# Developments in Primary Aldosteronism Subtyping Using Steroid Profiling

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## ABSTRACT

Adrenal venous sampling is the standard of care for identifying patients with unilateral primary aldosteronism, which is often caused by an aldosterone producing adenoma and can be cured with surgery. The numerous limitations of adrenal venous sampling, including its high cost, scarce availability, technical challenges, and lack of standardized protocols, have driven efforts to develop alternative, non-invasive tools for the diagnosis of aldosterone producing adenomas. Seminal discoveries regarding the pathogenesis of aldosterone producing adenomas made over the past decade have leveraged hypotheses-driven research of steroid phenotypes characteristic of various aldosterone producing adenomas. In parallel, the expanding availability of mass spectrometry has enabled the simultaneous quantitation of many steroids in single assays from small volume biosamples. Steroid profiling has contributed to our evolving understanding about the pathophysiology of primary aldosteronism and its subtypes. Herein, we review the current state of knowledge regarding the application of multi-steroid panels in assisting with primary aldosteronism subtyping.

## Introduction

Primary aldosteronism (PA) is the most common form of secondary hypertension, affecting 5–10 % of all hypertensive patients [1–3] and up to 20 % of those with resistant hypertension [4, 5]. PA is associated with higher prevalence of metabolic syndrome, and disproportionately higher cardiovascular and renal morbidity and mortality than essential hypertension [6–10]. Early diagnosis of PA and implementation of targeted therapy are, thus, essential for reducing its associated complications [6, 11, 12]. Conventionally, PA has been classified into two major subtypes: bilateral hyperaldosteronism (BHA), and unilateral PA. Unilateral PA accounts for 30–50 % of PA cases, and it is commonly caused by an aldosterone-producing adenoma (APA) [3]. APAs are ideally treated with laparoscopic adrenalectomy, which often cures PA and alleviates the cardiovascular morbidity in these patients [6, 7, 10]. Conversely, patients with BHA require life-long medical therapy that typically includes a mineralocorticoid receptor antagonist (MRA) [13].

Unlike other disorders of adrenal hormonal excess, the source(s) of aldosterone excess in PA cannot be reliably identified by conventional imaging studies. Two major factors contribute to the poor performance of adrenal imaging in PA subtyping: 1) areas producing aldosterone excessively can be smaller than the threshold for detection by cross-sectional imaging, or they might not distort the adrenal cortex anatomy at all [14] and 2) the prevalence of nonfunctional adrenal cortical nodules increases with age, as does that of PA [15, 16]. Indeed, CT findings can lead to erroneous subtyping in up to 50 % of PA cases [17–20]. Consequently, adrenal vein sampling (AVS) is endorsed by expert guidelines as the only reliable tool for PA subtyping [3, 21, 22]. AVS, however, is a costly, invasive, and operator-dependent procedure, with scarce availability. Further-

more, many aspects regarding AVS protocols and data interpretation remain controversial and differ among institutions [23, 24].

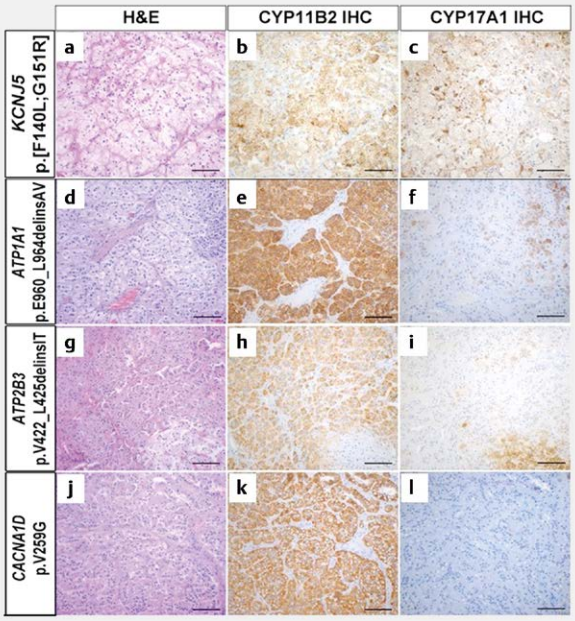
Recent progress in the understanding of PA pathogenesis has driven efforts to develop non-invasive tools for PA subtyping. One such approach is identifying steroid biomarkers pathognomonic of APAs. The transition from immunoassays to mass spectrometry has facilitated the simultaneous measurement of multi-steroid panels in small volume biospecimen samples. Steroid fingerprints specific to patients with APAs measured in peripheral blood could eliminate the need for AVS in all other PA patients, who are best served by medical therapy. Herein, we review the recent advances in the application of steroid mass spectrometry for PA subtyping.

## Steroid Profiles Characteristic of APAs with Different Underlying Aldosterone-Driver Somatic Mutations

With the implementation of next-generation sequencing (NGS), a series of APA tissue studies conducted over this past decade have identified several somatic mutations in genes with impact on aldosterone synthesis: *KCNJ5,* which encodes the Kir3.4 (GIRK4) potassium channel; *ATP1A1*, which encodes the $Na^+/K^+$ ATPase α-1 subunit; *ATP2B3,* encoding a $Ca^{2+}$ ATPase 3; and *CACNA1D*, which encodes a voltage-dependent L-type calcium channel subunit 1D [25–28]. Such mutations account for at least 90 % of sporadic APAs [29, 30], and they all facilitate inappropriate intracellular calcium entrance, which subsequently boosts aldosterone production.

Detailed histopathological studies of APAs have revealed morphologic and enzymatic heterogeneity across genotypes (▶**Fig. 1**). APAs harboring *KCNJ5* mutations are typically composed of large cells, with clear, lipid-rich cytoplasm, resembling zona fasciculata (ZF) cells [31–33]. Conversely, more variability has been reported in the histological features of APAs with other underlying somatic mutations [31, 34, 35]. Overall, APAs harboring *ATPase* or *CACNA1D* mutations display a higher proportion of compact eosinophilic, or lipid-poor, cells, similar to zona glomerulosa (ZG) cells (▶**Fig. 1**) [34]. Other features that distinguish APAs with *KCNJ5* mutations include their larger size and higher expression of CYP11B1 and CYP17A1 [34, 36, 37]. The co-localization within the same tumor of aldosterone synthase (CYP11B2), which is normally restricted to the ZG, and 17α-hydroxylase/17,20-lyase (CYP17A1), which is expressed in the ZF and zona reticularis, allows these *KCNJ5*-mutatated APAs to produce "hybrid steroids", such as 18-hydroxycortisol (18OHF) and 18-oxocortisol (18oxoF, ▶**Fig. 2**) [38, 39].

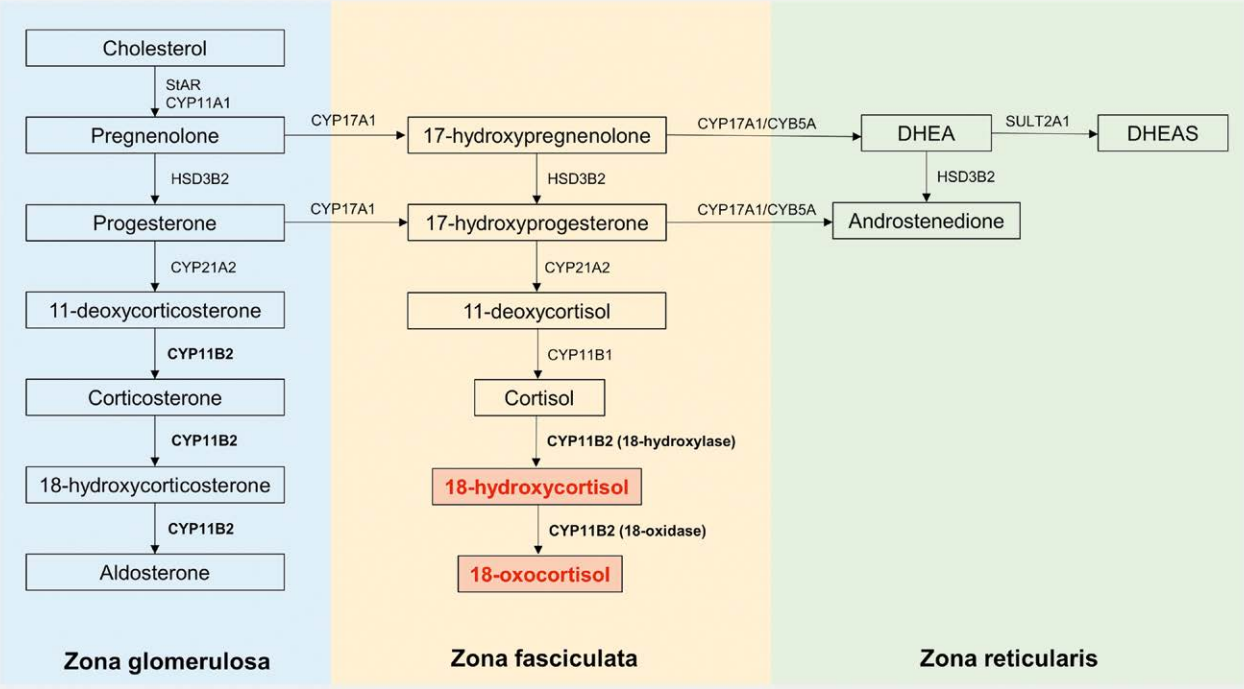
Evidence to support the previously suspected capability of *KCNJ5*-mutated APAs to over-produce hybrid steroid has only recently emerged. In a study of 79 patients with APAs of various genotypes (27 *KCNJ5*, 9 *ATPase*, 7 *CACNA1D*, and 36 "wild-type"), Williams et al. found that patients with APAs harboring *KCNJ5* mutations had abundant concentrations of 18oxoF in peripheral venous plasma: 21-fold higher compared to the wild-type group ($p < 0.05$) and 16-fold higher compared to all other groups combined ($p < 0.0001$). Patients with APAs harboring *ATPase* mutations displayed the highest peripheral concentrations of aldosterone, cortisol, 11-deoxycorticosterone (DOC) and corticosterone.




▶**Fig. 1** The histopathologic characteristics in APAs with different underlying aldosterone-driver somatic mutations. Figure reproduced from reference [30]. H & E: Hematoxylin and eosin; IHC: Immunohistochemistry [rerif].

Conversely, patients with *CACNA1D*-mutated APAs had lower concentrations of aldosterone, corticosterone, and DOC relative to all other groups combined. A panel of 7 steroids measured in peripheral plasma correctly classified 92 % of APAs based on their underlying mutations [40]. A limitation of this initial study was that the areas selected for DNA sequencing were not identified with CYP11B2 immunostaining, and consequently, 45.6 % of the APAs included were presumed to be "wild type." Subsequent studies that implemented CYP11B2 immunohistochemistry to guide the selection of tissue for NGS have shown that somatic mutations can be found in up to 90 % of APAs [29, 30, 41]. The incomplete stratification of APAs by mutation status has likely diluted the power of genotype-specific steroid signatures associated with APAs.

Further refining the steroid fingerprints of APAs according to their underlying pathology could be made possible by larger studies that include sufficient numbers of patients with APAs that harbor rare mutations. For instance, recent evidence suggests that *ATP2B3* mutations lead to distinct histopathological features [34] and PA phenotypes from those with *ATP1A1* mutations. In a study that included 61 patients with known APA mutation status (24 *KCNJ5,* 22 *CACNA1D*, 10 *ATP1A1*, 4 *ATP2B3*, and 1 *CTNNB1*), we found that peripheral venous aldosterone concentrations were higher in patients with *ATP2B3* and *KCNJ5* mutations than in those with *ATP1A1* or *CACNA1D* mutations [42]. In-depth understanding of the molecular mechanisms and histopathology of APAs will further leverage the development of non-invasive diagnostic tools.




▶ **Fig. 2** Adrenal steroidogenesis, illustrating the synthesis of "hybrid steroids", which require both 17α-hydroxylase/17,20-lyase (CYP17A1), which is expressed in the zona fasciculata (ZF) and reticularis (ZR), and aldosterone synthase (CYP11B2), which is expressed in the zona glomerulosa (ZG). DHEA: Dehydroepiandrosterone; DHEAS: Dehydroepiandrosterone sulfate; HSD3B2: 3β-Hydroxysteroid dehydrogenase type 2; SULT2A1: Sulfotransferase type 1A; CYB5A: Cofactor cytochrome $b_5$; StAR: Steroidogenic acute regulatory protein; CYP11A1: Cytochrome P450 cholesterol side-chain cleavage; CYP11B1: 11β-Hydroxylase; CYP21A2: 21α-Hydroxylase.

## Steroid Profiling as a Tool for PA Subtyping

Efforts to identify biomarkers specific to APAs preceded the discovery of aldosterone-driver mutations and characteristic histological features associated with these tumors. Early studies noted elevations of 18OHF and 18oxoF in patients with PA as compared to those with essential hypertension [43, 44], and particularly so in APA versus BHA [45–47]. The production of 18oxoF and 18OHF was recognized to require shared enzymatic features of the ZG and ZF (▶ **Fig. 2**) [48] as was observed in glucocorticoid remediable aldosteronism [49]. As shown more recently, APAs harboring *KCNJ5* mutation, which display such histological features, are globally the most common type of APAs.

In a Japanese study of 234 PA patients (113 with APA) LC-MS/MS was used to compare the peripheral concentrations of hybrid steroids and aldosterone between PA subtypes [50]. Both 18oxoF and 18OHF, as well as aldosterone were significantly higher in patients with APA than in those with BHA. Receiver operating characteristic (ROC) curve analyses showed that an 18oxoF peripheral venous concentration of 4.7 ng/dl had a sensitivity of 0.83 and specificity of 0.99 to discriminate APA from BHA. Aldosterone and 18OHF were also highly accurate for distinguishing between the two groups [area under the curve (AUC) of 0.917 and 0.85, respectively] [50].

In the subsequent year, Eisenhofer et al. published their findings from 216 European patients with PA (126 APA and 90 BHA) in whom 15 steroids were measured by LC-MS/MS [51]. Although peripheral venous 18oxoF was on average 8.5-fold higher in patients with APA than those in BHA, significant overlap between the two groups limited the discriminatory power of 18oxoF (AUC, 0.659). A panel incorporating all 15 steroids measured, however, achieved an AUC of 0.889 for distinguishing APA from BHA [51].

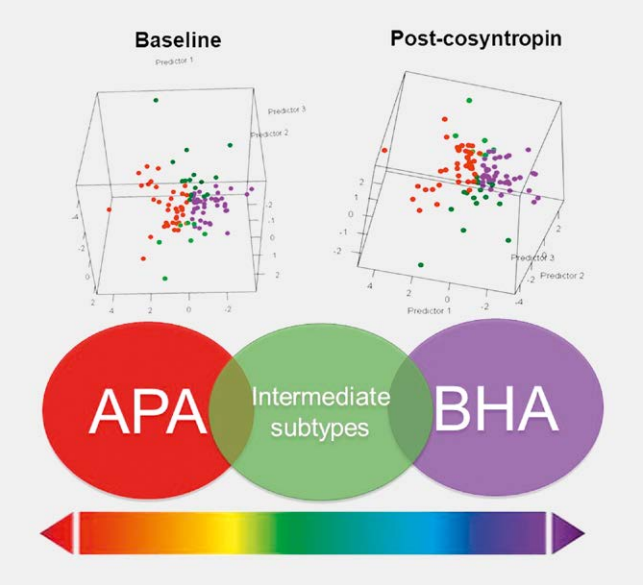
Racial differences in the prevalence of aldosterone-driver somatic mutation and genotype-specific steroid profiles in patients with APAs contribute to the variability in performance of steroid biomarkers for PA subtyping. As we have learned over the recent years, *KCNJ5* mutations are found in up to 80 % of East Asians populations [52, 53], but in only roughly 40 % of Europeans, Australians, and white Americans, who display a wider spectrum of APA genotypes [30, 54–58]. Notably, although somatic *CACNA1D* mutations are overall the most frequent in African American patients with APAs (43 %), *KCNJ5* mutations have the highest prevalence in women, regardless of race (55 % of African American and 70 % of Caucasian women) [29, 30]. Considering that *KCNJ5* mutated-APAs have the highest enzymatic capability to produce 18oxoF and 18OHF [38, 39], these hybrid steroids are expected to be highly accurate in identifying patients with APAs among Asians and women with PA.

A major challenge in establishing reliable biomarkers that can distinguish APA from BHA is the lack of a precise gold standard for PA subtyping. Unlike the explosion of knowledge derived from patients with APAs over the past decade, the pathogenesis of BHA is less well understood. As patients with BHA rarely undergo surgery,

the availability of tissue is scarce. A recent study of BHA adrenal tissue revealed that most such patients harbor micro-APAs and that 58 % of these micro-APAs have *CACNA1D* mutations [59].

In vivo, PA subtyping has been dependent on AVS. Considering that AVS protocols around the world are not uniform, it is conceivable that PA subtype classification is center-dependent, particularly for intermediate cases. Potential sources of variability in PA subtyping based on AVS include use of cosyntropin, differences in AVS data interpretation, and concomitant glucocorticoid excess [60]. Data from referral centers that perform AVS both prior to and after stimulation with cosyntropin have shown that subtyping can be discordant between the two protocols in approximately a quarter of PA patients [42, 61]. Notably, in a study of 222 PA patients who underwent AVS at the University of Michigan and had successful catheterization both pre- and post-cosyntropin stimulation [42], the proportion of patients with discordant AVS subtyping based on pre- and post-cosyntropin AVS results was higher in African American than in Caucasian patients (45 vs. 17 %, p = 0.003). African American patients with APAs commonly harbor *CACNA1D* mutations [29]. We found that, overall, patients with *CACNA1D*-mutated APAs had the least predictable impact of cosyntropin on the lateralization index [42], which could explain, at least in part, the higher proportion of discordant AVS results in this population. Stratified PA subtyping combining AVS results from both pre- and post-cosyntropin stimulation, revealed that cases consistently classified as unilateral had the highest adrenal vein and peripheral aldosterone concentrations, and the most profound contralateral suppression [42]. As the impact of cosyntropin on autonomous vs. normal aldosterone-producing cells remains elusive, cases with inconsistent lateralization might represent either small APAs or asymmetrical BHA. Other surrogates for aldosterone dominance derived from AVS data, such as the lateralization index and contralateral suppression index, have been relatively inconsistent as predictors of postoperative outcomes.[62–64] The lack of standardized criteria for adrenalectomy and only recent introduction of uniform postoperative follow up approaches [13] have further hampered our understanding of the spectrum of PA subtypes.

In a recent study, we used LC-MS/MS to compare the steroid profiles between stratified PA subtypes [65]. Out of 103 patients with valid AVS data both prior to and following cosyntropin administration, 20 patients met criteria for unilateral PA only at baseline and other 14 patients showed lateralization only after cosyntropin stimulation. Both baseline and cosyntropin-stimulated aldosterone and 18oxoF concentrations were highest in the peripheral serum of patients consistently classified as unilateral. Discriminant analysis based on all 17 steroids measured in peripheral serum demonstrated robust separation of patients with consistent subtyping regardless of cosyntropin use, with intermediate zones represented by cases with discrepant lateralization results (▶ **Fig. 3**). Indeed, even with multi-steroid panels measured in peripheral blood samples, the distinction between cases classified as unilateral or bilateral based on baseline AVS data alone was poor (AUC of 0.586). In contrast, the same 17-steroid panel measured in peripheral blood performed better in distinguishing patients with consistent unilateral or bilateral PA subtyping results based on pre- and post-cosyntropin stimulation AVS data (AUC of 0.768 with steroids measured at baseline, and 0.907 with steroids measured after cosyntropin-stimulation) [65]. Our results emphasize that PA subtypes are difficult to dichotomize (▶ **Fig. 3**), and that AVS offers only a snapshot of the relative contribution of the two adrenal glands to aldosterone production. Long-term follow up of ambiguous PA cases is likely to inform about their evolution towards unilateral dominance of aldosterone excess or BHA.




▶ **Fig. 3** Steroid profilies of PA subtypes in peripheral serum obtained at baseline (top left panel) and 20 minutes post-cosynropin stimulation (top right panel). Based on baseline and cosyntropin-stimulate adrenal vein sampling, PA was subtyped as consistently unilateral (APA, illustrated in red); bilateral (BHA, illustrated in purple); or having discrepant results (intermediate subtypes, illustrated in green). Figure adapted from reference [65].

While the development of steroid profiling for assisting with PA subtyping is still in its infancy, several aspects will mandate careful consideration as this tool evolves. Initial stages include establishing reliable reference ranges for steroids where such data is lacking, including for hybrid steroids, harmonization of standards, and quality assurance strategies. Incorporation of multi-steroid assays also adds complexity in results interpretation, possibly affecting the practical utility of multi-steroid panels. Although machine learning technology is frequently applied in research, designing simple interpretation algorithms will be of utmost importance for the busy clinician. Such algorithms might incorporate patients' demographics, other laboratory parameters (such as serum potassium), and/or imaging studies. Considering the numerous individual aspects to clinical care, it is likely that clinical decision making will use available tools complementarily. Finally, well-designed prospective studies, with randomization of treatment, and standardized long-term follow up, will be critical for establishing the utility of steroid profiling in PA subtyping and, hopefully, even in predicting postoperative outcomes.

## Conclusions

The development of non-invasive and widely available tools for PA subtyping is essential for leveraging personalized care and for maximizing the number of cured PA patients. Such innovations have been bolstered by major discoveries relevant to the pathogenesis and histopathological features of APAs emerged during the past decade. Steroids characteristic of APAs with *KCNJ5* mutations will be particularly relevant in Asians of both sexes and in Caucasian women with sporadic APA. Refining the steroid fingerprints of APAs with other underlying mutations and elucidating the pathogenesis of BHA will eventually increase the accuracy of peripheral blood tests for PA subtyping and will thus circumvent the need for AVS in a large number of PA patients.

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### Conflict of Interest

The authors declare that they have no conflict of interest.

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