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Mass Spectrometry–Based Adrenal and Peripheral Venous Steroid Profiling for Subtyping Primary Aldosteronism

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BACKGROUND: Differentiating patients with primary aldosteronism caused by aldosterone-producing adenomas (APAs) from those with bilateral adrenal hyperplasia (BAH), which is essential for choice of therapeutic intervention, relies on adrenal venous sampling (AVS)-based measurements of aldosterone and cortisol. We assessed the utility of LC-MS/MS–based steroid profiling to stratify patients with primary aldosteronism.

METHODS: Fifteen adrenal steroids were measured by LC-MS/MS in peripheral and adrenal venous plasma from AVS studies for 216 patients with primary aldosteronism at 3 tertiary referral centers. Ninety patients were diagnosed with BAH and 126 with APAs on the basis of immunoassay-derived adrenal venous aldosterone lateralization ratios.

RESULTS: Among 119 patients confirmed to have APAs at follow-up, LC-MS/MS–derived lateralization ratios of aldosterone normalized to cortisol, dehydroepiandrosterone, and androstenedione were all higher ($P < 0.0001$) than immunoassay-derived ratios. The hybrid steroids, 18-oxocortisol and 18-hydroxycortisol, also showed lateralized secretion in 76% and 35% of patients with APAs. Adrenal venous concentrations of glucocorticoids and androgens were bilaterally higher in patients with BAH than in those with APAs. Consequently, peripheral plasma concentrations of 18-oxocortisol were 8.5-fold higher, whereas concentrations of cortisol, corticosterone, and dehydroepiandrosterone were lower in patients with APAs than in those with BAH. Correct classification of 80% of cases of APAs vs BAH was thereby possible by use of a combination of steroids in peripheral plasma.

CONCLUSIONS: LC-MS/MS–based steroid profiling during AVS achieves higher aldosterone lateralization ratios in patients with APAs than immunoassay. LC-MS/MS also enables multiple measures for discriminating unilateral from bilateral aldosterone excess, with potential use of peripheral plasma for subtype classification.

Primary aldosteronism is the most frequent cause of secondary hypertension and is responsible for 5%–15% of cases of increased blood pressure among hypertensive populations *(1)*. Increased blood pressure results from excess adrenal production of aldosterone, also adversely impacting the cardiovascular system, resulting in increased mortality independent of and additive to related increases in blood pressure *(2–5)*. The cardiovascular risks of primary aldosteronism can be substantially mitigated by appropriate therapy, but this must be tailored according to the specific nature of the disease *(5–7)*.

The two main forms of primary aldosteronism are (*a*) unilateral excessive adrenal aldosterone secretion usually caused by an aldosterone-producing adenoma (APA)[10] and best treated by adrenalectomy, and (*b*) bilateral excessive aldosterone secretion, commonly ascribed to bilateral adrenal hyperplasia (BAH) and most appropriately treated with mineralocorticoid receptor antagonists. Distinguishing the 2 subtypes depends largely on adrenal venous sampling (AVS) studies to determine unilat-

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[10] Nonstandard abbreviations: APA, aldosterone-producing adenoma; BAH, bilateral adrenal hyperplasia; AVS, adrenal venous sampling; ARR, aldosterone-to-renin ratio; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate.

Table 1. Demographic and clinical data at study entry.[a]

	APA	BAH	*P*
n	126	90	
Munich	79	49	
Nijmegen	33	35	
Düsseldorf	14	6	
Gender, M/F	81/45	68/22	0.1005
Age, years	52 (44–60)	50 (43–59)	0.5119
Systolic BP, mmHg	152 (140–190)	153 (137–166)	0.5069
Diastolic BP, mmHg	93 (85–102)	93 (85–100)	0.9753
Daily defined dose	3.0 (2–4.3)	2.3 (1–3.8)	0.0267
Potassium, mmol/L	3.3 (3.0–3.6)	3.5 (3.3–3.9)	<0.0001
Aldosterone, ng/L	235 (168–392)	179 (128–239)	<0.0001
Renin, mU/L	3.6 (2.2–8.8)	4.7 (3.0–11.7)	0.0346
ARR[b]	61 (26–116)	32 (16–53)	<0.0001

[a] Data for continuous variables are median (interquartile range).
[b] Calculated using ng/L concentrations of aldosterone to mU/L concentrations of renin. To convert aldosterone from ng/L to nmol/L, divide by 360.44. To convert renin from mU/L to ng/L, multiply by 0.39.

eral vs bilateral adrenal sources of excess aldosterone *(8)*. Standard AVS procedures depend on comparisons of plasma aldosterone in each adrenal vein, normalized to concentrations of cortisol, to establish presence or absence of asymmetric aldosterone production *(9)*.

Apart from the difficulty, complexity, and expense of AVS *(10)*, there are several other limitations to the procedure. Concomitant autonomous adrenal cortisol overproduction can obscure the presence of unilateral aldosterone excess *(11, 12)*. Because cortisol has a long plasma half-life, relative differences in peripheral to adrenal venous concentrations do not always accurately indicate selectivity of AVS *(13)*. There are also well-known inaccuracies in immunoassay methods *(14–22)* that may particularly impact adrenal venous measurements of adrenal steroids, such as aldosterone, where immuno-cross-reactivity with high concentrations of other steroids could compromise results.

Recognizing the above shortcomings, we established steroid profiling with LC-MS/MS for subtyping patients with primary aldosteronism *(23)*. The hybrid steroids 18-oxocortisol and 18-hydroxycortisol may be useful for such subtyping *(24–27)* and were included in the panel. In the study reported here, we took advantage of samples collected during AVS studies at 3 tertiary referral centers where patients with BAH and APAs were distinguished on the basis of immunoassay measurements of aldosterone and cortisol. Because those immunoassay measurements provided the reference standard for subtyping, it was not possible in this retrospective series to establish whether LC-MS/MS provided superior discriminatory diagnostic power over immunoassay measurements. Instead, the study had 3 objectives: (*a*) to establish the impact of any differences between LC-MS/MS and immunoassay measurements on aldosterone lateralization ratio; (*b*) to investigate the utility of alternative steroids to cortisol as normalizers for estimation of aldosterone lateralization ratios; and (*c*) to characterize the presence of differences in adrenal steroid production among patients with APAs and BAH that may translate to differences in peripheral plasma concentrations useful for subtype classification.

Materials and Methods

PATIENTS

Patients were included in the study when the diagnosis of primary aldosteronism was confirmed by an intravenous saline infusion or a fludrocortisone-suppression test. Inclusion also required demonstration of bilateral selectivity of AVS, confirmed according to ratios of metanephrine or adrenal steroids in adrenal to peripheral venous plasma *(13, 23)*. Follow-up of patients with APAs was also required to verify the diagnosis.

On the basis of the above criteria, the study included 216 patients with primary aldosteronism who underwent AVS sampling procedures at 3 tertiary referral centers (Table 1). Study protocols were approved by institutional ethics committees at all centers. At Nijmegen, the ethics committee waived requirements for informed consent for the first 7 patients. All other patients provided written informed consent.

ADRENAL VEIN SAMPLING

All AVS studies were carried out after adjustment of antihypertensive medication according to accepted recommendations *(8)*. Patients received potassium supplementation to correct hypokalemia. Sequential catheterization of the adrenal veins was performed at all centers. Continuous cosyntropin stimulation (50 μg/h) was used during AVS procedures at Nijmegen, whereas procedures at the 2 other centers were performed without cosyntropin.

We collected blood samples (2–8 mL) from both adrenal veins by gravity or gentle negative pressure, with additional collections (8 mL) of peripheral venous blood for assessing selectivity of AVS and contralateral suppression of aldosterone secretion as described *(10, 13, 23)*. Samples were collected into tubes containing lithium heparin (Nijmegen and Düsseldorf) or EDTA (Munich) as anticoagulants, transported on ice to laboratories within 15 min of collection, and centrifuged at 3000–3500*g* (4 °C) for 10–15 min. We used a portion of the plasma immediately for routine immunoassay measurements of cortisol and aldosterone and stored another portion at −70 °C or less until it was sent on dry ice to Dresden for LC-MS/MS steroid profiling.

SUBTYPE DIAGNOSES

Subtyping patients to either BAH or APA groups was based on calculations of the lateralization ratio, according to immunoassay measurements of plasma aldosterone (*A*) and cortisol (*C*) concentrations in both adrenal veins and defined as the ratio of the higher (dominant) over the lower (nondominant) ratio by the following formula:

$$\text{Lateralization ratio} = \frac{(A_{\text{dominant}}/C_{\text{dominant}})}{(A_{\text{nondominant}}/C_{\text{nondominant}})}$$

Plasma aldosterone was measured at Munich and Düsseldorf with a commercial RIA (Coat-a-count, Siemens Healthcare), whereas aldosterone was measured at Nijmegen with an in-house RIA. Cortisol was measured with an automated chemiluminescence assay (Liaison, Diasorin) at Munich, whereas electrochemiluminescence immunoassays at Nijmegen and Düsseldorf used the Modular E170 and Elecsys (Roche Diagnostics) analyzers, respectively. Performance characteristics of these assays are detailed elsewhere *(13, 23, 28)*.

Patients subtyped to the APA group, on the basis of immunoassay-based lateralization ratios ≥4.0, all underwent laparoscopic adrenalectomy, whereas those diagnosed with BAH according to a lateralization ratio of <4.0 were treated with mineralocorticoid receptor antagonists (Fig. 1A). There were 5 exceptions involving patients with lateralization ratios <4.0 (3.8, 3.5, 3.4, 3.1, and 2.1) who underwent adrenalectomy. In these cases, the decision to operate was reached by consensus, on the basis of clinical details and computed tomographic scans indicating a high likelihood of APA: young age, hypokalemia, suppressed renin, and unilateral adrenal mass. Consequently, 90 patients were assigned a diagnosis of BAH and 126 a diagnosis of APA. These designations are an oversimplification, since it must be recognized that in some patients the diagnosis of BAH may reflect bilateral APAs, whereas in others there may be unilateral or asymmetric hyperplasia and classification by AVS as an APA. Furthermore, lateralization ratio cutoffs of 4.0 are somewhat arbitrary and may not always accurately distinguish BAH from APA. For these reasons, follow-up is mandatory for all patients operated for an APA.

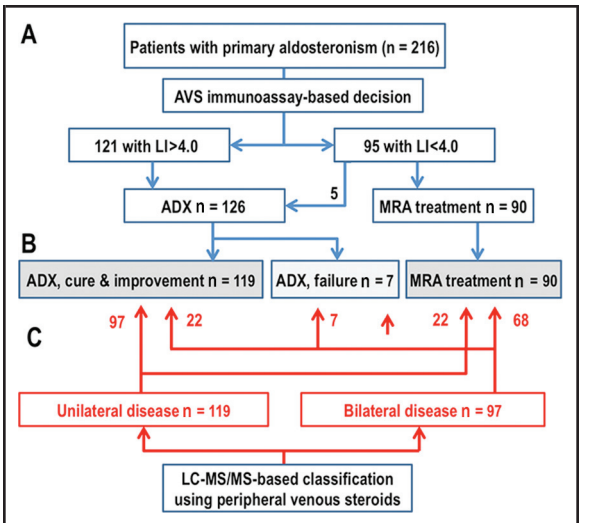

Fig. 1. Decision and outcome classification tree for AVS immunoassay-based subtyping and therapy of patients with primary aldosteronism (A) in relation to outcome assessed by patient follow-up (B) and separate classification of subtype and outcome on the basis of LC-MS/MS–based measurements of 12 adrenal steroids in peripheral venous plasma (C).

Five patients with immunoassay-derived lateralization indices <4.0 were nevertheless assigned to the group for adrenalectomy (ADX) rather than the group receiving mineralocorticoid receptor antagonist (MRA) therapy.

FOLLOW-UP

Among the patients subtyped with APAs, postoperative information was available at 6–12 months after surgery in all except 1 patient whose follow-up was at 3 months. Outcomes of surgery were categorized as cure, improvement, or failure. We defined cure as office systolic and diastolic blood pressure <140 and <90 mmHg, no use of antihypertensive medications, serum potassium >3.5 mmol/L, and a normalized aldosterone-to-renin ratio (ARR). We defined failure as a continuing increase of the ARR followed by a positive saline suppression test. In 1 of

14 patients with APAs in whom a follow-up saline infusion test was not possible, failure was based on a remaining increase of the ARR, low serum potassium, lack of decrease in blood pressure, and no change in antihypertensive medication use on follow-up. All other patients followed up postoperatively were defined as improved.

LC-MS/MS-BASED STEROID PROFILING

We measured 15 adrenal steroids simultaneously by LC-MS/MS, including aldosterone, cortisol, 18-oxocortisol, 18-hydroxycortisol, cortisone, 11-deoxycortisol, 21-deoxycortisol, corticosterone, 11-deoxycorticosterone, progesterone, 17-hydroxyprogesterone, pregnenolone, androstenedione, dehydroepiandrosterone (DHEA), and DHEA-sulfate (DHEA-S). Full details of the method, including validation and assay performance characteristics, are described elsewhere *(23)*.

STATISTICAL ANALYSES

For comparisons to APA groups, we defined dominance of adrenal venous lateralization in patients with BAH by a lateralization ratio >1.0 as measured by LC-MS/MS. Statistical analyses used the JMP statistics software package (SAS Institute). Data are expressed as medians and interquartile ranges. We used Mann–Whitney *U* and Wilcoxon matched paired sign-rank tests to assess significance of differences involving 2 groups (i.e., BAH vs APA) or paired comparisons (i.e., dominant adrenal vein vs nondominant adrenal vein). We used Kruskal–Wallis and Steel–Dwass all-pairs methods for nonparametric comparisons involving 3 groups (e.g., APA success vs failure vs BAH). Relationships were assessed by 1-tailed Spearman correlation coefficient (r_s). We used discriminant analysis to assess how combinations of steroids could be used to correctly classify tumors into BAH and APA groups as well as to subclassify patients with APAs according to cure, improvement, and treatment failure. Least squares multivariate analyses were used to assess relationships of different variables (e.g., cosyntropin vs no cosyntropin and BAH vs APA) to steroid profiles. Data were logarithmically transformed before parametric statistical tests.

Results

PATIENT CHARACTERISTICS AND OUTCOMES

Compared with the 126 patients subtyped with APA, the 90 patients with BAH had baseline characteristics at study entry indicating overall less severe disease (Table 1). Among the 126 patients with APAs, 32 (25%) showed complete cure after adrenalectomy, 87 (69%) showed improvement that included no remaining biochemical evidence of primary aldosteronism, and 7 patients (6%) showed remaining biochemical evidence of primary aldosteronism on follow-up (Fig. 1B). All 7 patients in the treatment-failure group also showed lack of postoperative decreases in blood pressure and no decrease in antihypertensive medications at follow-up (see Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue3). Potassium was also lower postoperatively in these patients than in the 119 patients with APAs who showed improvement or cure and were categorized together in a single treatment success group.

IMMUNOASSAY- VS LC-MS/MS-DERIVED LATERALIZATION RATIOS

Strong positive relationships ($P < 0.0001$) were observed between measurements of cortisol ($r_s = 0.976$, $P < 0.0001$) and aldosterone ($r_s = 0.932$, $P < 0.0001$) by immunoassay and LC-MS/MS, but overall both plasma cortisol and aldosterone were higher ($P < 0.0001$) by immunoassays than by LC-MS/MS, with the extent of these differences varying according to center, concentration range, and sampling site (see online Supplemental Results and Supplemental Fig. 1).

As a consequence of the differences between immunoassay and LC-MS/MS–based measurements, Bland–Altman plots indicated mean lateralization ratios for aldosterone normalized to cortisol that were 38% higher ($P < 0.0001$) by LC-MS/MS than by immunoassay (Fig. 2A). Lateralization ratios for LC-MS/MS measurements of aldosterone normalized to androstenedione and DHEA were ≤46% higher ($P < 0.0001$) than ratios derived from immunoassay measurements of aldosterone and cortisol (Fig. 2B,C). These differences remained significant ($P < 0.005$) for each center and, as shown by Bland–Altman plots, were larger at higher lateralization ratios. Thus, the largest differences were observed for the 119 patients with APAs, among whom lateralization ratios were 71%–101% higher by LC-MS/MS than by immunoassay (see online Supplemental Table 2).

Lateralization ratios of immunoassay-measured aldosterone normalized for cortisol showed strong positive ($P < 0.0001$) relationships with ratios of LC-MS/MS–measured aldosterone normalized for cortisol, androstenedione, and DHEA (see online Supplemental Results and Supplemental Fig. 2). Nevertheless, 10% of patients showed LC-MS/MS–derived lateralization ratios that differed from immunoassay-derived ratios according to the cutoff of 4.0; these included 17 patients (8%) in whom LC-MS/MS–derived ratios fell above and immunoassay-derived ratios fell below 4.0 and 4 patients (2%) consistently showing the opposite. The first group included 2 patients who were nevertheless operated for APAs, both showing LC-MS/MS–derived ratios consistently >50 and favorable responses to adrenalectomy. There were also 3 patients diagnosed with BAH on the basis of immunoassay-derived ratios who consistently showed lateralization ratios by LC-MS/MS of >12.

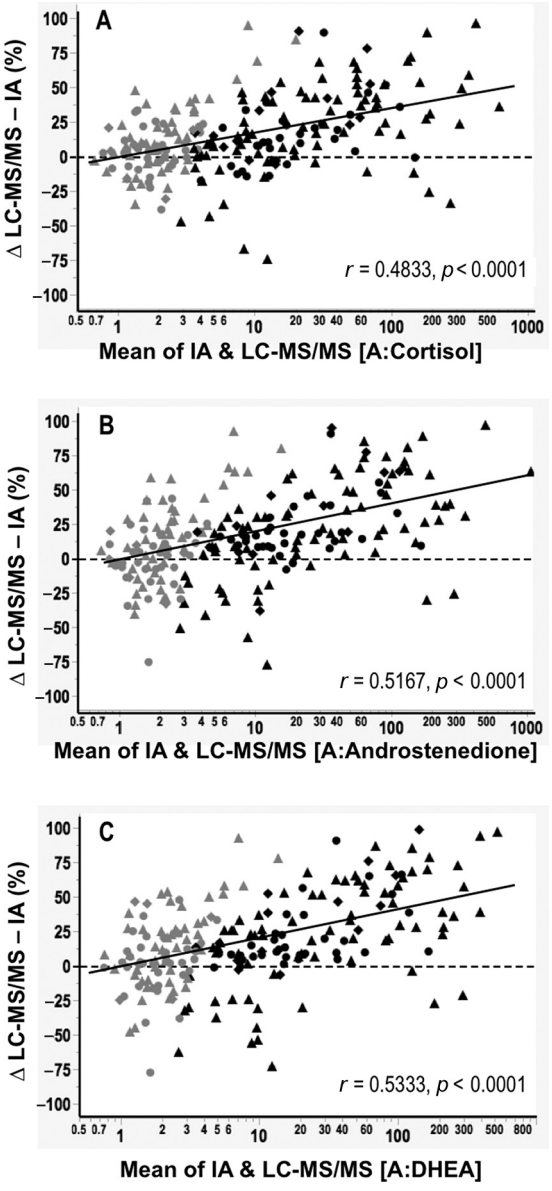

Fig. 2. Bland–Altman plot comparisons of immunoassay-derived lateralization ratios for aldosterone normalized to cortisol vs LC-MS/MS–derived lateralization ratios for aldosterone normalized to cortisol (A), androstenedione (B), and DHEA (C).

Bland–Altman plots are shown as means of lateralization ratios determined by LC-MS/MS and immunoassay measurements of aldosterone, on the *x* axes, vs differences in LC-MS/MS and immunoassay-derived ratios as a percent of mean values, on the *y* axes. The different symbols depict the different centers at which AVS was carried out, with gray symbols (▲, ●, ◆) indicating patients with BAH, and black symbols (▲, ●, ◆), those with APA.

LATERALIZATION RATIOS OF 18-OXOCORTISOL AND 18-HYDROXYCORTISOL

Lateralization ratios of 18-oxocortisol and 18-hydroxycortisol—normalized to cortisol, DHEA, or androstenedione—all were higher ($P < 0.001$) among the patients with APAs who showed a successful response to treatment compared with patients designated with BAH (see online Supplemental Table 2). The differences, however, were considerably more substantial for 18-oxocortisol than for 18-hydroxycortisol. By all methods of normalization, considerably lower ($P < 0.05$) lateralization ratios were estimated for both aldosterone and 18-oxocortisol for the 7 patients designated with APAs who failed to show a therapeutic response, compared with the 119 patients with APAs who showed a successful response to adrenalectomy.

Lateralization ratios for 18-oxocortisol and 18-hydroxycortisol showed positive ($P < 0.001$) relationships with those of aldosterone for measurements of the latter by both LC-MS/MS (Fig. 3, A and C) and immunoassay (Fig. 3, B and D). Among the APA group, 90 (76%) and 42 (35%) patients showed respective lateralization ratios of 18-oxocortisol and 18-hydroxycortisol above the cutoff of 4.0, compared to 6 (7%) and 2 (2%) patients in the BAH group. However, 3 of the 6 patients in the BAH group with lateralized production of 18-oxocortisol also showed lateralized production of aldosterone by LC-MS/MS, but not by immunoassay-derived lateralization ratios >4.0 (Fig. 3, A and B). Three other patients in the BAH group had strong lateralization of 18-oxocortisol (ratios of 6.7, 9.8, and 12.3) and LC-MS/MS–derived lateralization ratios for aldosterone between 2.7 and 3.2 (Fig. 3A), among whom 1 patient also had lateralized 18-hydroxycortisol production (ratio 6.6). The 2 patients with APAs who had immunoassay-derived lateralization ratios for aldosterone <4.0 but LC-MS/MS–derived lateralization ratios >40.0 (see online Supplemental Fig. 2) also had lateralization ratios for 18-oxocortisol of 52.3 and 119.3 (Fig. 3B) and for 18-hydroxycortisol of 5.9 and 25.4 (Fig. 3D).

ADRENAL VENOUS STEROID PROFILES

In contrast to the higher ($P < 0.01$) concentrations of aldosterone, 18-oxocortisol, and 18-hydroxycortisol in the dominant adrenal veins of patients with APAs than those with BAH, concentrations of these steroids in nondominant adrenal veins were higher ($P < 0.05$) in patients with BAH than those with APAs (Table 2). Concentrations of all 12 other steroids were also higher ($P < 0.05$) in the nondominant adrenal veins of patients with BAH than those with APAs. Furthermore, concentrations of cortisol, pregnenolone, 17-hydroxyprogesterone, 21-deoxycortisol, cortisone, androstenedione, DHEA, and DHEA-S were also higher ($P < 0.05$) in the dominant adrenal veins of patients with BAH compared to

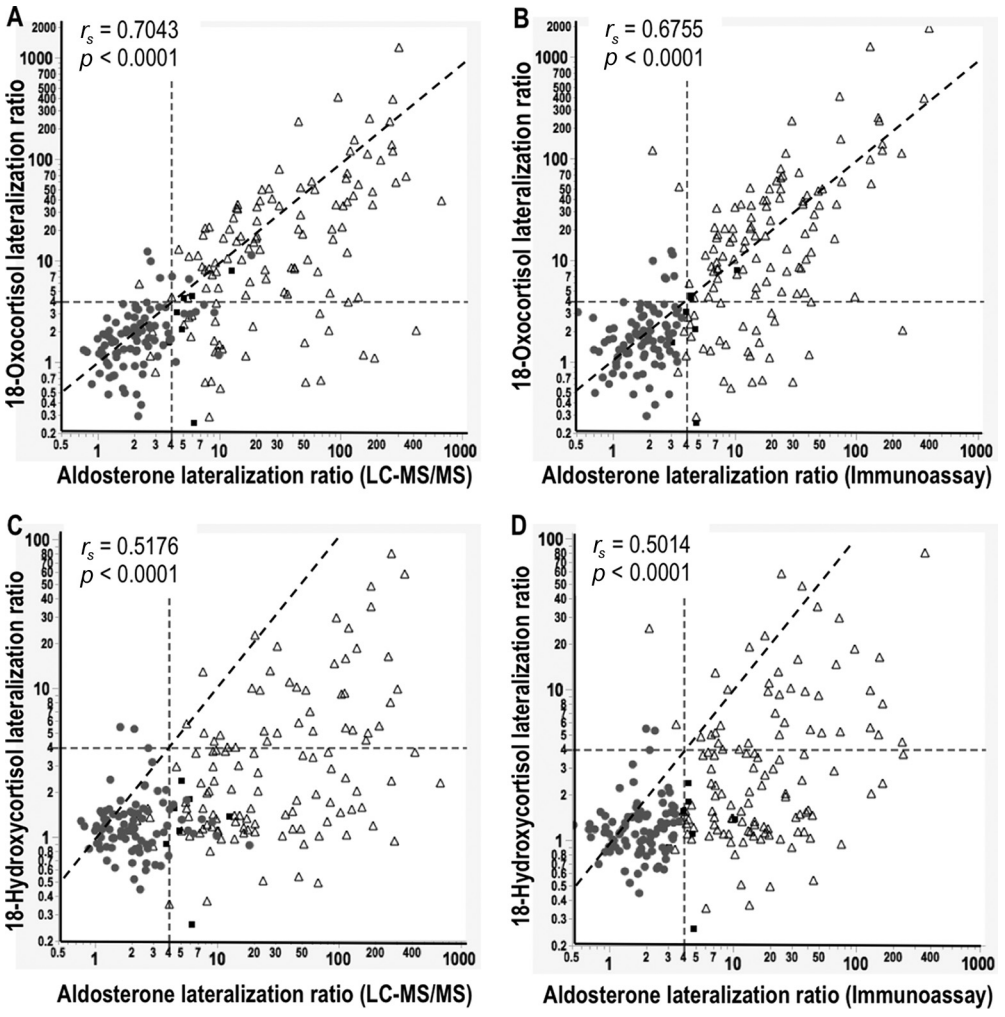

Fig. 3. Relationships of lateralization ratios of aldosterone normalized to cortisol (*x* axes) according to immunoassay (B,D) and LC-MS/MS (A,C) measurements compared to lateralization ratios of 18-oxocortisol (A, B) and 18-hydroxycortisol (C, D) normalized to means for cortisol, DHEA, and androstenedione (*y* axes) in patients with BAH (●) and APA (treatment success △; treatment failure ■).

Dashed vertical and horizontal gray lines illustrate cutoffs of 4.0 used to determine lateralized secretion. Lines of identity are also shown to clarify higher or lower lateralization ratios for LC-MS/MS–derived lateralization ratios for 18-oxocortisol and 18-hydroxycortisol and vs LC-MS/MS–derived (A,C) and immunoassay-derived (B,D) lateralization ratios for aldosterone.

those with APAs. These higher concentrations for patients with BAH compared to APAs remained significant ($P < 0.05$) for corticosterone, cortisol, progesterone, 21-deoxycortisol, cortisone, DHEA, and DHEA-S after correction for any confounding influence of cosyntropin stimulation.

The differences in adrenal venous concentrations of steroids between patients with APAs compared to BAH translated to differences in peripheral venous concentrations for several steroids. Specifically, whereas peripheral venous concentrations of aldosterone, 18-oxocortisol, and 18-hydroxycortisol were higher ($P < 0.05$) among patients with APAs than BAH, peripheral venous concentrations of corticosterone, cortisol, DHEA, and DHEA-S were higher ($P < 0.05$) among patients with BAH compared with APAs (Table 2).

DISCRIMINATION OF BAH AND APA BY USE OF PERIPHERAL VENOUS STEROIDS

Although concentrations of 18-oxocortisol in peripheral venous plasma were 8.5 times higher among patients with APAs than those with BAH, there was considerable overlap, so ROC curves indicated poor utility of this hybrid steroid for distinguishing patients with APAs

Table 2. Plasma concentrations of steroids in peripheral and adrenal veins of patients with APA (n = 119) and BAH (n = 90).[a]

	Dominant adrenal vein[b]			Nondominant adrenal vein[b]			Peripheral vein		
Concentration, ng/mL	APA	BAH	*P*	APA	BAH	*P*	APA	BAH	*P*
Aldosterone	24.93 (4.53–53.50)	5.43 (1.54–20.81)	<0.0001	0.55 (0.16–2.08)	2.67 (0.51–13.08)	<0.0001	0.20 (0.07–0.54)	0.12 (0.04–0.40)	<0.05
18-Oxocortisol	4.58 (0.64–13.58)	0.76 (0.27–2.66)	<0.0001	0.50 (0.07–0.65)	0.67 (0.11–1.86)	<0.05	0.119 (0.012–0.050)	0.014 (0.010–0.125)	<0.0001
18-Hydroxycortisol	35.5 (9.0–104.7)	21.0 (8.2–48.1)	<0.01	12.1 (4.1–30.7)	19.0 (5.5–46.9)	<0.05	1.69 (0.71–3.26)	1.34 (0.54–2.41)	<0.05
Deoxycorticosterone	7.53 (1.02–42.00)	10.99 (0.93–36.44)		1.42 (0.41–18.65)	8.80 (0.63–46.98)	<0.05	0.12 (0.05–0.36)	0.12 (0.05–0.35)	
Corticosterone	115 (33–1063)	548 (44–1354)		74 (17–930)	508 (39–1861)	<0.01	2.66 (1.14–20.00)	7.87 (1.81–25.65)	<0.05
Cortisol	1116 (335–4760)	3690 (566–5656)	<0.01	972 (338–4150)	3867 (507–7325)	<0.01	123 (72–208)	171 (102–256)	<0.05
Progesterone	3.85 (0.70–42.25)	13.93 (0.81–63.29)		1.27 (0.34–29.75)	16.68 (0.83–83.13)	<0.01	0.16 (0.08–0.60)	0.25 (0.08–0.54)	
Pregnenolone	3.23 (0.94–102.00)	30.00 (2.05–115.00)	<0.05	5.75 (0.91–53.25)	24.3 (2.08–139.75)	<0.05	0.35 (0.18–1.17)	0.46 (0.24–1.22)	
17-Hydroxyprogesterone	14.7 (4.1–129.5)	65.6 (7.5–184.0)	<0.05	16.6 (3.5–114.3)	78.9 (5.9–285.8)	<0.05	1.0 (0.5–2.2)	1.2 (0.6–2.5)	
11-Deoxycortisol	19.9 (8.2–91.5)	54.7 (8.4–103.3)		15.4 (5.3–66.5)	44.2 (5.3–154.9)	<0.05	0.38 (0.21–1.53)	0.73 (0.22–1.95)	
21-Deoxycortisol	1.34 (0.40–6.40)	3.47 (0.41–13.61)	<0.05	0.71 (0.24–6.80)	3.28 (0.44–15.90)	<0.01	0.08 (0.01–0.15)	0.09 (0.04–0.27)	
Cortisone	42.6 (20.2–98.0)	66.7 (35.1–108.0)	<0.01	41.8 (21.0–92.0)	63.4 (31.4–117.6)	<0.01	16.4 (13.0–23.4)	17.6 (14.3–21.5)	
Androstenedione	37.3 (11.8–92.0)	60.7 (20.0–119.8)	<0.05	36.0 (11.6–103.7)	59.7 (17.2–148.1)	<0.05	0.84 (0.51–1.63)	1.04 (0.67–1.78)	
DHEA	22.4 (6.4–60.0)	50.5 (16.0–91.5)	<0.001	21.7 (6.8–62.55)	45.9 (14.3–130.1)	<0.001	0.60 (0.34–1.13)	1.04 (0.56–1.55)	<0.001
DHEA-S	1420 (733–2118)	1655 (1001–4227)	<0.05	1419 (725–2078)	1823 (990–2893)	<0.01	1030 (566–1325)	1145 (734–1756)	<0.05

[a] Data are median (interquartile range). To convert to ng/mL to nmol/mL, divide by the molecular weight for each steroid (see Supplemental Table 3).
[b] Concentrations reflect lateralization ratios for aldosterone relative to cortisol respectively larger and smaller than 1.0.

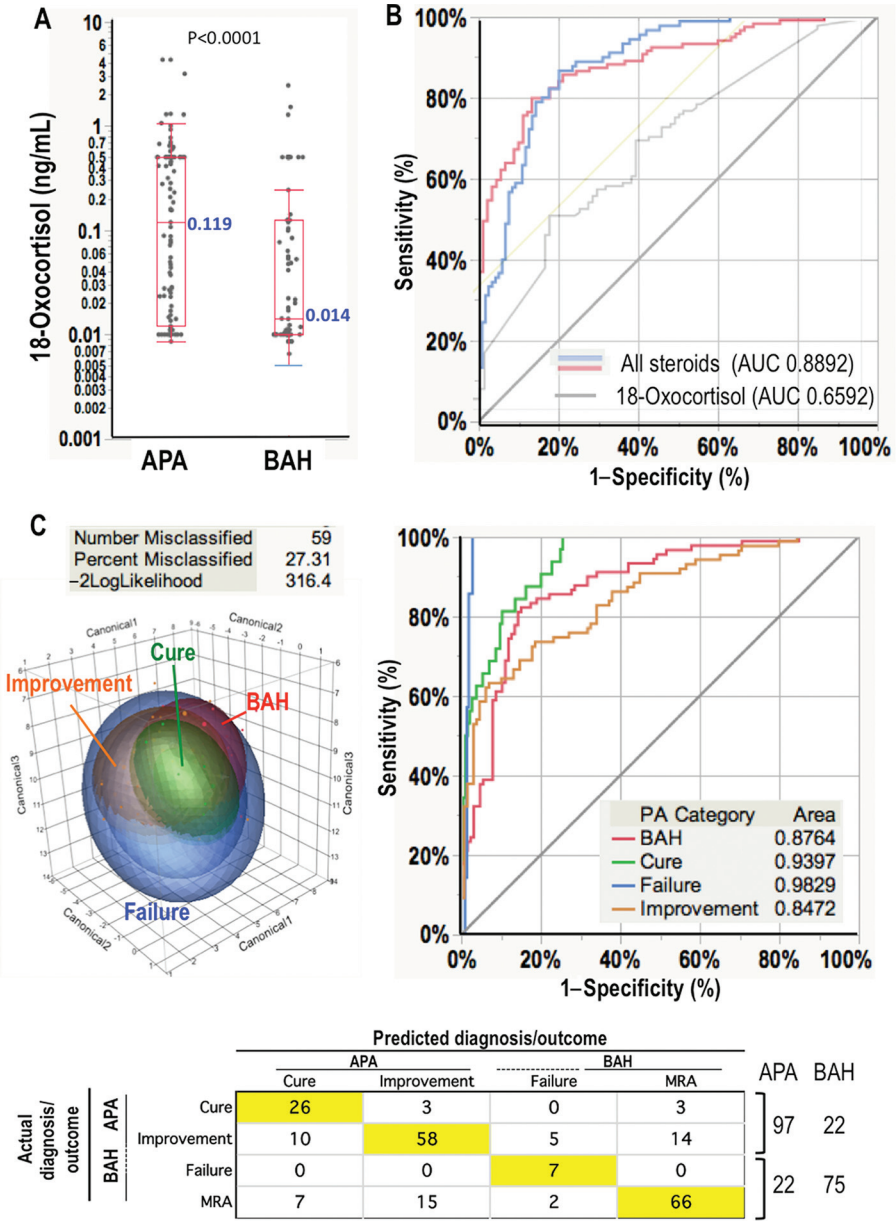

Fig. 4. Dot box and whisker plot of peripheral venous plasma concentrations of 18-oxocortisol in patients with APAs and BAH (A), ROC curves for distinguishing patients with APAs from BAH by use of peripheral venous 18-oxocortisol vs all 15 adrenal steroids (B), and results of discriminant analysis by use of peripheral plasma concentrations of steroids for classification according to outcome.

For the latter analysis, 12 adrenal steroids (aldosterone, 18-oxocortisol, 18-hydroxycortisol, 21-deoxycortisol, corticosterone, 11-deoxycorticosterone, progesterone, 17-hydroxyprogesterone, androstenedione, DHEA, and DHEA-S) were used to classify patients with primary aldosteronism according to diagnosis of BAH and response to adrenalectomy according to cure, improvement, and treatment failure among patients subtyped with APAs on the basis of immunoassay-derived lateralization ratios.

from those with BAH (Fig. 4, A and B). However, ROC curve analysis indicated considerably improved diagnostic utility for subtype discrimination when all 15 adrenal steroids were considered (Fig. 4B). With additional consideration of postadrenalectomy outcome data for subcategorizing patients with immunoassay-diagnosed APAs into groups showing cure or improvement vs treatment failure, it could be further determined with discriminant

analysis and stepwise variable selection that a selection of 12 adrenal steroids correctly classified 82% (97/119) and 100% (7/7) of patients into those 2 respective groups (Figs. 1C and 4C). However, 22 (17%) of the 126 patients subtyped with APAs according to immunoassay-derived lateralization ratios were classified as having BAH, and another 22 (24%) of the 90 patients subtyped with BAH were classified with APAs (Fig. 1C). With reclassification of cases of treatment failure to the BAH group, use of peripheral plasma steroids correctly classified 80% (172/216) of patients to APA and BAH groups.

Discussion

This study illustrates advantages of LC-MS/MS measurements over conventional immunoassay measurements of aldosterone and cortisol for AVS-based subtyping of patients with primary aldosteronism. We also establish alternative steroids to cortisol for correcting adrenal venous aldosterone for dilutional effects of imperfectly selective AVS, blood flow differences, or other influences that may erroneously affect interpretations of lateralized aldosterone production. Furthermore, we have expanded on existing knowledge concerning the utility of the hybrid steroids, 18-oxocortisol and 18-hydroxycortisol, as additional biomarkers for discriminating different subtypes of primary aldosteronism and provide data illustrating that differences in patterns of adrenal steroid production among patients with BAH and APAs translate to differences in peripheral venous steroid profiles that are potentially useful for subtype classification.

Although LC-MS/MS–based steroid profiling is increasingly showing utility for investigations of adrenal function and dysfunction *(22, 29–33)*, application of this technology to patients with hypertension caused by primary aldosteronism has to date been limited, particularly in the context of AVS. Perhaps the closest study to ours is that by Nakamura et al. *(34)*, who reported measurements of 10 adrenal steroids, including aldosterone, in adrenal venous plasma of 9 patients with primary aldosteronism before and after corticotropin stimulation. In that study, however, aldosterone was measured with an LC-MS/MS method separate from those used for the other steroids. A subsequent study involving LC-MS/MS–based steroid profiling during AVS did not include aldosterone or involve patients with primary aldosteronism *(35)*. Our study is unique in applying LC-MS/MS profiling of aldosterone, cortisol, and nearly all other principal adrenal steroids, in addition to 2 hybrid steroids, to a large population of patients with primary aldosteronism undergoing AVS for discriminating APA from BAH.

We previously established that cortisol is inferior to metanephrine for determining selectivity of adrenal venous catheterization *(13)*. In a subsequent study, we also established DHEA and androstenedione as further alternatives to cortisol for determining selectivity of AVS *(23)*. We show here that DHEA and androstenedione also provide useful alternatives to cortisol as normalizers to assess lateralized aldosterone production.

Our findings that lateralization ratios of aldosterone were considerably higher when measured by LC-MS/MS compared to immunoassays appears related to higher concentrations of aldosterone measured in nondominant adrenal veins (i.e., contralateral to adrenal veins with higher aldosterone concentrations) by immunoassays than by LC-MS/MS. This likely reflects relative inaccuracy of immunoassays to measure low concentrations of aldosterone in the venous outflows of adrenals with suppressed aldosterone production, possibly compounded by antibody cross-reactivity associated with high concentrations of other steroids in these outflows. Others have similarly reported higher concentrations of aldosterone measured by immunoassays than by LC-MS/MS *(14, 18–20, 22)*, with differences particularly prominent in the lower concentration range. Our data reiterate the advantages of LC-MS/MS over immunoassays for measurements of steroids and extend these advantages to use of AVS for subtyping patients with primary aldosteronism.

The utility of hybrid steroids, such as 18-oxocortisol and 18-hydroxycortisol, for subtyping patients with primary aldosteronism has been a subject of interest by numerous investigators *(24–27, 36–39)*. Most recently, Satoh et al. reported on the utility of measuring 18-oxocortisol in peripheral venous plasma to distinguish patients with BAH from APAs *(27)*. Although we could not establish similar utility in the present study, we could confirm 8.5-fold higher peripheral venous plasma concentrations of 18-oxocortisol in patients with APAs than BAH, a finding also consistent with earlier studies *(25, 26, 37)*. We have also established elsewhere that increases in plasma 18-oxocortisol among patients with APAs are particularly prominent and largely confined to tumors with somatic *KCNJ5* (potassium channel, inwardly rectifying subfamily J, member 5) mutations *(39)*. In earlier work, Gordon et al. established that increases of 18-oxocortisol are largely confined to patients with angiotensin-unresponsive APAs *(38)*. Because adenomas with mutations of *KCNJ5* have a zona fasciculata–like phenotype, it is possible that these tumors may correspond to the angiotensin-unresponsive APAs characterized by Gordon et al. *(38)*.

Although 18-oxocortisol appears to have limited value for subtype differentiation, as we now show, this can be considerably enhanced with inclusion of additional steroids. Indeed, our analyses showed that steroid profiles in peripheral samples could identify 75 of the 97 patients (77%) who did not benefit from adrenalectomy or who were indicated by AVS to have BAH (Fig. 1C).

This could thereby potentially avoid the need for AVS in such patients. However, it must also be recognized that not all patients with APAs could be distinguished from those with BAH, so that any use of peripheral steroids for subtyping and minimizing requirements for AVS would likely require additional consideration of imaging characteristics.

Although this study outlines promise for LC-MS/MS–based steroid profiling for subtyping of patients with primary aldosteronism, there are limitations. One concerns use of cosyntropin stimulation at 1 of the 3 centers where AVS was performed, which complicates interpretation of differences in steroid profiles. Nevertheless, even with this limitation, findings of differences between LC-MS/MS and immunoassays that were consistent among centers adds strength to the conclusion that LC-MS/MS achieves higher aldosterone lateralization ratios in patients with APAs than by immunoassay. The second, more important, limitation was that LC-MS/MS measurements were not used for diagnostic decision-making, which remained based on results of routine immunoassays. Thus, for some patients designated with BAH with immunoassay measurements, lateralization ratios on the basis of LC-MS/MS measurements were above the usual cutoff of 4.0, and it remains unclear whether these patients may have benefited from adrenalectomy. As pointed out elsewhere *(40)*, such cutoffs are somewhat arbitrary and potentially misleading. For example, therapeutic responses to adrenalectomy among patients of the present study were highly variable and included treatment failure in 7 patients, presumably a consequence of asymmetric bilateral disease. Among these 7 patients, it is noteworthy that all were correctly classified by peripheral steroid profiles.

With consideration of the above, clearly there is need for improved classification not only to distinguish patients with BAH from those with APAs, but also to identify which of the latter group will show the most benefit from surgical intervention. The present study addresses this need by establishing that compared with immunoassay-derived measurements, LC-MS/MS achieves enhanced magnitudes of measured aldosterone lateralization ratios in patients with APAs and provides multiple measures for discriminating APAs from BAH, the latter with potential application to measurements in peripheral plasma. With the necessary proof-of-principal data in hand, it is now possible to proceed to a prospective study in which LC-MS/MS–based steroid profiling guides diagnostic decision-making. From there, it might be possible for translation to routine clinical practice.

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