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Pharmacological and analytical interference in hormone assays for diagnosis of adrenal incidentaloma
Interférences pharmacologiques et analytiques des dosages hormonaux pour le diagnostic des incidentalomes surrénaliens

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Abstract
Adrenal incidentaloma refers to an asymptomatic adrenal mass detected through an imaging procedure performed for reasons unrelated to adrenal dysfunction or suspected dysfunction. In general, adrenal incidentalomas are non-functioning adrenal adenomas, but in some cases, may require therapeutic intervention: e.g. adrenocortical carcinoma, pheochromocytoma, primary aldosteronism, cortisol hypersecretion, or adrenal insufficiency. Hormone assessment is crucial to characterize adrenal incidentaloma. Nowadays, various hormone assay methods are available, such as immunoassay and mass spectrometry. However, there are several pitfalls that should be considered: e.g. circadian rhythm, gender/age dependency, preanalytical and analytical issues, and drug interactions. Pharmacological or analytical interference can lead to false serum concentrations and may result in misinterpretation of results and thus inappropriate treatment. The purpose of this review was to study the main interferences that may be observed in the different tumor types of adrenal incidentalomas in order to help physicians in their clinical decision-making and for the overall benefit of patients.

Résumé
Un incidentalome surrénalien est une masse asymptomatique découverte par un examen d'imagerie dont l'indication est indépendante de la pathologie surrénalienne. La plupart du temps, il s'agit d'adénomes surrénaliens non fonctionnels, mais dans certains cas, une intervention thérapeutique est nécessaire (corticosurrénalome, phéochromocytome, hyperaldostéronisme primaire, hypersécrétion de cortisol, insuffisance surrénalienne, par exemple). Le bilan hormonal est essentiel pour caractériser les incidentalomes surrénaliens. De nos jours, il existe différentes méthodes de dosages des hormones (techniques immunologiques, spectrométrie de masse...). Il y a, cependant, certains pièges dont il faut tenir compte : le rythme circadien, des corrélations avec l'âge ou le sexe, certaines questions analytiques ou pré-analytiques, et les interactions médicamenteuses. Les interférences pharmacologiques ou analytiques retrouvées dans ces techniques peuvent être responsables d'erreurs de dosages conduisant à des erreurs d'interprétation, et donc de prise en charge. L'objectif de cette revue est de présenter aux praticiens les principales interférences pouvant être observées dans le cadre du bilan biologique des différents types d'incidentalomes surrénaliens, pour améliorer la décision clinique et la prise en charge des patients.

Keywords:
Adrenal incidentaloma, interference, hormone assays

Mots Clés :
Incidentalomes surrénaliens, interférences, dosages hormonaux

1. Introduction

Adrenal incidentalomas are asymptomatic masses detected in absence of suspected adrenal diseases. The imaging examination is not executed for symptoms related to adrenal hormone excess, but rather for the evaluation of symptoms that are not related to an adrenal disease, e.g. back or abdominal pain. Physicians should perform additional investigations only in lesions ≥ 1cm [1-4]. The incidence and prevalence of adrenal incidentalomas can only be extrapolated from imaging or autopsy studies. Autopsy studies report a prevalence of around 2% of clinically unapparent adrenal masses (range 1.0–8.7%) [5-6]. Radiological studies suggest a prevalence of around 3% at the age of 50 years which increases with age (up to 10% in the elderly) [5,7-8]. Adrenal incidentalomas are extremely rare in childhood. Adrenal incidentalomas are composed of benign and malignant lesions derived from the adrenal cortex, the medulla or of extra-adrenal origin (Table 1).

Biological hormone assessment is crucial in characterizing adrenal incidentaloma. However, there are many pitfalls that should be considered (e.g. circadian rhythm, gender, age, drug interactions, preanalytical and analytical issues). Furthermore, since normal ranges depend on the method used, it is essential to interpret test results using appropriate reference ranges. Interferences occur when a substance or process falsely alters an assay result. Interferences are of pharmacological or analytical origin and can lead to falsely elevated or falsely low serum analyte concentrations. The consequences of such interferences can be devastating and may result in the misinterpretation of a patient's results leading to a wrong course of treatment. Immunoassay interferences are most commonly due to antibodies or cross-reaction. They may be autoantibodies or heterophile antibodies that predominantly interfere in two-site immunometric (sandwich) assays, between capture and detection antibodies. The purpose of this review was to study the main

interferences relative to the different tumor types of adrenal incidentalomas in order to help physicians in their clinical decision-making and for the overall benefit of patients.

2. Pheochromocytoma

A pheochromocytoma is a tumor arising from adrenal medulla chromaffin cells that produces catecholamines: adrenaline, noradrenaline, and dopamine. In some cases, these tumors can be biochemically silent. A paraganglioma (PPGL) is a tumor derived from the ganglia of the sympathetic chain in thorax, abdomen, and pelvis and from parasympathetic chain in head and skull base areas. Malignancy in PPGL is characterized by the presence of metastasis in lymph nodes or other distant sites: bones, lungs, and liver. The prevalence of PPGL in patients with hypertension is 0.2% to 0.6% [9], whereas in patients with an incidentaloma, it increases to 7% [3]. Initial biochemical assessments in case of clinical suspicion of a pheochromocytoma or PPGL should include measurements of plasma-free metanephrines or urinary metanephrines. Metanephrines exist in plasma and urine in free and mainly in sulfate-conjugated forms [11]. The last data established the superiority of plasma-free metanephrines because of better sensitivity and easier sampling conditions. However, specialized analytical methods as liquid chromatography with electrochemical detection (LC-ECD) or liquid chromatography with tandem mass spectrometry (LC-MS/MS), which are not available in all laboratories, are required. An increase in plasma metanephrines above 2-fold the upper cut-off suggests that the patient has a pheochromocytoma [9-11]. The determination of the dopamine metabolite i.e. 3-methoxytyramine (3MT) (both in urine and plasma) facilitates the diagnosis of malignant PPGL but can also be found in patients with neck and skull

PPGL. Nevertheless, this test is not commonly performed in all laboratories [12], and otherwise may have low specificity [13].

2.1. Sampling conditions

No dietary restrictions are needed for measurements of plasma metanephrines. Dietary use of amine rich foods might cause false-positive results for 3MT. Catecholamine-containing products can be found in bananas, nuts, tomatoes, and beans. Procedures to avoid dietary influences on metabolites measured are not commonly taken into account. Therefore, sampling should be collected after an overnight fast [14,17]. For measurements of plasma metanephrines, blood should be drawn after 30 minutes of supine rest [15]. This delay may reduce the risk of missing a PPGL. The strong influence of sympathetic activation and upright posture stimulate the release of noradrenaline with subsequent normetanephrine production, leading to an increase of +30% for normetanephrine, +12% for metanephrine but has no influence on 3MT levels [16]. Urinary assessment can help when supine sampling cannot be used. Nevertheless, it is important to ensure that patients provide a complete 24 h urine collection with simultaneous measurement of total volume and urinary creatinine determination [17].

2.2. Physiological or pathological situations

Stress situations associated with acute illness (intensive care, sepsis, heart failure, hypoglycemia) should be considered in interpreting marked elevations of plasma or urine metanephrines. Such comorbidities accompanied by strong elevations of sympathoneural activity are a source of falsely-elevated plasma normetanephrine levels [18]. Since an increase in normetanephrine levels is observed in the elderly,

reference values adjusted for age have been suggested [19-20]. Plasma total metanephrines are also increased in case of renal insufficiency; taken as a whole, the measurements of plasma-free metanephrines must be preferred [21].

2.3. Medical treatments

There are two kinds of drugs interfering with catecholamine metabolism and resulting in raised values of biochemical results due to analytical or pharmacological interferences. Some drugs directly interfere with measurement methods (e.g. acetaminophen, mesalamine and sulfasalazine in high pressure LC-ECD methods). Moreover, other medications used for hypertension or neurological diseases interfere with the secretion of catecholamines (e.g. tricyclic antidepressants block the neuronal uptake of catecholamines). About 40% of interferences are due to acetaminophen and tricyclic antidepressants, which are the most common medications in the care of pheochromocytoma [22-24]. To sum up, it is necessary to washout all interfering medications when possible (Table 2).

2.4. Analytical issues of measurement methods

The latest studies revealed that measurements of fractionated metanephrines by mass spectrometric methods as LC-MS/MS have negligible analytical interference compared to high pressure LC-ECD [3]. However, pharmacological interferences are still possible [25-26].

2.5. Practical approach

In most situations interferences in the biological assessment of pheochromocytoma are due to inappropriate sampling conditions. This issue is easily dealt with by repeat

sampling in supine position together with stopping medical treatment that may interfere. When clinical suspicion is low, patients can be monitored by biochemical follow-up. When clinical suspicion is moderate or high and plasma normetanephrine is elevated, clonidine suppression test is useful to exclude the presence of tumor [27]. However, this test has not been validated in any prospective study yet. Others have proposed the combination of measurements of chromogranin A and urinary fractionated metanephrines as follow-up tests in case of plasma metanephrine increase [28]. Nevertheless, the use of functional imaging modalities as ^{123}I-meta-iodobenzylguanidine (MIBG) scintigraphy can help to disprove a diagnosis of pheochromocytoma [29]. It has been reported that some pheochromocytomas are not associated with hypertension (normotensive incidentally discovered pheochromocytomas: NIPs). In approximately 25% of patients, pheochromocytomas are incidentalomas with normal blood pressure and normal values of metanephrines. In such cases, the CT scan characteristics of the incidentaloma are important to alert the clinician [30].

3. Primary aldosteronism

In patients with hypertension or hypokalemia, the latest guidelines recommend the use of the aldosterone-renin ratio (ARR) to exclude primary aldosteronism (PA). Numerous studies have demonstrated that this ratio is the parameter with the highest sensitivity (68–94%) and best negative predictive value [31-33]. The ARR has good within-patient reproducibility when performed under standardized conditions [34]. Like all biochemical investigations, the ARR is submitted to interferences [35].

3.1. Sampling conditions

The specificity of ARR is better with a sodium-normal diet. Low-sodium diet elevates renin and plasma aldosterone levels and lowers ARR [36]. Normal serum potassium concentration is advisable because low concentrations inhibit aldosterone secretion and may induce false negative screening. Aldosterone and renin levels rise in upright positions in relation with physiological adaptation. This stimulation is more important in the morning, thus, it is recommended to collect blood samples in the morning after patients have been out of bed for at least 2 hours, preferably after they have been seated for 5–15 minutes [37].

3.2. Physiological or pathological situations

Impaired renal function lowers renin concentration [38]. During pregnancy, ARR and especially plasma aldosterone levels are increased. Renin secretion decreases with age, probably in relation with progressive nephron loss. This may lead to false-positive ARR, and the ARR threshold for PA screening should ideally be revised after the age of fifty years [39].

3.3. Medical treatments

Many medications affect the ARR. It is necessary to washout all interfering medications when possible (Fig. 1). For example, estrogen and progesterone treatment (including oral contraceptive agents, hormonal replacement therapy in postmenopausal women) can induce false-positive ARR elevation if renin is assessed as “direct renin” concentration (DR). This interference is avoided with the measurement of plasma renin activity (PRA). Estrogenic impregnation increases liver angiotensinogen production and thus angiotensin-II, inhibiting renin secretion by negative feedback with little change in enzymatic activity. Some authors highlight the

increased precision obtained by the assessment of PRA, which takes into account individual angiotensinogen concentration, instead of DR [40-42]. With regard to antihypertensive treatment, it has been shown that beta-blockers reduce the secretion of renin and plasma aldosterone. Elsewhere, converting enzyme inhibitor (ACE) and angiotensin-II receptor antagonist (ARA-II) decrease plasma aldosterone and increase plasma renin concentrations; finally, diuretics induce renin elevation [32] (Table 3).

3.4. Analytical issues of measurement methods

Plasma aldosterone may be measured by radioimmunology (RIA) or chemiluminescence, the most common technique, or by LC-MS/MS, the latter method being already developed in several laboratories for its better sensitivity and specificity [43]. RIA methods undergo interferences from polar metabolites of aldosterone (tetrahydroaldosterone and aldosterone-18-glucuronide) [44]. In addition, plasma aldosterone measurements by immunoassay in patients with renal impairment can be overestimated due to an increase in aldosterone metabolites, which cross-react with reactive antibodies [45]. Renin may be assayed in two ways. PRA is assessed by RIA, but it is a manual and time-consuming method. Plasma renin concentration, also known as DR immunoassay is now the most common renin assay because of fast standardized analysis using an automated analyzer, with lower production costs [46]. Besides, the cut-off or ARR depends on the measurement method and the unit measure of renin and aldosterone. If standardized assay conditions are respected, the ARR threshold to screen PA is about 64 according to the latest guidelines (aldosterone in pmol/L, direct renin in mIU/L) (conversion

factors: aldosterone: pmol/L = pg/mL x 2.77 and direct renin: mIU/L = pg/mL x 1.67) [32]. However each laboratory should check its own cut-off [47].

3.5. Practical approach

In most situations, interferences in renin and aldosterone assays are due to inappropriate sampling and can be dealt with by repeat sampling in standardized conditions. To avoid overestimating ARR generally due to a very low renin concentration, a value of 5 mIU/l should be attributed to any direct renin result < 5 mIU/l [32]. With regard to blood sampling, standard conditions should be followed [32]:

- in the morning (out of bed for at least 2 hours)
- seated for 5–15 minutes
- under normal sodium diet / encourage patient to liberalize sodium intake (urinary sodium,100–200 mmol/24 h)
- potassium-replete / with normal serum potassium values / correct hypokaliemia
- washout of all interfering medications (anti hypertensive / estrogens-progestins)

Finally, when screening by ARR reveals conflicting results between plasma aldosterone and renin concentrations, dynamic tests as saline infusion may be undertaken in these ambiguous cases to confirm autonomous aldosterone secretion [32].

4. Cushing's syndrome

Cushing's syndrome (CD) is characterized by excessive cortisol secretion from the adrenocortical gland. The latest guidelines recommend that all patients with adrenal

incidentalomas undergo in first line a 1 mg overnight dexamethasone suppression test (DST) as a screening test to exclude cortisol hypersecretion [48-49]. A value ≤50 nmol/L (≤1.8 µg/dL) is considered as normal, allowing exclusion of cortisol hypersecretion [48]. To confirm cortisol secretory autonomy, some additional biochemical tests should be performed, such as 24 h urinary free cortisol (UFC), midnight salivary cortisol (MSC) assay repeated at least 2 times. However, the recent published literature is controversial and no clear statement has been made on these tests in patients with adrenal incidentaloma [48].

4.1. Pre-analytical conditions, physiopathological situations and medical treatments

Plasma cortisol is 80% bound to cortisol-binding globulin (CBG) and 10–15% to albumin. Some disorders can reduce (inflammation, rare genetic disorders) or increase CBG levels (estrogen, pregnancy, mitotane). Since most cortisol is bound to CBG, total serum cortisol levels are significantly affected by variation in CBG levels [50]. For example, patients with nephrotic syndrome, liver disease or malnutrition may have lower CBG levels, with a decrease in cortisol levels. Conversely, the use of oral estrogen contraceptives may result in increased CBG levels, leading to high serum cortisol concentrations. Whenever possible, estrogen-containing drugs should be withdrawn for 6 week before cortisol assessment [51]. Normal ranges vary substantially, depending on the method used, so it is essential to interpret test results in the context of the appropriate normal range. Hence, immunoassays may be affected by cross-reactivity with cortisol metabolites or synthetic glucocorticoids. In order to avoid cross-reactions and whenever possible, patients should be instructed to avoid any glucocorticoid intake for at least one week before each kind of cortisol sample collection (blood, urine collection and saliva test). Finally, cortisol

hypersecretion may be unrelated to any neoplasia, defined as non-neoplastic hypercortisolism. These “functional” hypercortisolism are induced by chronic hypersecretion of ACTH. These physiopathological situations include: alcoholic impregnation, neuropsychiatric disorder, chronic kidney disease, multiple sclerosis, and situations of insulin resistance (obesity, polycystic ovary syndrome and type 2 diabetes) [52].

4.1.1. Dexamethasone suppression test

The overnight test is easy to implement. Various doses of dexamethasone have been proposed, however, 1 mg dexamethasone is usually given between 11 p.m. and 12 p.m., and cortisol is measured between 8 a.m. and 9 a.m. the following morning. Variations in the absorption and metabolism of dexamethasone may influence the result of the overnight 1 mg dexamethasone suppression test (DST). Dexamethasone clearance may be reduced in patients with liver and/or renal failure. The measurement of plasma dexamethasone concentration may help to ensure adequate dexamethasone level, with low specificity [Cut-off > 5.6 nmol/l (0.22 g/dl)] [53]. Conversely, some drugs may induce hepatic enzymatic clearance of dexamethasone (mediated by CYP 3A4) leading to a decrease in dexamethasone concentrations, such as phenytoin, phenobarbitone, carbamazepine, rifampicin, and alcohol (Table 4).

4.1.2. Urinary free cortisol assay

Despite the high sensitivity of 1 mg DST in identifying cortisol status, the low specificity of this test leads to consideration of UFC levels. UFC provides an integrated assessment of cortisol secretion over a 24 h period. It is important to

ensure that patients provide a complete 24 h urine collection with appropriate total volume and to measure urinary creatinine levels. Since UFC is altered by renal filtration, UFC assessment is not indicated when creatinine clearance falls below 60 mL/min. Hence, UFC levels fall linearly together with the severity of renal failure [54].

4.1.3. Midnight salivary cortisol assay

Since UFC may be normal in some patients with mild Cushing's syndrome, salivary cortisol may be more useful in those cases. Patients are asked to collect a saliva sample on two separate evenings between 11 p.m. and 12 p.m. Saliva is collected either by passive drooling into a plastic tube or by placing a cotton pledget ("salivette") in the mouth and chewing for 1 – 2 min. In healthy individuals with stable conventional sleep-wake cycles, the level of serum cortisol follows a circadian rhythm. Cortisol rises at 3 – 4 a.m., reaches a peak at 7 – 9 a.m., and then falls to low levels for the rest of the day. An increase in blood cortisol is reflected by a change in salivary cortisol concentrations within a few minutes [55]. The loss of this circadian rhythm can lead to a diagnosis of Cushing's syndrome. It is important to note that the circadian rhythm is blunted in many patients with depressive illness and in shift workers [56]. Cigarettes smokers have been shown to exhibit higher late-night salivary cortisol levels than non-smokers. Although the duration of this effect is not known, it is advisable to avoid cigarette smoking on the day of saliva collection [57]. Moreover, abnormal elevated midnight salivary cortisol assay levels have been reported in elderly subjects and in those with diabetes mellitus [58]. Various methods have been used to measure cortisol in the saliva, resulting in different reference ranges and yielding differences in sensitivity and specificity. According to the low

concentrations measured, the best-validated assay is LC-MS/MS rather than RIA or chemiluminescence assays so far [59].

4.2. Analytical issues of measurement methods

The immunoassays most commonly used in some laboratories are affected by several limitations, especially those that require proper extraction and prepurification. High-pressure liquid chromatography (HPLC) or liquid chromatography tandem mass spectrometry assays are highly specific (especially LC-MS/MS) and provide reliable measurements of cortisol in plasma, urine and salivary samples. Immunoassays without extraction can be affected by cross-reaction with cortisol metabolites and synthetic glucocorticoids. In contrast, molecular structural based assays as HPLC and LC-MS/MS avoid this problem and are being used with increasing frequency [59]. However, there are also drugs (carbamazepine and fenofibrate) that may interfere with some chromatographic methods of free urinary cortisol assessment causing falsely elevated values [60]. In patients treated with metyrapone, the block effect on 11β-hydroxylase induces an increase in 11 β-desoxycortisol, which may result in positive interference with cortisol measurement when using some immunoassay, due to the very similar molecular structure of both steroids. In this case, analysis using mass spectrometry would be preferable [61]. Relative to the same mechanism, the upper limits of normal ranges are lower with HPLC or LC-MS/MS than with immunoassays, which may measure additional compounds with very similar molecular structures. To sum up, immunoassays should be preferred for serum cortisol assessment in emergency cases, and LC-MS/MS should be used for its high specificity when interferences are suggested or in specific situations as pregnancy.

4.3. Practical approach

Each cortisol test has its drawbacks and the choice of test should be individualized for each patient. UFC determination should not be used in patients with renal impairment. With regard to the 1 mg DST test, dexamethasone concentration may be measured in some difficult situations; if the concentration is too low at 8-9 a.m., the test should be repeated using a higher dose of dexamethasone to achieve an accurate morning level [49]. The measurement of serum CBG concentration could be of interest in patients with estrogenotherapy, but it's not usually used in routine. In case of an increase above the normal values, estrogen should be discontinued for 4–6 weeks [49]. Finally, if salivary cortisol is used in specific populations such as aged and diabetic patients (> 60 years old), night shift workers or cigarettes smokers, only normal results should be validated [49].

4.4. Adrenocortical carcinoma

Adrenocortical carcinoma (ACC) is less frequent but accounts for 2-11% of incidentally discovered adrenal tumors. The European Network for the Study of Adrenal Tumors (ENS@T) suggested a pre-operative hormonal biological assessment in case of suspected ACC. It includes assessment of dehydroepiandrosterone sulfate, 17-hydroxyprogesterone, androstenedione, testosterone, and 17-beta-estradiol (only in men and postmenopausal women) [62]. All steroid hormones are derived from cholesterol and share a basic cyclopentano-perhydrophenanthrene cycle structure. The small structural differences, unique to each steroid hormone (modifications to the basic 4-cycle steroid structure) explain why it is difficult to discriminate each steroid using a direct immunoassay method.

This analytical interference is known as cross reaction. The best way to overcome these issues is to use LC-MS/MS. A promising approach for differentiating adenomas from ACCs uses mass spectrometry-based steroid profiling of 24 h urine samples. Some recent studies have suggested that urine steroid metabolomic analysis could be a new tool to discriminate benign from malignant adrenocortical tumors, but this method has not yet been designed for routine use [63].

5. Adrenal insufficiency

In rare cases, adrenal incidentaloma can be bilateral. Bilateral adrenal enlargement due to metastatic diseases or adrenal hemorrhages can lead to adrenal insufficiency. Adrenal insufficiency is defined by the inability of the adrenal cortex to produce sufficient amounts of cortisol. It is a severe and potentially life-threatening disease due to the central role of these hormones in energy, salt, and fluid homeostasis. Primary adrenal insufficiency (PAI), also known as Addison's disease, should be distinguished from secondary adrenocortical insufficiency due to insufficient production of adrenocorticotropic hormone (ACTH) and without impact on the renin angiotensin-aldosterone system. Patients with autoimmune disease, systemic disorders or situations of increased cortisol metabolism induced by drugs have an increased risk of PAI. The diagnosis of adrenal insufficiency is based on low morning cortisol concentrations (<138 nmol/L (5 µg/dL)) and confirmed by low stimulation of cortisol secretion by corticotropin [64-65]. The corticotropin stimulation test (or short Synacthen standard dose: 250 µg) test is currently considered as the gold standard for the diagnosis of primary adrenal insufficiency. Usually, a peak of cortisol concentration after acute stimulation with corticotropin exceeding 500 nmol/L (18

µg/dL) is accepted as evidence of sufficient adrenocortical activity [65]. However, those cut-offs (morning cortisol level and after stimulation with corticotropin) are still debated and it is difficult to set up the best range [66-67]. In practice, since adrenal insufficiency can be a life-threatening disease, a higher threshold has been proposed to avoid eliminating a diagnosis of partial adrenal insufficiency (false negative) [64]. Otherwise, insulin tolerance test should be preferred for secondary adrenal insufficiency [65]. Dehydroepiandrosterone sulfate (DHEA-S) levels that are below the lower limit of normal range (adjusted for gender and age) may be a useful sign of PAI [65-66].

5.1. Pre-analytical conditions

Since cortisol base-line value is modulated by stress, physical activity and food, fasting and a period of rest of at least 15 minutes should be recommended before blood cortisol sampling. Since the peak level of cortisol is seen in the early morning, sampling should be performed at this time of day (between 6 a.m. and 10 a.m.) to detect a reduction of cortisol synthesis [65].

5.2. Physiological or pathological situations

There are several conditions requiring specific considerations since cortisol level is expected to be altered by nonadrenal pathologies, as critical illness and pregnancy. Reduced CBG levels in illness and elevated levels in pregnancy may alter the interpretation of cortisol levels [50]. The use of estrogen-containing oral contraceptives results in higher CBG with a corresponding rise in cortisol. Patients with nephrotic syndrome and liver disease as well as those who are in immediate

postoperative period or who require intensive care may have lower CBG and albumin and hence, lower cortisol measurements (Table 5).

5.3. Medical treatments

Immunoassays are more subject to interferences because of a lack of full specificity of the antibodies used. Several chemical structures that are similar to cortisol have been described to induce false-positive results (cross-reactivity). Those treatments include prednisolone, cortisone, and 6-methylprednisolone [68]. In case of pharmacological interferences, new drug classes actually used in the treatment of melanoma, renal cell carcinoma and non-small-cell lung cancer may also interfere in blood cortisol assessment. There are two kinds of cancer immunotherapy: anticytotoxic anti-T-cell antigen-4 antibodies (anti-CTLA4) or anti-programmed cell death-protein1 antibodies (anti-PD1). These antibodies are directed against inhibitory and co-stimulatory molecules to activate the immune system, in order to supress tumor cells [69-70]. Immune checkpoint blockade can induce endocrinopathies, including hypophysitis, thyroid dysfunction and diabetes mellitus. The incidence of secondary adrenal insufficiency by hypophysitis related to CTLA4 antibody therapy is around 6 % [71]. Some cases of ipilimumab-induced primary adrenalitis have been reported but seem to be exceptional [72-73]. Up to now, there are no data on analytical interference in cases of cancer immunotherapy. Physicians should report to the laboratory any discordance between clinical and laboratory data.

5.4. Analytical issues of measurement methods

The majority of laboratories in Europe use routine immunoassays with automated methods. Nevertheless, for some specific situations, methods based on

chromatographic separation and mass spectrometry are suitable. During pregnancy LC-MS threshold levels have been proposed for morning cortisol determination, i.e. 300 nmol/L (108 ng/mL) in the first trimester, 450 nmol/L (162 ng/mL) in the second and 600 nmol/L (216 ng/mL) in the third trimester [74].

5.5. Practical approach

Diagnosis of primary adrenal insufficiency can be difficult mainly in situations modulated by stress. First of all, it is important to check that blood cortisol sampling is done in calm conditions and confirm or exclude diagnosis by corticotropin stimulation test (short Synacthen standard dose: 250 µg). If a cross reaction by corticosteroid treatment is suspected, clinicians should investigate all medications given to the patient in the last months. Finally, LC-MS method may be used in cases with discordant data or in pregnant patients [75]. The measurement of serum free cortisol and CBG can also be used but these immunoassays are not routinely available. Morning salivary cortisol levels may be measured but there are insufficient data to propose accurate recommendations.

6. Conclusion

Interferences in immunoassays may lead to the misinterpretation of patients' results possibly leading to a wrong course of treatment. Laboratories should improve the processes to detect, test and report suspected analytical interferences. At the same time, it is important that physicians communicate to the laboratory any clinical suspicion of discordance relative to patients' preanalytical status and treatment in order to avoid any misinterpretation of biological results. The detection of interference

may require the use of an alternate analysis method or additional measurements, including dilution of the sample in a non-immune serum. Likewise, it is imperative that laboratories inform physicians of the follow-up procedure and report on the presence of any interference (Fig. 2). Finally, a permanent laboratory-physician collaboration is essential to ensure optimal clinical decision making based on pharmacological and analytical assays without interferences.

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Disclosure of interest

The authors declare that they have no competing interest.

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Table 1

Frequency of the different tumor types of adrenal incidentalomas. (Series including all patients with an adrenal mass)

Tumor entity	Median (%)	Range (%)
- Adenoma	80	33–96
Non-functioning	75	71–84
Autonomously cortisol-secreting	12	1.0–29
Aldosterone-secreting	2.5	1.6–3.3
- Pheochromocytoma	7.0	1.5–14
- Adrenocortical carcinoma	8.0	1.2–11
- Metastasis	5.0	0–18

Adapted from Terzolo M et al [3]

Table 2

Major medications that may cause falsely elevated test results for plasma and urinary metanephrines

	Plasma		**Urine**	
	NMN	MN	NMN	MN
Analytical interferences*				
Acetaminophen, sulfasalazine	++	-	++	-
α-methyldopa	++	-	++	--
Pharmacological interferences				
<u>Increase of catecholamine synthesis</u>				
Antiparkinsonian drugs: levodopa	+	+	++	+
<u>Inhibitor of catecholamine reuptake</u>				
Tricyclic antidepressants: amitriptyline	++	-	++	--
Cocaine	++	+	++	++
<u>Inhibitor of catecholamine catabolism</u>				
MAO-inhibitors	++	++	++	++
<u>Increase in catecholamine release</u>				
Alpha blocker: phenoxybenzamine	++	-	++	-
Amphetamines: ecstasy	+	+	+	+
Sympathomimetics: ephedrine, pseudoephedrine, phenylephrine	+	+	+	+

COMT: Catechol-O-methyltransferase, MAO: monoamine oxidase, MN: metanephrine, NMN: normetanephrine, ++: high increase / +: mild increase / -: no increase.

* These interferences are not observed with the use of chromatography-mass spectrometry methods

Adapted from Grouzmann E et al [25]

Table 3

Non-analytical factors that may affect the aldosterone-renin ratio and thus lead to false-positive or false-negative results

Factors	Effect on aldosterone levels	Effect on renin levels	Effect on ARR
Medications			
β-Adrenergic blockers	↓	↓↓	↑ (FP)
Central α-2 agonists (e.g. clonidine, α -methyldopa)	↓	↓↓	↑ (FP)
NSAIDs	↓	↓↓	↑ (FP)
K^+-wasting diuretics	→ ↑	↑↑	↓ (FN)
K^+-sparing diuretics	↑	↑↑	↓ (FN)
ACE inhibitors, ARBs	↓	↑↑	↓ (FN)
Ca^{2+} blockers (DHPs)	→ ↓	↑	↓ (FN)
Renin inhibitors	↓	↓↑	↑ (FP) / ↓ (FN)*
Serum potassium status			
Hypokalemia	↓	→ ↑	↓ (FN)
Potassium loading	↑	→ ↓	↑ (FP)
Dietary sodium			
Sodium restricted	↑	↑↑	↓ (FN)
Sodium loaded	↓	↓↓	↑ (FP)
Other conditions			
Advancing age	↓	↓↓	↑ (FP)
Renal impairment	→	↓	↑ (FP)
PHA-2	→	↓	↑ (FP)
Pregnancy	↑	↑↑	↓ (FN)
Renovascular HT	↑	↑↑	↓ (FN)
Malignant HT	↑	↑↑	↓ (FN)

ACE, angiotensin-converting enzyme; ARBs, angiotensin II type 1 receptor blockers; ARR, aldosterone-renin ratio; DHPs, dihydropyridines; FP, false positive; FN, false negative; HT, hypertension; NSAIDs, nonsteroidal anti-inflammatory drugs; SSRIs, selective serotonin reuptake inhibitors.

* Renin inhibitors lower plasma renin activity (PRA) but raise direct renin concentration (DRC). This is expected to result in false-positive ARR levels for renin measured as PRA and false-negatives for renin measured as DRC.

Adapted from Funder JW et al [31]

Table 4

Selected drugs that may interfere with the interpretation of biological results in the diagnosis of Cushing's syndrome.

Drugs that accelerate dexamethasone metabolism by induction of CYP3A4	**Drugs that impair dexamethasone metabolism by inhibition of CYP3A4**	**Drugs that increase cortisol binding globulin and may falsely elevate cortisol results**	**Drugs that increase urinary free cortisol results**
Pharmacological interferences Phenobarbitone Phenytoin Carbamazepine Primidone Rifampin Rifapentine Ethosuximide Pioglitazone	Pharmacological interferences Aprepitant/fosaprepitant Itraconazole Ritonavir Fluoxetine Diltiazem Cimetidine	Pharmacological interferences Estrogens Mitotane	Pharmacological interferences Drugs that inhibit 11β-HSD2 (licorice, carbenoxolone) Analytical interferences Carbamazepine Fenofibrate (LC-MS/MS) Glucocorticoids (Immunoassays)

LC-MS/MS: liquid chromatography with tandem mass spectrometry

Adapted from Nieman LK et al [49]

Table 5

Main origins of variations in corticosteroid binding globulin

Variations of serum CBG concentration	**Drugs**	**Physio-pathological conditions**
⇧	Estrogens Mitotane Antiepileptic drugs	Pregnancy / estrogenotherapy Hepatitis
⇩	Corticosteroid Interleukin-6	Inflammation Cushing's syndrome PCOS Cirrhosis Hypothyroidism Nephrotic syndrome CBG deficiency disease

CBG: corticosteroid binding globulin, PCOS: Polycystic ovary syndrome.

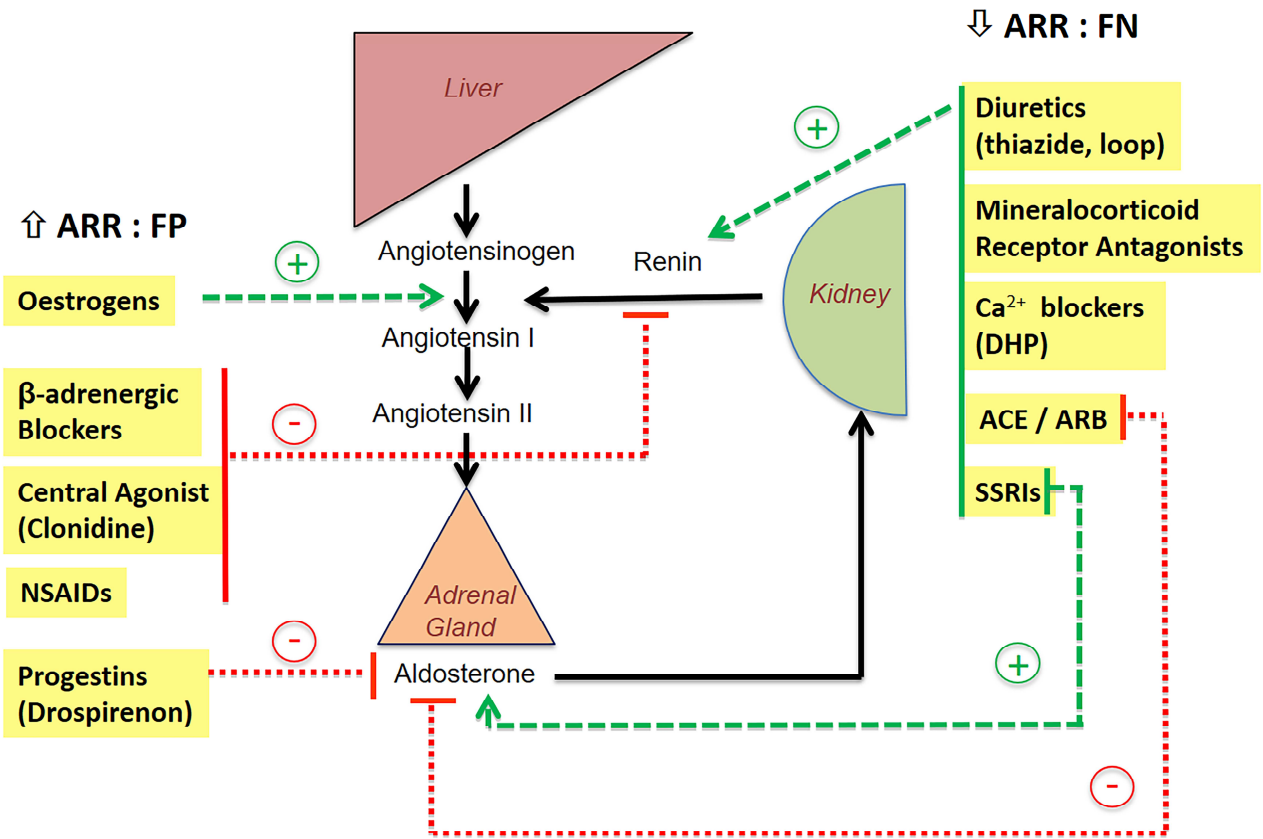

Fig. 1. Medications affecting the aldosterone-renin ratio

ACE, angiotensin-converting enzyme; ARBs, angiotensin II type 1 receptor blockers; ARR : aldosterone-renin ratio; DHPs, dihydropyridines; FP, false positive; FN, false negative; NSAIDs, nonsteroidal anti-inflammatory drugs; SSRIs, selective serotonin reuptake inhibitors. Adapted from Douillard C et al [32] and Funder JW et al [33]

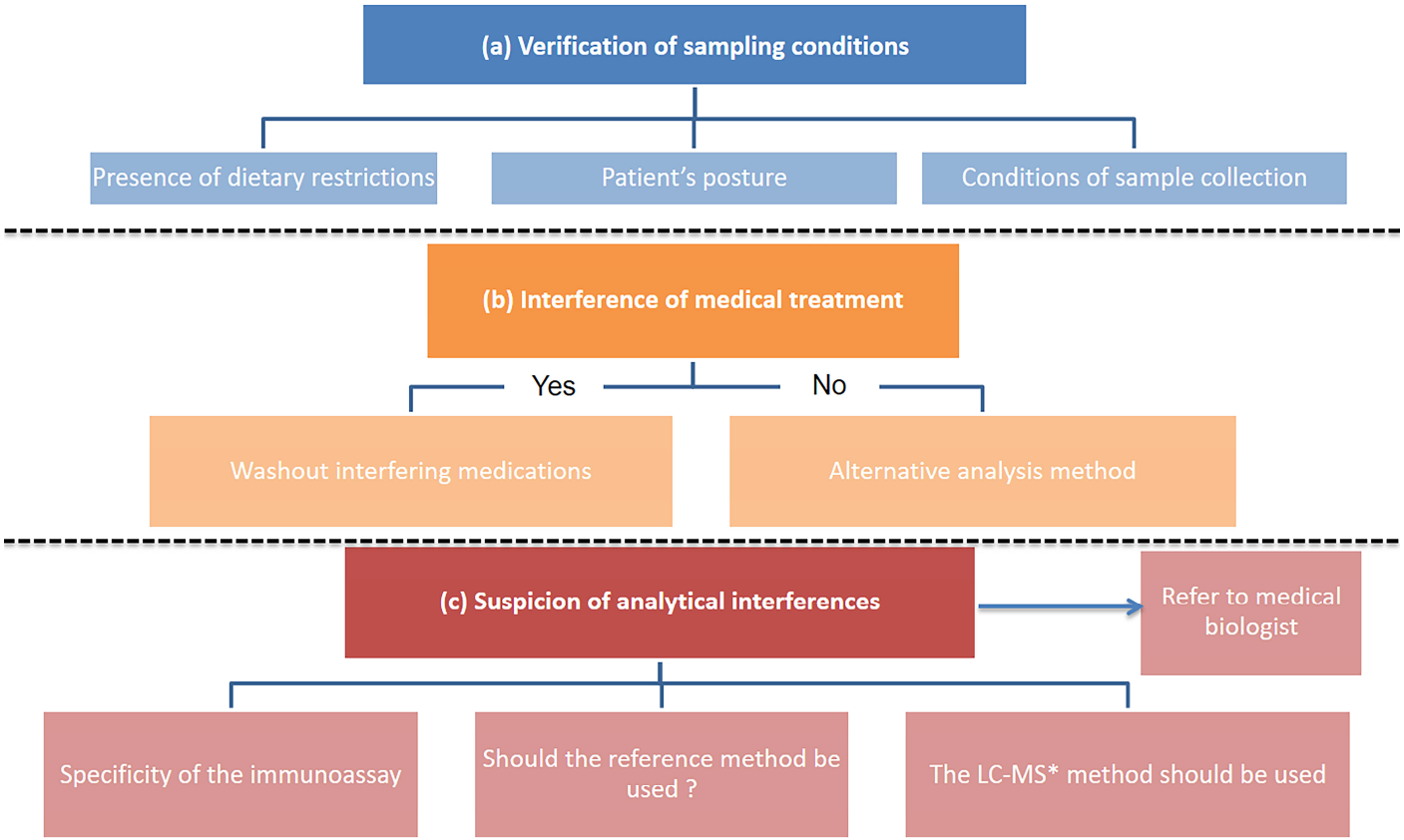

Fig. 2. Items that should be considered when inadequate results of hormonal biological assays are suspected.

*Liquid chromatography followed by tandem mass spectrometry