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

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**Abstract**

Unilateral primary aldosteronism is the most common surgically curable form of hypertension that must be accurately differentiated from bilateral primary aldosteronism for therapeutic management (surgical *versus* medical). Adrenalectomy results in biochemical cure (complete biochemical success) in almost all patients diagnosed with unilateral primary aldosteronism; the remaining patients with partial or absent biochemical success comprise those with persisting aldosteronism who were misdiagnosed as unilateral primary aldosteronism pre-operatively. To identify determinants of post-surgical 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 primary aldosteronism. A large multicentre 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 primary aldosteronism 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 primary aldosteronism ($N$=27). Steroid profiling was associated with histopathological phenotypes (solitary functional adenoma, hyperplasia and aldosterone-producing cell clusters) and classified patients according to biochemical outcome or diagnosis of bilateral primary aldosteronism. 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.

**Key words:** Hyperaldosteronism, aldosterone, adrenalectomy, postsurgical outcomes, adrenal gland, immunohistochemistry, steroid profiling

## Introduction

Primary aldosteronism (PA) is a form of endocrine hypertension caused by the overproduction of aldosterone from one or both adrenal glands mainly due to 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 (AVS) or adrenal computed tomography (CT) scanning is used to differentiate unilateral from bilateral PA although alternative approaches are currently being investigated including functional imaging using positron emission tomography/CT scanning with a radiolabelled tracer and peripheral venous steroid profiling. [5-10]

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 histopathological 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 (APCCs, aldosterone-producing cell clusters) 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 due to the medical, rather than surgical, management of bilateral PA. The Primary Aldosteronism Surgical Outcome (PASO) study 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

lateralisation performed by AVS 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 pre-operatively. In a large multicentre study with outcomes assessed in accordance with an international consensus, we analysed 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 upon reasonable request.

*Patient selection*

The study included surgically resected adrenals of 95 patients collected from 9 international centres that were diagnosed with unilateral PA and classified with absent, partial or complete biochemical success at 6-12 months after unilateral adrenalectomy in accordance with the PASO consensus (Table S1).[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 centre. Peripheral venous plasma samples from patients of the Munich cohort (*N*=70) were analysed 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 haematoxylin and eosin (H&E) 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), APCCs (clusters of *zona glomerulosa* cells, CYP11B2-positive and CYP11B1- and CYP17A1-negative, localised 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 (LC_MS/MS)*

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 LC-MS/MS 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 analysed using one-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 $\chi^2$ or Fisher's exact tests for categorical variables. A *P*-value of less than 0.05 was considered significant. Linear discriminant analyses were performed in R and decision tree analyses used MATLAB R2017b.

## Results

*Patient characteristics*

Patients with post-surgical 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 lateralisation 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 1). 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 1).

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 non-functional 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 1).

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 1).

*LC-MS/MS peripheral venous steroid profiling*

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

196 accuracy of prediction to 91% - 93% (misclassification rate, 0.07-0.09) (Figure
197 3C-F).
198
199 For the absent + partial group, concentrations of aldosterone were higher in
200 peripheral venous plasma compared with the bilateral PA group ($P$ <0.001) and
201 cortisone and 11-deoxycortisol concentrations were significantly higher than in
202 the complete group ($P$ = 0.021 and $P$ = 0.017, respectively) (Table S7).
203 Discriminant analysis correctly predicted biochemical outcome after
204 adrenalectomy and diagnosis of bilateral PA in 53 of 70 patients (76%) (Figure
205 4A). Decision tree analysis improved the correct classification to 60 of 70 cases
206 (86%, misclassification rate, 0.14): all 15 patients with an absent + partial
207 biochemical outcome after surgery were correctly predicted albeit 5 patients
208 with complete biochemical success were incorrectly classified with an absent or
209 partial biochemical outcome (Figure 4C). Linear discriminant analysis and
210 decision trees of steroid measurements resulted in a higher accuracy for the
211 classification of biochemical outcomes compared with predictive models using
212 AVS parameters (lateralisation index and contralateral ratio) (Table S8).
213
214 **Discussion**
215 We report the histopathology and peripheral venous steroid profiles associated
216 with biochemical outcome after adrenalectomy for unilateral PA. In a multicentre
217 international study with differentiation of unilateral from bilateral PA by AVS,
218 adrenalectomy for unilateral PA resulted in biochemical cure (complete
219 biochemical success) in 94% of patients thereby indicating the correct diagnosis
220 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) pre-operatively.[25] In the present study, the lower lateralisation 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 histopathological 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 multicentre 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 macro- 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 post-surgical 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 CT scanning included 13 adrenals from patients with bilateral PA that displayed an increased 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 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.

LC-MS/MS 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 post-surgical biochemical outcomes by steroid profiling which herein identified all patients with absent + partial biochemical success from patients with biochemical cure or from non-operated patients.

*Strengths and limitations of the study*

The strengths of the study are the large multicentre sample cohort comprising the largest reported sample set of resected adrenals from patients with post-surgical absent + partial biochemical success that were matched with a control group (complete biochemical success) and the strict standardised 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 post-surgical absent + partial biochemical success in all 15 patients, an association possibly driven by the underlying adrenal histopathological features. The 5 of 28 patients with biochemical cure, predicted by steroid profiling to have absent + partial biochemical success at 6-12 months post-adrenalectomy, 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 pre-operative diagnosis of unilateral PA.

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324

**Conflicts of Interest/Disclosures**

None

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**Novelty and Significance:**

*1) 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 PA
- Steroid profiling was associated with the presence or abence of solitary functional adenomas, cortical hyperplasia and APCCs
- Steroid profiling classifies the majority of patients according to complete or partial + absent biochemical success after unilateral adrenalectomy

*2) What is Relevant?*

- Peripheral venous steroid profiling may be useful to select patients with a pre-operative diagnosis of unilateral PA for surgery based on expectations of biochemical outcome

**Summary:**

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

**Figure Legends**

**Figure 1. Adrenal venous sampling 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 lateralisation indices (**Panel A**) and higher contralateral ratios (**Panel B**). Horizontal lines within boxes indicate the median, and box and whiskers represent the 25$^{th}$ to 75$^{th}$ and 5$^{th}$ to 95$^{th}$ percentiles, respectively. *n* indicates the number of patients in each group and a Mann-Whitney test was used to calculate *P* values.

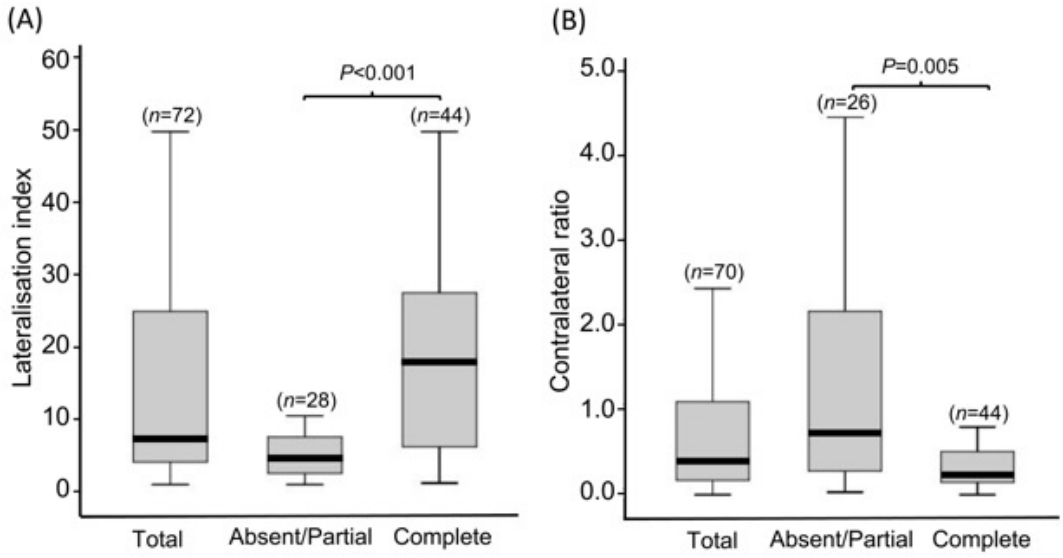


**Figure 2. Heterogeneous histopathology of resected adrenals from patients with PA**

The diverse histopathology of resected adrenals in this cohort is shown with H&E staining and CYP11B2 immunostaining as indicated. **Panels A-C:** adrenals without a functional adenoma showing the various histopathology of this subgroup. APCC indicated with a single arrow, hyperplasia with a double arrow. Scale bar represents 2 mm. These 3 adrenals were from patients with post-surgical absent or partial biochemical success; **Panels D-E**: examples of histopathological features classified in this study: solitary functional adenoma (**Panel D**), hyperplasia of the *zona glomerulosa* (**Panel E**) and an APCC (**Panel F**). Scale bars represent 200 μm.

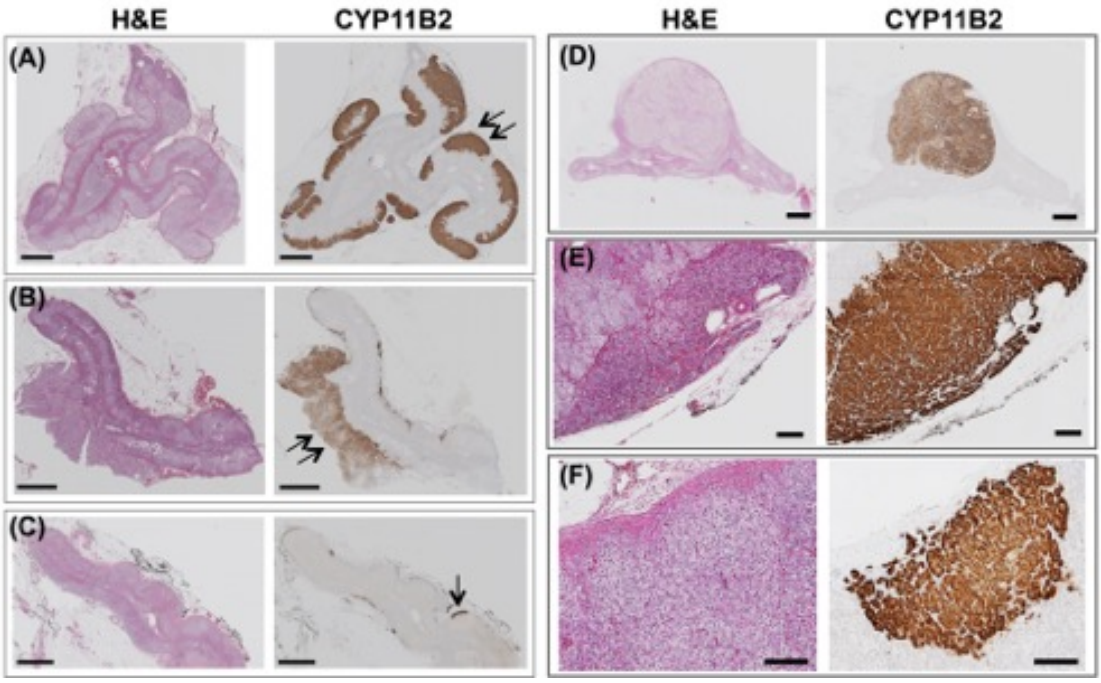


**Figure 3. Classification of adrenal histopathology in PA according to peripheral venous steroid concentrations**

The distribution of histopathological features (1, solitary functional adenoma; 2, hyperplasia; 3, APCC, aldosterone-producing cell clusters) stratified for biochemical outcome (indicated by an arrow) is represented in **Panel A**. Linear discriminant analyses using peripheral venous steroid concentrations was used to generate receiver-operating characteristic (ROC) curves with areas under the curves (inset) and tables showing the real and estimated presence (yes) or absence (no) of solitary functional adenomas (SF adenomas), hyperplasia and APCCs (**Panel B**). The steroids used in each model are shown in Table S9 with linear discriminant coefficients and cut-offs 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 (**Panel C**); hyperplasia (**Panel D**) and APCCs (**panel E**) are shown with an accompanying table with the real and estimated presence (yes) and absence (no) of each histopathological feature. Steroids used for decision tree analysis were selected from their estimated predictive performance (**Panel F**).

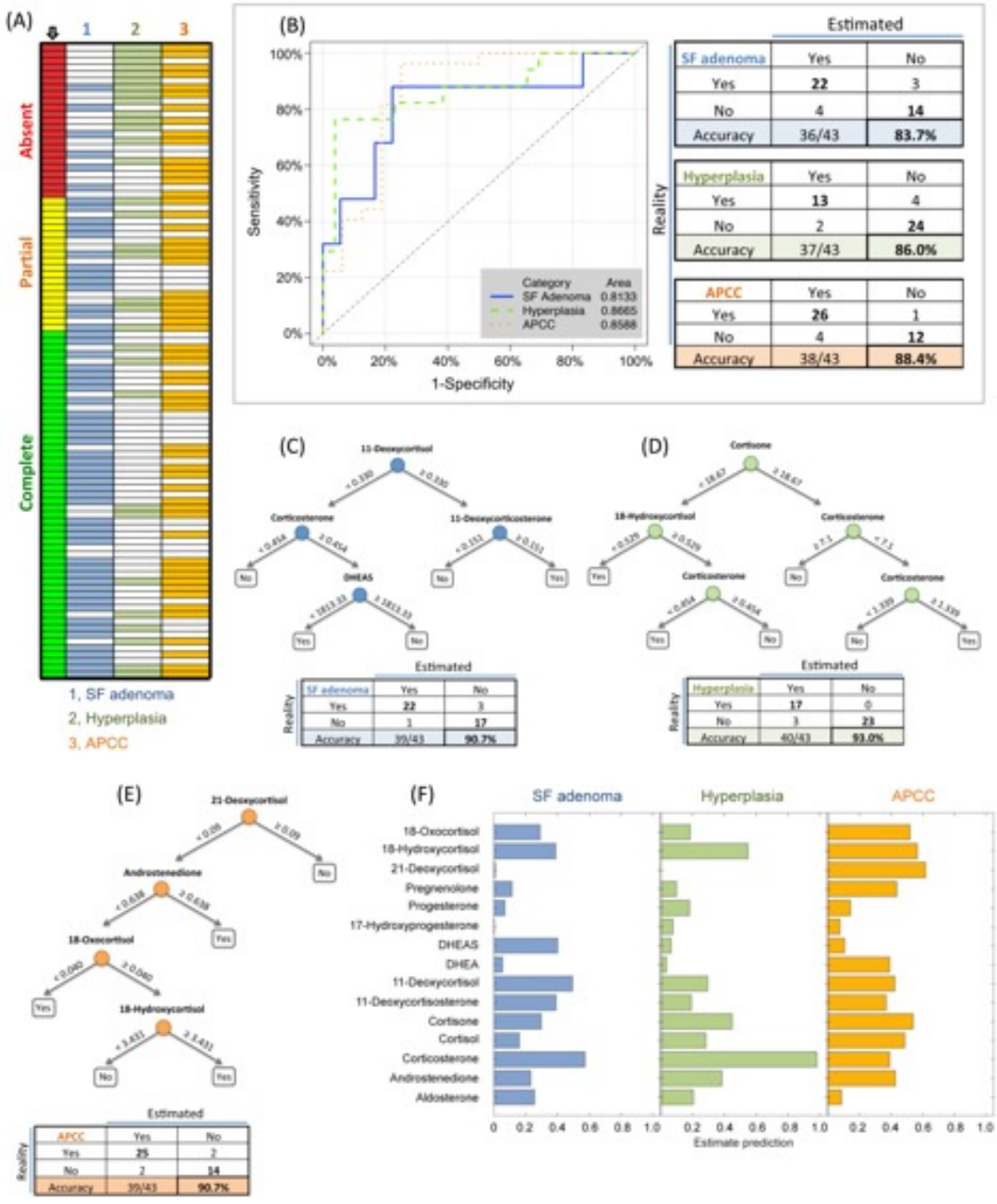
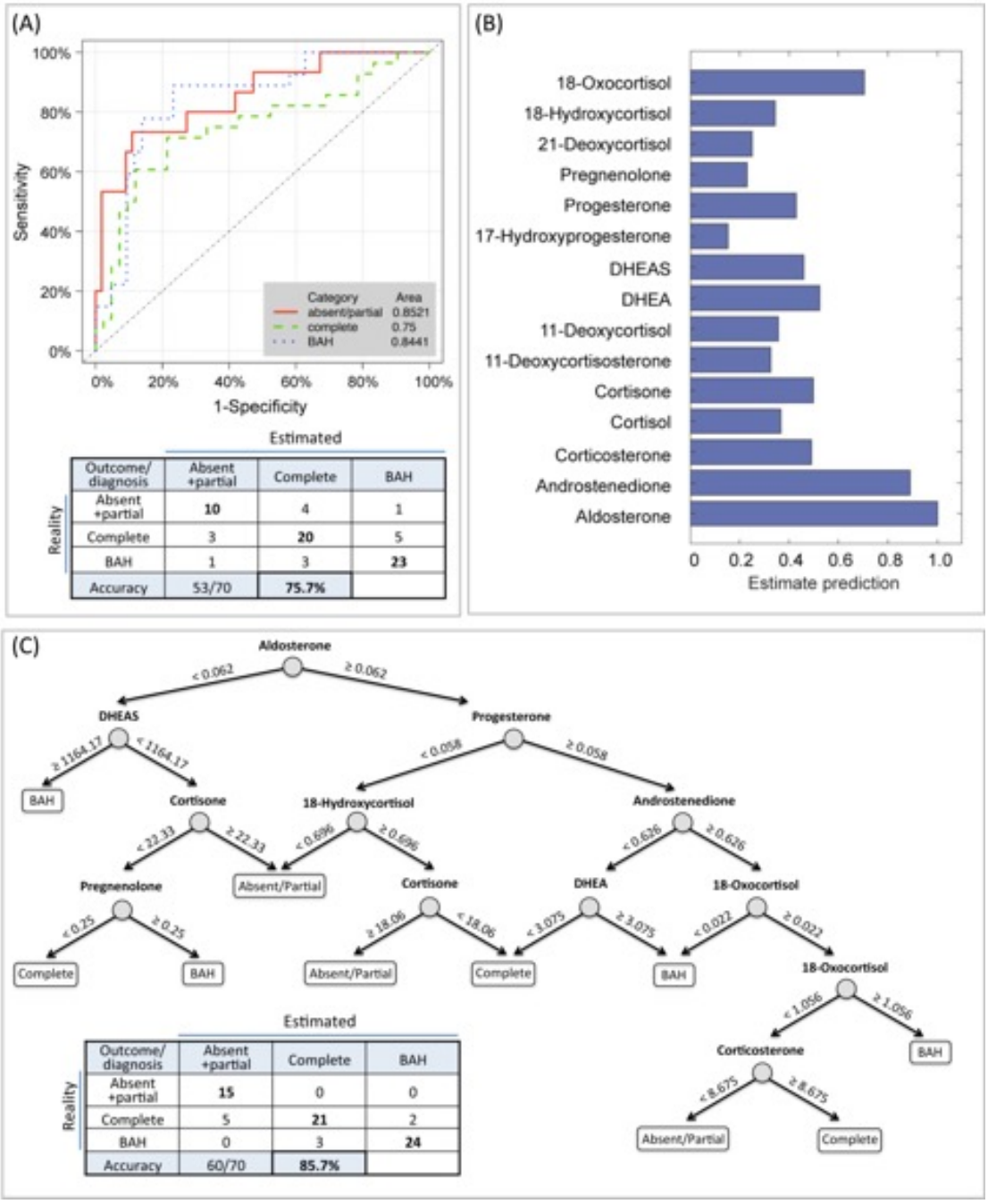

(A)
1
2
3
Absent
Partial
Complete
1, SF adenoma
2, Hyperplasia
3, APCC
(B)
Sensitivity
1-Specificity
Category Area
SF Adenoma 0.8133
Hyperplasia 0.8665
APCC 0.8588
Estimated
Reality
SF adenoma Yes No
Yes 22 3
No 4 14
Accuracy 36/43 83.7%
Hyperplasia Yes No
Yes 13 4
No 2 24
Accuracy 37/43 86.0%
APCC Yes No
Yes 26 1
No 4 12
Accuracy 38/43 88.4%
(C)
11-Deoxycortisol
Corticosterone
11-Deoxycorticosterone
DHEAS
Estimated
Reality
SF adenoma Yes No
Yes 22 3
No 1 17
Accuracy 39/43 90.7%
(D)
Cortisone
18-Hydroxycortisol
Corticosterone
Corticosterone
Corticosterone
Estimated
Reality
Hyperplasia Yes No
Yes 17 0
No 3 23
Accuracy 40/43 93.0%
(E)
21-Deoxycortisol
Androstenedione
18-Oxocortisol
18-Hydroxycortisol
Estimated
Reality
APCC Yes No
Yes 25 2
No 2 14
Accuracy 39/43 90.7%
(F)
SF adenoma
Hyperplasia
APCC
18-Oxocortisol
18-Hydroxycortisol
21-Deoxycortisol
Pregnenolone
Progesterone
17-Hydroxyprogesterone
DHEAS
DHEA
11-Deoxycortisol
11-Deoxycortisosterone
Cortisone
Cortisol
Corticosterone
Androstenedione
Aldosterone
Estimate prediction

**Figure 4. Classification of biochemical outcomes after adrenalectomy and diagnosis of bilateral adrenal hyperplasia according to peripheral venous steroid concentrations**

Discriminant analysis with 9 steroids (androstenedione, cortisol, cortisone, 11-deoxycortisol, DHEA, DHEA sulphate, pregnenolone, 18-hydroxycortisol and 18-oxocortisol) generated receiver-operating characteristic (ROC) 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 PA (BAH) (**Panel A**). The steroids used in each model are shown in Table S9 with linear discriminant coefficients and cut-offs for prediction of absent + partial biochemical success. Decision tree analysis using steroids based on estimated predictive performance (**Panel B**) improved the correct classification of biochemical outcomes and diagnosis of bilateral PA. Numbers in the decision tree indicate steroid concentrations in ng/mL (**Panel C**).




| Outcome/ diagnosis | Absent +partial | Complete | BAH |
|---|---|---|---|
| Absent +partial | **10** | 4 | 1 |
| Complete | 3 | **20** | 5 |
| BAH | 1 | 3 | **23** |
| Accuracy | 53/70 | **75.7%** | |

| Outcome/ diagnosis | Absent +partial | Complete | BAH |
|---|---|---|---|
| Absent +partial | **15** | 0 | 0 |
| Complete | 5 | **21** | 2 |
| BAH | 0 | 3 | **24** |
| Accuracy | 60/70 | **85.7%** | |

| 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 |

**Table 1. Histopathology of adrenals from patients stratified by biochemical outcome after adrenalectomy**

APA, aldosterone-producing adenoma; A, P and C refer to absent, partial and complete biochemical success after surgery; APCC, aldosterone-producing cell cluster; CYP11B2, aldosterone synthase. Values indicate absolute numbers with proportions in parenthesis (%) or average numbers ± SD. *P* values were calculated using a $\chi^2$ or Fisher's exact tests or Mann-Whitney tests as appropriate.

**Online-only supplement**

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

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**Contents:**

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

## References

1) Williams TA, Lenders JWM, Mulatero P, et al., Outcomes after adrenalectomy for unilateral primary aldosteronism: an international consensus on outcome measures and analysis of remission rates in an international cohort. *Lancet Diabetes Endocrinol*. 2017;5:689-699.

2) Mancia G, Fagard R, Narkiewicz K, et al; Task Force Members. 2013 ESH/ESC Guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *J Hypertens.* 2013;31: 1281-357.

3) Fernandes-Rosa FL, Williams TA, Riester A, et al., Genetic spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma. *Hypertension* 2014;64:354-61.

**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 [25$^{th}$-75$^{th}$ percentile]. *P* values were calculated using a $\chi^2$, 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 [25$^{th}$-75$^{th}$ 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 $\chi^2$ or t test as appropriate.

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

| VARIABLE | Total cohort | BIOCHEMICAL OUTCOME | | *P*-value |
|---|---|---|---|---|
| | | A + P | C | |
| **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: $([aldosterone]/[cortisol])_{dominant\ adrenal\ vein}$ /$([aldosterone]/[cortisol])_{non\text{-}dominant\ adrenal\ vein}$ ; CL ratio defined as: $([aldosterone]/[cortisol])_{non\text{-}dominant\ adrenal\ vein}$ /$([aldosterone]/[cortisol])_{peripheral\ vein}$; 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 [$25^{th}$-$75^{th}$ percentile]. *P* values were calculated by a $\chi^2$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 [25$^{th}$-75$^{th}$ 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 [25$^{th}$-75$^{th}$ 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**

| | COEFFICIENTS OF LINEAR DISCRIMINANTS | | |
|---|---|---|---|
| **STEROID** | **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)

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

**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:

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