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Steroid Profiling and Immunohistochemistry for Subtyping and Outcome Prediction in Primary Aldosteronism—a Review

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Abstract

Purpose of Review Steroid profiling and immunohistochemistry are both promising new tools used to improve diagnostic accuracy in the work-up of primary aldosteronism (PA) and to predict treatment outcomes. Herein, we review the recent literature and present an outlook to the future of diagnostics and therapeutic decision-making in patients with PA.

Recent Finding PA is the most common endocrine cause of arterial hypertension and unilateral forms of the disease are potentially curable by surgical resection of the overactive adrenal. Recent studies have shown that adrenal steroid profiling by liquid chromatography-tandem mass spectrometry (LC-MS/MS) can be helpful for subtyping unilateral and bilateral forms of PA, classifying patients with a unilateral aldosterone-producing adenoma (APA) according to the presence of driver mutations of aldosterone production in APAs, and potentially predicting the outcomes of surgical treatment for unilateral PA. Following adrenalectomy, immunohistochemistry of aldosterone synthase (CYP11B2) in resected adrenals is a new tool to analyze "functional" histopathology and may be an indicator of biochemical outcomes after surgery.

Summary Biochemical and clinical outcomes of therapy in PA vary widely among patients. Peripheral venous steroid profiling at baseline could improve diagnostic accuracy and help in surgical decision-making in cases of a suspected APA; results of "functional" histopathology could help determine which patients are likely to need close post-surgical follow-up for persistent aldosteronism.

Keywords Primary aldosteronism · Steroid profiling · Immunohistochemistry · Outcome · LC-MS · Adrenalectomy

Introduction

Since Jerome W Conn's first description of a new endocrine cause of hypertension in a young woman with hypokalemia in 1955 [1], primary aldosteronism (PA) has emerged as the most frequent endocrine cause of elevated blood pressure. Its estimated prevalence ranges from 5 to 12% in patients with hypertension among different study cohorts [2–4]. The excess of aldosterone is associated with severe cardiovascular morbidity, which is not only due to uncontrolled arterial hypertension but also due to the adverse effects of aldosterone overproduction independent of blood pressure [5]. Importantly, an adequate and early therapy for PA can lower the risk for cardiovascular complications significantly [6, 7, 8••]. Treatment options are dependent on the subtype of the disease. In unilateral adrenal aldosterone excess, commonly attributed to an aldosterone-producing adenoma (APA), adrenalectomy is preferred and can result in the remission of the disease [9, 10••, 11, 12]. In the bilateral subtype, mainly due to bilateral hyperplasia (BAH) of the adrenal cortex, mineralocorticoid receptor antagonists are used to block aldosterone effects.

Currently, adrenal vein sampling (AVS) is considered the gold standard for subtyping PA and is endorsed by current guidelines in therapeutic decision-making [2]. Alternatively, subtyping based on CT or MRI results has been advocated.

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According to the Primary Aldosteronism Surgical Outcome study, between 0 and 17% (mean, 6%) of patients will be incorrectly classified as having unilateral disease based on AVS results in expert centers [10••]. In contrast, CT-based subtyping will result in false classification of BAH as unilateral disease in 20% [13]. In a randomized head-to-head comparison of AVS- versus CT-based subtyping, the primary endpoint of the intensity of drug treatment for obtaining target blood pressure after 1 year of follow-up was not different between groups ($p = 0.87$). CT-based decision-making was not significantly inferior to AVS for any of the secondary endpoints although biochemical persistence of PA was observed in 20% of patients in the CT group and 11% in the AVS group [14]. Since current methods for lateralization lack optimal accuracy [13•, 14] and, moreover, AVS is invasive, technically demanding, and expensive, new diagnostic tools are being explored that could improve subtyping and treatment decision-making in PA. Steroid profiling by liquid chromatography-tandem mass spectrometry (LC-MS/MS) is evolving as a new tool for the potential subtyping of PA without invasive measures. Additionally, recent research has indicated that steroid profiling may be useful for the prediction of outcomes of adrenalectomy [15•]. The potential utility of steroid profiling is likely due to the influence of the genetic characteristics of adrenocortical cells on adrenal steroidogenesis. Therefore, immunohistochemistry of resected adrenals targeting key enzymes involved in steroidogenesis has become another popular area of research. It has led to a deeper understanding of the complex pathophysiology of mineralocorticoid excess and shed light on the underestimated multitude of subtypes of the disease [16, 17]. New findings suggest that the immunohistochemical analysis of resected adrenals could be useful to estimate surgical outcomes. Herein, we review the recent literature and present new diagnostic approaches that could influence the future of therapeutic decision-making in PA.

Pathophysiological Basis and New Developments

Recent developments in the diagnosis of PA reviewed herein require an understanding of the underlying physiological and pathophysiological basics of PA. The adrenal cortex is divided into three layers, a subcapsular *zona glomerulosa* where mostly mineralocorticoids are produced, *zona fasciculata* in the center where secretion of glucocorticoids takes place, and an inner *zona reticularis*, which is responsible for androgen production. In Fig. 1, the enzymatic processes of adrenal steroidogenesis in *zona glomerulosa* and *zona fasciculata* are presented. Shortly, cortisol is synthesized in *zona fasciculata* through hydroxylation of 11-deoxycortisol by CYP11B1 after earlier processing by CYP17A1 whereas aldosterone is produced in *zona glomerulosa* via hydroxylation and oxidation of deoxycorticosterone by CYP11B2 (aldosterone synthase). Aldosterone synthase is the key enzyme required for aldosterone production and in the normal adrenal is present exclusively in the *zona glomerulosa*. In adrenals from patients with PA, the source of aldosterone overproduction can be identified by CYP11B2 immunohistochemistry to visualize aldosterone-producing cells (see Fig. 2**)**. PA has classically been attributed to either a unilateral adenoma or bilateral hyperplasia. In recent years, immunohistochemistry of aldosterone synthase has led to a new understanding of the multitude of subtypes of PA [18]. Aldosterone can be produced by CYP11B2-expressing cells scattered throughout the *zona glomerulosa* as well as from tight clusters of cells with strong expression of CYP11B2 in the subcapsular region of the adrenal cortex. Consequently, PA can be due to multiple different histological entities: a singular CYP11B2-positive adenoma, multiple and enlarged clusters of cells expressing CYP11B2 (APCCs, aldosterone-producing cell clusters), diffuse uni- or bilateral (multinodular) hyperplasia of the CYP11B2-positive *zona glomerulosa*, or combinations of the above. The advances in immunostaining have also identified patients with PA with CYP11B2-negative adenomas in whom aldosterone production was really caused by surrounding CYP11B2-positive APCCs [19].

Interestingly, APCCs were also discovered in a series of adrenals from autopsies of normotensive individuals [20•]. Moreover, APCCs were shown to harbor somatic driver mutations in the calcium channel CACNA1D and the ion pumps ATP1A1 and ATP2B3 implicated in membrane depolarization, intracellular calcium influx, and aldosterone excess [21]. A current hypothesis suggests that APCCs are precursors of APAs, which is supported by findings of subcapsular APCC-like lesions with an inner APA-like lesion [22]. Interestingly, a driver mutation commonly present in APAs (mutation in the KCNJ5 gene) was only found in large APCC/APA-like lesions but not in APCCs or smaller APCC/APA-like lesions suggesting a possible role of driver mutations such as KCNJ5 in the transition of APCCs to APAs [18]. This concept is not experimentally verified and requires confirmation and a mechanistic explanation.

Moreover, histopathological studies have shown that APAs do not only contain CYP11B2-positive but also CYP11B1-positive cells, thought to be responsible for cortisol production [23]. This might partly explain the high prevalence of cortisol co-secretion detected in PA patients [24•]. Furthermore, analysis of additional adrenal steroids has led to the detection of high levels of the "hybrid steroids" 18-oxocortisol and 18-hydroxycortisol in blood serum from patients with APAs, particularly elevated in those harboring KCNJ5 mutations [25••]. 18-Oxocortisol is a physiologically occurring steroid, which is presumably converted from cortisol after enzymatic activities by both CYP11B2 (18-hydroxylation and 18-oxidation) and

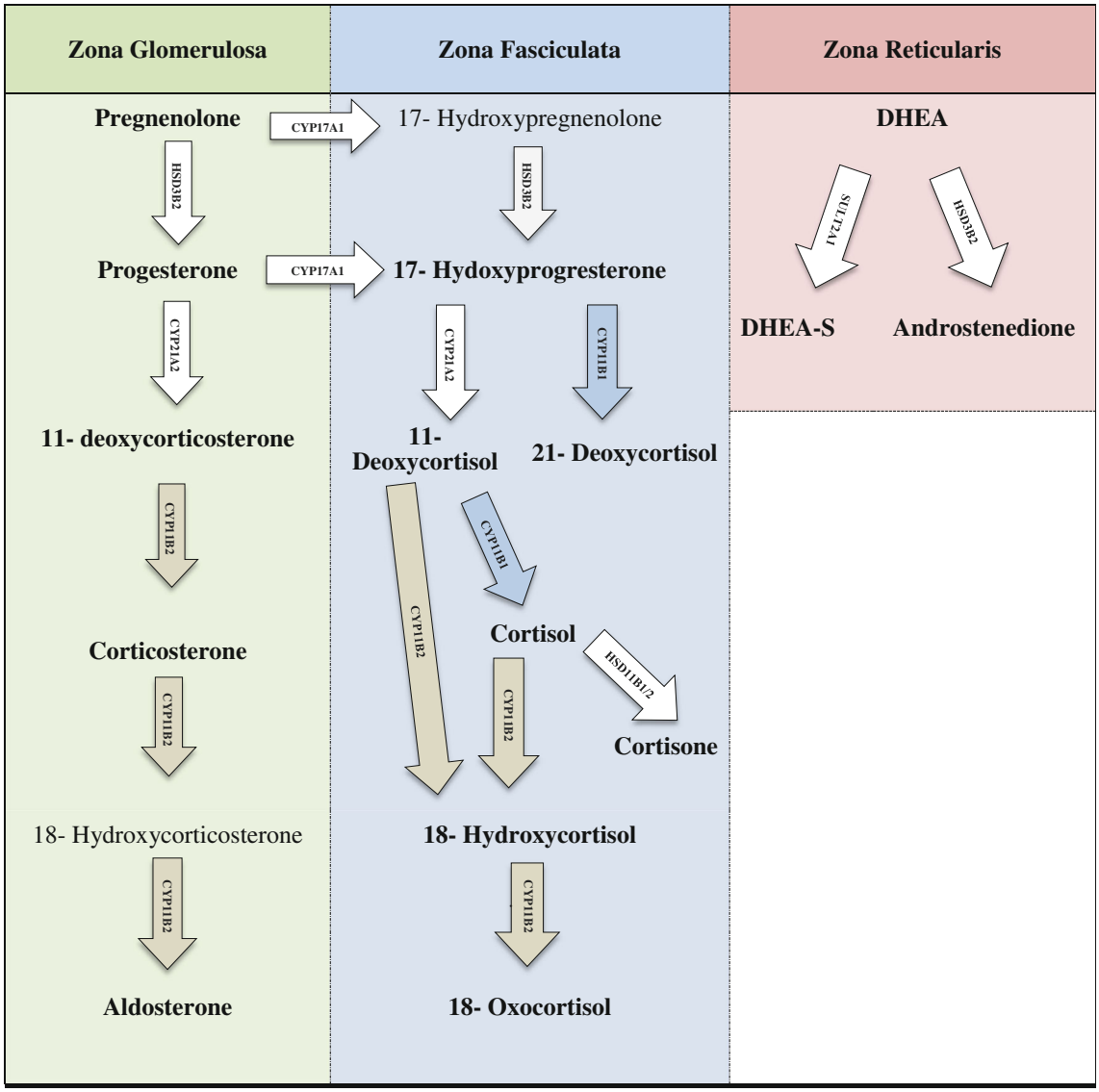

Fig. 1 Adrenal steroids and enzymes involved in adrenal steroidogenesis. Adrenal steroids marked *bold* are those generally included in steroid profiles measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

CYP11B1 (17-hydroxylation) or from 11-deoxycortisol by CYP11B2 with CYP17A1 providing the necessary precursor steroids [19, 26–28]. 18-Oxocortisol levels, therefore, are very low in healthy subjects with adrenal zonation which is intact (physiologic separation of CYP11B2-positive *zona glomerulosa* cells and CYP11B1-positive *zona fasciculata* cells). Higher levels of 18-oxocortisol are found in patients with an APA carrying a KCNJ5 mutation which is likely due

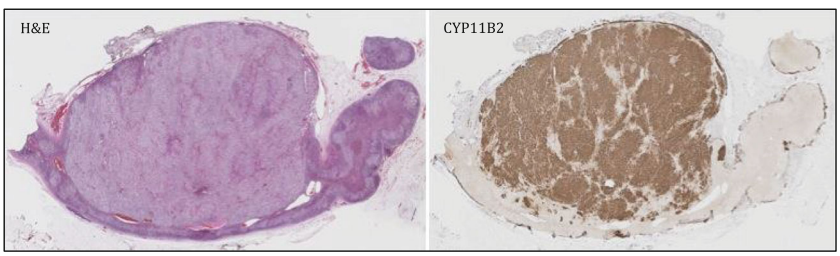

Fig. 2 Immunohistochemistry for aldosterone synthase. Hematoxylin and eosin (H&E, *left panel*) and CYP11B2 immunohistochemistry (*right panel*) staining showing a well-circumscribed adenoma with intense CYP11B2 (aldosterone synthase) expression. The adenoma carries a somatic KCNJ5 Leu168Arg mutation. The CYP11B2 monoclonal antibody was a kind gift from Prof. Celso Gomez-Sanchez, University of Mississippi, MS, USA. Scale bar = 2 mm

to the higher proportion of CYP11B1- and CYP17A1-expressing (*zona fasciculata*-type) cells compared with other APAs [29, 30]. In addition, patients with a familial form of PA, familial hyperaldosteronism type III, caused by germline mutations in KCNJ5, display greatly elevated production of the hybrid steroids [31].

In this context, 18-oxocortisol is just one prominent example of the biochemical consequences of genetic characteristics, which may be exploited in the diagnostic work-up of PA. Just as KCNJ5 mutations lead to high levels of 18-hydroxylated steroids, other genetic characteristics result in different but respectively specific steroid fingerprints. This important discovery entailed a number of studies analyzing adrenal genetics, immunohistochemistry, and steroid profiles as well as their potential use in the diagnostics of PA.

New Tools Needed to Shorten a Complicated Diagnostic Work-up

The current diagnostic work-up in patients with suspected PA involves a long and complicated multi-step process (see Fig. 3). First-line antihypertensive medications that influence the renin-angiotensin-aldosterone system must be changed to medications with little influence on the aldosterone-to-renin ratio (ARR), such as verapamil, doxazosin, or urapidil. If an elevated ARR is detected, patients are usually referred to an endocrinologist for confirmatory testing (salt loading test or other). If PA is confirmed, the 2016 Endocrine Society Clinical Practice Guideline recommends computed tomography in all and AVS in patients who are feasible candidates for surgery [2]. CT scanning ought to exclude the presence of a rare adrenocortical carcinoma in all patients and guide the therapeutic decision without AVS in young hypokalemic patients (< 35 years) with unilateral adenoma. In all other PA patients feasible for surgery, AVS by an experienced interventional radiologist is recommended in order to subtype into unilateral or bilateral disease.

Despite being the diagnostic gold standard, the current diagnostic measures have major weaknesses. The change of medication at the beginning of the diagnostic process is time-consuming and often difficult to achieve: it requires frequent contact with patients and primary care physicians, which often leads to unintended side-effects and sometimes even to hypertensive complications [32], especially in the context of salt loading testing (Heinrich et al., manuscript under review). Moreover, especially in early or mild normokalemic PA and low renin hypertension, the currently used confirmatory tests can lead to conflicting results [33–35].

Not only confirmatory testing but also the currently used methods for subtyping (CT and AVS) are problematic [13•, 14, 36]. The advantages and disadvantages of using imaging

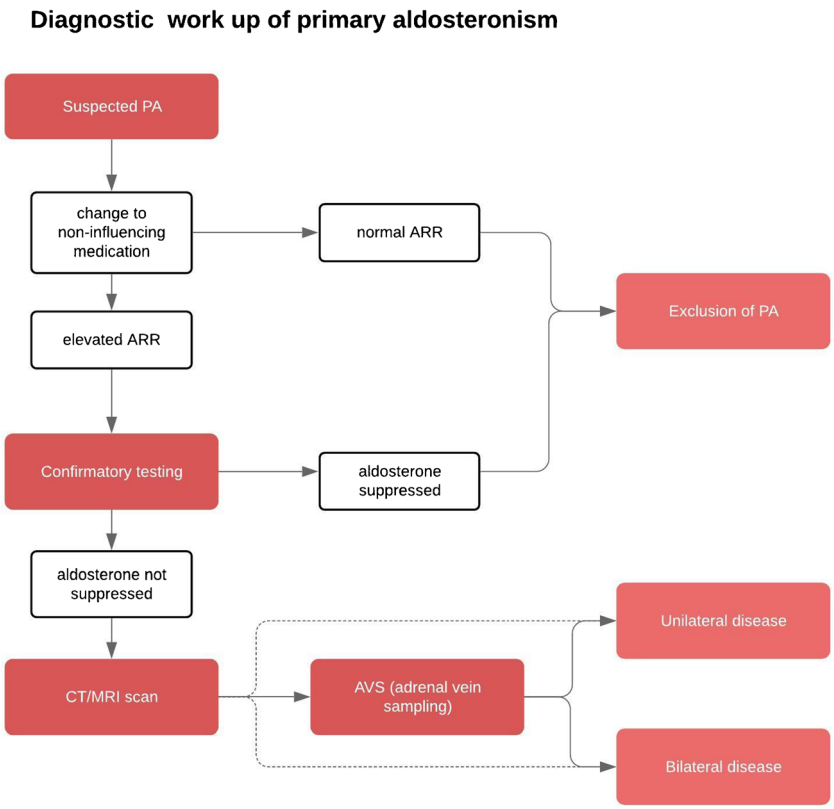

Fig. 3 Diagnostic work-up of primary aldosteronism. ARR, aldosterone-renin ratio; CT, computed tomography; MRI, magnetic resonance imaging; PA, primary aldosteronism

(CT or MR scan) alone or in combination with AVS for subtyping PA and for therapeutic decision-making have been studied intensively [13•, 14, 36] and are subject to ongoing controversies [37, 38]. CT scan alone is generally considered to have insufficient diagnostic accuracy due to its inability to detect aldosterone-producing micro-adenomas or mild hyperplasia and due to the high prevalence of inactive incidentalomas, which can be mistaken for APAs. In around 35–40% of cases, CT/MR scans lead to a different subtype diagnosis than AVS [36, 39, 40]. Under the assumption that AVS correctly identifies the source of aldosterone overproduction, studies found that CT-based adrenalectomy could lead to the removal of the wrong adrenal gland in ~ 2–4% of patients and to ~ 20–30% of patients missing the chance to be cured by surgery due to an incorrect diagnosis [13•, 36]. AVS is therefore considered the gold standard by experts [41] and current guidelines [2]. Nevertheless, AVS is only available at highly specialized centers and has been criticized for having too low success rates, being performed without standardization, being too expensive, and being associated with possible health risks [37].

A German multicenter study reported an association of significant radiation exposure with AVS, with some centers requiring a median of up to 29 mSv per AVS, corresponding to 1470 chest X-rays or 12 years of natural background radiation [42], posing a risk especially in younger patients. Moreover, when assessing clinical outcome (blood pressure levels and use of antihypertensive medication) of CT- versus AVS-based adrenalectomy, two international multicenter studies, the randomized SPARTACUS trial and the retrospective cohort PASO study, could not find a significant clinical benefit of AVS [13•, 14] (although this might be due to a relatively short follow-up of 6–12 months in both studies). However, in our international multicenter study, we found that biochemical remission after CT-based adrenalectomy was significantly lower than with a surgical decision based on AVS results (80% vs. 93%, $P < 0.001$) [13•]. Moreover, although AVS is generally considered expensive, the combination of CT and AVS has been proven to be more cost-efficient than using CT alone [43]. Regarding doubts about its safety and technical feasibility, different large studies have shown that AVS has a very low complication rate [44, 45] and success rates of AVS are increasing due to gradual implementation of supportive technical measures (such as intraprocedural cortisol measurement) [46] and increasing standardization [41, 44].

In summary, AVS remains a technically demanding procedure, which is not readily available in every hypertension center. This creates an "eye of the needle" diagnostic dilemma, posing a major barrier to widespread screening for PA. Therefore, new diagnostic tools that could help in the diagnosis and subtype differentiation of aldosterone excess are being studied extensively and have become a growing field of research [47, 48].

Radiolabeled Imaging of Aldosterone Production

Functional imaging using radiolabeled tracers binding to enzymes involved in aldosterone synthesis, especially CYP11B2 (aldosterone synthase), is evolving as a promising tool to subtype PA [49–51] and shall therefore be mentioned shortly. Using this approach, aldosterone-producing adenomas might be differentiated from bilateral hyperplasia, non-functional adenoma, and normal adrenals by a noninvasive procedure. Different tracers have been examined as potential ligands to the aldosterone synthase used for imaging. In the late 1990s and early 2000s, inhibitors of adrenal 11ß-hydroxylase carbon-11-etomidate and especially carbon-11-metomidate (C-MTO) were reported as promising tracers for PET imaging of the adrenal cortex [52, 53]. While subsequent studies observed that C-MTO and its iodine-123-labeled analog 123I-iodometomidate (I-MTO) could not distinguish between benign and malignant adrenal masses, it was demonstrated that they were specific for the differentiation of adrenocortical from non-cortical lesions [49, 54, 55]. Burton et al. found that C-MTO PET-CT scan had a reasonably high specificity (87%) and sensitivity (76%) to distinguish unilateral from bilateral disease [56] (when relying on a lateralization index of > 4 in AVS to confirm lateralization). However, the availability of C-MTO PET imaging is limited due to the short half-life of carbon-11 requiring a nearby cyclotron. Therefore, fluorine-18-labeled analogues of metomidate such as 18F-FAMTO are being studied in vitro and in animal studies [57], although their safety and utility in humans remain unknown. Moreover, metomidate has a low specificity for CYP11B2 (aldosterone synthase) over CYP11B1 (involved in cortisol synthesis) and, therefore, metomidate-labeled PET requires pre-treatment with dexamethasone [58] in order to suppress CYP11B2 activity.

Abe et al. developed a fluorine-18-labeled imaging agent (18F-CDP2230) and demonstrated higher selectivity for CYP11B2 over CYP11B1 in vitro and successful application in PET/MRI imaging in rats. Whether the tracer is useful in humans to distinguish between unilateral and bilateral disease remains to be seen. Recently, the CXC chemokine receptor type 4 (CXCR4) and its ligand 68Ga-pentixafor have also been shown to correlate strongly with the expression of CYP11B2 and less with CYP11B1 in vitro [59, 60]. In a recent study by Heinze et al., CXCR4 PET imaging correctly identified unilateral aldosteronism in 9 patients (sensitivity 88.9%, specificity 87.2%), in whom AVS was technically not successful and CYP11B2-positive adenomas were later confirmed by immunohistochemistry [60]. In our experience, the currently available CXCR4 PET technique can be helpful in decision-making in difficult individual cases but—due to its limited availability—cannot be used for subtyping PA in a broad scale. Currently, this imaging technique should be

considered experimental but the general promising methodical approach of functional imaging has the potential to one day replace CT and AVS as standard methods for lateralization in PA.

Adrenal Steroid Profiling for Subtype Differentiation and Genotype Prediction

A promising area of research related to subtyping and lateralization of PA addresses the individual expression of the "steroid metabolome." The measurement of up to 15 different adrenal steroids using liquid chromatography with tandem mass spectrometry (LC-MS/MS) [61] has shed new light on pathophysiological mechanisms in PA [24•, 62] and other adrenal disorders such as Cushing syndrome [63]. As an example, we used LC-MS/MS steroid profiling to reveal a significantly increased cortisol and total glucocorticoid excretion in patients with PA [24•]. The glucocorticoid output was associated with intratumoral expression of CYP11B1, the main enzyme involved in glucocorticoid synthesis, supporting our hypothesis of cortisol co-secretion within the Conn adenoma. Our findings of a high correlation between different surrogate parameters of metabolic risk and glucocorticoid (but not mineralocorticoid) excretion might also explain the high burden of metabolic diseases like diabetes mellitus and osteoporosis in this cohort. These and other pathophysiological findings have thus brought up hope for a more effective diagnostic work-up in different adrenal pathologies. Not only in PA but also in Cushing disease steroid profiling has been investigated as a potential tool for diagnosing and subtyping [63].

The steroids currently measured by LC-MS/MS include aldosterone, cortisol, 18-oxocortisol, 18-hydroxycortisol, cortisone, 11-deoxycortisol, 21-deoxycortisol, corticosterone, 11-deoxycorticosterone, progesterone, 17-hydroxyprogesterone, pregnenolone, androstenedione, dehydroepiandrosterone (DHEA), and DHEA-sulfate (DHEA-S) (see bold marked steroids in Fig. 1).

Especially the "hybrid steroids" 18-oxocortisol and 18-hydroxycortisol have been investigated thoroughly in the past decades due to early studies showing their potential in differentiating the subtypes of PA. They shall therefore be discussed here in more detail. As mentioned above, 18-oxocortisol and 18-hydroxycortisol are naturally occurring steroids requiring enzymatic processing by CYP11B2 (aldosterone synthase) usually expressed in *zona glomerulosa* as well as CYP11B1 and CYP17A1 found in *zona fasciculata* [64–66]. After the discovery of elevated C-18-oxygenated steroids in a patient with aldosterone-producing adenoma in the 1960s [67], different studies in the early and mid-1980s found that the newly discovered adrenal steroids 18-hydroxycortisol [68] and 18-oxocortisol [69] were elevated in APA patients' serum but not in BAH patients. This led to many studies in the following decades examining the utility of different adrenal steroids in order to subtype PA [70].

In 2011, Nakamura and colleagues were the first to demonstrate that levels of 18-oxocortisol were significantly higher in the adrenal veins of APAs compared with either adrenal vein of patients with BAH [71]. They found that in all but one of 14 APA patients (and in all 14 after ACTH stimulation), the 18-oxocortisol/cortisol ratios of the dominant adrenal vein were higher than those of either adrenal vein of all 7 BAH cases or the contralateral vein of APA cases. In a later study by Mulatero et al., peripheral serum levels of 18-oxocortisol and 18-hydroxycortisol and of the adrenal (*zona glomerulosa*) steroid 18-hydroxycorticosterone as well as 24 h-urine levels of 18-oxocortisol and 18-hydroxycortisol were compared between 20 APA patients, 61 BAH patients, and 62 patients with essential hypertension. They found that median levels of serum 18-hydroxycorticosterone, 18-oxocortisol, and 18-hydroxycortisol were significantly higher in patients with APA than with BAH (1090 vs. 654 pg/ml, 1742 vs. 1103 pg/ml, and 93 vs. 45 pg/ml respectively). However, there was significant overlap between all groups and it was not possible to establish cutoff values to allow the differentiation of patients with APA from those with BAH [72]. They further demonstrated that 18-hydroxycortisol and 18-oxocortisol levels were significantly higher in 24-h urine collections in patients with APA compared with BAH. Using cutoffs for urinary 18-hydroxycortisol measurements, the majority of patients with PA could be subtyped without AVS. The authors claimed that patients with PA with urinary 18-hydroxycortisol concentrations under 130 μg/day could have avoided AVS because none of the patients with APA had levels below this cutoff. Moreover, concentrations above 510 μg/day were specific to patients with an APA and all patients with a clear nodule at CT and urinary 18-hydroxycortisol levels above this cutoff could have undergone adrenalectomy without AVS. The authors suggested that only patients with values between 130 and 510 μg/day required AVS to subtype aldosterone excess and proposed the inclusion of urinary 18-hydroxycortisol in the diagnostic work-up of PA. Interestingly, they also discovered that the salt loading test may lead to the reduction of 18-hydroxycorticosterone, 18-oxocortisol, and 18-hydroxycortisol in all patients emphasizing that production of these steroids is under partial control of the renin-angiotensin system. The reduction of the three steroids after salt loading test was significantly less prominent in patients with APA than with BAH and essential hypertension, which could be interpreted as a more autonomous production of these steroids in patients with APA.

Satoh et al. were the first to use liquid chromatography with tandem mass spectrometry (LC-MS/MS) as a more reliable method to measure peripheral plasma 18-oxocortisol and 18-hydroxycortisol in PA [73•]. Using a certain cutoff for 18-oxocortisol alone (6.1 ng/dl), they were able to distinguish

an aldosterone-producing adenoma (excluding CT-undetectable microadenomas of < 10 mm) from bilateral hyperplasia with a diagnostic accuracy of 84%. This was higher than when relying on CT scan alone to subtype aldosterone excess (accuracy of 72% in their study). The high diagnostic accuracy for subtype differentiation with 18-oxocortisol alone in their study is most likely due to the high prevalence of underlying KCNJ5 mutations in Asia of up to 70% [74, 75]. These results can, therefore, not be translated to the worldwide population.

In a multicenter study including 119 APA and 90 BAH patients, we measured complete steroid profiles (15 adrenal steroids) by LC-MS/MS in peripheral and adrenal venous Peripheral plasma [76••]. Plasma 18-oxocortisol levels were 8.5-fold higher in patients with APA than in patients with BAH. Despite the clear elevation of 18-oxocortisol in patients with APA, the use of this steroid alone was not useful for subtyping patients with PA due to significant overlap between groups. Conversely, plasma cortisol, corticosterone, and dehydroepiandrosterone (DHEA) and DHEA-S levels were significantly higher in patients with BAH than in patients with APA. Using a combination of all measured steroids, a correct differentiation between unilateral and bilateral disease was possible in 80% of cases (Table 1).

In a recent study (2019), we analyzed in detail the differences in LC-MS/MS-based peripheral venous steroid profiles of BAH, micro-APA (< 10 mm), and macro-APA (≥ 10 mm) patients in a large cohort ($n = 197$) and created an online tool allowing the prediction of the respective subtype on the basis of a single blood draw [77]. We found that patients with macro-APAs had higher levels of 18-oxocortisol, 18-hydroxycortisol, and aldosterone than both patients with micro-APA or BAH. Moreover, patients with macro-APAs displayed higher levels of 11-deoxycorticosterone and pregnenolone than those with BAH. Concentrations of DHEA-S were more elevated in patients with a micro-APA or BAH than patients with a macro-APA, contributing to the hypothesis that larger APAs may suppress ACTH-dependent androgen synthesis due to an increased glucocorticoid co-secretion. We did not find significant differences in concentrations of single steroids between patients with BAH and micro-APAs but a trend towards higher levels of aldosterone and 11-deoxycorticosterone in micro-APAs than in BAH. Using "random forest classification trees," we were able to correctly classify 97% of macro-APAs and 87% of BAH solely based on peripheral venous steroid profiles. In contrast, only 11 of 33 micro-APAs were correctly categorized (33%). Adding CT and AVS results into the analysis, correct classification of micro-APAs was raised to 68% and steroid profiling improved the diagnostic accuracy compared with CT and AVS alone. All in all, this study supports the high potential of steroid profiling for subtype differentiation of patients with PA.

The potential use of steroid profiling for subtype differentiation in PA is likely due to the association of steroid

Table 1 Relative concentrations of different adrenal steroids found in plasma of patients with different subtypes of primary aldosteronism

	Steroid hormone	Type of histopathology		
		BAH	Micro-APA	Macro-APA
Relative hormone concentrations in peripheral venous plasma	11-Deoxycorticosterone [77]	+	(+)	++
	11-Deoxycortisol [76]	=	=	=
	17-Hydroxyprogesterone [76]	++	(++)	+
	18-Hydroxycortisol [77]	+	+	++
	18-Oxocortisol [76], [77]	+	+	++
	21-Deoxycortisol [76]	=	=	=
	Aldosterone [77]	+	+	++
	Androstenedione [76]	=	=	=
	Corticosterone [76]	++	(+)	+
	Cortisol [76]	++	(+)	+
	Cortisone [76]	=	=	=
	DHEA [76]	++	(++)	+
	DHEA-S [77]	++	++	+
	Pregnenolone [77]	+	(+)	++
	Progesterone [76]	=	=	=

APA, aldosterone-producing adenoma; *BAH*, bilateral adrenal hyperplasia. Micro-APAs indicates < 10 mm in diameter; macro-APAs indicates ≥ 10 mm in diameter; "+" indicates significantly lower than ++; "++" indicates significantly higher than +, "=" indicates no significantly different concentrations detected in either subgroup, "(+)" indicates no significantly different concentrations observed in micro-APA compared with macro-APA or BAH but significantly lower in BAH than in macro-APA, "(++)" indicates no significantly different concentrations observed in micro-APA compared with macro-APA or BAH but significantly higher in BAH than in macro-APA

measurements with somatic APA mutations driving autonomous aldosterone production [25••]. The most thorough investigation came from our study analyzing genotype-phenotype associations in a series of 79 patients with APA. Adenoma DNA was analyzed for the presence of activating somatic driver mutations in the genes for *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D*. Baseline plasma samples were analyzed by LC-MS/MS for the measurement of 15 adrenal steroids. The genetic study demonstrated the typical prevalence of mutations (34.1% KCNJ5, 8.8% CACNA1D, 6.3% ATPase). The prevalence of KCNJ5 mutations in our study was similar to that in other studies (outside Asia), which estimated a prevalence of around 40% in all APAs worldwide and significantly lower when compared with studies in Eastern Asia (up to 70%) [74, 75, 78]. In this study of adrenal and peripheral venous steroid profiles, we detected that levels of 18-oxocortisol, aldosterone, 18-hydroxycortisol, and 11-deoxycorticosterone were up to 18-fold higher (18-oxocortisol) in the affected adrenal vein of KCNJ5-mutated APAs than in all other mutations and the wild-type combined. When analyzing peripheral venous blood, we found 21-fold higher concentrations of 18-oxocortisol and 2.9-fold higher levels of 18-hydroxycortisol in APAs harboring a KCNJ5 mutation than in the wild-type. Using *adrenal* venous steroid profiles of the dominant and non-dominant veins, we were able to correctly classify 95% of all driver mutations including 100% accuracy of detection of KCNJ5 and CACNA1D mutations. When *peripheral* venous blood was analyzed, a correct categorization was still possible in 92% of cases. These results emphasize that each driver mutation leads to an individual biochemical fingerprint that can be detected by measuring adrenal or peripheral venous steroid profiles.

This new diagnostic approach was recently taken advantage of in the case of a 55-year-old female patient with PA and a left-sided adrenal mass in whom AVS was unsuccessful (case report to be published). Steroid profiling of a peripheral venous sample revealed elevated levels of aldosterone, 18-oxocortisol, 18-hydroxycortisol, 11-deoxycorticosterone, and 11-deoxycortisol. These results were strongly suggestive of a macro-APA due to a KCNJ5 mutation [25••], which led to the decision for adrenalectomy and was later confirmed by histopathology and genetic testing.

These important advances in the diagnostics of PA should be taken into account in all centers screening for and treating patients with PA. In our experience, while AVS still is considered the gold standard for subtyping PA, steroid profiling can and should be used in those cases in which AVS is not possible, inconclusive, or unsuccessful.

Outcome

Treatment outcomes for PA can be defined in different ways. Firstly, outcome can refer to the treatment response of hormonal or biochemical parameters (biochemical outcome). Secondly, outcomes can be assessed by the clinical response such as the change of systolic and/or diastolic blood pressure and antihypertensive medications (clinical outcome). Both were used in the 2017 international consensus paper (study on Primary Aldosteronism Surgery Outcome, PASO), which laid out generally accepted criteria for the definition of outcomes of treatment for surgically treated patients with unilateral PA [10••]. According to the PASO study, biochemical and clinical outcomes are classified as absent, partial, or complete (Table 2).

A complete biochemical outcome is herein defined as normalization of potassium values (if low before surgery) and normalization of aldosterone/renin ratio (ARR) 6–12 months after surgery. If ARR is pathological, a normal confirmatory test defines complete biochemical success, a decrease of baseline aldosterone levels ≥ 50% compared with preoperative levels is defined as partial biochemical success, and a decrease

Table 2 Definition of clinical and biochemical outcomes of adrenalectomy according to the Primary Aldosteronism Surgery Outcome (PASO) study [10]

	Complete success	Partial success	Absent success
Clinical outcome	Normal blood pressure without the aid of antihypertensive medication	Unchanged blood pressure with less antihypertensive medication *or* a reduction in blood pressure with the same or less antihypertensive medication	Unchanged or increased blood pressure with the same or more antihypertensive medication
Biochemical outcome	Correction of hypokalemia (if present pre-surgery) and normalization of the aldosterone-to-renin ratio *or* suppressed aldosterone secretion in a confirmatory test	Correction of hypokalemia (if present pre-surgery) and a raised aldosterone-to-renin ratio with ≥ 50% decrease in baseline plasma aldosterone concentration (compared to pre-surgery) *or* abnormal but improved post-surgery confirmatory test result	Persistent hypokalemia (if present pre-surgery) *and/or* persistent raised aldosterone-to-renin ratio with failure to suppress aldosterone secretion with a post-surgery confirmatory test

of < 50% and/or persistent hypokalemia is defined as absent biochemical success. Complete clinical success is defined as normalization of blood pressure without antihypertensive medication and partial clinical success as having either the same levels of blood pressure as before surgery using less antihypertensive medication or a reduction in blood pressure with either the same amount or less antihypertensive medication. Absent clinical success is considered when levels of blood pressure are not different or increased (using the same amount or more antihypertensive medications) compared with before surgery.

Besides this consensus definition of short-term biochemical and clinical characteristics, outcomes can refer to the long-term rate of cardiovascular morbidity and mortality, which is generally relatively high in patients with PA [5–7, 8••]. The latter understanding of outcome is not part of this review due to the lack of studies investigating associations between steroid profiling or immunohistochemistry and cardiovascular outcome.

Outcome Prediction

Despite high rates (83–100%) of biochemical remission after adrenalectomy [10••, 11], cure of hypertension (complete clinical success) after surgery is only achieved in less than half of patients [6, 10••, 79]. Therefore, investigators have looked for ways to predict biochemical and clinical outcomes after adrenalectomy [80]. As an example, in a recent study, we used clinical characteristics (known duration of hypertension, sex, antihypertensive medication dosage, body mass index, target organ damage) as well as the size of largest adrenal nodule at imaging in a model to predict the probability of complete clinical outcome after adrenalectomy with an accuracy of 79.2% [81•]. While these prediction models rely on empiric data, more individual parameters for outcome prediction are being studied. Steroid profiling as well as the immunohistochemistry of resected adrenals (although the latter not being useful before therapy decision) could be more precise variables for outcome prediction because of their correlation with the individual genetic characteristics of a patient.

Steroid Profiles Associated with Outcomes

Studies on the association of steroid profiling and outcome of therapy in PA are scarce. In 2018, we published a detailed analysis of steroid profiles of patients with PA (APA and BAH) measured by LC-MS/MS (before therapy) and examined the association of 15 different adrenal steroids with both biochemical outcomes and histopathological results of adrenalectomy [15•]. We found that levels of cortisone and 11-deoxycortisol at baseline were significantly lower in patients whose surgery resulted in complete biochemical success than in those having partial or absent biochemical success. Using linear discriminant analysis and decision tree analysis of different steroid profile concentrations at baseline in a group of PA patients undergoing adrenalectomy, we could classify biochemical outcomes after surgery (complete vs. partial/absent) in 88% (35/40, linear discriminant analysis) and 100% (40/40, decision tree analysis) of patients respectively. Interestingly, these outcome classification models were more accurate than previous models using the lateralization index and contralateral ratio in AVS (using linear discriminant analysis 88% vs. 75%; decision tree analysis 100% vs. 95%).

Immunohistochemical Expression of CYP-11B1 and CYP-11B2 Associated with Outcomes

Earlier studies have assessed histopathology but not immunohistochemistry in resected adrenals as well as outcomes after adrenalectomy [82–84]. In a retrospective study of 126 patients, the authors could not find significantly different histopathological characteristics between 122 biochemically cured patients and 6 patient with persistent PA or 3 patient with recurrent aldosterone excess after surgery [83]. In contrast, a more recent Norwegian study detected clear correlations between histopathology and outcome. In all patients who were cured of hypertension after adrenalectomy, an adenoma was found in the resected adrenal whereas all cases of hyperplasia belonged to the group of patients with persistent hypertension [84].

Volpe et al. were the first to examine functional, immunohistochemical features of resected adrenals and biochemical and clinical outcomes [85]. In their study, all but one patient (93/94) with a single CYP11B2-positive adenoma were biochemically cured after adrenalectomy. Forty-six percent of APA patients in their study showed complete clinical success with normalization of blood pressure levels. In contrast, only 10 of 26 patients with functional hyperplasia (CYP11B2-positive nodules, including 5 patients with non-functional adenoma in the resected adrenal) reached biochemical long-term remission (after a median of 4 years follow-up). Accordingly, normalization of blood pressure levels was documented in only 19% of these patients after surgery.

In our outcome study mentioned above, we also examined histopathology and immunohistochemistry and its correlation with absent, partial, and complete biochemical success according to standardized PASO criteria [15•]. Our cohort consisted of 43 patients with absent or partial and 52 patients with complete biochemical success. We found a higher prevalence of cortical hyperplasia (21/43, 49% vs. 11/52, 22%) and CYP11B2-negative (non-functional) adenomas (24/43, 56% vs. 11/52, 21%) in the absent/partial success group than in sex- and age-matched patients with complete biochemical success. On the other side, the complete biochemical success group consisted of a significantly higher rate of solitary

CYP11B2-positive adenomas (44/52, 79% vs. 19/43, 41%) than the absent/partial success group. Interestingly, neither the characteristics of the adjacent adrenal cortex (normally appearing vs. hyperplastic adjacent cortex) nor the existence or number of APCCs correlated with either biochemical outcome.

In a different study, we analyzed biochemical and clinical outcomes according to the PASO criteria as well as histopathological and immunohistochemistry findings in 128 patients with unilateral aldosteronism who had undergone adrenalectomy [77]. We detected that complete clinical and biochemical success was more frequent in patients with CYP11B2-positive macro-APAs (> 10 mm) than in patients with micro-APAs (< 10 mm). When CYP11B2 immunohistochemistry was performed in micro-APA patients with partial or absent biochemical success, we mostly found more than one micronodule. However, we contributed the significant difference in *clinical* success mainly to a higher prevalence of cardiovascular risk factors (higher BMI, more male patients) in the micro-APA subgroup.

Consequently, although limited data exists on the association of functional histopathology with outcome in PA, patients with diffuse hyperplasia or (multiple) micronodules/ micro-APAs in immunohistochemistry of resected adrenals seem to have a higher risk of persistence or recurrence of aldosterone excess (Table 3).

A Clinical Case

At our center, steroid profiles and immunohistochemistry are being used increasingly in everyday clinical practice. To illustrate the clinical benefits of these techniques, we present the recent case of a 27-year-old woman with severe refractory arterial hypertension due to PA.

Table 3 Association of immunohistochemistry and outcomes

Immunohistochemistry of resected adrenals	Biochemical outcome	
	Incomplete	Complete
Solitary CYP11B2-pos. adenoma [15, 77]	+	++
CYP11B2-negative adenoma [15]	++	+
Diffuse Hyperplasia [15]	++	+
Multiple mironodules/ micro-APAs [77]	++	+

++ indicates more likelihood of being linked to this type of outcome than +, + indicates less likelihood of being linked to this type of outcome than ++

The student initially presented to our outpatient center with severe and recurrent headache due to hypokalemic hypertension. After confirmation of PA by salt loading and captopril challenge tests, an abdominal MRI was performed. It showed a typical adrenal adenoma of 25 mm on the right side. However, the left adrenal gland also showed an irregular configuration, attributed to left-sided hyperplasia. In order to predict the subtype (uni- vs. bilateral aldosterone excess) and possibly a driver mutation, a baseline peripheral blood draw with adrenal steroid profiling by LC-MS/MS was conducted.

As indicated in Table 4, the steroid profile predicted a macro-APA due to a KCNJ5 mutation as described in recent studies [25••, 77]. In order to confirm the diagnosis, AVS was performed. AVS demonstrated a strong lateralization of aldosterone production to the left side (lateralization index left/right, 94.3). After adrenalectomy, immunohistochemistry confirmed a CYP11B2-positive macroadenoma of 32 mm with normally appearing tissue in the adjacent adrenal. Genetic analysis of the adenoma confirmed a KCNJ5 mutation, as predicted by the baseline steroid profile. Postoperative follow-up revealed normal blood pressure levels as well as normalization of potassium, aldosterone, and aldosterone-renin ratio, indicating a complete clinical and biochemical remission after surgery.

This case illustrates that a highly predictive steroid profile could have replaced AVS and even predicted a specific driver mutation in this young patient. Moreover, the presence of a single CYP11B2-positive adenoma after immunohistochemistry correctly predicted the complete outcome of surgery.

Conclusions

Recent advances in the diagnostics of PA have brought up two clinically relevant new tools. On the one hand, the measurement of different adrenal steroids by liquid chromatography-tandem mass spectrometry (LC-MS/MS) allows characterization of the individual steroid metabolome of a patient. Since underlying genetic mutations lead to specific histopathological phenotypes and different adrenal steroid concentrations in peripheral plasma, the analysis of steroid profiles can be used to predict the subtype of PA and in case of a KCNJ5 mutation the genotype underlying aldosterone excess. Combined with an unequivocal adrenal imaging result, this simple and noninvasive diagnostic measure could, in many cases, replace adrenal vein sampling (see Fig. 4). This could simplify the diagnostic flow chart and reduce health care costs, radiation

Table 4 Peripheral venous plasma concentrations of different adrenal steroids in a 27-year-old patient with primary aldosteronism due to an aldosterone-producing adenoma with a KCNJ5 mutation

Steroid hormone	Plasma concentrations		
	< 2.5 percentile	2.5 – 97.5 percentile	> 97.5 percentile
11-Deoxycorticosterone			
11-Deoxycortisol			
17-Hydroxyprogesterone			
18-Hydroxycortisol			
18-Oxocortisol			
21-Deoxycortisol			
Aldosterone			
Androstenedione			
Corticosterone			
Cortisol			
Cortisone			
DHEA			
DHEA-S			

Concentrations are shown as bar graphs relative to the 2.5 and 97.5 percentiles established by Eisenhofer et al. [76]

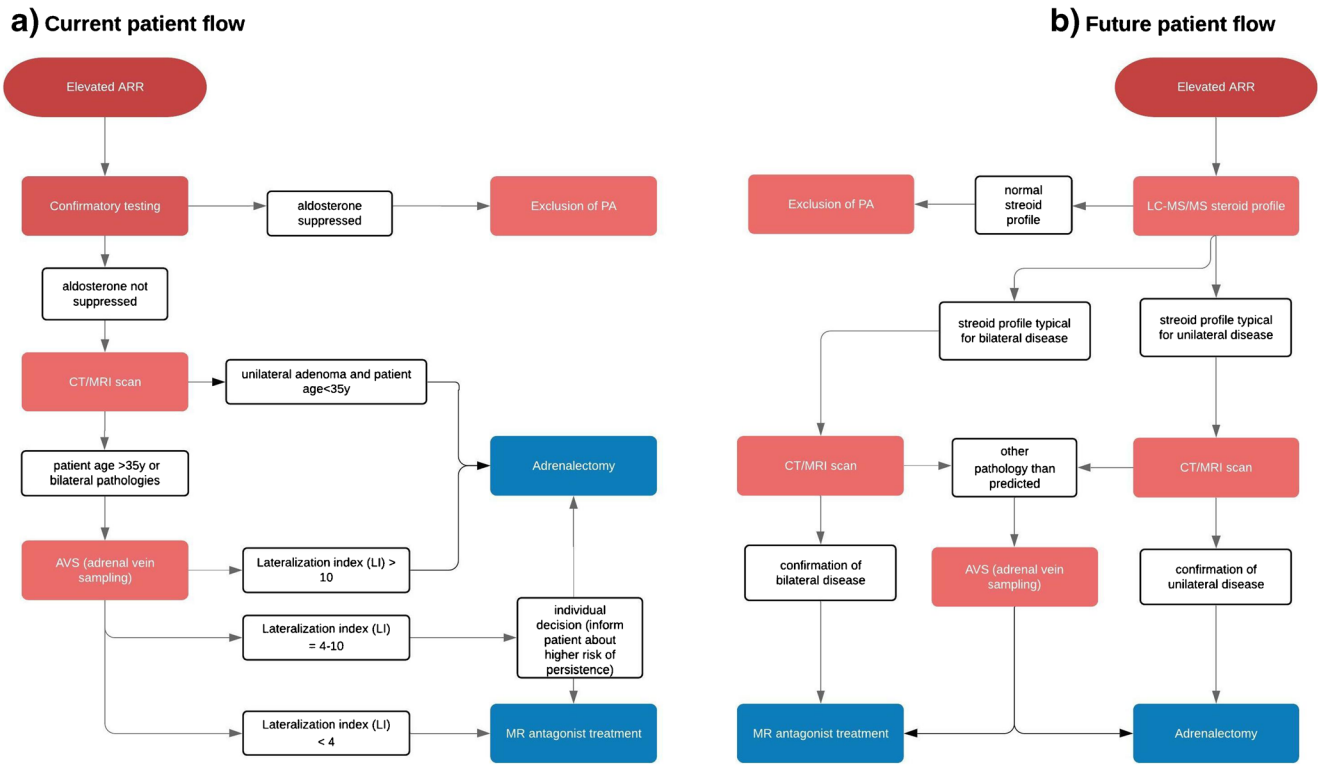

Fig. 4 Flow chart of **a** current and **b** potential future patient flow (including steroid profiling) of patients undergoing diagnostic work-up of primary aldosteronism. ARR, aldosterone-renin ratio; CT, computed tomography; MR, mineralocorticoid receptor; MRI, magnetic resonance imaging; PA, primary aldosteronism

exposure, and periprocedural complications. Nonetheless, randomized studies are needed to evaluate and validate this technique. Moreover, steroid profiles by LC-MS/MS are presently only measured in highly specialized centers. At this time, they should be considered a helpful diagnostic measure to subtype aldosterone excess in cases where AVS cannot be performed and surgery is considered a feasible therapeutic option.

The second diagnostic tool in the work-up of PA is functional histopathology by immunohistochemistry. Immunostaining of sections of resected adrenals for aldosterone synthase (CYP11B2), the enzyme involved in the terminal steps of aldosterone biosynthesis, allows the localization of aldosterone synthesis. The application of this technique has demonstrated that the pathophysiology of aldosterone excess is a lot more complex than previously thought. It has revealed that PA can be due not only to an aldosterone-producing adenoma or multinodular hyperplasia but also to aldosterone-producing cell clusters (APCCs). Moreover, it has shown that relevant aldosterone production sometimes takes place in the adjacent adrenal tissue surrounding an adenoma. Additionally, the adrenal CYP11B2 immunohistochemistry after adrenalectomy for PA maybe associated with outcomes of surgery. Solitary functional (CYP11B2-positive) adenomas are associated with higher rates of complete biochemical and clinical outcomes whereas the presence of (multinodular) hyperplasia in the resected adrenal is linked to persistence of aldosterone excess after surgery. The type of immunohistochemistry could and should therefore determine the intensity of follow-up of PA patients after surgery. Thus, immunohistochemistry should be used on a routine basis in all patients undergoing surgery for PA in order to confirm the preoperative diagnosis and define the interval of planned follow-up visits.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national research committee standards, and international/national/institutional guidelines).

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