Aldosterone LC-MS/MS Assay-Specific Threshold Values in Screening and Confirmatory Testing for Primary Aldosteronism

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Context: Current threshold values for primary aldosteronism (PA) diagnostic testing are based on measuring aldosterone (PAC) using immunoassays. Quantification of PAC by liquid chromatography-tandem mass spectrometry (LC-MS/MS) yields lower values.

Objective: To compare aldosterone measurement by radioimmunoassay (RIA) with LC-MS/MS and evaluate performances of proposed LC-MS/MS–specific cutoffs for PA screening and confirmatory testing.

Patients and Intervention: Forty-one patients underwent aldosterone/renin ratio (ARR) testing to screen for, and fludrocortisone suppression testing (FST) to confirm or exclude, PA. Renin (DRC) was measured by chemiluminescent immunoassay.

Results: Median serum $PAC_{LC\text{-}MS/MS}$ was 27.8% lower ($P < 0.05$) than plasma PAC_{RIA} in 164 pairs of FST samples. A positive correlation (Spearman coefficient, 0.894, $P < 0.01$; Pearson r coefficient, 0.861, $P < 0.01$) was observed between the two assays. Thirty-seven patients showed consistent FST diagnoses (29 positive, 8 negative), whereas four showed inconsistent FSTs by the two assays. Good agreement (κ coefficient, 0.736; $P < 0.01$) was observed between the current FST diagnostic PAC_{RIA} cutoff of 165 pmol/L and the proposed $PAC_{LC\text{-}MS/MS}$ cutoff of 133 pmol/L. Among 37 patients with consistent FST results, no differences were observed in sensitivity (89.7% *vs* 93.1%) or specificity (87.5% *vs* 87.5%) for PA screening between the current ARR cutoff of 70 pmol/mU (PAC_{RIA}/DRC) and the proposed cutoff of 55 pmol/mU ($PAC_{LC\text{-}MS/MS}$/DRC).

Conclusions: Adjustment of the current cutoffs for PA diagnostic testing is necessary if PAC is measured by LC-MS/MS. Our preliminary results suggest that the proposed LC-MS/MS cutoffs for ARR and FST perform as well as current RIA cutoffs. (***J Clin Endocrinol Metab*** **103: 3965–3973, 2018)**

As the main salt-retaining hormone in humans, aldosterone plays an important role in regulating sodium and potassium handling within the distal nephron (1). The biosynthesis of aldosterone by adrenal glands is physiologically regulated by the renin-angiotensin system (RAS), which is activated in response to low blood

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received 15 May 2018. Accepted 15 August 2018.
First Published Online 20 August 2018

Abbreviations: AD, Attoquant Diagnostics; ADX, adrenalectomy; ARR, aldosterone/renin ratio; AVS, adrenal venous sampling; CLIA, chemiluminescent immunoassay; CV, coefficient of variation; DRC, direct renin concentration; FST, fludrocortisone suppression testing; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PA, primary aldosteronism; PAC, peripheral aldosterone concentration; PQ, Pathology Queensland; PRA, plasma renin activity; RAS, renin-angiotensin system; RIA, radioimmunoassay; ROC, receiver operating characteristic; SIT, saline infusion testing.

pressure and low salt status (2). However, in primary aldosteronism (PA), overproduction of aldosterone, caused by adenoma or hyperplasia of one or both adrenal glands, is relatively autonomous of the RAS, levels of which are usually suppressed (3). Such inappropriate aldosterone production in PA leads to (1) excessive sodium retention, which causes volume expansion and hypertension; (2) increased potassium excretion which, if severe and prolonged enough, may lead to hypokalemia (4); and (3) adverse cardiovascular and renal consequences (5, 6).

PA is now recognized as the most common endocrine cause of hypertension with a prevalence approaching 5% to 13% (7–9). Early diagnosis of PA is of considerable potential benefit to affected individuals, because unilateral adrenalectomy (ADX) results in cure or improvement of hypertension in patients with unilateral PA, whereas specific drugs (such as mineralocorticoid receptor antagonists) that antagonize aldosterone action usually have substantial beneficial effects on control of hypertension in bilateral PA (10). The diagnostic workup of PA includes: (1) screening for PA by aldosterone/renin ratio (ARR) testing, (2) confirmatory testing [*e.g.*, by fludrocortisone suppression testing (FST) or saline infusion testing (SIT)], and (3) determining the PA subtype, primarily involving distinguishing unilateral from bilateral PA by adrenal CT and adrenal venous sampling (AVS) (11). Accurate measurement of peripheral (plasma or serum) aldosterone concentration (PAC) is essential for all stages of PA diagnostic workup, with ARR reliant on accurate assays of both aldosterone and renin [direct renin concentration (DRC) or plasma renin activity (PRA)], FST dependent on precise quantification of aldosterone (as well as renin and cortisol), and AVS reliant on reliable assays of aldosterone and cortisol.

Because peripheral aldosterone circulates at picomolar concentrations, accurate measurement of PAC requires highly sensitive and specific assays. Currently, PAC is most often measured by antibody-based immunoassays (12) that have demonstrated lack of high specificity (causing overestimation of PAC) (13) and variability in assay performance among different laboratories. Therefore, in the past decade, there has been growing interest in quantifying PAC by using liquid chromatography-tandem mass spectrometry (LC-MS/MS), which has been reported to be more reliable than traditional radioimmunoassay (RIA) (14). However, studies evaluating this emerging approach with regard to assay-specific cutoffs for PA screening and confirmatory testing are rare (15).

In 2009, our center reported on the development of an aldosterone LC-MS/MS assay that was highly accurate and reproducible (16, 17). Subsequent analysis within our laboratory has revealed a lower value of PAC measured by this approach ($PAC_{LC\text{-}MS/MS}$) compared with RIA (PAC_{RIA}), probably at least in part because of the improved specificity of LC-MS/MS. These results suggest that when undertaking screening and confirmatory testing for PA, reduction in current threshold values for the ARR and FST (which were both established using PAC_{RIA}) would be necessary if PAC is measured by LC-MS/MS.

In the current study, we applied regression equations derived from our comparison results of two aldosterone assays as well as consideration of clinical factors to calculate $PAC_{LC\text{-}MS/MS}$–specific threshold values for the ARR and FST, and then evaluated the performance of these threshold values by measuring both $PAC_{LC\text{-}MS/MS}$ and PAC_{RIA} in patients who had undergone FST in our center.

Subjects and Methods

Study design and participants

This study was approved by the Human Research Ethics Committees of the Princess Alexandra Hospital, the Greenslopes Private Hospital, and the University of Queensland (HREC/13/QPAH/229). Forty-one patients (19 males and 22 females; age range, 37 to 73 years) were recruited according to the following inclusion criteria: patients who were hypertensive with positive ARR screening results (PAC_{RIA}/DRC >70 pmol/mU) who were undergoing FST to definitely confirm or exclude PA as the cause of their hypertension (n = 33) and patients with previously confirmed unilateral PA who had undergone unilateral ADX (n = 8) and were completing postsurgical FST to determine whether PA had been biochemically (or clinically) cured (Supplemental Table 1) or residual disease was still present. For each FST study, four samples were collected (at 7:00 AM after overnight recumbency and 10:00 AM after 3 hours' upright posture on both the basal day and on day 4 of FST). Patients with severe, uncontrolled hypertension, heart failure, or impaired liver or renal function were excluded because of concerns about the risk of fluid overload associated with oral salt loading during FST. To permit meaningful analysis of the diagnostic performance of ARR and FST PAC cutoff values, only FST studies that yielded conclusive results (PA positive or PA negative) were included in this study. A detailed description of the FST procedure and definitions of positive and negative FST results are provided in Online Supplemental Data. Clinical characteristics of the study population are displayed in Table 1.

Routine clinical measurement of plasma PAC and DRC

Plasma PAC_{RIA} and DRC were measured routinely during FST in the laboratory of Pathology Queensland (PQ, Brisbane, Australia). PAC_{RIA} was determined by the Coat-a-Count™ aldosterone RIA kit (Diagnostic Products Corp.). The inter-assay coefficient of variations (CVs) was 11% at 164, 6% at 690, and 7.1% at 1409 pmol/L. The intra-assay CVs were 6.8% at 171, 4.9% at 705, and 5.9% at 1467 pmol/L. DRC was determined by the LIAISON® XL immunoanalyzer (DiaSorin, Italy) using a chemiluminescent immunoassay (CLIA) kit (DiaSorin, LIAISON® Direct Renin, Italy). The

Table 1. Clinical Characteristics of 41 Studied Patients

	FST Negative	FST Positive	FST Inconsistent	*P*
Demographics				
Number of patients[a]	8	29	4	—
Age, y	61 ± 9	56 ± 10	58 ± 6	0.295
Female, n (%)	4 (50.0)	16 (55.2)	2 (50.0)	0.955
BMI, kg/m^2 (IQR)	26.4 (24.6–29.8)	28.0 (25.9–31.8)	24.8 (22.4–31.6)	0.184
SBP, mm Hg (IQR)	126 (120–134)[b]	142 (128–146)[c]	133 (130–145)	0.044
DBP, mm Hg (IQR)	81 (73–95)	80 (76–88)	84.0 (73–98)	0.838
HR, beats/min (IQR)	65 (50–69)	64 (62–70)	67 (62–71)	0.669
Antihypertensives				
Verapamil SR, n (%)	5 (62.5)	23 (79.3)	4 (100.0)	0.320
Hydralazine, n (%)	1 (12.5)	17 (58.6)	2 (50.0)	0.069
Prazosin, n (%)	0	7 (24.1)	0	0.174
Moxonidine, n (%)	0	1 (3.4)	0	0.809
Diltiazem, n (%)	0	2 (6.9)	0	0.647
Total DDD (IQR)	0.63 (0.0–1.0)[b]	1.25 (0.9–2.7)[c]	1.13 (0.81–1.25)	0.047

Categorical data are displayed as number (percentage). The age distribution was compared by one-way ANOVA, numbers (percentages) by the χ^2 test, and medians by the Kruskal-Wallis and Mann-Whitney *U* tests. *P value in boldface type: $P < 0.05$.

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; DDD, defined daily dose (antihypertensive medications); HR, heart rate; IQR, interquartile range; SBP, systolic blood pressure; SR, slow release.

[a]Among the total 41 patients, 37 showed consistent FST results by the two aldosterone assays (LC-MS/MS and RIA) and their corresponding cutoffs, whereas four patients showed inconsistent FST results.

[b]$P < 0.05$ *vs* FST-positive group.

[c]$P < 0.05$ *vs* FST-negative group.

interassay CVs were 7.4% at 27 mU/L and 6.0% at 107 mU/L. The intra-assay CVs were 3.7% at 15, 2.0% at 82, and 1.2% at 258 mU/L. The functional sensitivities for the PAC_{RIA} and DRC assays reported by the manufacturer were 30 pmol/L and 2 mU/L, respectively. Among the total of 164 EDTA plasma samples collected from the 41 patients during FST (4 samples per FST), none was found to have PAC_{RIA} <30 pmol/L, whereas 76 (46.3%) samples were reported with DRC <2 mU/L.

LC-MS/MS–based quantification of serum PAC

For this evaluation study, gel-free serum was collected prospectively during FST and was stored at −20°C immediately after collection. All 164 FST serum samples underwent measurement of $PAC_{LC\text{-}MS/MS}$ in the laboratory of Attoquant Diagnostics (AD, Vienna, Austria). When ready for assay, samples (200 μL) were spiked with stable isotope-labeled internal standard for aldosterone at a concentration of 1387 pmol/L (500 pg/mL). Following C18-based solid-phase extraction and fractionated elution of aldosterone, samples were subjected to LC-MS/MS analysis using a reversed-phase analytical column (Acquity UPLC® C18, Waters) operating in line with a XEVO TQ-S triple quadrupole mass spectrometer (Waters Xevo TQ/S, Milford, MA) in multiple reaction monitoring mode. The internal standard was used to correct for analyte recovery across the sample preparation procedure in each sample. Analyte concentrations were calculated from integrated chromatograms considering the corresponding response factors determined in appropriate calibration curves in serum matrix, on condition that integrated signals exceeded a signal-to-noise ratio of 10. The functional sensitivity for $PAC_{LC\text{-}MS/MS}$ assay was 14 pmol/L, and there were only two (1.2%) serum samples from one postsurgical patient that were reported with $PAC_{LC\text{-}MS/MS}$ <14 pmol/L. At 50 pmol/L serum, the interassay and intra-assay CVs for PAC were 7.9% and 5.2%, respectively.

Choices of aldosterone assay-specific cutoffs for the ARR and FST used in this study

In our center, as previously reported (18), if PAC was measured by RIA, (1) the PA screening result was considered positive if the ARR_{RIA} (PAC_{RIA}/DRC) was >70 pmol/mU; and (2) PA was confirmed or excluded by showing positive or negative FST results, for which a cutoff PAC_{RIA} (measured in samples collected in the upright position at 10:00 AM on day 4 of FST) of 165 pmol/L was used. If PAC was measured by LC-MS/MS, based on a previous in-house comparison between ARR_{RIA} and $ARR_{LC\text{-}MS/MS}$ ($PAC_{LC\text{-}MS/MS}$/DRC) from 311 plasma samples in the PQ laboratory, we derived a regression equation of $ARR_{LC\text{-}MS/MS} = 0.81 \times ARR_{RIA} - 1.7$ ($r^2 = 0.92$) and accordingly adjusted the $ARR_{LC\text{-}MS/MS}$ screening cutoff to 55 pmol/mU.

To establish a diagnostic cutoff for FST $PAC_{LC\text{-}MS/MS}$, we first confirmed that the adjusted cutoff should be lower than that for PAC_{RIA} by deriving a regression equation for PAC. For the purpose of the current study, our previous assay comparison of $PAC_{LC\text{-}MS/MS}$ between the two laboratories (PQ's plasma $PAC_{LC\text{-}MS/MS}$ *vs* AD's serum $PAC_{LC\text{-}MS/MS}$) was undertaken using 124 pairs of plasma and serum samples and demonstrated excellent agreement (Supplemental Figure 1), in particular within a PAC range between 0 and 600 pmol/L, within which no potential outliers were present. Hence, in this study, an extended comparison between aldosterone LC-MS/MS and RIA results was carried out by combining the data derived from our previous in-house PQ's analysis (plasma PAC_{RIA} *vs* plasma $PAC_{LC\text{-}MS/MS}$, n = 311) described above for ARR and the current 164 pairs of FST samples in which plasma PAC_{RIA} was measured by PQ and serum $PAC_{LC\text{-}MS/MS}$ was analyzed by AD, giving a total of 475 results available for comparative analysis (Fig. 1). This yielded a regression equation of $PAC_{LC\text{-}MS/MS} = 0.82 \times PAC_{RIA} - 24.4$ ($r^2 = 0.85$). We then assessed the performance characteristics of a range of cutoffs approximating

that predicted by this regression equation (for PAC_{RIA} = 165 pmol/L, $PAC_{LC\text{-}MS/MS}$ = 111 pmol/L) among a larger FST database to ensure that the final selected cutoff was most relevant clinically. In particular, we sought to determine a cutoff that (1) would minimize the rate of false positives in patients who had clearly been cured of PA by unilateral ADX using the criteria established by the Primary Aldosteronism Surgical Outcomes study (19), and (2) minimize the rate of false negatives in patients with unequivocal unilateral PA, defined by lateralization on AVS. The reasoning for this approach was based on the notions that (1) the primary purpose for confirmatory testing is to identify among patients with raised ARR those who do not have PA and who therefore can be spared AVS, an invasive and costly procedure not without risk; (2) the main role of AVS is to identify those subjects with unilateral PA who are potentially curable by, and therefore candidates for, unilateral ADX; and therefore that (3) confirmatory testing should ideally accurately identify patients who do not have PA, but not miss those with unilateral, surgically correctable PA. Applying these clinical considerations, we found that a cutoff of 133 pmol/L correctly ruled out PA in all patients cured of PA by unilateral ADX and correctly identified PA in all patients who lateralized on AVS. We therefore chose 133 pmol/L as the FST $PAC_{LC\text{-}MS/MS}$ cutoff in this study.

Statistical analysis

SPSS 23.0 Statistics for Windows (IBM Corp., Armonk, NY) was used to analyze the data. Nonnormally distributed data are presented as median and interquartile range. Spearman nonparametric correlation and simple linear regression analysis were used to assess the relationship between PAC_{RIA} and $PAC_{LC\text{-}MS/MS}$. Bland-Altman analysis was used to evaluate the bias and agreement between two aldosterone assays by using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Independent samples Mann-Whitney *U* test was used to compare the data between the FST-negative and FST-positive groups. McNemar test was used to compare the difference in sensitivity and specificity of different threshold values. $P < 0.05$ was considered statistically significant. For statistical analysis, DRC and $PAC_{LC\text{-}MS/MS}$ below 2 mU/L and 14 pmol/L were rounded up to 2 mU/L and 14 pmol/L, respectively.

Results

Comparison of PAC_{RIA} and $PAC_{LC\text{-}MS/MS}$

Our previous in-house comparison between the two aldosterone assays (PQ, n = 311) revealed an ~28.0% higher estimation ($P < 0.01$) of median PAC by RIA (403.0 pmol/L; range, <30.0 to 2110.0) than by LC-MS/MS (290.0 pmol/L; range, <14.0 to 1873.0). The Spearman correlation and Pearson *r* coefficients between plasma PAC_{RIA} and $PAC_{LC\text{-}MS/MS}$ were 0.956 ($P < 0.01$) and 0.938 ($P < 0.01$), respectively. In the current study, aldosterone testing results from 164 pairs of plasma and serum samples collected during FST showed an ~27.8% higher estimation ($P < 0.01$) of median PAC by RIA (300.0 pmol/L; range, 40.0 to 2170.0) than by LC-MS/MS assay (216.7 pmol/L; range, <14.0 to 1277.0). The Spearman correlation and Pearson *r* coefficients between plasma PAC_{RIA} and serum $PAC_{LC\text{-}MS/MS}$ were 0.894 ($P < 0.01$) and 0.861 ($P < 0.01$), respectively.

Performance of two aldosterone threshold values for FST

If using FST day 4 upright PAC_{RIA} ≥165 pmol/L and $PAC_{LC\text{-}MS/MS}$ ≥133 pmol/L as PAC cutoffs, among the total of 41 patients, 37 (90.2%) demonstrated consistent

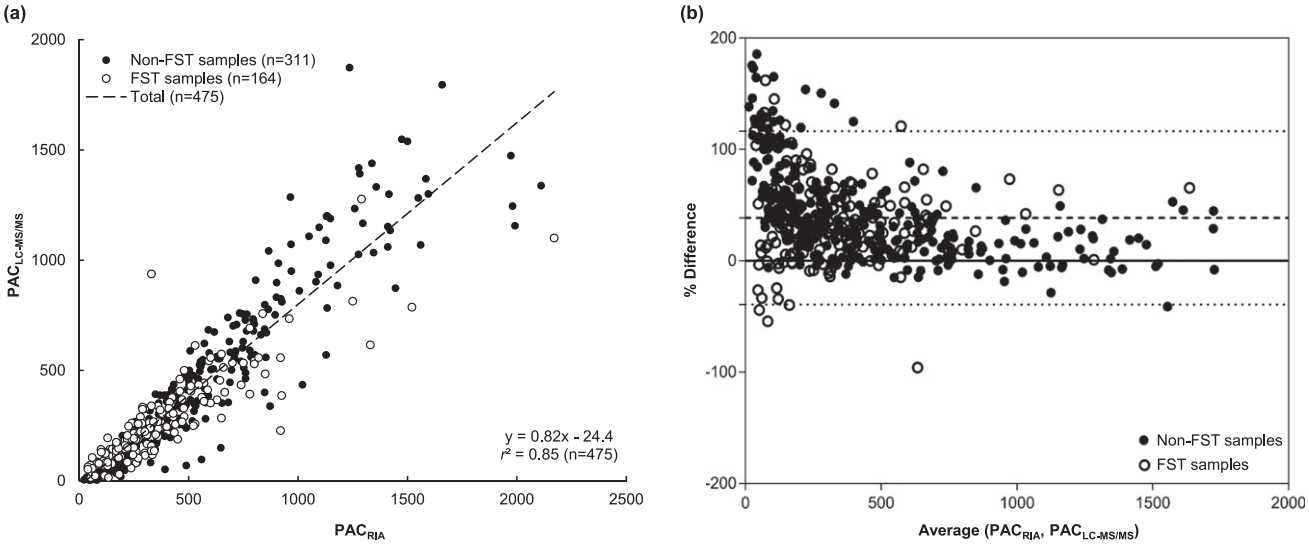

Figure 1. Aldosterone assay comparison using combined data from PQ and AD. Comparison between aldosterone RIA and LC-MS/MS assays, consisting of our previous in-house data from PQ (plasma PAC_{RIA} *vs* plasma $PAC_{LC\text{-}MS/MS}$, n = 311; solid circles) and current PAC data during FST (PQ's plasma PAC_{RIA} *vs* AD's serum $PAC_{LC\text{-}MS/MS}$, n = 164 pairs; open circles) yielded a total regression equation of $PAC_{LC\text{-}MS/MS} = 0.82 \times PAC_{RIA} - 24.4$ ($r^2 = 0.85$, n = 475). (a) Scatter plot showed a good linear relationship ($P < 0.01$) between PAC_{RIA} and $PAC_{LC\text{-}MS/MS}$, with a Spearman correlation coefficient of 0.937 ($P < 0.01$) and Pearson *r* coefficient of 0.921 ($P < 0.01$). (b) Bland-Altman analysis exhibited a bias (average percentage difference) of 38.5% (dashed line, SD = 39.7%) higher for RIA, with 95% limits of agreement from −39.3% to 116.4% (two dotted lines). No outliers were excluded (n = 475).

FST results (29 positive and 8 negative) by the two aldosterone assays, whereas four showed inconsistent results (Table 2). A good agreement (κ coefficient = 0.736, $P < 0.01$) and no difference ($P > 0.05$) in distinguishing positive and negative FSTs were observed between the two FST PAC_{RIA} and $PAC_{LC\text{-}MS/MS}$ cutoffs. Further comparison between patients who were FST-negative (n = 8, including 7 post-ADX) and patients who were FST-positive (n = 29, none post-ADX) showed that, as expected, patients who were FST-positive (PA) had higher ($P < 0.01$) levels of PAC and ARR than patients who were FST-negative (non-PA or PA cured) on both days 0 and 4 of FST, but in this study, the differences of DRC between two groups were not significant ($P > 0.05$) on both days (Table 3).

Performance of two ARR threshold values for screening PA

Among the 37 patients whose FST results were consistent, patients' ARR data at 10:00 AM on basal day (before FST) was used to examine the screening performance of two ARR threshold values. If using ARR_{RIA} >70 pmol/mU and $ARR_{LC\text{-}MS/MS}$ >55 pmol/mU as cutoffs, four (10.8%) patients showed false screening results, including two patients with PA (FST positive) who displayed both false-negative ARR_{RIA} and $ARR_{LC\text{-}MS/MS}$, one patient who was non-PA (FST-negative, none post-ADX) who displayed both false-positive ARR_{RIA} and $ARR_{LC\text{-}MS/MS}$, and one patient with PA who demonstrated correct positive $ARR_{LC\text{-}MS/MS}$ but false-negative ARR_{RIA}. False-negative rates for the two assay-specific ARR cutoffs were 10.3% (RIA) and 6.9% (LC-MS/MS), whereas false-positive rates for these two cutoffs were identical at 12.5%. The differences in sensitivity (89.7% for RIA *vs* 93.1% for LC-MS/MS) and specificity (87.5% for both assays) between the two assay-specific ARR cutoffs were not notable.

Discussion

Because ARR screening results can be falsely positive (20–25), confirmatory testing is necessary to definitively confirm or exclude the diagnosis of PA. Compared with other widely used confirmatory tests for PA, FST is regarded by our center to be the most sensitive and reliable (26–28). The basis of FST as a confirmatory test for PA involves demonstration of ongoing aldosterone secretion in the face of suppression of renin achieved by fludrocortisone administration and oral salt loading. As mentioned earlier, a reliable aldosterone assay is important for judging whether FST is positive or negative for PA; this hinges mostly on day 4 PAC levels. At present, PAC is most often measured by RIA (29) but can also be assayed by a faster and automated CLIA method (30, 31). However, concerns have been raised about the weaknesses of these immunometric techniques. First, the specificity of antibodies for RIA and CLIA varies between assays, which is likely from interfering substances in tested samples such as structurally related steroids (as well as their precursors and metabolites) that have the potential for cross-reactivity with the aldosterone assay antibody (13), thereby causing overestimation of PAC, which is especially problematic because the PAC is relatively low compared with many potentially interfering steroids. Second, inadequate standardization of assay procedures in different laboratories, poor interlaboratory reproducibility, and limited comparability of immunoassays remain problematic and impose barriers in defining a uniform cutoff for PA diagnostic workup (32). Schirpenbach *et al.* (33) compared four aldosterone immunoassays (in-house RIA after extraction and chromatography *vs* two commercial RIA kits without extraction *vs* automated CLIA) and reported that they gave markedly different results.

A major recent advance in aldosterone quantification has been the development of a highly accurate and reproducible mass spectrometric or LC-MS/MS method, which has been proven to be highly reliable within the clinically relevant range (34). The main advantages of the LC-MS/MS assay are high specificity and relatively rapid throughput (35, 36) while allowing for internal standardization by using stable isotopes, thereby normalizing for specific matrix effects and variations in analyte recovery during sample preparation, which are both features neglected by immunoassays. Unlike immunoassay,

Table 2. Four Patients' FST Results Inconsistent According to the Two Aldosterone Assays

				Day 0						Day 4		
Patient ID	ADX	SBP/DBP, mm Hg	DDD	PAC_{RIA}, pmol/L	$PAC_{LC\text{-}MS/MS}$, pmol/L	DRC, mU/L	ARR_{RIA}, pmol/mU	$ARR_{LC\text{-}MS/MS}$, pmol/mU	K^+, mmol/L	PAC_{RIA}, pmol/L	$PAC_{LC\text{-}MS/MS}$, pmol/L	Saline Infusion Testing
2	No	130/92	0.75	362.0	236.4	<2.0	181.0	118.2	3.5	246.0	117.1	Positive
5	No	155/100	1.25	593.0	543.1	11.0	53.9	49.4	3.6	237.0	57.4	Negative
15	No	136/76	1.25	290.0	324.0	2.0	145.0	162.0	3.4	100.0	141.0	—
18	Post	130/72	1.00	330.0	135.3	13.0	25.4	10.4	4.3	220.0	83.8	—

Abbreviation: DBP, diastolic blood pressure.

Table 3. Measured Parameter Comparison Between FST-Negative and FST-Positive Groups

	FST Negative (n = 8)	FST Positive (n = 29)	*P*
Day 0, upright			
PAC_{RIA}, pmol/L	190.0 (116.0–277.8)	460.0 (310.0–695.0)	0.01
$PAC_{LC\text{-}MS/MS}$, pmol/L	133.5 (70.4–240.5)	338.3 (239.7–521.6)	0.01
Renin, mU/L	4.5 (3.0–16.0)	3.0 (2.0–5.5)	NS
ARR_{RIA}, pmol/mU	40.9 (12.0–61.1)	140.0 (103.3–223.35)	0.01
$ARR_{LC\text{-}MS/MS}$, pmol/mU	23.8 (9.8–38.6)	100.0 (73.0–139.3)	0.01
Day 4, upright			
PAC_{RIA}, pmol/L	95.5 (63.3–127.5)	330.0 (220.0–610.0)	0.01
$PAC_{LC\text{-}MS/MS}$, pmol/L	72.2 (53.0–113.1)	226.9 (166.8–380.6)	0.01
Renin, mU/L	2.0 (2.0–6.5)	2.0 (2.0–3.0)	NS
ARR_{RIA}, pmol/mU	40.8 (14.4–63.8)	130.0 (88.4–199.5)	0.01
$ARR_{LC\text{-}MS/MS}$, pmol/mU	30.6 (10.9–41.0)	102.6 (69.8–139.5)	0.01

Abbreviation: NS, not significant ($P > 0.05$).

LC-MS/MS does not require a specific antibody to target the analyte and permits differentiation and measurement of aldosterone and other steroids together with their corresponding internal standards simultaneously in a single run from one sample, thereby providing more accurate and powerful clinical information (37). It could be anticipated that, for aldosterone testing, the gold method should be based on LC-MS/MS detection (38). However, the accessibility to LC-MS/MS equipment remains an obstacle to many clinical laboratories, and using LC-MS/MS also requires specific technical experience.

In the current study, our previous in-house aldosterone assay comparison (PQ, plasma PAC_{RIA} *vs* plasma $PAC_{LC\text{-}MS/MS}$, n = 311) and current extended comparison during FST (PQ's plasma PAC_{RIA} *vs* AD's serum $PAC_{LC\text{-}MS/MS}$, n = 164 pairs) both revealed a lower ($P < 0.01$) value of $PAC_{LC\text{-}MS/MS}$ than PAC_{RIA} (28.0% and 27.8% differences in the median, respectively), and yielded a total regression equation of $PAC_{LC\text{-}MS/MS} = 0.82 \times PAC_{RIA} - 24.4$ ($r^2 = 0.85$, n = 475). These results are in keeping with other recent similar studies. Juutilainen *et al.* (15) reported an average of 15% lower values of $PAC_{LC\text{-}MS/MS}$ than PAC_{RIA}, with an equation of $PAC_{LC\text{-}MS/MS} = 0.8 \times PAC_{RIA} + 18.3$ ($r^2 = 0.97$, n = 42). Hinchliffe *et al.* (38) reported an equation of $PAC_{LC\text{-}MS/MS} = 0.79 \times PAC_{RIA} - 41.7$ ($r^2 = 0.88$, n = 54). Van Der Gugten *et al.* (39) reported $PAC_{LC\text{-}MS/MS}$ to be a median of 12.2% lower than PAC_{RIA}, with an equation of $PAC_{LC\text{-}MS/MS} = 1.17 \times PAC_{RIA} - 63.9$ ($r^2 = 0.88$, n = 110). Taken together, our results confirmed again that PAC is lower if measured by LC-MS/MS than by RIA, presumably because of the higher specificity of LC-MS/MS.

How did we select the aldosterone LC-MS/MS assay-specific threshold values for PA screening (ARR) and confirmatory (FST) testing in this study? The proposed $ARR_{LC\text{-}MS/MS}$ cutoff value of 55 pmol/mU was deduced by applying above ARR regression equation (in which the current ARR_{RIA} cutoff value of 70 pmol/mU was used), derived from a direct comparison of ARR_{RIA} and $ARR_{LC\text{-}MS/MS}$ performed in-house (PQ, n = 311) when we established this aldosterone LC-MS/MS assay for routine use in our hospital. This $ARR_{LC\text{-}MS/MS}$ cutoff proved to be highly sensitive (93.1%), a major prerequisite of a screening test, and with reasonable specificity (87.5%). When determining the FST $PAC_{LC\text{-}MS/MS}$ threshold value, we were mindful that both sensitivity and specificity (more so than screening) are important considerations for confirmatory testing in PA, given the undesirable consequences of overdiagnosing PA (which may lead to patients needlessly undergoing AVS, which is costly, difficult and invasive) and missing unilateral PA (which is potentially curable by unilateral ADX). Additionally, given that many factors (*e.g.*, outliers, normality of data distribution) could affect the PAC equation and 95% confidence interval existed for the equation's slope (0.79 to 0.86) and intercept (−43.4 to −5.4), we therefore assessed a range of cutoffs and settled on one (133 pmol/L) that was slightly higher than that predicted directly by our PAC regression equation (111 pmol/L, at which two patients, including one patient who was post-ADX, with negative FST results by RIA would be misdiagnosed as having PA), but that differentiated without overlap patients with unequivocal unilateral PA from those who were unequivocally cured of PA by unilateral ADX.

In this study, a good agreement (κ coefficient = 0.736, $P < 0.01$) between PAC cutoff values of 165 (by RIA) and 133 (by LC-MS/MS) was observed in distinguishing FST-positive and FST-negative cases, with 37 patients showing consistent FST results. Importantly, among the remaining four patients with inconsistent FST results (Table 2) by the two aldosterone assays, one with normal potassium, unsuppressed renin, both negative ARR_{RIA} and $ARR_{LC\text{-}MS/MS}$, and negative SIT result showed positive FST by RIA but negative FST by LC-MS/MS; one with hypokalemia, suppressed renin, and both positive ARR_{RIA} and $ARR_{LC\text{-}MS/MS}$ before FST demonstrated

negative FST by RIA but positive FST by LC-MS/MS; and one patient who was post-ADX with evidence of complete biochemical cure (normal potassium, unsuppressed renin, both negative ARR_{RIA} and $ARR_{LC\text{-}MS/MS}$ before FST) and partial clinical cure (improved blood pressure with reduced antihypertensive medications) of PA by unilateral ADX showed positive FST by RIA but negative FST by LC-MS/MS. Hence, in these three subjects, the FST diagnosis using $PAC_{LC\text{-}MS/MS}$ appeared to more likely to be correct (based on other clinical characteristics of these patients) than that by PAC_{RIA}. However, the fourth patient, who displayed normal potassium, suppressed renin, both positive ARR_{RIA} and $ARR_{LC\text{-}MS/MS}$, and a positive SIT result, exhibited positive FST by RIA but negative FST by LC-MS/MS (day 4 upright $PAC_{LC\text{-}MS/MS}$ = 117.1 pmol/L).

Although the ARR has been recommended by guidelines (11) as the most reliable approach for PA screening, its cutoff is not standardized among different laboratories and clinical centers, mainly because of lack of uniformity in assay methods and in the units used for reporting aldosterone (ng/dL or pmol/L for PAC) and renin (ng/mL/h or pmol/L/min for PRA; ng/L or mU/L for DRC). It is likely that this variability will diminish with time as more laboratories change from PRA to DRC and adopt the Systeme Internationale method of reporting PAC and DRC. Hopefully, the use of LC-MS/MS will further facilitate the standardization of aldosterone assay approaches and measured values. Indeed, in the current study, a comparison of $PAC_{LC\text{-}MS/MS}$ values measured by two different laboratories (PQ, AD) showed excellent agreement with an acceptable bias of 4.7% (or 1.0% if focusing on a PAC range between 0 and 600 pmol/L) (Supplemental Figure 1). Currently, only a few studies have reported data on determining ARR screening cutoff by using $PAC_{LC\text{-}MS/MS}$. Juutilainen *et al.* (15) recommended a cutoff value of 44 pmol/ng (sensitivity = 100%, specificity = 84%) derived from receiver operating characteristic (ROC) curve analysis, which equates to 27.7 pmol/mU and is therefore much lower than our figure of 55 pmol/mU. However, antihypertensive medications, which are reported to affect the ARR, were used by >70% of the patients in Juutilainen's study. In our study, the estimates of sensitivity and specificity for the ARR_{RIA} cutoff value of 70 pmol/mU (89.7%, 87.5%) and the $ARR_{LC\text{-}MS/MS}$ cutoff value of 55 pmol/mU (93.1%, 87.5%) were similar ($P > 0.05$), indicating the potential usefulness of this proposed cutoff value of 55 if $PAC_{LC\text{-}MS/MS}$ is measured.

Strengths of this study are that (1) PA diagnosis was confirmed by using FST; (2) potentially interfering factors (including antihypertensive medications, salt intake, hypokalemia, and time of day for sampling) to renin and aldosterone levels were all controlled and standardized before and during testing, which therefore improved the accuracy of the day 0 ARR results for PA screening; and (3) that this study attempted to validate an aldosterone LC-MS/MS assay-specific cutoff for FST. However, this study is not without limitations. No healthy (normotensive) subjects (other than those cured of hypertension post-ADX) and no patients who were hypertensive with consistently negative ARR were included in this study because FST requires 5 days of hospitalization, rendering it unfeasible to undertake in patients for whom the test is not clinically indicated. Among the 41 total participants, 10 (including all 8 patients who were post-ADX and 2 patients who were FST-positive) showed negative ARR screening results on day 0 by the two aldosterone assays, and one patient who was FST-positive showed negative ARR_{RIA} but positive $ARR_{LC\text{-}MS/MS}$ results. Because of the relatively low number of patients with raised ARR who ended up with negative FST results, we elected to include patients who were post-ADX as a means of expanding the FST-negative cohort. In the current study, of eight patients who were FST-negative (confirmed by the two assays), seven had been previously diagnosed with unilateral PA and had undergone unilateral ADX, leading to the complete biochemical (or clinical) cure of PA (Supplemental Table 1). This probably explains why there was no difference in DRC on day 0 between the FST-positive and FST-negative groups because the RAS activity of these patients who were post-ADX may not have fully recovered from its chronically suppressed state. The difference in sample matrix may also raise concerns. In this study, EDTA plasma was used for measuring PAC_{RIA}, whereas serum was used to measure $PAC_{LC\text{-}MS/MS}$; however, Taylor *et al.* (16) and Van Der Gugten *et al.* (39) reported that the aldosterone assay results were not affected significantly by these specimen types. Finally, although we previously calculated an optimal $ARR_{LC\text{-}MS/MS}$ screening cutoff value of 52.4 pmol/mU using ROC curve analysis (40), the currently proposed $ARR_{LC\text{-}MS/MS}$ and FST $PAC_{LC\text{-}MS/MS}$ cutoffs were derived from the comparison results between PAC_{RIA} and $PAC_{LC\text{-}MS/MS}$ rather than from ROC curve analysis. Therefore, further studies involving larger populations and healthy controls and patients with essential hypertension are required to further validate our recommended LC-MS/MS cutoffs. Furthermore, because this analysis was limited to FST, additional studies are necessary to establish and validate cutoff $PAC_{LC\text{-}MS/MS}$ values for other commonly used confirmatory tests (including SIT).

Conclusions

In summary, our results provide preliminary evidence supporting the satisfactory performance of proposed

cutoffs for PA diagnostic workup specific for PAC measurement by LC-MS/MS. There was no difference in sensitivity or specificity between the current ARR_{RIA} cutoff value of 70 pmol/mU and the proposed $ARR_{LC\text{-}MS/MS}$ cutoff value of 55 pmol/mU in PA screening or between the current FST day 4 PAC_{RIA} cutoff value of 165 pmol/L and proposed $PAC_{LC\text{-}MS/MS}$ cutoff value of 133 pmol/L in PA definitive testing by FST. These data emphasize the need for adjustment of current threshold values for PA screening and confirmatory testing if aldosterone is measured by LC-MS/MS.

Acknowledgments

Financial Support: This work was funded by the Irene Patricia Hunt Memorial Hypertension Research Fund (to Z.G., A.A., M.W., and M.S.). Z.G. is supported by the International Postgraduate Research Scholarship and University of Queensland Centennial Scholarship from the University of Queensland.

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Disclosure Summary: M.P. is the director of Attoquant Diagnostics GmbH. The remaining authors have nothing to disclose.

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