



Primary aldosteronism diagnostics: *KCNJ5* mutations and hybrid steroid synthesis in aldosterone-producing adenomas

Juilee Rege¹, Adina F. Turcu², William E. Rainey^{1,2}

¹Department of Molecular and Integrative Physiology, ²Division of Metabolism, Endocrinology, and Diabetes, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA

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Correspondence to: William E. Rainey, PhD. Departments of Molecular and Integrative Physiology and Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA. Email: WER@umich.edu.

Abstract: Primary aldosteronism (PA) is characterized by autonomous aldosterone production by renin-independent mechanisms and is most commonly sporadic. While 60–70% of sporadic PA can be attributed to bilateral hyperaldosteronism, the remaining 30–40% is caused by a unilateral aldosterone-producing adenoma (APA). Somatic mutations in or near the selectivity filter the *KCNJ5* gene (encoding the potassium channel GIRK4) have been implicated in the pathogenesis of both sporadic and familial PA. Several studies using tumor tissue, peripheral and adrenal vein samples from PA patients have demonstrated that along with aldosterone, the hybrid steroids 18-hydroxycortisol (18OHF) and 18-oxocortisol (18oxoF) are a hallmark of APA harboring *KCNJ5* mutations. Herein, we review the recent advances with respect to the molecular mechanisms underlying the pathogenesis of PA and the steroidogenic fingerprints of *KCNJ5* mutations. In addition, we present an outlook toward the future of PA subtyping and diagnostic work-up utilizing steroid profiling.

Keywords: Aldosterone-producing adenomas; *KCNJ5*; *CYP11B2*; *CYP17A1*; 18-hydroxycortisol; 18-oxocortisol; aldosterone

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Introduction

Primary aldosteronism (PA) is the most common form of secondary hypertension, and it accounts for 5–8% of hypertension (1–6) and 11–20% of resistant hypertension (7–9). PA is characterized by inappropriate autonomous production of aldosterone via renin-independent mechanisms (10). Most PA patients exhibit a sporadic form, whereas 5–6% of the cases are caused by familial disease (11). Approximately 60–70% of PA cases can be attributed to bilateral hyperaldosteronism (BHA), with the remaining 30–40% being caused by unilateral aldosterone-producing adenomas (APA) (2,12). Uncommon forms of PA include unilateral adrenal hyperplasia and adrenal

carcinoma (13).

Differentiation of the uni- and bilateral forms of PA is important for guiding therapy. Unilateral PA can benefit from adrenalectomy and BHA requires indefinite medical therapy, which typically incorporates a mineralocorticoid receptor antagonists (MRA) (14,15). Adrenal vein sampling (AVS) is the most reliable method for distinguishing between APA and BHA (10). However, there are several caveats to the AVS methodology and interpretation of hormonal data. Despite being highly predictive of outcome (10), this procedure is laborious, invasive, and expensive. Additionally, AVS is performed in a limited number of referral centers, and it is dependent on highly-skilled interventional radiologists with large annual AVS volume (16–18).

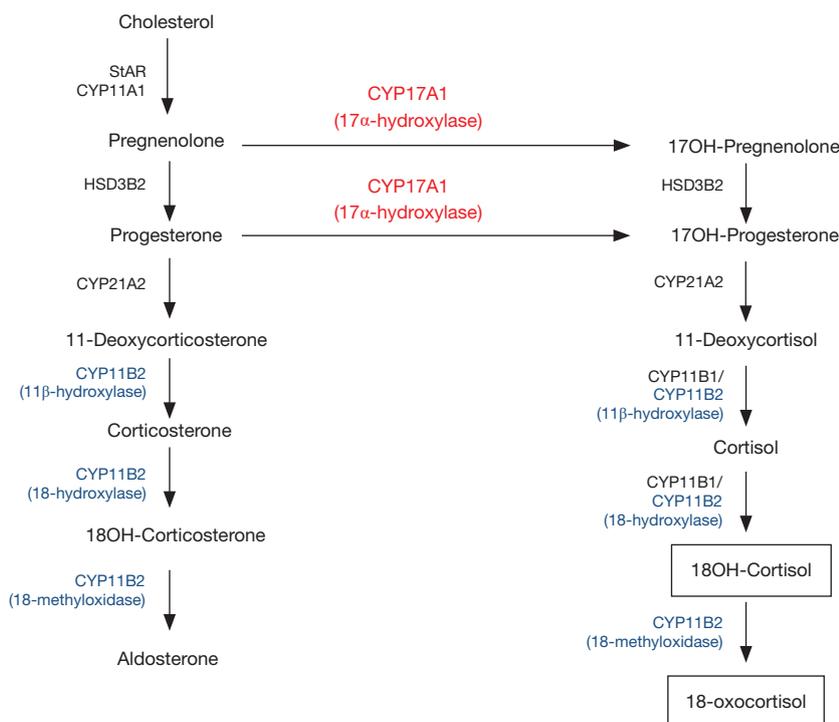


Figure 1 The steroidogenic pathway for aldosterone and the ‘hybrid steroids’—18OH-cortisol and 18-oxocortisol. While only CYP11B2 is required for aldosterone production, both CYP17A1 and CYP11B2 contribute to the biosynthesis of the hybrid steroids.

A simple blood test that identifies APA-derived serum steroid biomarkers would help distinguish patients who will benefit from adrenalectomy from those who should be treated with medical therapy. Such biomarkers could also conserve healthcare resources by sparing many patients expensive imaging and invasive studies. Finally, the utility of such biomarkers would increase the rate of PA screening, facilitate PA diagnosis and appropriate treatment, and thus reduce the burden of cardiovascular and renal complications which affect PA patients disproportionately more than those with essential hypertension.

Hybrid steroids as diagnostic markers for differentiation of APA from BHA

Over the past 25 years, the applicability of the 18-oxygenated derivatives of cortisol, 18-oxocortisol (18oxoF) and 18-hydroxycortisol (18OHF) for subtyping PA as uni- or bilateral, has been of interest (19-27). Several studies have shown higher levels of 18OHF and 18oxoF in PA patients compared to those with essential hypertension (23,28-30). High concentrations were also demonstrated

in patients with familial hyperaldosteronism type 1 (FH type I) (31,32). FH type I accounts for <1% of cases of PA (11) and is caused by a chimeric gene that is composed of the promoter of 11 β -hydroxylase (*CYP11B1*) fused with the coding region of aldosterone synthase (*CYP11B2*) (31). Like *CYP11B1*, this *CYP11B1/CYP11B2* chimeric enzyme is present in the zona fasciculata (ZF) of the adrenal cortex and is regulated by ACTH. As a result, the *CYP11B1/CYP11B2* chimera is able to use cortisol as a substrate to produce 18OHF, which is further metabolized to 18oxoF (19,23,30,32-34) (Figure 1). These metabolites of cortisol are designated as “hybrid” steroids owing to their molecular structure comprising features of steroid metabolism which typically occur in the zona glomerulosa (ZG) (18-hydroxylation and 18-oxidation) and the ZF (17-hydroxylation) (31) (Figure 1).

In the normal adrenal gland, expression of *CYP11B2* is restricted to the ZG, while 17 α -hydroxylase/17,20-lyase (*CYP17A1*) and *CYP11B1* are expressed exclusively in the ZF and zona reticularis (Figure 2), thereby leading to low production of 18OHF and 18oxoF in normal subjects. Histologic studies have shown that some APA display a ZG-

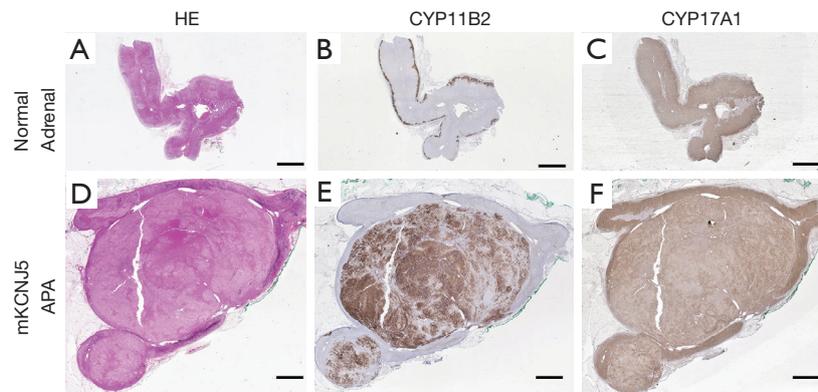


Figure 2 Histologic findings in a normal adrenal (A,B,C) and an APA harboring a *KCNJ5* mutation (D,E,F). Immunohistochemical analysis illustrates that *CYP11B2* expression is localized in the zona glomerulosa of the normal adrenal (B), while CYP17A1 is expressed in the zona fasciculata and zona reticularis (C). A *KCNJ5* mutation-harboring APA showed intratumoral *CYP11B2* expression heterogeneity, with distinct *CYP11B2* positive and negative regions within the tumor (E). The APA also exhibits elevated CYP17A1 expression in the tumor region. Scale: 5 mm.

like phenotype, with small, compact cells, while other APA are composed of large, lipid-rich cells, similar to those seen in ZF (35-38). Although transcriptomic analysis has not detected any differences in *CYP11B2* mRNA expression between the different APA subtypes (39), higher *CYP11B2* protein expression has been observed in the APA with ZG-like cells in some studies (35,37). Specific to APA with ZF-like histology is a higher expression of steroidogenic enzymes required for cortisol biosynthesis, such as CYP17A1 and CYP11B1 (40-42). The co-expression of CYP17A1 and CYP11B2 in these APA facilitates the production of hybrid steroids (21,33,43) (Figures 1,2).

Ulick *et al.* performed the initial PA hybrid steroid studies in 1993 and demonstrated that urinary 18OHF and 18oxoF were elevated in patients with APA compared to those with BHA (21,22). Numerous immunoassay studies followed that indicated higher plasma and urinary 18OHF and 18oxoF levels in subjects with APA *vs.* those with BHA (19,23,29). More recently liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to quantify the hybrid steroids in APA and BHA, confirming some of the previous immunoassay analyses (20,25). Satoh *et al.* measured 18OHF and 18oxoF in the peripheral plasma of 234 Japanese PA patients by LC-MS/MS and found that these steroids could discriminate APA from BHA with considerable specificity and sensitivity (20). In contrast, a European study of 216 PA patients indicated that both 18oxoF and 18OHF displayed significant overlap between APA and BHA, thereby suggesting a limitation in the

utility of these steroids as discriminators between the two PA subtypes (24). Nevertheless, this analysis presented a composite of 12 steroids that was able to correctly classify the PA subtype in 80% of the patients (24). A subsequent study from the same group revealed that APA with different underlying somatic mutations produce specific steroid fingerprints (44).

The hybrid steroids and *KCNJ5* somatic mutation connection in APA

In 2016, Williams *et al.* identified specific steroid fingerprints in adrenal vein (AV) and peripheral vein (PV) plasma from patients with APA with various underlying mutations (44). Of the 79 PA patients with unilateral PA included, 34% had APA harboring *KCNJ5* (encoding the G protein-coupled inward-rectifying potassium channel 4, GIRK4) mutations, 11% had ATPase (*ATP1A1* (Na^+/K^+ ATPase α 1-subunit), *ATP2B3* (Ca^{2+} ATPase 3) mutations and 9% had *CACNA1D* (encoding the voltage-dependent L-type calcium channel subunit α -1D, Cav1.3) mutations. In the remaining 46% of APA no mutations were identified. Patients with *KCNJ5*-harboring APA had the highest concentrations of 18OHF and 18oxoF in both the AV and PV plasma (44). The elevated levels of the hybrid steroids produced by the *KCNJ5*-mutated APA could be explained by their predominantly ZF phenotype (elevated CYP17A1) along with CYP11B2 expression (35-38) (Figure 2). Conversely, APA with *ATP1A1*, *ATP2B3*, and *CACNA1D* mutations, were shown to be

smaller in size and to be composed principally of ZG-like cells (35-38). Assembling a 7-steroid panel measured in PV plasma, including aldosterone, 18oxoF, 18OHF, 11-deoxycorticosterone (11-DOC), corticosterone, cortisol, and 21-deoxycortisol, William *et al.* were able to classify 92% of APA according to the underlying somatic mutation (44). In a subsequent study, the same group used steroid profiling to differentiate patients with micro-APA, macro-APA and BHA (45). Patients with macro-APAs, which frequently harbor *KCNJ5* mutations, displayed higher concentrations of aldosterone and the hybrid steroids as compared with patients with micro-APA and BHA (45). These findings were in concordance with a recent analysis of APA tissue by comprehensive mass spectrometry imaging in relation to mutation status, immunohistochemical reports of steroidogenic enzymes and steroid profiles from 139 patients (46). Increased intratumoral intensities of 18OHF and 18oxoF were seen in *KCNJ5*-mutated APA. Additionally, two *in vitro* studies corroborated the finding that expression of a *KCNJ5* mutation in the adrenocortical HAC15 cell line results in a significant increase in *CYP11B2* gene transcription, and elevation in the production of aldosterone and the hybrid steroids (47,48).

The utility of hybrid steroids as promising discriminators between APA and BHA in Japanese patients with PA (20) could be attributed to the high prevalence of APA *KCNJ5* mutations in this population (49,50). Tezuka *et al.* recently highlighted the potential of 18oxoF as biomarker for *KCNJ5*-harboring APA in Japanese patients in a study that measured its intratumoral and peripheral serum levels in patients with PA who underwent unilateral adrenalectomy (51). This study showed that APA harboring *KCNJ5* mutations demonstrated enhanced synthesis of 18oxoF owing to elevated intratumoral cortisol production which could be used as substrate by *CYP11B2*. These tumors also had increased *CYP11B1* and *CYP11B2* double-positive hybrid cells compared with APA harboring the wild-type *KCNJ5* gene (51). In addition to aberrant *KCNJ5*-related sporadic PA, the hybrid steroids have also been shown to be elevated in familial hyperaldosteronism type III (FH type III) (52,53) which was described by Geller *et al.* in 2008 as an early onset and severe form of primary aldosteronism (52) and was shown to be caused by germline mutations in *KCNJ5* (53).

Somatic *KCNJ5* mutations in APA—the mechanics

The product of *KCNJ5*, GIRK4 is a member of the G

protein-activated inwardly rectifying K⁺ channel subfamily and is localized on the plasma membrane of tissues such as the heart, central and peripheral neurons along with various endocrine tissues (54,55). Tissue transcriptome analysis, however, suggests that the adrenal is by far the tissue with highest levels of the transcript encoding GIRK4. ‘Inward rectifiers’ are a class of K⁺ channels that conduct large currents in the inward direction at membrane voltages negative to the K⁺ equilibrium potential. The primary structure of this channel consists of 2 membrane spanning helices flanking one extracellular pore-forming region in between and cytoplasmic N- and C-termini that contribute to the pore structure (56,57). The pore-forming domain constitutes the K⁺ ion selectivity filter of the channel which is characterized by the signature sequence Gly-Tyr-Gly (57,58). This sequence allows stringent passage of the larger K⁺ ions through the channel into the cell and prevents the entry of smaller, more abundant Na⁺ ions (59). Immunohistochemical studies have shown that GIRK4 is localized mainly to the ZG of the human adrenal cortex and to the outer part of the ZF (40,53,60). GIRK4 and other K⁺ channels maintain the hyperpolarized state of the ZG cell by allowing an outward flow of K⁺ conductance (54,55) (*Figure 3*).

The advent of large-scale methods of analyses such as gene sequencing [e.g., next generation sequencing (NGS) and whole exome sequencing] in the last decade has helped to elucidate the genetic landscape and molecular mechanism of PA pathogenesis. The genetic basis of PA was largely an uncharted territory until Choi *et al.* used exome sequencing to first report a role for somatic mutations in driving autonomous aldosterone production in APA in 2011 (53). This study identified two “hot-spot” somatic mutations (p.Gly151Arg (G151R) and p.Leu168Arg (L168R) substitutions) in *KCNJ5* (53) (*Figure 4*). These originally described *KCNJ5* somatic gain-of-function mutations were located near or within the selectivity filter and disrupt its ion selectivity by facilitating indiscriminate entry of Na⁺ through the pore of the outer tunnel (53). The resulting depolarization of the cell membrane induces the opening of the voltage-gated Ca²⁺ channels leading to elevated intracellular Ca²⁺, increased activation of the calcium signaling pathway, augmented *CYP11B2* transcription and aldosterone biosynthesis (*Figure 3*). Two cell-based analyses from our group established that mutated *KCNJ5* activates the acute and chronic regulatory steps of aldosterone production and that increased aldosterone production occurs along with elevations in *CYP11B2*, and

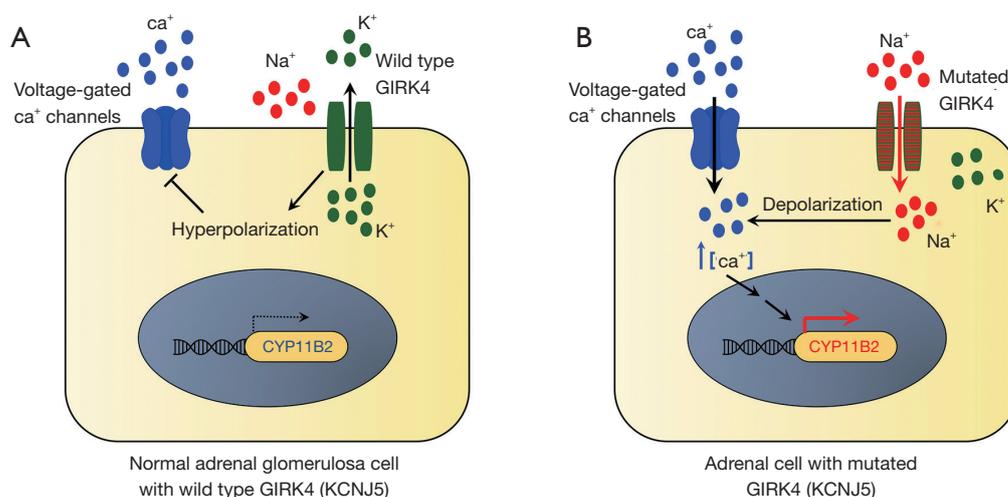


Figure 3 Regulation of adrenal aldosterone production by wild type and mutated GIRK4 (*KCNJ5*). (A) Adrenal glomerulosa cells with wild type GIRK4 (*KCNJ5*) are in a hyperpolarized state as a result of high resting K⁺ conductance. (B) In pathological conditions, adrenal cells carrying mutations in GIRK4 (*KCNJ5*) demonstrate indiscriminate conductance of Na⁺, resulting in chronic cell membrane depolarization and constitutive activation of *CYP11B2* and aldosterone production.

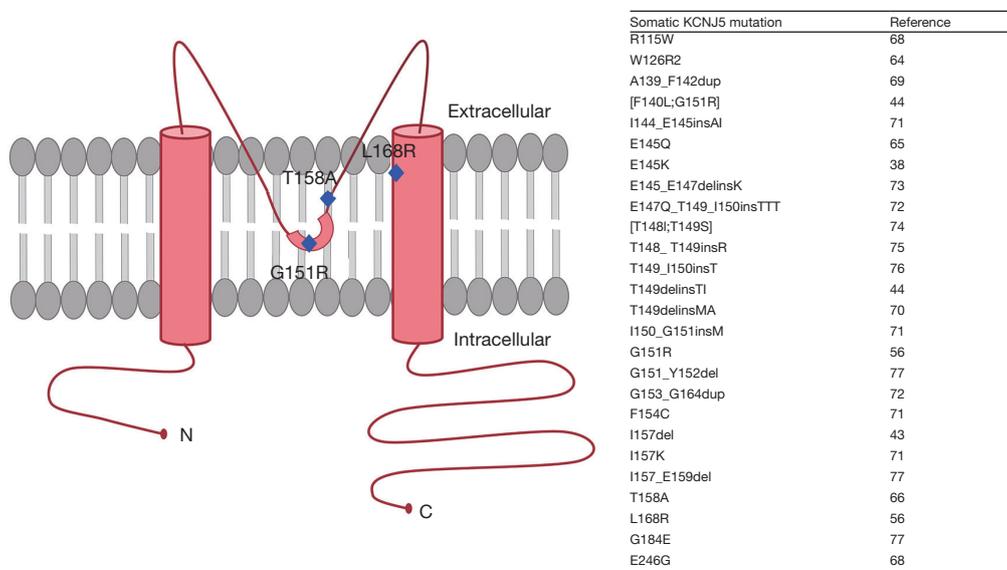


Figure 4 *KCNJ5* mutations in APA. The most common *KCNJ5* mutations (G151R, L168R and T158A) occur in or near the selectivity filter (38,43,44,56,64-66,68-77). Other reported mutations are listed in the table.

its regulatory transcription factors nuclear receptor related 1 protein (*NURR1*) and activating transcription factor 2 (*ATF2*) (47,60). Besides G151R and L168R which constitute 90% of all the *KCNJ5* APA mutations, 24 other *KCNJ5* mutations have also been detected in APA (40,41,53,61-70,74-77) (Figure 4).

Along with their studies on the genetic causes of somatic APA, Choi *et al.* also established the genetic basis of FH type III by identifying a novel gain-of-function *KCNJ5* germline mutation in a father and his two daughters, all with PA (53). This substitution mutation—p.Thr158Ala (T158A)—is located near the selectivity filter of the channel pore. The

T158A mutation was later also detected in sporadic APA by Mulatero *et al.* in 2012 (76). Adrenocortical cell line studies demonstrated that the p.Thr158Ala mutation in *KCNJ5* causes an increase in aldosterone production via membrane depolarization and Na⁺ and Ca²⁺ influx (47,48). While patients carrying the germline mutations G151R (similar to the recurrent somatic *KCNJ5* mutation in APA), T158A and p.Ile157Ser, presented with early onset and a severe PA phenotype with drug-resistant hypertension and adrenal hyperplasia (71,72), those with the p.Gly151Glu (G151E) (72) and p.Tyr152Cys (73) variants exhibited a remarkably milder phenotype.

Somatic *KCNJ5* mutations in APA—the demographics

The last 10 years have resulted in a plethora of studies in the field of PA that have investigated the presence and racial prevalence of the various aldosterone-driving somatic mutations in APA, including *KCNJ5*. Collaborating investigators from the European Network for the Study of Adrenal Tumors (ENS@T) have conducted the largest mutation prevalence studies to date assessing 474 APA with the sequencing directed at the previously reported mutation hotspots (39). This multicenter study demonstrated the presence of somatic mutations in 54% of APA, with genetic abnormalities in *KCNJ5* representing 38% (39). This study corroborated the frequency of somatic mutations in *KCNJ5* that were identified in previous smaller studies in European populations (35,74,75,78,79). Of note, the prevalence of reported somatic mutations in APA has been shown to vary by race. In particular, somatic *KCNJ5* mutations are much more common in East Asian patients than in Europeans (70% *vs.* 38%) (36,49,50,65,68,80–82). We recently conducted two studies wherein we determined the incidence of APA somatic mutations in White Americans and Americans of African descent (Blacks) (41,77). Instead of using grossly dissected snap frozen tumor tissue as was done in previous studies, we applied a unique sequencing approach by utilizing a CYP11B2 immunohistochemistry (IHC)-guided comprehensive NGS protocol targeting the entire coding regions of a panel of genes frequently mutated in APA. While the *KCNJ5* gene aberrations were most frequently seen in White Americans (43%) (41), Blacks showed a different APA somatic mutation profile with *KCNJ5* (34%) being the second-most mutated gene after *CACNA1D* (77). Notably, the unique approach of utilizing the CYP11B2 IHC-directed adenoma selection

and a comprehensive, full coding sequence-based NGS approach overturned the previously published prevalence by demonstrating that over 90% of APA have a known aldosterone-driver gene. However, in agreement with previous studies that utilized non-IHC-directed, selected exon sequencing-based approaches, aldosterone-driving mutations were not identified in CYP11B2-negative tumor samples by the IHC-guided method (41,77,83,84). Collectively, this suggests that the heterogeneity of CYP11B2 expression in PA adrenals could have led to discrepancies between macroscopic-guided sequencing and the CYP11B2 IHC-guided sequencing results.

While *KCNJ5* mutations in APA have consistently been reported to be more frequent in females in Western populations (35,39,41,74,75,77–79), this distinction was not clear in East Asians where the prevalence was very high in both men and women (36,49,50,65,68,80–82,85). The association of *KCNJ5* mutations with sex (women higher than men), younger age, pronounced hyperaldosteronism and larger tumor size was also demonstrated in a meta-analysis study comprising 1,636 APA patients from 13 studies (86).

Conclusions and perspectives

The current diagnostic work-up for PA is a complex multitiered process (10). Although the Endocrine Society Guidelines recommend measurement of the aldosterone-to-renin ratio (ARR) as an appropriate initial screening test for PA, ARR is only about 80% specific and sensitive (87–89). Furthermore, it requires confirmatory testing of aldosterone production following sodium loading and volume expansion (10). More importantly, the ARR is elevated in patients with both BHA and APA. Consequently, the biochemical diagnosis of PA does not differentiate between the two primary causes of PA, and follow-up procedures are needed to classify patients with PA. The major impediments to screening for and treating PA are the complexities in the later stages of the evaluation. Currently available laboratory tests can confirm the diagnosis of PA, but neither these tests, nor current imaging studies can determine which adrenal gland(s) is (are) the source(s) of aldosterone. There is general agreement that cross-sectional imaging studies such as computed tomography scanning or magnetic resonance imaging cannot distinguish between most APA and BHA cases. This failure derives from both the small size of most APA and the high prevalence of nonfunctional adrenocortical adenomas in

the general population, most of which do not produce aldosterone. Thus, the expensive, technically cumbersome and invasive AVS technique remains the gold standard for the subtyping of patients with PA. The variable success rates of AVS methodology from center-to-center has prompted researchers to consider alternative non-invasive diagnostic tools that could aid in diagnosing and classifying the different forms of PA.

Recent advances in the diagnostics of PA have been possible with the emergence of the LC-MS/MS methodology which allows steroid profiling in an individual patient. Increasing evidence suggests that steroid fingerprinting might be a major determinant in not only differentiating APA from BHA, but also in providing genotype-phenotype associations of APA, thereby making it a useful tool in simplifying the complex diagnostic work-up in patients with suspected PA. The utility of hybrid steroids—18OHF and 18oxoF—as potential differentiators between APA and BHA, has been tested for the past three decades with encouraging results. In fact, a panel of 12 steroids including the hybrid steroids in peripheral plasma that was put forth by Eisenhofer *et al.* was successful in classifying 80% of the PA cases as APA or BHA (24). The same research group also demonstrated that the putative application of steroid profiling for subtype classification of PA is likely due to the association of the steroid metabolome with somatic APA aldosterone-driver mutations (44). Notably, they showed that the high levels of hybrid steroids could be a signature for *KCNJ5*-mutated APA. Moreover, steroid profiling of peripheral blood was able to correctly categorize 92% of the somatic APA mutations. They recently took a similar diagnostic route in the case of a 55-year-old female patient with left adrenal mass in whom AVS was a failure (90). The patient's peripheral plasma displayed increased levels of aldosterone, 18OHF, 18oxoF, 11-DOC, 11-deoxycortisol (90) which were indicative of a macro-APA as a result of a *KCNJ5* mutation (45). Adrenalectomy was recommended for this patient and the diagnosis was later confirmed by genetic testing and histopathology (90). This example highlights the clinical applicability of steroid fingerprinting in the PA work-up.

The inclusion of steroid profiling in the PA diagnostic chart has several advantages. Sixty percent to 70% of patients with PA have BHA and thus receive no benefit from the AVS procedure, because they are treated medically rather than surgically. Certain populations would benefit from the new diagnostic approach following ARR wherein

steroid profiling could help identify patients that need to undergo the CT and AVS and bypass invasive testing in patients with BHA, who require lifelong personalized medical treatment. *KCNJ5* mutations, which are more prevalent in women and constitute the majority of East Asian APA cases of both sexes, could be diagnosed rapidly by serum hybrid steroids. Given the high prevalence of *KCNJ5* mutations in PA patients, macrolides might potentially be used to identify patients that bear APA with *KCNJ5* mutations (91) and as targeted personalized treatments for these patients. Inclusion of steroid fingerprinting in the PA diagnostic work-up will reduce healthcare cost and increase the impact on patient safety for almost 60–70% PA patients by reducing radiation exposure and application of invasive procedures. Prospective studies will, however, be needed to validate whether this method is a better alternative to the invasive and technically onerous AVS.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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