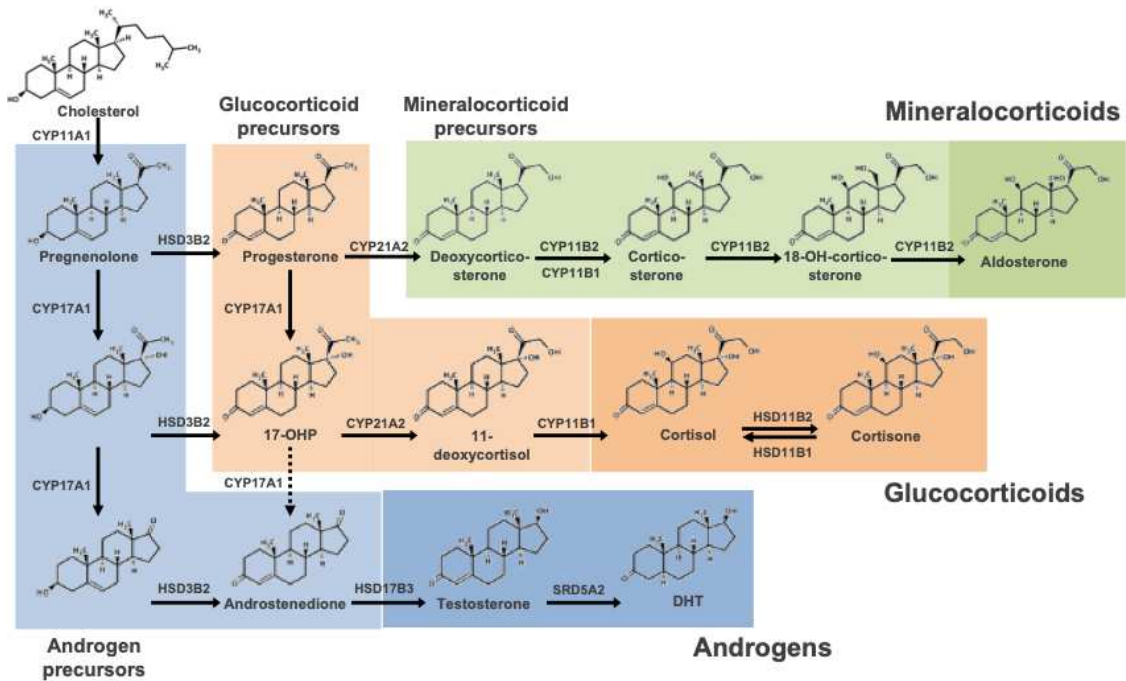


Figure 2: Diagnostic algorithm, adapted from the EURINE-ACT study, Bancos et al. (16)

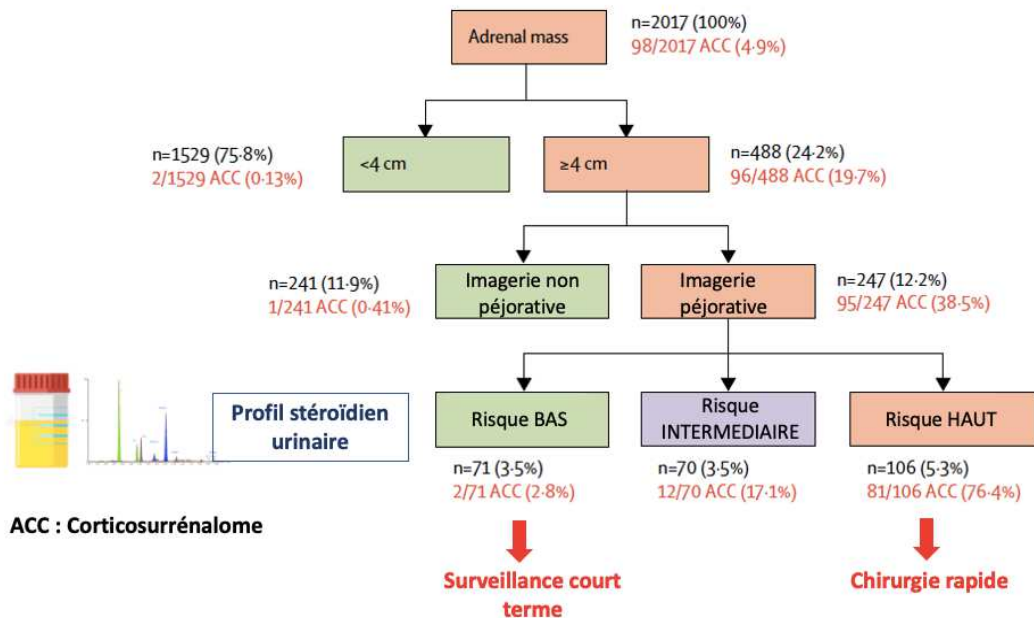
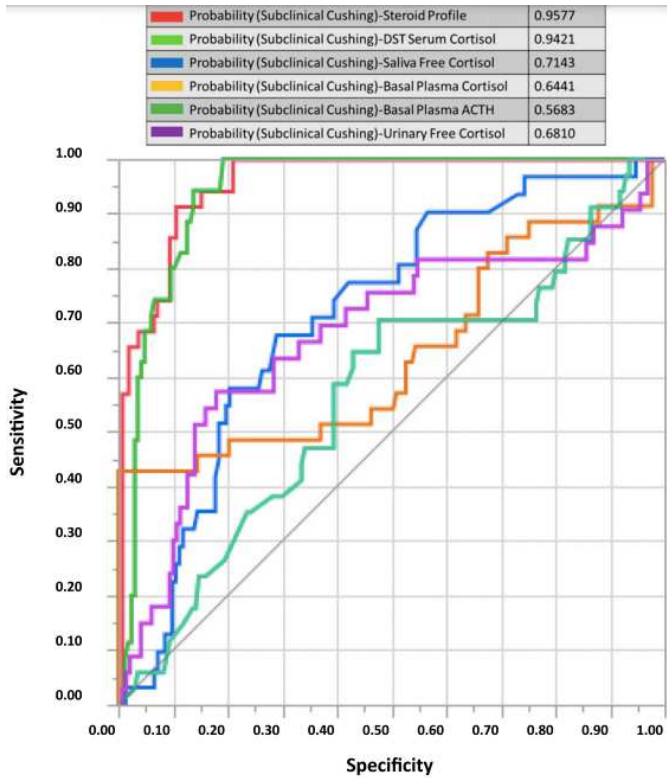


Figure 3: ROC curves showing diagnostic performances for each biochemical test used in ACSA, compared to the ROC curve for the steroid profile in red (adapted from Majkur et al (21)).





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