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Expanding spectrum of primary aldosteronism: Exploring new grounds

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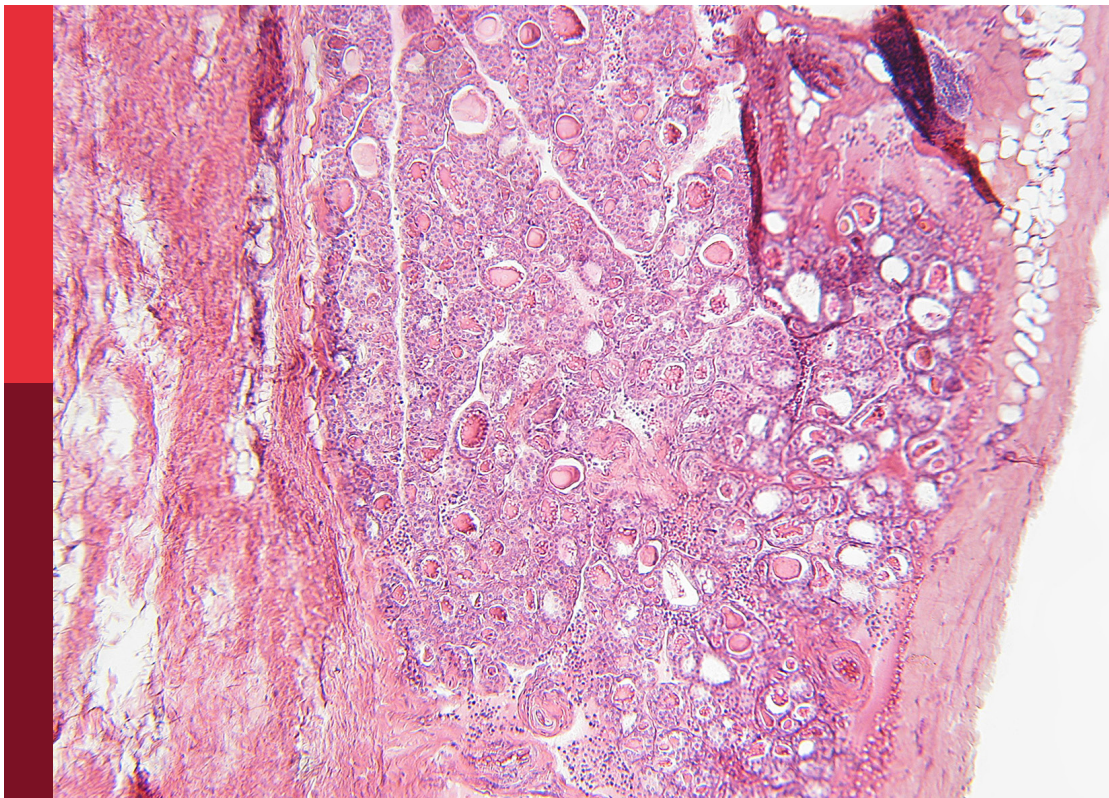

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Expanding spectrum of primary aldosteronism: Exploring new grounds

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Editorial: Expanding spectrum of primary aldosteronism: exploring new grounds

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Editorial on the Research Topic
Expanding spectrum of primary aldosteronism: exploring new grounds

Seven decades have lapsed since the first description of primary aldosteronism (PA) by Jerome W. Conn (1). Despite a significant time-lapse, PA remains an underrated cause of hypertension with devastating cardiovascular and renal complications (2, 3).

This Research Topic gathers different contributions highlighting the latest advances in the diagnosis and management of PA. In the first article by Parasiliti-Caprino et al., the accuracy of simple and adjusted aldosterone indices for assessing selectivity and lateralization of adrenal vein sampling (AVS) is evaluated. AVS is regarded as the gold standard test for PA subtyping (4). However, AVS is technically difficult and failure to cannulate both adrenal veins render the test inconclusive. The authors demonstrated the utility of aldosterone indices to determine both selectivity and lateralization. These indices could potentially be useful when only one adrenal vein is successfully cannulated, and their utility should be determined in future prospective trials.

The second article highlights the importance of accurate catheter tip placement during AVS which is critical in the interpretation of the results. Traditionally, the ratio of plasma cortisol concentration in the adrenal vein to peripheral vein defines the selectivity index (SI). However, with no standardized cut-off levels, this limits the applicability of AVS when cortisol is being utilized (5, 6). The use of free metanephrine (FMN) has been shown to be superior to cortisol in assessing SI (7, 8). This study shows that FMN is a better analyte than cortisol in confirming the correct placement of the catheter's tip. The main limitation, however, was that no confirmatory tests were performed. Other studies have also explored the use of different analytes such as aldosterone-to-renin ratio (ARR) in combination with cortisol SI (9) and plasma metanephrines in addition to cortisol (10). While the results are promising, further research is needed to establish the utility of these markers especially in those with confirmed PA.

The diagnosis of PA requires the demonstration of an unsuppressed aldosterone level by various suppression tests (11). The oral salt loading test (OSLT) has been widely used in the diagnostic work-up, albeit its accuracy has been challenged in recent years. In the third article, Ozeki et al. present a new chemiluminescent enzyme immunoassay (CLEIA) based on a two- step sandwich method to measure 24-hour urine aldosterone excretion. The accuracy of aldosterone measurement in urine samples using various methods have also been examined previously. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was noted to have the highest accuracy (12). The lower cut-off value suggested for PA diagnosis needs to be validated in a larger clinical trial.

The fourth article focuses on the role of urinary extracellular vesicles (uEVs) sodium chloride cotransporter (NCC) in PA subtyping. In view of the invasive nature and technical difficulties of AVS, identifying a non-invasive alternative is crucial. The authors show that NCC can be potentially used to distinguish between different subtypes of PA as the expression of phosphorylated form of NCC (pNCC) in uEVs are different in various subtypes and genotypes. Previous studies have explored the use of uEVs as a biomarker in lupus nephritis and diabetic nephropathy where the presence of uEVs provide insights into the pathophysiology and aid the diagnosis and management (13, 14). The main limitations of this study include the small sample size and the lack of a validation cohort. Although the study highlights the potential of using uEVs as a non-invasive biomarker to subtype PA, further research in this field is warranted.

In the fifth article, Nguyen et al. recruited 300 patients with a recent stroke and reported the prevalence of PA amongst those with hypertension, resistant hypertension and hypertension with atrial fibrillation (AF) as 4%, 11% and 30% respectively. Previous studies have shown that patients with PA are at increased risk of stroke and AF (15, 16). This study highlights the importance of screening for PA, particularly in high-risk groups of patients. This approach will enable early detection and treatment of a potentially reversible cause of hypertension and stroke.

The sixth article by Puar et al. demonstrated that PA treatment improved subclinical left ventricular (LV) systolic function, using speckle-tracking echocardiography to assess LV global longitudinal strain (GLS). While previous studies have shown that patients with PA have impaired LV systolic function (17), this study demonstrated that surgery led to better improvement compared to medical therapy, and that reversal of renin suppression may be a key factor in improving systolic function.

The seventh article by Huang et al. investigates the relationship between vascular ageing and left ventricular concentric geometry (LVCG) in patients with newly diagnosed PA. In this study carotid intima-media thickness (cIMT) is significantly associated with left ventricular hypertrophy (LVH) whilst brachial-ankle pulse wave velocity (baPWV) is significantly associated with LVCG. While the study has some limitations, such as its retrospective and single-center design, its findings are consistent with previous research. Wang et al. (18) demonstrate a positive correlation between baPWV and left ventricular mass index in hypertensive patients. This study provides important insights into the early assessment of cardiac damage in newly diagnosed PA.

The review article by Ahmed and Hundemer provides a comprehensive literature search encompassing studies from the past two decades supporting surgical adrenalectomy as the preferred treatment option for unilateral PA compared to medical therapy. Adrenalectomy is highly successful in reversing the clinical and biochemical abnormalities, mitigating long-term risks, and offering the potential for disease cure. Several other studies also favored surgical approach with significant reductions in blood pressure, glucose, number of medications used, and cardiovascular events and mortality (2, 3, 19). This review underscores the importance of personalized treatment plans for improved patient outcomes.

Tetti et al. in their review provide an extensive overview of the current knowledge on the molecular and cellular mechanisms that contribute to the pathogenesis of PA. The authors focus on recent advances in the understanding of the disrupted cell growth mechanisms in PA through the combined application of transcriptomics, metabolomics, and epigenetics. The review is a valuable resource for clinicians as it highlights the key findings in the field, fill up the gaps in the literature and explore areas where further research is needed.

Loh and Sukor presented a review article that collates and puts into perspective current available research on the association between PA and obstructive sleep apnea (OSA). Given the high prevalence of hypertension, PA, and OSA, understanding their potential association and clinical implications is important. The authors critically analyzed the existing literature and identified several limitations in the currently available studies, which include heterogeneity in the study designs and populations, lack of uniform criteria for diagnosis and treatment of PA and OSA, and potential confounding factors such as obesity and diabetes. Although other studies have also suggested a potential link between PA and OSA (20, 21), there is a need for further research to elucidate the relationship between these two conditions.

A large majority of operated aldosterone-producing adenomas (APAs) harbour known somatic mutations, which are associated with membrane depolarisation and increased aldosterone production. However, mechanisms driving cell proliferation of these adenomas remained unknown. Abdellatif et al. summarizes the current knowledge on known regulators of adrenal growth and function. In addition, they have focused on the interplay between the hormonal and vascular interfaces which may explain the development of APAs and PA. The strength of this review lies in its comprehensive coverage of the current knowledge on the topic, drawing from various research disciplines including endocrinology, molecular genetics and vascular biology.

In conclusion, this Research Topic provides a timely overview of the latest advances in the diagnosis and treatment of PA and its associated sequelae. The articles included in this Research Topic provide valuable insights into the expanding pathophysiology of PA

and the need for ongoing research to fill up the gap of this important disease entity.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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The Accuracy of Simple and Adjusted Aldosterone Indices for Assessing Selectivity and Lateralization of Adrenal Vein Sampling in the Diagnosis of Primary Aldosteronism Subtypes

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Objective: This study aimed to evaluate the reliability of simple and corrected aldosterone indices for assessing the selectivity and lateralization of adrenal vein sampling (AVS) in patients with primary aldosteronism.

Methods: Data of all consecutive patients with primary aldosteronism who underwent AVS for subtype diagnosis, followed at two Italian referral centers, were analyzed retrospectively.

Results: AVS achieved bilateral selectivity in 112/144 patients. Unilateral disease was diagnosed in 60 cases (53.6%) and idiopathic hyperaldosteronism in 52 individuals (46.4%). The aldosterone index (aldosterone ratio between an adrenal vein and the inferior vena cava) showed a high accuracy in predicting selectivity, compared to a cortisol selectivity index of 1.1, and a moderate accuracy, compared to cortisol cut-offs of 2 and 3. The simple aldosterone index showed a moderate accuracy in predicting ipsi/contralateral aldosterone hypersecretion, while lesion side- and hypokalemia-corrected aldosterone index revealed a significant improvement in predicting ipsi/contralateral disease. Moreover, the comparative aldosterone index (aldosterone ratio in the dominant vs the non-dominant adrenal vein) revealed a high accuracy in predicting unilateral primary aldosteronism. For an immediate clinical application of our results, the adjusted cut-offs were calculated, according to the Youden's criterion and to a pre-established specificity of 90%, for all possible combinations of lesion side at imaging and presence/absence of hypokalemia.

Conclusions: This study demonstrated the diagnostic accuracy of simple and clinical-/imaging-corrected aldosterone indices for adrenal vein sampling in subtype diagnosis of primary aldosteronism and suggests the potential application of these tools to select patients for adrenalectomy when standard indices cannot be performed.

Keywords: aldosterone, cortisol, adrenal tumor, hypokalemia, adrenal glands, adrenalectomy, secondary hypertension, endocrine hypertension

INTRODUCTION

Primary aldosteronism (PA) is a heterogeneous group of disorders caused by an excessive aldosterone secretion that seems autonomous from renin (1–3) and is the most frequent form of secondary hypertension. Its prevalence increases with the severity of hypertension (4, 5), reaching over 29% in individuals with resistant hypertension (6). Diagnosing PA is important to give patients the opportunity of a specific surgical or medical treatment in order to reduce cardiovascular risk (7–9).

The last step of the diagnostic process is the subtype differentiation, in which the adrenal vein sampling (AVS) is currently considered the gold standard, also given the absence of alternative strategies (10). Due to the technical difficulties of its execution, AVS has low diffusion among centers worldwide, with a reported success rate ranging from 26 to 81% (11–14). The reason mainly lies in the difficulty of cannulation of the right adrenal vein, and many studies have demonstrated that an expert radiologist is crucial for improving sampling success (11, 15–17).

The major diagnostic indices for the assessment of lateralization of aldosterone hypersecretion are the lateralization index [LI - dominant vs non dominant adrenal vein aldosterone/cortisol ratio (A/C)], and the contralateral index [CI - non-dominant adrenal vein vs inferior vena cava A/C] (18). These indices, however, can be reliably interpreted only when AVS is bilaterally selective. Therefore, during last years, some authors proposed the use of simple unconventional indices with a moderate (19, 20), and often not reproducible, accuracy for determining PA lateralization (21–24). Most recently, Burrello et al. developed two scores (25, 26) for predicting PA subtypes, using a machine learning approach and reaching a high accuracy; similarly, our group proposed the use of adjusted unconventional AVS indices in order to determine the lateralization of aldosterone secretion when adrenal vein cannulation is not bilaterally selective (27).

The diagnostic performance of absolute aldosterone ratios has been analyzed only in few studies so far; Mailhot et al. (28) described the application of aldosterone index (AI), defined as the ratio of aldosterone levels between an adrenal vein and the inferior vena cava, for the assessment of selectivity; Liu et al. (29) reported a moderate accuracy of the simple AI, in sequential non-cosyntropin stimulated AVSs, for the assessment of lateralization; finally, El Ghorayeb et al. (30) demonstrated the high diagnostic accuracy of an AI <1.5 in predicting contralateral suppression and positive postoperative outcomes.

In the present study, we evaluated the performance of the aldosterone indices in predicting the selectivity of the AVS and the lateralization of aldosterone hypersecretion. The novelties of the present study were:

- The determination of AI cut-offs for the assessment of selectivity in unstimulated AVS;
- The attempt to integrate the information derived from the AI in terms of lateralization with that carried by other recognized predictors of unilateral hypersecretion, in order to create a unique clinical-/imaging-adjusted diagnostic score;
- The evaluation of the accuracy of the comparative aldosterone index (CAI), defined as the ratio of aldosterone levels in the dominant vs non-dominant adrenal vein.

METHODS

Design and Study Population

Data of all patients that achieved a biochemical diagnosis of PA and underwent AVS for subtype differentiation, referred between 01/2009 and 12/2020 to two tertiary referral centers in northern Italy (the Division of Endocrinology, Diabetes and Metabolism of the University of Turin and the Endocrinology Unit of the University of Padua), were collected from prospective registries and analyzed retrospectively. The study followed the Standards for Reporting Diagnostic accuracy studies (STARD) (31). The protocol included patients who underwent AVS, with at least 6-12 months of follow-up after eventual adrenalectomy. Exclusion criteria were: diagnosis of aldosterone- and cortisol-co-secreting adrenal tumors and ACTH-stimulated AVS.

Approvals from local ethics committees were obtained (no. 0029680) with a central coordination by the Ethics Committee of the City of Health and Science University Hospital of Turin (ClinicalTrials.gov no. NCT04378387). The participants provided their written informed consent to participate in this study. The patients of this series were involved in a previous and different investigation (27).

Clinical and Biochemical Investigations

Clinical and biochemical evaluations at diagnosis were collected. Office blood pressure (BP) values were measured according to guidelines (32). BP control was defined as an average office BP <140/90 mmHg. Hypokalemia was defined as serum potassium levels <3.5 mmol/L. Plasma aldosterone concentration (PAC), plasma renin activity (PRA) and PAC after saline infusion test (SIT) were determined in all patients after the replacement of interfering drugs, according to

Endocrine Society guidelines (1). Furthermore, before the hormonal testing, hypokalemia was corrected, and patients were advised to maintain a normal dietary sodium intake. Patients remained in the same therapy during the entire diagnostic workup (from first-line tests to AVS). PA was diagnosed when the following criteria were met at the same time: 1) aldosterone-to-renin ratio (ARR) >300 (pg/mL)/(ng/mL/h) and 2) plasma aldosterone concentration (PAC) after saline infusion test (SIT) >100 pg/mL or ARR after captopril challenge test (CCT) >300 (pg/mL)/(ng/mL/h) or PAC reduction after CCT <30% compared to the pre-test value.

Differentiation of PA Subtypes

In all patients with proven PA, contrast-enhanced computed tomography (CT) of the adrenal glands was performed to identify any adrenal lesions and to localize adrenal veins. Patients were considered eligible for AVS if they agreed to undergo unilateral adrenalectomy in case of demonstration of lateralized aldosterone hypersecretion. AVS was performed by two experienced radiologists between 8:00 and 11:00 AM, using sequential cannulation and without cosyntropin stimulation. No more than 15 minutes elapsed between sampling of the left and right adrenal veins. AVS was performed *via* a percutaneous femoral vein approach and the adrenal veins were cannulated using a fluoroscopy guide.

For clinical purposes, a cortisol selectivity index (CSI) >2 was considered as indicative of selective sampling. Intraprocedural cortisol measurement was routinely used in our centers to evaluate the correct placement of catheter. A LI >3 was considered to demonstrate lateralization of aldosterone production. When results of AVS were inconclusive, the repetition of AVS was offered to the patient.

All patients with lateralization of aldosterone secretion (LI >3) underwent adrenalectomy; the diagnosis of unilateral PA was confirmed after surgery by histological examination, cure or significant amelioration of hypertension, normokalemia, normal ARR and low aldosterone levels, and/or the normal suppressibility of aldosterone. Outcomes of adrenalectomy were assessed according to the PASO criteria (33).

Aldosterone Indices

For each adrenal gland, we calculated the aldosterone index (AI, aldosterone in the adrenal vein divided by aldosterone in IVC) and the comparative aldosterone index (CAI, the ratio between aldosterone levels in the dominant and non-dominant adrenal veins, respectively). The diagnostic accuracy of AI was tested in predicting the selectivity of AVS and the final diagnosis of unilateral PA; to enhance clarity, when using the AI for these two purposes, we will refer to it as aldosterone selectivity index (ASI), and aldosterone lateralization index (ALI), respectively. The diagnostic accuracy of CAI was assessed only for the subtype diagnosis of PA. For this purpose, single adrenal glands were classified into three secretive conditions: glands with ipsilateral hypersecretion, with contralateral hypersecretion or glands within a setting of bilateral hypersecretion.

Analytical Methods

Serum aldosterone levels (pg/mL) were measured by RIA (ALDOCTK-2, Sorin Biomedica, Saluggia, Italy). The sensitivity of the assay was 10 pg/mL; the intra- and interassay coefficient of variation ranges from 1.7% to 5.3% and from 3.4% to 7.0%, respectively. PRA (ng/mL/h) was assessed by radioimmunoassay (RENCTK, Sorin Biomedica, Saluggia, Italy). The sensitivity of the assay was 0.20 ng/mL; the intra- and interassay coefficient of variation ranges from 5.4% to 9.9% and from 7.7% to 11.5%, respectively. Serum cortisol levels (μg/L) were determined by a competitive electrochemiluminescence immunoassay automated on Cobas e601 instrument (Roche Diagnostics GmbH, Germany). Analytical sensitivity was 0.018 μg/dL (0.500 nmol/L). Intra- and inter-assay precision for serum cortisol ranged from 3.0% to 5.7% and from 2.4% to 6.2%, respectively. All other biochemical variables were assayed in plasma or serum using standard methods.

Statistical Analysis

Baseline characteristics of all patients are summarized using median and interquartile range (IQR) for continuous non-normally distributed variables, or mean and standard deviation for normally distributed ones. Categorical variables are summarized using percent values. Shapiro-Wilk test was used to assess normality. Between-group differences in personal and clinical features were evaluated by the Student t-test or Mann-Whitney U test for continuous variables and by the chi-square test or Fisher's exact test for categorical variables. Paired t-test or Wilcoxon signed-rank test were used for paired data comparison in patients before and after adrenalectomy. A p-value <0.05 was considered statistically significant.

Receiver-Operating Characteristic (ROC) curves were computed to evaluate the diagnostic performances of the unconventional aldosterone indices (ASI, ALI and CAI). The accuracy of ASI was evaluated in predicting selectivity according to the most diffused cut-offs for CSI (1.1, 2 and 3). The accuracy of ALI was evaluated for the reciprocal distinction between three different gland secretive conditions; more specifically, a pairwise classification analysis was performed in order to distinguish (i) glands with ipsilateral disease from those with bilateral hypersecretion and (ii) glands with contralateral disease from those with bilateral hypersecretion. Finally, the accuracy of CAI was evaluated for the distinction between unilateral and bilateral forms of the disease.

In order to refine our analysis, ALI, after a logarithmic transformation, was then included in multivariate logistic regression models, together with lesion side at imaging and the presence of hypokalemia, in order to improve its performance in the prediction of the outcome. The performances of the multivariate classifiers (the covariate-adjusted indices), obtained by the weighted combination of these predictors according to the regression coefficients of each model, were again evaluated using ROC curves. For an immediate clinical application of our results, we then calculated the thresholds of these covariate-adjusted unconventional indices in an explicit form, for all possible combinations of the other covariates (i.e.,

lesion side at imaging and presence/absence of hypokalemia); these thresholds were computed both according to the Youden's criterion and after setting a pre-established specificity of 90%. Statistical analysis was performed using R 3.5.3 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2019).

RESULTS

General Characteristics of the Study Population

One hundred and sixty-one AVSs were performed in our centers from 01/2009 to 12/2020. As shown in **Figure 1**, 16 cosyntropin-stimulated and 1 case with partial data were excluded from the study. Thus, 144 procedures (112 bilaterally selective and 32 non bilaterally selective) were included for the evaluation of selectivity, while only the 112 selective AVSs were considered for the assessment of lateralization. The subtype diagnosis of PA revealed 60 cases (53.6%) of lateralized aldosterone secretion (66.7% on the left and 33.3% on the right side) and 52 individuals (46.4%) with idiopathic hyperaldosteronism (IHA).

No differences between unilateral PA and IHA groups were found in gender, center of diagnosis, age at diagnosis of PA, duration of hypertension, weight, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), number of antihypertensive drugs, sodium, PRA, lesion size and right/left CSI (**Table 1**).

As expected, when compared with IHA patients, unilateral PA individuals had lower levels of potassium (2.9 ± 0.5 vs 3.6 ± 0.5 mmol/L; p<0.001), higher values of PAC (median 445, IQR 303-714 vs 348, 246-481 pg/mL; p=0.012), ARR [1950, 826-3063 vs 1220, 606-3009 (pg/mL)/(ng/mL/h); p=0.049], PAC after SIT (199, 129-309 vs 141, 110-203 pg/mL; p=0.005), PAC after CCT (437, 330-594 vs 236, 147-262; p<0.001), peripheral vein (383, 228-567 vs 192, 148-331 pg/mL; p<0.001) and left adrenal vein (7105, 1877-18434 vs 3709, 1179-6350 pg/mL; p=0.013) aldosterone levels. Unilateral PA group showed lower levels of right adrenal vein aldosterone (2482, 937-12909 vs 7554, 3162-14865 pg/mL; p=0.047), lower rate of bilateral adrenal hyperplasia/normal adrenal glands at CT (8.3 vs 48.1%; p<0.001) and higher rate of left (55.0 vs 26.9%; p<0.001) and right adrenal nodules (25.0 vs 17.3%; p<0.001), compared to IHA patients (**Table 1**).

Post-Surgery Outcomes

Histological examination discovered 51 cases of APA, 6 adrenal hyperplasia and 3 micronodular hyperplasia. The re-evaluation at 6-12 months after adrenalectomy, according to PASO criteria (33), demonstrated the positive outcome of surgical treatment on BP, sodium, potassium, number of antihypertensive drugs, ARR, PAC and PAC after SIT, without significant effect on weight and BMI (**Table S1**). Complete biochemical success was achieved in 96.7% and partial in 3.3% of patients. Complete clinical success was reached in 41.7% and partial in 58.3% of patients. Outcomes were not different between patients with APA, adrenal hyperplasia and micronodular hyperplasia, but this observation is limited by the small number of patients with other than APA forms of unilateral PA. All patients that achieved complete clinical success were amongst those who also achieved complete biochemical success (data not shown).

Accuracy of Aldosterone Indices

Aldosterone Selectivity Index (ASI)

The accuracy of ASI was assessed for determining the correct cannulation of adrenal veins, using as reference standards the most diffused cut-offs for CSI (1.1, 2 and 3). As shown in **Figure 2** and **Table 2**, ASI demonstrated a high accuracy in predicting the selectivity using CSI cut-off of 1.1 as the reference standard (AUC 0.93, 95% CI 0.85-0.97, cut-off >1.67, Se 85%, Sp

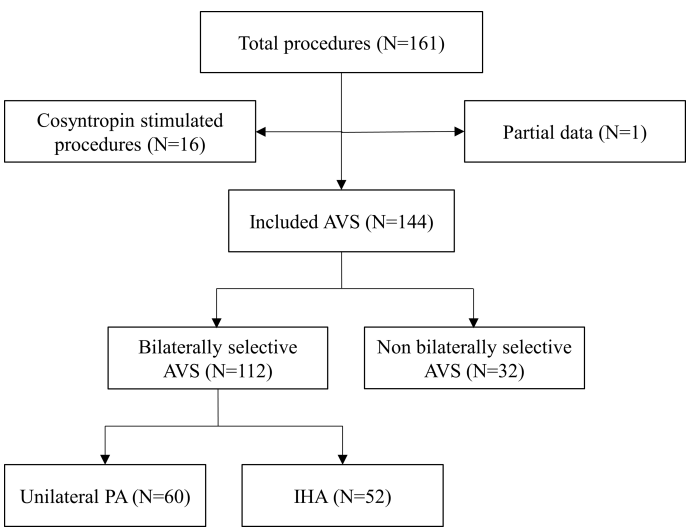

FIGURE 1 | Study flow chart. AVS, adrenal vein sampling; IHA, idiopathic hyperaldosteronism; PA, primary aldosteronism.

TABLE 1 | Clinical characteristics for all patients and for the IHA, the unilateral PA and the unclassified groups.

Variables/Parameters	Overall data (N=144)	Diagnosis			p-value
		Unclassified (N=32)	IHA (N=52)	Unilateral PA (N=60)	
Center					
Turin	59%	62.5%	67.3%	50%	0.161
Padua	41%	37.5%	32.7%	50%	
Male gender	61.8%	53.1%	71.2%	58.3%	0.197
Age at diagnosis of PA (years)	49 ± 11	48 ± 12	49 ± 10	48 ± 12	0.709
Duration of AH (years)	5 (2-12)	3.5 (0-9)	8.5 (3-15)	5 (2-13)	0.346
Weight at diagnosis (Kg)	79 ± 16	80 ± 17	82 ± 17	78 ± 16	0.211
BMI (Kg/m^2)	27.38 ± 4.35	27.66 ± 4.76	27.91 ± 4.64	26.87 ± 3.92	0.094
SBP (mmHg)	160 (140-170)	160 (148-180)	160 (143-175)	155 (140-170)	0.218
DBP (mmHg)	100 (90-109)	100 (94-105)	100 (90-110)	100 (90-101)	0.131
No. of antihypertensive drugs	2 (1-3)	2 (0-3)	2 (1-3)	2 (1-3)	0.213
Sodium (mmol/L)	142 ± 2	142 ± 3	143 ± 3	143 ± 2	0.843
Potassium (mmol/L)	3.2 ± 0.7	3.2 ± 0.8	3.6 ± 0.5	2.9 ± 0.5	<0.001
PAC (pg/mL)	380 (279-574)	374 (278-493)	348 (246-481)	445 (303-714)	0.012
PRA (ng/mL/h)	0.20 (0.10-0.60)	0.20 (0.1-0.46)	0.21 (0.10-0.68)	0.20 (0.10-0.62)	0.806
ARR [(pg/mL)/(ng/mL/h)]	1547 (694-3027)	1537 (784-3117)	1220 (606-3009)	1950 (826-3063)	0.049
PAC after SIT (pg/mL)	188 (120-271)	219 (153-285)	141 (110-203)	199 (129-309)	0.005
PAC after CCT (pg/mL)	358 (206-473)	272 (176-416)	236 (147-262)	437 (330-594)	<0.001
Lesion size (mm)	14 (10-19)	15 (10-19)	14 (10-15)	13 (10-18)	0.742
Lesion side					
Absent/hyperplasia	30.6%	43.8%	48.1%	8.3%	<0.001
Left	38.2%	25%	26.9%	55.0%	
Right	22.2%	25%	17.3%	25.0%	
Bilateral	9.0%	6.2%	7.7%	11.7%	
Peripheral vein cortisol	127 (107-213)		156 (106-228)	156 (108-206)	0.320
Peripheral vein aldosterone	209 (180-454)	236 (162-401)	192 (148-331)	383 (228-567)	<0.001
Left adrenal vein cortisol	1068 (450-3437)	303 (193-575)	1915 (896-3705)	1952 (581-3685)	0.556
Left adrenal vein aldosterone	3406 (833-8697)	650 (353-2451)	3709 (1179-6350)	7105 (1877-18434)	0.013
Right adrenal vein cortisol	1405 (524-5536)	290 (138-895)	2945 (1594-7516)	2997 (771-5865)	0.320
Right adrenal vein aldosterone	3918 (937-12124)	777 (126-8517)	7554 (3162-14865)	2482 (937-12909)	0.047
Left CSI	8.9 (3.1-19.6)	1.8 (1.3-3.9)	13.1 (5.7-22.1)	13.1 (5.7-20.7)	0.746
Right CSI	14.6 (4.5-29.8)	1.74 (1.1-4.9)	21.5 (8.2-38.8)	17.1 (7.1-29.8)	0.355
Left AI	14.2 (2.8-39.0)	2.5 (1.1-19.0)	15.6 (6.2-32.9)	18.1 (5.2-51.8)	0.378
Right AI	14.4 (2.7-46.8)	1.8 (0.9-29.6)	29.3 (12.9-72.4)	6.9 (2.6-30.2)	<0.001

Statistical differences were calculated between the IHA and the unilateral PA groups. AH, arterial hypertension; ARR, aldosterone-to-renin ratio; BMI, body mass index; CCT, captopril challenge test; CSI, cortisol selectivity index; DBP, diastolic blood pressure; IHA, idiopathic hyperaldosteronism; PA, primary aldosteronism; PAC, plasma aldosterone concentration; PRA, plasma renin activity; SBP, systolic blood pressure; SIT, saline infusion test.

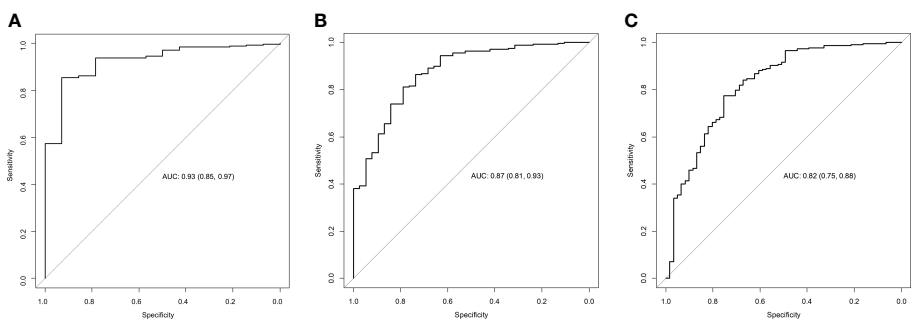

FIGURE 2 | Accuracy of the AI for predicting selectivity of AVS. ROC curves describing the accuracy of AI for predicting selectivity using as reference a cortisol selectivity cut-off of 1.1 **(A)**, 2 **(B)** and 3 **(C)**. AUC, area under the curve; AVS, adrenal vein sampling; AI, aldosterone index.

TABLE 2 | Accuracy of aldosterone indices.

	Cut-off	AUC (95% CI)	Se	Sp	+LR	-LR
CSI cut-offs			**ASI**			
1.1	>1.67	0.93 (0.85-0.97)	85	93	5.00	0.16
2	>2.87	0.87 (0.80-0.93)	81	79	3.50	0.24
3	>4.83	0.82 (0.75-0.88)	78	75	3.10	0.29
Predicted side of hypersecretion			**Simple ALI**			
Ipsilateral[#]	>26.63	0.71 (0.62-0.78)	77	61	1.97	0.38
Contralateral[#]	<9.20	0.84 (0.78-0.90)	80	76	3.33	0.26
Predicted subtype			**CAI**			
Unilateral PA	>5.02	0.92 (0.87-0.96)	82	92	10.3	0.20

Cut-offs, AUC, sensitivity, specificity, +LR and -LR of ASI, simple ALI and CAI, according to the Youden's criterion. ALI, aldosterone lateralization index; AUC, area under the curve; ASI, aldosterone selectivity index; CAI, comparative aldosterone index; CSI, cortisol selectivity index; -LR, negative likelihood ratio; +LR, positive likelihood ratio; PA, primary aldosteronism; Se, sensitivity; Sp, specificity.

[#]*Versus bilateral hypersecretion.*

93%, +LR 5, -LR 0.16; **Figure 2A** and **Table 2**), and a moderate accuracy if compared with CSI of 2 (AUC 0.87, 95% CI 0.81-0.93, cut-off >2.87, Se 81%, Sp 79%, +LR 3.9, -LR 0.24; **Figure 2B** and **Table 2**) and 3 (AUC 0.82, 95% CI 0.75-0.88, cut-off >4.83, Se 78%, Sp 75%, +LR 3.1, -LR 0.29; **Figure 2C** and **Table 2**).

Aldosterone Lateralization Index (ALI)

The diagnostic accuracy of ALI was assessed to distinguish 1) glands with ipsilateral disease from those with bilateral hypersecretion and 2) glands with contralateral disease from those with bilateral hypersecretion. ALI showed a moderate accuracy in predicting ipsilateral disease (AUC 0.71, 95% CI 0.63-0.79, cut-off >26.63, Se 77%, Sp 61%, +LR 1.97, -LR 0.38) and contralateral aldosterone hypersecretion (AUC 0.84, 95% CI 0.78-0.89, cut-off <9.20, Se 80%, Sp 76%, +LR 3.33, -LR 0.26) (**Figure 3** and **Table 2**).

Covariate-Adjusted ALI

Multivariate logistic regression models, considering as covariates lesion side at CT and hypokalemia, were used to create lesion- and hypokalemia-corrected ALI.

Lesion side at imaging was initially considered as a three-level (ipsilateral lesion, contralateral lesion, bilateral/no lesions) unordered categorical variable; however, in all models predicting ipsilateral disease, no statistically significant differences were found between the "contralateral lesion" and the "bilateral/no lesions" categories; similarly in all models predicting contralateral disease, no statistically significant differences were found between the "ipsilateral lesion" and the "bilateral/no lesions" categories. Therefore, all logistic regression models were simplified by merging the lesion categories that did not significantly differed, thus reducing lesion side at imaging to a binary variable. Complete data about the final logistic regression models are available in the supplementary material (**Tables S2, S3**).

The ROC curves, evaluating the performances of the lesion-/hypokalemia-corrected ALI, revealed a significant improvement in the accuracy of predicting ipsilateral (AUC 0.88, 95% CI 0.82-0.93, Se 85%, Sp 81%, +LR 4.5, -LR 0.19; **Figure 3A**) and contralateral (AUC 0.89, 95% CI 0.83-0.93, Se 92%, Sp 77%, +LR 4.0, -LR 0.10; **Figure 3B**) disease, in comparison with simple ALI. The improvement of covariate-adjusted unconventional indices was statistically significant in all models (**Figure 3**). In

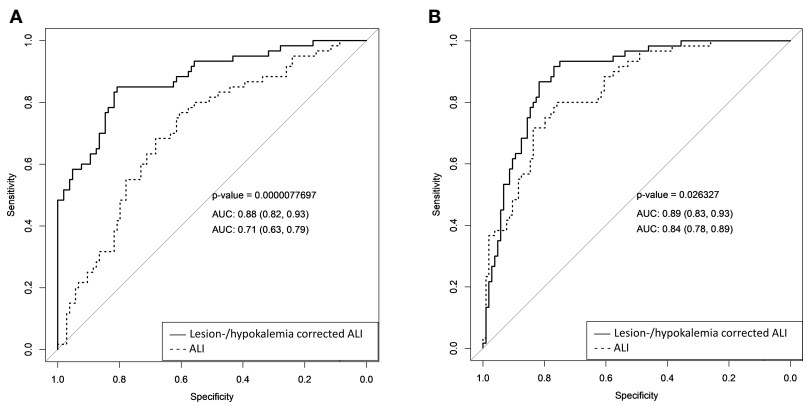

FIGURE 3 | Comparison of ROC curves of simple and corrected AI for predicting lateralization of aldosterone hypersecretion. Comparison of ROC curves of simple and lesion-/hypokalemia-corrected AI in the prediction of ipsilateral **(A)** and contralateral **(B)** aldosterone hypersecretion. AUC, area under the curve; AI, aldosterone index.

addition to the cut-offs identified by Youden's criterion, those corresponding to a pre-established specificity of 90% were also considered (**Table S4**). For an immediate clinical application of our results, we then calculated the thresholds of these covariate-adjusted unconventional indices in an explicit form, for all possible combinations of the other covariates (i.e., lesion side at imaging and presence/absence of hypokalemia); these thresholds were computed both according to the Youden's criterion (**Table S5**) and after setting a pre-established specificity of 90% (**Table 3**).

Comparative Aldosterone Index (CAI)

CAI revealed a high accuracy in predicting the subtype diagnosis (AUC 0.92, 95% CI 0.87-0.96, Se 82%, Sp 92%, +LR 10.3, -LR 0.20) with a cut-off >5.02 for unilateral aldosterone hypersecretion (**Figure 4** and **Table 2**).

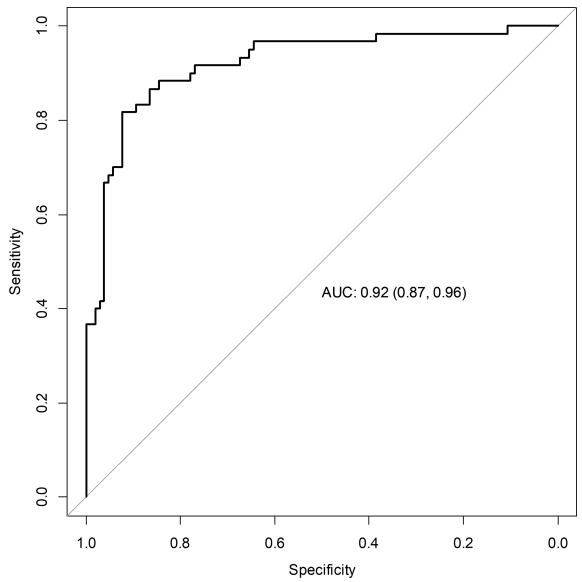

FIGURE 4 | Accuracy of CAI for predicting lateralization of aldosterone hypersecretion. ROC curve describing the accuracy of CAI for predicting the lateralization of aldosterone hypersecretion. AUC, area under the curve; CAI, comparative aldosterone index.

DISCUSSION

The present study seemed to endorse the application of the AI in predicting selectivity and lateralization of AVS in patients with PA. We studied the AI as a selectivity index (ASI) and as lateralization index (ALI), which both provided a satisfactory accuracy, particularly for ALI, that became even more accurate after the correction for hypokalemia and lesion side. Moreover, we introduced the CAI, a straightforward aldosterone ratio between the adrenal glands, that showed a high accuracy in the assessment of lateralization of aldosterone hypersecretion.

International guidelines and expert consensus (1, 2, 34–36) recognize AVS as the gold standard for the diagnosis of PA subtypes, however, AVS is used only in few centers worldwide, due to technical difficulties in the interventional radiology approach. Without a bilaterally selective procedure, the LI, which is the most widely accepted index in the literature, is not applicable to guide the diagnosis of lateralization of aldosterone hypersecretion and the subsequent opportunity of surgical treatment. Therefore, the introduction of unconventional indices and statistical models (25–27, 37, 38) could help in determining the lateralization of aldosterone secretion in case of suboptimal AVS. Our group recently demonstrated the high accuracy of corrected unconventional indices in the prediction of unilateral disease in a large series of patients with PA subtyped with AVS (27). In the present study, we focused on the reliability of aldosterone indices for both the selectivity of the sampling and the lateralization of aldosterone hypersecretion. In the literature, Mailhot et al. (28) described the utility of ASI and CSI (alone or combined) during unstimulated AVS in predicting selectivity, using the same pre-established cut-offs used for the CSI (1.1, 2 and 3) and comparing their accuracy with a composite standard (CSI and/or ALI >5 during ACTH-stimulated AVS). In our study, we derived 3 new cut-offs for ASI: 1.67 compared to a CSI of 1.1, 2.87 compared to a CSI of 2, and 4.83 compared to a CSI of 3. It is clear how the retrieved aldosterone cut-offs are quite different in comparison to the cortisol ones.

Regarding the role of AI in determining lateralization of aldosterone secretion, Liu et al. (29) tried to prove the utility of AI in determining the dominant adrenal gland. While El Ghorayeb et al. (30) demonstrated the high diagnostic accuracy of AI <1.5 in predicting contralateral suppression and positive postoperative outcomes.

In the present study, the performance of simple ALI was higher in the prediction of contralateral (rather than ipsilateral) aldosterone hypersecretion, but the correction for lesion side and hypokalemia provided a significant improvement in the diagnostic accuracy of this index for the prediction of both

TABLE 3 | Thresholds of lesion side- and potassium-corrected ALI for the diagnosis of ipsilateral/contralateral aldosterone hypersecretion, setting a specificity of 90%.

Index	Predicted side of hypersecretion	Normokalemia			Hypokalemia		
		Ipsilateral lesion	Bilateral/no lesions	Contralateral lesion	Ipsilateral lesion	Bilateral/no lesions	Contralateral lesion
ALI	**Ipsilateral**[#]	>157.51	>1063.91	>1063.91	>13.08	>88.35	>88.35
	Contralateral[#]	<0.53	<0.53	<3.34	<1.34	<1.34	<8.36

ALI, aldosterone lateralization index.
[#]*Versus bilateral hypersecretion.*

ipsi- and contralateral lateralization. It has to be highlighted that we should be sure of a correct cannulation of the adrenal vein when we use the ALI for predicting contralateral hypersecretion; in fact, a low ALI value could be present also in the case of an inadequate selectivity of the AVS. To avoid false positive results that may lead to the surgical removal of an adrenal gland in the context of IHA, we also decided to provide cut-offs with optimized specificity. Moreover, we derived the thresholds of covariate-adjusted ALI in an explicit form, in order to give an immediate clinical applicability to the study.

In this study, we also introduced the CAI, a straightforward ratio of aldosterone in the two adrenal veins, which demonstrated a high accuracy in predicting lateralization of aldosterone hypersecretion. However, being a ratio between aldosterone values in the two adrenal veins, the application of this index needs a bilaterally selective AVS.

The strengths of our study are the very low rate of false positive diagnosis of PA, due to the diagnosis of florid cases of PA and to the diagnostic experience of our centers, which confers homogeneity to the studied population; the longstanding expertise in performing AVS of dedicated radiologists working in our centers; the presence of rigidly defined AVS criteria to assess lateralization (using a LI >3) and to guide the surgical indication; the presence of subsequent follow-up and the application of widely accepted criteria for the evaluation of outcomes in surgical treated patients, obtaining outcomes that proved to be consistent with literature (33).

It should be noted that in the present study the reliability of unconventional indices was derived only in comparison with the IHA group, with a potential underestimation of the real accuracy of these indices. Despite this choice, we showed that the reached reliability is highly satisfactory.

The present study has some limitation. First, its retrospective design, even if data were collected from prospective registries. Second, the potential for selection bias due to the tertiary nature of our centers. Third, our results apply to unstimulated AVS with a correct selectivity in at least one adrenal vein. Moreover, data should be validated in an external cohort before the introduction in clinical practice.

Contribution of the Study to the Field and Conclusions

Our study suggests the possibility of accurately determining selectivity (ASI) and lateralization of the aldosterone secretion (ALI and CAI) without using cortisol-corrected indices and, exclusively for ALI, even if AVS is not bilaterally selective. We know that the lack of accepted standards for the performance of selective AVS and the interpretation of its results contributes to the hesitancy towards the adoption of the unconventional indices in the case of non-conclusive AVS data. In this way, however, many PA patients are denied curative surgery or undergo adrenalectomy without the evidence of lateralization, which may result in the removal of an adrenal gland in a patient with IHA. PA is a frequent cause of organ damage among patients with arterial hypertension, and aldosterone has a key role in the pathogenesis of cardiovascular disease. Considering the low probability of spontaneous remission of florid PA (39), the possibility to cure PA with adrenalectomy, the improvement of organ damage (40–42) and the reduced incidence of cardiometabolic complications with the resolution of hyperaldosteronism (43–45), the research of alternative/unconventional indices for the interpretation of AVS, even in case of interfering factors or suboptimal sampling, is of crucial importance.

Given our results, we suggest the use of the proposed unconventional aldosterone indices (ASI, ALI, CAI) to further confirm the selectivity and the lateralization of AVS, in addition to conventional SI and LI. Moreover, they could help in the interpretation of unilaterally or bilaterally selective AVS and in the selection of patients for adrenalectomy in all cases in which the measurement of cortisol levels could be unreliable, such as in patients taking chronic steroid treatment, and in the case of pre-analytical errors that denied a complete hormonal assessment. These indices may be also considered in patients with aldosterone/cortisol cosecretion or the so-called Conn-shing syndrome, even if patients with cosecretion have been excluded from this study. Further *ad hoc* studies are necessary for a definitive validation of this last indication.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Approvals from local ethics committees were obtained (no. 0029680) with a central coordination by the Ethics Committee of the City of Health and Science University Hospital of Turin (ClinicalTrials.gov no. NCT04378387). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: MP, FB, FC, and MM. Methodology: MP and FB. Validation: CL, MB, MC, and GV. Resources: MB, DR, and GG. Data Curation: FB, FC, CL, and GV. Writing – Original Draft Preparation: MP and FB. Writing – Review & Editing: DR, GG, CS, EG, and MM. Supervision: CS, EG, and MM. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.801529/full#supplementary-material

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Accurate Location of Catheter Tip With the Free-to-Total Metanephrine Ratio During Adrenal Vein Sampling

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Background: The selectivity index (SI) of cortisol is used to document correct catheter placement during adrenal vein sampling (AVS) in patients with primary aldosteronism (PA). We aimed to determine the cutoff values of the SIs based on cortisol, free metanephrine, and the free-to-total metanephrine ratio (FTMR) using an adapted AVS protocol in combination with CT.

Methods: Adults with PA and referred for AVS were recruited in two hypertension centers. The cortisol and free metanephrine-derived SIs were calculated as the concentration of the analyte in adrenal veins divided by the concentration of the analyte in the distal vena cava. The FTMR-derived SI was calculated as the concentration of free metanephrine in the adrenal vein divided by that of total metanephrine in the ipsilateral adrenal vein. The AVS was classified as an unequivocal radiological success (uAVS) if the tip of the catheter was seen in the adrenal vein. The SI cutoffs of each index marker were established using receiver operating characteristic curve analysis.

Results: Out of 125 enrolled patients, 65 patients had an uAVS. The SI cutoffs were 2.6 for cortisol, 10.0 for free metanephrine, 0.31 for the FTMR on the left side, and 2.5, 9.9, and 0.25 on the right side. Compared to free metanephrine and the FTMR, cortisol misclassified AVS as unsuccessful in 36.6% and 39.0% of the cases, respectively.

Conclusion: This study is the first to calculate the SIs of cortisol, free metanephrine, and the FTMR indices for the AVS procedure. It confirms that free metanephrine-based SIs are better than those based on cortisol.

Keywords: primary aldosteronism, secondary hypertension, aldosterone, adrenal vein sampling (AVS), metanephrines (plasma), cortisol

INTRODUCTION

Primary aldosteronism (PA) is one of the most common forms of secondary hypertension (1–3). Identification of patients with this disease is important because they have an increased risk of target organ damage and carry a worse cardiovascular prognosis as compared to patients with essential hypertension with the same level of blood pressure (BP) (4). Two therapeutic strategies may be proposed to patients with PA: blockade of the mineralocorticoid receptor or surgical removal of one adrenal gland if there is lateralized overproduction of aldosterone in one of the adrenal glands. Unilateral adrenalectomy may offer a potential cure for patients with favorable conditions (5, 6).

The standard reference technique to show lateralization of aldosterone secretion is adrenal vein sampling (AVS) (7, 8). However, this procedure is not standardized across reference centers and countries (8). The technique may differ in several ways such as sequential or bilateral simultaneous measurement of hormones, cosyntropin stimulation, or no stimulation. Historically, cortisol with or without cosyntropin stimulation has been used as a marker of correct catheter positioning in the adrenal veins; and, traditionally, adrenal vein cortisol concentration to peripheral vein (antecubital fossa or inferior vena cava below the adrenal veins or iliac vein) plasma cortisol concentration ratio defines the selectivity index (SI). However, several cutoffs have been used to define the SI, which may have limited the use of AVS due to the absence of clear consensus and may have excluded patients from a curative strategy when too stringent cutoffs were applied (9, 10).

The use of free metanephrine (FMN) has been shown to be superior to cortisol in assessing SI, particularly in the setting of non-stimulated sampling of the adrenal veins (11, 12). Sulfotransferase 1A3, the enzyme that sulfate-conjugates FMN, is mainly found in the intestine, but its expression in the adrenal gland has yet to be reported to the best of our knowledge (13). Consequently, FMN, which is released continuously from adrenal chromaffin cells and independently of any stimulation, is then conjugated peripherally (14, 15). We thus hypothesized that the free-to-total metanephrine ratio (FTMR) in the adrenal veins would be a better marker of correct catheter placement than cortisol, like FMN alone, but with the advantage of reducing the number of sampling sites during AVS. Indeed, the closer the vein sampling is to the adrenal gland, the higher the FTMR will be.

The first objective of the study was to determine SI cutoffs for the FTMR, FMN, and cortisol using a contrast-enhanced multi-detector CT. An adapted AVS protocol allowed us to perform receiver operating characteristic (ROC) analysis. The second objective was to compare the performance of cortisol, FMN, and the FTMR for the successful assessment of adrenal vein catheterization.

Abbreviations: AVS, adrenal vein sampling; eAVS, equivocal radiological success of adrenal vein catheterization; uAVS, unequivocal radiological success of adrenal vein catheterization; BP, blood pressure; FMN, free metanephrine; FTMR, free-to-total metanephrine ratio; PA, primary aldosteronism; ROC, receiver operating characteristic; SI, selectivity index.

METHODS

This was a prospective study conducted from 2013 to 2018 with patients from two reference centers for hypertension: the Geneva University Hospital and the Lausanne University Hospital. Eligible participants were men and women older than 18 years with a diagnosis of PA based on two increased aldosterone/plasma renin activity ratios or one increased ratio associated with a concomitant increased urinary 24-h aldosterone excretion and referred for an AVS at the Lausanne University Hospital. No confirmatory suppression test was carried out, as lateralization of aldosterone secretion is not excluded by the positive saline infusion suppression test (16). A single experienced interventional radiologist performed all procedures in a hybrid interventional operating room equipped with a 128-slice CT Philips Ingenuity (Philips Systems, Cleveland, OH, USA) and C-Arm Philips Veradius Unity (Philips Systems, Cleveland, OH, USA). Each patient was lying down on the table of the CT scan. After rigorous asepsis and surgical field placement, a common right femoral 6F access was introduced using the Seldinger technique, under local anesthesia and with US guidance. Under fluoroscopy, the radiologist catheterized each adrenal vein and the proximal and the distal vena cava and obtained blood samples. The procedure was performed sequentially without cosyntropin stimulation. The order of catheterization and blood sampling was the proximal vena cava (suprarenal), the distal vena cava (infrarenal), the left adrenal vein, and finally the right adrenal vein. The position of the tip of the catheter was confirmed by gently injecting contrast media to show retrograde adrenal parenchymography for both sides. For the right side, a small helical CT acquisition was also conducted to prove the placement of the catheter in the right adrenal vein. When the operator had difficulties locating the adrenal veins, additional CT acquisitions were performed (**Supplementary Figure 1**). The procedure was conducted in an outpatient setting. Antihypertensive drugs other than the alpha-blocker doxazosin or calcium channel blockers were stopped 2 weeks prior to AVS, except for spironolactone, which was stopped 6 weeks before the procedure.

The study was approved by the local ethics committee (Commission cantonale d'éthique de la recherche sur l'être humain, www.cer-vd.ch). Each participant provided written informed consent. The procedures followed in this study were in accordance with institutional guidelines.

Plasma renin activity (PRA) and plasma aldosterone were measured with commercial radioimmunometric assays (RENCTK (Angiotensin I) RIA, DiaSorin, Saluggia, Italy, and ALDO RIACTR Kit, Cisbio Bioassays, Codolet, France, respectively). Serum cortisol levels were measured with fluorescence polarization immunoassay (FPIA) on an AxSYM instrument (Abbott Diagnostics, Lake Forest, IL, USA). Plasma free and total metanephrines were measured using ultrahigh-performance liquid chromatography–tandem mass spectrometry (17, 18).

The cortisol-derived SI was calculated as the concentration of cortisol in adrenal vein samples divided by that in distal vena cava samples. The FMN-derived SI was calculated as the

concentration of FMN in adrenal vein samples divided by that in distal vena cava samples. The FTMR-derived SI was calculated as the concentration of FMN in adrenal vein samples over total metanephrine in the ipsilateral adrenal vein. The SI cutoffs of each index marker were established using ROC curve analysis. Placement of the tip of the catheter in the proximal vena cava confirmed by angiography was defined as a negative test, and placement of the tip of the catheter in the adrenal vein confirmed by angiography coupled with CT was defined as a positive test for the reference test. We used the FTMR in the proximal vena cava and the proximal vena cava to distal cava cortisol or FMN ratio as the corresponding negative index test. The AVS was classified as an unequivocal radiological success (uAVS) if the radiologist had the visual certainty of correct adrenal vein placement using angiography coupled with CT. If the success of the adrenal vein catheterization was doubtful, the procedure was classified as equivocal radiological success (eAVS).

Adrenal Gland Metanephrine Content

Free and total metanephrines (i.e., free + sulfated metanephrine) were extracted from tissues of four human adrenal glands obtained from kidney transplant organ donors after disruption in perchloric acid 0.1 M and were sonicated using a Branson Sonifier 450 (Branson, Danbury, CT, USA) at full power for 30 s. Sulfated metanephrines were hydrolyzed with sulfatase from *Aerobacter aerogenes* (S1629) purchased from Sigma-Aldrich (St. Louis, MO, USA) as previously reported (19). Values are expressed in nanomoles of metanephrine per gram of tissue.

Statistics

Data are presented as means ± SD or medians with interquartile range (IQR) if variables were non-normally distributed. A t-test was used to compare normally distributed variables. A rank-sum test was used to compare data not normally distributed. A chi-square test was used to compare categorical variables. ROC curve analysis was used to establish the most appropriate SI for each marker in the subset of patients with uAVS. The point chosen to establish the SIs was the point providing the maximum sensitivity for a specificity of 100%. Agreement between index markers was tested with a kappa test in the subset of patients with eAVS. Statistical analysis was performed using Stata 15.0 (Stata Corp), and the nominal level of statistical significance was set at a p-value of <0.05.

RESULTS

Between 2014 and 2018, 125 patients with PA were referred for an AVS and signed their informed consent (**Figure 1**, flowchart). The percentage of patients with normal CT was 45.6%, while 30.4% had a left nodule, 16.0% had a right nodule, 3.2% had left hyperplasia, 1.6% had bilateral nodules, and 2.4% had bilateral hyperplasia. Nine patients were excluded from the analysis because they had a previous unsuccessful (prior to 2013) AVS, suggesting a potential difficult procedure. Of the remaining 116 patients, 65 had radiological unequivocal bilateral successful AVS and were used to determine cutoff values of markers of selectivity, while 51 had radiologically equivocal bilateral successful AVS and were used together with 9 patients, who were referred for a second try, in the secondary analysis to compare the performance of the different analytes. Of these 51 participants, the uncertainty of correct placement of the tip of the catheter in the right adrenal vein by the radiologist was the most common reason for eAVS.

Patients' characteristics are shown in **Table 1**. More than half of the participants were men, and the majority were of Caucasian ethnicity. Despite the use of oral potassium in patients with hypokalemia diagnosed before AVS, mean serum potassium was still below the local laboratory reference range (3.5–4.5 mmol/L).

Hormonal Concentration at Specific Sampling Site (Distal Vena Cava, Proximal Vena Cava, Left Adrenal Vein, Right Adrenal Vein, and Adrenal Gland)

Hormonal concentrations at specific sampling sites are shown in **Table 2** for patients with uAVS and eAVS. In patients with uAVS, aldosterone, FMN, and the FTMR were all higher in the proximal vena cava than in the distal vena cava. Conversely, total metanephrine was higher in the distal vena cava. The median ratios of the right adrenal vein to the distal vena cava of cortisol, FMN, and total metanephrine were respectively 3.0 (1.6–7.2), 54.5 (27.4–107.5), and 2.3 (1.7–4.0) (**Supplementary Figure 2**). The ratios from the left adrenal vein were 6.3 (3.1–149), 100.3 (72.8–130.8), and 3.6 (2.8–4.5). The ratios from the left side were significantly higher, except for total metanephrine. In the adrenal gland, 96.8 (median) of the total metanephrine consisted of FMN (**Table 2**). Hormonal concentrations and ratios from the whole cohort (equivocal and unequivocal AVS) are shown in **Figure 2**.

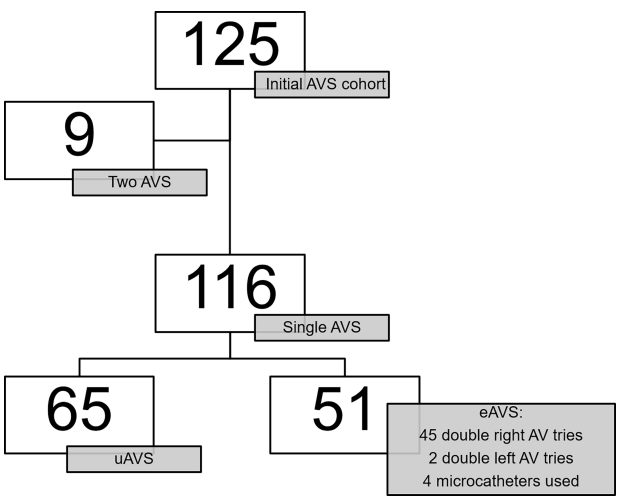

FIGURE 1 | Flowchart. uAVS, radiological unequivocal adrenal vein sampling; eAVS, radiological equivocal adrenal vein sampling; AV, adrenal vein.

TABLE 1 | Baseline patients' characteristics.

	Total (n = 116)	uAVS (n = 65)	eAVS (n = 51)	*p*-Value
Number	116	65	51	
Sex (women, %)	50 (43)	28 (43)	22 (43)	0.995
Ethnicity (Caucasian, %)	101 (87.1)	55 (84.6)	46 (90.2)	0.791
Age (years)	49.2 ± 10.2	50.5 ± 10.2	47.6 ± 10.1	0.129
BMI (kg/m^2)	30.0 ± 6.9	30.0 ± 6.6	30.1 ± 7.5	0.949
Office SBP (mmHg)	151.9 ± 17.7	153.8 ± 18.8	149.4 ± 15.8	0.185
Office DBP (mmHg)	92.8 ± 12.7	93.7 ± 12.4	91.7 ± 13.1	0.4
Office heart rate (bpm)	73.5 ± 12.1	74.7 ± 12.3	71.7 ± 11.7	0.229
eGFR (ml/min/1.73 m^2)	87.9 ± 22.5	87.4 ± 22.5	88.5 ± 23.4	0.8
Plasma K^+ (mmol/L)	3.35 ± 0.45	3.31 ± 0.41	3.40 ± 0.49	0.266
PRA (ng/ml/h)	0.1 (0.10–0.26)	0.1 (0.10–0.19)	0.1 (0.10–0.4)	0.039
Aldosterone (pg/ml)	145 (94–225)	145 (94–194)	141 (93–241)	0.824

Data are n (%), median (IQR), and mean (SD), unless otherwise specified.
uAVS, unequivocal radiologic successful adrenal vein sampling; eAVS, equivocal radiologic successful adrenal vein sampling; SBP, systolic blood pressure; DBP, diastolic blood pressures; eGFR, estimated glomerular filtration rate; PRA, plasma renin activity.

Selectivity Indices for Cortisol, Free Metanephrine, and the Free/Total Metanephrine Ratio

Using ROC curves analysis, with CT-confirmed correct catheter tip placement used as the reference test (positive if the catheter is in the adrenal vein and negative if the catheter is in the proximal vena cava), the area under the curve was 1.0 (95% CI 1.000–1.000) for FMN, 1.0 (95% CI 1.000–1.000) for the FTMR, and 0.913 (95% CI 0.981–1.000) for cortisol on the left side. The area under the curve was 0.998 (95% CI 0.990–1.000) for FMN, 0.995 (95% CI 0.988–1.000) for the FTMR, and 0.882 (95% CI 0.813–953) on the right side. ROC curves are shown in **Supplementary Figure 3**. The SI cutoff values of each marker are shown in **Table 3**.

Concordance and Agreement Between Markers of Selectivity in Participants With Equivocal Radiological Success

Bilaterally successful and unsuccessful AVS based on the newly defined cutoffs for each selectivity marker and applied to the group with eAVS (51 patients) and those with double AVS (9 patients) is shown in **Table 4**. The use of FMN and the FTMR as selectivity markers enabled the reclassification of bilateral AVS wrongly labeled as unsuccessful based on cortisol SI in 36.6% and 39.0%, respectively. Bilateral disagreement between the FMN and the FTMR was found in two cases in which FMN labeled AVS as unsuccessful and where the FTMR labeled AVS as successful. The levels of agreement between cortisol and FMN were 68.3% with a kappa of 0.367 ± 0.120. The level of agreement between cortisol and the FTMR was 68.3% with a kappa of 0.382 ± 0.117. The level of agreement of FMN with the FTMR was 96.7% with a kappa of 0.933 ± 0.129.

DISCUSSION

This study is the first to report and compare the SIs of cortisol, FMN, and the FTMR using contrast-enhanced multi-detector CT as a reference test for positivity. Using a modified AVS protocol, which included an additional blood sampling in the proximal part of the vena cava, we were also able to collect data reflecting a true negative test. The study confirms that FMN is a better analyte than cortisol to confirm the successful placement of the catheter's tip during AVS. In addition, it shows that the FTMR performs as well as FMN and does not necessitate an additional peripheral sample (infrarenal inferior vena cava) for SI determination.

AVS is the reference test to determine lateralization of aldosterone secretion, although some have questioned its

TABLE 2 | Adrenal, adrenal veins, and proximal and distal vena cava plasma concentrations.

	Group	Distal VC	Proximal VC	Left AV	Right AV	Adrenal gland (N = 4)
Aldosterone (pg/ml)	uAVS	145 (94; 194)	179 (116; 340)*	1,263 (711; 4,687)	834 (462; 1,700)	.
	eAVS	140 (93; 251)	166 (95; 351)	1,138 (407; 6,227)	294 (84; 1,205)	
Cortisol (nmol/L)	uAVS	307 (252; 416)	339 (256; 433)*	2,235 (887; 6,408)	1,184 (515; 2,040)	.
	eAVS	280 (211; 394)	290 (201; 415)	1,671 (689; 4,011)	507 (295; 1,169)	
Free metanephrine (nM)	uAVS	0.11 (0.08; 0.13)	0.22 (0.15; 0.29)*	10.0 (6.6; 15.1)	5.54 (3.1; 12.2)	17.8 (12.5; 20.7)
	eAVS	0.11 (0.09; 0.14)	0.23 (0.18; 0.32)	9.55 (6.94; 13.3)	1.56 (0.3; 4.65)	
Total metanephrine (nM)	uAVS	4.33 (3.06; 5.56)	4.18 (2.89; 5.82)*	15.0 (9.9; 21.5)	10.2 (6.5; 18.3)	18.1 (12.5; 21.0)
	eAVS	4.06 (3.1; 5.24)	3.90 (3.1; 5.06	14.9 (10.8; 18.8)	6.3 (3.8; 9.13)	
FTMR	uAVS	0.025 (0.021; 031)	0.054 (0.043; 0.069)*	0.69 (0.634; 0.729)	0.57 (0.430; 0.694)	0.968 (0.903; 1.02)
	eAVS	0.025 (0.022; 0.031)	0.056 (0.045; 0.072)	0.688 (0.626; 0.749)	0.3 (0.086; 0.495)	

Data are medians with interquartile ranges.
VC, vena cava; AV, adrenal vein; uAVS, unequivocal adrenal vein sampling; eAVS, equivocal adrenal vein sampling.
**p < 0.05 distal VC vs. proximal VC in patients with uAVS.*

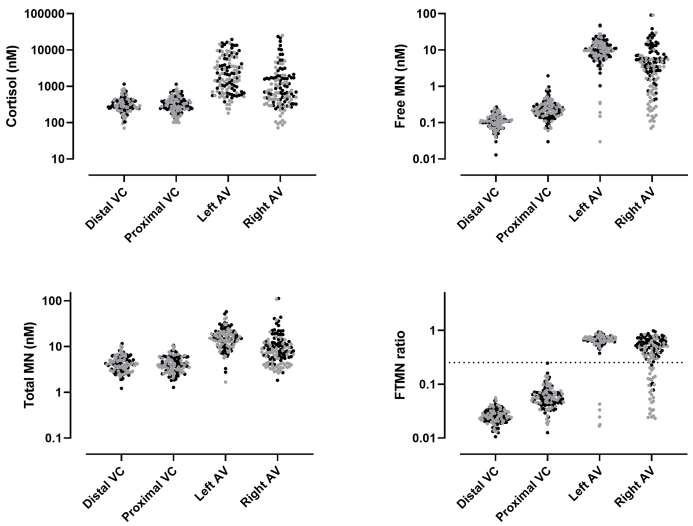

FIGURE 2 | Hormonal concentrations and free/total metanephrine ratio (FTMR) at specific sites with calculated selectivity index for FTMR (dashed line). VC, vena cava; AV, adrenal vein; MN, metanephrine; AVS, adrenal vein sampling. Black dot: unequivocal AVS. Gray dots: equivocal AVS.

superiority in terms of outcomes after surgery when compared to CT (20). As such, the successful placement of the catheter tip in the draining adrenal vein is of paramount importance, and it is usually assessed using cortisol SI. However, no consensus exists regarding the SI of cortisol, and experts advise choosing an SI superior to two or three at the expense of excluding the right positioning of the catheter (21). We show from ROC curve analysis that the SI for cortisol is >2.6 for the left adrenal vein and >2.5 for the right adrenal vein. Interestingly, this cutoff value is very close to the cutoff value of 2.7, which was determined in a multicentric study focusing on the reproducibility of subtype diagnosis in patients with PA, who had two AVS, using different cutoffs (22). The SI of FMN of 9.9 for the right adrenal vein and 10.0 for the left adrenal vein is only marginally lower than the SI proposed by Dekkers et al., who calculated an SI of 12 (11). Their methodology differed from ours since they used a cosyntropin-stimulated adrenal vein/peripheral vein ratio of FMN to assess the selectivity AVS, with a cortisol-derived SI of 3.0 as a reference index. The definite proof of the superiority of FMN or FTMR-derived SI would be to study the biochemical and clinical outcomes in patients with discordant selectivity results with cortisol and these two other markers.

The FTMR is an interesting marker of selectivity since we show that almost all the metanephrines (>96%) in the adrenal gland are composed of FMN. This ratio progressively decreases along the venous drainage with secondary dilution by peripheral venous circulation and supply of sulfated metanephrine (**Figure 3**) (23). We show for the first time that an FTMR of >0.25 in the right adrenal vein and a ratio >0.31 in the left adrenal vein are sensitive and specific indicators of selectivity.

Historically, cortisol with or without cosyntropin stimulation has been used as the reference analyte for the SI. Cortisol levels are however dependent on stress during the procedure in unstimulated conditions. Secondly, co-secretion of cortisol in PA may influence the SI and the lateralization (21). Subclinical hypercortisolism is not rare, and a prevalence rate of 28% has been reported in the Asian population especially (24–26). Co-secretion may induce negative feedback on the contralateral adrenal gland and therefore generate a false negative SI. It may also decrease the lateralization index on the affected side and

TABLE 3 | Range and cutoff values for selectivity indices for cortisol, free metanephrine, and the free-to-total metanephrine ratio.

	Range	Cutoff value	Sensitivity%	95% CI	Specificity%	95% CI
Cortisol left	0.635–83.7	>2.63	79.0	66.8% to 88.3%	100	94.5% to 100.0%
Cortisol right	0.502–241	>2.50	57.8	44.8% to 70.1%	100	94.5% to 100.0%
Free MN left	0.75–579	>10.00	100.0	94.5% to 100.0%	100	94.5% to 100.0%
Free MN right	0.727–1,014	>9.92	95.4	87.1% to 99.0%	100	94.5% to 100.0%
Free/total MN ratio left	0.167–0.937	>0.31	100.0	94.5% to 100.0%	100	94.5% to 100.0%
Free/total MN ratio right	0.233–0.984	>0.25	93.9	85.0% to 98.30%	100	94.5% to 100.0%

The cortisol-derived SI was calculated as the concentration of cortisol in adrenal vein samples divided by that in distal vena cava samples. The free metanephrine-derived SI was calculated as the concentration of free metanephrine in adrenal vein samples divided by that in distal vena cava samples. The FTMR-derived SI was calculated as the concentration of free metanephrine in adrenal vein samples divided by that of total metanephrine in ipsilateral adrenal vein samples. The SI cutoffs of each index marker were established using receiver operating characteristic curve analysis.

MN, metanephrine; AV, adrenal vein; SI, selectivity index.

TABLE 4 | Agreement between the markers of selectivity in the right, left, and both AVS.

		Free MN		FTMR		Side
		Failure	Success	Failure	Success	
Cortisol	Failure	26	15	25	16	Both
	Success	4	15	3	16	
FTMR	Failure	28	0			
	Success	2	30			
Cortisol	Failure	22	17	23	16	Right
	Success	3	18	4	17	
FTMR	Failure	25	0			
	Success	2	33			
Cortisol	Failure	4	12	4	12	Left
	Success	1	43	1	43	
FTMR	Failure	5	0			
	Success	0	55			

FTMR, free-to-total metanephrine ratio; MN, metanephrine; AVS, adrenal vein sampling.

result in a false-negative test of lateralization (21). In our study, we confirm that FMN has a better sensitivity than cortisol in determining correct catheter tip location in the adrenal veins in non-stimulated conditions and show that the FTMR performs similarly to FMN with a very good agreement between the two methods. This improved sensitivity, compared to cortisol, allowed the reclassification of bilateral success in 36% (FMN) and 39% (FTMR) of cases classified as bilaterally unsuccessful with cortisol.

The FTMR as the SI needs only two sites of sampling (both adrenals veins) instead of three (both adrenal veins and a peripheral site). It may reduce the duration of the procedure. However, additional laboratory work needs to be performed, and expenses increase as both free and total metanephrines have to be measured. Steroids other than cortisol, such as 17-alpha-hydroxyprogesterone and particularly androstenedione, have been proposed as SI markers, as they have a higher step-up between the adrenal vein and the inferior vena cava than cortisol (12, 27). Indeed, Ceolotto et al. have recently compared the SI of cortisol, FMN, and androstenedione using a bilateral simultaneous unstimulated technique. They have also shown the superiority of FMN and androstenedione over cortisol, allowing the use of AVS data despite an AVS incorrectly labeled as unsuccessful with cortisol. In their comparison, they have, however, used a single arbitrarily but recognized SI cutoff of ≥2 for all analytes. Local expertise in steroids or metanephrine assays may guide the choice of the selectivity marker.

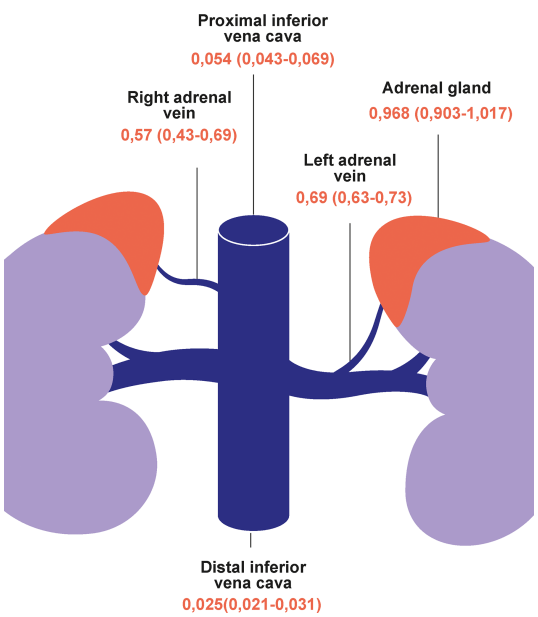

FIGURE 3 | The free-to-total metanephrine ratio from the adrenal glands to the vena cava. Data are median and interquartile ranges.

Of note, the ratios of analytes between the adrenal veins and the peripheral veins (distal vena cava) were higher on the left side (**Table 2**), which probably reflects easier and more distal cannulation of the left side. This phenomenon has been observed in some but not all studies (11, 12, 27). In addition, total metanephrine was higher in the distal vena cava than in the proximal vena. This inversion of proportion may be explained by the fact that total metanephrine is more diluted in the suprarenal vena cava.

The monocentric location of the AVS procedure may limit the external validation of the study. Patients were however included in two different tertiary referral hypertension centers, and their clinical characteristics were similar to other studies including patients referred for AVS (11, 27). In addition, the results concerning the SI of FMN are in good agreement with the study of Dekkers et al. (11). A possible limitation of the study was that a confirmatory test such as a saline load was not performed systematically in all patients as recommended by some guidelines (7). The guidelines, however, recognize that the evidence for a confirmatory test is low (7). In addition, this confirmatory test would not have influenced the results since the objective of the study was to determine the cutoff values of cortisol, FMN, and the FTMR. Another limitation of this study is that cosyntropin was not used in this study, limiting the comparison to unstimulated AVS only. The use of cosyntropin during the

AVS procedure adds, however, a level of complexity and time to a procedure already known to be challenging (8).

In summary, the FTMR is an SI with high sensitivity and specificity with very good agreement compared to FMN. Both have a better sensitivity than cortisol in unstimulated AVS. In addition, precise cutoffs for all markers were established using a dedicated procedure, compared to the arbitrary cutoffs that have been used so far for cortisol.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CER-VD. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FC captured the data and wrote and reviewed the manuscript (co-first author). EP made the statistical analysis and reviewed the manuscript (co-first author). AD performed the AVS and reviewed the manuscript. KA analyzed the adrenal content. TZ and MauM sampled the adrenal gland from deceased kidney donor and reviewed the manuscript. AP-B selected the patients and reviewed the manuscript. MarM performed the aldosterone and PRA analyses and reviewed the manuscript. EG performed the metanephrine analysis and reviewed the manuscript. GW designed the protocol, selected the patients, supervised the statistical analysis, and wrote the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.842968/full#supplementary-material

Supplementary Figure 1 | (A) Axial computed tomography angiography of the right adrenal gland. **(B)** Coronal computed tomography angiography of the right adrenal gland.

Supplementary Figure 2 | Comparison of selectivity indices (SI) in **(A)** The left adrenal vein. **(B)** The right adrenal vein. **(C)** Both adrenal veins. Total metanephrine (TMN); free metanephrine (FMN). * indicates $p<0.05$.

Supplementary Figure 3 | Receiver operation characteristic curves analysis exploring the diagnostic performance of cortisol, free metanephrine and the free to total metanephrine ratio. AV, adrenal vein; VC, vena cava; MN, metanephrine.

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ORIGINAL RESEARCH
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Re-Assessment of the Oral Salt Loading Test Using a New Chemiluminescent Enzyme Immunoassay Based on a Two-Step Sandwich Method to Measure 24-Hour Urine Aldosterone Excretion

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Since April 2021, the plasma aldosterone concentration has been measured by chemiluminescent enzyme immunoassay (CLEIA) in Japan. In the present study, we developed a new CLEIA using a two-step sandwich method to measure the 24-hour urine aldosterone level. We collected 115 urine samples and measured 24-hour urine aldosterone levels employing radioimmunoassay (RIA), CLEIA, and liquid chromatography–tandem mass spectrometry (LC-MS/MS). The results showed that the 24-hour urine aldosterone levels measured using CLEIA and LC-MS/MS were significantly correlated ($\rho = 0.992$, $P < 0.0001$). Based on the results of Passing–Bablok regression analysis, the slope was 0.992 and the intercept –19.3. The 24-hour urine aldosterone levels measured using CLEIA and RIA were also significantly correlated ($\rho = 0.905$, $P < 0.0001$). However, the aldosterone level measured by CLEIA was lower than that measured by RIA (slope, 0.729; intercept, 120.9). In Japan, a new guideline for primary aldosteronism has been announced, with changes in the aldosterone measurement method. The cutoff values for oral sodium loading test (OSLT) were changed, but clinical verification using real-world urine samples has not been performed. Therefore, we examined the cut-off value of the 24-hour urine aldosterone level after the OSLT. Receiver operating characteristic analysis revealed a cut-off value for primary aldosteronism of 3 μg/day.

Keywords: primary aldosteronism, 24-hour urine aldosterone level, CLEIA, two-step sandwich method, oral sodium loading test

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INTRODUCTION

Primary aldosteronism (PA) is widely recognized as the most frequent cause of secondary hypertension, and its prevalence ranges from 5% to 10% in hypertensive patients (1–3). PA is caused by either unilateral hyperaldosteronism (UHA) due to an aldosterone-producing adenoma or bilateral adrenal hyperplasia (4, 5). Patients with PA have an increased risk of cardiovascular events, such as cerebral infarction, myocardial infarction, and chronic kidney disease compared to those with essential hypertension, and early diagnosis makes it a treatable disease (6, 7).

PA is diagnosed by a screening test, followed by a confirmatory or exclusionary test and a subtype test using computed tomography and adrenal vein sampling (AVS). The aldosterone to renin ratio (ARR) is used to screen for PA (8, 9). The number of patients diagnosed with PA has increased due to more frequent screening (10, 11). In Japan, a plasma aldosterone concentration (PAC) > 120 pg/mL, and PAC (pg/mL) to plasma renin activity (ng/mL/h) ratio >200 or a PAC (pg/mL) to active renin concentration (ARC, pg/mL) ratio>40, have been the screening cut-off values for PA (12).

We performed an oral sodium loading test (OSLT), captopril challenge test (CCT), saline infusion test (SIT), and furosemide upright posture test to screen for PA (2–4, 8, 9). When at least one confirmatory test was positive, we diagnosed the patient with PA. When a patient wants to undergo curative adrenal surgery, it is crucial to conduct subtype testing using AVS (2, 3). Measuring of PAC or the urinary aldosterone concentration is important in screening tests, confirmatory tests and AVS. Therefore, an accurate aldosterone measurement method is required for clinical practice.

The PAC and 24-hour urine aldosterone level are conventionally measured by radioimmunoassay (RIA) (13, 14). However, PAC assay kits using the RIA method have been unavailable since March 2021 in Japan. A chemiluminescent enzyme immunoassay (CLEIA) makes it possible to measure aldosterone concentrations more rapidly (12). A relatively strong correlation is observed in aldosterone concentrations between RIA and CLEIA. Some discrepancies in aldosterone concentrations have been reported between the current CLEIA method and liquid chromatography-tandem mass spectrometry (LC-MS/MS), which is the gold standard measurement method according to The Japan Endocrine Society (15). As a result, new CLEIA measurement kits using the sandwich method were developed (16–18). We reported that the PAC measured by the CLEIA using a two-step sandwich method was significantly correlated with the levels measured by LC-MS/MS (17). Therefore, PAC has been measured by the new CLEIA method since April 2021 in Japan.

A screening cut-off value for PA and specific criteria for the confirmatory test may be required to adjust the assay, because the aldosterone levels were lower with the CLEIA method and LC-MS/MS than the RIA method. As a provisional response, the "Japan Endocrine Society Clinical Practice Guideline for the Diagnosis and Management of Primary Aldosteronism 2021" was announced (19). In this guideline, the cut-off values for the PAC and 24-hour urine aldosterone levels using the screening test and confirmatory test changed.

We developed a CLEIA kit using the two-step sandwich method to measure 24-hour urine aldosterone. In this study, we measured 24-hour urine aldosterone levels in patients using RIA, the new CLEIA kit, and LC-MS/MS. The results showed that the 24-hour urine aldosterone levels measured with the new CLEIA kit were highly correlated with those of LC-MS/MS. Similar to PAC, 24-hour urine aldosterone levels measured with the new CLEIA kit were lower than those obtained by RIA. Furthermore, we examined the cut-off value of the 24-hour urine aldosterone level after the OSLT.

MATERIALS AND METHODS

Patients and Study Design

We collected 115 urine samples (60 males and 55 females) from patients hospitalized at Oita University Hospital between October 2018 and July 2020. We diagnosed 48 patients with PA based on a positive screening test and at least one positive confirmatory test for PA (PA group) according to the Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2019) (20). Sixty patients were not diagnosed with PA based on negative screening results for PA (non-PA group). Seven patients did not belong to either group, because they were positive on the screening test, and the confirmatory test had not been performed. The screening test was considered positive when the PAC/ARC ratio was > 40 and the PAC was > 120 pg/mL. The SIT involved intravenous infusion of 2 L of saline over 4 hours, with the patient in a supine position. The test was considered positive when the PAC was > 60 pg/mL after saline loading. The CCT was performed by administering 50 mg captopril, and blood was drawn after 90 min. When the PAC/ARC ratio was > 40 at 90 min after administration, the test was considered positive. The OSLT results were determined by analyzing 24-hour urine samples after the patients had consumed a high salt diet. The test was considered positive when the 24-hour urinary aldosterone and Na excretion levels were > 8 μg/day and > 170 mEq/day, respectively. Urine Na < 170 mEq/day reflected insufficient NaCl loading; those cases were excluded from the analysis. In the present study, 77 of 81 patients with hypertension were taking antihypertensive medications (calcium channel blockers, 65 patients; angiotensin II receptor blockers, 23 patients; a mineralocorticoid receptor antagonist, 4 patients; or others, 14 patients).

All urine samples were collected into a plastic container; the last urine sample was collected after 24 hours. We measured 24-hour urine aldosterone levels in those samples using RIA, CLEIA, and LC-MS/MS.

The study protocol followed the Declaration of Helsinki and was approved by the Ethical Committee of Oita University. All subjects gave informed consent to participate in the study.

Chemiluminescent Enzyme Immunoassay

We used the Accuraseed Aldosterone S kit (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) in this study. The analyzer was used "Accuraseed", an IVD-approved fully automated immunoassay platform in Japan. This analyzer can measure both PAC and ARC in 10 minutes. This assay uses a two-step sandwich method based on the CLEIA, and an antibody that recognizes an immune complex of aldosterone and an anti-aldosterone antibody. We have previously reported that this assay shows a good correlation with LC-MS/MS in serum and plasma samples (17). Detailed verification data for the new reagent appear in the **Supplementary Material**. Urine samples must be pretreated; in this study, they were mixed with 0.2 N hydrochloric acid in a 1:2 ratio and reacted at 25°C for 24 hours to deconjugate glucuronic acid. Subsequently, the hydrochloric acid-treated urine was mixed nine volumes of buffer solution to neutralize it. The urine sample was analyzed in the same way as serum and plasma samples. The aldosterone concentration was determined by multiplying the measured value by 30. The detection range was 3–3,200 pg/mL.

Radioimmunoassay

We used the SPAC-S Aldosterone kit (Fujirebio Co., Ltd., Tokyo, Japan) in this study. This assay is based on the competitive method. The aldosterone in the sample competes with iodine 125 (tracer)-labeled aldosterone for the antibody coated on the tubes. After aspiration, the level of radioactivity in the tubes is measured with a gamma counter. The degree of binding is inversely proportional to the aldosterone concentration in the sample. Similar to the CLEIA method, urine samples must be pretreated. In particular, the primitive urine sample was mixed with 0.2 N hydrochloric acid at a 1:2 volume ratio and reacted at room temperature for 16–24 hours to deconjugate glucuronic acid. Subsequently, the hydrochloric acid-treated urine was mixed with nine volumes of standard solution to neutralize the urine samples. The urine sample was analyzed in the same way as serum and plasma samples. The aldosterone concentration was calculated by multiplying the measured value by 30.

LC-MS/MS

As a control, we compared the new assay results with those of LC-MS/MS. We outsourced the LC-MS/MS measurements to ASKA Pharmaceutical Co., Ltd. (Tokyo, Japan).

Statistical Analysis

The data are presented as mean ± standard deviation and were analyzed using JMP16 software (SAS Institute, Cary, NC, USA). A P-value < 0.05 was considered significant. The correlations were analyzed by Passing–Bablok regression and the Bland–Altman analysis using XLSTAT statistical software (Addinsoft, New York, USA).

RESULTS

Baseline Clinical Characteristics of the Patients

The clinical characteristics of the subjects are shown in **Table 1**. Systolic and diastolic blood pressure were significantly higher in the PA than non-PA patients. The PAC and the ARR were significantly higher in PA than non-PA patients, and the ARC was significantly lower.

Twenty-six patients in the PA group underwent subtype testing using AVS. According to The Japan Endocrine Society, the AVS cut-off values after ACTH stimulation are a lateralized ratio (LR) > 4 and contralateral ratio (CR) < 1 (21). The LR is the ratio of aldosterone to cortisol in the dominant adrenal vein by that in the nondominant adrenal vein. The CR is the ratio of

TABLE 1 | Baseline Clinical Characteristics.

	PA (n=48)	Non-PA (n=60)	p
Age (years)	53.8 ± 11.7	54.5 ± 17.1	N.S
Male/Female	22/26	34/26	
BMI (kg/m^2)	25.5 ± 4.5	27.3 ± 7.1	N.S
Systolic blood pressure (mmHg)	129.8 ± 17.7	119.0 ± 74.3	<0.01
Diastolic blood pressure (mmHg)	80.6 ± 10.3	74.3 ± 12.7	<0.01
HR (bpm)	67.1 ± 10.6	79.4 ± 12.8	N.S
BUN (mg/dL)	13.6 ± 3.8	14.4 ± 4.9	N.S
Cr (mg/dL)	0.8 ± 0.3	0.8 ± 0.3	N.S
K (mmol/L)	3.7 ± 0.5	4.6 ± 0.4	<0.01
eGFR (mL/min/1.73m^2)	74.5 ± 18.5	70.9 ± 24.0	N.S
Plasma aldosterone concentration (pg/mL)	250.7 ± 141.8	135.3 ± 60.9	<0.01
Active renin concentration (pg/mL)	2.9 ± 1.8	21.3 ± 35.9	<0.01
ARR	115.7 ± 98.4	17.8 ± 16.5	<0.01
Hypertensive medication			
Calcium channel blocker	42/48 (88%)	23/60 (38%)	
Angiotensin II Receptor Blocker	4/48 (8%)	19/60 (32%)	
Mineralocorticoid receptor antagonist	2/48 (4%)	2/60 (3%)	
Others	4/48 (8%)	10/60 (17%)	

Date were shown as Average (mean ± SD).
BMI, Body mass index; PA, Primary aldosteronism.
ARR, The ratio of aldosterone to renin concentrations; N.S, not significant.

aldosterone to cortisol in the nondominant adrenal vein by that in the inferior vena cava. We diagnosed 16 patients with bilateral hyperaldosteronism (BHA) and 10 with UHA.

Correlation of 24-Hour Urine Aldosterone Levels Between the CLEIA and LC-MS/MS

This correlation analysis was performed using 115 urine samples. Aldosterone concentrations measured by the CLEIA were significantly correlated with those by LC-MS/MS ($\rho = 0.994$, $P < 0.001$).

The results of Passing–Bablok regression analysis of the aldosterone concentrations obtained with the CLEIA and LC-MS/MS are shown in **Figure 1A**. The slope was 0.993 and the intercept −20.0, and 95% confidence intervals were also calculated. Bland–Altman analysis showed that the mean difference in aldosterone concentration between the two assays was −33.2 pg/mL, with a 95% confidence interval of −69.0 to 2.6 pg/mL (**Figure 1C**).

To include concentrations of up to 5,000 pg/mL, the analysis was performed using 102 urine samples. Aldosterone concentrations measured by the CLEIA were significantly correlated with those measured by LC-MS/MS ($\rho = 0.992$, $P < 0.0001$). The result of Passing–Bablok regression analysis of the aldosterone concentrations obtained with the CLEIA and LC-MS/MS are shown in **Figure 1B**. The slope was 0.992 and the intercept −19.3; 95% confidence intervals were also calculated. Bland–Altman analysis showed that the mean difference in the aldosterone concentration between the two assays was −28.9 pg/mL, with a 95% confidence interval of −62.4 to 4.6 pg/mL (**Figure 1D**).

Correlation of 24-Hour Urine Aldosterone Levels Between the CLEIA and RIA

This correlation analysis was performed using 88 urine samples. The 24-hour urine aldosterone levels measured by CLEIA were significantly correlated with those determined by RIA ($\rho = 0.956$, $P < 0.0001$). The aldosterone levels measured by the CLEIA were lower than those measured by RIA.

The results of Passing–Bablok regression analysis between the 24-hour urine aldosterone levels determined by the CLEIA and RIA are shown in **Figure 2A**. The slope was 0.751 and the intercept 37.1; 95% confidence intervals were also calculated. Bland–Altman analysis showed that the mean difference in aldosterone concentration between the two assays was −905.2 pg/mL, with a 95% confidence interval of −1,074.7 to −735.6 pg/mL (**Figure 2C**).

At concentrations up to 5,000 pg/mL, the analysis was performed using 67 urine samples. Aldosterone concentrations measured by the CLEIA were significantly correlated with those measured by RIA ($\rho = 0.905$, $P < 0.0001$). The results of Passing–Bablok regression analysis of 24-hour urine aldosterone levels determined by the CLEIA and RIA are shown in **Figure 2B**. The slope was 0.729 and the intercept 120.9; 95% confidence intervals were also calculated. Bland–Altman analysis showed that the

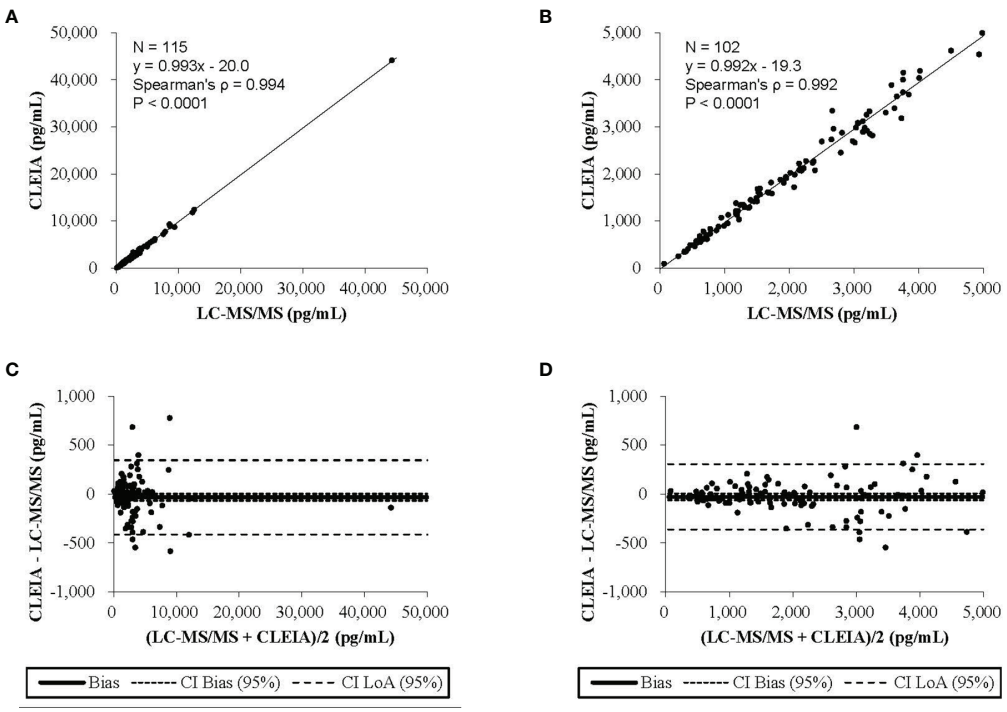

FIGURE 1 | The results of the Passing-Bablok regression analysis and the Bland-Altman analysis **(A)** The CLEIA versus the liquid chromatography-tandem mass spectrometry(LC-MS/MS). **(B)** The CLEIA versus the LC-MS/MS in a concentration range of up to 5000 pg/ml. **(C)** The CLEIA versus the LS-MS/MS. **(D)** The CLEIA versus the LS-MS/MS in a concentration range of up to 5000 pg/ml.

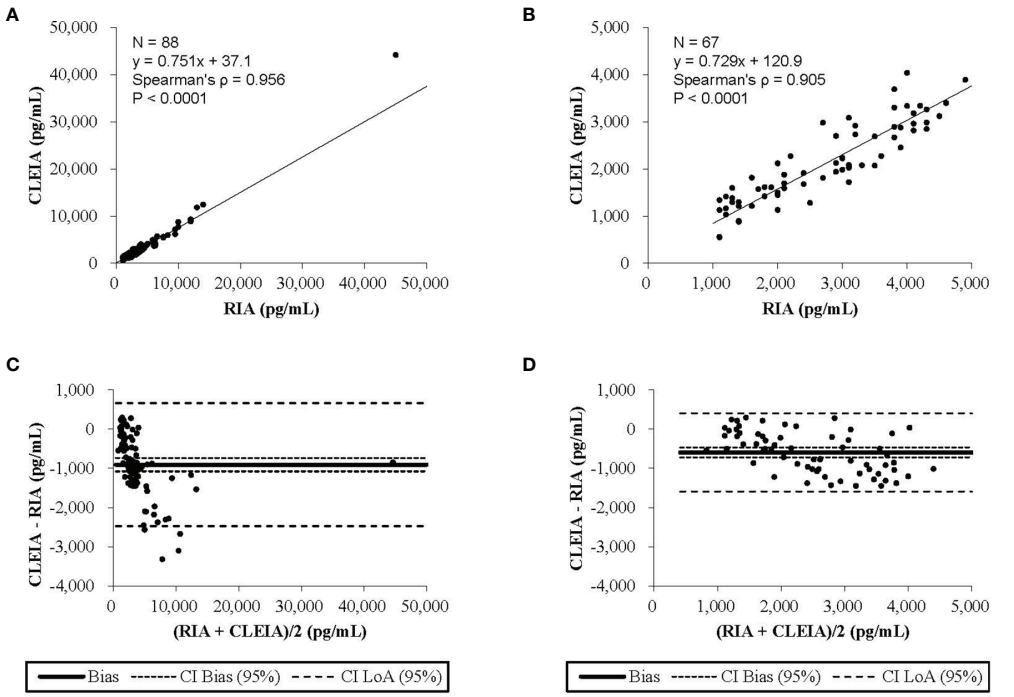

FIGURE 2 | The results of the Passing-Bablok regression analysis and the Blond-Altman analysis. **(A)** The chemiluminescent enzyme immunoassay (CLEIA) versus the radioimmunoassay (RIA). **(B)** The CLEIA versus the RIA in a concentration range of up to 500 pg/ml. **(C)** The CLEIA versus the RIA. **(D)** The CLEIA versus the RIA in a concentration range of up to 5000 pg/ml.

mean difference in the aldosterone concentration between the two assays was −591.8 pg/mL, with a 95% confidence interval of −716.3 to −467.3 pg/mL (**Figure 2D**).

Comparison of the 24-Hour Urine Aldosterone Levels After the OSLT Between PA and Non-PA Groups Using the CLEIA

This analysis was performed using 51 urine samples (8 PA and 43 non-PA samples, respectively) from patients who had Na excretion levels > 170 mEq/day. Receiver operating characteristic (ROC) curve analysis revealed a cut-off value for the PA diagnosis of 3.1 μg/day (sensitivity, 63%; specificity, 82%, **Figure 3**).

DISCUSSION

We recently developed a new CLEIA using a two-step sandwich method to measure plasma and serum aldosterone concentrations. PAC has been measured by CLEIA using the sandwich method since April 2021 in Japan.

As was true of based on the changes of the measuring method, the cutoff value for the SIT has been changed to 12 pg/mL from 60 pg/mL (17, 19). But, the recent study showed that the cutoff value for the SIT in the seated position was PAC > 61.6 pg/mL, which was measured by high-performance liquid chromatography mass spectrometry (22). The reason why our assay indicates much lower cut-off value than the previous report might be due to the different assay methods. Our new CLEIA uses double sandwich

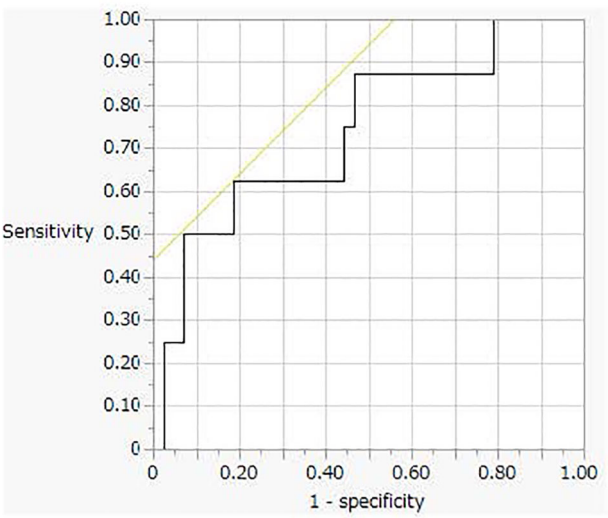

FIGURE 3 | The receiver operating characteristic (ROC) curve analysis for oral sodium loading test cut off value.

antibodies, whereas the former report uses single antibody for CLEIA.

In the present study, we verified the utility of a new CLEIA method that measures urine aldosterone excretion. Several studies have shown that the PAC and 24 hour urine aldosterone concentration determined by the CLEIA using the sandwich method was highly correlated with the LC-MS/MS value but lower than the RIA value (16, 17). This study demonstrated that 24-hour urine aldosterone levels measured by CLEIA using a two-step sandwich method can be used in clinical practice, but the cut-off 24 hour urine aldosterone level of the OSLT must be adjusted for the new method.

We collected 115 urine samples (PA group, 48 patients; non-PA group, 60 patients; other group, 7 patients). We confirmed that both the PAC and ARR levels were significantly higher in PA than non-PA patients, and that the ARC was significantly lower.

PA management should proceed step-wise from screening to confirmatory testing, subtype testing, and finally treatment. The OSLT, a confirmatory test for PA, can be performed by measuring the 24-hour urine aldosterone level and NaCl excretion. According to the Japanese Society of Hypertension Clinical Practice Guideline 2019, PA is present when the 24-hour urine aldosterone and Na excretion levels exceed 8 μg and 170 mEq, respectively. In the new Japanese guidelines for PA, the cut-off level of 24-hour urine aldosterone excretion was revised to > 6 μg/day from > 8 μg/day based on the changes in the aldosterone measurement method. However, this cut-off value is tentative; it has not been validated using clinical samples. We therefore investigated 24-hour urine samples from both PA and non-PA patients. In this study, only 20 of the 43 urine samples collected after the OSLT tested positive (PAC ≥ 6 μg/day) by the CLEIA method. The ROC analysis revealed a cut-off value of 3 μg/day with 63% sensitivity and 82% specificity, which was lower than that of the new guidelines.

Second, OSLT is recommended as a confirmatory test for PA according to the clinical practice guidelines of both the Endocrine Society and the Japanese Society of Hypertension (20, 23). The OSLT has both advantages and disadvantages. As the OSLT measures 24-hour urine aldosterone excretion, it reflects that severity of PA. Moreover, we found that a positive OSLT result was significantly associated with both higher 24-hour blood pressure and albuminuria (24). We therefore consider that the OSLT not only serves as a PA confirmatory test but also reflects the organ damage caused by PA. However, the OSLT is cumbersome, and 24-hour urine collection is required. Also, when the 24-hour urine Na excretion level is < 170 mEq, the OSLT result is cannot be evaluated because of a lack of salt loading. It is crucial to educate patients to consume more salt than usual prior to undergoing the OSLT.

Third, UHA, an important pathology of PA, is attributable principally to aldosterone-producing adenomas. Patients with unilateral aldosterone-producing adenomas often present with resistant hypertension, a markedly high PAC, and spontaneous hypokalemia; patients with BHA usually present with mild hypertension, a normal serum potassium level, and a normal-to-high PAC. As UHA is curable *via* unilateral adrenalectomy, it is essential to effectively distinguish UHA from BHA. After diagnosing PA with a confirmatory test, AVS is the gold standard for diagnosing the PA subtype (25–27). We diagnosed 16 patients with BHA, and 10 with UHA employing AVS. The clinical characteristics of the BHA and UHA patients are listed in **Table 2**.

Several studies have shown that both confirmatory testing as well as AVS can be avoided in full-blown UHA patients with a markedly high PAC, a reduced plasma renin level, spontaneous hypokalemia, and a unilateral hypodense adrenal tumor (28–30). The 24-hour urinary aldosterone level reflected the total daily level of aldosterone secretion and is useful when evaluating PA pathology (31). In this study, ROC analysis revealed a cut-off value after the OSLT for both UHA and BHA of 9.4 μg/day, with 70% sensitivity and 81% specificity. The calculated positive and negative predictive values for diagnosing UHA were 64% and 80%, respectively. Therefore, the 24-hour urine aldosterone assay may be one of the method of UHA diagnosis.

TABLE 2 | Baseline Clinical Characteristics of the patients with bilateral hyperaldosteronism and unilateral hyperaldosteronism.

	BHA (n=16)	UHA (n=10)	p
Age (years)	49.6 ± 9.7	53.3 ± 13.3	N.S
Male/Female	9/7	5/5	
BMI (kg/m^2)	25.6 ± 4.3	24.0 ± 3.4	N.S
Systolic blood pressure (mmHg)	130.1 ± 14.5	133.3 ± 18.5	N.S
Diastolic blood pressure (mmHg)	82.8 ± 7.5	79.7 ± 11.3	N.S
HR (bpm)	69.5 ± 10.4	66.5 ± 8.9	N.S
BUN (mg/dL)	13.7 ± 2.8	12.6 ± 3.6	N.S
Cr (mg/dL)	0.8 ± 0.2	0.7 ± 0.3	N.S
K (mmol/L)	3.9 ± 0.5	3.3 ± 0.5	<0.01
eGFR (mL/min/1.73m^2)	73.3 ± 14.0	82.2 ± 21.8	N.S
Plasma aldosterone concentration (pg/mL)	229.0 ± 73.5	401.8 ± 184.5	<0.01
Active renin concentration (pg/mL)	2.7 ± 2.0	2.1 ± 1.9	N.S
ARR	120.6 ± 71.3	410.9 ± 420.7	N.S
24-hour urine aldosterone levels (μg/day)	7.2 ± 4.2	13.8 ± 10.9	N.S

Date were shown as Average (mean ± SD).
BMI, Body mass index; ARR, The ratio of aldosterone to renin concentrations.
BHA, bilateral hyperaldosteronism; UHA, unilateral hyperaldosteronism.
N.S, not significant.

In conclusion, we re-assessed the OSLT cut-off values in patients with PA using a new CLEIA method to measure urine aldosterone excretion. The cut-off level of 24-hour urine aldosterone excretion for a positive OSLT is 3 μg when the 24-hour urine Na level exceeds 170 mEq.

The new CLEIA aldosterone assay can handle serum, plasma and urine samples, and it is automated, reproducible, and rapid in terms of PA diagnosis.

Limitations

The present study had several limitations. First, the number of cases was small, particularly the number of patients in the PA group with Na excretion levels > 170 mEq/day. Second, many patients in the non-PA group were on drugs that affected aldosterone levels. These limitations should be addressed in a future study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Oita University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Methodology, YO, TM, and HS. Formal Analysis, YO and TM. Investigation, YO, MK, SM, and YY. Data curation YO, MK, and TM. Writing—original draft preparation, YO, MO, and KG. Visualization, YO and KK. Review and Editing, YO and HS. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.859347/full#supplementary-material

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The Role of Urinary Extracellular Vesicles Sodium Chloride Cotransporter in Subtyping Primary Aldosteronism

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Background: Adrenal venous sampling (AVS) is recognized as the gold standard for subtyping primary aldosteronism (PA), but its invasive nature and technical challenges limit its availability. A recent study reported that sodium chloride cotransporter (NCC) in urinary extracellular vesicles (uEVs) is a promising marker for assessing the biological activity of aldosterone and can be treated as a potential biomarker of PA. The current study was conducted to verify the hypothesis that the expression of NCC and its phosphorylated form (pNCC) in uEVs are different in various subtypes and genotypes of PA and can be used to select AVS candidates.

Methods: A total of 50 patients with PA were enrolled in the study. Urinary extracellular vesicles (uEVs) were isolated from spot urine samples using ultracentrifugation. NCC and pNCC expressions were tested in patients diagnosed with PA who underwent AVS. Sanger sequencing of *KCNJ5* was performed on DNA extracted from adrenal adenoma.

Results: pNCC (1.89 folds, $P<.0001$) and NCC (1.82 folds, $P=0.0002$) was more abundant in the uEVs in the high lateralization index (h-LI, ≥ 4) group than in the low LI (l-LI, < 4) group. Carriers of the somatic *KCNJ5* mutations, compared with non-carriers, had more abundant pNCC expression (2.16 folds, P=0.0039). Positive correlation between pNCC abundance and plasma aldosterone level was found in this study ($R = 0.1220$, $P = 0.0129$).

Conclusions: The expression of pNCC in uEVs in patients with PA with various subtypes and genotypes was different. It can be used as biomarker of AVS for PA subtyping.

Keywords: primary aldosteronism, adrenal venous sampling, extracellular vesicles, NCC, *KCNJ5*

Abbreviations: PA, primary aldosteronism; uEVs, urinary extracellular vesicles; EVs, extracellular vesicles; AVS, adrenal venous sampling; NCC, sodium chloride cotransporter; pre-SSIT, pre-supine saline infusion test; pNCC, phosphorylated sodium chloride cotransporter; h-LI, high lateralization index; l-LI, low lateralization index; PAC, plasma aldosterone concentration.

INTRODUCTION

Primary aldosteronism (PA) is the most common form of secondary hypertension, characterized by aldosterone overproduction and suppression of plasma renin activity (1, 2). Patients with unilateral aldosteronism confirmed by adrenal venous sampling (AVS) can be treated with adrenalectomy. Those with bilateral hyperaldosteronism are treated mainly with mineralocorticoid receptor antagonists. More than 30% of operated patients have clinical success, and 94% have biochemical success according to the PASO study (3). Potassium inwardly-rectifying channel subfamily J member 5 (*KCNJ5*) was the first identified gene mutated in aldosterone-producing adenoma (APA) (4). Recent studies have suggested that *KCNJ5* mutation is a protective factor for complete clinical success (5–7). To date, AVS is generally regarded as the gold standard test for distinguishing between unilateral and bilateral aldosteronism; the lateralization rate has been reported as 50-60%, but it is not widely available due to technical difficulties, cost, and the invasive nature of operation (8–10). Therefore, it is necessary to find alternative criteria or biomarkers that can predict lateralization and accordingly decrease the number of AVS candidates.

The sodium chloride cotransporter (NCC), expressed in the proximal part of the convoluted tubule, is involved in blood pressure regulation by mediating the reabsorption of filtered sodium and water (11). Its function is highly regulated by aldosterone *via* phosphorylation (12). To explore the expression of NCC and phosphorylated NCC (pNCC) *in vivo* in human renal tubules, urinary extracellular vesicles (uEVs) are an ideal non-invasive diagnostic tool compared to renal biopsy. Urinary extracellular vesicles (EVs) are lipid bilayer vesicles, which are released into the urine by cells from all nephron segments *via* exocytosis (13). They are enriched in a variety of proteins, nucleic acids, and lipids. These biomolecules reflect the pathophysiological status of parental cells and can be used as disease biomarkers (14, 15). Recently, Van der Lubbe et al. reported that pNCC in uEVs are promising markers for assessing the biological activity of aldosterone and potential clinical biomarkers for PA (16). Nevertheless, the relationship between NCC or pNCC expression and subtypes of PA has not been clarified.

In light of the above information, the present study was conducted to explore the hypothesis that NCC and pNCC expression in uEVs are different in different PA subtypes or genotypes, and uEVs could be a non-invasive predictive tool to decrease the number of AVS candidates.

METHODS

Patients

We recruited hospitalized patients who were diagnosed with PA between September 2020 and September 2021 in the Hypertension Department (Ruijin Hospital, Shanghai Jiao Tong University School of Medicine). The workup for PA complied with the 2016 Endocrine Society clinical guidelines for the diagnosis and management of PA, as reported in our previous study (10). In brief, before workup, patients were advised to withdraw mineralocorticoid receptor antagonists for at least 6 weeks, non-potassium sparing diuretics for 4 weeks, and β-blockers, angiotensin-converting enzyme inhibitors, and angiotensin II type 1 receptor blockers (ARBs) for 2 weeks. Non-dihydropyridine Ca^{2+} blockers and/or α_1-blockers were prescribed for blood pressure (BP) control as necessary. Patients with an aldosterone-renin ratio (ARR) of > 30 (ng/dL)/(ng/mL/h) at least twice at the outpatient clinic were considered as PA candidates and were then admitted to the hospital for a confirmatory test. Patients with hypokalemia were corrected with an oral potassium chloride supplement to reach a serum potassium level of > 3.5 mmol/L. A supine saline infusion test was performed. Patients with post-test plasma aldosterone concentration (PAC) of > 10 ng/dL were diagnosed with PA. All patients underwent thin-sliced (3 mm) adrenal computed tomography (CT). AVS was performed in all patients who were willing to undergo unilateral adrenalectomy. A selectivity index (adrenal cortisol to peripheral cortisol) of ≥ 2.0 was considered correct catheterization and a lateralization index (LI, aldosterone/cortisol ratio from high to low side) of ≥ 4.0 without cosyntropin stimulation was regarded as lateralization. Patients with LI ≥ 4 and < 4 were classified into high LI (h-LI) and low LI (l-LI) groups, respectively. Patients with LI > 4 underwent unilateral adrenalectomy, as well as those with 3 ≤ LI < 4 and a contralateral suppression index of < 1. The remaining patients received medical treatment, mainly mineralocorticoid antagonists. APAs were confirmed using hematoxylin and eosin and *CYP11B2* staining.

Urinary EVs Study

Urine Collection and uEVs Isolation

Briefly, approximately 50 ml first-void morning urine sample was collected during the day of the saline infusion test. Urine sample (10 ml of urine sample was directly used for the measurement of creatinine content using a creatinine [urinary] Assay Kit, 500701-96, Cayman, USA), and the remaining sample was treated with 3 ml PMSF (10 mM) and 50 μl leupeptin (1 mg/ml) before freezing at -80°C (16, 17). Urinary EVs were isolated *via* ultracentrifugation, as previously described by Salih et al. (18). All centrifugations were performed in an ultracentrifuge (Optima XPN-100, Beckman Coulter, USA) with an angle rotor (Type 70 Ti Rotor, Beckman Coulter, USA) at 4°C. Briefly, frozen urine was quickly thawed at 32°C and vortexed for 90 s. The urine was first centrifuged at 17,000×g for 15 min to remove high-density particles. The supernatant obtained above was ultracentrifuged at 200,000×g for 90 min to obtain a gel-like precipitate and the new supernatant was discarded. This gel-like pellet was resuspended in 1× PBS buffer (Sangon Biotech, China), and then ultracentrifuged at 200,000 × g for 90 min to obtain a new pellet containing EVs. The pellet was resuspended in 160 μL 1× PBS buffer and froze at -80°C after aliquoting.

Nanoparticle Tracking Analysis

The particle size and concentration of uEVs were measured using nanoparticle tracking analysis (NTA) at VivaCell Biosciences with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany)

and the corresponding software ZetaView 8.04.02. Briefly, uEVs were appropriately diluted in 1× PBS buffer (Biological Industries, Israel) to measure particle size and concentration. After the sample cell was cleaned using 1× PBS buffer (Biological Industries, Israel), the ZetaView system was calibrated using 110 nm polystyrene particles.

Transmission Electron Microscopy

The exosomal suspension was dripped into the copper mesh (4406, TEDpella, USA) and left for more than one minute, and then the droplet was negatively stained with 2% phosphotungstic acid solution (G1871, Solarbio, China) and fixed for 10 min. Finally, the samples were analyzed using an electron microscope (Tecnai G2 Spirit BioTWIN, Tecnai, USA) operated at 120 kV.

Immunoblotting

The total protein content of uEVs was quantified using the BCA method. Urine creatinine was used for normalization of the samples, as previously described (14, 19). The protein samples were loaded based on the same amount of protein vs creatinine. Protein samples were separated using SDS-PAGE (4 to 12%, Biofuraw™ Precast Bis-Tris Gel), transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories), and blocked with 5% nonfat milk for 1 h at room temperature. Blots were incubated with primary antibodies against CD9 (1:1000, Abcam, ab92726), CD63 (1:1000, Abcam, ab134045), ALIX (1:1000, Cell Signaling Technology, 2171S), NCC (1:1000, StressMarq, SPC-402), and pNCC (1:1000, PhosphoSolutions, p1311-53) at 4°C overnight. Horseradish peroxidase-conjugated secondary antibodies were diluted in 5% BSA to detect the bound primary antibodies. Immunoreactive bands were detected using ECL reagent (Thermo Scientific, Waltham, MA), and densitometry measurements were performed using Image J (Version 1.53, NIH, USA). CD9 was used as a measure of EVs, and we compared the densitometry of protein vs CD9 between lanes.

DNA Isolation and Sanger Sequencing of KCNJ5

DNA was obtained from 35 mm tumor slices using Qiagen column separation according to the manufacturer's instructions (Qiagen, Hilden, Germany). *KCNJ5* sequences were amplified using a previously described primer (20). PCR conditions were as follows: 95°C for 5 min; 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 min in a 96-well GeneAmp PCR system 9700 (Applied Biosystems) using 20 ng of template DNA. All PCR amplimers were analyzed using 1.2% agarose gel electrophoresis. Sequencing reactions were performed using a BigDye Terminator Cycle Sequencing Kit (Thermo Fisher, Waltham, MA, USA) and analyzed on a 24-capillary 3500 DX DNA Analyzer (Applied Biosystems, USA). Exonic sequences were read and aligned using Chromas software (version 1.62, Technelysium).

Statistical Analysis

Statistical analyses were performed using SAS software (version 9.4; SAS Institute, Cary, NC, USA) and GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA). Results were expressed as the mean and standard deviation or the median, as appropriate. Data were logarithmically transformed before analysis in the case of a non-normal distribution. Student's t-test or analysis of variance was used for group comparison. Pearson correlation was used to analyze correlations between different biochemical indicators and urinary sodium transporters (NCC and pNCC). Statistical significance was set at $P<0.05$.

RESULTS

Clinical Characteristics in Patients With PA

A total of 50 patients were enrolled in this study (**Figure 1**), with 32 and 18 patients in h-LI and l-LI groups, respectively. As shown in **Table 1**, there were no significant differences in age, sex, body mass index (BMI), hypertension duration, number of antihypertensive medications, ambulatory BP, and serum potassium levels between the two groups. Compared with the l-LI group, the h-LI group had higher levels of 24 h urinary aldosterone (26.8 ± 11.3 μg/24 h vs 16.7 ± 6.9 μg/24 h, P = 0.0003) and supine PAC (308 (205–423) pg/mL vs. 185 (128-217) pg/mL, P = 0.0003) on admission. In addition, the pre-supine saline infusion test (pre-SSIT) supine PAC (after correction of hypokalemia) in the h-LI group was higher than that in the l-LI group (356 ± 150 pg/mL vs. 251 ± 104 pg/mL, P =0.0114).

Characterization of uEVs

The results of the characterization of uEVs are shown in **Figure 2**. The particles extracted from urine were characterized using NTA and transmission electron microscopy. The results showed that these particles were cup-shaped in morphology and ranged from 70 to 150 nm in diameter (**Figures 2A, B**). These are two typical characteristics of uEVs. Furthermore, the results of western blotting showed that all uEVs samples were positive for EVs markers (CD9, CD63, and Alix), which were not present in the supernatant (**Figure 2C**).

NCC and pNCC Expression for Different Lateralization Indices

Compared with the l-LI group, pNCC and NCC are more abundant in the uEVs of the patients in the h-LI group (P <.0001, P =0.0002, respectively). The abundance of pNCC and NCC in patients of the h-LI group was approximately 1.89 and 1.82 folds higher than those in the l-LI group (**Figure 3A**), respectively. Higher NCC abundance in uEVs was observed in the h-LI group (1.82 folds, P = 0.0002). There was no significant difference in the pNCC/NCC ratio between the two groups. Furthermore, plasma PAC correlated positively with pNCC (R = 0.1220, P = 0.0129), but not with NCC (**Figure 3B**). There was no correlation between serum potassium and pNCC or NCC expression (**Figure 3C**).

NCC and pNCC Expression in Patients With or Without KCNJ5 Somatic Mutation

Twenty seven patients underwent unilateral adrenalectomy in our hospital, and four patients received operations in other hospitals (the pathological sections were not obtained). Sanger

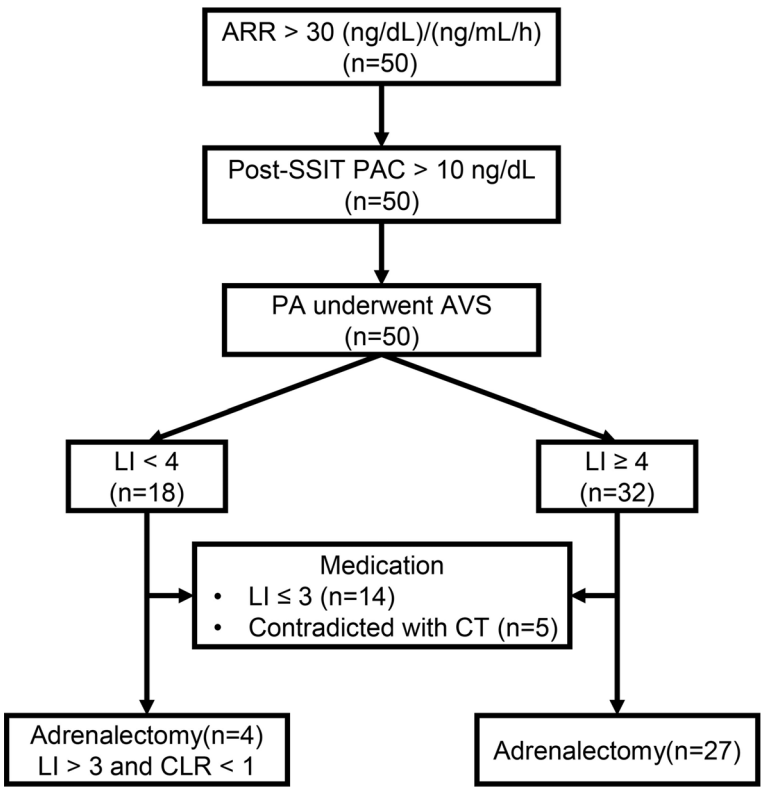

FIGURE 1 | The workup of primary aldosteronism (PA). ARR, Aldosterone-renin-ratio; Post-SSIT, Post-supine saline infusion test; PA, Primary aldosteronism; AVS, Adrenal venous sampling; LI, Lateralization index; CLR, Contralateral ratio; CT, Computed tomography.

TABLE 1 | Clinical characteristics of patients stratified by different lateralization index (LI).

Characteristic	All patients (n=50)	LI≥4 (N=32)	LI<4 (N=18)
Age (years)	49.5 ± 10.9	49.1 ± 10.1	50.3 ± 12.6
Male sex (%)	36 (72.0)	22 (68.8)	14 (77.8)
BMI (kg/m^2)	26.4 ± 3.7	25.7 ± 3.4	27.7 ± 4.0
Hypertension duration (years)	10.0 (4.0-20.0)	10.0 (3.5-13.0)	13.0 (5.0-20.0)
Antihypertensive drugs (n)	3.0 (2.0-3.0)	3.0 (2.0-3.0)	3.0 (2.0-3.0)
On admission			
24 h SBP (mm Hg)	142 ± 13	142 ± 13	140 ± 11
24 h DBP (mm Hg)	89 ± 10	91 ± 9	86 ± 9
24 h urinary Na^+ (μg/24 h)	158.7 ± 65.5	148.6 ± 63.4	176.6 ± 67.2
Plasma creatinine (mmol/L)	74.1 ± 19.9	74.7 ± 22.8	73.0 ± 13.9
eGFR (mL/min·1.73 m^2)	100.9 ± 19.2	99.5 ± 20.7	103.3 ± 16.6
24 h urinary protein (mg/24 h)	153 (123-234)	154 (123-243)	148 (117-221)
Serum K^+ (mmol/L)	3.34 ± 0.30	3.32 ± 0.31	3.38 ± 0.28
Supine PAC (pg/mL)	231 (178-367)	308 (205-423) **	185 (128-217)
Supine PRA (ng/mL·h)	0.32 (0.16-0.58)	0.32 (0.17-0.62)	0.35 (0.14-0.51)
Supine ARR ([pg/mL]/[ng/mL·h])	809 (436-1801)	987 (526-2454)	459 (336-1316)
24 h urinary aldosterone (μg/24 h)	23.2 ± 11.0	26.8 ± 11.3 **	16.7 ± 6.9
Pre-SSIT			
Serum K^+ (mmol/L)	3.84 ± 0.32	3.90 ± 0.31	3.76 ± 0.33
PAC (pg/mL)	318 ± 143	356 ± 150 *	251 ± 104
PRA (ng/mL·h)	0.36 (0.15-0.73)	0.36 (0.17-0.67)	0.40 (0.15-0.87)
ARR ([pg/mL]/[ng/mL·h])	749 (338-1669)	806 (473-1915)	521 (230-1326)

Values are indicated as the mean ± standard deviation or as median (25th and 75th).

SBP, systolic blood pressure; DBP, diastolic blood pressure; K^+, potassium ions; eGFR, estimated glomerular filtration rate (Chronic Kidney Disease Epidemiology Collaboration); PAC, plasma aldosterone concentration; PRA, plasma renin activity; ARR, aldosterone-to-renin ratio; Pre-SSIT, Pre-supine saline infusion test.

**P < 0.05, **P < 0.001 (LI ≥ 4 vs LI<4).*

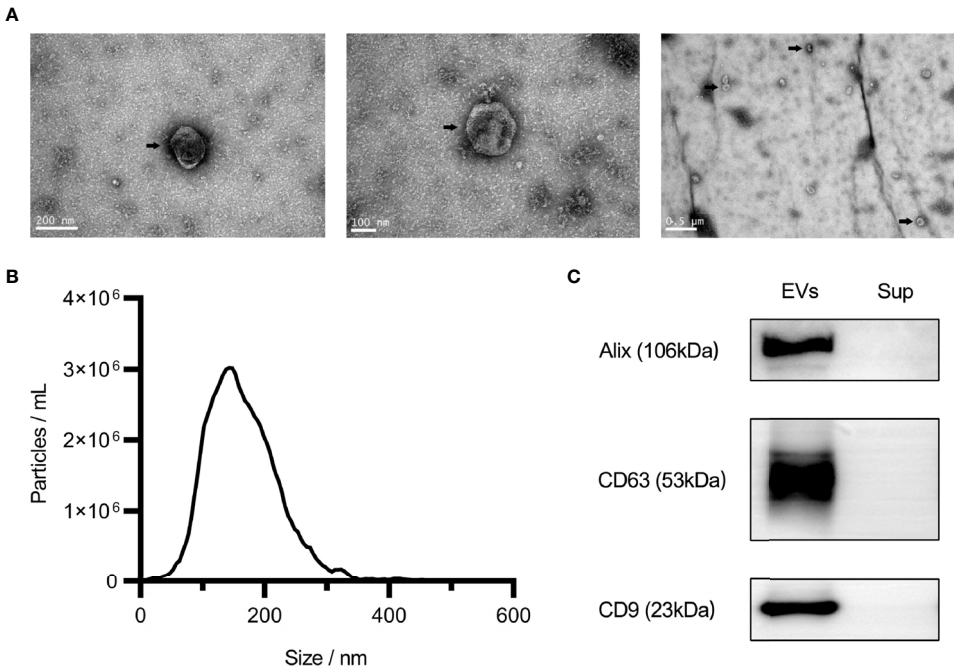

FIGURE 2 | Characteristics of urinary extracellular vesicles (EVs). Electron microscopy analysis of EVs under different magnifications, as shown by the arrow **(A)**. Nanoparticle tracking analysis of EVs **(B)**. Western blot analysis of Alix, CD63, and CD9 **(C)**. Sup, Supernatant.

sequencing was performed for 27 patients. Among them, 26 cases were confirmed as aldosterone-producing adenoma (APA) and one (LI≥ 4) as adrenal hyperplasia (**Table 2**). Somatic KCNJ5 mutations were detected in 17 (65.4%) of the 26 APA cases (**Table 2**), but no mutations were detected in one hyperplasia case. KCNJ5 mutations included G151R and L168R mutations in eleven and five cases, respectively, and an insertion mutation (p.T148_149insW) in one patient. As shown in **Table S1**, at baseline, a trend toward higher level of supine PAC and pre-SSIT PAC in the KCNJ5 mutation group was observed but without statistical significance. Carriers of somatic KCNJ5 mutations, compared with non-carriers, had a higher abundance of pNCC in uEVs (2.16 folds, P=0.0039) (**Figures 4A, B**). There was no significant difference between the two groups in pNCC-to-NCC ratio and NCC abundance.

DISCUSSION

In this study, we investigated whether pNCC and NCC in uEVs, important indicators of true aldosterone status, can be used as biomarkers to distinguish between unilateral and bilateral aldosterone overproduction in patients with PA. The results revealed that pNCC and NCC expression in the h-LI group were higher than those in the l-LI group (1.89 folds and 1.82 folds, respectively). In addition, pNCC content increased in uEVs in patients with a KCNJ5 mutation. This finding indicates that pNCC and NCC could be potential indicators of lateral hyperaldosteronism before AVS.

This is the first study in humans to document the expression of pNCC and NCC in uEVs in two main subtypes of PA. The observation regarding the relationship between pNCC and NCC in uEVs and PAC in this study merits discussion. To the best of our knowledge, early studies suggest that pNCC and NCC in uEVs of humans or animals are possible markers of aldosteronism. Van der Lubbe et al. reported that the infusion of aldosterone in rats increased pNCC abundance by three folds and NCC abundance by 1.5 folds in uEVs, respectively. The pNCC expression in uEVs of patients with PA was 2.6 folds higher than patients with essential hypertension (16). Subsequently, Wolley et al. reported that mineralocorticoid administration in humans increases the levels of NCC and pNCC in uEVs rapidly and sustainably (17). Consistent with the above results, in our study, the abundance of pNCC and NCC in uEVs increased by approximately 1.89 folds and 1.82 folds respectively in the h-LI group, while the simultaneous pre-supine saline infusion test PAC level was 1.42 folds higher than that in the l-LI group. To further support this concept, a 2.16-fold increase in pNCC abundance was observed in patients with *KCNJ5* somatic mutations compared to those without *KCNJ5* mutations. These mutation carriers also had higher PAC levels. Additionally, pNCC, but not NCC, manifested significant positive correlation with plasma PAC level, which suggest that pNCC was a better indicator of true aldosterone status.

The expression and modification of NCC have been attributed to the effects of aldosterone. More recently, several studies in animal and cell models have demonstrated an important role for serum potassium in regulating NCC (12). *In vitro*, high-K^+ exposure

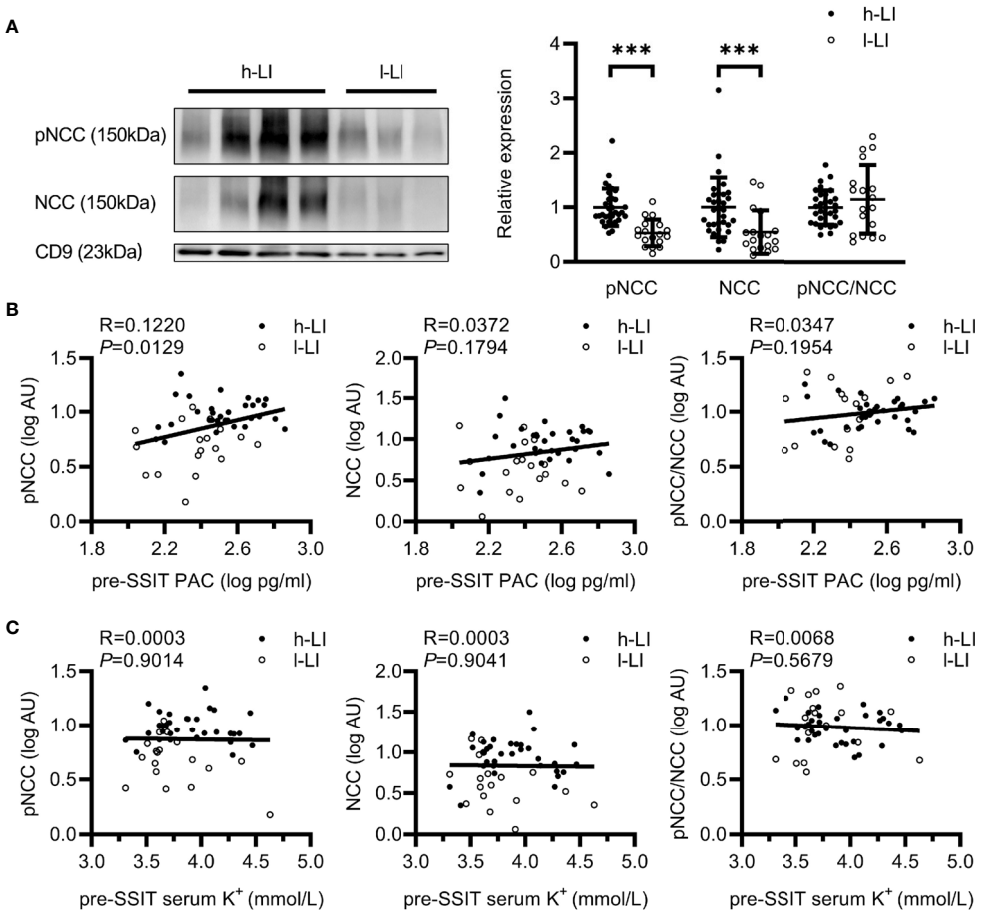

FIGURE 3 | Expression of renal sodium transporters in patients with different lateralization index (LI). Western blot analysis of pNCC and NCC in uEVs of patients with PA. Patients with PA were divided into h-LI (n=32) and l-LI (n=18) groups **(A)**. Correlations between pNCC or NCC expression and PAC or serum potassium **(B, C)**. PA, Primary aldosteronism; h-LI, high lateralization index; l-LI, low lateralization index; PAC, plasma aldosterone concentration; pre-SSIT, pre-supine saline infusion test. ***$P < 0.001$.

TABLE 2 | Somatic mutation detection.

Genotype	Adrenal adenomas (N=26)	Adrenocortical hyperplasia (N=1)
KCNJ5 mutation	17	.
G151R	11	.
L168R	5	.
T148_149insW	1	.
KCNJ5 wild type	9	1

reduces NCC abundance in a ubiquitin-mediated manner in renal cortical tubules; conversely, K^+ deficiency stimulates NCC via a kinase cascade involving no lysine (WNK) kinases (21, 22). Similarly, low- and high-K^+ diets can rapidly increase or reduce NCC phosphorylation by changing the serum K^+ concentration in mice (22). In our study, no correlation was found between NCC or pNCC abundance and serum potassium. Van der Lubbe et al. once reported that upon either high-dose or low-dose (long-term) aldosterone infusion in rats, pNCC expression in uEVs increases, followed by a plateau, suggesting that the expression and activation of NCC by long-term aldosterone infusion will eventually reach saturation (16). The evolution of PA is a relative long way that hypokalemia secondary to hyperaldosteronism only presented in the late phase of PA in around 30% patients (10). When hypokalemia was corrected by KCL supplementation, the level of PAC increased further, as observed in our study. In this context, the expression and activation of NCC in uEVs in patients with PA were mainly affected by long-term pathological aldosterone overproduction instead of hypokalemia, which is secondary to hyperaldosteronism. Recently, Wolley et al. reported that serum

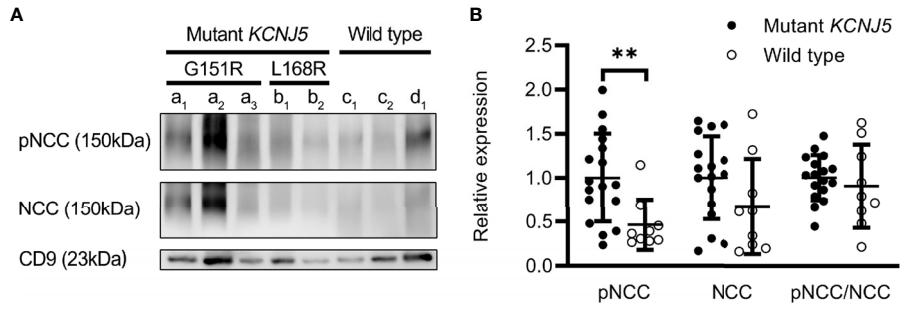

FIGURE 4 | Expression of renal sodium transporters in patients with different genotypes. Western blot analysis of pNCC and NCC in patients with (n=17) or without (n=9) *KCNJ5* mutation **(A)**. Aldosterone-producing adenomas (a–c). G151R mutation on *KCNJ5* (a). L168R mutation on *KCNJ5* (b). No *KCNJ5* mutation (c). Adrenocortical hyperplasia (d). Quantification of western blotting analysis **(B)**. $^{**}P < 0.01$.

potassium levels correlate negatively with NCC and pNCC abundance in uEVs of patients with PA (17). This inconsistency of the effects of serum potassium on NCC may be attributable to different study methods and subjects. In our study, hypokalemia was corrected as much as possible by KCL supplementation before supine saline infusion test, whereas in the study of Wolley's, no KCL supplementation was performed before fludrocortisone suppression test and not all patients were finally diagnosed to have PA.

Our study reported that the prevalence of KCNJ5 mutations in APAs was 65.4%, which is relatively lower than that reported in our previous study (76.8%) (20). In addition, the abundance of pNCC was higher in mutation carriers, which implied that pNCC in uEVs might be an indicator of KCNJ5 mutations. Recently, several studies have indicated that somatic KCNJ5 mutations are an independent predictor of favorable outcomes after adrenalectomy (5–7, 23). High pNCC expression in uEVs might, therefore, be an indicator of KCNJ5 mutations and AVS.

This study had several limitations. First, the number of patients included in this study was relatively small. These results should be further confirmed in a larger cohort using further quantitative methods such as mass spectrometry. Second, the spot urine sample was collected for the convenience of the patients instead of 24 h urine, which reflects the expression of renal sodium channel protein more comprehensively; however, we corrected it with urinary creatinine. Third, our study did not reflect the changes of pNCC or NCC levels in uEVs after PA treatment. Therefore, further investigations are required to study the expression of pNCC or NCC in uEVs of patients with PA after adrenalectomy or mineralocorticoid antagonist treatment to clarify the impact of different treatment on these uEVs.

CONCLUSION

In conclusion, our results revealed that the abundance of pNCC in uEVs was 1.89 folds higher in lateralized aldosteronism and 2.16 folds higher in patients with somatic KCNJ5 mutations. The expression of pNCC in the uEVs may serve as an indicator of AVS and effectively decrease the number of AVS candidates during the workup of PA.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ruijin Hospital Ethics Committee of Shanghai Jiao Tong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LK performed the experiments, interpreted the data, and drafted the manuscript. XT and YK collected samples and clinical data. LD analyzed and interpreted the pathological and molecular genetic data. JX performed the adrenal venous sampling. JT, PJG, and J-GW contributed to manuscript writing. LZ and WS designed the study and edited the manuscript. All authors contributed to the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.834409/full#supplementary-material

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Primary Aldosteronism More Prevalent in Patients With Cardioembolic Stroke and Atrial Fibrillation

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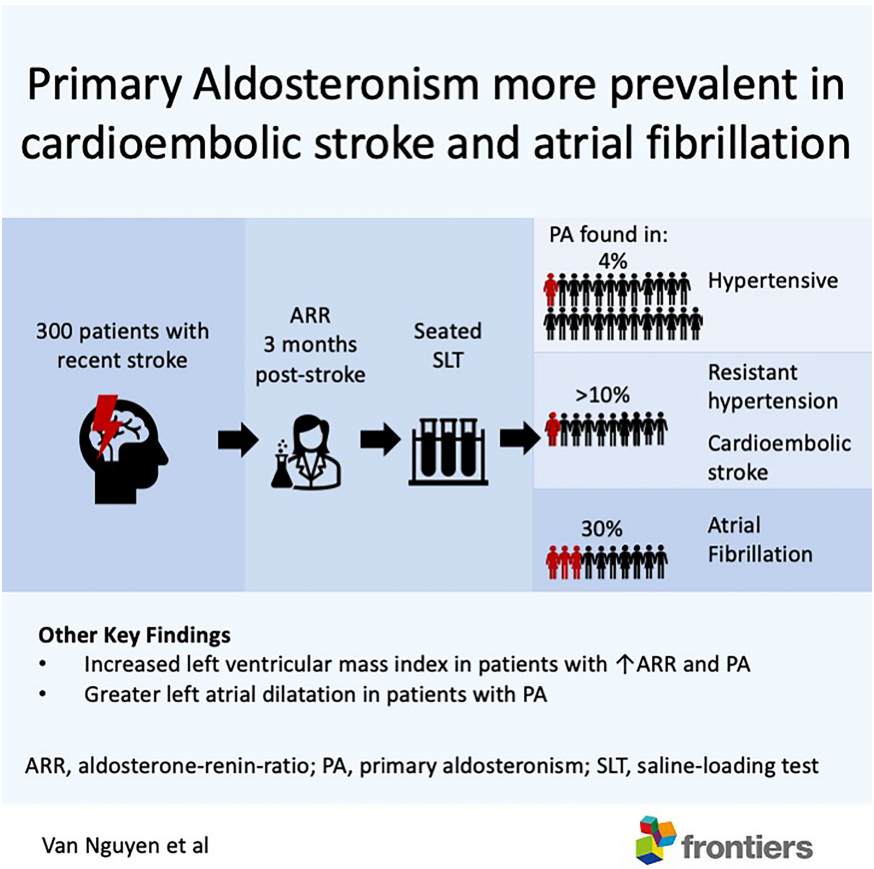
Background: Primary aldosteronism (PA) is the most common cause of secondary hypertension, and patients are at an increased risk of atrial fibrillation (AF) and stroke. We assessed the prevalence of PA in patients with recent stroke.

Methods: We recruited 300 patients admitted to an acute stroke unit with diagnosis of cerebrovascular accident (haemorrhagic/ischaemic) or transient ischaemic attack. Three months post-stroke, plasma renin and aldosterone were measured. Patients with an elevated aldosterone–renin ratio proceeded to the confirmatory saline loading test.

Results: Twenty-six of 192 (14%) patients had an elevated aldosterone–renin ratio. Three of 14 patients who proceeded to saline loading were confirmed with PA (post-saline aldosterone >138 pmol/l). Another three patients were classified as confirmed/likely PA based on the markedly elevated aldosterone–renin ratio and clinical characteristics. The overall prevalence of PA amongst stroke patients with hypertension was 4.0% (95% confidence interval (CI): 0.9%–7.1%). Prevalence of PA was higher amongst patients with cardioembolic stroke, 11% (95% CI: 1.3%–33%), resistant hypertension, 11% (95% CI: 0.3%–48%), and hypertension and AF, 30% (95%CI: 6.7%–65%). If only young patients or those with hypokalaemia were screened for PA, half of our patients with PA would not have been diagnosed. Our decision tree identified that stroke patients with AF and diastolic blood pressure ≥83mmHg were most likely to have PA.

Conclusion: We found that amongst hypertensive patients with stroke, PA was more prevalent in those with AF, or cardioembolic stroke. Screening for PA should be considered for all patients with stroke.

Keywords: hyperaldosteronism, cerebrovascular accident, transient ischaemic attack, atrial fibrillation, secondary hypertension, endocrine hypertension



GRAPHICAL ABSTRACT | Amongst patients with recent stroke, primary aldosteronism was more prevalent in those with atrial fibrillation, cardio-embolic stroke and resistant hypertension. Patients with primary aldosteronism had greater left ventricular mass and larger left atrial volume. Patients with good functional recovery after a stroke should be screened for primary aldosteronism.

INTRODUCTION

Primary aldosteronism (PA) is the most common treatable cause of hypertension, affecting 5% of all patients with hypertension (1), and up to 25% amongst those with severe or resistant hypertension (2). Compared to patients with essential hypertension of similar blood pressure (BP), patients with PA are at a greater risk of renal and cardiovascular events (3, 4), due to the direct deleterious effects of aldosterone. Since hypertension is the most important risk factor for stroke (5), PA is likely as prevalent amongst patients with stroke.

Importantly, PA has been associated with increased risk of atrial fibrillation (AF), due to the presence of mineralocorticoid receptors on the myocardium (6, 7). A recent study by Seccia and colleagues found a prevalence of PA in 42% of hypertensive patients with unexplained AF, which may further compound the risk of stroke in PA (8). While hypertension is the main risk factor for both haemorrhagic stroke, and ischaemic strokes from large or small vessel disease (9), AF is the major risk factor for cardioembolic strokes (10).

Treatment of PA can ameliorate the risk of cardiovascular events, renal disease, and even improve quality of life, which underlies the importance of diagnosing and treating PA (11). Furthermore, patients with unilateral PA may be cured with surgery, leading to better outcomes compared with medical therapy (12, 13). There is currently no neurology consensus on which patients with stroke should be screened for secondary causes of hypertension. While some guidelines recommend screening young patients with stroke, the definition of a young age differs between guidelines (14). Current Endocrine Society guidelines recommend screening for PA in patients with severe or resistant hypertension, and those with family history of stroke below 40, but do not have a recommendation for patients with stroke (15). Hence, we hypothesized that PA is prevalent amongst patients with stroke and conducted a prospective trial in patients admitted with an acute stroke to assess the prevalence of PA.

METHODS

We prospectively recruited 300 patients admitted to the acute stroke unit of Changi General Hospital, Singapore, between August 2018 and October 2020. Inclusion criteria were age 21 to 80 years, diagnosis of transient ischaemic attack (TIA), or

ischaemic/haemorrhagic acute cerebrovascular accident (CVA). Exclusion criteria were limited life expectancy (e.g. terminal illness), pregnancy, impaired renal function (estimated glomerular filtration rate (eGFR) <45ml/min), and impaired cardiac function (ejection fraction 45% or lower). Chronic kidney disease was defined as eGFR <60 ml/min. Dyslipidaemia was defined as LDL ≥1.8 mmol/l, or use of lipid-lowering medications. The study was approved by the local ethics committee, registered with Clinicaltrials.gov (NCT03789357), and informed consent was obtained in all patients. Baseline characteristics such as demographics, medical history, drug information, and comorbidities were collected.

Stroke Diagnosis and Management

All patients were managed for stroke in accordance with best clinical practice (16). Patients underwent further tests to determine the aetiology of stroke, which included computed tomography or magnetic resonance imaging of the brain, ultrasound of carotid arteries, 2-dimensional transthoracic echocardiogram (2DE), and 24-h Holter monitoring as indicated. All of the above test results were reviewed independently by two investigators (VN, TT), and subtypes of stroke were classified based on the Trial of Org 10172 in Acute Stroke Treatment (TOAST) Classification (17). Functional recovery status at 3 months or more were determined using the Modified Rankin Scale (MRS) (18).

BP Medications and Tests for PA

In the acute and post-acute management, choice of antihypertensive medications and BP targets were left to the managing neurologist, according to current treatment guidelines. Patients with newly diagnosed hypertension were started on first-line antihypertensive medications, usually angiotensin-converting enzyme (ACE) inhibitors or calcium-channel blockers. Two to four months post-stroke, all patients had a morning seated blood test for plasma aldosterone concentration (PAC), plasma renin activity (PRA), and renal function. All antihypertensive medications were continued prior to the screening test, and fifteen patients with hypokalaemia (serum potassium <3.5 mmol/l) prior to the screening test were prescribed potassium supplementation. Clinic BP was taken in the seated position after 5 min of rest. Resistant hypertension was defined as clinic systolic BP ≥140 mmHg, or diastolic BP ≥90 mmHg, while on three antihypertensive medications. The screening test was positive if the aldosterone–renin ratio (ARR) was greater than 277 (pmol/l per ng/ml/h). Patients with a positive screening test underwent a confirmatory seated saline-loading test (SLT). Patients were confirmed with PA if post-saline PAC was >138 pmol/l or had spontaneous hypokalaemia with undetectable plasma renin activity (PRA) and PAC was >277 pmol/l (15). PAC and PRA were analysed by Mayo Clinic Laboratories, Rochester, MN, USA, for determination using LC–MS/MS, and the reference ranges were 0.6–3.0 ng/ml/h and 581 pmol/l or less, respectively, with the lowest level of detection 0.6 ng/ml/h and 110 pmol/l, respectively. For calculation of ARR, PRA was taken to be 0.6 ng/ml/h in those with undetectable PRA. Patients with PA were managed by an endocrinologist in accordance with the Endocrine Society guidelines.

Statistical Analysis

Statistical analysis was conducted using R (A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R–project.org/). Continuous variables were expressed as a mean and standard deviation (SD) or median, minimum, and maximum or first and third quartile as appropriate and analysed using the independent-sample *t*-test or Mann–Whitney U test, depending on whether normality assumption was tenable. Categorical variables were presented as number (percent) and compared using the chi-squared test or Fisher exact test (where appropriate). The primary outcome was to determine the prevalence of PA in our cohort of patients with stroke. Secondary outcomes included the prevalence of PA in certain subgroups, and differences in characteristics of patients: those with PA versus those without PA, those with positive ARR versus negative ARR. A decision tree analysis *via* recursive partitioning for the classification method was performed to investigate how the baseline characteristics can help in discriminating patients with and without PA. Significance level was set at p value < 0.05.

RESULTS

From August 2018 to October 2020, 886 eligible patients were admitted with the primary diagnosis of TIA or CVA to the acute stroke unit (**Figure 1**). We recruited 300 patients, of which two patients were subsequently excluded from analysis as their final clinical diagnosis was not stroke and 106 patients were lost to follow-up (41 patients were uncontactable, 22 patients were discharged to primary care, 35 patients withdrew consent, five patients went overseas, three patients died). At 3 months post-stroke, 192 patients underwent ARR screening, median age 58.0 years, with 137 (71.4%) females (**Table 1**). Amongst 192 patients, 150 (78.1%) had hypertension, 55 (28.6%) had diabetes, 60 (31.3%) had history of smoking, and 24 (12.5%) had a previous stroke. Stroke subtypes were ischaemic in 156 (81.3%), haemorrhagic in 20 (10.4%), and TIA in 16 (8.3%) patients. Patients who underwent ARR screening were slightly younger (58.0 vs. 61.0 years, $p = 0.03$) compared to those who did not, but otherwise there were no other significant differences between the two groups (**Supplementary Table 1**).

Patients With Positive ARR

Twenty-six of 192 patients (14%) had a positive ARR screening test. The positive ARR group had higher diastolic blood pressure compared to the negative ARR group, 84.5 vs. 79.5 mmHg, $p = 0.003$, despite the similar number and type of antihypertensive medications (**Table 2**). Calcium-channel blockers and ACE inhibitors were the most commonly used. Patients with positive ARR were more likely to have AF, 19.2% vs. 3.0%, $p = 0.005$, and had greater left ventricular mass index (g/m^2), 79.1 (69.8–99.8) vs. 67.8 (57.4–81.5), $p = 0.02$. There was no difference in stroke

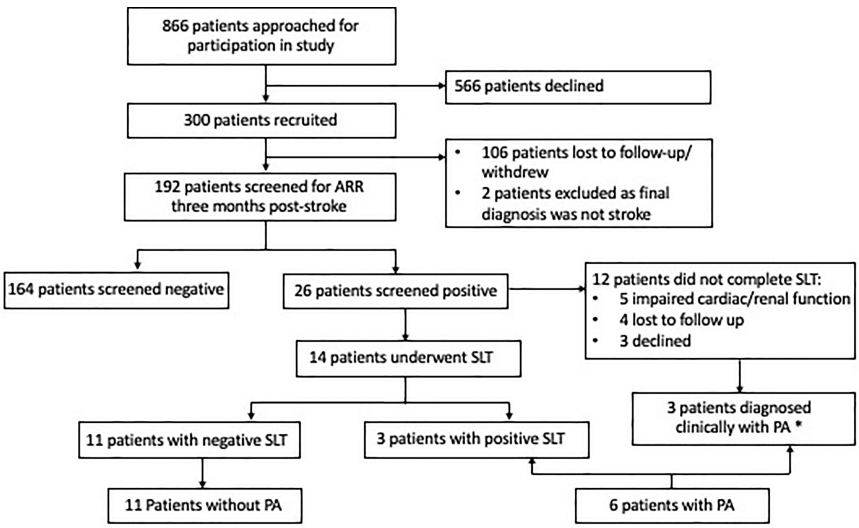


FIGURE 1 | Consort diagram of 300 patients with acute stroke recruited. *One patient did not require confirmatory testing in view of the elevated aldosterone–renin ratio and spontaneous hypokalemia; two patients had elevated aldosterone and elevated aldosterone–renin ratio and were started on spironolactone. ARR, aldosterone–renin ratio; CVA, cerebrovascular accident; GFR, glomerular filtration rate; PA, primary aldosteronism; SLT, saline-loading test; TIA, transient ischaemic attack.

subtype, and MRS score post-stroke between the groups. We repeated this analysis after restricting only to patients with hypertension (**Supplementary Table 2**) since hypertension may independently increase the risk of AF and left ventricular mass. We similarly found that hypertensive patients with positive ARR were more likely to have AF, 5 (21.7%) vs. 4 (3.2%), $p = 0.005$, and had greater left ventricular mass index (g/m^2), 84.7 (69.9–101.1) vs. 68.9 (57.9–85.4), $p = 0.016$.

Patients With PA

Fourteen patients proceeded with confirmatory SLT, with three patients having post-SLT PAC >138 pmol/l, consistent with diagnosis of PA (**Figure 1**). Of the 12 patients who did not proceed with the confirmatory SLT test, five patients had poor cardiac or renal function (which developed/worsened after recruitment) which were contraindications to SLT, four were lost to follow-up, and three declined. One patient had baseline aldosterone ≥554 pmol/l, undetectable PRA, and spontaneous hypokalaemia and did not require additional tests to confirm PA. One patient was not able to undergo SLT due to poor cardiac function, and in view of repeated elevated ARRs (PAC 15 and 13 ng/dl, with undetectable renin), the patient was treated with spironolactone. One final patient had an inappropriate PAC (7.9 ng/dl) and undetectable renin despite being on an ACE inhibitor. Due to newly diagnosed AF and impaired cardiac function, spironolactone was started with clinical improvement. In total, six patients had confirmed or likely PA. All six were treated with spironolactone and did not pursue subtype testing: one patient migrated, three patients declined surgery (two were >65 years), and two were subsequently diagnosed with cardiac or renal impairment which made them poor surgical candidates.

All six patients with PA had hypertension, three had AF, and two had hypokalaemia. Patients with PA had a higher diastolic BP at the 3-month follow-up (87.0 vs. 80.0 mmHg, $p = 0.01$) and were more likely to use calcium-channel blockers (83.5% vs. 36.6%, p = 0.03), and the median number of BP medications used was 1.7 vs. 1.0, $p = 0.06$ **(Table 3)**. Patients with PA were more likely to have AF (50% vs. 3.8%, p = 0.002) compared to those without PA. Patients with PA had a higher left ventricular mass index (g/m^2), 90.7 vs. 69.2, $p = 0.01$, and were more likely to have left atrial dilatation (defined as left atrial volume index >34 ml/m^2), 50.0% vs. 11.8%, $p = 0.03$.

The prevalence of PA in the cohort was 3.1%, 95% CI:1.2–6.7%, amongst all patients, and 4.0%, 95% CI: 0.9%–7.1%, amongst patients with hypertension (**Figure 2**). Prevalence rates were higher in subgroups: age ≤50 years, 6.1% (3 of 49), 95% CI: 1.3%–16.9%, cardioembolic strokes, 10.5% (2 of 19), 95% CI: 1.3%–33.1%, resistant hypertension, 11.1% (1 of 9), 95% CI: 0.3%–48.3%, hypertension with hypokalaemia, 13.3% (2 of 15), 95% CI: 1.7%–40.5%, hypertension and AF, 30.0% (3 of 10), 95% CI: 6.7%–65.3%.

Decision Tree Algorithm

Our decision tree algorithm was derived from the 192 patients for whom complete data existed for all predictor and outcome variables, and it identified two main factors: diastolic BP and presence of AF. Patients with a diastolic BP ≥83 mmHg were more likely to have PA, and this increased to 100% amongst those with both diastolic BP ≥83 mmHg and presence of AF at 3 months post-stroke (**Figure 3**). In the 169 patients with 2D-echocardiography data, the decision tree algorithm yielded similar results.

TABLE 1 | Baseline characteristics of patients who completed the screening aldosterone–renin-ratio test.

Baseline characteristics	N = 192
Demographics	
Age, years	58.0 [21.0, 78.0]
Male	137 (71.4)
Body mass index, g/m^2	25.5 [13.8, 45.7]; N = 183
Potassium, mmol/L	4.0 [2.9, 5.8]
Estimated glomerular filtration rate, mL/min/1.73 m^2	94.6 [41.3, 130]
Systolic blood pressure, mmHg	157 [80, 247]; N = 187
Diastolic blood pressure, mmHg	90 [51, 142]; N = 187
Serum aldosterone, pmol/L	155 [38, 1,551]
Plasma renin activity, ng/ml/h	1.7 [0.6, 24.0]
Aldosterone–renin ratio, pmol/L per ng/ml/h	122 [6, 922]
Comorbidities	
Hypertension	150 (78.1)
Diabetes	55 (28.6)
Dyslipidaemia	192 (100)
History of smoking	60 (31.3)
History of drinking	34 (17.7)
Ischaemic heart disease	27 (14.1)
Atrial fibrillation	10 (5.2)
Chronic kidney disease	15 (7.8)
Stroke history	24 (12.5)
Stroke subtypes	**N (%)**
Ischaemic stroke	156 (81.3)
Small-artery occlusion	50 (26.0)
Large-artery atherosclerosis	25 (13.0)
Cardioembolic	19 (10.0)
Undetermined	62 (32.3)
Haemorrhagic stroke	20 (10.4)
Transient ischaemic attack	16 (8.3)
Modified Rankin Scale Score	1.2 (1.4); N = 183
Use of antihypertensive medication	
ACE-inhibitor or angiotensin II receptor blocker	69 (35.9)
Beta-blocker	46 (24.0)
Calcium channel blocker	73 (38.0)
Diuretics	2 (1.0)
Alpha blocker	1 (0.52)
Number of antihypertensive medication	1.0 (0.91)
2D echocardiogram parameters	
Left ventricular ejection fraction, %	60.0 [15.0, 66.0]; N = 169
Presence of left ventricular hypertrophy	9 (5.33%); N = 169
Relative wall thickness, h/r	0.4 [0.2, 0.7]; N = 165
Left atrium volume index, ml/m^2	24.4 [13.2, 134]; N = 162
Left ventricular mass index, m^2	69.8 [39.3, 173]; N = 164

Data presented as median [min, max], mean (SD), or number (%) as appropriate.

DISCUSSION

In our prospective cohort study, PA was prevalent in at least 4% of hypertensive patients with stroke, which increased to 11% amongst those with cardioembolic stroke and 30% amongst those with atrial fibrillation. There has been a growing body of evidence that PA is associated with increased risk of AF (7, 13), and our study identifies another high-risk group of patients that should be screened for PA, namely those with cardioembolic stroke, or stroke with concomitant atrial fibrillation. We also found that patients with PA or elevated ARR had a greater left ventricular mass index, which highlights the deleterious effects of hyperaldosteronism, as well as importance of appropriate diagnosis and treatment of PA.

AF is the main predisposing factor for embolic strokes, which accounts for 15%–20% of all strokes (19). Excessive aldosterone is a major contributor to AF pathogenesis by inducing both structural cardiac remodelling through atrial dilatation and fibrosis, and electrical remodelling through arrhythmogenicity (20, 21). In our study, patients with positive ARR had higher rates of AF and greater left ventricular mass index compared to those with negative ARR, suggesting that the effects of hyperaldosteronism may not be restricted only to patients with PA, as has been previously reported (22). Patients with elevated ARR are particularly responsive to mineralocorticoid antagonists. Hence, patients with elevated ARR but normal response to saline suppression (false-positive ARR) may still benefit from therapy with spironolactone, similar to patients with PA (23). In addition, our patients with PA had a larger left atrial volume index, which predisposes to development of AF. Milliez and colleagues found a 12-fold higher risk of prevalent AF amongst patients with PA (6), compared to patients with essential hypertension, which has been supported by subsequent studies (24). Most recently, Seccia and colleagues found a high prevalence of PA in 42% of hypertensive patients with unexplained AF (8), which is not dissimilar from our prevalence of 30% amongst hypertensive patients with AF and stroke. Hence, our study confirms findings from previous studies that patients with PA are at a higher risk of stroke. Of particular note, one of our patients with PA was classified as embolic stroke of undetermined source (ESUS), because repeated cardiac monitoring (24-hHolter and extended 28-day Spyder) did not reveal any episodes of AF. ESUS may be attributed to various potential embolic sources, including occult atrial cardiopathy, and some patients may go on to develop AF (25). Hence, it is plausible that PA may be similarly common amongst patients with ESUS, and these patients should be offered screening for PA.

Hypertension is the main risk factor for haemorrhagic strokes, as well as ischaemic strokes from small- or large-vessel disease (9). Patients with PA have a 4–5-fold greater odds of stroke compared to patients with essential hypertension (6, 26), which is attributed to the deleterious effects of aldosterone on cerebral vasculature through oxidative stress and endothelial dysfunction (27). In our cohort, patients with either elevated ARR or PA had a greater left ventricular mass index, which underlines the effects of hyperaldosteronism on the myocardium (28). While all of our PA patients had hypertension, less than half had hypokalaemia, or were aged below 50 years, highlighting that hypokalaemia and age thresholds should not be prerequisites to screen for PA. One previous study on young stroke patients (below 45 years) found the prevalence rate of PA to be 12% (21). If we restricted screening to patients below 45 years of age, we would have only diagnosed one patient. Miyaji and colleagues found the prevalence of PA to be 4% amongst a cohort of patients with recent stroke, and 4.9% when restricted to those with hypertension, but did not report on the prevalence of AF (29). In their study, patients were screened for PA in the first 2 weeks post-stroke, which is not ideal as malignant hypertension or acute illness may alter PAC and PRA, and cosyntropin

TABLE 2 | Characteristics of patients with positive ARR (N = 26) versus patients with negative ARR (N = 166).

Characteristics	Patients with ARR + N = 26 (13.5%)	Patients with ARR– N = 166 (86.5)	*p* value
Demographics			
Age, years	56.0 [47.5, 63.5]	58.0 [51.0, 64.1]	0.43
Male	16 (61.5)	121 (72.9)	0.25
Body mass index, g/m^2	25.6 [25.0, 27.0]	25.5 [23.0, 28.3] N = 157	0.76
Systolic blood pressure, mmHg	145 [133, 148]	137 [125, 149] N = 162	0.11
Diastolic blood pressure, mmHg	84.5 [80.0, 92.0]	79.5 [74.0, 85.0] N = 162	0.003
Estimated glomerular filtration rate, mL/min/1.73 m^2	94.3 [78.1, 100]	94.6 [82.5, 104]	0.56
Sodium, mmol/L	140 [139, 142]	140 [138, 141]	0.17
Potassium, mmol/L	3.9 [3.6, 4.1]	4.0 [3.7, 4.2]	0.25
Bicarbonate, mmol/L	23.0 [22.0, 25.8]	23.0 [21.0, 24.0]	0.26
Total cholesterol mmol/L	4.8 [4.1, 5.3]	4.7 [3.9, 5.4] N = 158	0.62
LDL mmol/L	3.2 [2.8, 3.8]	3.2 [2.6, 3.8] N = 158	0.73
HBA1c, %	5.9 [5.5, 6.7]	6.1 [5.5, 7.1] N = 161	0.37
Serum aldosterone, pmol/L	269 [213, 410]	139 [111, 222]	<0.001
Plasma renin activity, ng/ml/h	0.60 [0.60, 0.78]	1.95 [0.90, 3.38]	<0.001
Aldosterone-renin-ratio, pmol/L per ng/ml/h	355 [321, 452]	108 [47, 186]	<0.001
Comorbidities			
Hypertension	23 (88.5)	127 (76.5)	0.21
Diabetes mellitus	7 (26.9)	48 (29.3)	1.0
Dyslipidaemia	26 (100)	166 (100)	1.0
History of smoking	7 (26.9)	53 (31.7)	0.82
History of drinking	4 (15.4)	30 (18.3)	1.0
Ischaemic heart disease	4 (15.4)	23 (14.0)	0.77
Atrial fibrillation	5 (19.2)	5 (3.0)	0.005
Chronic kidney disease	3 (11.5)	12 (7.2)	0.44
Stroke history	1 (3.9)	23 (13.9)	0.21
Stroke subtypes			
Ischaemic stroke	23 (88.5)	133 (80.1)	0.78
Undetermined	9 (34.6)	53 (31.9)	
Others	14 (53.9)	82 (48.2)	
Haemorrhagic stroke	2 (7.7)	18 (10.8)	
Transient ischaemic attack	1 (3.9)	15 (9.0)	
Modified Rankin Scale Score	0.92 (1.3) N = 15	1.2 (1.4) N = 159	0.33
Antihypertensive medication			
ACE-inhibitor or Angiotensin II receptor blocker	8 (30.8)	61 (36.7)	0.66
Beta–blocker	8 (30.8)	38 (22.9)	0.46
Calcium channel blocker	12 (46.2)	61 (36.7)	0.39
Diuretics	1 (3.9)	1 (0.60)	0.25
Alpha blocker	1 (3.9)	0 (0)	0.14
Number of antihypertensive medication	1.2 (1.2)	1.0 (0.86)	0.75
2D echocardiogram parameters			
Left ventricular ejection fraction, %	60.0 [55.0, 60.0] N = 15	60.0 [55.0, 60.0] N = 144	0.92
Presence of left ventricular hypertrophy	3 (12.0) N = 15	6 (4.2) N = 144	0.13
Relative wall thickness, h/r	0.42 [0.35, 0.48] N = 15	0.40 [0.34, 0.45] N = 140	0.37
Left atrium volume index, ml/m^2	25.7 [22.2, 30.0]	24.0 [19.7, 28.5]	0.25
Left ventricular mass index, m^2	79.1 [69.8, 99.8] N = 25	67.8 [57.4, 81.5] N = 139	0.016

Data presented as median [min, max], mean (SD), or number (%) as appropriate.
ARR, aldosterone–renin ratio.

stimulation was used as a confirmatory test for PA, which is not currently recommended by guidelines (15).

Our findings suggest that all patients with previous stroke may benefit from screening for PA. Treatment of PA has been shown to reduce the risk of developing AF, cardiovascular, and cerebrovascular diseases (30). Although earlier studies found both surgical and medical therapy equally effective, more recent data suggest that surgical treatment leads to better outcomes (12, 13). None of our patients underwent subtype testing with adrenal vein sampling and surgery, similar to previous studies (29, 31), due to poor functional status or comorbidities in the patients. This emphasizes the importance of early diagnosis of PA, which unfortunately remains suboptimal, with less than 1% of all patients at risk of PA being screened (32). Early diagnosis and treatment of PA reduces target organ damage and increases the likelihood of cure of hypertension (33, 34). While patients diagnosed with PA post-stroke may not be good candidates for surgery, they are at high cardiovascular risk and can still benefit from aldosterone antagonists. Eplerenone treatment has been shown in animal models to reduce the aldosterone-induced damage to the cerebral cortex (35). In our retrospective cohort, 14 of our 154 (9%) patients with PA had a prior diagnosis of CVA (36). Hence, while there should be sustained efforts in the early diagnosis and

TABLE 3 | Characteristics of patients with primary aldosteronism (N = 6) versus patients without primary aldosteronism (N = 186).

Characteristics	Patients with PA N = 6	Patients without PA N = 186	*p* value
Demographics			
Age, years	57.3 [44.5, 65.9]	58.0 [51.0, 64.0]	0.91
Male *N (%)*	4 (66.7)	133 (71.5)	1.0
Body mass index, g/m^2	24.8 [21.8, 27.4]	25.5 [23.0, 28.2] N = 177	0.58
Systolic blood pressure, mmHg	145 [138, 147]	137 [126, 149] N = 182	0.36
Diastolic blood pressure, mmHg	87.0 [84.3, 92.8]	80.0 [74.0, 86.0] N = 182	0.011
Estimated glomerular filtration rate, mL/min/1.73 m^2	78.2 [66.1, 89.6]	94.9 [82.5, 103]	0.060
Serum aldosterone, pmol/L	416 [271, 521]	150 [111, 238]	0.0014
Plasma renin activity, ng/ml/h	0.65 [0.60, 1.15]	1.7 [0.7, 3.2]	0.048
Aldosterone–renin ratio, pmol/L per ng/ml/h	343 [319, 609]	116 [53, 188]	<0.001
Comorbidities			
Hypertension	6 (100)	144 (77.4)	0.34
Diabetes mellitus	2 (33.3)	53 (28.5)	1.0
Dyslipidaemia	6 (100)	186 (100)	1.0
History of smoking	2 (33.3)	58 (31.2)	1.0
History of drinking	0 (0)	34 (18.3)	0.59
Ischaemic heart disease	1 (16.7)	26 (14.0)	1.0
Atrial fibrillation	3 (50.0)	7 (3.8)	0.0019
Chronic kidney disease	1 (16.7)	14 (27.5)	0.39
Stroke history	0 (0)	24 (12.9)	1.0
Stroke subtypes			
Ischaemic stroke	6 (100)	150 (80.6)	1.0
Undetermined	2 (33.3)	60 (32.3)	
Others	4 (66.7)	90 (67.7)	
Haemorrhagic stroke	0 (0)	20 (10.8)	
Transient ischaemic attack	0 (0)	16 (8.6)	
Modified Rankin Scale Score	0.33 (0.82)	1.2 (1.4) N = 178	0.10
Antihypertensive medication			
ACE-inhibitor or angiotensin II receptor blocker	2 (33.3)	67 (36.0)	1.0
Beta-blocker	3 (50.0)	43 (23.1)	0.15
Calcium channel blocker	5 (83.3)	68 (36.6)	0.030
Diuretics	0 (0)	2 (1.1)	1.0
Alpha blocker	0 (0)	1 (0.5)	1.0
Number of anti-HTN meds, *mean*	1.7 (0.82)	1.0 (0.9)	0.06
2D echocardiogram parameters			
Left ventricular ejection fraction, %	52.5 [30.0, 60.0]	60.0 [55.0, 60.0] N = 163	0.11
Presence of left ventricular hypertrophy	0 (0)	9 (5.5) N = 163	1.0
Relative wall thickness, h/r	0.41[0.33, 0.42]	0.40 [0.35, 0.45] N = 159	0.61
Left atrium volume Index, ml/m^2	31.2 [25.6, 44.0]	24.0 [19.8, 28.2]	0.032
Left ventricular mass index, m^2	90.7 [83.9, 102]	69.2 [57.9, 83.7]	0.012

Data presented as median [min, max], mean (SD), or number (%) as appropriate.
PA, primary aldosteronism.

treatment of PA, the event of a stroke should prompt clinicians to consider screening for secondary causes, particularly in patients with good functional recovery.

We recognize several limitations of our study. First, as our number of patients diagnosed with PA was small, we included 95% CI to reflect the strength of our prevalence estimates. Second, one-third of patients enrolled did not undergo a screening test. However, their baseline characteristics did not differ greatly from patients who proceeded with screening and are unlikely to affect our estimates. Third, not all patients with a positive screening test proceeded with a confirmatory SLT, due to cardiac or renal dysfunction. Hence, there are likely more patients with PA and a higher prevalence of PA. Fourth, a single PAC assessment may not fully reflect aldosterone status, and studies using 24-h urinary aldosterone have found a higher prevalence of PA (2). Finally, ARR can be affected by use of antihypertensive medications, such as ACE inhibitors and diuretics, leading to false-negative results (15). To reduce false-negative screening rates, we adopted a lower ARR threshold for case detection. Furthermore, other studies have similarly screened for PA with these medications on-board (1), and we had to avoid changing antihypertensive medications soon after a recent stroke as stringent BP control is of critical importance (37). Despite these known limitations when screening patients with stroke (38), we were able to diagnose a significant proportion of patients with PA. This underlies the fact that PA is common and often missed early on in its natural history. Following the call that more patients with stroke should be screened for PA (38), our study offers clinicians a feasible strategy to do so.

In conclusion, we found PA to be prevalent in patients with a recent stroke, similar to cohorts of hypertensive patients, and particularly higher in those with cardioembolic stroke, and concomitant AF. Restricting screening of PA to young patients or those with hypokalaemia would have missed a majority of patients with PA. Hence, we suggest screening all hypertensive patients with stroke for PA as a possible underlying cause, particularly if they have good functional recovery. The impetus

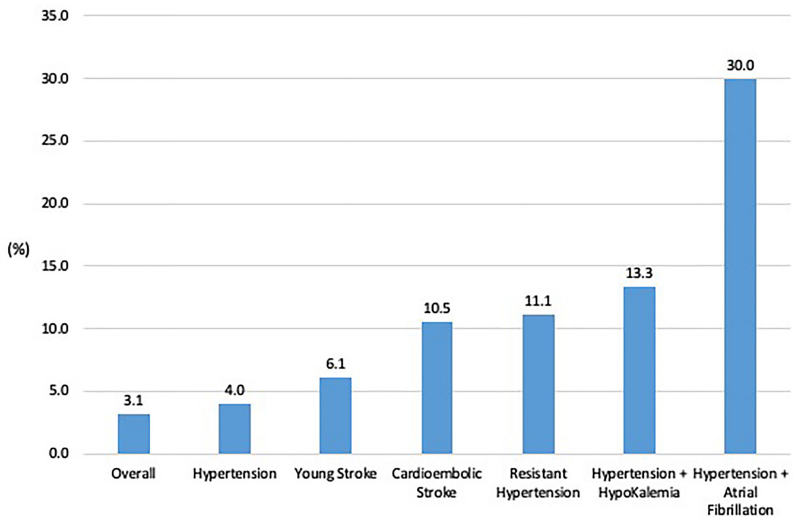


FIGURE 2 | Prevalence rate of primary aldosteronism in the entire cohort and various subgroups.

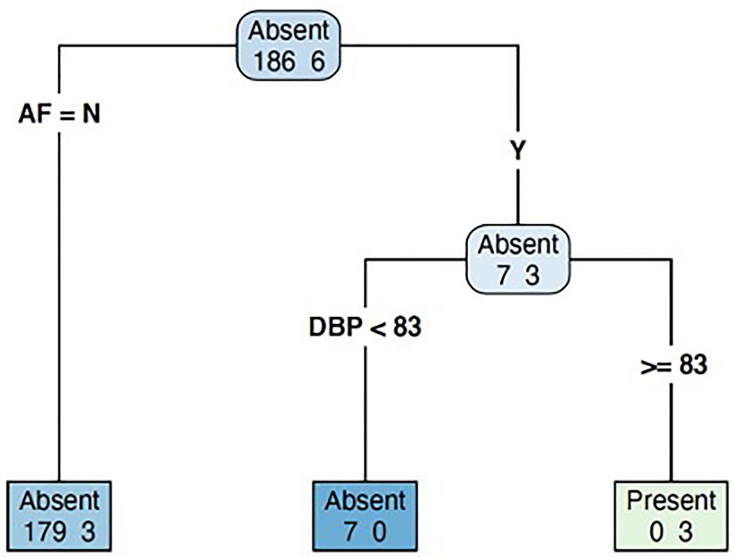


FIGURE 3 | Decision tree model to predict the likelihood of primary aldosteronism in patients with recent stroke. AF, atrial fibrillation; DBP, diastolic blood pressure.

for screening these patients is that appropriate diagnosis and treatment can ameliorate BP control, potentially identify a treatable and potentially curable cause of hypertension, and prevent a recurrent stroke, which may be catastrophic.

Perspectives

Current guidelines recommend screening for PA in patients with severe or resistant hypertension, and hypertension with hypokalaemia. Our study supports the increasing call that presence of AF should be included as another indication for screening. In addition, patients with previous strokes are more likely to have PA. While these patients may not be ideal candidates for surgical treatment, they are at a high cardiovascular risk, and specific medical treatment for hyperaldosteronism is warranted. Early diagnosis and treatment for all patients with PA are the ultimate goal.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article can be made available by the authors upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by SingHealth Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VN, SS, and TP had full access to the data in the study and take responsibility of the integrity of the data and accuracy of data analysis. TT, MM, JL, MZ, and TP recruited patients in the study and performed the data collection. TT, TA, LO, SF, MZ, and TP worked on the study concept and design. VN, TT, SF, and TP were involved in drafting of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.869980/full#supplementary-material

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Benefits of Surgical Over Medical Treatment for Unilateral Primary Aldosteronism

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Primary aldosteronism is the most common and modifiable form of secondary hypertension. Left untreated, primary aldosteronism leads high rates of cardiovascular, metabolic, and kidney disease. Therefore, early diagnosis and targeted therapy are crucial to improve long-term patient outcomes. In the case of unilateral primary aldosteronism, surgical adrenalectomy is the guideline-recommended treatment of choice as compared to alternative medical therapies such as mineralocorticoid receptor antagonist medications. Surgical adrenalectomy is not only highly successful in reversing the biochemical abnormalities inherent to primary aldosteronism, but also in mitigating the long-term risks associated with this disease. Indeed, as opposed to medical treatment alone, surgical adrenalectomy offers the potential for disease cure. Within this review article, we review the existing evidence highlighting the benefits of surgical over medical treatment for unilateral primary aldosteronism.

Keywords: primary aldosteronism, hyperaldosteronism, Conn syndrome, surgery, adrenalectomy, unilateral

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INTRODUCTION

Primary aldosteronism (PA) is a condition defined by aldosterone secretion from one or both adrenal glands, independent of renin and angiotensin II. Though historically considered a rare condition, PA is now widely recognized as the most common and modifiable form of secondary hypertension (1–5). However this condition remains highly under-diagnosed in modern-day clinical. The significance of missing this diagnosis is highlighted by the fact that left untreated, PA leads to disproportionately high rates of cardiovascular, metabolic, and kidney disease. Therefore, early disease identification and targeted therapy are crucial to reduce these risks.

The final step in the diagnostic algorithm of PA is lateralization; i.e., determining whether the source of autonomous aldosterone secretion is from the left adrenal gland, right adrenal gland, or both (6). Lateralization guides which targeted therapy is recommended to the patient. Traditionally, lifelong mineralocorticoid receptor (MR) antagonist therapy is the treatment of choice for bilateral PA. In contrast, surgical adrenalectomy (where the source of aldosterone excess is removed) is the treatment of choice for unilateral PA. Historically, the question has been raised about whether adrenalectomy is indeed superior to lifelong MR antagonist therapy in the treatment of unilateral PA. While no randomized controlled trials have served to provide this answer, a number of recent observational studies have suggested a substantial biochemical and clinical benefit of adrenalectomy

over MR antagonists in the treatment of unilateral PA. Herein, we review the available evidence to date of the benefits of surgical over medical treatment for unilateral PA.

BIOCHEMICAL AND CLINICAL IMPACT OF PRIMARY ALDOSTERONISM

Biochemical Impact of Primary Aldosteronism

PA is defined by a number of biochemical abnormalities that can be readily explained by the underlying disease pathophysiology including suppressed renin, elevated aldosterone, hypokalemia, metabolic alkalosis, and elevated glomerular filtration rate (GFR) (7). In normal physiology, aldosterone secretion is dependent upon three primary regulators: angiotensin II, potassium, and adrenocorticotropic hormone (ACTH). In contrast, PA is defined by aldosterone being secreted at high levels independent of these regulators. Aldosterone binds to and activates the MR of the principal cell of the distal nephron. MR activation leads to sodium reabsorption *via* the epithelial sodium channel (ENaC) and corresponding excretion of potassium and hydrogen ions; hence why patients often develop hypokalemia and/or metabolic alkalosis. ENaC-mediated sodium reabsorption leads to volume expansion and glomerular hyperfiltration (↑ GFR) (8) thereby leading to suppression of both renin and angiotensin II; hence why the aldosterone-to-renin ratio (ARR) is employed as a screening test as it represents high aldosterone relative to suppressed renin. Suppression of angiotensin II leads to decreased sodium reabsorption in the proximal nephron. In turn, this results in increased sodium delivery to the distal nephron which further amplifies the aldosterone-mediated sodium reabsorption *via* the ENaC channel leading to chronic volume expansion which perpetuates this vicious cycle.

Clinical Impact of Primary Aldosteronism

The sodium retention and chronic volume expansion underlying the pathophysiology of PA along with extra-renal aldosterone-mediated MR activation lead to disproportionately high rates of cardiovascular, metabolic, and kidney disease. The extra-renal MR-mediated effects of excess aldosterone include activation of vascular endothelial and smooth muscles cells resulting in vascular fibrosis and stiffness (9–13). These extra-renal aldosterone effects help to explain why many of the long-term adverse clinical outcomes associated with PA occur even independent of blood pressure as discussed below.

It is now well-known that PA is associated with disproportionately high rates of adverse cardiovascular outcomes including coronary artery disease, congestive heart failure, atrial fibrillation, and stroke (2, 14–26). A meta-analysis which consolidated many observational studies on this topic found that PA was associated with 77% higher odds of coronary artery disease, 2-fold higher odds of congestive heart failure, 3.5-fold higher odds of atrial fibrillation, and 2.5-fold higher odds of stroke compared with essential hypertension (27). Notably, this finding of increased cardiovascular risk with PA persisted even when the analysis was restricted to only studies where patients were matched based upon blood pressure thereby highlighting the blood pressure-independent effects of PA. Several studies have further reported an increased cardiovascular mortality with PA (20, 22).

The adverse long-term health outcomes associated with PA are not limited to only cardiovascular disease. A number of adverse metabolic and kidney outcomes have also been reported. PA is associated with a higher risk of both diabetes mellitus (2, 22, 27–36) as well as metabolic syndrome (27, 29, 37) compared with essential hypertension. A meta-analysis of observational studies showed that PA was associated with 33% higher odds of diabetes mellitus and 53% higher odds of metabolic syndrome as compared with essential hypertension (27). These associations are, at least in part, related to the fact that excess glucocorticoid co-secretion often occurs along with excess aldosterone secretion in patients with PA (30). Further, the autonomous aldosterone secretion inherent to PA decreases insulin secretion and increases insulin clearance (35). In regard to kidney disease, PA has been shown to lead to early hyperfiltration followed by a steeper decline in estimated glomerular filtration rate (eGFR) along with a higher incidence of proteinuria and chronic kidney disease (CKD) (8, 38–45).

Treatment Approach to Primary Aldosteronism

Based on the high risks of cardiovascular, metabolic, and kidney disease associated with PA, early diagnosis and targeted therapy are important to improve long-term patient outcomes. The longstanding convention for treating PA has been dependent on whether the disease is found to be unilateral or bilateral based on lateralization testing. For bilateral PA, the mainstays of treatment are dietary sodium restriction plus lifelong MR antagonist therapy (6). For unilateral PA, adrenalectomy is the recommended treatment of choice for patients healthy enough and willing to undergo surgery (6). Adrenalectomy is now typically performed *via* a laparoscopic, rather than an open, approach which has resulted in lower perioperative complication rates and shorter hospital stays (46–48). For patients with unilateral PA who are unable or unwilling to undergo adrenalectomy, the recommended treatment is the same as for bilateral PA: dietary sodium restriction plus lifelong MR antagonist therapy (6).

However, a natural question that arises from this conventional PA treatment algorithm is: 'Does surgical adrenalectomy provide clinical benefit beyond MR antagonist therapy in the treatment of unilateral PA?' Intuitively, adrenalectomy would be preferable in unilateral PA as it entails completely removing the source of autonomous aldosterone excess whereas MR antagonists would simply be blocking the interaction between aldosterone and the MR which is reliant on a number of factors including drug pharmacokinetics, optimal dosing, and patient compliance. However, there have been no randomized controlled trials to compare surgical versus medical therapy in unilateral PA. Such a trial has not been pursued due to the perceived lack of clinical

equipoise as the longstanding notion amongst PA experts is that surgery is superior. Moreover, such a study would require the more uniform use of AVS (which is typically reserved for patients suspected of possible unilateral PA who would be amenable to surgery) to definitively evaluate disease lateralization. This would incur both increased costs to the healthcare system as well as procedure-related risks to the patient. Instead, the majority of evidence informing the benefits of surgical versus medical therapy relies upon comparing outcomes with surgical adrenalectomy for unilateral PA versus MR antagonist therapy in bilateral PA (or with unconfirmed lateralization). This comparison is somewhat confounded as unilateral and bilateral PA differ in both their underlying pathophysiology as well as in their clinical presentation. For instance, somatic mutations known to contribute to autonomous aldosterone secretion are present in the vast majority of cases of unilateral PA (49, 50). In contrast, aldosterone-producing micronodules (previously termed aldosterone-producing cell clusters), which increase in prevalence with age, lead to the autonomous aldosterone secretion underlying many cases of bilateral PA (51, 52). Additionally, unilateral PA typically presents at a younger age and with a more overt clinical phenotype than bilateral PA (2, 5, 53). Bearing these caveats in mind, the following sections summarize the existing observational evidence of the benefits of surgical adrenalectomy in the treatment of unilateral PA.

BIOCHEMICAL OUTCOMES FOLLOWING SURGICAL ADRENALECTOMY FOR UNILATERAL PRIMARY ALDOSTERONISM

The biochemical changes that occur following surgical adrenalectomy for unilateral PA correlate with the physiologic sequelae that would be anticipated to arise from complete removal of the source of autonomous aldosterone excess (**Table 1**). Aldosterone levels are immediately reduced post-adrenalectomy. In turn, MR-mediated sodium reabsorption *via* the ENaC channel is reduced. The reduction in sodium reabsorption leads to volume contraction (i.e., reversal of the chronic volume expansion inherent to PA) and cessation of glomerular hyperfiltration thereby leading to 'un-suppression' of renin and angiotensin II. Also, with reduced MR-mediated sodium reabsorption *via* the ENaC channel, the concurrent excretion of potassium and hydrogen ions is similarly reduced. In sum, adrenalectomy for unilateral PA often results in normalization of the ARR, resolution of hypokalemia and metabolic alkalosis, and a decline in eGFR consistent with resolution of glomerular hyperfiltration. Standardized criteria defining biochemical success post-adrenalectomy have been set forth by the Primary Aldosteronism Surgery Outcomes (PASO) study (54). Below, we discuss the existing evidence for the rates of success in these biochemical outcomes post-adrenalectomy including PASO and other recent studies.

TABLE 1 | Biochemical outcomes following surgical adrenalectomy for unilateral primary aldosteronism.

BIOCHEMICAL OUTCOMES FOLLOWING ADRENALECTOMY
↓ Aldosterone
↑ Renin
Normalization of the Aldosterone-to-Renin Ratio (ARR)
Resolution of Hypokalemia
Resolution of Metabolic Alkalosis
Reversal of Glomerular Hyperfiltration (↓ eGFR)

Normalization of the Aldosterone-to-Renin Ratio

The PASO study defined 'complete biochemical success' post-adrenalectomy as normalization of the ARR as well as correction of hypokalemia (if present pre-surgery), assessed 6-12 months post-surgery (54). Among this multi-national cohort of 699 PA patients, a striking 94% of patients met the criteria of 'complete biochemical success'. Therefore, the vast majority of PA patients achieve a normal ARR with adrenalectomy thereby demonstrating resolution of renin-independent aldosterone secretion. The finding of a high success rate of ARR normalization with adrenalectomy has been confirmed by a number of other observational studies (55–63).

Resolution of Hypokalemia

While hypokalemia is certainly not a universal biochemical finding in PA (64), the vast majority of unilateral PA patients who do have hypokalemia become normokalemic post-adrenalectomy. For instance, a recent Japanese retrospective cohort study of 166 unilateral PA patients demonstrated that 82% of patients who were hypokalemic prior to adrenalectomy had resolution of the hypokalemia post-operatively (59). A similar study out of the United Kingdom reported that the median serum potassium levels increased from 3.2 (IQR 2.3-4.7) mmol/L to 4.4 (IQR 3.5-5.3) mmol/L following adrenalectomy (65).

Reduction in Glomerular Filtration Rate

As discussed above, adrenalectomy reverses the chronic glomerular hyperfiltration inherent to PA. This is reflected in decline in eGFR which should be anticipated post-adrenalectomy and usually does not indicate acute kidney injury (AKI) (8, 39–42, 44, 66). In fact, this sometimes results in CKD being 'unmasked' post-operatively. In a study of 25 unilateral PA patients who underwent adrenalectomy where GFR was measured pre- and six months post-adrenalectomy, the mean decline in GFR was 15 mL/min/1.73m^2 (8). Though on the surface, this initial GFR decline post-operatively may be viewed as a negative clinical effect, it should be noted that these changes are simply hemodynamic and that longer-term kidney outcomes improve following adrenalectomy. For instance, the GFR decline in the aforementioned study (8) correlated with a concurrent decline in albuminuria, a finding that has been confirmed by a number of other observational studies (8, 39, 41, 42). Further, longitudinal eGFR decline is slowed compared to treatment with MR antagonist therapy among patients with PA. A longitudinal cohort study of 120 PA patients treated with adrenalectomy, 400 PA patients treated with MR antagonists, and a control group of 15,474 patients with essential hypertension compared rates of

eGFR decline between these three groups (39). The results demonstrated that the mean annual rate of eGFR decline while similar between PA patients treated with adrenalectomy (-0.8 mL/min/1.73m^2) and patients with essential hypertension (-0.9 mL/min/1.73m^2) were slower than that of PA patients treated with MR antagonists (-1.6 mL/min/1.73m^2) (39). Taking this another step further, Kobayashi et al. demonstrated that the steeper the acute fall in eGFR with PA targeted treatments, the less steep the subsequent long-term decline in eGFR (66).

CLINICAL OUTCOMES FOLLOWING SURGICAL ADRENALECTOMY FOR UNILATERAL PRIMARY ALDOSTERONISM

While no randomized trials on this topic exist, the clinical benefits of surgical adrenalectomy versus MR antagonist therapy (**Table 2**) have been implied by a number of observational studies. These studies must be interpreted within the limitations of their observational design including selection and referral biases, a lack of uniformity in regard to how PA is defined, and inherent pathophysiologic differences between unilateral and bilateral PA. Moreover, the fact that patients treated with MR antagonists likely reflect a mix of unilateral and bilateral PA whereas adrenalectomy is generally reserved for true unilateral PA may further add some degree of bias to these findings.

Blood Pressure Control

The most commonly reported clinical benefits of surgical adrenalectomy for unilateral PA are in regard to blood pressure control. The PASO study defined 'complete clinical success' as normal blood pressure without the use of any antihypertensive medication at 6-12 months post-adrenalectomy (54). 'Partial clinical success' was defined as either a reduction in the number of antihypertensive medications or a reduction in blood pressure with the same number of antihypertensive medications at 6-12 months post-adrenalectomy. Among the 705 patients in the PASO study, 37% and 47% experienced complete or partial clinical success, respectively (54). Therefore, 84% of patients experienced either cure or significant improvement in blood pressure control with adrenalectomy. Reported rates of complete cure of hypertension following adrenalectomy for unilateral PA have ranged from 20-66% (55, 57, 58, 65, 67–71). These studies show that the vast majority of patients who do not achieve complete cure still have a significant improvement in blood pressure control as demonstrated by reduced blood pressure readings and/or reduced antihypertensive medication requirements. The finding that hypertension persists (though the severity is typically reduced) in many patients likely reflects the long delay, and concurrent exposure to excess aldosterone, that often exists between the timing of the onset of hypertension and the ultimate diagnosis of PA (62).

TABLE 2 | Clinical outcomes following surgical adrenalectomy for unilateral primary aldosteronism.

CLINICAL OUTCOMES FOLLOWING ADRENALECTOMY
Cure of Hypertension or Significant Improvement in Blood Pressure Control
↓ Cardiovascular Disease Risk
↓ Kidney Disease Risk
↓ Diabetes Risk
↓ Mortality
↑ Quality of Life

Cardiovascular Outcomes

Observational data also suggests superiority of adrenalectomy to MR antagonist therapy in regard to cardiovascular outcomes. This was demonstrated in a retrospective study comparing 205 patients with unilateral PA treated with adrenalectomy, 602 patients with PA treated with MR antagonists, and a comparator group of >40,000 patients with essential hypertension (22). The study defined incident cardiovascular events as a composite of myocardial infarction, coronary revascularization, hospital admission for congestive heart failure, or stroke. Despite similar blood pressure control between the groups, PA patients treated with MR antagonists experienced substantially higher rates of cardiovascular events as compared with PA patients treated with adrenalectomy (HR 3.27 [95% CI 1.93-5.55]) (22). Notably, the PA patients treated with adrenalectomy experienced lower rates of cardiovascular events than even patients with essential hypertension (HR 0.58 [95% CI 0.35-0.97]) (22). Similar benefits to adrenalectomy over MR antagonist therapy for PA has also been demonstrated in atrial fibrillation (21, 26) and left ventricular hypertrophy (72, 73).

End-Stage Kidney Disease Outcomes

In addition to the effects of adrenalectomy on eGFR discussed above, there is some evidence that adrenalectomy may lower the long-term risk of end-stage kidney disease (ESKD) compared with MR antagonist therapy. A Taiwanese study using population-wide administrative health data compared rates of ESKD between 2699 PA patients (including 657 who underwent adrenalectomy) and propensity score-matched essential hypertension patients (74). While accounting for the competing risk of death, the study found that PA patients treated with adrenalectomy had a lower risk of ESKD compared with patients with essential hypertension (sub-distribution HR 0.55, P = 0.02) (74). In contrast, PA patients treated with MR antagonists had a similar risk of ESKD compared with patients with essential hypertension (sub-distribution HR 1.08, P = 0.58) (74).

Metabolic Outcomes

Surgical adrenalectomy is also associated with a reduced risk of incident diabetes mellitus compared with MR antagonist therapy. A longitudinal population-based Taiwanese study of 2,367 PA patients without diabetes mellitus at baseline found that targeted therapy with an MR antagonist was associated with an increased risk of incident diabetes mellitus (HR 1.16, P < 0.001) whereas targeted therapy with adrenalectomy was associated with a reduced risk of incident diabetes mellitus (HR 0.61, P < 0.001) (31). These associations were confirmed

in a large United States observational study (22). The reduction in diabetes risk with adrenalectomy may be related to the fact that cortisol is frequently co-secreted from aldosterone producing adenomas (30, 75). Indeed, targeted treatment in PA is associated with increased insulin sensitivity and decreased insulin clearance (35).

Mortality

The aforementioned Taiwanese population-based studies also suggest a reduction in mortality among patients with unilateral PA treated with adrenalectomy compared with patients with essential hypertension (23, 74). Chen et al. reported no significant difference in mortality comparing PA treated with MR antagonists versus essential hypertension (HR 1.05 [95% CI 0.94-1.14]) (74). In contrast, PA treated with adrenalectomy was associated with a reduced risk in mortality compared with essential hypertension (HR 0.22 [95% CI 0.15-0.34]) (74).

Quality of Life

While both adrenalectomy and MR antagonists improve quality of life measures for patients with PA, adrenalectomy has been shown to improve these measures both more rapidly and more robustly (63, 76–78). For instance, two Australian studies examined changes in quality of life among patients with unilateral PA treated and patients with PA treated with spironolactone or amiloride (63, 76). At the time of diagnosis and prior to targeted treatment, patients with PA had lower quality of life scores as assessed by the Medical Outcomes Study Short Form 36 General Health Survey (SF-36). While quality of life measures were seen to improve within three months among patients with unilateral PA treated with adrenalectomy (63), improvement was not seen until six months among patients with bilateral PA treated with medical therapy (76). Notably, the degree of improvement in quality of life seen after six months for bilateral PA treated with medical therapy was lower than that

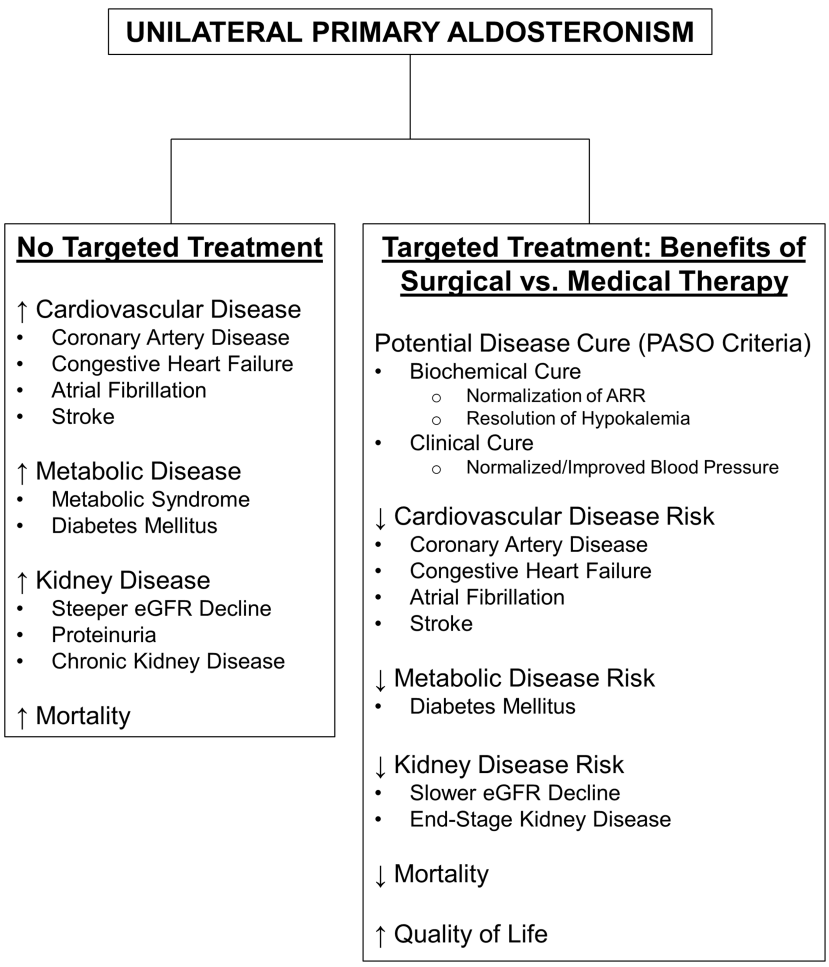

FIGURE 1 | Summary of the Health Impact of Untreated Primary Aldosteronism and the Benefits of Surgical vs. Medical Therapy in the Treatment of Unilateral Primary Aldosteronism. ARR, aldosterone-to-renin ratio; eGFR, estimated glomerular filtration rate; PASO, Primary Aldosteronism Surgery Outcomes study (54).

seen within the same time frame for unilateral PA treated with adrenalectomy (63, 76).

CONCLUSION

Early diagnosis and targeted treatment for PA are critical given the disproportionate morbidity and mortality linked with this condition. Moreover, identifying disease lateralization early in the diagnostic algorithm is necessary to provide optimal, personalized care for PA patients. Guidelines recommend surgical adrenalectomy over medical therapy with MR antagonists for patients with unilateral PA who are healthy enough and willing to undergo surgery. In addition to a high degree of success in reversing the biochemical abnormalities associated with PA, a key benefit of adrenalectomy over MR antagonists for unilateral PA is the potential for disease cure as the source of autonomous aldosterone excess is fully removed rather than its effect simply being blocked. More importantly, despite biases inherent to the existing observational literature, the sheer abundance of evidence suggesting improved clinical outcomes with adrenalectomy versus MR antagonists continues to mount. These include significant reductions in blood pressure, cardiovascular events, kidney disease, diabetes mellitus, and mortality along with improvements in quality of life as summarized in **Figure 1**. As a whole, these findings strongly support the current guideline recommendations advising surgical adrenalectomy to treat unilateral PA.

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Conception and design of the review and drafting of the manuscript: SA and GH. All authors contributed to the article and approve the submitted version.

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Treatment of Primary Aldosteronism and Reversal of Renin Suppression Improves Left Ventricular Systolic Function

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Introduction: Primary aldosteronism (PA) is associated with increased risk of cardiovascular events. However, treatment of PA has not been shown to improve left ventricular (LV) systolic function using the conventional assessment with LV ejection fraction (LVEF). We aim to use speckle-tracking echocardiography to assess for improvement in subclinical systolic function after treatment of PA.

Methods: We prospectively recruited 57 patients with PA, who underwent 24-h ambulatory blood pressure (BP) measurements and echocardiography, including global longitudinal strain (GLS) assessment of left ventricle, at baseline and 12 months post-treatment.

Results: At baseline, GLS was low in 14 of 50 (28.0%) patients. On multivariable analysis, GLS was associated with diastolic BP ($P = 0.038$) and glomerular filtration rate ($P = 0.026$). GLS improved post-surgery by −2.3, 95% CI: −3.9 to −0.6, $P = 0.010$, and post-medications by −1.3, 95% CI: −2.6 to 0.03, $P = 0.089$, whereas there were no changes in LVEF in either group. Improvement in GLS was independently correlated with baseline GLS ($P < 0.001$) and increase in plasma renin activity ($P = 0.007$). Patients with post-treatment plasma renin activity ≥1 ng/ml/h had improvements in GLS ($P = 0.0019$), whereas patients with persistently suppressed renin had no improvement. Post-adrenalectomy, there were also improvements in LV mass index ($P = 0.012$), left atrial volume index ($P = 0.002$), and mitral E/e' ($P = 0.006$), whereas it was not statistically significant in patients treated with medications.

Conclusion: Treatment of hyperaldosteronism is effective in improving subclinical LV systolic dysfunction. Elevation of renin levels after treatment, which reflects adequate reversal of sodium overload state, is associated with better systolic function after treatment.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier: NCT03174847.

Keywords: hyperaldosteronism, adrenalectomy, secondary hypertension, adrenal vein sampling (AVS), myocardial strain analysis, ejection fraction (EF)

INTRODUCTION

Up to 20% of all patients with hypertension have primary aldosteronism (PA), making it the most common treatable cause of hypertension (1). Compared with patients with essential hypertension (EH) matched for age, gender, and blood pressure (BP), patients with PA are at higher risk of cardiovascular disease, renal failure, and poorer quality of life (2–4), attributed to the direct deleterious effects of aldosterone. Hyperaldosteronism induces left ventricular (LV) hypertrophy and fibrosis, which leads to LV remodeling and dysfunction. Patients with PA have increased LV mass index (LVMI), wall thickness, concentric remodeling, and LV diastolic dysfunction, which can be reversed with treatment (5). However, there are limited data on benefits of treatment on systolic function.

Systolic function is usually assessed with LV ejection fraction (LVEF), which has poor sensitivity, particularly in the presence of LV hypertrophy (6). LVEF impairment only occurs in end stage hypertensive heart disease. Other limitations include suboptimal reproducibility, inability to reflect regional LV function, and being a volume-centric measurement. Previous studies using LVEF have not found differences in patients with PA, compared with those with EH (7). In contrast, systolic function can also be assessed using myocardial strain, which represents a change in myocardial length from relaxed to contractile state, and assessed in different spatial components, e.g., longitudinal, circumferential, or radial. Myocardial strain is most commonly assessed using speckle-tracking echocardiography (8) on conventional B-mode echocardiography images, which is widely available. Speckle-tracking echocardiography offers high temporal and spatial resolutions, with good correlation with tagged magnetic resonance imaging, the reference method of strain assessment (9). Echocardiography-measured peak systolic global longitudinal strain (GLS) has been most extensively studied and can demonstrate impairment of systolic function before reduced LVEF.

Chen and colleagues demonstrated that patients with PA have impaired LV systolic function using GLS, compared with patients with EH (8), but did not perform repeat assessment after treatment. Although PA can be treated with mineralocorticoid (MR) antagonists, patients with unilateral PA can be offered curative adrenalectomy. Recent studies have found that surgery ameliorated the excess risk of cardiovascular events (10) and progression of renal disease (11). However, in patients with PA treated medically, the excess risk was only ameliorated in patients with unsuppressed renin levels post-treatment, suggesting that adequate medical treatment is important to reverse the deleterious effects of hyperaldosteronism. Hence, we conducted a prospective study in patients with PA, to assess for changes in LV GLS after both surgical and medical treatment.

METHODS

We prospectively recruited 57 patients with PA from February 2017 to October 2019 in Changi General Hospital, Singapore, in PA_PACES study (Primary Aldosteronism Prospective study Assessing Cardiovascular, Endothelial and other outcomes post-Surgical and medical treatment). The study was approved by local ethics committee, and informed consent was obtained from all patients (Clinicaltrials.gov:NCT03174847). Detailed baseline demographic characteristics and medical history were recorded.

Diagnostic Tests for PA

Inclusion criteria were age 18 years and older and diagnosis of PA in accordance with the Endocrine Society guidelines (12). Exclusion criteria were patients with a terminal condition or glucocorticoid-remediable hyperaldosteronism. Plasma aldosterone concentration (PAC) and plasma renin activity (PRA) were measured as previously reported (13). Before hormonal measurements, MR antagonists were withdrawn at least 6 weeks in all patients, whereas medications that may interfere with PAC and PRA (e.g., ACE inhibitors and diuretics) were withdrawn at least 2 weeks when possible. Patients with aldosterone-renin ratio (ARR) greater than 554 (pmol/L per ng/ml/h) underwent confirmatory testing with seated intravenous saline infusion test, and all patients had a post-saline PAC of ≥138 pmol/L.

Subtype Tests

Thin-sliced computed tomography (CT) of the adrenal glands was performed in all patients. In patients keen to undergo unilateral adrenalectomy, adrenal vein sampling (AVS) was performed sequentially under continuous corticotropin infusion by an experienced interventional radiologist (KSN) in majority of cases (14). Samples were taken from both adrenal veins and infrarenal vena cava (peripheral vein). AVS was successful if cortisol levels in both adrenal veins were at least three times that of peripheral vein. Lateralization ratio was determined by the higher adrenal aldosterone-cortisol ratio, divided by the contralateral adrenal aldosterone-cortisol ratio. Lateralization ratio of >4 was consistent with unilateral PA and of <3 was consistent with bilateral PA. Patients with ratio

between 3 and 4 were discussed at a multidisciplinary meeting to determine subtype diagnosis and management. Patients treated surgically underwent unilateral adrenalectomy *via* minimally invasive transabdominal approach.

Ambulatory Blood Pressure and 2D Echocardiography

All patients were scheduled to undergo a 24-h ambulatory BP monitoring and two-dimensional (2D) echocardiography (2DE) at baseline and 12 months post-treatment. Echocardiographic ultrasound systems equipped with speckle tracking, Philips, and General Electric were used to perform echocardiographic examinations. Transthoracic echocardiographic images were acquired at the enrolling centre following a study-specific acquisition protocol. Conventional measurements were analyzed by an independent core laboratory in our clinical measurement unit comprising senior sonographers. LV dimensions, septal, and posterior wall thickness were measured *via* the parasternal long axis view in accordance with the guidelines (15). Echocardiographic LVMI was calculated by applying the ASE M-mode equation. LVEF and left atrial volume index (LAVI) were measured by biplane area–length method (16). Speckle-tracking results were analyzed offline using a semi-automated algorithm (Cardiac Performance Analysis, a module of TomTec Arena; TomTec Imaging Systems, Unterschleissheim, Germany). The endocardial border was automatically detected and then tracked throughout the cardiac cycle using speckle-tracking technology. Manual adjustments were applied when needed to optimize boundary position only when necessary. GLS was calculated as the average of the magnitude of peak longitudinal strain from 17 ventricular segments, which were obtained from apical four-chamber, three-chamber, and two-chamber views (17). All strain analyses were performed by an experienced sonographer who was familiar with strain analysis and blinded to the clinical status of the patients. Normal LV GLS at our laboratory is less than −18%. Intra- and inter-observer reproducibility was estimated in 10 randomly selected subjects, and the coefficients of variation for GLS were 4.2% and 7.5% respectively.

Outcomes

After at least 6 months post-treatment, all patients were reassessed for biochemical and clinical outcomes according to Primary Aldosteronism Surgery Outcome (PASO) consensus (18). Antihypertensive medications were recorded as number of medications and defined daily dosage (DDD) (https://www.whocc.no/atc_ddd_index/). Clinic BP was taken with an automated machine in all patients after five minutes of rest, from the arm in a seated position. In patients without a pre- or post-treatment 24-h ambulatory BP, clinic BP readings were used to calculate changes after treatment. Cure of hypertension was defined as post-treatment ambulatory daytime systolic BP below 135 mmHg and diastolic BP below 85 mmHg (systolic BP below 140 mmHg and diastolic BP below 90 mmHg, if clinic BP was used), without use of antihypertensive medications.

Statistical Analysis

Statistical analysis was conducted using R (a language and environment for statistical computing; R Foundation for Statistical Computing, Vienna, Austria; https://www.R–project.org/). Continuous variables were expressed as a mean and standard deviation (SD) or median, minimum and maximum, or first and third quartile, as appropriate, and categorical variables were presented as number (percent). Groups were compared using independent samples *t*-test or Mann–Whitney U-test for continuous variables and chi-squared test or Fisher exact test for categorical variables, as appropriate. Post-treatment changes were compared using paired *t*-test or Wilcoxon signed-rank test for continuous variables and McNemar's test for categorical variables, as appropriate. Univariate linear regression analysis was performed to investigate the association between GLS and clinical parameters at baseline. Significant determinants in the univariate linear regression analysis ($P < 0.1$) were then examined using multivariable linear regression analysis. This was similarly conducted to test for association between change in GLS and clinical parameters. Significance level was set at *P*-value of <0.05.

RESULTS

Eighty-six consecutive patients with ARR of >554 underwent confirmatory saline-infusion test, whereas two patients had PAC of >554 pmol/L, suppressed PRA, and spontaneous hypokalemia and did not require confirmatory testing. Of 61 patients confirmed with PA, we recruited 57 patients, mean age 54.8 ± 11.0 years, and 17 females (29.8%) (**Figure 1**). Four patients had chronic kidney disease, whereas five patients were not keen for adrenalectomy. The remaining 48 patients underwent AVS, with all patients having successful AVS. Among 29 patients with lateralization on AVS, 25 patients proceeded to unilateral adrenalectomy, whereas four patients opted for medical treatment despite lateralization on AVS: one for personal reasons and three had lateralization ratios of 4.0, 4.1, and 4.1, of which two patients' AVS lateralized to the opposite side of an adrenal adenoma. Hence, 32 patients were treated with medications: 28 patients with spironolactone, and four patients with eplerenone and/or amiloride. The final median daily dose of spironolactone in patients medically treated was 37.5 (IQR: 25–75) mg. Patients treated with surgery were younger, had more severe hypokalemia, and were less likely to have ischemic heart disease and hyperlipidemia (**Table S1**) (19). In addition, patients treated surgically had a higher baseline daytime systolic and diastolic BP, 153 ± 16 mmHg and 94 ± 9 mmHg, respectively, compared with those on medications, 144 ± 12 and 85 ± 10 mmHg, respectively, $P = 0.025$ and $P = 0.001$.

2DE Parameters and Changes With Treatment

At baseline, 50 patients underwent 2DE (**Figure 1**). Baseline GLS was low (above −18) in 14 (28.0%) patients, of which 8 of 24 (33.3%) were treated surgically, and 6 of 26 (23.1%) were treated

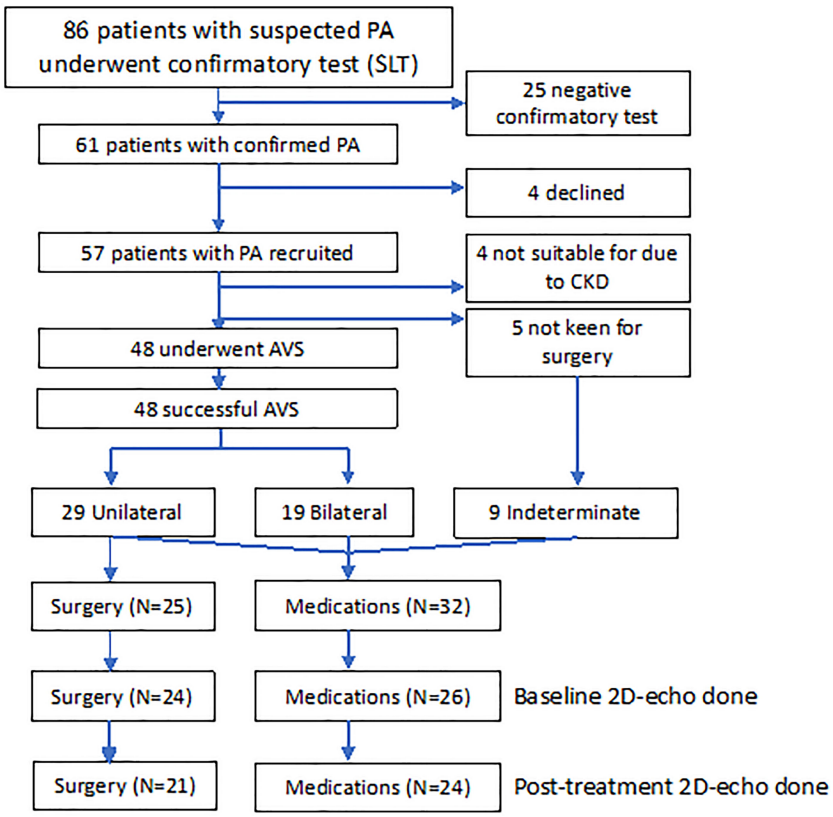

FIGURE 1 | Consort diagram of 57 patients with PA recruited into study treated with adrenalectomy (n = 25) and medications (n = 32). AVS, adrenal vein sampling; CKD, chronic kidney disease; SLT, saline-loading test.

medically, with no statistical difference between the groups, P = 0.53. Absolute baseline GLS also did not differ between the surgical and medical groups, −19.3 ± 3.5 versus −20.0 ± 3.1, respectively, P = 0.31.

LV GLS improved post-surgery by −2.3, 95% CI: −3.9 to −0.6, P = 0.010, and post-medications by −1.3, 95% CI: −2.6 to 0.03, P = 0.056. Overall, the proportion of patients with low GLS also improved post-treatment, from 12 (26.7%) to 5 (11.1%), P = 0.039. Post-adrenalectomy, there were improvements in LVMI by −10.0, 95% CI: −18.0 to −2.4, P = 0.012, LAVI by −5.0, 95% CI: −9.1 to −2.2, P = 0.002, and mitral E/e' −1.9, 95% CI: −4.1 to −0.5, P = 0.006 (**Figure 2**). Post-medications, there was trend toward similar improvements in LVMI, LAVI, and mitral E/e', but these did not reach statistical significance (**Figure 3**).

Other Post-Treatment Changes

In both treatment groups, there were increases in potassium and PRA and declines in ARR and eGFR post-treatment (**Table 1**). In addition, PAC levels decreased in patients post-surgery. Surgical patients had higher baseline BP, and there was improvement in daytime and nighttime systolic and diastolic BP (all $P \leq 0.01$) post-surgery. Medical patients did not have significant BP decline post-medications. However, post-treatment BP achieved was similar in both groups. Patients treated with surgery showed reduction in number of antihypertensive medications by 1.0 antihypertensive medication, 95% CI: 0.8 to 1.7, $P < 0.001$, wereas patients with medications had an increase by 1.0 antihypertensive medication, 95% CI: 0.02 to 0.9, P = 0.033.

Linear Regression Analysis

On univariate analysis, baseline GLS was associated with baseline diastolic BP (**Table 2**), with an unadjusted coefficient 0.090, 95% CI: 0.005 to 0.175, P = 0.038. On multivariable analysis, after including baseline diastolic BP, both baseline diastolic BP, adjusted coefficient 0.090, 95% CI: 0.005 to 0.175, P = 0.038, and baseline glomerular filtration rate (GFR), adjusted coefficient −0.059, 95% CI:−0.111 to −0.008, P = 0.026, were correlated with baseline GLS.

On univariate analysis, improvement in GLS was associated with baseline daytime diastolic BP, baseline GLS, and change in log PRA (**Table 3**). On multivariable analysis, only baseline GLS, adjusted coefficient −0.534, 95% CI: −0.781 to −0.282, $P < 0.001$, and increase in log PRA, adjusted coefficient −1.428, 95% CI:−2.438 to −0.417), P = 0.007, were associated with improvement in GLS.

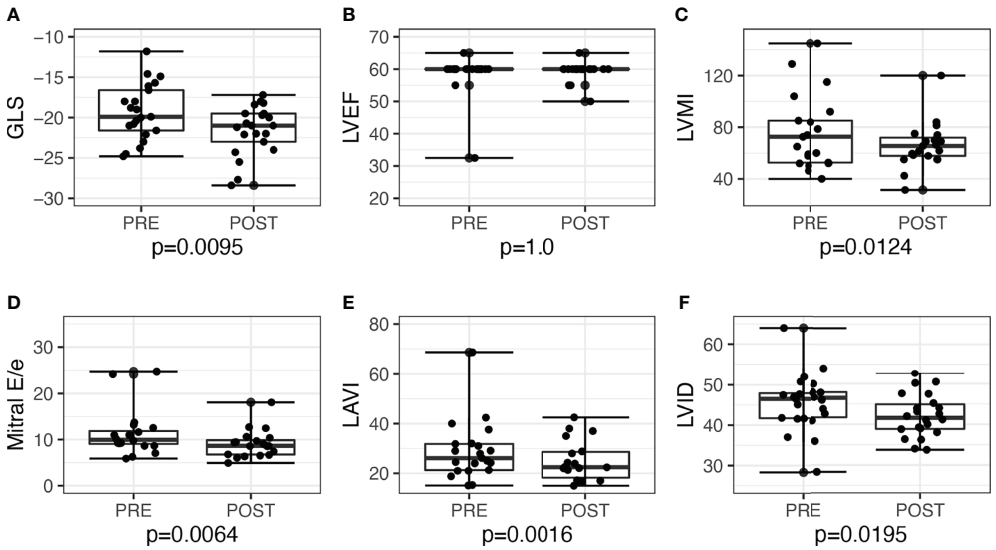

FIGURE 2 | Echocardiographic parameters before and after adrenalectomy among patients with unilateral primary aldosteronism (n = 21). Improvement of left ventricular systolic function seen when assessed with **(A)** global longitudinal strain (GLS), but not with **(B)** left ventricular ejection fraction (LVEF). Improvements seen post-adrenalectomy in other parameters as well: **(C)** left ventricular mass index (LVMI); **(D)** mitral E/e'; **(E)** left atrial volume index (LAVI); **(F)** left ventricular internal dimension (LVID).

Changes of GLS in Subgroups

When stratified by post-treatment renin response, there was a significant decline in post-treatment GLS of −2.45, 95% CI: −3.48 to −0.84, P = 0.0019, among 30 patients who achieved post-treatment PRA ≥1 ng/ml/h, whereas there was no significant change in GLS of −0.7, 95% CI −2.53 to 0.72, P = 0.18, among 13 patients with persistent PRA <1 ng/ml/h (six treated with surgery and seven treated with medications) (**Figure 4**). We further restricted analysis only to medically–treated patients with similar results, demonstrating in 15 patients with PRA

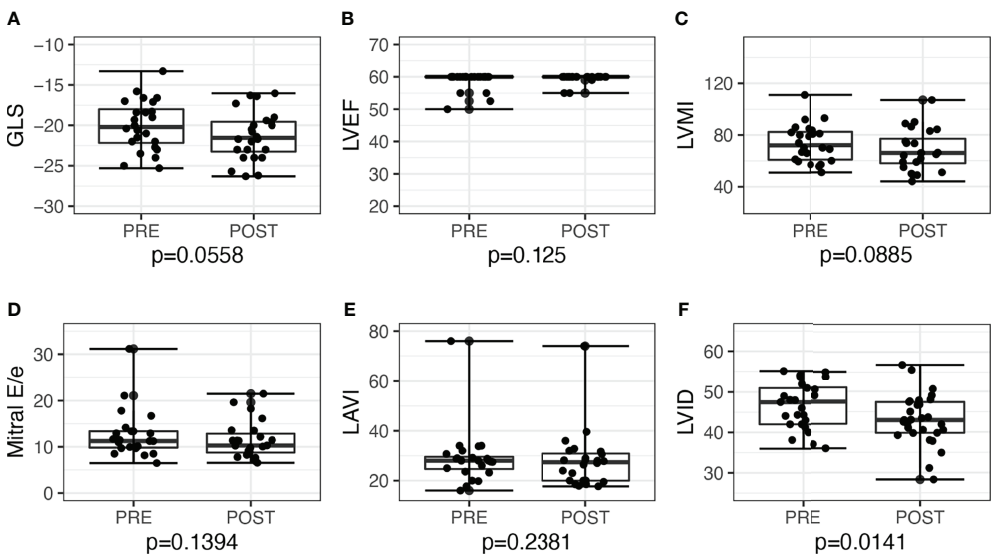

FIGURE 3 | Echocardiographic parameters before and after medical treatment among patients with primary aldosteronism (n = 24). **(A)** Global longitudinal strain (GLS) but not in **(B)** left ventricular ejection fraction (LVEF). **(C)** Left ventricular mass index (LVMI); **(D)** mitral E/e'; **(E)** left atrial volume index (LAVI); **(F)** Left ventricular internal dimension (LVID).

TABLE 1 | Changes in biochemical, 24-h ambulatory blood pressure and echocardiographic parameters from baseline to 12 months after surgical and medical treatment in patients with primary aldosteronism.

Variable	Surgical, n = 25					Medical, n = 32				
	n	Pre	Post	Diff (95% CI)	p-value	n	Pre	Post	Diff (95% CI)	p-value
Potassium, mmol/L	25	3.7 ± 0.4	4.3 ± 0.4	0.6 (0.4, 0.8)	<.0001	32	3.7 ± 0.4	4.1 ± 0.6	0.4 (0.1, 0.6)	0.005
eGFR	24	85.4 (51.5 to 111.1)	81.2 (31.6 to 106.5)	–5.18 (–10.6, –0.70)	0.037	32	76.2 (28.4 to 101.8)	67.1 (11.6 to 97)	–5.01 (–11, –2.51)	0.003
PAC	25	948 (388 to 1939)	552 (213 to 2354)	–804 (–1030, –672)	<.0001	24	491 (252 to 1496)	363 (139 to 2314)	–82 (–171, 397)	0.89
PRA	25	0.6 (0.2 to 2.3)	1.8 (0.6 to 19)	1 (0.8, 4.7)	<.0001	30	0.6 (0.2 to 2.5)	1.3 (0.6 to 14)	0.9 (0.9, 3.1)	<.0001
ARR	25	1790 (554 to 8367)	79.8 (7 to 205)	–1,689 (–3,295, –1,600)	<.0001	24	1229 (216 to 7133)	448 (8 to 2409)	–565 (–1861, –522)	<.0001
Daytime Systolic BP, mmHg	22	151.8 ± 16.4	136.4 ± 14.1	–15.4 (–25.0, –5.9)	0.003	26	142.1 ± 12.1	137.2 ± 17.1	–4.9 (–12, 2.2)	0.17
Daytime Diastolic BP, mmHg	22	93.1 ± 9.3	86.5 ± 11.1	–6.7 (–11.6,–1.8)	0.010	26	85.4 ± 10.5	85.2 ± 11	–0.2 (–4, 3.6)	0.92
Night–time Systolic BP, mmHg	22	145.1 ± 14.6	130.3 ± 14.9	–14.8 (–22.0, –7.6)	<.0001	26	135.8 ± 16.0	131.3 ± 27.2	–4.5 (–13.5, 4.5)	0.31
Night–time Diastolic BP, mmHg	22	87.4 ± 8.3	78 ± 10.3	–9.4 (–14.3, –4.5)	<.0001	26	79.3 ± 1 0.5	77.8 ± 13.4	–1.5 (–6.2, 3.2)	0.53
LVEF, %	21	60 (32.5 to 65)	60 (50 to 65)	0 (–1.5, 2.7)	1.0	24	60 (50 to 60)	60 (55 to 60)	0 (0.0, 1.4)	0.13
IVS, mm	21	9.22 ± 2.1	9.25 ± 2.24	0.03 (–0.53, 0.58)	0.92	24	9.22 ± 1.59	8.94 ± 1.62	–0.28 (–1.03, 0.46)	0.44
LVPW, mm	21	8.96 ± 1.94	8.51 ± 1.93	–0.45 (–1.07, 0.16)	0.14	24	8.27 ± 1.17	8.42 ± 1.3	0.15 (–0.46, 0.76)	0.62
LVMI, g/m^2	21	72.6 (40 to 144.9)	65.5 (31.4 to 120)	–10.0 (–18.0, –2.4)	0.012	24	72 (51 to 111)	66 (44 to 107)	–5 (–11.2, 0.3)	0.089
RWT	21	0.40 ± 0.09	0.42 ± 0.14	0.02 (–0.03, 0.06)	0.45	24	0.37 ± 0.08	0.40 ± 0.09	0.03 (–0.02, 0.07)	0.21
GLS	21	–19.3 ± 3.5	–21.6 ± 3.0	–2.3 (–3.9, –0.6)	0.010	24	–20.0 ± 3.1	–21.3 ± 3.0	–1.3 (–2.6, 0.03)	0.056
LAVI, ml/m^2	21	26.1 (15.1 to 68.6)	22.1 (11.5 to 42.5)	–5 (–9.1, –2.2)	0.002	24	28.0 (16.0 to 76.0)	27.4 (17.7 to 74.0)	–0.7 (–3.4, 1.4)	0.24
LVID, mm	21	45.2 ± 7.2	42.3 ± 5.3	–2.9 (–5.2, –0.5)	0.020	23	46.2 ± 5.9	43 ± 6.9	–3.3 (–5.8, –0.7)	0.014
Mitral E/e'	20	10 (5.9 to 24.7)	8.6 (4.9 to 18.1)	–1.9 (–4.1, –0.5)	0.006	23	11.3 (6.5 to 31.2)	10.3 (6.6 to 21.5)	–1.5 (–2.8, 0.5)	0.14
Patients with impaired LVEF <50%, %	21	2 (9.5%)	1 (4.8%)	–	0.32	24	0 (0%)	0 (0%)	–	NA
Patients with low GLS >–18, %	21	6 (28.6%)	1 (4.8%)	–	0.025	24	6 (25.0%)	4 (16.7%)	–	0.32
Antihypertensive medications, number	25	2 (1 to 5)	1 (0 to 2)	–1 (–1.7, –0.8)	<.0001	32	2 (0 to 4)	3 (0 to 5)	1 (0.02, 0.9)	0.033
Antihypertensive medications, number, DDD	25	3 (0.4 to 10.8)	1 (0 to 6)	–2 (–3.2, –1.3)	<.0001	32	2 (0 to 4)	3 (0 to 5)	0.2 (–0.7, 0.6)	0.80

ARR, aldosterone-renin ratio; BP, blood pressure; DDD, defined daily dose; E/e', early diastolic transmitral and myocardial velocity on tissue Doppler imaging ratio; eGFR, estimated glomerular filtration rate; IVS, interventricular septum; LAVI, left atrium volume index; GLS, global longitudinal strain; LVEF, left ventricular ejection fraction; LVID, left ventricular internal dimension; LVMI, left ventricular mass index; LVPW, left ventricular posterior wall thickness; PAC, plasma aldosterone concentration; PRA, plasma renin activity; RWT, relative wall thickness in diastole.

Continuous variables reported as mean ± standard deviation or median (minimum to maximum) and compared between pre and post-treatment using paired t-test or Wilcoxon signed-rank test depending on normality assumption; categorical variable presented as frequency (%), and compared between pre and post treatment using McNemar's test.

NA, Not Applicable.

≥1ng/ml/h an improvement in GLS of –1.7, 95% CI: –3.4 to –0.1, P = 0.042, compared with no change in seven patients with persistent PRA <1 ng/ml/h, of +0.1, 95% CI: –2.8 to 3.1, P = 0.91.

When stratified by post-treatment BP changes, patients who showed improvements in both systolic and diastolic BP had improvements in GLS of –2.1, 95% CI: –3.6 to –0.7, P = 0.006, whereas patients without improvement in BP had a tendency toward improvement in GLS of –1.1, –2.5 to 0.2, P = 0.088.

PASO Outcome

Among 25 patients who underwent surgery, all 25 patients had complete biochemical cure as defined by PASO, with resolution

TABLE 2 | Univariate and multivariable linear model of baseline global longitudinal strain (GLS) in all patients treated for primary aldosteronism (n = 45).

Variable	Univariate		Multivariable *	
	Un-Adjusted BetaCoefficient (95% CI)	***p*-value**	**Adjusted BetaCoefficient (95% CI)**	***p*-value**
Female gender	–0.200 (–2.44, 2.042)	0.86	–0.114 (–2.282, 2.054)	0.92
Age	–0.004 (–0.096, 0.089)	0.94	0.065 (–0.039, 0.169)	0.22
BMI, g/m2	0.056 (–0.144, 0.256)	0.58	0.009 (–0.190, 0.208)	0.93
Systolic BP, mmHg	0.013 (–0.051, 0.078)	0.68	–0.049 (–0.128, 0.031)	0.23
Diastolic BP, mmHg	**0.090 (0.005, 0.175)**	**0.038**	**0.090 (0.005, 0.175)**	**0.038**
Baseline antihypertensive medications, number	0.032 (–0.852, 0.916)	0.94	0.376 (–0.523, 1.276)	0.40
Baseline potassium	–1.45 (–3.90, 0.998)	0.24	–1.26 (–3.64, 1.121)	0.29
Baseline eGFR	–0.034 (–0.087, 0.018)	0.19	**–0.059 (–0.111, –0.008)**	**0.026**
Baseline log PAC	–0.177 (–2.07, 1.719)	0.85	–0.734 (–2.62, 1.154)	0.44
Baseline log PRA	–1.150 (–5.749, 3.45)	0.62	–0.813 (–5.274, 3.649)	0.72
Baseline log ARR	0.139 (–1.07, 1.343)	0.82	–0.156 (–1.35, 1.041)	0.79

ARR, aldosterone-renin ratio; BP, blood pressure; eGFR, estimated glomerular filtration rate; PAC, plasma aldosterone concentration; PRA, plasma renin activity.
Linear regression analysis was performed to calculate the beta coefficients and 95% confidence intervals.
** Adjusted for DBP.*
Bold values are statistically significant.

of hypokalemia and normalization of ARR. Three patients had complete clinical success, 17 had partial clinical success, and five had no clinical response. Among 32 patients treated medically, 12 had persistent renin suppression (PRA <1ng/ml/h), whereas four had hypokalemia at their 1-year clinic visit.

DISCUSSION

We demonstrated that treatment of PA can improve subclinical LV systolic function, and this improvement was associated with a rise in renin levels. This provides further support that reversal of renin suppression is important in ameliorating the excess cardiovascular risk seen in PA. In addition, baseline GLS was associated with baseline renal function and diastolic BP, suggesting either an interplay between cardiac and renal dysfunction (cardiorenal syndrome) or more likely hyperaldosteronism contributing to pathology in both organs. Assessment with speckle-tracking echocardiography was more sensitive in detecting improvement in systolic function compared with conventional LVEF assessment. In our study, surgery led to greater improvements in cardiac function

TABLE 3 | Univariate and multivariable linear model of Δglobal longitudinal strain (GLS) before and after treatment in all patients treated for primary aldosteronism (n = 45).

Variable	Univariate		Multivariable*	
	Un-Adjusted BetaCoefficient (95% CI)	**p-value**	**Adjusted BetaCoefficient (95% CI)**	**p-value**
Female Gender	0.593 (–1.66, 2.843)	0.60		
Age, years	0.032 (–0.059, 0.124)	0.48		
BMI, kg/m^2	–0.067 (–0.271, 0.137)	0.51		
Baseline systolic BP, mmHg	–0.044 (–0.109, 0.022)	0.19		
Baseline diastolic BP, mmHg	–0.089 (–0.179, 0.001)	0.053	–0.068 (–0.143,.008)	0.077
Baseline antihypertensive medications, number	–0.216 (–1.12, 0.692)	0.63		
Baseline potassium	0.490 (–2.05,3.028)	0.70		
Baseline eGFR	–0.011(–0.067,0.043)	0.68		
Baseline log PAC	–0.118 (–2.08, 1.845)	0.90		
Baseline log PRA	1.325 (–3.41, 6.06)	0.56		
Baseline log ARR	–0.235 (–1.50,1.026)	0.71		
Baseline GLS	**–0.610 (–0.864, –0.355)**	**<.0001**	**–0.534 (–0.781, –0.282)**	**<.0001**
Δ potassium	0.607 (–0.925, 2.138)	0.43		
Δ eGFR	0.023 (–0.069,0.114)	0.62		
Δ systolic BP, mmHg	0.007 (–0.044,0.058)	0.77		
Δ diastolic BP, mmHg	0.080 (–0.014, 0.174)	0.094		
Δ log PAC	0.120 (–1.340, 1.580)	0.87		
Δ log PRA	**–1.36 (–2.62, –0.097)**	**0.036**	**–1.428 (–2.438, –0.417)**	**0.007**
Δ LVMI	0.045 (–0.021, 0.110)	0.18		
Δ LAVI	–0.047 (–0.195, 0.100)	0.52		
Δ antihypertensive medications, number	0.159 (–0.562, 0.879)	0.66		

ARR, aldosterone-renin ratio; BP, Blood Pressure; eGFR, estimated glomerular filtration rate; PAC, plasma aldosterone concentration; PRA, plasma renin activity.
Linear regression analysis was performed to calculate the beta coefficients and 95% confidence intervals.
** Backward selection criteria: significant level of stay = 0.1.*

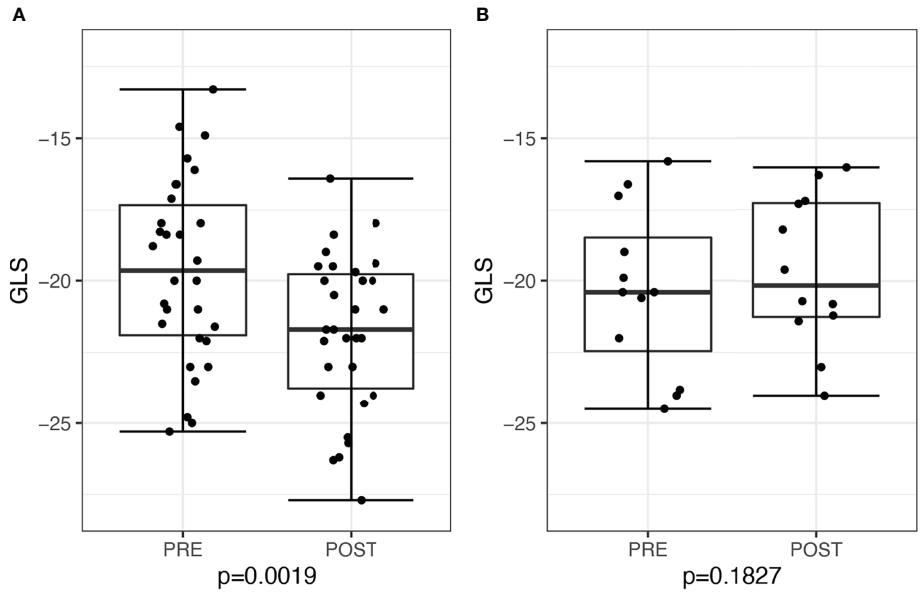

FIGURE 4 | Global longitudinal score (GLS) in patients before and after treatment for primary aldosteronism stratified by post-treatment plasma renin activity (PRA). **(A)** Patients with post-treatment PRA >1 ng/ml/h (n = 30) and **(B)** patients with post-treatment PRA ≤1 ng/ml/h (n = 13).

compared with medical treatment, which underlines the importance of subtyping PA and offers curative adrenalectomy for unilateral disease.

Although patients with PA are at increased risk of cardiac failure, there are limited data to show that treatment of PA can improve LV systolic function (20). A previous study found that patients with PA had a subclinical LV systolic dysfunction compared with those with EH, using GLS (8). We have now demonstrated improvement of LV systolic function with adrenalectomy for PA and, to a lesser extent, with medications. Similar to previous studies, we did not find changes in LVEF post-treatment (7, 13), highlighting the limited sensitivity of LVEF. GLS analysis using 2D speckle-tracking echocardiography is more sensitive and more reproducible (9), leading expert committees to recommend its routine use to monitor patients during chemotherapy for cardiotoxicity (21). Improvements in GLS have also been observed in treatment of patients with other forms of secondary hypertension. Dobrowolski and colleagues demonstrated that resection of a pheochromocytoma or paraganglioma led to improvements in LV subclinical systolic dysfunction assessed by GLS, highlighting the deleterious effects of catecholamine excess on the myocardium (22).

Of particular interest, we found that improvements in systolic function correlated with reversal of renin suppression and baseline GLS. Studies by Hundemer and colleagues found that patients with PA treated with medications with persistent renin suppression (PRA < 1 ng/ml/h) remained at high cardiovascular risk of ischemic events, atrial fibrillation, and cardiac death, whereas those with unsuppressed renin (PRA ≥ 1 ng/ml/h) had similar risk to patients with EH (10, 23). In our study, improvement in GLS was only observed in the group of patients with post-treatment PRA ≥ 1 ng/ml/h, and not among those with persistent renin suppression. These findings support the notion that adequate reversal sodium and volume overload, reflected by a rise in PRA, is important to improve LV systolic function and lower the risk of further cardiac sequelae. In addition, when stratified by BP response, we found that patients with improvement in BP had improvement in subclinical systolic function. Our patients treated with surgery had higher baseline BP and greater BP reductions post-treatment, although the final BP achieved was similar in both groups. This may explain the greater improvement seen in the surgery group. These findings suggest that, in patients with EH, improvement of BP control may have similar positive effects on cardiac function, whereas in patients with PA, it highlights the importance of attaining both BP and biochemical control.

We also found that, at baseline, lower GLS was associated with lower GFR and higher diastolic BP. High diastolic BP likely leads to increased afterload, resulting in poorer systolic function (24). The association of GLS with GFR is particularly interesting. Cardiorenal syndrome is a classification of patients with both organ dysfunction based on the presumed primary diseased organ (25). In PA, dysfunction in both organs is likely due to a common pathway of aldosterone excess. Elevated aldosterone levels lead to myocyte hypertrophy, chronic inflammation, and dysregulation of the extracellular matrix (20). Resultant LV hypertrophy and remodeling predisposes to systolic and diastolic dysfunction, and patients with PA are twice as likely to develop cardiac failure (3), whereas treatment reduces the incidence of heart failure (13). Similarly, hyperaldosteronism leads to glomerular hyperfiltration, falsely elevated GFR (4), increased risk of fibrosis, and renal disease. Most of our

patients had normal GFR at baseline, but significant decline in GFR post-treatment was observed, due to unmasking of the underlying renal impairment. Our findings highlight a current challenge in managing these patients, with subclinical multiorgan dysfunction early in the disease. GLS has been shown to identify subclinical LV dysfunction, before onset of heart failure with reduced EF (9), and has important prognostic value (26). Hence, our study suggests that GLS may be useful in detecting more patients with subclinical LV dysfunction compared with the conventional LVEF.

In addition to improvement of GLS, other cardiac benefits were seen in our patients post-adrenalectomy. Regression of LVH is concordant with findings from a recent meta-analysis (5). Reduction in LA volume index could be due to improvement in LV diastolic function (reflected by decline in mitral E/e') and decreased LVMI that improves ventricular compliance. Aldosterone has been shown to induce atrial fibrillation through electrophysiological dysfunction, inflammation, and vascular remodeling (27), and treatment of PA can reduce risk of new onset atrial fibrillation (3, 23). Overall, we found that surgery led to greater improvement in cardiac parameters compared with medical treatment, consistent with some, but not all, previous studies (28, 29). In addition to differences in extent of BP improvement as mentioned earlier, there are other possible reasons for this. First, some studies have shown that a longer duration of follow-up eventually lead to similar outcomes, suggesting that the benefits of medical treatment may be less immediate (5). In a recent study, Chen and colleagues found that GLS improved after adrenalectomy but not after medical treatment for PA, whereas we found a trend toward improvement in GLS in patients after medical treatment. This may be because our study had a longer follow-up duration of 12 months compared with 3–6 months in the earlier study. Second, our medically treated patients had improvements in serum potassium and PRA, but no changes to BP. More aggressive up-titration of MR antagonists has been shown to provide greater improvements in BP and albuminuria (30) and may have led to improvements in subclinical systolic dysfunction in our patients. It has to be noted that medical treatment may be fraught with dose-limiting side effects, leading to suboptimal dosages and efficacy, as other investigators (31) and we have previously reported (32). Finally, current MR antagonists may not completely block aldosterone effects or concomitant glucocorticoid excess, which can occur in PA (13).

Our prospective cohort study of patients with confirmed PA had several strengths, including successful AVS in all patients, which allowed appropriate treatment according to underlying PA subtype. This high AVS success rates have been achieved only in a few centers worldwide (33) and were attained by an experienced and dedicated interventional radiologist (KSN) leading the team. We also performed pre- and post-treatment 24-h ambulatory BP measurements, which are superior to office BP for reflecting hemodynamic status. We recognize several limitations. First, we did not have information on dietary salt intake although this is

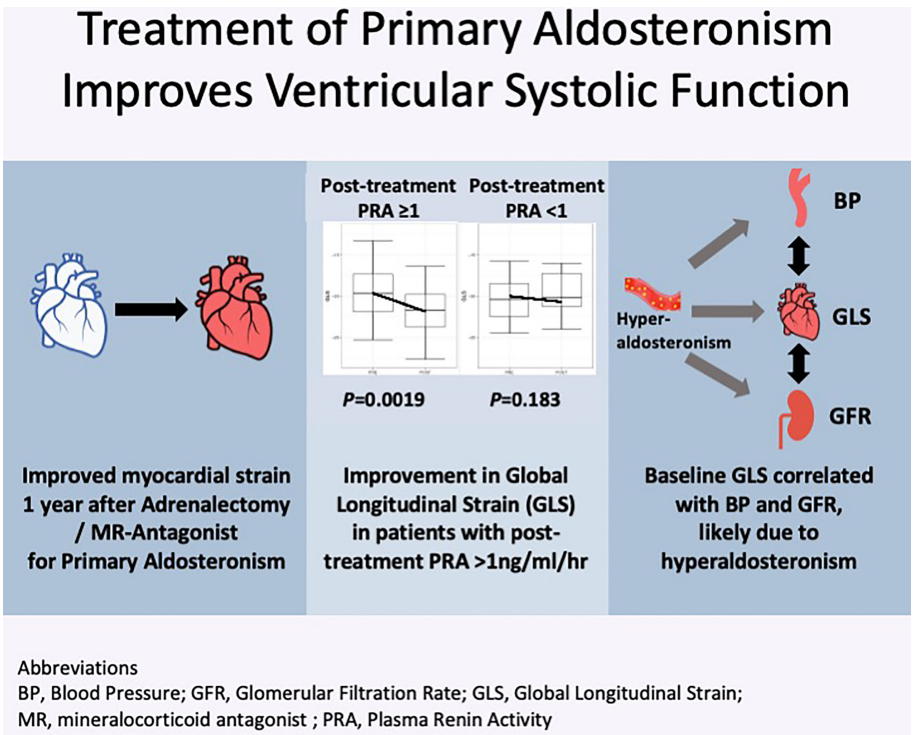

FIGURE 5 | Visual Abstract.

likely high (34). Excessive dietary salt is important in cardiac deterioration induced by hyperaldosteronism (35), through sodium retention and volume expansion. However, we demonstrated that changes in renin levels, which reflect volume status, correlated with improvement in LV systolic dysfunction. Second, with a larger patient cohort, we may have been able to identify other variables correlated with GLS, such as severity of hyperaldosteronism. Using 24-h urine aldosterone may also be better to quantify the total aldosterone production (1). Third, we did not routinely assess for autonomous cortisol secretion. Using metabolome analysis, cortisol excess has been reported in PA and has been associated with increased LV hypertrophy (13, 36). Fourth, we did not have a control group of patients with EH for comparison, but a previous study has already demonstrated that patients with PA have an impaired LV systolic function compared with patients with EH with similar BP (8). Finally, although we found improved LV systolic dysfunction, further studies will be required to demonstrate that this improvement in LV GLS leads to clinical significant outcomes for patients with PA. However, there is currently evidence that LV GLS has important prognostic implications in other patient populations (21, 26).

In conclusion, we demonstrated that appropriate treatment of PA can improve LV subclinical systolic function, with either unilateral adrenalectomy or medical treatment after adequate MR blockade, as reflected by unsuppression of renin. Furthermore, the co-occurrence of cardiac and renal dysfunction highlights the various sequalae of hyperaldosteronism. This underlines the importance of early diagnosis and treatment of PA, to prevent further organ damage.

PERSPECTIVES

We have shown with speckle-tracking echocardiography that patients with PA have subclinical LV systolic dysfunction (**Figure 5**). This is associated with impaired renal function, highlighting the myriad of complications attributed to hyperaldosteronism. Specific treatment of PA, with adequate reversal of renin suppression and sodium overload state, is able to improve subclinical LV systolic function. With PA affecting up to 20% of all patients with hypertension, this reinforces the call for increased screening and early treatment of primary aldosteronism.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by SingHealth Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TP, CC, and SCC had full access to the data in the study and take responsibility of the integrity of the data and accuracy of data analysis. TP, MZ, JK, LG, TK, WL, SS, VA, TT, ET, LM, and JY recruited patients in the study and performed data collection. TP, RF, YT, KT, SL, and S-CC worked on the study concept and design. TP, SS, TT, MC, KT, SL, and SCC were involved in data collection and analysis. TP, CC, TT, MC, and SCC were involved in drafting of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.916744/full#supplementary-material

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Primary aldosteronism: Pathophysiological mechanisms of cell death and proliferation

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Primary aldosteronism is the most common surgically curable form of hypertension. The sporadic forms of the disorder are usually caused by aldosterone overproduction from a unilateral adrenocortical aldosterone-producing adenoma or from bilateral adrenocortical hyperplasia. The main knowledge-advances in disease pathophysiology focus on pathogenic germline and somatic variants that drive the excess aldosterone production. Less clear are the molecular and cellular mechanisms that lead to an increased mass of the adrenal cortex. However, the combined application of transcriptomics, metabolomics, and epigenetics has achieved substantial insight into these processes and uncovered the evolving complexity of disrupted cell growth mechanisms in primary aldosteronism. In this review, we summarize and discuss recent progress in our understanding of mechanisms of cell death, and proliferation in the pathophysiology of primary aldosteronism.

KEYWORDS

adrenal adenoma, adrenal gland, aldosterone, cell death, endocrine hypertension, ferroptosis, hyperaldosteronism, proliferation

Introduction

Primary aldosteronism (PA) is the most common form of secondary hypertension that accounts for 5 to 15% of patients with hypertension (1). The disorder encompasses a group of pathological conditions of the adrenal glands that trigger aldosterone overproduction associated with a higher risk of cardiovascular events (2). Thus, a prompt diagnosis of PA with accurate subtyping of unilateral from bilateral cases is essential to initiate specific treatment strategies (adrenalectomy or pharmacotherapy with a mineralocorticoid receptor antagonist) for optimal patient outcomes (1). Bilateral forms include familial forms of PA, which are rare monogenic forms of hypertension caused by germline pathogenic variants. To date, 4 genetically distinct forms of familial

hyperaldosteronism have been identified (FH types 1 to 4), classified according to the affected gene (3, 4). Sporadic aldosterone-producing adenomas (APAs) carry somatic mutations in aldosterone-driver genes and in genes that drive the development of multiple adrenal tumors. In addition to genetic insights, a wealth of data has accumulated from transcriptomics and metabolomics analyses of aldosterone-producing lesions to gain a deeper understanding of PA pathophysiology.

Genetics of primary aldosteronism

An extensive review on the genetics of PA has been published recently (4). In brief, FH type 1 is caused by a chimeric *CYP11B1/CYP11B2* gene composed of the adrenocorticotropic hormone (ACTH)-responsive promoter region of *CYP11B1* fused to the *CYP11B2* coding region (5). As a result, transcription of *CYP11B2* (aldosterone synthase) is under the regulatory control of ACTH instead of angiotensin II and potassium as in normal aldosterone physiology. The dysregulated aldosterone production in FH types 2, 3 and 4 is caused by variants in the ion channels encoded by *CLCN2* (CIC-2 chloride channel), *KCNJ5* (GIRK4 potassium channel), and *CACNA1H* (Cav3.2 calcium channel), respectively, that function in intracellular ion homeostasis. In addition, germline pathogenic variants in *CACNA1D* can cause a complex form of PA with seizures and neurologic abnormalities (PASNA) (Table 1).

The landscape of somatic APA mutations overlaps with the germline pathogenic variants. Thus, *KCNJ5*, *CACNA1D*, *CACNA1H*, and *CLCN2* can carry somatic or germline mutations in PA. In APAs, this set of aldosterone-driver genes is complemented by somatic mutations in *ATP1A1* and *ATP2B3*, which encode the ion pumps Na^+/K^+-ATPase and Ca^{2+}-ATPase, and in genes that are targeted in multiple adrenal tumors such as *CTNNB1* (β-catenin), and *GNA11*, *GNAQ*, and *GNAS*, encoding (guanine nucleotide-binding proteins), *PRKACA* (catalytic subunit of protein kinase A), and *ARMC5* (a member of the armadillo/β-catenin-like repeat family) (Table 1). The potential function of PA-related mutations in cell growth mechanisms is discussed below.

Aldosterone-driver mutations and deregulated cell growth

The role of recurrent PA mutations in aldosterone overproduction is clearly demonstrated but less evident is their role in perturbing regulatory mechanisms of adrenal cell growth that lead to an increase of the adrenal cell mass and tumor development.

GIRK4 potassium channel

Choi et al. (6) identified somatic APA mutations and an inherited germline mutation in *KCNJ5* (encoding Gly151Arg and Leu168Arg somatic, and Thr158Ala germline variants in GIRK4, G-protein-gated inwardly rectifying K^+ channel 4). This triggered a rapid evolution of research and the description of somatic mutations in other genes (7, 8, 12, 16, 17, 22–27) and the identification of additional familial forms of PA (10, 11, 13, 14). The *KCNJ5* variants result in single amino acid substitutions in or close to the selectivity filter of the encoded GIRK4 potassium channel. The channel properties are consequently altered causing loss of potassium selectivity and abnormal sodium influx (instead of exclusive K^+ efflux) from glomerulosa cells. The resultant depolarization of zona glomerulosa cells causes opening of voltage-gated calcium channels, increased intracellular calcium concentrations and activation of calcium signal transduction and *CYP11B2* (aldosterone synthase) gene transcription and increased aldosterone production (6) (Figure 1). *KCNJ5* mutations are highly prevalent in APAs (3, 4), associated with larger adenoma size, female sex, and more severe aldosteronism (28). The application of genotype analysis to CYP11B2 (aldosterone synthase) immunopositive regions of paraffin-embedded adrenal sections combined with next generation sequencing (29) has resulted in an increased detection of somatic APA mutations such that prevalence rates are now described in around 90% of cases (22–25, 27, 30).

Germline *KCNJ5* mutations can cause FH type 3 which presents with a variable clinical phenotype that might be explained by the functional properties of the mutated GIRK4 potassium channel. This is supported by the report of 4 families with FH type 3 caused by either of 2 different *KCNJ5* mutations (31). Two families with GIRK4-Gly151Arg mutations presented with massive adrenocortical hyperplasia and severe aldosteronism requiring bilateral adrenalectomy. The other 2 families carried a Gly151Glu mutation associated with a milder PA phenotype without evident adrenal hyperplasia on imaging and easily controlled hypertension with pharmacotherapy. The Gly151Glu mutation caused higher sodium influx and greater consequent adrenal cell death than Gly151Arg accounting for the absence of structural changes at adrenal imaging in patients with the GIRK4-Gly151Glu germline variant (31). The cell toxicity of GIRK4 mutants can be circumvented by low levels of gene transcription and expression of the mutated GIRK4 channel (32).

Sodium/potassium-transporting ATPase

The Na^+/K^+-ATPase pump couples the hydrolysis of an ATP molecule to the transport of 3 Na^+ out and 2 K^+ into the cell. Azizan et al. (8) and Beuschlein et al. (16), reported somatic

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TABLE 1 Effect Of Gene Variants In Primary Aldosteronism Driver Genes.

GENE	PROTEIN	MECHANISM IN PA PATHOPHYSIOLOGY	PA SUBTYPE			REFERENCE
Ion Channels						
KCNJ5	Potassium inwardly rectifying channel subfamily J member 5	Activation of Ca^{2+} signaling and dysregulated aldosterone production; deregulated cell growth	Somatic Germline	Unilateral Bilateral	APA FH-III	Choi M, 2011 (6)
CACNA1D	Calcium voltage-gated channel subunit alpha1 D	Activation of Ca^{2+} signaling and dysregulated aldosterone production	Somatic Somatic Germline	Unilateral Bilateral Bilateral	APA APM PASNA	Scholl UI, 2013 (7) Azizan EA, 2013 (8) Omata K, 2018 (9)
CACNA1H	Calcium voltage-gated channel subunit alpha1 H	Activation of Ca^{2+} signaling and dysregulated aldosterone production	Germline Somatic	Bilateral Unilateral	FH-IV APA	Scholl UI, 2015 (10) Daniil G, 2016 (11) Nanba K, 2020 (12)
CLCN2	Chloride voltage-gated channel 2	Activation of Ca^{2+} signaling and dysregulated aldosterone production	Germline Somatic	Bilateral Unilateral	FH-II APA	Scholl UI, 2018 (13) Fernandes-Rosa FL, 2018 (14) Dutta RK, 2019 (15)
Ion Transporters						
ATP1A1	ATPase Na^+/K^+ transporting subunit alpha 1	Activation of Ca^{2+} signaling and dysregulated aldosterone production; deregulated cell growth	Somatic Somatic	Unilateral	APA APM	Beuschlein F, 2013 (16) Azizan EA, 2013 (8)
ATP2B3	ATPase plasma membrane Ca^{2+} transporting 3	Intracellular acidification and dysregulated aldosterone production	Somatic Somatic	Unilateral	APA APM	Beuschlein F, 2013 (16)
Cell Signaling Systems						
CTNNB1	Catenin beta 1	Aldosterone overproduction; deregulated cell growth	Somatic	Unilateral	APA	Åkerström T, 2016 (17)
GNA11 *GNAQ*	G protein subunit alpha 11 G protein subunit alpha Q	Aldosterone overproduction; deregulated cell growth	Somatic	Unilateral (when coincident with *CTNNB1* variant)	APA	Zhou J, 2021 (18)
GNAS	GNAS complex locus	Deregulated cell growth	Somatic	Unilateral	APA	Nakajima Y, 2016 (19)
PRKACA	Protein kinase cAMP-activated catalytic subunit alpha	Deregulated cell growth	Somatic	Unilateral	APA	Rhayem Y, 2016 (20)
ARMC5	Armadillo repeat containing 5	Deregulated cell growth	Somatic	Unilateral	APA	Zilbermint M, 2015 (21)
Cytochrome P450 Enzyme						
CYP11B1/B2	Ectopically expressed aldosterone synthase	Dysregulated aldosterone production	Germline	Bilateral	FH-I	Lifton RP, 1992 (5)

APA, aldosterone-producing adenoma; APM, aldosterone-producing micronodule; FH, familial hyperaldosteronism; PA, primary aldosteronism; PASNA, PA, seizures, and neurologic abnormalities.

gain-of-function mutations in *ATP1A1* (Na^+/K^+-ATPase alpha-1 subunit). *ATP1A1* variants are found in 5-17% of APAs (3) and many have been shown to severely impair K^+ binding and ATPase activity conferring membrane depolarization in the cells which express them (8, 16, 33). Adrenal cells expressing *ATP1A1* mutations show normal intracellular Ca^{2+} concentrations suggesting that activated Ca^{2+} export pathways, *via* a Na^+/Ca^{2+} exchanger or Ca^{2+}-ATPase, or inactivation of Ca^{2+} channels might function as compensatory mechanisms (34). However, adrenal cells expressing *ATP1A1* mutants exhibit abnormal H^+ leak currents that cause intracellular acidification that can lead to increased expression of *CYP11B2* (aldosterone synthase) (34) (Figure 1).

Na^+/K^+-ATPase can display a dual function not only as an ion pump but also as a signaling complex relaying extracellular signals to intracellular compartments (35–37). For example, ouabain- the cardiotonic steroid- can bind to Na^+/K^+-ATPase which in turn interacts, *via* its alpha-1 subunit, with the Src proto-oncogene tyrosine kinase. Subsequent Src activation by phosphorylation then enhances downstream signaling pathways (37). Human adrenal cells expressing Na^+/K^+-ATPase-p.Leu104Arg (a recurrent somatic APA mutation) show

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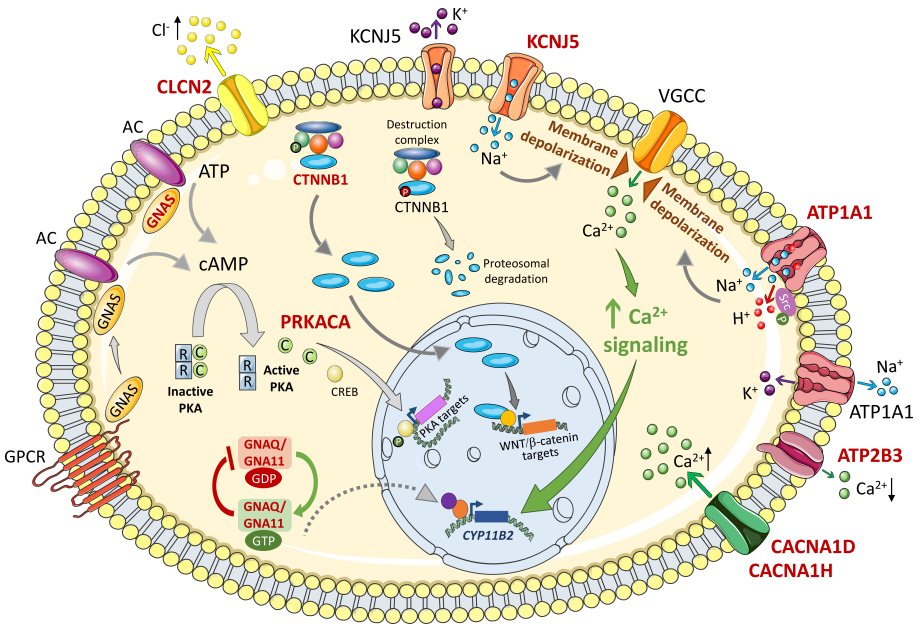

FIGURE 1
Mechanisms of deregulated cell growth of variants related to primary aldosteronism. The figure shows intracellular signaling pathways activated by APA somatic mutations implicated in cell growth mechanisms. Wild type and mutated forms are labeled in black and red respectively. Membrane depolarization triggered by Na+ or H+ influx causes opening of voltage gated ion channels (VGCC), Ca2+ influx and activation of Ca2+ signaling that activates CYP11B2 gene transcription to drive increased aldosterone biosynthesis and cell proliferation. ATP1A1 is also reported to activate cell proliferation by phosphorylated Src. Intracellular β-catenin concentrations are kept low under basal conditions by continuous proteosomal degradation. Activating mutations in exon 3 of the β-catenin gene bypass this control mechanism resulting in cytoplasmic β-catenin accumulation and nuclear translocation to activate specific gene transcription programs. In many adrenal tumours including some rare APA examples, cAMP/PKA signaling is activated by mutations in GNAS and PRKACA for activation of target genes. GNAQ and GNA11 mutations can cause upregulated aldosterone production *in vitro* and are associated with hyperplasia of glomerulosa cells adjacent to an APA. They are clinically silent *in vivo* except when concomitant with CTNNB1 (β-catenin) mutations. See text for details. Somatic mutations in CLCN2, CACNA1D, CACNA1H, and ATP2B3 have been described in APAs which result in increased Cl- efflux (CLCN2), increased Ca2+ influx (CACNA1D, CACNA1H), and decreased Ca2+ efflux (ATP2B3). AC, adenylyl cyclase; ATP, adenosine triphosphate; ATP1A1, ATPase Na +/K+ transporting subunit α1; ATP2B3, ATPase plasma membrane Ca2+ transporting 3; C, catalytic subunit; CACNA1D, calcium voltage-gated channel subunit alpha1 D; CACNA1H, calcium voltage-gated channel subunit alpha1 H; cAMP, cyclic adenosine monophosphate; Ca2+, calcium ions; CLCN2, chloride voltage-gated channel 2; CREB, cAMP response element-binding protein; CYP11B2, cytochrome P450 family 11 subfamily B member 2 (aldosterone synthase); GDP, guanosine diphosphate; GNA11, G protein subunit α11; GNAS, GNAS complex locus; GNAQ, G protein subunit αQ; GTP, guanosine triphosphate; GPCR, G protein coupled receptor; H +, hydrogen ions; K+, potassium ions; KCNJ5, potassium inwardly rectifying channel subfamily J member 5; Na+, sodium ions; PKA, protein kinase A; PRKACA, protein kinase cAMP-activated catalytic subunit α; R, regulatory subunit; VGCC, voltage-gated calcium channel; WNT, wingless-related integration site. Figure produced using Servier Medical Art (https://smart.servier.com/).

increased levels of Src phosphorylation and enhanced cell proliferation implicating a potential novel mechanism of the Na^+/K^+-ATPase-Src functional complex in the progression and development of APAs with an *ATP1A1* mutation (38) (Figure 1).

Calcium-transporting ATPase, Cav1.3 and Cav3.2 calcium channels, and the CIC-2 chloride channel

As mentioned above, somatic APA mutations have been identified in other genes involved in intracellular ion homeostasis (*ATP2B3*, *CACNA1D*, *CACNA1H*, and *CLCN2*). However, there is no experimental evidence to demonstrate a potential role for mutations in these genes in cell proliferation or cell death. *ATP2B3* encodes the Ca^{2+}-ATPase which pumps intracellular Ca^{2+} across the plasma membrane to the extracellular environment. The Ca^{2+}-ATPase mutations in PA interfere with Ca^{2+} ion binding resulting in decreased export and increased Ca^{2+} influx.

Heterozygous somatic mutations in the L-type voltage gated Ca^{2+} channel Cav1.3 (encoded by *CACNA1D*) are frequently described in aldosterone-producing lesions associated with PA (in APAs and in aldosterone-producing micronodules [APMs]) (7–9) and more rarely in the T-type Ca^{2+} channel Cav3.2 (encoded by *CACNA1H*) (12). PA-driver mutations in *CACNA1D* and *CACNA1H* confer a gain-of-function and result in increased Ca^{2+} influx. Somatic APA mutations in

CLCN2 encoding the CIC-2 chloride channel have been described in just a few cases (15).

Driver mutations of tumorigenesis in primary aldosteronism

Somatic mutations that constitutively activate WNT/β-catenin (*CTNNB1*), G-protein (*GNAS*, *GNAQ*, *GNA11*), and cAMP/protein kinase A (PKA) (*GNAS*, *PRKACA*) signaling can drive unrestrained cell proliferation and survival in diverse tumors, including adrenal tumors. Activating mutations in β-catenin (encoded by *CTNNB1*) that cause WNT-independent signaling are widely reported in APAs (17, 39). Constitutive cAMP/PKA signaling due to *PRKACA* and *GNAS* activating mutations has been reported in a limited number of APAs (19, 20). Mutations in G protein α subunits, particularly those encoded by *GNAS* and *GNAQ*/*GNA11*, have been reported in many tumours including a wide variety of endocrine tumors (40, 41).

Activation of WNT/β-catenin signaling

The regulated destruction of the transcriptional co-activator β-catenin is central to the WNT-mediated β-catenin signaling cascade. This process involves a multi-subunit destruction complex, comprising AXIN (axis inhibitor protein), APC (tumor suppressor adenomatous polyposis coli), and GSK-3β (glycogen synthase kinase-3), which phosphorylates β-catenin for ubiquitylation and proteasomal degradation. In contrast, WNT-mediated cell stimulation disrupts β-catenin degradation causing its accumulation and subsequent nuclear translocation to co-activate the transcription of WNT target genes (42).

Mutations that drive continuous activation of β-catenin signaling are widely reported in human cancers and in several adrenal tumors (43). Consistent with the proliferative advantage of adrenal cells with activating *CTNNB1* mutations, transgenic mice with constitutively active β-catenin display adrenal cell hyperproliferation and adrenal hyperplasia (44) (Figure 1). Many studies implicate disrupted WNT/β-catenin signaling as a key mechanism in APA pathogenesis. This pathway is constitutively activated in 70% APAs, an observation related to the decreased expression of an endogenous inhibitor of the WNT/β-catenin signaling called SFRP2 (secreted frizzled related protein 2) (45). Activating somatic *CTNNB1* mutations located in exon 3 have been identified in 1% to 5% of APAs (17, 18, 46). Of note, 16 of 27 (59%) APAs with a *CTNNB1* activating mutation carry a concurrent G protein α subunit substitution mutation of a highly conserved glutamine residue (p.Gln209) in GNAQ or GNA11 (18) (discussed in more detail below). Overall, *CTNNB1* mutations are found in only a small proportion of APAs with β-catenin cytoplasmic accumulation and nuclear translocation (17, 45, 47) thereby suggesting a role for other factors, other than mutations, in the disruption of WNT/β-catenin signaling in the pathogenesis of APAs (48).

Inactivation of the ARMC5 tumour suppressor protein

Germline and somatic inactivating mutations in the putative tumor-suppressor gene *ARMC5* (armadillo repeat containing 5)-a member of the armadillo/β-catenin-like repeat superfamily-can cause primary bilateral macronodular adrenal hyperplasia (a rare form of primary adrenal Cushing syndrome) (49, 50). ARMC5 has multiple binding partners (51) and may function in PKA, and the WNT/β-catenin signaling pathways (52). Armc5 plays a vital role in early mouse embryonic development, and the T-cell immune response. In addition, the development of adrenal hyperplasia with increasing age in Armc5 knock out mice illustrates the key role of Armc5 in the adrenal (51, 52).

Zilbermint et al. (21) identified germline *ARMC5* variants in 22 of 56 (39.3%) patients with PA. These included variants predicted to be damaging by in silico analysis in 6 of 56 (10.7%) patients. All patients carrying a damaging *ARMC5* variant were African Americans suggesting that ARMC5 pathogenic variants might contribute to the known increased predisposition of this population to low renin hypertension (21). In contrast, *ARMC5* mutations in the coding sequence or intron-to-exon boundaries in a cohort of 37 Caucasian patients with PA due to bilateral adrenal lesions were not identified (53).

Activation of G protein signaling

Abnormal expression and activation of G proteins (guanine nucleotide-binding proteins) are frequent features of tumorigenesis (40) with alterations in the G protein α subunits GNAS, GNAQ, and GNA11 commonly detected (41). G protein α subunits are components of the heterotrimeric G protein complex (comprising a Gα, Gβ, and Gγ subunit) which mediates G protein coupled receptor signal transduction. Signal activation is achieved by GDP for GTP exchange on the Gα subunit, hydrolysis of GTP to GDP terminates the signal. Recurrent mutations in GNAS, GNAQ and GNA11 disturb GTPase activity thus driving constitutively active and prolonged signaling (Figure 1).

GNAS is one of the most recurrently mutated G proteins in human cancer (40). GNAS mutations frequently alter either a p.Arg201 or a p.Gln227 residue, which are required for GTPase activity. GNAS mutations are found in a substantial proportion of cortisol-producing adenomas associated with subclinical mild autonomous cortisol excess (5 of 7, 71.4%) (54). Only a few APA

with GNAS mutations (GNAS p.Arg201Cys) have been described and these were in cases of PA associated with autonomous cortisol secretion and activation of cAMP/PKA (protein kinase A) signaling (19).

Hotspot somatic mutations in the G protein α subunits GNAQ and GNA11 are found in 295 of 8778 (3.4%) and 155 of 6237 (2.5%) of human tumors, respectively (40, 55). Most are substitution mutations of residues p.Arg183 and p.Gln209 (homologous to p.Arg201 and p.Gln227 in GNAS) that impair GTP hydrolysis. However, mutations of p.Arg183 in GNAQ and GNA11 maintain some sensitivity to regulators of G protein signal termination and are less damaging variants.

As mentioned above, Zhou et al. (18) reported a high prevalence of APA *GNA11* or *GNAQ* mutations coexisting with *CTNNB1* mutations. The *GNA11* or *GNAQ* mutations caused substitution of the conserved p.Gln209 residue for either a His, Pro, or Leu amino acid, thereby driving their constitutive activation. Transfection of primary adrenocortical cells with *CTNNB1* and *GNA11* mutants, individually or combined, caused increased aldosterone production. Solitary *GNA11* mutations were also identified in the hyperplastic zona glomerulosa layer of the adrenal cortex adjacent to APAs with *GNA11* and *CTNNB1* mutations (18). This supports the 2-hit model of tumorigenesis in APA development (56) in which a first event stimulates adrenocortical cell proliferation, and a second event (a somatic aldosterone-driver mutation) drives autonomous aldosterone production (4, 57, 58).

Activation of cAMP/protein kinase A signaling

Cyclic adenosine monophosphate (cAMP) regulates multiple cellular functions in most cell types *via* the control of target gene transcription mainly mediated by protein kinase A (PKA). Adenylyl cyclase is activated by GNAS binding thus enabling the conversion of adenosine triphosphate (ATP) to cAMP, which in turn activates PKA. The PKA heterotetramer comprises two regulatory subunits (PRKAR1A) and two catalytic subunits (PRKACA). The regulatory subunits maintain the inactivity of the catalytic subunits in the tetrameric complex. cAMP binding to each regulatory subunit causes their dissociation and activation (59). PKA regulates transcription by direct phosphorylation of transcription factors such as CREB (cAMP-response element-binding protein), and CREM (cAMP-responsive modulator) so they can bind to cAMP-response elements in target genes.

Constitutive activation of cAMP/PKA signaling due to *GNAS* or *PRKACA* mutations is linked to the formation of many tumors including endocrine tumors (60, 61). The rare examples of APA *GNAS* mutations are discussed above (under G protein signaling). Somatic *PRKACA* mutations, encoding a p.Leu206Arg substitution in the catalytic PKA subunit, have been identified in a substantial proportion of cortisol-producing adenomas (54, 60, 62, 63). PRKACA Leu206 directly interacts with the PKA regulatory subunit. The PRKACA p.Leu206Arg mutation disrupts this interaction causing PRKACA constitutive activation with consequent increased phosphorylation of downstream targets (60). Expression of PRKACA p.Leu206Arg *in vitro* causes increased basal PKA activity and cAMP signaling (60, 62), which can account for the dysregulated cortisol production and cell proliferation of these tumors. In PA, PRKACA p.Leu206Arg mutations are rare (20, 60) and seem to occur in some patients with cortisol co-secretion. In addition, the adenoma with the PRKACA p.Leu206Arg mutation can be negative for CYP11B2 (aldosterone synthase) immunostaining (64, 65) indicating the likely function of this mutation in tumor formation.

Transcriptomics of aldosterone-producing adenomas

APA transcriptome analyses show considerable heterogeneity in methodology (different reference tissues, different gene expression platforms). Despite this, many studies report consistent findings, which have helped define the panorama of transcriptome changes in APA formation and have highlighted genes and signaling pathways that function in dysregulated aldosterone production (Table 2). Below we focus on differentially expressed genes in APAs which are implicated in mechanisms of cell death and proliferation. The application of transcriptomics to identify mechanisms of aldosterone overproduction are described elsewhere (66, 67).

Mechanisms of cell death

Early reports relied on microarray gene expression studies. Transcriptome comparison of 8 APAs with those of 3 normal adrenals identified a range of differentially expressed genes which included *TDGF1* (teratocarcinoma-derived growth factor 1)- also reported as upregulated by a SAGE (serial analysis of gene expression) study (68)- and *VSNL1* (visinin-like 1) encoding a calcium binding protein (69, 70). Overexpression of *TDGF1* and *VSNL1* in human adrenocortical cells *in vitro* suggested that each gene played a role in cell survival by protection against cell death by apoptosis (69, 70). The nuclear transcription factor and WNT/β-catenin target *AFF3*, also protects adrenal cells from apoptosis and is a positive regulator of adrenal cell proliferation (71). mRNA-seq analysis of 15 APAs suggested a role for *AFF3* in APA pathophysiology by demonstrating upregulated *AFF3* gene expression in *CTNNB1* mutated APAs versus those without a *CTNNB1* mutation (72) (Table 2).

Transcriptome analysis of APAs and paired adjacent zona glomerulosa highlighted a potential role for oxidative stress in

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TABLE 2 Potential function of differentially expressed genes and Mirnas in Aldosterone-Producing Lesions.

GENE or miRNA	PROTEIN	TRANSCRIPTOME COMPARISON		FUNCTIONAL EFFECT IN ADRENOCORTICAL CELLS	GENE EXPRESSION PLATFORM	VALIDATION	REFERENCE
		SAMPLE TISSUE	REFERENCE TISSUE				
Upregulated gene expression							
AFF3	AF4/FMR2 Family Member 3	APA with *CTNNB1* variant (*n*=3)	APA without *CTNNB1* variant (*n*=12)	Negative regulation of cell death by apoptosis. Positive regulator of cell proliferation.	RNAseq	Yes (49 APAs)	Backman S, 2019 (72)
BEX1	Brain expressed X-linked 1	APA < 10 mm diam. (*n*=12)	APA ≥ 30 mm diam. (*n*=9)	Negative regulation of cell death by ferroptosis.	RNAseq	Yes (71 APAs)	Yang Y, 2021 (73)
		APA with *CACNA1D* or *ATP1A1* variant (*n*=5)	APA with *KCNJ5* variant (*n*=8)		Microarray	No	Azizan EA, 2013 (8)
		APM (*n*=4)	Paired adjacent zG (*n*=4)		Microarray	No	Nishimoto K, 2015 (74) Yang Y, 2021 (73)
NEFM	Neurofilament medium chain	APA without *KCNJ5* variant (with compact eosinophilic cells) (*n*=7)	APA with *KCNJ5* variant (with clear cells) (*n*=7)	Negative regulator of cell proliferation. Suppressor of aldosterone production	Microarray	Yes	Zhou J, 2016 (75) Maniero C, 2017 (76)
SHH	Sonic hedgehog	APA (*n*=12)	Normal adrenals (*n*=6)	Positive regulator of cell proliferation.	*In situ* hybridization	No	Boulkroun S, 2011 (47) Werminghaus P, 2014 (77) Gomes DC, 2014 (78)
TDGF1	Teratocarcinoma-derived growth factor 1	APA (*n*=8)	Normal adrenals (*n*=3)	Negative regulation of cell death by apoptosis. Stimulation aldosterone production.	Microarray	Yes (19 APAs versus 10 normal adrenals)	Williams 2010 (69)
		APA (*n*=1)	Paired adjacent cortex (*n*=1)		SAGE	–	Assié G, 2005 (68)
VSNL1	Visinin like 1	APA (*n*=8)	Normal adrenals (*n*=3)	Negative regulation of cell death by apoptosis. Stimulation aldosterone production.	Microarray	Yes (19 APAs versus 10 normal adrenals)	Williams TA, 2010 (69) Williams TA, 2012 (70)
YPEL4	Yippee Like 4	APA (*n*=39)	Nonfunctional adrenoma (*n*=12)	Positive regulator of cell proliferation. Stimulation of aldosterone production. Positive correlation with APA diameter.	qRT-PCR	–	Oki K, 2016 (79)
Downregulated gene expression							
LGR5	Leucine rich repeat containing G protein-coupled receptor 5	APA (*n*=14)	Paired adjacent zG (*n*=14)	Positive regulation of apoptosis. Negative regulator of cell proliferation. Suppresses aldosterone production.	Microarray	Yes	Shaikh LH, 2015 (80) Zhou J, 2016 (75)
Downregulated miRNA expression							
miR-193a-3p	–	APA (*n*=15)	Paired adjacent cortex (*n*=15)	Negative regulation of cell proliferation. Suppresses aldosterone production	qRT-PCR	–	Zhang G, 2018 (81)
miR-203	–	APA (*n*=10)	Paired adjacent cortex (*n*=10)	Negative regulation of cell proliferation. Negative correlation with APA diameter	Microarray	Yes (40 APAs versus 40 paired adjacent cortex)	Peng KY, 2018 (82)
miR-375	–	APA (*n*=6)	Normal adrenals (*n*=4)	Negative regulation of cell proliferation. Negative correlation with APA diameter.	Microarray	Yes (88 APAs, 16 normal adrenals)	He J, 2015 (83)

APA pathogenesis (75). The top canonical biological pathway associated with the differentially expressed genes was NRF2 (nuclear factor erythroid 2–related factor 2)-mediated oxidative stress, which is a critical cellular mechanism to maintain intracellular redox homeostasis and limit oxidative damage (84). An imbalance in redox-based metabolic processes can result in inappropriate production of reactive oxygen species (ROS) and oxidative damage to membrane lipids. If cellular antioxidant systems are unable to inhibit oxidative damage, a chain reaction of lipid peroxidation occurs, and cells commit to a form of regulated cell death called ferroptosis (85). GPX4 (glutathione peroxidase 4) is an essential antioxidant peroxidase which catalyzes the reduction of lipid hydroperoxides to protect cells from ferroptosis. Adrenocortical cells are particularly sensitive to inducers of ferroptosis which act *via* GPX4 inhibition (86). Functional enrichment analysis of differentially expressed genes in APAs compared with adjacent zona glomerulosa identified a ferroptosis-related gene set (87). The upregulated genes included *SCD* and *GCLC*, which encode key enzymes with a ferroptosis protective function in the catalysis of monounsaturated fatty acids and glutathione biosynthesis.

To detect mechanisms of APA tumorigenesis, Yang et al. (73) compared APA transcriptomes of highly diverse sizes (9 macro APAs with adenoma diameter ≥30 mm versus 12 micro APAs ≤10 mm). Over-representation analysis of the transcriptome dataset from Yang et al. (73) illustrates enrichment of cell survival pathways (negative regulation of cell growth, positive regulation of cell death, and positive regulation of apoptotic processes) in the *KCNJ5* mutation-negative macro versus micro APAs but not in *KCNJ5*-mutated APAs (Figures 2A, B). Thus, activation of gene expression programs with a negative impact on cell survival can limit the size of APAs without a *KCNJ5* mutation. In contrast, this is not a feature of APAs with a *KCNJ5* mutation which are characterized by their larger tumor size (28).

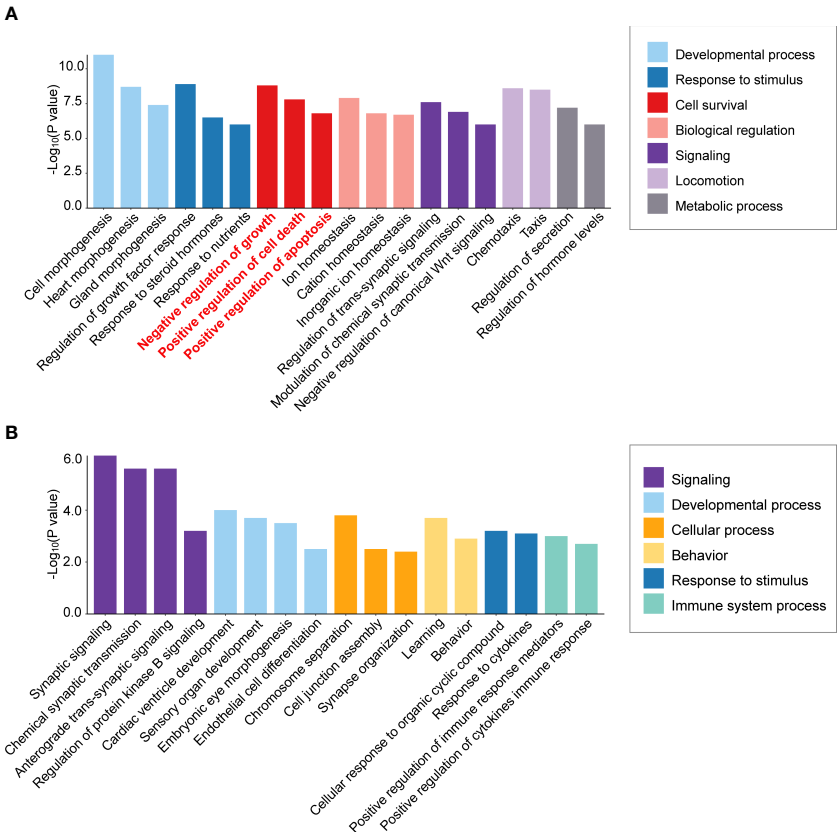

FIGURE 2
Differential enrichment of cell survival pathways in aldosterone-producing adenomas according to *KCNJ5* (GIRK4) mutation status. Biological process enrichment analysis was performed of significantly differentially expressed genes in macro versus micro APAs (≥30 mm versus ≤10 mm adenoma diameter) without a KCNJ5 mutation **(A)** and with a KCNJ5 mutation **(B)** using Metascape (http://metascape.org/accessed on 26 February 2022) analysis of publicly available dataset (https://github.com/MedIVLMUMunich/MacroMicroAPA_RNAseq). Enrichment visualization was performed using R package ggplot2 (v3.3.5).

Validation of expression levels of genes associated with processes of cell death and cell proliferation in an expanded sample set of 71 APAs identified a subset of genes with transcription profiles strongly correlated with adenoma diameter in *KCNJ5*-mutation negative APAs (73). These included *BEX1* (brain expressed X-linked 1) which was inversely correlated with APA diameter and was upregulated in APAs relative to paired adjacent cortex. A previous study (8) reported upregulated *BEX1* gene expression in APAs with a *CACNA1D* or *ATP1A1* mutation (that tend to be small adenomas) compared with the larger *KCNJ5*-mutated APAs (8, 28). Flow cytometry analyses of human adrenocortical cells with stable BEX1 overexpression demonstrated its role in protection from cell death by ferroptosis, rather than by apoptosis or by interference with cell cycle progression (73). The relatively high *BEX1* gene expression in smaller versus larger APAs and in aldosterone-producing micronodules (APMs) compared with adjacent zona glomerulosa cells (73, 74) implicates a role for *BEX1* in the promotion of cell survival in the initiation of adenoma formation.

Mechanisms of cell proliferation

Genes involved in calcium signaling were first highlighted by some of the first APA gene expression studies (88), consistent with the later discovery of activation of the calcium signaling system by aldosterone-driver mutations (89). Signaling cascades initiated by intracellular Ca^{2+} are ubiquitously employed for the regulation of cell proliferation. This is achieved at several levels including by the promotion of resting G0 cell entry to the cell cycle, activation of the initiation of DNA synthesis at the G1 to S phase transition, and by stimulation of mitosis (90). Thus, in addition to a role in aldosterone production, genes which function in calcium signaling might also mediate cell proliferation, such as those with a calcium binding function such as *VSNL1* (visinin-like), *CALN1* (calneuron 1) and *CLGN* (calmegin), which are all significantly upregulated in APAs (70, 91, 92).

The *YPEL* (yippee-like) gene family encodes proteins with putative zinc-finger-like metal-binding domains (yippee domains). The encoded proteins localize to the centrosome and nucleolus and have been proposed to regulate cell division and proliferation (93). Transcriptome analysis of freshly isolated rat adrenal zona glomerulosa cells treated with angiotensin II (100 nM) or potassium (16 mM KCl) identified a set of genes, comprising *YPEL4*, that might function in aldosterone production (94). The upregulation of *YPEL4* in response to angiotensin II or K^+ stimulation in rat adrenal cells was successively observed in human adrenocortical cells which caused increased adrenal cell proliferation (79). A role for *YEPL4* in adrenal cell proliferation in APAs was also suggested by the slight but significant positive correlation of *YPEL4* gene expression levels with APA diameter (79) (Table 2).

In a microarray study of 14 APAs, *LGR5* (leucine rich repeat containing G protein-coupled receptor 5) was downregulated in APA compared with adjacent zona glomerulosa (75, 80). LGR5 overexpression in human adrenal cells caused increased apoptosis, a reduction of proliferation, and decreased aldosterone production (80). Moreover, *NEFM* (neurofilament medium chain) was upregulated in APAs without a *KCNJ5* mutation relative to those with a *KCNJ5* mutation (75) and transfection of mutated *KCNJ5* into adrenal cells significantly decreased *NEFM* gene expression levels (60). Consistently, *NEFM* gene silencing resulted in increased adrenal cell proliferation and amplified aldosterone production (Table 2).

A large transcriptome analysis of APAs identified the potential role of the retinoic acid receptor alpha (RARα) in adrenal cell proliferation. Inactivation of *Rarα* in transgenic mice caused an inhibition of non-canonical Wnt signaling and increased proliferation in male mice (95). The study suggests that RARα might function in the structural maintenance of the adrenal cortex and that disrupted RARα signaling could be factor which contributes to APA pathogenesis.

In situ metabolomics of aldosterone-producing adenomas and micronodules by mass spectrometry imaging

In situ matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) is a novel technique that can simultaneously measure up to thousands of molecules in a single tissue section. The detected molecules encompass metabolites, lipids, glycans, peptides and proteins, as well as drugs and their metabolites (96). The approach allows co-integration of the detected molecules with conventional hematoxylin and eosin staining or immunohistochemistry and thus enables the spatial visualization of metabolite distribution, and other molecules including hormones, in the context of morphology or protein expression.

According to classical adrenal morphology, the adrenal comprises 3 distinct concentric zones of the cortex (glomerulosa, fasciculata, and reticularis) and the adrenal medulla. Using MALDI-Fourier transform-ion cyclotron resonance-MSI (MALDI-FT-ICR-MSI) applied to fresh frozen normal human adrenal sections, Sun et al. (97) determined a new molecular definition of adrenal gland anatomy. The study established the unexpected complexity of the adrenal by visualization of 10 clearly distinct molecular zones (6 cortical and 4 medullary substructures). The functional and physiological relevance of this multi-layered molecular anatomy remains to be determined but this technique has been extensively applied to extend our knowledge of adrenal pathology (98–100).

In addition to fresh frozen tissue sections, MALDI-FT-ICR MSI can reliably be performed on formalin-fixed paraffin embedded

(FFPE) archived tissue samples, including individual biopsy specimens, and tissue microarrays comprising thousands of tissue cores. Comparison of mass spectrometry peaks from tissue specimens that had been fresh frozen or paraffin-embedded, revealed that similar metabolites were detected, with a 72% overlap, of comparable peak intensities, particularly for non-lipid low molecular weight metabolites (101).

MALDI-FT-ICR MSI analysis of a tissue microarray representing 132 APAs with genotype data showed that samples did not cluster according to genotype in the total dataset. However, restricting analysis to adenomas with either a *KCNJ5* or a *CACNA1D* mutation classified these 2 genotype groups with significant differences of 137 metabolites (98). Biological pathway analysis revealed enrichment of purine metabolism with increased purine synthesis in *KCNJ5*-mutated APAs, which are generally larger, than those with a *CACNA1D* mutation. Thus, the increased purine synthesis might conceivably result from cell cycle promotion and enhanced cell proliferation (98).

Sugiura et al. (99) used MALDI-MSI with chemical derivatization to visualize specific steroids in fresh frozen adrenal tissue sections. The study generally confirmed the production of aldosterone in areas of CYP11B2 immunostaining in APAs and in aldosterone-producing micronodules (APMs) (99). APMs are small (<10 mm) CYP11B2-positive adrenal lesions located under the adrenal capsule (102, 103). They frequently carry somatic mutations in *CACNA1D*, *ATP2B3* and *ATP1A1* corresponding to those found in APAs (104). In contrast, *KCNJ5* mutations are largely absent in APMs, despite their high frequency in APAs (74). A small number of adrenal lesions with hybrid immunohistological features composed of an outer APM-like part, characterized by CYP11B2 but not CYP11B1 immunostaining, and an inner APA-like part, with both CYP11B2 and CYP11B1 immunostaining have been described (105). The occurrence of mutations common to both APMs and APAs and the observation of hybrid lesions led to the proposal that APMs might be precursors of APAs in some cases (105, 106) (Figure 3).

MALDI-FT-ICR MSI was used to determine the metabolomic phenotypes of 27 APMs and 6 APAs from surgically resected adrenals from patients with PA (100). The study established 2 subgroups of APMs (subgroups 1 and 2) with distinct metabolic phenotypes. The pattern of metabolites in APM subgroup 1 (20 of

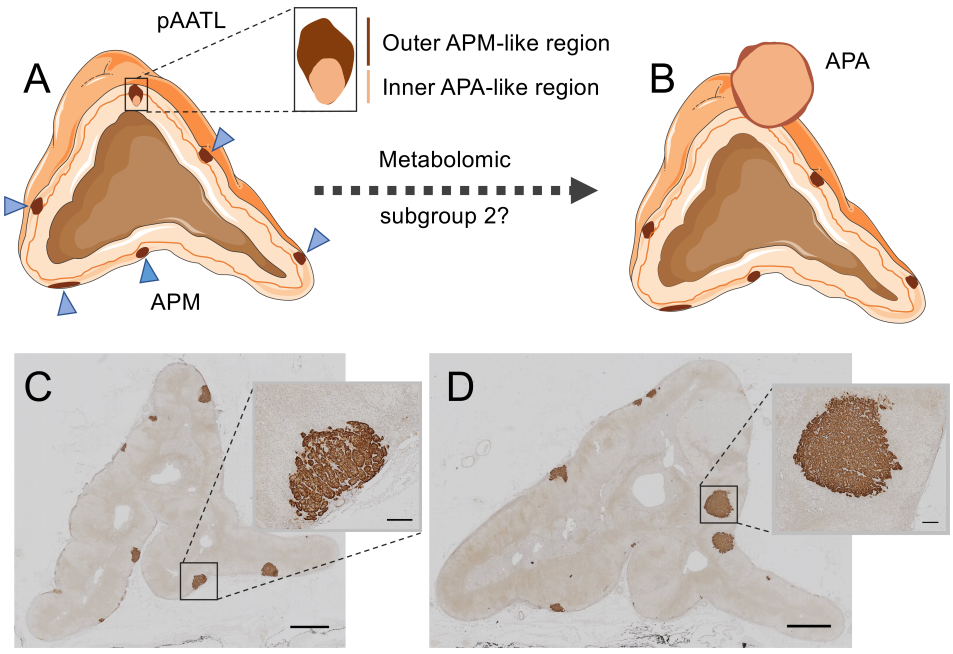

FIGURE 3
Hybrid lesions in the transition of aldosterone-producing micronodules to adenomas. The pAATL is a lesion composed of an outer APM-like region (with positive immunostaining for CYP11B2 but negative for CYP11B1) and an inner APA-like region (with positive immunostaining for both CYP11B2 and CYP11B1) that can have a different mutation status for PA-driver genes **(A)**. Thus, pAATLs have been proposed as hybrid APM-APA lesions and might represent intermediary lesions in the transit of APMs to APAs. Subgroups of APMs can be differentiated by their highly divergent metabolic profiles. A subset of APMs (metabolic subgroup 2) display a metabolic signature like that of APAs and might suggest progression to APAs **(B)**. CYP11B2 (aldosterone synthase) immunostaining of an adrenal surgically removed from a patient with PA **(C, D)**. The adrenal in Panel C shows multiple APMs (magnified in inset), a different sample block of the same adrenal in Panel D shows formation of aldosterone-producing nodules (magnified in inset). Bars = 2mm (main image) or 200 µm (inset). Blue triangles indicate APMs in Panel **(A)** APA, aldosterone-producing adenoma; APM, aldosterone-producing micronodule; pAATL, possible APM-to-APA transitional lesion; CYP11B1, 11β-hydroxylase; CYP11B2, aldosterone synthase. Figure produced using Servier Medical Art (https://smart.servier.com/).

27 APMs) was clearly separated from that of both subgroup 2 (7 of 27 APMs) and APAs. In contrast, the metabolic phenotype of APMs in subgroup 2 closely resembled that of APAs and displayed enrichment of biological pathways supporting cell proliferation and potentially tumour progression with increased purine synthesis characterizing APM subgroup 2 (100). Mutational status did not appear to account for the difference in metabolic signatures between the 2 APM subgroups and they were indistinguishable by immunohistology. Despite the apparent absence of hybrid lesions, these observations support the hypothesis of the "transitional lesion" in which a subgroup of APMs progress to APAs (100, 105) (Figure 3). Such a model in which an APM can transit to form an APA is an alternative to the 2-hit hypothesis of APA development (57) in which an initial event stimulates cell proliferation [such as a genetic variant (107) or circulating factor (108, 109)] and a subsequent somatic mutation in a stimulated cell, drives the aldosterone overproduction.

Emerging role of epigenetics and micro RNAs

Increasing numbers of studies report the disruption of epigenetic and post-transcriptional mechanisms, notably by DNA methylation and microRNAs (miRNA/miR), as mechanisms which might facilitate APA pathogenesis.

DNA methylation in the pathophysiology of aldosterone-producing adenomas

Several studies highlight deregulated DNA methylation as an epigenetic mechanism in APA pathogenesis. Gene expression is typically repressed by cytosine methylation of CpG (5'-cytosine-guanine-3' dinucleotide) islands in target gene promoter regions. APAs display a distinct pattern of methylation (hypomethylated status) compared with their adjacent adrenal cortex and non-functioning adrenal adenomas (110, 111). *CYP11B2* was hypomethylated and upregulated, a process which was bypassed in the presence of either APA *KCNJ5* or *ATP1A1* mutations (110–112). Murakami et al. (113) integrated a genome-wide methylome analysis with transcriptome data and demonstrated that many genes involved in tumorigenesis (*HOX* family genes, *PRRX1*, *RAB38*, *FAP*, *GCNT2*, and *ASB4*) and steroidogenesis (*CYP11B2*, *MC2R*, and *HPX*) were hypomethylated and upregulated in APAs compared with adjacent cortex.

Micro RNAs in the pathophysiology of aldosterone-producing adenomas

MicroRNAs (miRs, miRNAs) are small molecules, 18-22 nucleotides in length, that regulate post-transcriptional gene expression levels usually *via* downregulating the expression of specific genes by binding to the 3′untranslated regions of their corresponding mRNAs (114, 115). miRNA expression profiling can characterize diseased states and, as such, might be promising as a diagnostic tool (114). In PA, changes in circulating plasma miRNA expression levels have been reported in patients according to subtype with significant levels of miR-30e-5p, miR-30d-5p, and miR-7-5p overexpression in patients with bilateral adrenal hyperplasia versus those with a unilateral APA (116, 117).

In addition to circulating miRNAs, several studies have reported downregulated miRNAs in APA tissue samples compared with the adjacent adrenal cortex or relative to other resected adrenal specimens (Table 2). Downregulated miR-375 expression was reported in APA relative to both unilateral adrenal hyperplasia and normal adrenals. A role for miR-375 in tumorigenesis is supported by the reduction in adrenal cell viability in response to miR-375 overexpression *in vitro*. In addition, miR-375 expression levels are negatively correlated with APA diameter and are also downregulated in other tumors (82, 83). The putative tumorigenic function of miR-375 might be mediated by decreased expression of the target gene *MTDH* (metadherin) and subsequent suppression of Akt signalling (83). Peng et al. identified miRNA-203 as a candidate functional miRNA in APA pathophysiology from microarray-based expression analysis of 10 APA tissues and paired adjacent adrenal cortex (82). Treatment of human adrenal cells with miR-203 inhibitors caused increased aldosterone production and cell proliferation. Moreover, miR-203 mimics resulted in decreased adrenal cell proliferation as well as aldosterone hypersecretion from primary cell cultures derived from APA tissue. *WNT5A* was identified as a direct target of miR-203 implicating WNT5A/β-catenin signaling in mediating the observed functional effects (82).

Perspectives

Diverse mechanisms alter the balance between adrenal cell death and proliferation to favor APA formation and development. These processes can vary according to genotype. Unrestrained proliferation of adrenal cells carrying a *KCNJ5* mutation contrasts with specific gene expression programs elicited in *KCNJ5* mutation negative APAs which influence adrenal cell viability and potentially regulate tumor size. The application of advanced omics technologies has tremendous potential to advance our understanding of the underlying biology of APA tumorigenesis. Accordingly, single-cell and spatially resolved transcriptomics will provide a detailed cellular atlas of PA adrenals and define gene expression profiles and genotype in selected cell populations. Integration of spatial transcriptomics with metabolic phenotyping using *in situ* mass spectrometry imaging will achieve a higher-level

definition of biological pathways in cell subpopulations and elucidate the fundamental pathological processes in APA tumor cells.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Relationship between vascular ageing and left ventricular geometry in patients with newly diagnosed primary aldosteronism

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Background: Changes in left ventricular (LV) geometry are early manifestations of cardiac damage. The relationship between vascular aging and LV geometry has been reported. However, in newly diagnosed primary aldosteronism (PA), with more severe target organ damage than essential hypertension, the relationship between vascular aging and LV geometry has never been described.

Methods: We conducted a retrospective study among newly diagnosed PA from 1 January 2017 to 30 September 2021 at the Third Xiangya Hospital. The data of vascular aging parameters were collected, including ankle–brachial index (ABI), brachial–ankle pulse wave velocity (baPWV), and carotid intima-media thickness (cIMT). Echocardiography data were collected to assess LV geometry patterns.

Results: A total of 146 patients with newly diagnosed PA were included. The mean age was 44.77 ± 9.79 years, and 46.58% participants were women. Linear regression analysis adjusting all potential confounders showed that cIMT was significantly associated with LV mass index (LVMI) (β=0.164, P=0.028) and baPWV was significantly associated with relative wall thickness (RWT) (β= 0.00005, P=0.025). Multifactorial adjusted logistic regression analysis demonstrated that cIMT was significantly associated with LV hypertrophy (LVH) (OR=7.421, 95%CI: 1.717–815.688, P=0.021) and baPWV was significantly associated with LV concentric geometry (LVCG) (OR=1.003, 95% CI: 1.001–1.006, P=0.017).

Conclusion: baPWV was significantly associated with LVCG and cIMT was significantly associated with LVH in newly diagnosed PA. This study provides insights on the importance of baPWV measurement and cIMT measurement in early assessment of cardiac damage in newly diagnosed PA.

KEYWORDS

vascular ageing, ankle–brachial index, brachial–ankle pulse wave velocity, carotid intima-media thickness, left ventricular geometry, primary aldosteronism

Introduction

Primary aldosteronism (PA) is defined as increased secretion of aldosterone by the adrenal cortex and suppressed activity of the renin–angiotensin system, which is clinically manifested as hypertension and hypokalemia (1). The prevalence of PA varied according to the population studied: it ranged from 3.2% to 12.7% in primary care and from 1% to 29.8% in referral centers (2, 3). Studies have demonstrated that patients with PA have more worse target organs of the heart and kidneys compared to patients with essential hypertension (4–7). Changes in left ventricular (LV) geometry, as an important target organ injury induced by elevated blood pressure, are independent predictors of cardiovascular events (8, 9). Early identification of the potential risk factors of changes in LV geometry plays an important role in preventing cardiovascular disease.

Vascular aging reflects the structural and functional changes of the large conduit arteries (10). Carotid intima-media thickness (cIMT), brachial–ankle pulse wave velocity (baPWV), and ankle–brachial index (ABI) are main evaluation indicators to detect the degree of vascular aging (11). The relationship between vascular aging and LV geometry patterns has been reported in a previous study. A recent study based on Northern Shanghai general populations revealed that baPWV, carotid-femoral PWV (cfPWV), and cIMT were associated with structural measurements of LV including relative wall thickness (RWT) with LV mass index (LVMI), and increased cfPWV and increased baPWV and decreased ABI were significantly associated with LV concentric geometry (LVCG) (12). A previous study found that cfPWV and baPWV were significantly correlated with LV hypertrophy (LVH) in a community-based elderly cohort (13). A prospective cohort study showed that baPWV had significant correlations with RWT and LVMI in untreated hypertensive patients, and baPWV was significantly increased in patients with LVH (14). Now, available studies are based on data from the general population and essential hypertension.

However, in PA, a population at high risk for cardiovascular events, the relationship between vascular aging and LV geometry has never been explored. In the present study, we aimed to investigate the association of the vascular aging parameter (baPWV, ABI, and cIMT) and LV geometry in new diagnosed patients with PA and to provide support for the assessment of target organ injury in the preliminary evaluation of PA.

Methods

Study population

The study participants were recruited from 1 January 2017 to 30 September 2021 at the Department of Cardiology, The Third Xiangya Hospital, Central South University, Changsha, China. The records of 146 patients with newly diagnosed PA were retrospectively collected in the database of the Third Xiangya Hospital. The inclusion criteria were as follows: (1) age ≥18 years old and (2) confirmed diagnosed with PA on this admission and not previously diagnosed with PA. The exclusion criteria were as follows: (1) missing echocardiography data; (2) simultaneously missing data of baPWV, ABI, and cIMT; (3) a definite diagnosis of other secondary hypertension; (4) serious heart disease (New York Heart Association functional classification ≥III) or severe chronic kidney disease [estimated glomerular filtration rate (eGFR) < 30 ml/min/1.73 m^2]; (5) cardiovascular events within the last 6 months, including myocardial infarction or stroke; (6) suffered from cancer and other systemic diseases, including acute or chronic inflammatory diseases; and (7) pregnant or lactating women. The present study was approved by the Medical Ethics Committee of the Third Xiangya Hospital (Approval ID: I22013). All the patients signed informed consent at admission and agreed to share their health information for medical research.

Screening and confirmatory tests for primary aldosteronism

Before screening and confirmatory tests, antihypertensive medicine with great influence on the aldosterone-to-renin ratio (ARR) was withdrawn at least 4 weeks according to the Endocrine Society's clinical practice guideline (15). Patients with poor blood pressure control may be treated with alpha blockers or non-dihydropyridine calcium channel blockers, which have a lesser effect on ARR. Blood samples were collected to measure plasma aldosterone concentration (PAC) and plasma renin activity (PRA) in the morning after patients woke up and maintained a non-supine position (sitting, standing, or walking) for at least 2 h and sitting for 5–15 min. ARR was calculated as PAC/PRA. For patients with positive screening (ARR>20 $ng{\cdot}ml^{-1}/ng{\cdot}ml^{-1}{\cdot}h^{-1}$), further confirmatory tests were performed to confirm the diagnosis.

All patients with positive screening tests underwent seated saline suppression testing (SSST). PAC was measured basally at 08:00 and after the completion of an infusion of 2 L of normal saline in 4 h, and PA was diagnosed when PAC >10 $ng{\cdot}ml^{-1}$ after the infusion of 2 L of normal saline (15). Studies have confirmed that SSST is more sensitive than recumbent saline suppression testing (RSST) in the diagnosis of PA (16).

Data collection and variable definitions

Information on sociodemographic characteristics and clinical data were obtained from electronic medical records, including age, gender, height, weight, smoking status (current, never, or past), drinking status (current, never, or past), serum

potassium level, blood pressure (BP), heart rate, the duration of hypertension, a family history of premature cardiovascular disease, and BP-lowing drug treatment. BP measurements were taken in the sitting position after a rest period of at least 5 min using an automated electronic device (Omron HEM-7200, OmronCo, Dalian, China). The body mass index (BMI) is calculated by dividing the body weight in kilograms by the square of the height in meters. Biochemical parameters included fasting blood glucose (FBG), blood lipid levels (total cholesterol, triglycerides, high-density lipid cholesterol, and low-density lipid cholesterol), and serum creatinine. The eGFR was calculated using the Modification of Diet in Renal Disease formula (17).

Measurement of vascular aging parameters

The ABI and baPWV were measured simultaneously with an automatic waveform analyzer (BP-203 RPE III; Omron, Dalian, China). The measurement was taken in the supine position after a rest period of at least 5 min. ABI was measured as the ratio of the systolic BP of the ankle artery to that of the brachial artery. The pulse waves of the brachial and posterior tibial arteries of the left and right limbs were measured to assess the transmission time between brachial and posterior tibial artery waveforms. The baPWV was calculated as the brachial–ankle distance divided by the transmission time (18). The average values of the left- and right-side assessments were calculated and used for analysis. ABI and baPWV were measured in 105 patients. Carotid ultrasonography was performed to measure cIMT using color Doppler ultrasound (Vivid E95; GE Healthcare, Massachusetts (MA), USA; or EPIQ 7C; Philips Medical Systems, Massachusetts (MA), USA). The cIMT represents the distance between two parallel echogenic lines corresponding to the lumen-intima interface and media-adventitia interface (19). The cIMT value used for analysis was defined as the average value of the left and right assessment. The cTMT was measured in 135 patients.

Echocardiography assessment of left ventricular geometry

Echocardiography was performed by trained cardiologists using an available machine (Vivid E95; GE Healthcare, MA, USA or EPIQ 7C; Philips Medical Systems, MA, USA), according to the recommendation from the American Society of Echocardiography and the European Association of Cardiovascular Imaging (20). The LV internal diameter (LVID) at the end-diastole, interventricular septum (IVS), and posterior wall thickness (PWT) at the end-diastole were measured at the standard parasternal window. LV mass was calculated by a validated formula (20). LVMI was calculated with the formula (LV mass/body surface area) and RWT with the formula (2 × PWT/LVID) (20, 21).

LV geometry patterns were defined into normal, concentric remodeling, concentric LVH, and eccentric LVH. LVH was defined as LVMI >115 g/m² in men and >95 g/m² in women, including concentric LVH (RWT > 0.42), and eccentric LVH (RWT ≤ 0.42). RWT > 0.42 without LVH was considered diagnostic for concentric remodeling. Concentric LVH and concentric remodeling were defined as LVCG.

Statistical analysis

Continuous variables were presented as weighted means ± standard deviation or median (interquartile range) and categorical variables as numbers (percentages). Continuous variables were compared using Student's t-test or Wilcoxon rank sum test, and categorical variables were compared using chi-square analysis. Spearman correlation analysis was performed to assess the correlation of vascular ageing parameters with LVMI and RWT. The linear regression model was applied to compare the relationship between vascular aging parameters with LVMI and RTW. When the dependent variable was not normally distributed data, we performed logarithmic transformation on the data. When the association of vascular ageing parameters and LV geometry was investigated, logistic regression analysis was used to calculate odds ratios (ORs) and 95% confidence intervals (95%CIs) by controlling age, gender, BMI, smoking status, drinking status, BP, heart rate, FBG, TG, HDL-C, eGFR, serum potassium level, PAC, the duration of hypertension, a family history of premature cardiovascular disease, and BP-lowering drug treatment. All statistical analyses were performed using Stata version 16.0 (StataCorp, College Station, TX, USA). P-value < 0.05 (two sided) was considered to be statistically significant.

Results

Characteristics of study patients

This study included 146 patients with newly diagnosed PA. The characteristics of these patients are shown in Table 1. The participants' mean age was 44.77 ± 9.79 years, and 46.58% of participants were female, with an elevated median value of systolic BP (160 mm Hg) and diastolic BP (96 mmHg). The duration of the hypertension of patients was 2.00 years, and approximately 60% of the patients were receiving BP-lowering drug treatment. The median of the baPWV value and cIMT value were 1,618 cm/s and 0.9 mm, respectively. The mean value of ABI was 1.15. Approximately 87.67% of the patients had abnormal LV geometry patterns. Approximately 50.68% of patients had LVH, and 83.56% had LVCG.

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TABLE 1 Characteristics of study patients.

Variables	Value
Age (years)	44.77 ± 9.79
Female, n(%)	68(46.58)
BMI (kg/m2)	25.41 ± 3.60
Current smoking, n(%)	33(22.60)
Current drinking, n(%)	13(8.90)
Systolic blood pressure (mmHg)	160(143–170)
Diastolic blood pressure (mmHg)	96(87–106)
Heart rate (beats/min)	71(65–76)
TC (mmol/L)	4.31 ± 0.92
TG (mmol/L)	1.55(0.99–2.35)
LDL-C (mmol/L)	2.25 ± 0.67
HDL-C (mmol/L)	1.10(0.95–1.27)
FBG (mmol/L)	5.02(4.54–5.44)
eGFR (ml/min/1.73 m^2)	99.14(85.07–112.98)
Serum K^+ (mmol/L)	3.51 ± 0.52
PAC (ng/ml)	29.62(25.08–33.55)
PRA (ng ml^{-1}/h)	0.89(0.74–1.17)
ARR (ng ml^{-1}/ng ml^{-1} h^{-1})	31.82(25.43–37.50)
Family history of premature CVD, n(%)	48(32.88)
Duration of hypertension, year	2.00(0.25–5.00)
Blood pressure–lowering drug treatment, n(%)	89(60.96)
baPWV (cm/s)	1,618(1,456–1,830)
ABI	1.15 ± 0.07
cIMT (mm)	0.90(0.75–1.05)
LVMI (g/m^2)	104.67(92.39–124.24)
RWT	0.49 ± 0.07
Left ventricular geometry patterns	
Normal	18(12.33)
Concentric remodeling	54(36.99)
Concentric LVH	68(46.57)
Eccentric LVH	6(4.11)
LVH	74(50.68)
LVCG	122(83.56)

Continuous variables were presented as weighted means ± standard deviation or median (interquartile range) and categorical variables as numbers (percentages).
BMI, body mass index; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipid cholesterol; HDL-C, high-density lipid cholesterol; FBG, fasting blood glucose; eGFR, estimated glomerular filtration rate; CVD, cardiovascular disease; PAC, plasma aldosterone concentration; PRA, plasma renin activity; ARR, aldosterone-to-renin ratio; baPWV, brachial–ankle pulse wave velocity; ABI, ankle–brachial index; cIMT, carotid intima-media thickness; LVMI, left ventricular mass index; RWT, relative wall thickness; LVH, left ventricular hypertrophy; LVCG, left ventricular concentric geometry.

Correlation of vascular parameters with left ventricular mass index and relative wall thickness

The correlation of vascular parameters with LVMI and RWT is demonstrated in Table 2. ABI (r=0.313, P=0.001) and cIMT (r=0.346, P<0.001) were positively correlated with LVMI. BaPWV was positively correlated with RWT (r=0.238, P=0.014). The correlation plots of vascular parameters with LVMI and RWT are shown in Figure 1.

Association of vascular parameters with left ventricular mass index and relative wall thickness

The results of the linear regression analysis of the relationship of vascular parameters with LVMI and RWT are summarized in Table 3. **A** crude model showed that ABI and cIMT were positively associated with LVMI and baPWV was positively associated with RWT. After adjusting all potential confounders, cIMT was still significantly associated with LVMI (β=0.164, P=0.028) and baPWV was still significantly associated with RWT (β= 0.00005, P=0.025).

Association of vascular parameters with left ventricular hypertrophy and left ventricular concentric geometry

We compared the values of vascular parameters between LVH and non-LVH and LVCG and non-LVCG. As shown in Figure 2, patients with LVH had significantly higher cIMT values compared to those without LVH (P=0.001), and patients with LVCG had significantly higher baPWV values compared to those without LVCG (P=0.022).We further stratified the values of vascular parameters into four groups of LV geometry patterns. There was no statistical difference in the values of vascular parameters between the four LV geometry patterns.

Table 4 shows the logistic regression analysis results of the association of vascular parameters with LVH and LVCG. The crude model showed that cIMT was positively associated with LVH and baPWV was positively associated with LVCG. After adjusting all potential confounders, cIMT was still significantly associated with LVH (OR=37.421, 95%CI: 1.717–815.688, P=0.021) and baPWV was still significantly associated with LVCG (OR=1.003, 95%CI: 1.001–1.006, P=0.017).

Discussion

To the best of our knowledge, this is the first study to explore the relationship of baPWV, ABI, and cIMT with LV geometry in patients with newly diagnosed PA. In the present study, we found that baPWV was significantly associated with RWT and LVCG and cIMT was significantly associated with LVMI and LVH in newly diagnosed PA. The finding of this study provided evidence for the significant contribution of baPWV and cIMT to abnormal LV geometry in newly diagnosed PA.

The change of LV geometry is a response to systemic and local hemodynamic changes and has also been described as target organ

TABLE 2 Correlation of vascular parameters with LVMI and RWT.

Parameters	LVMI		RWT	
	r	P	r	P
baPWV(cm/s)	-0.013	0.897	0.238	0.014
ABI	0.313	0.001	0.074	0.452
cIMT(mm)	0.346	<0.001	0.161	0.062

baPWV, brachial–ankle pulse wave velocity; ABI, ankle–brachial index; cIMT, carotid intima-media thickness.

injury (22, 23). It is widely recognized that LV geometry changes are independent risk factors for cardiovascular events (24–26). A number of studies have examined the relationship between vascular aging parameters and LV geometry and found that they are closely related (12–14, 27–34). The Northern Shanghai Study (NSS) involving a community-dwelling older population suggested that baPWV, cfPWV, and cIMT were significantly associated with LVMI and RWT, and cIMT was significantly related to LVMI, even adjusting for conventional cardiovascular risk factors and diseases and treatments (12). A study investigated the association of baPWV with LV geometry in treatment-naive hypertensive patients, which found that baPWV had significant associations with RWT and LVMI and showed a fair discrimination ability of LVH (14). There were two studies that have assessed the relationship between cIMT and LV geometry in hypertensive patients (27, 28). A study on Italian hypertensive patients showed a significant correlation of cIMT and LVMI (28), and another study on Korean hypertensive patients found that cIMT was independently associated with RWT (20). Linde et al. demonstrated that increased cfPWV was also associated with LVH in non-elderly ischemic stroke survivors (32). However, those epidemiological results on the relationship of the vascular aging parameter and LV geometry were based on the general population or hypertensive patients.

In this current study, we analyzed the association of baPWV, ABI, and cIMT with LV geometry in patients with newly diagnosed PA. Our study found that baPWV was positively correlated with RWT; ABI and cIMT were positively correlated with LVMI, which was consistent with previous studies (12, 28). Moreover, we found that when the potential covariables were adjusted, baPWV was still significantly associated with RWT and cIMT was still significantly associated with LVMI. Based on these findings, we analyzed the association of vascular aging parameters with LVH and LVCG; we found the significant association of baPWV with LVCG and cIMT with LVH. In untreated hypertensive patients, baPWV showed a significant association of LVH (14); however, our research did not find that baPWV was associated with LVH. The Possible causes were considered: there were confounding factors between the association of baPWV and LVH and the population in this study was from a single center.

Vascular remodeling in patients with PA mainly involves the increase of cells and extracellular matrix in the subintimal space and middle layer, which is manifested by vascular wall thickening, arterial stiffness, and subsequent vascular dysfunction (35, 36). BaPWV is an evaluation of arterial stiffness and reflects the elasticity of middle-sized and large arteries (37–39); cIMT is more

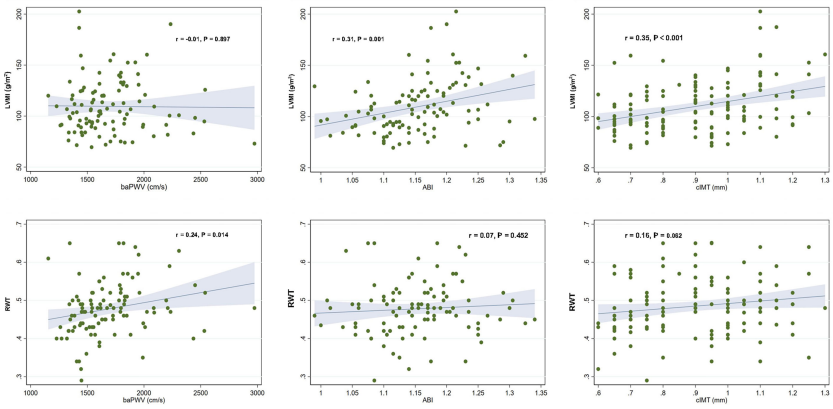

FIGURE 1
Correlation plots showing the associations of vascular parameters with left ventricular mass index and relative wall thickness. Each dot indicates an individual patient's data. The linear regression line (blue line) and 95% confidence interval (shaded area) are depicted. LVMI, left ventricular mass index; baPWV, brachial–ankle pulse wave velocity; ABI, ankle–brachial index; cIMT, carotid artery intima-media thickness; RWT, relative wall thickness.

TABLE 3 Linear regression analysis showing the association of LVMI and RWT with vascular parameters.

	Crude Model[a]		Adjusted Model[b]	
	β(95%CI)	P	β(95%CI)	P
logLVMI				
baPWV(cm/s)	0.00004(-0.00005–0.00013)	0.377	0.00003(-0.00005–0.00012)	0.436
ABI	0.392(0.010–0.773)	0.044	0.041(-0.340–0.423)	0.830
cIMT(mm)	0.196(0.066–0.326)	0.003	0.164(0.018–0.311)	0.028
RWT				
baPWV(cm/s)	0.00005(0.00001–0.00009)	0.014	0.00005(0.00001–0.00011)	0.025
ABI	0.073(-0.118–0.263)	0.452	-0.053(-0.268–0.161)	0.623
cIMT(mm)	0.067(-0.003–0.137)	0.062	0.044(-0.043–0.131)	0.316

[a]without adjustment.
[b]adjusted for age, gender, body mass index, smoking status, drinking status, blood pressure, heart rate, triglycerides, high-density lipid cholesterol, fasting blood glucose, estimated glomerular filtration rate, serum potassium level, plasma aldosterone concentration, the duration of hypertension, a family history of premature cardiovascular disease, and blood pressure–lowering drug treatment.
LVMI, left ventricular mass index; baPWV, brachial–ankle pulse wave velocity; ABI, ankle–brachial index; cIMT, carotid intima-media thickness; RWT, relative wall thickness.

reflective of the arterial wall structure throughout the body (40). A novel finding of our study is that baPWV was significantly associated with LVCG and cIMT was significantly associated with LVH in newly diagnosed PA. The study will provide a preliminary evidence of the association of the vascular aging parameter and LV geometry in patients with newly diagnosed PA. It is suggested that in newly diagnosed PA, early baPWV measurement has important clinical significance in assessing LV geometry changes and carotid vascular ultrasound in assessing LVH. The most plausible explanation for the association between vascular aging parameters and LV remodeling is that arterial stiffness and systemic changes in the vascular wall structure reflect the pressure pulse wave back to the LV more quickly, resulting in increased afterload of LV (41–43).

This study also has some limitations. First, since this study was retrospective, it could not provide a causal relationship between vascular parameters and LV geometry. Future studies could follow up the effects of increased baPWV or cIMT on LV geometry using a longitudinal design. Furthermore, we used baPWV as the indicator of arterial stiffness, while cfPWV is considered a gold indicator of arterial stiffness (18). However, the value of baPWV has been shown to be strongly associated with the value of cfPWV (44) and baPWV was better correlated with LV mass than cfPWV (45), suggesting baPWV as an acceptable indicator of vascular stiffness. In addition, our study participants were from a single center and the inclusion was Chinese patients, so that our results cannot be generalized to other groups of subjects with different demographics.

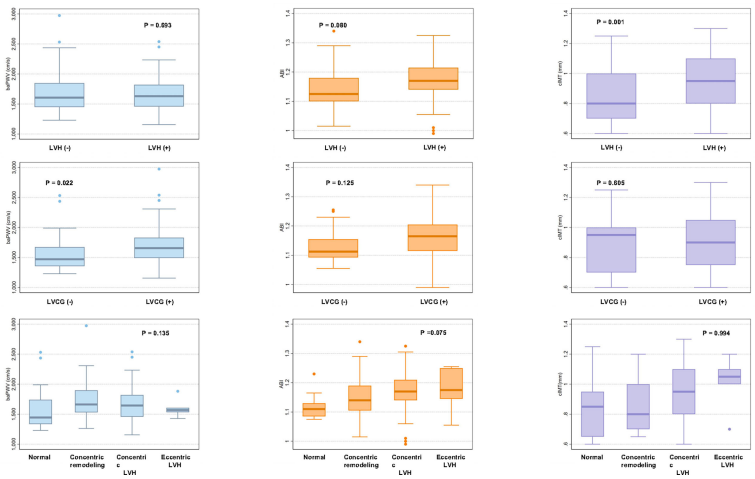

FIGURE 2
Box plots showing brachial–ankle pulse wave velocity, ankle–brachial index, and carotid intima-media thickness by left ventricular geometry patterns. BaPWV, ABI and cIMT values are shown as box plots with the median and interquartile range. BaPWV, brachial–ankle pulse wave velocity; ABI, ankle–brachial index; cIMT, carotid intima-media thickness; LVH, left ventricular hypertrophy; LVCG, left ventricular concentric geometry.

TABLE 4 Logistic regression analysis of LVH and LVCG with vascular parameters.

	Crude Model[a]		Adjusted Model[b]	
	β(95%CI)	P	β(95%CI)	P
LVH				
baPWV(cm/s)	1.000(0.999–1.001)	0.775	0.998(0.996–1.000)	0.074
ABI	142.149(0.520–38870.310)	0.083	1.601(0.001–4107.266)	0.906
cIMT(mm)	26.480(3.375–207.745)	0.002	37.421(1.717–815.688)	0.022
LVCG				
baPWV(cm/s)	1.001(1.000–1.003)	0.045	1.003(1.001–1.006)	0.017
ABI	278.184(0.202–382199.400)	0.127	82.108(0.004–1824029.000)	0.388
cIMT(mm)	1.879(0.153–23.021)	0.622	2.853(0.092–88.122)	0.549

[a]without adjustment.
[b]adjusted for age, gender, body mass index, smoking status, drinking status, blood pressure, heart rate, triglycerides, high-density lipid cholesterol, fasting blood glucose, estimated glomerular filtration rate, serum potassium level, plasma aldosterone concentration, the duration of hypertension, a family history of premature cardiovascular disease, and blood pressure–lowering drug treatment.
LVH, left ventricular hypertrophy; baPWV, brachial–ankle pulse wave velocity; ABI, ankle–brachial index; cIMT, carotid intima-media thickness; LVCG, left ventricular concentric geometry.

Conclusion

The present study suggested that baPWV was significantly and independently associated with LVCG and cIMT was significantly and independently associated with LVH in patients with newly diagnosed PA. This study provides insights on the importance of early baPWV and cIMT measurement in the assessment of LV geometry change in newly diagnosed PA.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was reviewed and approved by the Medical Ethics Committee of the Third Xiangya Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

WJ and XL contributed to conception and design of the study. MH and JL wrote the first draft of the manuscript. MH and JL performed the statistical analysis. XZ and SC wrote the sections of the manuscript. All authors contributed to article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Primary aldosteronism and obstructive sleep apnea: What do we know thus far?

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Both primary aldosteronism and obstructive sleep apnea are well-known causes of hypertension and contribute to increased cardiovascular morbidity and mortality independently. However, the relationship between these two entities remains unclear, with studies demonstrating contradictory results. This review aims to collate and put into perspective current available research regarding the association between primary aldosteronism and obstructive sleep apnea. The relationship between these two entities, clinical characteristics, clinical implications, outcomes of treatment, potential causal links and mechanisms are hereby presented.

KEYWORDS

renin, angiotensin, aldosterone, sleep disorders, obesity, hypertension, RAAS

Introduction

Primary aldosteronism (PA) is now recognized as one of the most common causes of secondary hypertension (1). Patients with PA were demonstrated to have higher risk of cerebrovascular and cardiovascular events, cardiovascular mortality and renal injuries compared to patients with essential hypertension, independent of blood pressure levels (2). Unfortunately, this disorder of the adrenal gland is substantially under-diagnosed (3). For decades, it is believed that the activation of renin-angiotensin-aldosterone system (RAAS) regulated the biosynthesis of aldosterone (4–7). There is now growing evidence that aldosterone secretion is not solely under RAAS regulation.

Obstructive sleep apnea (OSA) is a chronic and potentially life-threatening sleep-related breathing disorder caused by periodic narrowing and obstruction of upper airway during sleep, leading to repetitive apnea and hypopnea episodes (8). The prevalence has continued to rise over the years, mainly driven by an increase in prevalence of obesity, which is one of the major causes of OSA. It is another well-known risk factor for hypertension and is demonstrated to affect more than 80% of patients with resistant hypertension (9). In addition, OSA is associated with multiple long-term health complications, which include cardiovascular diseases and metabolic disorders (10).

Many studies have attempted to demonstrate a relationship between OSA and RAAS. Activation of RAAS, especially excess aldosterone, has been implicated to play a pathophysiological role in the relationship between OSA and hypertension, particularly resistant hypertension and PA (11). In fact, the Endocrine Society has identified OSA in the presence of hypertension as one of the groups with high prevalence of PA, and thus now recommends screening of PA among this cohort of patients (12).

In animal studies, episodic hypoxia led to elevated blood pressure which was prevented by renal artery denervation or treatment with angiotensin receptor blockers, suggesting a relationship between OSA and RAAS (13). Subsequently, this led to few human studies examining this relationship, which are illustrated below. Nevertheless, to date, the relationship between these two entities remains unclear. As both PA and OSA are known to contribute to increased cardiovascular morbidity and mortality, understanding the relationship between these two entities is essential in improving healthcare management of these patients by reducing cardiovascular-associated risks.

Hence, this review aimed to gather and put into perspective current available research regarding the association between PA and OSA. It focused on the relationship between these two entities, and presented evidence for their clinical characteristics with its implications, outcomes of treatment as well as the potential causal links and mechanisms.

OSA in PA

Prevalence

In a cohort of 207 patients with confirmed PA, 67.6% were found to have OSA (64.4% in White, 70.0% in Chinese), of which 27.1% were mild, 21.7% moderate and 18.8% severe (14). An almost similar prevalence of 55% was also observed in a retrospective analysis of 71 Japanese patients with PA (15). The prevalence of OSA seems to be much higher in PA than non-PA population (59.5% vs. 42.4%), albeit not significant (p=0.058) (16). This is probably due to the small sample size. Nevertheless, apnea hypopnea index (AHI) was demonstrated to be higher among patients with PA compared to those without PA (p=0.024) (16).

The prevalence of OSA in patients with confirmed PA is summarized in Table 1.

Clinical characteristics

Patients with PA and OSA were more likely to be males, older, with larger neck circumference, more abdominal obesity, higher body mass index (BMI) and worse metabolic profile. These individuals have higher blood glucose and triglyceride levels with lower HDL-cholesterol concentrations (14–16). The elevated plasma aldosterone level was noted to be significantly correlated with OSA severity. However, this correlation was only observed among the White, but not in the Chinese and Japanese cohort (14, 15), despite Chinese PA population demonstrated a more severe phenotype of OSA compared to the White (14). The discrepancy observed could probably be attributed to differences in craniofacial anatomy, adiposity and salt intake between Asians and Caucasians (18–20).

Outcomes of treatment

When patients with OSA and co-existent essential hypertension, resistant hypertension or PA were given mineralocorticoid receptor (MR) blockade for total duration of 8 weeks to 8 months, a significant reduction in AHI, hypoxic index and oxygen desaturation index was observed along with a decrease in body weight, neck circumference and blood pressure in all three groups of patients (17, 21, 22). Nevertheless, the reduction in AHI among patients with PA was not uniform and the

TABLE 1 Prevalence of OSA in PA.

Author	Number of patients with confirmed PA, n	Prevalence of confirmed OSA, n (%)	OSA severity, n (%)
Prejbisz 2013 (16)	32	19 (59.4)	NA
Wolley 2017 (17)	34	27 (79.4)	Mild 9 (33.3) Moderate 8 (29.6) Severe 10 (37.0)
Buffolo 2019 (14)	207	140 (67.6)	Mild 56 (40.0) Moderate 45 (32.1) Severe 39 (27.9)
Nakamura 2021 (15)	71	39 (54.9)	Mild 12 (30.8) Moderate 16 (41.0) Severe 11 (28.2)

PA, Primary aldosteronism.
OSA, Obstructive sleep apnea.
NA, Not available.

difference seen could be confounded by difference in population studied, methodologies and sample sizes.

Compared to medical therapy, the effect of surgical treatment among patients with PA and OSA is less studied. Adrenalectomy performed among patients with co-existing PA and OSA led to reduction in AHI and neck circumference (17). Nevertheless, the small number of patients (n=7) in this study limited statistical significance and certainty. In a larger cohort of patients with PA and OSA (n=48), the probability of OSA reduced significantly (Berlin score 1.69 pre-operation vs 1.33 post-operation, $p<0.001$) after adrenalectomy (23). However, the absence of OSA confirmation with sleep study might have explained the non-significant difference on the reduction of OSA probability between the surgical and medical therapies.

Possible mechanisms

Hyperaldosteronism may worsen the clinical course of OSA in patients with PA due to aldosterone-induced fluid accumulation in the neck (24). Aldosterone excess leads to salt and water retention in the distal tubules (25) causing rostral fluid shifts and para-pharyngeal edema. With the presence of neck tissue congestion, this increases upper airway resistance and subsequently collapse, thus worsening OSA (26, 27).

Besides, in rat models, infused aldosterone acted centrally to increase brain RAAS activity, oxidative stress, and sympathetic drive (28). This aldosterone-induced activation of central receptors may also lead to abnormal regulation of central breathing mechanisms, leading to deterioration of OSA.

Aldosterone excess has detrimental effects on β cell function leading to hyperglycemia. Hence it is commonly associated with metabolic dysregulation including type 2 diabetes (2, 29). Moreover, hyperaldosteronism is reported to induce insulin resistance by several other mechanisms such as autonomous cortisol secretion, impairment of glucose uptake into the liver, increment of hepatic glucose release, and enhancement of insulin-like growth factor-1 signaling (2). This echoes the results from Framingham Offspring study which demonstrated that aldosterone level is positively correlated with development of metabolic syndrome and increment of systolic blood pressure (30, 31). Patients with type 2 diabetes were reported to have an almost 50% increased risk in developing OSA compared to those without diabetes, especially among insulin-treated cohort, suggesting the role of insulin resistance in development of OSA (32, 33). This could be contributed by several mechanisms, including mixed apneic events seen in patients with type 2 diabetes (34), increased oxidative stress, autonomic dysfunction (35) and weight gain secondary to anti-diabetic medications (33).

Soluble plasma pro-renin receptors, which are specific receptors for both renin and pro-renin, were found to be significantly higher in male patients with OSA compared to age matched non-OSA male (36). This might explain the higher prevalence of OSA seen in male patients with PA. These receptor levels were also demonstrated to be positively correlated with severity of OSA, but not BMI, which further supports the association between OSA and RAAS (36).

MR antagonists augment diuresis and reduce leg-to-neck fluid redistribution. This leads to reduction in pharyngeal edema and upper airway resistance, causing improvement in OSA severity (14).

PA in OSA

Prevalence

Among 203 multi-ethnic cohort of patients diagnosed with OSA and hypertension, the prevalence of PA was reported to be 8.9% (11.8% in White, 5.9% in Chinese) (14). The authors concluded that this prevalence is not significantly different compared to the prevalence of PA observed in earlier studies (8.9% vs 5.9% in general hypertensive population and 11.2% from referral centers). However, in these earlier studies, OSA was not screened for in the subjects, which may explain the comparable prevalence. Furthermore, the prevalence of PA in OSA reported in this study needs to be interpreted with caution as the study was performed in different centers, using different confirmatory tests, different kits for aldosterone and renin, with lack of single scoring center, which could have biased the results.

In another study of 94 patients with moderate-to-severe OSA and hypertension, PA was confirmed in 21.3% compared to 8% of those without OSA (37). The prevalence of other metabolic disorders was noted to be high in this studied population (diabetes and impaired fasting glucose 90%, difficult-to-treat hypertension 70%, resistant hypertension 60%). These show that PA is a common part of multi-morbidity in patients with OSA, including diabetes and resistant hypertension (38).

The prevalence of PA in patients with confirmed OSA is presented in Table 2.

Clinical characteristics

Majority of patients with OSA who were subsequently diagnosed with PA presented with uncontrolled blood pressure ≥150/100mmHg, resistant hypertension or hypokalemia (14, 37). The frequency of PA in patients who presented only with OSA symptoms is low (1/18 and 4/20 respectively) (14, 37). Plasma aldosterone level in patients with OSA and metabolic syndrome was significantly higher compared to patients with OSA without metabolic syndrome, and this level was significantly related to AHI, waist circumference, triglyceride and HDL-cholesterol levels (11). Among patients with

TABLE 2 Prevalence of PA in OSA.

Author	Number of patients with confirmed OSA, n	Prevalence of confirmed PA, n (%)	Subtype classification, n (%)
DiMurro 2010 (39)	53	18 (34.0)	APA 5 (27.8) IHA 13 (72.2)
Buffolo 2019 (14)	230	18 (8.9)	APA 7 (38.9) IHA 10 (55.6) Undetermined 1 (5.5)
Dobrowolski 2021 (37)	94	20 (21.3)	APA 7 (10.0) IHA 18 (90.0)

OSA, Obstructive sleep apnea.
PA, Primary aldosteronism.
APA, Aldosterone-producing adenoma
IHA, Idiopathic hyperaldosteronism.

moderate-to-severe OSA and type 2 diabetes, plasma aldosterone, plasma renin and urinary aldosterone levels were higher compared to non-OSA patients with type 2 diabetes, although no correlation was found between AHI and the RAAS components in this cohort (40).

Outcomes of treatment

The use of CPAP therapy, ranging from 1 week to 12 months, among different cohort of patients with OSA, *ie* presence of metabolic syndrome (11), normotension (41), essential (42–45) and resistant hypertension (46, 47), and type 2 diabetes (40), showed significant reduction in RAAS components. Several studies which demonstrated lack of reduction in the RAAS components were mostly limited by a small sample size or short duration of CPAP use (48–53). To date, there are no studies evaluating the effect of CPAP therapy on RAAS components amongst OSA and PA patients.

Possible mechanisms

Intermittent hypoxia was shown to increase plasma levels of renin and aldosterone. It also enhanced angiotensin (Ang) I expression and resulted in AngII stimulation of carotid body receptors in animal models (13, 54–57). Similarly, sleep fragmentation and repetitive arousals in patients with OSA may lead to activation of the RAAS, causing an increased secretion of AngI, AngII and subsequently aldosterone.

In animal studies, acute hypercapnia or hypoxia separately increased plasma aldosterone levels, which was independent of increases in plasma renin activity, suggesting a renin-independent pathway in aldosterone secretion (58, 59). Sleep fragmentation and repeated arousal induce stress which stimulates the release of ACTH from the pituitary (11). The persistent activation of sympathetic nerve during both sleep and wakefulness not only stimulates the RAAS (53), but also the hypothalamic-pituitary-adrenal axis in releasing cortisol (40). Both RAAS and ACTH synergistically regulate aldosterone pulse wave. While RAAS plays a major role at night when plasma cortisol concentration is low, elevated cortisol concentration controls the pulse amplitude of aldosterone during the daytime (40).

OSA is commonly found in patients who are obese. The adipose tissue present in obesity is an important source of RAAS hormone secretion, which is independent from the classical RAAS activation (60, 61). This is demonstrated in experimental studies which showed that adipocytes release adipokines and free fatty acids that could stimulate aldosterone secretion from the adrenocortical cells (59, 62, 63). The finding of renin-binding protein gene in adipocytes, which acts as renin inhibitor, might be involved in modulation of renin activity. AngI and AngII receptors were obtained in rodent and human adipocytes with increased expression of AngI gene, especially in visceral adipocytes (64).

The angiotensin converting enzyme (ACE) is a vital enzyme in RAAS, playing a major role in development of cardiovascular diseases with I/D polymorphism of the ACE gene. Interaction between OSA and the ACE gene I/D polymorphism was significantly associated with presence of hypertension among Swedish patients (65) but not in the Turkish cohort (66). Furthermore, it is shown that Caucasian individuals with DD genotype are more prone to develop hypertension (66), in contrast to II genotype in Asians (67). Hence, ethnic differences in the genotype distribution for ACE gene I/D polymorphism may explain the differences seen in RAAS dysregulation in patients with OSA of different ethnicity.

As intermittent hypoxia is closely related to activation of RAAS, the resolution of intermittent hypoxia by CPAP may decrease the activity of RAAS leading to a reduction in aldosterone level (45). Additionally, CPAP improves ventilation, reduces sleep interruption, reduces sympathetic excitability, and increases insulin sensitivity, which in turn reduces aldosterone level (40).

The proposed mechanisms of this bi-directional relationship which are understood so far are summarized in Figure 1.

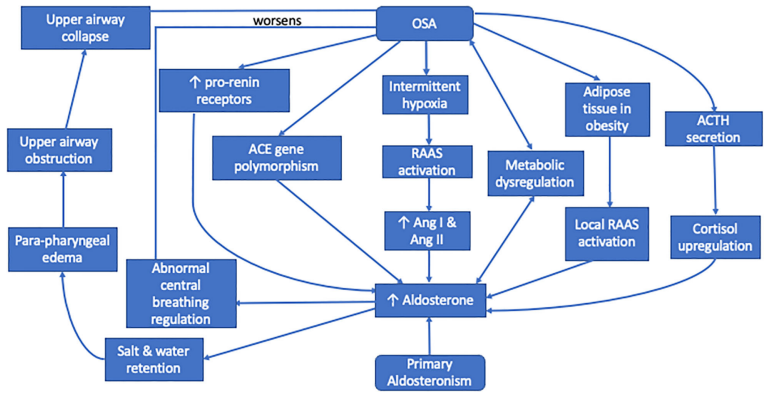

FIGURE 1
Relationship between primary aldosteronism and obstructive sleep apnea.

Research gap and future direction

Despite the studies summarized above depicting the relationship between PA, RAAS and OSA, there remains gaps in knowledge with regards to the associations among these entities. Given that clear relationships between PA and OSA have yet to be established, there is a need for further larger prospective studies to examine these links, especially among patients with hypertension, to determine if OSA is truly more prevalent among patients with PA. Likewise, large scale studies to screen the RAAS hormones among patients with OSA are needed to elucidate the true prevalence of PA among this cohort of patients. Risk factors for these patients to have co-existent PA and OSA should be determined to enable clinicians to screen more effectively for the presence of these disorders in high risk patients.

Furthermore, as data of a few studies point toward a likelihood of adipocyte-derived factor releasing adipokines and free fatty acids that could stimulate aldosterone secretion from the adrenocortical cells, independent from the systemic RAAS circulation, studies which examine this local RAAS effect on patients with OSA can further contribute to the knowledge of the relationship between PA and OSA.

It is shown that ACE enzyme is involved in RAAS regulation and ACE gene I/D polymorphism may play a role in hypertension development. As genotype distribution could be contributed by ethnic differences, studies examining ethnic factors can further explain the mechanisms of association between PA and OSA. This might lead to exploration of the role of ethnicity in the complex relationship between PA and OSA, including diagnosis and response to treatment. For example, those with ACE gene polymorphism may respond better to anti-aldosterone treatment for hypertension or blockade of different aspects of the RAAS, hence may benefit more from this class of treatment. Some of the studies have also demonstrated the association between PA and OSA to be more prevalent in specific phenotype with male predominance. Hence studies examining gender differences of this relationship may provide further insights in this field.

Data on the effect of treatment, whether CPAP on RAAS or PA-directed treatment on OSA severity, is substantially insufficient to date. This is especially true for long term outcomes of these treatment, not only on the disease per se, but on cardiovascular outcomes and reduction of target organ damage. This is an important aspect to be explored as it can lead to targeted therapy, which can be beneficial in this cohort of patients. The use of MR antagonists, ACE inhibitor or angiotensin receptor blockers remains low and clinical trials exploring the effect of these treatment are essential towards optimal blood pressure control.

In clinical context, for patients who are confirmed to have PA, OSA screening needs to be considered especially among males and those who are older, with larger neck circumference, greater abdominal obesity, higher BMI and worse metabolic profile. On the other hand, among patients with confirmed OSA, PA should be screened especially if blood pressure is ≥150/100mmHg, or in the presence of resistant hypertension or hypokalemia.

The evidence presented herein further underscores the necessity of early recognition and diagnosis of PA in patients with OSA and hypertension, as well as OSA in patients with PA, in line with the current Endocrine Society Guideline recommendations.

Conclusion

Current evidence suggests a bi-directional relationship between PA and OSA *via* aldosterone-induced worsening of OSA and OSA-associated dysregulation of RAAS. The beneficial effect seen with treatment of OSA on RAAS as well as PA-

directed treatment on OSA severity further supports the role of RAAS-driven pathogenesis in worsening of OSA, as well as the role of OSA-driven pathogenesis in worsening of PA. Nevertheless, given that clear relationship between PA and OSA has yet to be established, there is a need for further studies to examine this link, particularly the role of genotypic and phenotypic relationship, as well as long term beneficial effects of PA-directed therapy in OSA and vice versa.

Author contributions

HL conceived, designed and drafted the work. NS revised it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Vascular and hormonal interactions in the adrenal gland

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Primary aldosteronism is the most common form of secondary arterial hypertension, due to excessive aldosterone production from the adrenal gland. Although somatic mutations have been identified in aldosterone producing adenoma, the exact mechanisms leading to increased cell proliferation and nodule formation remain to be established. One hypothesis is that changes in vascular supply to the adrenal cortex, due to phenomena of atherosclerosis or high blood pressure, may influence the morphology of the adrenal cortex, resulting in a compensatory growth and nodule formation in response to local hypoxia. In this review, we will summarize our knowledge on the mechanisms regulating adrenal cortex development and function, describe adrenal vascularization in normal and pathological conditions and address the mechanisms allowing the cross-talk between the hormonal and vascular components to allow the extreme tissue plasticity of the adrenal cortex in response to endogenous and exogenous stimuli. We will then address recent evidence suggesting a role for alterations in the vascular compartment that could eventually be involved in nodule formation and the development of primary aldosteronism.

KEYWORDS

adrenal gland, aldosterone, vascularization, primary aldosteronism (PA), aldosterone producing adenoma

Introduction

The adrenal gland is an endocrine tissue composed of two distinct zones with different functions: the cortex, responsible for steroid biosynthesis, and the medulla, where catecholamine biosynthesis occurs. In human, the adrenal cortex is subdivided into three distinct functional zones: the outer part of the adrenal cortex is formed by the zona glomerulosa (ZG) responsible for mineralocorticoid biosynthesis, the intermediate and thickest part is formed by the zona fasciculata (ZF), responsible for glucocorticoids biosynthesis, and the inner part is formed by the zona reticularis (ZR) responsible for the biosynthesis of adrenal androgens (1). Both adrenal glands receive arterial blood supply

from the ventral aorta and the renal artery; the left adrenal is also supplied by the caudal branch of the aorta and the right adrenal by the phrenic artery (2)

Interestingly, the adrenal gland is one of the most vascularized organs. It has been shown that this organized vascular network played an important role during embryogenesis to ensure adrenal growth and differentiation, but also during whole life to provide precursors necessary for the biosynthesis of steroid hormones and to allow their secretion in blood flow (3). The specific ramification of adrenal cortex vasculature suggests strong interactions between endothelial and adrenal cells (4), allowing their coordinated development. Moreover, the proximity between endothelial and endocrine cells allows a rapid release of steroids into the blood flow.

Primary aldosteronism (PA) is the most common form of secondary arterial hypertension due to autonomous aldosterone production from the adrenal cortex. The two major causes are unilateral aldosterone producing adenoma (APA) or bilateral adrenal hyperplasia (BAH, also called idiopathic hyperaldosteronism). Patients show increased blood pressure, often associated with hypokalemia. Diagnosis is made in the presence of suppressed renin levels and increased aldosterone to renin ratio and is confirmed by one of different suppression tests. Adrenal imaging and adrenal vein sampling allow to distinguish between unilateral and bilateral forms and to introduce optimal treatment, either adrenalectomy for APA or treatment with mineralocorticoid receptor antagonists for bilateral forms (5). PA is found in up to 10% of patients with hypertension (6, 7) and its prevalence increases with the severity of hypertension (8). Over the past ten years, major progress has been made in elucidating genetic defects underlying familial and sporadic forms of PA. In particular, somatic mutations have been identified in genes coding for ion channels (*KCNJ5*, *CACNA1D*, *CACNA1H*, *CLCN2*) and ATPases (*ATP1A1*, *ATP2B3*) in up to 96% of APA (9–13). These mutations lead to cell membrane depolarization (*KCNJ5*, *ATP1A1*, *CLCN2*) or increase intracellular calcium (*ATP2B3*, *CACNA1D*, *CACNA1H*), leading to activation of calcium signaling that is the main trigger for aldosterone biosynthesis. In addition, mutations in *CTNNB1* coding for β-catenin, à key regulator of adrenal cortex development and function, have been identified in a subset of patients with PA, either alone or in association with mutations of *GNAQ*/*GNA11* in patients presenting with PA at puberty, pregnancy or menopause (14). However, it is still unclear, whether those mutations, in addition to promoting aldosterone biosynthesis, also increase cell proliferation and nodule formation and different hypotheses have emerged in recent years. Among them it has been postulated that changes in vascular supply to the adrenal cortex, due to phenomena of atherosclerosis or high blood pressure, may influence the morphology of the adrenal cortex, resulting in a compensatory growth and nodule formation in response to local hypoxia (15). This could eventually lead to the development of APA in extreme cases.

In this review we will briefly summarize the mechanisms regulating aldosterone biosynthesis in the adrenal gland, describe adrenal vascularization in normal conditions and how the cross-talk between the hormonal (epithelial) and vascular (endothelial) components ensures adrenal cortex growth and function under physiological conditions. We will then address recent evidence suggesting a role for alterations in the vascular compartment that could eventually be involved in nodule formation and the development of PA.

Regulation of aldosterone biosynthesis in the adrenal cortex

Aldosterone is synthesized in the ZG of the adrenal cortex from the precursor cholesterol through a series of enzymatic steps involving in particular the enzyme aldosterone synthase, encoded by *CYP11B2*, which is specifically expressed in this zone. Regulation of aldosterone biosynthesis is aimed at maintaining its essential functions as one of the principal regulators of extracellular fluid and electrolyte homeostasis as well as blood pressure, due to its effects on sodium reabsorption and potassium secretion in the kidney. Thus, aldosterone biosynthesis is regulated by the renin-angiotensin system (RAS), potassium concentrations and, to a lesser extent, by the adrenocorticotropic hormone (ACTH) (1). Following dehydration or salt loss, activation of the RAS regulates aldosterone biosynthesis *via* angiotensin II (AngII) binding to its type 1 receptor (AT1R) in ZG cells. This activates the inositol triphosphate pathway that stimulates Ca^{2+} release from the endoplasmic reticulum; alternatively, AngII inhibits potassium channels and the Na^{+},K^{+}-ATPase, inducing cell membrane depolarization, followed by opening of voltage-gated calcium channels. Both pathways increase intracellular calcium concentrations and activate calcium signaling, which regulates different steps involved in aldosterone biosynthesis, including expression of *CYP11B2 via* calcium/calmodulin-dependent protein kinases (16). Similarly, increased extracellular potassium concentrations induce cell membrane depolarization followed by activation of voltage-gated calcium channels and activation of calcium signaling (17).

ACTH binds to its receptor (melanocortin type 2 receptor, MC2R) and activates adenylate cyclase (AC), with subsequent activation of downstream signaling pathways, in particular the cAMP-dependent protein kinase (PKA) pathway (Figure 1). PKA activates StAR (steroidogenic acute regulatory protein) either directly, or by increasing its expression *via* CREB (cAMP response element binding protein) phosphorylation, thus increasing the amount of cholesterol delivered to the inner mitochondrial membrane. The conversion of cholesterol to pregnenolone in the mitochondria is one of the principal limiting steps in steroid biosynthesis (18) catalyzed by the P450 side chain cleavage enzyme (P450scc or CYP11A1) which is located at the inner mitochondrial membrane. The P450scc

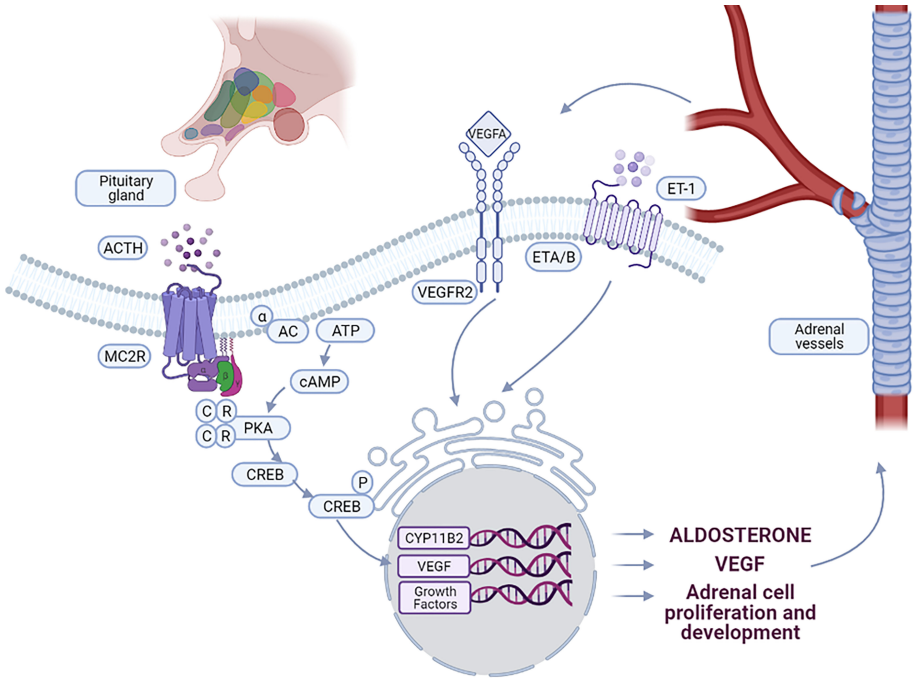

FIGURE 1
Interaction between vascular and steroidogenic cells in adrenal. ACTH, produced by the pituitary gland, controls the interactions between steroidogenic cells and vessels. Binding of ACTH to MC2R leads to the activation of PKA signaling pathway, stimulating CYP11B2 expression and aldosterone biosynthesis. ACTH stimulates also the expression of VEGF-A and specific growth factors by adrenal cells leading to vessel growth. By binding to VEGFR2, VEGF-A stimulates on one hand endothelial cell growth and on the other hand expression of growth factors involved in the maintenance and function of the adrenal cortex. Aldosterone biosynthesis is also induced by ET-1 through binding to the endothelin receptor ETA and ETB, expressed in the ZG of the adrenal cortex.

catalyzes the 20α-hydroxylation, the 22-hydroxylation and the cleavage of the bond between C-20 and the C-22 of cholesterol to obtain pregnenolone (19). In addition, ACTH also increases the expression of other enzymes of the steroidogenic cascade, such as *CYP11A1*, increasing the amount of precursors for aldosterone biosynthesis (20, 21).

In addition to these endocrine regulatory loops, autocrine and paracrine regulation of aldosterone production has been described (Table 1). Different factors produced by steroidogenic cells, such as renin and AngII (22–24), epoxyeicosatrienoic acid (EET) or prostaglandin E2 (PGE2), may modulate aldosterone secretion though an autocrine mechanism (25–27). Components of the RAS have been detected in the adrenal, however, their role in adrenal function is still unclear (36). It has been proposed that the adrenal RAS could play a role in the control of aldosterone production under potassium stimulation (23). Interestingly, in

TABLE 1 Endocrine, paracrine and autocrine regulators of aldosterone biosynthesis.

Secretagogue	Localization	Effect	References
Local Renin Angiotensin System	Steroidogenic cells	Stimulation	(22–24)
Epoxyeicosatrienoic acid (EET)	Steroidogenic cells	Stimulation	(25)
Prostaglandin E2 (PGE2)	Steroidogenic cells	Stimulation	(26, 27)
Epinephrine, Norepinephrine	Chromaffin cells	Stimulation	(28)
Dopamine	Chromaffin cells	Inhibition	(28)
Endothelin 1 (ET-1)	Endothelial cells	Stimulation	(29)
Cytokine C1q/TNF related protein	Adipocytes	Stimulation	(30)
Leptin	Adipocytes	Stimulation	(31, 32)
Substance P	Nerve fibers	Stimulation	(33)
Serotonin (5-HT)	Immune cells	Stimulation	(34, 35)

wild-type mice, adrenal production of renin is observed during embryonic development while kidneys are immature (37), but down-regulated after birth. However, in specific mouse models, expression of renin is observed even in adult mice to compensate deficiency in proteins involved in the control of aldosterone biosynthesis. This is the case in Task3 potassium channel knock-out mice (38) or aldosterone synthase deficient mice (39), as well as in mast cell deficient mice under low salt diet (40). Deletion of Task3 in mice leads to low-renin salt-sensitive hypertension, with suppressed plasma renin and aldosterone biosynthesis that is not-suppressible by increasing salt intake (38). Furthermore, paracrine regulation of aldosterone production is mediated by factors released by components of the microenvironment both in normal human adrenals and adrenals with APA, i.e. chromaffin cells, endothelial cells, adipocytes, nerve fibers and immune cells (28, 33, 41, 42). Interestingly, serotonin (5-HT) released by perivascular mast cells is known to induce aldosterone production by activating the 5-hydroxytryptamine receptor 4 (5-HT4) expressed in ZG cells (34). Chromaffin cells and nerve fibers stimulate aldosterone production by secreting neurotransmitters (NT), including catecholamines and various neuropeptides (35). In particular, it has been shown recently that the neuropeptide substance P released by intraadrenal nerve fibres is able to regulate aldosterone biosynthesis in the human adrenal cortex by binding to neurokinin type 1 receptors (33). Cytokine C1q/TNF related protein and the adipokine leptin are also able *in vitro* to activate aldosterone production (30–32). ZG cells express leptin receptors, thus leptin released by adrenal adipocytes may have a direct effect on aldosterone production (43). Finally, endothelial cells secrete endothelin 1 (ET-1) which by binding to endothelin receptor type A and B (ET-A, ET-B) on adrenocortical cells can stimulate aldosterone production (29, 44, 45).

Vascularization of the adrenal cortex

As an endocrine organ, the adrenal gland is highly vascularized, allowing each endocrine cell to be in contact with an endothelial cell (3). Part of the arterial flow to both adrenal glands is provided by the ventral aorta. The remaining arterial supply is provided bilaterally by the renal artery, completed by the phrenic artery for the right adrenal and by a caudal branch of the aorta for the left adrenal (3, 4). In the middle of the medulla, the central vein is responsible for venous drainage. This vein merges with the vena cava in the right adrenal or the renal vein in the left adrenal. Three adrenal arteries are distinguished: the superior adrenal artery; the middle adrenal artery and the inferior adrenal artery.

From the capsule, two types of arteries emerge from the arteriolar capsular plexus and enter the cortex and medulla: 1) The arteriae medullae, responsible for medulla arterial supply after passing directly through the adrenal cortex; and 2) The arteriae cortices, that arise directly from the capsule plexus, form an anastomotic network in the ZG, then cross the ZF as longitudinal capillary sinusoids between the columns of ZF cells (3, 4). The vasculature of the adrenal gland is composed of fenestrated sinusoids (46) that are highly permeable to fluids and small molecules. This facilitates the supply in nutrients, oxygen and cholesterol to the gland and the secretion of steroid hormones into the blood flow.

The widely branched capillary bed of the adrenal cortex strongly suggests an interaction between endothelial cells (ECs) and adrenal epithelial cells (4, 47). Indeed, ACTH controls the coordinated development of vessels and endocrine cells (Figure 1). The interaction between adrenocortical cells and endothelial cells enables a coordinated development of the vascular network with the proliferation of adrenal cells and organ growth. Endocrine glands are characterized by the high expression of vascular endothelial growth factor (VEGF) even in adults, regardless of the absence of active angiogenesis. In this context, the role of VEGF-A, whose expression is controlled by ACTH, is to maintain a high density of stable fenestrated microvessels (48). The combined secretion of angiogenic factors by endocrine cells and trophic factors by endothelial cells makes it possible to maintain a "symbiosis" between these cellular compartments. Regulation of adrenal vascularization and growth must be coordinated to ensure that the cortical mass has appropriate vascular support essential for both growth of the adrenal cortex and its endocrine function (49).

Regulation of vascularization in the adrenal cortex

Maintenance of the vascularization of the adrenal cortex and the regulation of blood flow by vasoconstriction involves different signaling pathways. AngII plays an important role in the regulation of blood pressure through its direct action on vasoconstriction (50). Interestingly, in the adrenal, vasodilatation or vasoconstriction may occur depending on the levels of AngII. Low concentration of AngII induces vasodilatation, *via* AT2R activation, production and release of nitric oxide (NO) by endothelial cells, whereas increased concentration of AngII leads to vasoconstriction due to activation of smooth muscle AT1R, resulting in decreased adrenal blood flow (51). ACTH, on the other hand, plays a role in the development and maintenance of this vascularization, and regulates blood flow to the adrenal gland through the release of vasorelaxant agents by adrenocortical cells such as metabolites of arachidonic acid (EETs) (52), but also through the release of histamine and serotonin by adrenal mast cells, factors modulating the tonicity of adrenal arterioles (53), indirectly influencing the production of steroids. Also, in response to ACTH, adrenal cortex cells secrete Thrombospondin-2 (TSP2), a large matricellular protein (54). It has been shown that TSP2

may act as an inhibitor of angiogenesis. *In vitro*, TSP2 inhibits the migration of capillary endothelial cells and, *in vivo*, neovascularization (55). It has been also shown that NO induces angiogenesis *via* the suppression of TSP2 expression, confirming the anti-angiogenic role of TSP2 (56). In addition, TSP2 may mediate ACTH-dependent centripetal adrenocortical cell migration (57). However, in mice lacking TSP2 no alterations in adrenal cortex morphology were observed (58, 59).

In the adrenal gland, ACTH also stimulates the release of VEGF and stabilization of its mRNA by the HuR protein (60). Conversely, the suppression of ACTH by dexamethasone in mice induces a progressive decrease in the expression of VEGF in the cells of the adrenal cortex and the regression of the vascularization (3). Interestingly, studies have also shown the role of mast cells in the development and maintenance of vascularization and in vasoconstriction. Mast cells are important cells in the immune system that originate from hematopoietic stem cells, which secrete serotonin, chondroitin, histamine and protease (61). Resident adrenal mast cells modulate the blood flow by the release of histamine and serotonin (5-hydroxytryptamine; 5-HT) (62). These cells are also a source of angiogenic factors including VEGF, fibroblast growth factor (FGF) 2, transforming growth factor β (TGF-β), tumor necrosis factor-α (TNF-α) and interleukin 8 (IL8) (63). They also induce the expression of VEGF by the release of cytokines and growth factors (TNF-α, TGF-β, platelet-derived growth factor (PDGF), FGF2 and IL-6) (62). Growth factors and cytokines released by mast cells have the ability to modulate endothelial cell function by increasing the expression of E-selectin but also by stimulating other cells that facilitate angiogenesis such as fibroblasts, epithelial cells and macrophages (64). The activation of mast cells also allows an increase in microvascular permeability, which has proangiogenic effects following the release of histamine, prostaglandin D2, Leukotriene B4, Leukotriene C4, VEGF and platelet-activating factor (65). Finally, the adrenal gland is a richly innervated organ, allowing innervation of chromaffin cells of the adrenal medulla. This innervation is, therefore, under the control of the sympathetic nervous system and allows the innervation of the internal part of the adrenal cortex. The adrenal cortex is also thought to be innervated by sympathetic fibers originating from extra-adrenal neurons, which, together with the blood vessels form the subcapsular plexus (66).

Adrenal cortex and medulla develop from two separate embryological tissues: the medulla is derived from the neural crest, while the cortex develops from the intermediate mesoderm. During development, the human fetal adrenal (HFA) cortex that develops from the adrenogonadal primordium, is composed of two zones: the inner zone, referred to as the fetal zone (FZ) with high expression of steroidogenic enzymes and a smaller outer zone, called definitive zone (DZ) where expression of steroidogenic enzymes is lower (67). The FZ of the adrenal cortex is the principal site of VEGF synthesis and one of the most vascularized organs in the human fetus (68). This pattern of VEGF localization is consistent with the fetal zone being the most vascular compartment in the cortex and the primary site of adrenal cortical growth. Thus, VEGF may act as a local regulator of fetal zone vascularization. The fetal zone vasculature comprises an extensive sinusoidal plexus. In contrast, the vasculature of the definitive zone is composed of distinct arterioles that arise from terminal branches of the capsular arterial network and enter the gland along connective tissue trabeculae. Therefore, as the cortex grows, the bulk of neovascularization would be expected to occur in the fetal zone (69). This vascular arrangement results in centripetal blood flow from the capsule through the definitive zone and into the sinusoidal network of the fetal zone to eventually drain into the central vein. Angiogenesis is essential for the rapid growth of the HFA. In addition, the HFA requires the development of an extensive vascular system for the delivery of steroid hormone precursors to the gland and the secretion of hormone products into the peripheral circulation. Various factors are involved in the regulation of angiogenesis. The evaluation of the expression and regulation of angiogenic factors specific to vascular endothelial cells, VEGF family members, angiopoietins (Angs) 1 and 2 in HFA medium showed that these factors are expressed in the HFA and that ACTH regulates them in isolated HFA cortical cells, suggesting that these factors may be key local regulators of HFA angiogenesis (70). Thus, they can mediate the tropic action of ACTH, exerting parallel control over the vascular system. In particular, ACTH induces an altered balance in which Ang2 predominates over Ang1. In addition, the Ang2 protein is mainly localized in the periphery of the HFA (i.e. the DZ and the outer region of the FZ). Its expression has been restricted to vascular remodeling sites, and Ang2 has been proposed to make endothelial cells sensitive to angiogenic stimuli, such as VEGF-A and FGF-2 (71). Furthermore, Steroidogenic factor-1 (SF-1) and Ang2 were found to be coexpressed in early stages of HFA development (72). It is demonstrated that despite the role of SF-1 in adrenal development and function, it plays a crucial role also in its angiogenesis by activating the Ang2 gene promoter in HFA (73). By using chromatin immunoprecipitation (ChIP) microarrays, it has been shown that vascular remodeling is a mechanism regulated by SF-1 in adrenal development and tumorigenesis (72).

Coordinated development of steroidogenesis and angiogenesis in the adrenal cortex by ACTH

The adrenal cortex is a highly plastic organ, in which environmental stimuli are translated to hormonal responses that can involve extreme tissue remodeling. Example of this is the ZG expansion observed under a low salt diet, which stimulates the renin-angiotensin system to promote aldosterone biosynthesis (74). On the other hand, endogenous

or exogenous glucocorticoid excess, such as treatment with dexamethasone, leads to a major regression of the ZF and suppression of glucocorticoid production (75). Both these changes are reversible and may involve major modification of adrenal vascularization.

ACTH is the main hormone regulating the function of the zona fasciculata and zona reticularis and stimulating glucocorticoid biosynthesis; it also stimulates, to a lesser extent, aldosterone production by the ZG. In addition, ACTH when binding to its receptor MC2R in the adrenals also induces the adrenal production of factors affecting adrenal growth and its blood flow. Indeed, ACTH controls angiogenesis and vascularization in the adrenal gland by stimulating the intra-adrenal production of VEGF and the vaso-relaxant EETs. On the other hand, VEGF can act on adrenal cells by binding on VEGF receptors present on adrenocortical cells and stimulates aldosterone production. Vascularization and adrenal cortex development must be coordinated to ensure that adrenocortical cells have access to blood vessels as the adrenal growths.

The vasculature of the HFA is established by the eighth week of gestation when the adrenal is supplied by arteries from the descending aorta, and the capillary sinusoids within the gland form a continuum with the systemic circulation. This stimulation is mediated by specific angiogenic factors like VEGF (76). Shifren et al. (69) showed that the HFA cortex is highly vascularized, consistent with its function as an endocrine organ, and that the HFA cortex expresses VEGF, which may regulate cortical vascular development (76). ACTH increases the steady state abundance of mRNA encoding VEGF. This may suggest that VEGF expression and secretion by human fetal adrenal cortical cells are up-regulated by ACTH and factors that increase intracellular cAMP production. In the same study, the authors also demonstrated that forskolin and ACTH are able to stimulate VEGF expression and secretion by HFA adrenocortical cells. This suggests that adenylate cyclase and cAMP pathways are the main regulators of ACTH-dependent VEGF production. Therefore, ACTH induces steroidogenic enzymes, cortisol and aldosterone production and expression of different growth factors *via* the same pathway, suggesting that ACTH may coordinate vascularization, adrenocortical development and steroidogenesis in the adrenal gland.

Cross-talk between aldosterone and the vascular system

In addition to AngII, extracellular potassium and ACTH, endothelin and VEGF have been shown to stimulate aldosterone production in a paracrine manner (28, 77). In particular, VEGF has been shown to stimulate aldosterone production indirectly by maintaining endothelial integrity but also directly by stimulating aldosterone synthase expression in ZG cells (77). The action of VEGF is either synergistic or independent of Ang II. Interestingly, in contrast to Ang II, VEGF does not increase the expression of StAR. The stimulatory role of VEGF is restricted to enhance aldosterone production, but does not modify cortisol biosynthesis in H295R adrenocortical cells (77). In addition, the inhibition of VEGF by overexpression of soluble fms-like tyrosine kinase-1 (sFlt-1) in rats is associated with reduced adrenal cortex vascularization (reduction of CD31 endothelial cell marker) that is accompanied by a reduction of aldosterone production (77). These results suggest that VEGF may have a role in aldosterone production independently of the RAS and may play a role in the autonomous overproduction of aldosterone in PA.

It has also been demonstrated that endothelial cell-conditioned medium stimulates aldosterone production in human adrenocortical H295R cells (78, 79). The interaction between endothelial and steroidogenic cells was demonstrated at the molecular level. *In vitro* studies in cultured human adrenocortical cells revealed that cytokines like IL-6 and ET-1, as well as NO produced by endothelial cells, are the main actors in this interaction (33, 42, 72). Adrenocortical cells express big ET-1, the precursor of endothelin, and its specific proteolytic enzyme endothelin converting enzyme, which generates the 21 amino acid peptide ET-1 (45, 80). Interestingly, ET-1 stimulates aldosterone biosynthesis in human and in rats (81). This action is mediated by the two endothelin receptor subtypes ETA and ETB expressed in the ZG of the adrenal cortex (33, 42).

On the other hand, aldosterone can act not only on renal epithelial cells to regulate blood pressure but also on vascular endothelial and smooth muscle cells. Indeed, activation of its receptor, the mineralocorticoid receptor (MR), has been shown to induce endothelial dysfunction, inflammation, remodeling, stiffening and atherosclerosis (82–88). In addition, it has been demonstrated in mice that aldosterone increases vessel density in response to ischemia, a phenomenon mediated by MR activation, AngII signaling and VEGF (89). Aldosterone also increases placental growth factor (PGF, a member of the VEGF family) expression in human atherosclerotic vessels, leading to inflammation and proliferation of vascular cells (90). Aldosterone may also up-regulates VEGF-A production in human neutrophils by activating PI3 kinases, ERK1/2, and to a lesser extent p38 MAPK pathways, suggesting that aldosterone has an active role on neovascularization (91).

Interplay between vascular and hormonal components in primary aldosteronism

Adrenals with APA show increased nodulation and reduced vascularization in the peritumoral adrenal cortex, as well as ZG hyperplasia (92). In addition, different studies indicate that the

structure and function of the adrenal cortex changes with age and it has been suggested that this may be a consequence of vascular dysfunction (93). Ageing is associated with an increase in adrenal nodulation in the general population, which may represent compensatory growth in response to ischemic changes due to localized atherosclerosis or hypertension (15). In addition, the adrenal cortex contains special structures, called aldosterone producing cell clusters (APCCs), in which somatic mutations have been identified in genes responsible for APA in normal subjects (94) and in adrenals with APA (12). It has been postulated that APCCs represent structures of autonomous aldosterone production. Interestingly, the number of APCCs increases with age (95), in parallel with a dysregulation of aldosterone production (93).

Mutations in different genes increase aldosterone production in PA, but additional mechanisms may contribute to increased cell proliferation and APA development. We have recently shown that retinoic acid receptor α (RARα) contributes to the maintenance of normal adrenal cortex structure and cell proliferation in mice, by modulating non-canonical Wnt signaling, extracellular matrix composition and angiogenesis. Dysregulation of this interaction may contribute to abnormal cell proliferation, creating a propitious environment for the emergence of specific driver mutations in PA (96). Indeed, RARα was identified as a central molecular network involved in adrenal nodulation in a transcriptome study comparing 48 APA and 11 control adrenals. Inactivation of Rarα in mice induced a major structural disorganization of the adrenal cortex in both sexes, with increased adrenal cortex size in female mice and increased cell proliferation in males. These changes were associated with abnormalities of vessel architecture and extracellular matrix. At the molecular level, Rarα inactivation led to decreased expression of components of the non-canonical Wnt signalling pathway, with decreased expression of *Wnt4*, *Tcf3*, *Lef1*, without affecting the canonical Wnt pathway nor PKA signaling. Rarα inactivation also reduced the expression of VEGF-A, while other angiogenesis factors such as VEGF-C and Hif1α were not affected. In contrast, the expression of components of the extracellular matrix like fibronectin 1, microfibrillar associated protein 2 and 5 (Mfap2 and Mfap5) and collagen 3α1 were increased. Altogether, these data highlight the important role of the interplay between the vascular and hormonal components in the adrenal cortex and suggest that alterations affecting this interplay, such as modifications of the extracellular matrix composition, may contribute to the disorganization of the adrenal cortex by modulating adrenocortical and vascular cell migration (96).

As observed in normal adrenals, it is expected that endothelin secreted by adrenal vessels and its signaling pathway in steroidogenic cells can stimulate aldosterone production and may have a role in its autonomous overproduction and by consequence contribute to the development of PA (80). Rossi et al. showed that selective endothelin receptors ETA and ETB antagonists lowered blood pressure in patients with PA and high to normal renin hypertension. Interestingly, in PA patients, these selective antagonists induced also a decrease in aldosterone biosynthesis (97, 98). However, in peripheral blood samples, ET-1 levels were similar in control subjects and in patients with APA; similar expression of prepro-ET-1, the endothelin-converting enzyme and the endothelin receptors ETA and ETB was found in APA and in normal adrenal gland (99). Moreover, ET-1 was demonstrated to have the same stimulatory effect as AngII on aldosterone production in APA (97). However, ET-1 receptors were found to be partially downregulated in APA in another study (45). These observations suggest that the endothelin signaling pathway in adrenocortical cells may play a role on aldosterone biosynthesis in APA and normal adrenals, but that this system is not crucial in the pathogenesis of this disease.

Recently, we have investigated the relationship between different signaling pathways and components of the microenvironment in adrenals with APA. We have applied multiplex immunofluorescence and multispectral image analysis to investigate the colocalization of proteins involved in aldosterone (CYP11B2) and cortisol (CYP11B1/CYP17A1) biosynthesis, markers of Wnt/β-catenin (β-catenin) and ACTH/cAMP/PKA (MC2R, pCREB) signaling, as well as paracrine pathways of the tumor microenvironment (Tryptase, S100) and vascularization (CD34) (100). Our results show a dense vascularization in APA, which is independent of the somatic mutation status of the tumor. Although the vascular surface was similar in areas expressing aldosterone synthase and areas not expressing aldosterone synthase in APA, VEGF-A expression analyzed by RT-qPCR in three APA was higher in areas expressing aldosterone synthase. This difference may be explained by the presence of perivascular mast cells, which was higher in areas positive for aldosterone synthase expression, which are involved in the maintenance of angiogenesis; alternatively, activation of the ACTH/cAMP pathway *via* MC2R may be involved, as MC2R was highly expressed in these same regions.

Vascularization and angiogenesis were also studied in other types of benign adrenocortical adenomas as well as in malignant adrenocortical tumors (ACC). Whether it was a benign or a malignant tumor, carriers of adrenocortical tumors showed higher circulating VEGF levels in comparison with healthy subjects (101, 102). Interestingly, VEGF expression was shown to be very high in patients with adrenocortical carcinomas and higher in APA in comparison to non-functional adenomas (103). Despite the high expression of VEGF, APA did not present higher vascular density than normal adrenals, but remarkably APA presented higher vascular density than non-functional adenomas, cortisol producing adenomas and adrenal cortical carcinomas (103). The same study also demonstrated that in APA, vascular density is positively correlated to aldosterone levels and negatively correlated to plasma renin activity. These results suggest that angiogenesis and the functional status of adrenocortical tumors are closely associated.

Conclusions

In conclusion, the coordinated interaction between steroidogenic and endothelial cells plays a crucial role in adrenal development and function and allows adrenal cortex remodeling in response to different physiological stimuli, such as modifications of sodium diet or stress response. Key players in this interaction appear to be ACTH and VEGF, which cross-talk to regulate hormone biosynthesis and vessel growth (Figure 1). Alterations in this interaction or factors affecting adrenal cortex vascularization may modify adrenocortical cell growth and promote cell proliferation and nodule formation, creating a propitious environment for the occurrence of somatic mutations in genes involved in the development of PA. Further studies will allow deciphering how this interplay may be altered in physiological or pathological conditions.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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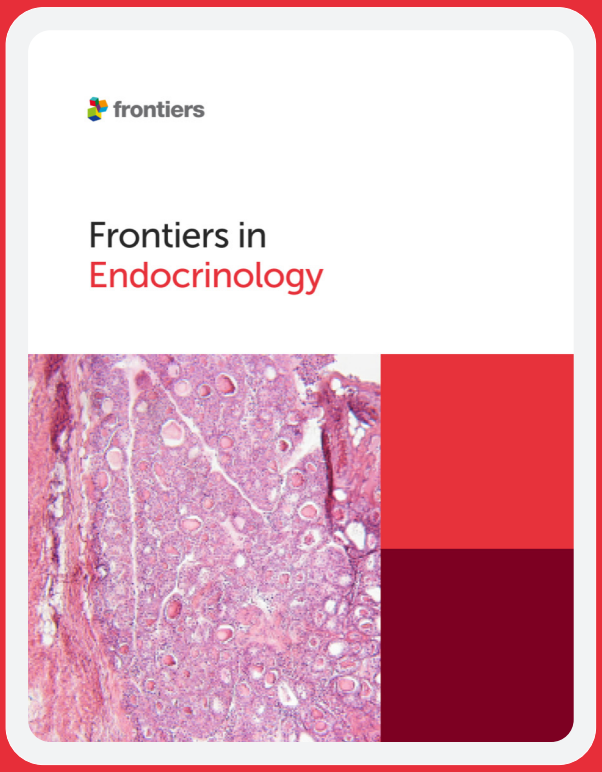

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