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Original Article

Immunohistopathology and Steroid Profiles Associated With Biochemical Outcomes After Adrenalectomy for Unilateral Primary Aldosteronism

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Abstract—Unilateral primary aldosteronism (PA) is the most common surgically curable form of hypertension that must be accurately differentiated from bilateral PA for therapeutic management (surgical versus medical). Adrenalectomy results in biochemical cure (complete biochemical success) in almost all patients diagnosed with unilateral PA; the remaining patients with partial or absent biochemical success comprise those with persisting aldosteronism who were misdiagnosed as unilateral PA preoperatively. To identify determinants of postsurgical biochemical outcomes, we compared the adrenal histopathology and the peripheral venous steroid profiles of patients with partial and absent or complete biochemical success after adrenalectomy for unilateral PA. A large multicenter cohort of adrenals from patients with absent and partial biochemical success (n=43) displayed a higher prevalence of hyperplasia (49% versus 21%; *P*=0.004) and a lower prevalence of solitary functional adenoma (44% versus 79%; *P*<0.001) compared with adrenals from age- and sex-matched patients with PA with complete biochemical success (n=52). We measured the peripheral plasma steroid concentrations in a subgroup of these patients (n=43) and in a group of patients with bilateral PA (n=27). Steroid profiling was associated with histopathologic phenotypes (solitary functional adenoma, hyperplasia, and aldosterone-producing cell clusters) and classified patients according to biochemical outcome or diagnosis of bilateral PA. If validated, peripheral venous steroid profiling may be a useful tool to guide the decision to perform surgery based on expectations of biochemical outcome after the procedure. (*Hypertension*. 2018;72:00-00. DOI: 10.1161/HYPERTENSIONAHA.118.11465.) ● Online Data Supplement

Key Words: adrenalectomy ■ adrenal glands ■ aldosterone ■ hyperaldosteronism ■ immunohistochemistry

Primary aldosteronism (PA) is a form of endocrine hypertension caused by the overproduction of aldosterone from one or both adrenal glands mainly because of a unilateral aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia.[1] Specific treatment by unilateral laparoscopic adrenalectomy (unilateral PA) or medical therapy with mineralocorticoid antagonists (bilateral PA) reverses the increased risk of stroke and heart disease in patients with PA compared with patients with essential hypertension.[2–4] Adrenal venous sampling or adrenal computed tomographic scanning is used to differentiate unilateral from bilateral PA although alternative approaches are currently being investigated, including functional imaging using positron emission tomography/computed tomographic scanning with a radiolabeled tracer and peripheral venous steroid profiling.[5–10]

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Immunohistochemistry using polyclonal[11] or monoclonal antibodies[12–14] to key enzymes involved in adrenal steroidogenesis (CYP11B2, CYP11B1, and CYP17A1) has aided identification of pathological features contributing to aldosterone overproduction.[15–17] The histopathologic abnormalities present in unilateral PA are highly heterogeneous and include solitary functional unilateral adenoma, adenoma with adjacent hyperplasia of the zona glomerulosa, or unilateral diffuse hyperplasia with functional micronodules or hyperplasia with macronodules.[17–21] Nests of CYP11B2-positive cells (aldosterone-producing cell clusters [APCCs]) have been identified beneath the adrenal capsule and are present in normal adrenals and in the adrenal cortex adjacent to an APA.[11,16,19,22,23] The occurrence of APCCs increases with age, and they frequently have somatic *CACNA1D*, *ATP1A1*, and *ATP2B3* mutations that drive dysregulated aldosterone production in APAs and have been proposed as a likely source of constitutive aldosterone production and possible precursors to APAs.[6,22,24]

Unlike unilateral PA, the pathophysiology of bilateral PA remains poorly understood, hampered, in part, by the scarce availability of resected adrenal specimens because of the medical, rather than surgical, management of bilateral PA. The PASO study (Primary Aldosteronism Surgical Outcome) established criteria to assess outcomes (complete, partial, or absent clinical and biochemical success) of patients after adrenalectomy for unilateral PA.[25] Clinical outcomes were defined by blood pressure measurements and antihypertensive medication dosage, biochemical outcomes by plasma potassium, aldosterone, and renin measurements.[25] Biochemical outcomes provide a quality measure of patient diagnosis with complete biochemical success defining the correct diagnosis and appropriate treatment, whereas absent and partial biochemical success indicate persistent aldosteronism after surgery. Absent and partial biochemical success combined comprises around 1 in every 20 patients with aldosterone lateralization performed by adrenal venous sampling, which presumably results from bilateral asymmetrical aldosterone overproduction.[25]

We hypothesised that patients with an absent or partial biochemical outcome comprise mainly cases of bilateral PA misdiagnosed as unilateral preoperatively. In a large multicenter study with outcomes assessed in accordance with an international consensus, we analyzed the histopathology of 95 adrenals from patients operated for unilateral PA (43 from patients with absent and partial biochemical success matched with 52 cases of complete biochemical success) and determined peripheral venous steroid profiles in a subgroup of these patients compared with patients diagnosed with bilateral PA.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Patient Selection

The study included surgically resected adrenals of 95 patients collected from 9 international centers that were diagnosed with unilateral PA and classified with absent, partial, or complete biochemical success at 6 to 12 months after unilateral adrenalectomy in accordance with the PASO consensus (Table S1 in the online-only Data Supplement).[25] Patients with absent and partial biochemical success

were matched for age (±5 years) and sex with patients with complete biochemical success from the same center. Peripheral venous plasma samples from patients of the Munich cohort (n=70) were analyzed by steroid profiling (absent or partial biochemical success, n=15; age and sex matched with complete success, n=28; and bilateral PA, n=27). All patients were diagnosed according to the Endocrine Society Guideline or the Japan Endocrine Society Guideline.[1,26]

Baseline and follow-up parameters of patients providing resected adrenals are shown in Table S2. Baseline parameters at study entry of patients providing peripheral plasma samples are shown in Table S3. Blood pressure measurements were recorded as described previously.[25] Study approval was obtained from the appropriate institutional review committees, and all patients gave informed consent in accordance with local ethical guidelines.

Histopathology

Successive paraffin-embedded adrenal tissue sections (4-μm thick) were immunostained for CYP11B2 (clone 41-17B), CYP11B1 (clone 80-7-5), and CYP17A1 (clone 10-19-G6) developed by C.E.G-S.[12,13] All hematoxylin and eosin–stained sections and immunostained sections were independently assessed by a specialist in adrenal histopathology (C.E.G-S.) and an experienced pathologist (I.C.). Both investigators were blinded for the surgical outcome of enrolled patients, and agreement was reached in cases of discordant scoring. The samples were scored for solitary functional adenoma (a single well-defined adenoma with positive CYP11B2 staining), hyperplasia (multiple CYP11B2-positive macronodules, focal thickening of the zona glomerulosa with CYP11B2-positive immunostaining or CYP11B2-positive diffuse hyperplasia with or without CYP11B2-positive micronodules), and APCCs (clusters of zona glomerulosa cells, CYP11B2 positive and CYP11B1 and CYP17A1 negative, localized in the subcapsular region extending into the zona fasciculata).[17,23] There were no significant differences in sex distribution or the average age of patients with a solitary functional adenoma, hyperplasia, or APCCs (Table S4).

Steroid Profiling Using Liquid Chromatography–Tandem Mass Spectrometry

Blood was drawn by venipuncture at time of diagnosis of PA between 8:00 and 10:00 AM in a fasting state and processed according to standard operational procedures. The measurement of 15 adrenal steroids using liquid chromatography–tandem mass spectrometry was performed in plasma of 15 patients with absent or partial biochemical success, 28 patients with complete biochemical success, and 27 patients with a diagnosis of bilateral PA as described.[9,10]

Statistical Analysis

Statistical analyses were performed using SPSS, version 24. Data are shown as mean±SD, as medians and interquartiles, or as absolute numbers and percentages. Quantitative normally distributed variables were analyzed using 1-way ANOVA with a post hoc Bonferroni or a *t* test, group differences by Kruskal-Wallis or Mann-Whitney *U* tests for quantitative non-normally distributed variables, and χ^2 or Fisher exact tests for categorical variables. A *P* value of <0.05 was considered significant. Linear discriminant analyses were performed in R, and decision tree analyses used MATLAB R2017b.

Results

Patient Characteristics

Patients with postsurgical complete biochemical success (n=52) had lower serum potassium concentrations at baseline relative to patients with an absent+partial biochemical outcome (*P*=0.035; Table S2). No significant differences were detected in nodule size (at pathology or imaging) and in the appearance of the contralateral adrenal at imaging with respect to biochemical outcome. However, patients with absent+partial success displayed a lower lateralization

index and a higher contralateral ratio compared with patients with a complete biochemical outcome (Figure 1; Table S5). Genotype data were available for 46 of the 95 specimens; the proportion of adrenals with a *KCNJ5* mutation was not significantly higher in the complete biochemical outcome group (18 adrenals carrying a *KCNJ5* mutation of 30 [58%] genotyped samples compared with 7 of 16 [37%] in the absent group; *P*=0.292; Table S5).

Adrenal Histopathology of Resected Sample Specimens According to Biochemical Outcome

The distribution of solitary functional adenoma, hyperplasia, or APCCs in the complete, partial, and absent biochemical success groups is shown in Figure 2A. In the total sample set, the majority of adrenals showed a solitary functional adenoma (60 of 95 samples, 63%) with 50% (30 of 60) displaying concurrent APCC in the adjacent cortex, 15% (9 of 60) associated with cortical hyperplasia, and 48% (29 of 60) without hyperplasia or APCC (normal-appearing adjacent cortex). The complete biochemical outcome group displayed a significantly higher prevalence of solitary functional adenomas compared with the absent+partial group (79% versus 44%; *P*<0.001; Table). The immunohistopathology of the adjacent cortex surrounding a functional solitary adenoma was not perceivably different in patients with complete biochemical success compared with an absent+partial biochemical outcome (Table).

Adrenals without a functional adenoma (without CYP11B2-positive immunostaining) comprised 37% of the total sample set (35 of 95 samples) with a higher prevalence noted in the absent+partial compared with the complete biochemical success group (56% [24 of 43] versus 21% [11 of 52]; *P*<0.001).

These adrenals showed a combination of mainly APCC and cortical hyperplasia, but nonfunctional adenomas (CYP11B2 negative), without a concurrent functional adenoma, were present in 9 adrenals with 7 in the absent+partial group and 2 in the complete biochemical success group (Table).

Adrenals from the absent+partial group had a higher prevalence of cortical hyperplasia (49% versus 21%; *P*=0.004), but no differences were observed in the proportion of adrenals with APCC or the average number of APCC per tissue

section compared with the complete biochemical success group (Table).

Liquid Chromatography–Tandem Mass Spectrometry Peripheral Venous Steroid Profiling

There were no significant differences in concentrations of peripheral venous adrenal steroids according to histopathologic feature (Table S6). Linear discriminant analyses of adrenal steroids correctly classified the presence or absence of solitary functional adenoma, hyperplasia, or APCC in 84% to 88% of samples (misclassification rate, 0.12–0.16; Figure 3B), and decision tree analysis using steroids selected from estimate prediction certainties improved the accuracy of prediction to 91% to 93% (misclassification rate, 0.07–0.09; Figure 3C through 3F).

For the absent+partial group, concentrations of aldosterone were higher in peripheral venous plasma compared with the bilateral PA group (*P*<0.001), and cortisone and 11-deoxycortisol concentrations were significantly higher than in the complete group (*P*=0.021 and *P*=0.017, respectively; Table S7). Discriminant analysis correctly predicted biochemical outcome after adrenalectomy and diagnosis of bilateral PA in 53 of 70 patients (76%; Figure 4A). Decision tree analysis improved the correct classification to 60 of 70 cases (86%; misclassification rate, 0.14): all 15 patients with an absent+partial biochemical outcome after surgery were correctly predicted albeit 5 patients with complete biochemical success were incorrectly classified with an absent or partial biochemical outcome (Figure 4C). Linear discriminant analysis and decision trees of steroid measurements resulted in a higher accuracy for the classification of biochemical outcomes compared with predictive models using adrenal venous sampling parameters (lateralization index and contralateral ratio; Table S8).

Discussion

We report the histopathology and peripheral venous steroid profiles associated with biochemical outcome after adrenalectomy for unilateral PA. In a multicenter international study with differentiation of unilateral from bilateral PA by adrenal venous sampling, adrenalectomy for unilateral PA resulted in biochemical cure (complete biochemical success) in 94% of
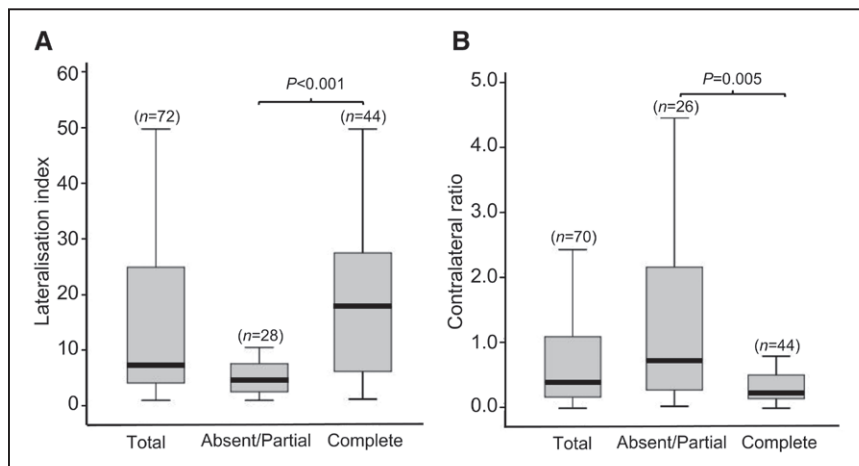
Figure 1. Adrenal venous sampling (AVS) results stratified for biochemical outcomes. Box and whisker plots showing AVS results stratified for biochemical outcomes. Patients with absent or partial biochemical success compared with complete success after adrenalectomy have lower lateralization indices (**A**) and higher contralateral ratios (**B**). Horizontal lines within boxes indicate the median, and box and whiskers represent the 25th to 75th and 5th to 95th percentiles, respectively. n indicates the number of patients in each group, and a Mann-Whitney *U* test was used to calculate *P* values.
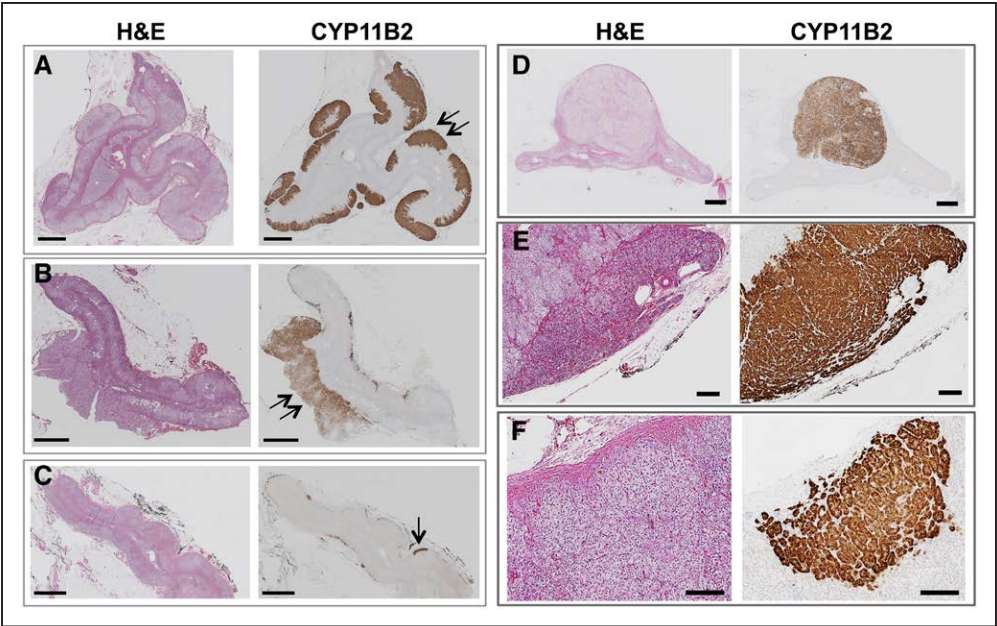
Figure 2. Heterogeneous histopathology of resected adrenals from patients with primary aldosteronism. The diverse histopathology of resected adrenals in this cohort is shown with hematoxylin and eosin (H&E) staining and CYP11B2 immunostaining as indicated. **A**–**C**, Adrenals without a functional adenoma showing the various histopathology of this subgroup. aldosterone-producing cell cluster (APCC) is indicated with a single arrow and hyperplasia with a double arrow (scale bar=2 mm). These 3 adrenals were from patients with postsurgical absent or partial biochemical success. **D** and **E**, Examples of histopathologic features classified in this study: solitary functional adenoma (**D**), hyperplasia of the zona glomerulosa (**E**), and an APCC (**F**; scale bars=200 μm).

patients thereby indicating the correct diagnosis and appropriate treatment.[25,27,28] Partial or absent biochemical success classifies patients with bilateral aldosterone excess who were presumably misdiagnosed as unilateral (instead of bilateral) preoperatively.[25] In the present study, the lower lateralization index and the higher contralateral ratio of patients with absent+partial biochemical outcomes would be consistent with the higher aldosterone production from the contralateral adrenal compared with the complete biochemical success group, despite a similar incidence of abnormalities detected by adrenal imaging.

The development of specific antibodies to CYP11B2 and CYP11B1 has revealed the complex heterogeneity of adrenal histopathology in PA.[11,12,17] Immunostaining of CYP11B2 identifies cells comprising the likely origin of constitutive aldosterone production and classifies diverse histopathologic subtypes of PA.[15–17] Unilateral aldosterone excess is usually produced from an APA[29] frequently accompanied by APCCs in the hyperplastic adjacent cortical tissue.[15,19]

In a multicenter study of patients diagnosed with unilateral PA, Åkerström et al[21] reported adenomas without associated hyperplasia in 287 of 348 (82%), adenomas with associated hyperplasia in 52 of 348 (15%), and hyperplasia with macronodules or micronodules in 9 of 348 (3%) of sample specimens. A higher prevalence of cortical hyperplasia has been reported by others[30,31] with multinodular hyperplasia or diffuse hyperplasia present in 54 and 12 resected adrenals, respectively, from 122 patients with postsurgical biochemical cure.[31] No association of histopathology with persistent PA was found in 6 patients with persistent PA and recurrent PA reported in 3 of 79 patients with long-term follow-up data who were previously biochemically cured.[31] Few studies have addressed the histopathology of bilateral PA. A study on 25

resected adrenals from patients with undetectable abnormalities by computed tomography scanning included 13 adrenals from patients with bilateral PA that displayed an increased

Table. Histopathology of Adrenals From Patients Stratified by Biochemical Outcome After Adrenalectomy

Variable	Total Cohort, n (%)	Biochemical Outcome		P Value
		A+P	C	
Total	95 (100%)	43 (45%)	52 (55%)	
Solitary functional adenoma	60 (63%)	19 (44%)	41 (79%)	<0.001
Normal-appearing adjacent cortex	29 (48%)	9 (47%)	20 (49%)	0.919
Hyperplasia	9 (15%)	5 (26%)	4 (10%)	0.200
APCC	30 (50%)	10 (53%)	20 (49%)	0.781
No functional adenoma	35 (37%)	24 (56%)	11 (21%)	<0.001
CYP11B2-negative adenoma	9 (26%)	7 (29%)	2 (18%)	0.403
Hyperplasia	23 (66%)	16 (67%)	7 (64%)	0.576
APCC	27 (77%)	18 (75%)	9 (81%)	0.508
Hyperplasia	32 (34%)	21 (49%)	11 (21%)	0.004
APCC	57 (60%)	28 (65%)	29 (56%)	0.355
APCC number (sample section)	3.2±2.9	3.2±3.1	3.3±2.8	0.641

Values indicate absolute numbers with proportions in parenthesis (%) or average numbers±SD. *P* values were calculated using χ^2 or Fisher exact tests or Mann-Whitney *U* tests as appropriate. A indicates absent biochemical success after surgery; APCC, aldosterone-producing cell cluster; C, complete biochemical success after surgery; CYP11B2, aldosterone synthase; and P, partial biochemical success after surgery.
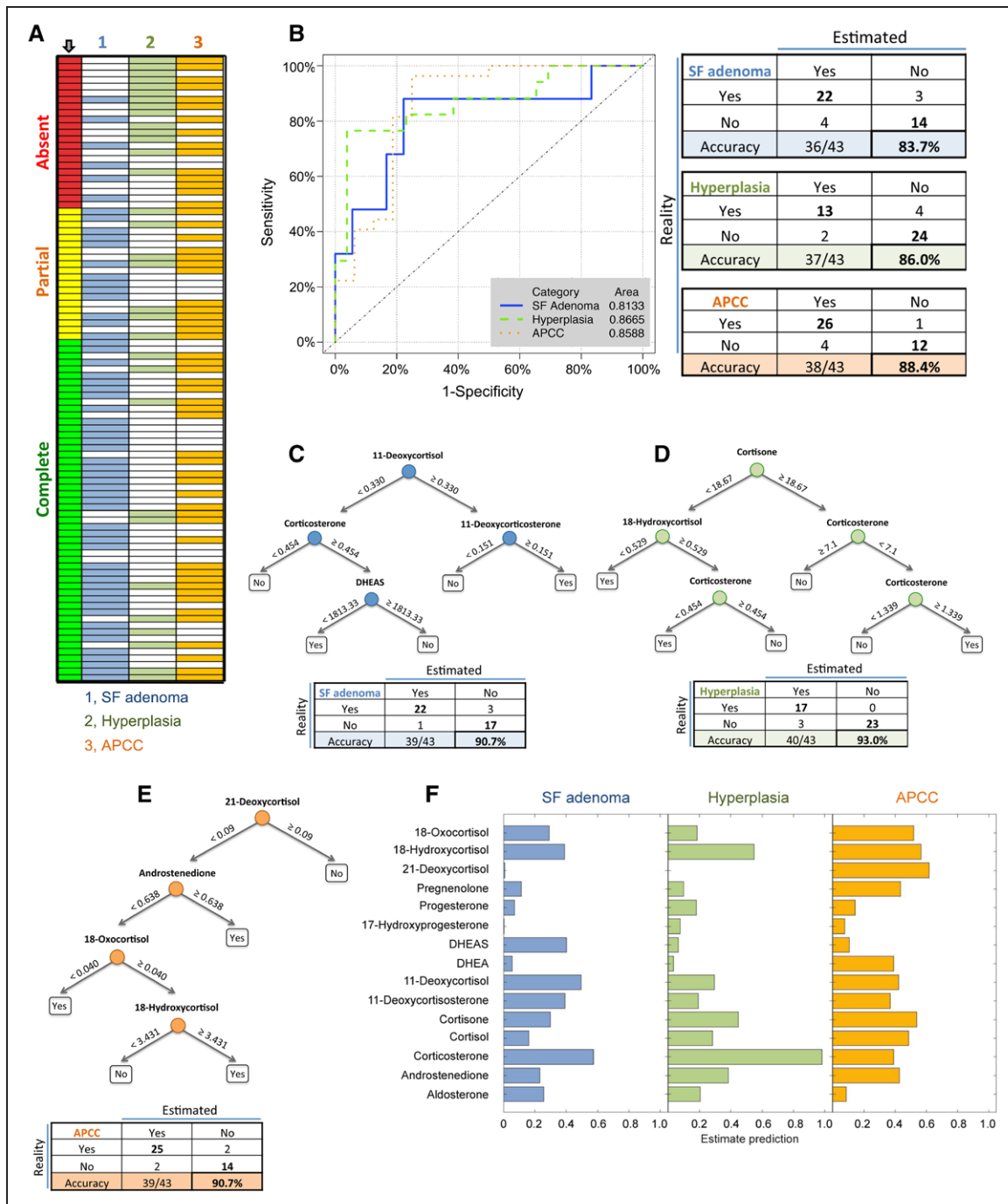
Figure 3. Classification of adrenal histopathology in primary aldosteronism according to peripheral venous steroid concentrations. The distribution of histopathologic features (1, solitary functional adenoma; 2, hyperplasia; and 3, aldosterone-producing cell cluster [APCC]) stratified for biochemical outcome (indicated by an arrow) is represented in (**A**). Linear discriminant analyses using peripheral venous steroid concentrations was used to generate receiver operating characteristic curves with areas under the curves (inset) and tables showing the real and estimated presence (yes) or absence (no) of solitary functional (SF) adenomas, hyperplasia, and APCCs (**B**). The steroids used in each model are shown in Table S9 with linear discriminant coefficients and cutoffs for prediction of the presence of SF adenoma, hyperplasia, or APCC. Decision tree analyses improved the prediction of histopathology by steroid measurements: decision trees with numbers indicating steroid concentrations in ng/mL predicting the presence (yes) or absence (no) of a solitary functional (SF) adenoma (**C**); hyperplasia (**D**) and APCCs (**E**) are shown with an accompanying table with the real and estimated presence (yes) and absence (no) of each histopathologic feature. Steroids used for decision tree analysis were selected from their estimated predictive performance (**F**). DHEA indicates dehydroepiandrosterone; and DHEAS, dehydroepiandrosterone sulfate.

incidence of diffuse functional hyperplasia compared with adrenals from unilateral PA.[16]

In a large sample set of 43 resected adrenals from patients with absent+partial biochemical success after adrenalectomy for unilateral PA, we show an increased prevalence of cortical hyperplasia in adrenals in agreement with the proposal that nodular hyperplasia may comprise a risk factor for persistent aldosteronism after surgery.[32] We also show the increased incidence of solitary functional adenomas (APAs) in the complete biochemical success group. Functional adenomas were
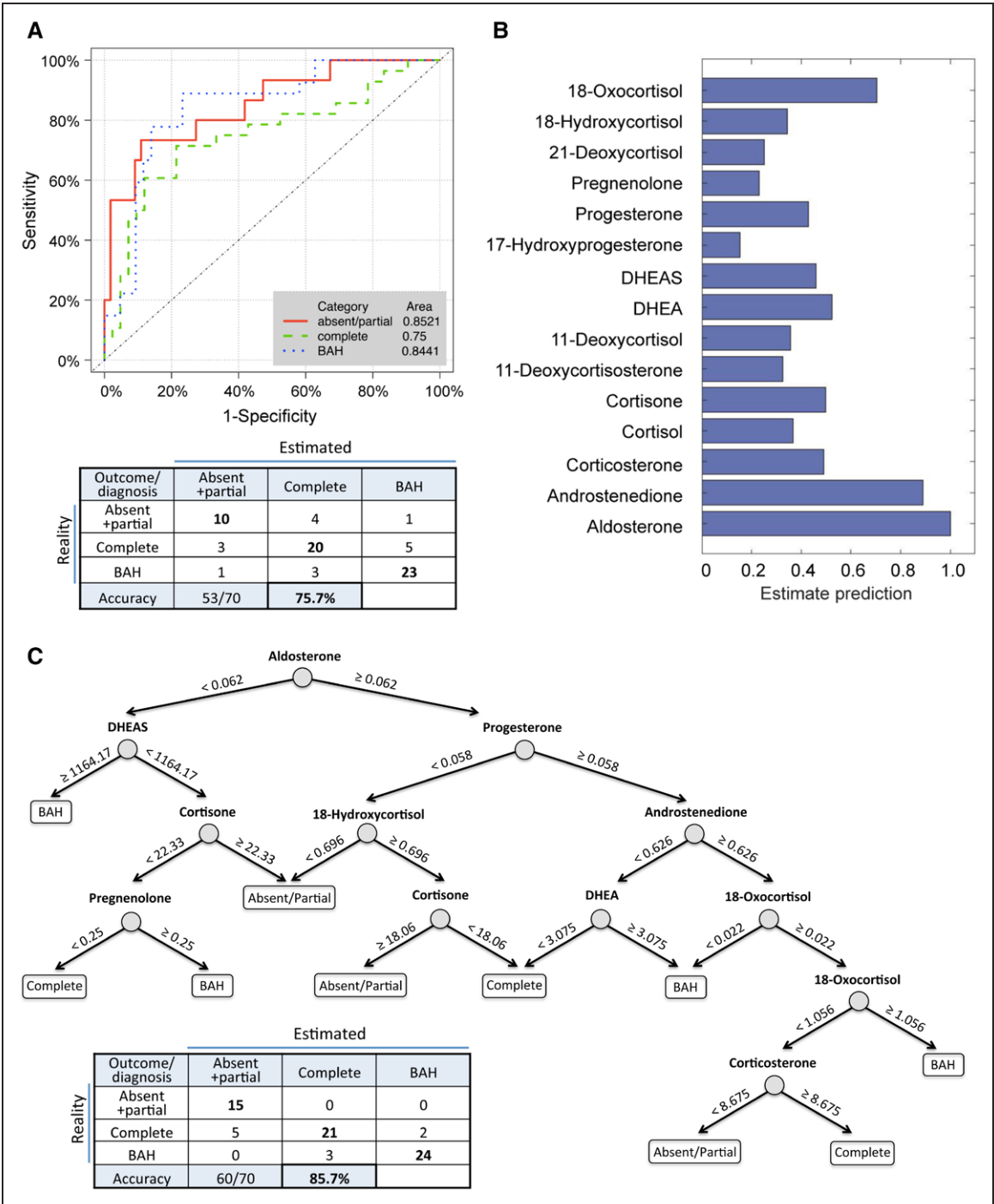
Figure 4. Classification of biochemical outcomes after adrenalectomy and diagnosis of bilateral adrenal hyperplasia (BAH) according to peripheral venous steroid concentrations. Discriminant analysis with 9 steroids (androstenedione, cortisol, cortisone, 11-deoxycortisol, dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), pregnenolone, 18-hydroxycortisol, and 18-oxocortisol) generated receiver operating characteristic curves with areas under the curves (inset) and a table showing real and estimated biochemical outcomes (absent+partial and complete biochemical success) and diagnosis of bilateral primary aldosteronism (PA; BAH) (**A**). The steroids used in each model are shown in Table S9 with linear discriminant coefficients and cutoffs for prediction of absent+partial biochemical success. Decision tree analysis using steroids based on estimated predictive performance (**B**) improved the correct classification of biochemical outcomes and diagnosis of bilateral PA. Numbers in the decision tree indicate steroid concentrations in ng/mL (**C**).

often associated with APCCs in the adjacent cortex, more frequently than with hyperplasia. There were no perceivable differences in the prevalence or numbers of APCC per sample section between biochemical outcomes although the potential association of somatic mutations in APCCs with biochemical outcomes cannot be excluded.

Liquid chromatography–tandem mass spectrometry measurements of plasma adrenal steroids predicted the presence or absence of a solitary functional adenoma, hyperplasia, or APCCs. The association of histopathology in PA with adrenal steroid concentrations ostensibly underlies or contributes to the classification of postsurgical biochemical outcomes

by steroid profiling, which herein identified all patients with absent+partial biochemical success from patients with biochemical cure or from nonoperated patients.

Strengths and Limitations of the Study

The strengths of the study are the large multicenter sample cohort comprising the largest reported sample set of resected adrenals from patients with postsurgical absent+partial biochemical success that were matched with a control group (complete biochemical success) and the strict standardized PASO criteria used to assess biochemical outcomes. A limitation is the small size of the study population used for steroid profiling, in particular, the number of patients with absent+partial biochemical success. Nonetheless, in the Munich cohort, adrenal steroid concentrations in peripheral plasma correctly predicted postsurgical absent+partial biochemical success in all 15 patients—an association possibly driven by the underlying adrenal histopathologic features. The 5 of 28 patients with biochemical cure, predicted by steroid profiling to have absent+partial biochemical success at 6 to 12 months postadrenalectomy, potentially comprise patients who develop long-term recurrent PA.[30,31] A prospective validation study with long-term follow-up should address this possibility.

Perspectives

The histopathology of adrenals from patients who are biochemically cured after adrenalectomy for unilateral PA is quantitatively different from the adrenals from patients with absent+partial biochemical success. The absence of a functional adenoma at pathology or the presence of cortical hyperplasia may indicate patients in whom follow-up, including assessment of biochemical parameters, should be considered mandatory. Measurements of adrenal steroids in peripheral venous plasma are associated with adrenal histopathology and biochemical outcomes after surgery. This highlights the potential application of steroid profiling to guide the decision to perform surgery in patients who are being considered for adrenalectomy on the basis of a preoperative diagnosis of unilateral PA.

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Disclosures

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Novelty and Significance

What Is New?

- The absence of a functional solitary adenoma or the presence of cortical hyperplasia is associated with partial+absent biochemical success after surgery for unilateral primary aldosteronism.
- Steroid profiling was associated with the presence or abence of solitary functional adenomas, cortical hyperplasia, and aldosterone-producing cell clusters.
- Steroid profiling classifies the majority of patients according to complete or partial+absent biochemical success after unilateral adrenalectomy.

What Is Relevant?

- Peripheral venous steroid profiling may be useful to select patients with a preoperative diagnosis of unilateral primary aldosteronism for surgery based on expectations of biochemical outcome.

Summary

Immunohistopathology may help determine which patients are likely to need ongoing follow-up for persistent primary aldosteronism, and steroid profiling may be useful to guide the decision to perform surgery.

Hypertension

American Heart Association®

Immunohistopathology and Steroid Profiles Associated With Biochemical Outcomes After Adrenalectomy for Unilateral Primary Aldosteronism

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Immunohistopathology and steroid profiles associated with biochemical outcome after adrenalectomy for unilateral primary aldosteronism

Lucie S. Meyer, Xiao Wang, Eva Sušnik, Jacopo Burrello, Alessio Burrello, Isabella Castellano, Graeme Eisenhofer, Francesco Fallo, Gregory A. Kline, Thomas Knösel, Tomaz Kocjan, Jacques, W.M. Lenders, Paolo Mulatero, Mitsuhide Naruse, Tetsuo Nishikawa, Mirko Peitzsch, Lars C. Rump, Felix Beuschlein, Stefanie Hahner, Celso E. Gomez-Sanchez, Martin Reincke, Tracy Ann Williams

Contents:

Table S10. Linear discriminant analysis for the classification of biochemical outcomes

and BAH

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Table S1. Centres providing adrenals for Immunohistopathology

COUNTRY	CENTRE	BIOCHEMICAL OUTCOME		AVS/CT	AVS protocol
		Absent/Partial	Complete		
Canada	Calgary	2	2	AVS	ACTH stimulated
Germany	Düsseldorf	1	1	AVS	Unstimulated
	Munich	15	29	AVS	Unstimulated
	Würzburg	6	1	CT	NA
Italy	Padua	1	1	CT	NA
	Torino	2	2	AVS	Unstimulated
Japan	Kyoto	7	7	AVS	ACTH stimulated
	Yokohama	7	7	AVS	ACTH stimulated
Slovenia	Ljubljana	2	2	AVS	Unstimulated
	Total	**43**	**52**		

Each centre participating to the study is shown with the number of resected adrenals analysed from patients with either an absent + partial combined or complete biochemical success after surgery for unilateral PA. Adrenals from patients with complete biochemical success were selected by matching for age (± 5 years) and sex compared with patients with absent + partial biochemical success. Peripheral venous steroid profiling was performed on plasma samples from patients in the Munich cohort.

AVS, adrenal venous sampling; ACTH, adrenocorticotropic hormone; CT, computed tomography; NA, not applicable

Table S2. Baseline and follow-up characteristics of patients included for adrenal immunohistopathology

VARIABLE	N	TOTAL	BIOCHEMICAL OUTCOME		P-value
			A+P (N=43)	C (N=52)	
Biochemical outcomes		95 (100 %)	43 (45 %)	52 (55 %)	
Clinical outcomes		88 (100 %)	40 (45 %)	48 (55 %)	0.157
Complete		19 (22 %)	6 (15 %)	13 (27 %)	
Partial		44 (50 %)	19 (48 %)	25 (52 %)	
Absent		25 (28 %)	15 (38 %)	10 (21 %)	
Age at surgery (years)	94	51.2 ± 10.3	52.4 ± 10.7	50.2 ± 10.1	0.309
Sex (Female/Male)	44/51	44/51 (46/54%)	19/24 (44/56%)	25/27 (48/51%)	0.705
BMI (kg/m^2)	94	27.3 ± 5.4	27.2 ± 5.6	27.3 ± 5.2	0.749
Known duration of HT (months)	76	96 [47-181]	120 [50-216]	94 [22-154]	0.161
BASELINE PARAMETERS					
Aldosterone (pmol/L)	94	578 [386-1056]	697 [374-1176]	571 [390-1023]	0.523
PRA (pmol/L/min)	41	3.8 [1.3-5.1]	2.6 [1.3-3.8]	3.8 [1.3-7.3]	0.439
ARR_PRA	41	268 [122-1002]	263 [118 -1030]	268 [112-1001]	0.825
DRC (mU/L)	53	4.0 [2.0-9.6]	4.7 [2.0-9.3]	3.1 [2.0-10.2]	0.519
ARR_DRC	53	125 [49-259]	110 [62-237]	140 [44-325]	0.942
Lowest serum K$^+$ (mmol/L)	94	3.0 ± 0.5	3.2 ± 0.5	2.9 ± 0.5	0.035
Systolic BP (mmHg)	94	149 ± 23	148 ± 22	151 ± 23	0.508
Diastolic BP (mmHg)	94	91 ± 14	91 ± 16	92 ± 13	0.654
Antihypertensive medication (DDD)*	93	3.0 [1.5-4.8]	3.0 [2.0-5.1]	3.0 [1.3-4.7]	0.349
FOLLOW-UP PARAMETERS					
Aldosterone (pmol/L)	95	283 [161-413]	355 [277-499]	183 [98-287]	< 0.001
PRA (pmol/L/min)	41	9.0 [2.6-28.2]	2.6 [1.3-5.1]	23 [9.8-49.3]	< 0.001
ARR_PRA	41	44 [8-135]	135 [58-317]	8 [5-25]	< 0.001
DRC (mU/L)	54	11.4 [6.0-18.2]	7.3 [2.7-13.9]	15.1 [9.6-26.7]	0.002
ARR_DRC	54	26 [8-46]	46 [29-126]	10 [6-19]	< 0.001
Lowest serum K$^+$ (mmol/L)	95	4.1 ± 0.5	4.0 ± 0.6	4.2 ± 0.4	0.019
Systolic BP (mmHg)	94	134 ± 16	137 ± 15	131 ± 16	0.066
Diastolic BP (mmHg)	94	85 ± 11	86 ± 12	85 ± 11	0.706
Antihypertensive medication (DDD)	90	1.0 [0.0-3.0]	2.0 [0.5-3.5]	0.6 [0.0-2.4]	0.016

A, P and C refer absent, partial and complete biochemical success after surgery as defined in the PASO study[1]; BMI, body mass index; BP, blood pressure, (office);[2] DDD, daily defined dose; HT, hypertension; PRA, plasma renin activity; ARR_PRA, aldosterone-to-renin ratio assessed using the PRA; DRC, direct renin concentration; ARR_DRC,

aldosterone-to-renin ratio assessed using the DRC; *Antihypertensive drug doses (DDD, daily defined dose) is the assumed average maintenance dose per day for a drug used for its main indication in an adult and is calculated according to the ATC/DDD Index 2010. Values are shown as absolute numbers with proportions in parenthesis (%), as averages ± SD or as medians [25th-75th percentile]. *P* values were calculated using a χ^2, t-test or Mann-Whitney test as appropriate.

Table S3. Baseline characteristics of patients included for peripheral venous steroid profiling

VARIABLE	A + P (*N* = 15)	C (*N* = 28)	Bilateral PA (*N* = 27)	*P*-value
Age at study entry	48 ± 13*	47 ± 9.7	49 ± 10	0.915
Sex (Female/Male)	8 (53%)/7 (47%)	14 (50%)/14 (50%)	12 (44%)/15 (56%)	0.844
BMI	28.9 ± 6.9*	29.0 ± 5.4	29.4 ± 5.6	0.907
Aldosterone (pmol/L)	616 [304-929]*	483 [325-639]	449 [283-706]	0.371
DRC (mU/L)	7.9 [2.0-11.2]	2.7 [2.0-11.0]	4.3 [2.8-13.4]	0.371
ARR_DRC	93 [43-260]	123 [40-312]	71 [42-143]	0.552
Lowest serum K+ (mmol/L)	3.0 ± 0.5*‡	3.0 ± 0.5**	3.6 ± 0.5	< 0.001
Systolic BP (mmHg)	152 ± 11*	149 ± 15	152 ± 23	0.665
Diastolic BP (mmHg)	96 ± 10*	93 ± 11	95 ± 11	0.606
Antihypertensive medication (DDD)†	3.0 [2.3-4.6]*	3.0 [1.0-4.9]	2.8 [1.5-3.5]	0.635

A, P and C refer absent, partial and complete biochemical success after surgery as defined in the PASO study[1]; BMI, body mass index; BP, blood pressure (office);[2] DDD, daily defined dose; HT hypertension; PRA, plasma renin activity; ARR_PRA, aldosterone-to-renin ratio assessed using the PRA; DRC, direct renin concentration; ARR_DRC, aldosterone-to-renin ratio assessed using the DRC; * Data available for 14 patients; † Antihypertensive drug doses (DDD, daily defined dose) is the assumed average maintenance dose per day for a drug used for its main indication in an adult and is calculated according to the ATC/DDD Index 2010. ‡ Difference (*P*=0.002) from bilateral PA ** Difference (*P*<0.001) from bilateral PA. Values are shown as absolute numbers with proportions in parenthesis (%), as averages ± SD or as medians [25th-75th percentile]. *P* values were calculated by Kruskal-Wallis or a one-way ANOVA with a post hoc Bonferroni as appropriate.

Table S4. Sex and age distribution according to histopathology

Histopathological feature	Female	Male	*P* value
SF adenoma (*N*=60)	29 (48 %)	31 (52 %)	0.606
Age (years)	49.7 ± 9.59	52.8 ± 9.21	0.195
Hyperplasia (*N*=32)	17 (53 %)	15 (47 %)	0.343
Age (years)	47.0 ± 15.24	53.7 ± 8.59	0.135
APCC (*N*=57)	29 (51 %)	28 (49 %)	0.275
Age (years)	48.3 ± 11.50	52.8 ± 8.44	0.103

SF, solitary functional; APCC, aldosterone-producing cell cluster; *N*, total number. Values are shown as absolute numbers with proportions in parenthesis (%) or as averages ± SD. *P* values were calculated using a χ^2 or t test as appropriate.

Table S5. Adrenal characteristics and AVS results according to biochemical outcome

VARIABLE	Total cohort	BIOCHEMICAL OUTCOME		
		A + P	C	*P*-value
Adrenal characteristics				
Nodule size at pathology (mm) (*N*= 89)	12 [7-16]	9 [6-15]	14 [8-17]	0.052
Nodule size at imaging (mm) (*N*= 68)	14 [10-20]	14 [11-18]	14 [10-21]	0.582
CL gland (abnormal, %) (*N*= 88)	17 (19 %)	7 (8 %)	10 (11 %)	0.772
Genotype	46 (48 %)	16 (37 %)	30 (58 %)	
No mutation detected	18 (39 %)	7 (44 %)	11 (37 %)	0.639
KCNJ5 mutation	25 (54 %)	7 (44 %)	18 (60 %)	0.292
Other	3 (7 %)	2 (12.5 %)	1 (3.3 %)	
AVS results				
Lateralisation index (*N*= 72)	7.5 [4.2-26.9]	4.7 [2.5-7.8]	18.3 [6.1-29.4]	< 0.001
CL ratio (*N*= 70)	0.4 [0.2-1.1]	0.7 [0.3-2.2]	0.3 [0.1-0.5]	0.005
CL suppression (present, %) (*N*= 70)	52/70 (74 %)	16/26 (62 %)	36/44 (82 %)	0.061

A, P and C refer to absent, partial and complete biochemical success after surgery; AVS, adrenal venous sampling; CL, contralateral; lateralisation index defined as: $([aldosterone]/[cortisol])_{\text{dominant adrenal vein}}/([aldosterone]/[cortisol])_{\text{non-dominant adrenal vein}}$; CL ratio defined as: $([aldosterone]/[cortisol])_{\text{non-dominant adrenal vein}}/([aldosterone]/[cortisol])_{\text{peripheral vein}}$; contralateral suppression defined as a CL ratio <1; nodule size refers to diameter of largest nodule at pathology or imaging as indicated. Genotype analysis was performed by direct sequencing using genomic DNA extracted from the largest nodule as described (3). Values are shown as absolute numbers with proportions in parenthesis (%) or as medians [25th-75th percentile]. *P* values were calculated by a χ^2 or Mann-Whitney test as appropriate.

Table S6. Peripheral venous adrenal steroid concentrations according to histopathology

STEROID	SF adenoma (N = 25)	Hyperplasia (N = 17)	APCC (N = 27)	P-value
Aldosterone	0.13 [0.07-0.28]	0.16 [0.08-0.49]	0.15 [0.07-0.31]	0.739
Androstenedione	0.52 [0.44-1.17]	1.02 [0.46-1.46]	0.80 [0.44-1.33]	0.299
Corticosterone	1.94 [0.98-4.97]	2.39 [1.17-4.34]	2.26 [1.15-5.33]	0.657
Cortisol	88.6 [60.0-137.5]	108.7 [66.9-140.2]	111.1 [61.3-140.7]	0.872
Cortisone	16.9 [12.7-20.7]	21.1 [14.6-22.7]	18.0 [11.9-22.9]	0.337
11-Deoxycorticosterone	0.05 [0.03-0.16]	0.05 [0.05-0.11]	0.06 [0.04-0.12]	0.490
11-Deoxycortisol	0.24 [0.15-0.43]	0.37 [0.23-0.58]	0.27 [0.20-0.50]	0.410
DHEA	2.53 [1.33-3.90]	2.71 [0.94-5.12]	2.69 [1.10-3.77]	0.889
DHEAS	900 [556-1307]	843 [710-1570]	830 [500-1680]	0.902
17-Hydroxyprogesterone	0.52 [0.30-0.90]	0.64 [0.30-0.89]	0.56 [0.30-0.89]	0.997
Progesterone	0.12 [0.05-0.17]	0.07 [0.05-0.53]	0.10 [0.05-0.25]	0.771
Pregnenolone	0.33 [0.15-0.76]	0.21 [0.16-1.32]	0.38 [0.18-1.22]	0.507
21-Deoxycortisol	0.02 [0.01-0.09]	0.05 [0.01-0.08]	0.03 [0.01-0.08]	0.828
18-Hydroxycortisol	1.05 [0.66-2.50]	0.65 [0.51-2.90]	0.78 [0.52-1.70]	0.362
18-Oxocortisol	0.05 [0.03-0.33]	0.03 [0.02-0.10]	0.03 [0.01-0.12]	0.205

Peripheral venous plasma concentrations (ng/mL) shown as medians [25th-75th percentile]. *P* values indicate group differences by the Kruskal-Wallis test or one-way ANOVA with a post hoc Bonferroni. There were no significant pairwise differences. To convert concentrations in ng/mL to pmol/L, concentrations should be divided by the molecular weight of each steroid. Molecular weights: aldosterone, 360.44; androstenedione, 286.41; corticosterone, 346.46; cortisol, 362.46; cortisone, 360.44; 11-deoxycorticosterone, 330.46; 11-deoxycortisol, 346.46; DHEA, 288.42; DHEA-sulphate, 367.50; 17-hydroxyprogesterone, 330.46; progesterone, 314.46; pregnenolone, 316.48; 21-deoxycortisol, 346.46; 18-hydroxycortisol, 378.46; 18-oxocortisol, 376.45
FS adenoma, functional solitary adenoma; APCC, aldosterone-producing cell cluster; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate.

Table S7. Peripheral venous adrenal steroid concentrations according to biochemical outcome and diagnosis of bilateral PA

STEROID	BIOCHEMICAL OUTCOME A+P (n = 15)	C (n = 28)	Diagnosis Bilateral PA (n = 27)	P-value
Aldosterone	0.20 [0.10-0.44]*	0.12 [0.07-0.25]*	0.05 [0.03-0.07]	< 0.001
Androstenedione	0.81 [0.49-1.46]	0.56 [0.44-1.18]	0.79 [0.54-1.09]	0.417
Corticosterone	3.11 [1.47-5.33]	1.82 [0.85-2.98]	2.00 [1.07-4.23]	0.402
Cortisol	120 [73-140]	98 [58-155]	111 [76-164]	0.713
Cortisone	21.7 †[15.8-22.9]	16.6 [11.8-19.9]	18.3 [14.9-21.7]	0.042
11-Deoxycorticosterone	0.05 [0.04-0.12]	0.06 [0.04-0.13]	0.06 [0.03-0.08]	0.523
11-Deoxycortisol	0.44 [0.33-0.64] †	0.24 [0.15-0.38]	0.28 [0.16-0.64]	0.076
DHEA	2.49 [1.06-5.67]	2.54 [1.28-3.71]	3.26 [1.83-4.60]	0.284
DHEAS	830 [521-1680]	982 [528 -1289]	1183 [649-1738]	0.359
17-Hydroxyprogesterone	0.75 [0.34-0.97]	0.54 [0.28-0.90]	0.62 [0.36-0.97]	0.569
Progesterone	0.06 [0.03-0.16]	0.11 [0.06-0.17]	0.11 [0.07-0.21]	0.158
Pregnenolone	0.63 [0.17-1.83]	0.23 [0.16-0.61]‡	0.27 [0.13-0.37]	0.086
21-Deoxycortisol	0.05 [0.01-0.08]	0.03 [0.01-0.09]	0.08 [0.01-0.10]	0.674
18-Hydroxycortisol	0.69 [0.52-2.22]	1.19 [0.60-1.95]	0.74 [0.48-1.51]	0.292
18-Oxocortisol	0.03 [0.01-0.08]	0.05 [0.02-0.20]*	0.01 [0.01-0.05]	0.022

Peripheral venous plasma concentrations (ng/mL) shown as medians [25th-75th percentile]. P values indicate group differences by the Kruskal-Wallis test or one-way ANOVA with a post hoc Bonferroni. For pairwise comparisons a Mann-Whitney or t test was used as appropriate. To convert concentrations in ng/mL to pmol/L, concentrations should be divided by the molecular weight of each steroid. Molecular weights: aldosterone, 360.44; androstenedione, 286.41; corticosterone, 346.46; cortisol, 362.46; cortisone, 360.44; 11-deoxycorticosterone, 330.46; 11-deoxycortisol, 346.46; DHEA, 288.42; DHEA-sulphate, 367.50; 17-hydroxyprogesterone, 330.46; progesterone, 314.46; pregnenolone, 316.48; 21-deoxycortisol, 346.46; 18-hydroxycortisol, 378.46; 18-oxocortisol, 376.45. A, P and C indicate absent, partial and complete post-surgical biochemical outcomes; DHEA, dehydroepiandrosterone. * Difference (P<0.01) from bilateral PA; † Difference (P<0.05) from C; ‡ Difference (P<0.05) from bilateral PA.

Table S8. Classification of biochemical outcomes after unilateral adrenalectomy: predictive modelling using steroid profiling *versus* AVS parameters

(A) Classification with steroid profiling

		Estimated					Estimated	
	LDA	1	2		DT	1	2
Reality	1	11	2	Reality	1	13	0
	2	3	24		2	0	27
	Accuracy	35/40	88 %		Accuracy	40/40	100 %

(B) Classification with AVS parameters (LI and CL ratios)

		Estimated					Estimated	
	LDA	1	2		DT	1	2
Reality	1	3	10	Reality	1	13	0
	2	0	27		2	2	25
	Accuracy	30/40	75 %		Accuracy	38/40	95 %

Classification of 40 patients according to biochemical outcomes after unilateral adrenalectomy using adrenal steroid measurements in peripheral plasma (**Panel A**) or AVS parameters (lateralisation index and CL ratio) (**Panel B**). There were 13 patients with an absent + partial outcome (**Group 1**) and 27 patients with a complete biochemical outcome (**Group 2**). All patients with steroid measurements and a pre-operative diagnosis of unilateral PA and AVS results were included in the analyses. Unilateral PA was diagnosed by AVS under bilateral unstimulated conditions and a lateralisation index ≥ 4. Patients with bilateral PA were not included because they were differentiated from patients with unilateral disease using AVS parameters.

Linear discriminant analysis using steroid profiling displayed a higher accuracy of classification of biochemical outcomes compared with AVS parameters (LI and CL ratio) (88% *versus* 75%). Linear discriminant analysis using AVS parameters misclassified 10 of 13 patients with absent + partial biochemical success compared with 2 of 13 patients using steroid measurements. Decision trees with steroid measurements correctly classified all 40 patients according to biochemical outcome. Decision trees using AVS parameters correctly classified all patients with an absent + partial biochemical outcome but 2 of 27 patients with complete success were misclassified as absent + partial biochemical success.

AVS, adrenal venous sampling; CL ratio, contralateral ratio; DT, decision trees; LDA, linear discriminant analysis; LI, lateralisation index

Table S9. Linear discriminant analyses for the classification of histopathological phenotype

(A) Linear discriminant coefficients for histopathological features

STEROID	COEFFICIENTS OF LINEAR DISCRIMINANTS		
	SF adenoma LD1	Hyperplasia LD1	APCC LD1
Aldosterone	-	-3.37	-1.103
Androstenedione	1.395	-	-1.77
Corticosterone	-	-	-0.30
Cortisol	-	0.0149	-
Cortisone	0.0835	-0.1082	-
11-Deoxycorticosterone	-8.495	7.6096	13.895
11-Deoxycortisol	-	-2.23	-
DHEA	-0.3228	-	0.144
DHEAS	-	-	0.000388
17-Hydroxyprogesterone	-0.6760	-	1.236
Progesterone	-0.1749	-	-0.308
Pregnenolone	-	-0.092	-
21-Deoxycortisol	10.93377	4.4116	17.78
18-Hydroxycortisol	-	-	-0.139
18-Oxocortisol	-2.24	1.8528	2.490

(B) Interpretation of combined adjusted linear discriminant coefficients for the presence of a solitary functional adenoma, cortical hyperplasia or aldosterone-producing cell cluster

SF ADENOMA			HYPERPLASIA			APCC		
Cut-off	Spec	Sens	Cut-off	Spec	Sens	Cut-off	Spec	Sens
-2.5	1	0	-3.7	1	0	-2.1	1	0
-0.35	1	0.32	-2.1	1	0.29	-0.1	1	0.22
-0.32	0.94	0.32	-1.8	0.96	0.29	-0.06	0.94	0.22
0.18	0.94	0.48	**-1.26**	0.96	0.76	0.25	0.94	0.41
0.45	0.83	0.48	-1.1	0.77	0.76	0.3	0.88	0.41
0.6	0.83	0.68	-0.6	0.62	0.82	0.35	0.88	0.44
0.74	0.78	0.68	-0.57	0.62	0.88	0.4	0.81	0.44
1	0.78	0.88	0.1	0.35	0.88	1.13	0.81	0.85
2	0.17	0.88	0.15	0.35	0.94	1.15	0.75	0.85
2.1	0.17	1	0.2	0.31	0.94	**1.4**	0.75	0.96
3.2	0	1	0.32	0.31	1	1.6	0.5	0.96
			2.5	0	1	1.8	0.5	1
						4.4	0	1

Linear discriminant (LD1) coefficients derived from the linear discriminant analysis (LDA) model for the classification of the presence or absence of a solitary functional (SF) adenoma, cortical hyperplasia and aldosterone-producing cell clusters (APCC) are shown in **Panel A**. Cut-offs for the presence of each histopathological feature derived from the ROC curves in Figure 3B of the main manuscript are shown in **Panel B**.

To estimate the presence of SF adenoma, cortical hyperplasia or APCC, each steroid concentration should be multiplied by its corresponding coefficient (LD1) (**Panel A**) and

adjusted coefficients for all steroids used in each LDA model should be summed to derived value *x*. If *x* is less than the cut-offs indicated for SF adenoma, hyperplasia or APCC indicated in **Panel B** (shown in red bold), obtained from the ROC curves shown in Figure 3B of the main manuscript, then the presence of that histopathological feature is predicted.

Therefore, an estimation of the presence of SF adenoma, hyperplasia or APCC is given by the following equation (where the cut-off is specific for each histopathologial feature)

$$= LDA_{coeff1} * Steroid_1 + LDA_{coeff2} * Steroid_2 \; . \quad . \quad . \; LDA_{coeffn} * Steroid_n < cut-off$$

Table S10. Linear discriminant analysis for the classification of biochemical outcomes and BAH

(A) Linear discriminant coefficients for biochemical outcomes and BAH

STEROID	COEFFICIENTS OF LINEAR DISCRIMINANTS	
	LD1	LD2
Androstenedione	0.055	1.13
Cortisol	-0.0255	-0.026
Cortisone	0.242	0.1893
11-Deoxycortisol	2.63	2.03
DHEA	-0.0998	-0.39
DHEAS	0.000275	-0.0005
Pregnenolone	0.77	1.61
18-Hydroxycortisol	-0.14	0.29
18-Oxocortisol	-0.66	-1.46

(B) Interpretation of combined adjusted linear discriminant coefficients for absent + partial biochemical success

Cut-off 1	Cut-off 2	ABSENT + PARTIAL	
		Specificity	Sensitivity
7	6	1	0
4.2	3.7	1	0.2
4.1	3.7	0.98	0.2
3.8	2.8	0.98	0.47
3.77	2.51	0.96	0.47
3.75	2.51	0.96	0.6
3.7	2.5	0.95	0.6
3.67	2.5	0.95	0.67
3.6	2.5	0.93	0.67
3.5	**2.5**	0.93	0.73
2.7	1.7	0.75	0.73
2.6	1.7	0.75	0.8
1.87	0.87	0.6	0.8
1.87	0.85	0.6	0.87
1.8	0.65	0.53	0.87
1.8	0.6	0.53	0.93
1.2	0.6	0.47	0.93
1.2	0.5	0.47	1
-2	-4	0	1

The linear discriminant analysis (LDA) model used 9 steroids to separate the 3 groups (absent + partial, complete biochemical success and BAH) in 2 dimensions using 2 LD coefficients (LD1 and LD2) as indicated in **Panel A** (a model using LDA to separate 4 or more groups would require 3 dimensions with coefficients LD1, LD2 and LD3). To estimate an absent + partial biochemical outcome compared with a complete biochemical outcome + a diagnosis of BAH, each steroid concentration should be multiplied separately by each of the corresponding LD1 and LD2 coefficients and summed to derived values *x* and *y*. If *x* is greater than cut-off 1 (for the adjusted LD1) and *y* is greater than cut-off 2 (for the adjusted LD2), shown in red bold in **Panel B**, then an absent + partial biochemical outcome is predicted. Therefore, an absent + partial biochemical outcome is estimated by the following equations:

$$(LDA1_{coeff1} * Steroid_1 + LDA1_{coeff2} * Steroid_2 \ldots LDA1_{coeffn} * Steroid_n > cut-off1) \ AND$$
$$(LDA2_{coeff1} * Steroid_1 + LDA2_{coeff2} * Steroid_2 \ldots LDA2_{coeffn} * Steroid_n > cut-off2)$$