Renin-Angiotensin-Aldosterone System Triple-A Analysis for the Screening of Primary Aldosteronism

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Abstract—Primary aldosteronism is recognized as the most frequent cause of secondary hypertension, and its screening is expected to become a routine evaluation in most patients with hypertension. The interference of antihypertensive therapies with the aldosterone-to-renin ratio during screening process is a major confounder. Renin-angiotensin-aldosterone system Triple-A analysis is a novel liquid chromatography/tandem mass spectrometry diagnostic assay that allows simultaneous quantification of aldosterone, equilibrium Ang I (angiotensin I), and Equilibrium Ang II in a single sample of serum. We performed a comparative evaluation of the diagnostic performance of the aldosterone-to-Ang II ratio and 5 renin-based diagnostic ratios, differing in methods to determine aldosterone levels and renin activity in a cohort of 110 patients with hypertension (33 patients with confirmed primary aldosteronism and 77 with essential hypertension). All ratios showed comparable areas under the curves ranging between 0.924 and 0.970 without significant differences between each other. The evaluation of the Ang II-to-Ang I ratio revealed persistent drug intake in some patients as cause for suppressed renin-based diagnostic ratios, while aldosterone-to-Ang II ratio remained unaffected. The Youden index optimal cutoff value for the aldosterone-to-Ang II ratio was 6.6 ([pmol/L]/[pmol/L]) with a sensitivity of 90% and a specificity of 93%, proving noninferiority compared with the aldosterone-to-renin ratio while pointing to the potential for an interference-free application in patients under ACE (angiotensin-converting enzyme) inhibitor therapy. This study shows for the first time the accuracy and reliability of renin-angiotensin-aldosterone system triple-A analysis for the screening of primary aldosteronism that can be applied in clinical routine. **(*Hypertension*. 2020;75:163-172. DOI: 10.1161/HYPERTENSIONAHA.119.13772.)** ● **Online Data Supplement**

Key Words: adrenal glands ■ aldosterone ■ angiotensin II ■ mass spectrometry ■ renin-angiotensin system

Primary aldosteronism (PA) is a frequent form of secondary hypertension[1,2] that is caused by inappropriate aldosterone production for the renin status. Patients with PA, more often than patients with essential hypertension (EH), display cardiovascular and metabolic complications, such as stroke, myocardial infarction, heart failure, atrial fibrillation, metabolic syndrome, type 2 diabetes mellitus, and increased renal damage.[3,4] However, an early diagnosis and correct subtypes distinction allow establishing correct therapies that result in cure or control of the disease and thus revert the cardiovascular risk excess.[5] Therefore, the current guideline suggests extensive screening for PA in all the categories of patients with increased risk of this disease.[1] Unfortunately, guidelines are often not applied by general practitioners, one of the reasons being the difficulty of performing the screening under not-interfering therapy.[6]

The suggested screening test is the aldosterone-to-renin (measured as direct renin concentration [DRC] or plasma renin activity [PRA]) ratio (ARR)[1]; ideally, patients should be tested before the beginning of antihypertensive therapy, but this is often unfeasible because high blood pressure levels require immediate treatment or because the patient is seen by a specialist when is already under interfering antihypertensive therapy. α-1-adrenergic receptor blockers and nondihydropyridine calcium channel blocker, which display minimal or no effect on the ARR, are used to controlled hypertension during the screening and subsequent diagnostic work-up.[1] However, in some patients, the withdrawal of other antihypertensive drugs is considered unsafe, for example, in patients with previous myocardial infarction.[7]

AGT (Angiotensinogen), the precursor hormone of angiotensins, is primarily secreted by the liver and serves as a prehormone for all angiotensin metabolites. Ang 1–10, Ang I is produced from AGT by the enzymatic action of renin. The levels of active renin and of AGT contribute to the Ang I formation, also referred to as PRA. Ang I serves as a substrate to multiple plasma enzymes converting it to metabolites, including Ang 1–8, Ang II, the major effector molecule of the renin-angiotensin-aldosterone system (RAAS). These biochemical features build up the basis for a novel approach for the evaluation of the RAAS. The underlying principle is based on the condition that all components required for

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in vivo angiotensin metabolite formation are present in the plasma. Using a controlled ex vivo equilibration procedure followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) based angiotensin quantification, RAAS equilibrium analysis can be used to generate angiotensin metabolite profiles that reflect biochemical features of the circulating RAAS.[8–10]

The assessment of the RAAS has been claimed to be useful not only to diagnose secondary form of hypertension including PA and other rare genetic conditions[11] but also to guide antihypertensive therapy in patients with EH.[12,13]

Here, we introduce and validate a novel diagnostic test for application in patients with hypertension: RAAS triple-A testing is based on the LC-MS/MS quantification of equilibrium Ang I (eqAng I), equilibrium Ang II (eqAng II), and aldosterone in clinical samples and allows for stratification of hypertensive patients in terms of RAAS activity (sum of Ang I and Ang II, PRA-S), ACE (angiotensin-converting enzyme) activity (Ang II-to-Ang I ratio, ACE-S), and PA screening (aldosterone-to-Ang II ratio [AA2R]).

Methods

The authors declare that all supporting data are available within the article and its online-only Data Supplement files.

Patients Selection

We prospectively recruited 110 hypertensive patients with suspected PA referred to the Hypertension Unit of the University of Torino from April 2014 to January 2015. Of the 110 patients, 79 were included in the previous RENATO study (Renin and Aldosterone Measurements in Hypertensive patients in Torino).[14] Patients underwent to screening and confirmatory/exclusion testing and subtype diagnosis (including adrenal vein sampling) according to the Endocrine Society Guideline,[1] as previously described.[14] Briefly, patients were screened for PA after withdrawal of interfering medications. All antihypertensive drugs were stopped at least 3 weeks before the screening test; diuretics and spironolactone were stopped at least 6 and 8 weeks before measurements, respectively; when it was not possible to stop all antihypertensive drugs, patients received the α-blocker doxazosin and the nondihydropiridine calcium channel blocker verapamil. The cutoff levels for a positive screening test were an ARR of 30 (ng/dL·ng·mL·h; 832.2 pmol/L·ng·mL·h), together with an aldosterone concentration ≥10 ng/dL (277.4 pmol/L). Patients with a positive screening test underwent confirmatory testing. The confirmatory saline infusion test (N=33) consisted of an intravenous saline load (2 L of 0.9% NaCl infused over 4 hours) that was carried in seated position[15] and was considered positive if post-test aldosterone levels were higher than 5 ng/dL (138.7 pmol/L). For patients undergoing captopril test (N=8), PA was considered confirmed when the ARR was higher than 30 (ng/dL·ng·mL·h; 832.2 pmol/L·ng·mL·h) 120' after captopril 50 mg. The approval for the RENATO-II study was obtained by the local Ethics Committees and fully informed written consent was signed by all patients.

Biochemical Measurements

For the hypertensive cohort, a sample of serum for each screened patient was sent to Attoquant Diagnostics GmbH laboratory in Vienna for Ang I, Ang II, PRA, and aldosterone measurements with LC-MS/MS. The samples were collected at the time of screening, in the morning after patients had been out of bed for at least 2 hours and then been seated for at least 15 minutes before venipuncture; blood was collected at room temperature into anticoagulant-free tubes, centrifuged (3.000 rpm for 15 minutes at 27°C–28°C), frozen at −20°C and never thawed before analysis. Details on biochemical analyses are reported in the online-only Data Supplement (Methods in the online-only Data Supplement).

Novel RAAS Activity Markers and Diagnostic Ratios

For each patient, the determination of PRA on the basis of Ang I formation, as well as RAAS triple-A testing (Ang I, Ang II, and Aldosterone), was performed followed by calculation of the diagnostic ratios investigated. Recently described novel angiotensin-derived markers for PRA (PRA-S, [eqAng I + eqAng II], and ACE activity [ACE-S]: eqAng II/eqAng I)[8] were calculated and compared between cohorts. Different diagnostic ratios between aldosterone and RAAS activity markers were compared in terms of diagnostic performance in PA screening: 3 variants of the classic ARR using PRA as denominator were compared by combining aldosterone and PRA values either determined by radioimmunoassay in the course of RENATO-I, or by LC-MS/MS (M) following re-analysis of stored serum samples in the course of RENATO-II study. The concentration of active renin (DRC) was determined in the course of RENATO-I study,[14] using a chemiluminescence-based immunoassay (CLIA, C). ARR (R/R) indicates the ratio initially determined on the basis of radioimmunoassay measurements for PRA and aldosterone. As recent data suggested that LC-MS/MS-based aldosterone quantification is more accurate than antibody-based detection methods, we further calculated an ARR form LC-MS/MS-based aldosterone values and radioimmunoassay PRA values, which was termed ARR (M/R). As a third ARR, we combined LC-MS/MS-based aldosterone levels with LC-MS/MS-based PRA measurements, which we termed ARR (M/M). Combining LC-MS/MS-based aldosterone levels with CLIA based active renin levels, we obtained a ratio termed ARR (M/C). Moreover, 2 novel angiotensin based diagnostic ratios were introduced and compared in terms of diagnostic performance in the given cohort. The ARR-S using the angiotensin-based PRA marker PRA-S as denominator, and the AA2R, which uses eqAng II directly as the denominator of the diagnostic ratio for PA.

We retrospectively analyzed screening samples from RENATO-I in a blinded manner and compared the results with historical data obtained from radioimmunoassay-based previous measurements of aldosterone and PRA, and CLIA based measurement of DRC. Moreover, different methods for aldosterone quantification and RAAS activity assessment were compared directly.

Statistics

Prism 6 was used as a software tool for statistical analyses. Data were expressed as median (25th to 75th percentile) or mean ± SD and analyzed by Mann-Whitney or Student *t* test, when appropriate. Categorical variables were compared through a χ^2 test (Fisher exact test when sample size was ≤5). Diagnostic ratios and their denominators were compared through correlation analysis (Spearman *R* correlation test) and Bland-Altman plot to assess the within-patient relationship and detect systematic error, proportional error, or a magnitude dependent bias. To assess the diagnostic accuracy of ARR, ARR-S, and AA2R for PA diagnosis, we used receiver operator characteristics (ROC) curves. ROC curves were compared by the area under the curve: a value of *z* above the critical level of 1.96 was used to accept the hypothesis that 2 areas were different. The Youden index was used to determine the optimal diagnostic thresholds for PA diagnosis.

Results

Description of the Population

The characteristics of the hypertensive cohort of our study are reported in Table 1. The mean age of the 77 patients (43 males and 34 females) with EH and of the 33 patients with PA (17 males and 16 females) was not significantly different. Systolic blood pressure and aldosterone concentration were significantly higher, whereas potassium levels, PRA, DRC, PRA-S, and eqAng II were significantly lower in patients with PA compared with patients with EH ($P<0.001$ for all comparison except DRC, with a P of 0.03). ACE-S revealed a significant

Table 1. Cohort Characteristics

Variable	EH (n=77)	PA (n=33)	*P* Value
Sex (male/female)	43/34	17/16	0.68
Age, y	48±10.7	52±9.2	0.06
SBP, mm Hg	145±15.8	156±19.7	0.01
DBP, mm Hg	92±9.1	95±13.2	0.23
K, mEq/L	4.1±0.5	3.5±0.6	<0.001
PRA (R), (ng·Ang I/mL)/h	0.9 (0.4–2.0)	0.1 (0.1–0.3)	<0.001
DRC (C), mU/L	20.3 (10.3–33.1)	3.8 (2.9–7.4)	0.03
PRA (M), (ng·Ang I/mL)/h	0.5 (0.2–1.2)	0.1 (0.1–0.2)	<0.001
PRA-S (eqAng I + eqAng II), pmol/L	165.6 (80.6–328.3)	40.5 (18.2–57.5)	<0.001
eqAng II, pmol/L	100.9 (56.3–227.0)	24.2 (13.3–42.0)	<0.001
Aldosterone (R), pmol/L	385.6 (246.9–590.9)	693.6 (507.7–885.0)	<0.001
Aldosterone (M), pmol/L	186.2 (102.7–293.2)	391.5 (331.1–486.3)	<0.001
ACE-S, (pmol/L)/(pmol/L)	2.6 (1.9–3.4)	3.5 (2.7–5.3)	<0.001
ARR (R/R), ng/dL/([ng/mL]/h)	17.4 (7.6–31.5)	178.5 (96.5–239.0)	<0.001
ARR (M/R), ng/dL/([ng/mL]/h)	8.7 (3.5–16.8)	98.3 (46.8–136.5)	<0.001
ARR (M/C), (ng/dL)/(mU/L)	0.4 (0.2–0.8)	3.9 (2.1–5.0)	<0.001
ARR (M/M), (ng/dL)/([ng/mL]/h)	12.4 (5.8–23.4)	126.3 (81.2–281.5)	<0.001
ARR-S, (pmol/L)/(pmol/L)	1.2 (0.5–2.0)	10.7 (7.0–22.9)	<0.001
AA2R, (pmol/L)/(pmol/L)	1.8 (0.7–3.1)	15.6 (9.5–43.1)	<0.001

Means ± SD, or medians (interquartile range) are given for indicated variables, when appropriate. Corresponding units are shown in as unit. EH (n=77) and PA (n=33) patients are statistically compared. *P* values are shown on the right. AA2R indicates aldosterone-to-Ang IIratio; ACE, angiotensin-converting enzyme; ARR, aldosterone-to-renin ratio; C, chemiluminescence-based immunoassay; DBP, diastolic blood pressure; DRC, direct renin concentration; EH, essential hypertensive; M, liquid chromatography-tandem mass spectrometry; PA, primary aldosteronism; PRA, plasma renin activity; R, radioimmunoassay; RAAS, renin-angiotensin-aldosterone system; -S, RAAS triple-A derived marker; and SBP, systolic blood pressure.

increase in patients with PA compared with patients with EH. Across all patient subcohorts, radioimmunoassay-based measurements resulted in significantly higher median values for PRA as well as aldosterone when compared with data obtained from LC-MS/MS analysis. All 6 diagnostic ratios investigated (ARR [R/R], ARR [M/R], ARR [M/C], ARR [M/M], ARR-S, and AA2R) were significantly higher in patients with PA compared with patients with EH ($P<0.001$). Among patients with PA, 23 were diagnosed with bilateral PA, whereas 10 had an aldosterone-producing adenoma. Except for blood pressure, potassium, and radioimmunoassay-based aldosterone levels, none of the molecular markers investigated was significantly different between patients with bilateral PA versus aldosterone-producing adenoma (Table S1 in the online-only Data Supplement).

Comparative Analysis of PA Screening Performance for Different Diagnostic Ratios (ROC Analysis)

Diagnostic ratios for PA were calculated using 5 different denominators (PRA [radioimmunoassay], DRC (CLIA), PRA (MS), PRA-S, and eqAng II) and 2 different numerators (aldosterone [radioimmunoassay] and aldosterone [MS]) in 6 combinations: ARR (R/R), ARR (M/R), ARR (M/C), ARR (M/M), ARR-S, and AA2R, as shown in Table 2, together with corresponding units. ROC analysis was performed for each individual ratio considering the final diagnosis obtained in RENATO-I study, as gold standard. All 6 diagnostic ratios showed comparable area under the curves ranging between 0.924 and 0.970 without significant differences between each other (Figure 1). ARR ratios involving PRA values measured by radioimmunoassay but differently measured aldosterone levels rapidly reached 100% sensitivity (Figure 1A and 1B), while the ARR (M/C), calculated on basis of the active renin concentration measured by CLIA, showed a small number of persisting false negatives (Figure 1C). Ratios involving RAAS denominators obtained in sample re-analysis revealed minimal differences in terms of false-negative and false-positive rates without significantly changing the area under the curves (Figure 1D through 1F). Seeking to get behind the cause of this observation, we decided to investigate the correlations between the ARR (M/R) and the ARR (M/C) with the AA2R as well as the correlation between different denominators and numerators in more detail.

In-Depth Analysis of Correlations Between ARR (M/R and M/C) and the AA2R

ARR (M/R) and ARR (M/C) both showed a good correlation with the AA2R with a Spearman *R* of 0.86 and 0.75, respectively (Figure 2A and 2D). In both correlation analysis, patient samples showing suppressed ACE-S, which were identified in PRA/ACE-S blots (Figure 2C and 2F), were labeled in red. When stratifying patients according to their ACE-S value in

Table 2. Diagnostic Performance and Cutoff Values

Diagnostic Ratio			Cutoff	YI	FP	FN	Sensitivity, %	Specificity, %
ARR (R/R)=	Aldosterone (RIA)	ng/dL	62.1	0.82	10	1	97	86
	PRA (RIA)	(ng/mL)/h	48.0	0.81	14	0	100	81
ARR (M/R)=	Aldosterone (MS)	ng/dL	28.1	0.86	11	1	100	86
	PRA (RIA)	(ng/mL)/h	23.0	0.81	14	0	100	81
ARR (M/C)=	Aldosterone (MS)	ng/dL	1.3	0.81	4	5	87	94
	DRC (CLIA)	mU/L	0.9	0.71	13	4	90	81
ARR (M/M)=	Aldosterone (MS)	ng/dL	61.1	0.87	3	4	90	97
	PRA (MS)	(ng/mL)/h	30.6	0.75	15	3	93	81
ARR-S=	Aldosterone (MS)	pmol/L	5.1	0.86	4	4	90	96
	Ang I + Ang II (MS)	pmol/L	2.6	0.78	15	2	97	81
AA2R=	Aldosterone (MS)	pmol/L	6.6	0.83	8	4	90	93
	Ang II (MS)	pmol/L	3.4	0.78	17	1	97	81
Aldosterone (MS)		ng/dL	9.8	0.61	21	4	90	71
			13.0	0.41	13	14	60	81

YI, number of FP, number of FN, sensitivity, and specificity for 6 diagnostic ratios for PA and for aldosterone are shown for indicated cutoff values obtained from ROC analysis of patients with ACE-S >1.0 (pmol/L)/(pmol/L) (n=100).The higher cutoff indicates the value corresponding to the maximum of the YI curve shown in Figure S5. To allow for direct comparability between different ratios, the lower cutoff value was set to match the specificity of the ARR (R/R) as a comparator (specificity =81%). AA2R indicates aldosterone-to-Ang II ratio; ACE, angiotensin-converting enzyme; ARR, aldosterone-to-renin ratio; C, chemiluminescence-based immunoassay; DRC, direct renin concentration; FN, false negative; FP, false positive; M, liquid chromatography-tandem mass spectrometry; PA, primary aldosteronism; PRA, plasma renin activity; R, radioimmunoassay; RAAS, renin-angiotensin-aldosterone system; ROC, receiver operator characteristic; -S, RAAS triple-A derived marker; and YI, Youden index.

2 groups of 100 and 10 patients respectively, a significant suppression of each ARR (M/R and M/C) compared with the AA2R was observed in patients with suppressed ACE activity (Figure 2B and 2E) with *P* values of 0.015 and 0.006, respectively. This effect became particularly evident when analyzing the correlation between ARR (M/M) and the AA2R along with ACE-S and median adjusted Bland-Altman analysis for these 2 diagnostic ratios (Figure S1), which are exclusively composed of LC-MS/MS-based aldosterone, PRA, and eqAng II values obtained during RENATO-II. Investigating patient records of these individuals revealed that 9 of the 10 patients with ACE-S <1 (pmol/L)/(pmol/L) received ACE inhibitors before drug withdrawal for PA screening. Ultra-high-performance liquid chromatography (MS/MS) based quantification of drug levels[16] further revealed that ramipril was present in these 9 samples at pharmacologically active concentrations ranging between 5.7 and 35 ng/mL, indicating noncompliance with the withdrawal procedure. The one remaining sample did not contain detectable concentrations of ramipril. Both, eqAng I and eqAng II were below the lower limit of quantification in this sample, causing an artificial suppression of ACE-S by calculation with indiscrete values eqAng I and eqAng II, indicating that ACE inhibition was correctly detected in 100% patients with detectable angiotensin levels.

Correlation Analysis of Different RAAS Denominators Confirms Suppressed ACE to be the Cause of Suppression of Renin-Based Diagnostic Ratios

We further analyzed the correlation of the denominators PRA (radioimmunoassay) and DRC (CLIA) and eqAng II with each other (Figure 3), which revealed a reduced recovery of eqAng II from renin in red highlighted patient samples (Figure 3A and 3B), while highlighted samples were evenly distributed when comparing PRA and DRC (Figure 3C). To normalize for differences in absolute values for PRA, DRC, and eqAng II and allow for subsequent Bland-Altman analysis, calculated individual values for the denominators were adjusted to obtain a similar median compared with the denominator to be compared with. Previous observations suggesting a specific bias for samples with suppressed ACE activity were confirmed by median adjusted Bland-Altman analysis, showing an accumulation of these samples at high overestimation biases for PRA as well as DRC (Figure 3D and 3E) while the median adjusted Bland-Altman comparison between PRA and DRC did not reveal a specific bias for samples with low-ACE activity (Figure 3F).

Correlation and Bland-Altman analyses revealed a generally weaker correlation at low denominator values as indicated by lower Spearman *R* coefficients (Table S2) and a broader 95% confidence band shown in all graphs (Figure 3A through 3C; Figure S2A through S2F). Of note, comparison of LC-MS/MS-based denominators reflecting PRA (PRA [MS] and PRA-S) with eqAng II revealed the clearest separation of low-ACE samples from a very close correlation line (Figure S2E and S2F), suggesting a high robustness of LC-MS/MS-based denominator assays, which was further supported by Bland-Altman analysis (Figure S3C, S3E, and S3F) and in particular by correlation analysis at low renin values (Table S2). Comparing the technical variability between radioimmunoassay (RENATO-I) and LC-MS/MS (RENATO-II) on the basis of aldosterone and PRA assays by correlation and Bland-Altman analysis revealed an overestimation of radioimmunoassay-based measurements of 49.3% and 68.2% for

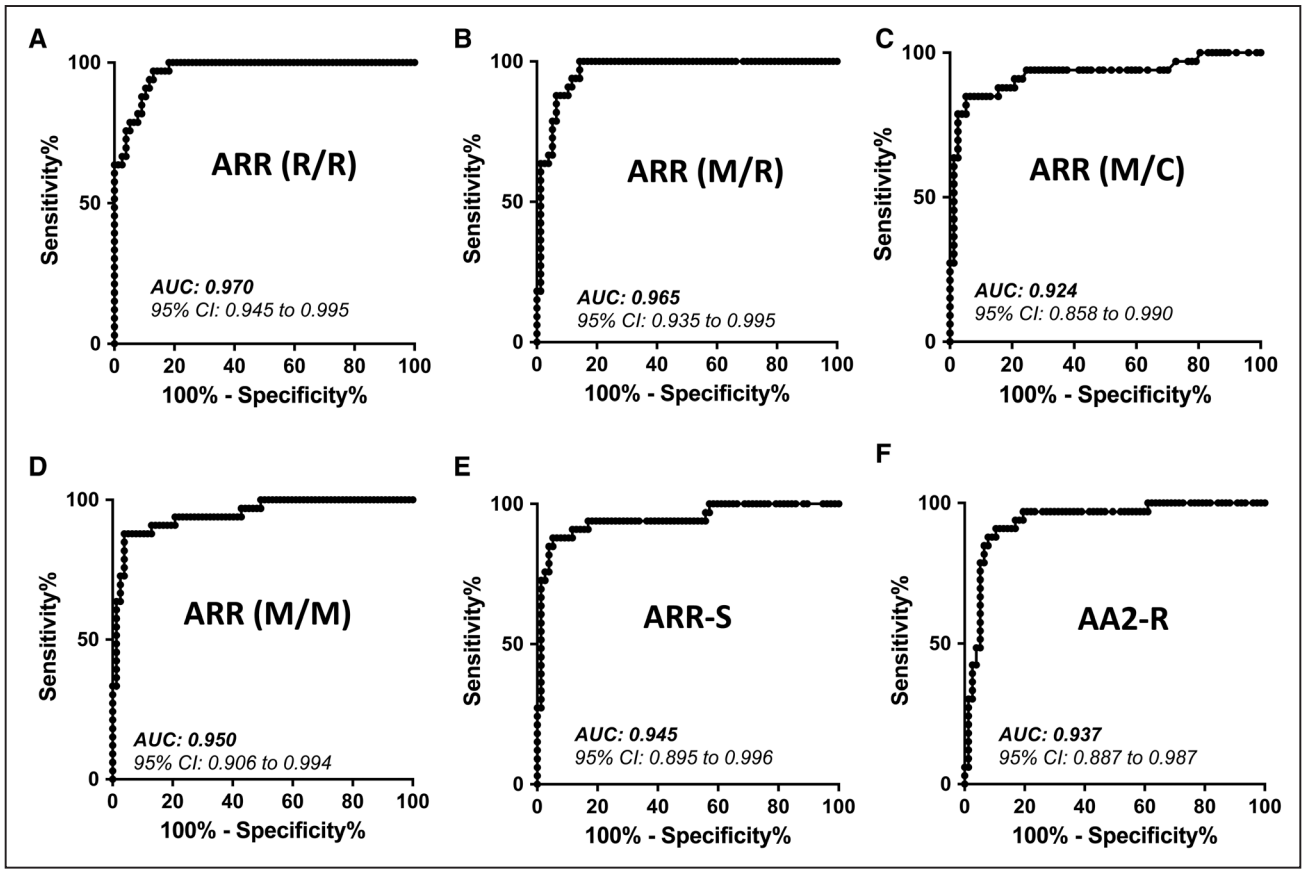

Figure 1. Comparative analysis of primary aldosteronism (PA) screening performance. Receiver operator characteristics (ROC) curves for individual diagnostic ratios are shown. ARR indicates aldosterone-to-renin ratio (**A**-**E**); AA2R, aldosterone-to-Ang II ratio (**F**); AUC, area under the curve; C, chemiluminescence based immunoassay; M, liquid chromatography-tandem mass spectrometry; R, radioimmunoassay; RAAS, renin-angiotensin-aldosterone system; and -S, RAAS triple-A derived marker.

PRA and aldosterone, respectively (Figure S4). As a result of our observations identifying unexpected ACE suppression at the time of PA screening to be the cause of potential differences between the AA2R and renin-based diagnostic ratios in screening procedures, we decided to repeat the ROC analysis after exclusion of these 10 patients with confounded ARR values potentially interfering with ROC analysis to see whether these qualitative differences persist.

Exclusion of Low-ACE Patients From ROC Evaluation Results in Improved Curve Shape for the AA2R

We replotted the ROC curves for all 6 diagnostic ratios following exclusion of patients with ACE-S <1 ([pmol/L]/[pmol/L]; Figure S5). As shown by the overlay graphs in Figure 4 comparing ROC curves before and after excluding low-ACE patients from the ROC evaluation, the minor discrepancies observed for the AA2R disappear when excluding low-ACE patients, while the ROC curves for ARR (M/R), ARR (M/C) and ARR-S remain unaffected. The optimal diagnostic cutoff values for PA screening were determined for all 6 ratios based on the Youden index analyzed in the cohort excluding low-ACE patients, consisting of 71 patients with EH and 29 patients with PA (Figure S6). The optimal cutoff for the AA2R was 6.6 ([pmol/L]/[pmol/L]), corresponding to a sensitivity of 90% and a specificity of 93% (Youden index: 0.83). Table 2 shows sensitivity and specificity for indicated cutoff values and number of false-positives and false-negative screening results are shown for each diagnostic ratio investigated and for aldosterone without relating to an RAAS denominator. Ratios involving LC-MS/MS-based denominators generally showed a lower number of false-positive screening results and a higher number of false-negative screening results. Aiming to allow for direct comparability between different screening approaches, we set a second cutoff value to match the specificity of the ARR (R/R) as a comparator (specificity =81%) and compared the resulting sensitivity. While ARR (R/R) and ARR (M/R) showed 100% sensitivity, ARR (M/C), and ARR (M/M) stayed clearly below with 90% and 93% sensitivity, respectively. ARR-S and AA2R both showed a sensitivity of 97% when accepting a specificity of 81%. The single false-negative patient accounting for the remaining 3% (ID: 80) remained negative with an AA2R clearly below the cutoff (1.4 [pmol/L]/[pmol/L]). Of note, all 3 ratios involving LC-MS/MS-based numerators and denominators stayed clearly below the cutoff values, while the aldosterone (radioimmunoassay) value appeared to >5-fold higher compared with the aldosterone (MS) value in this patient (Tables S3 and S4). Considering this single donor as a technical discrepancy caused by strongly differing aldosterone levels between radioimmunoassay and LC-MS/MS, the optimal cutoff value for screening for PA using the AA2R was set to 3.9 (pmol/L)/

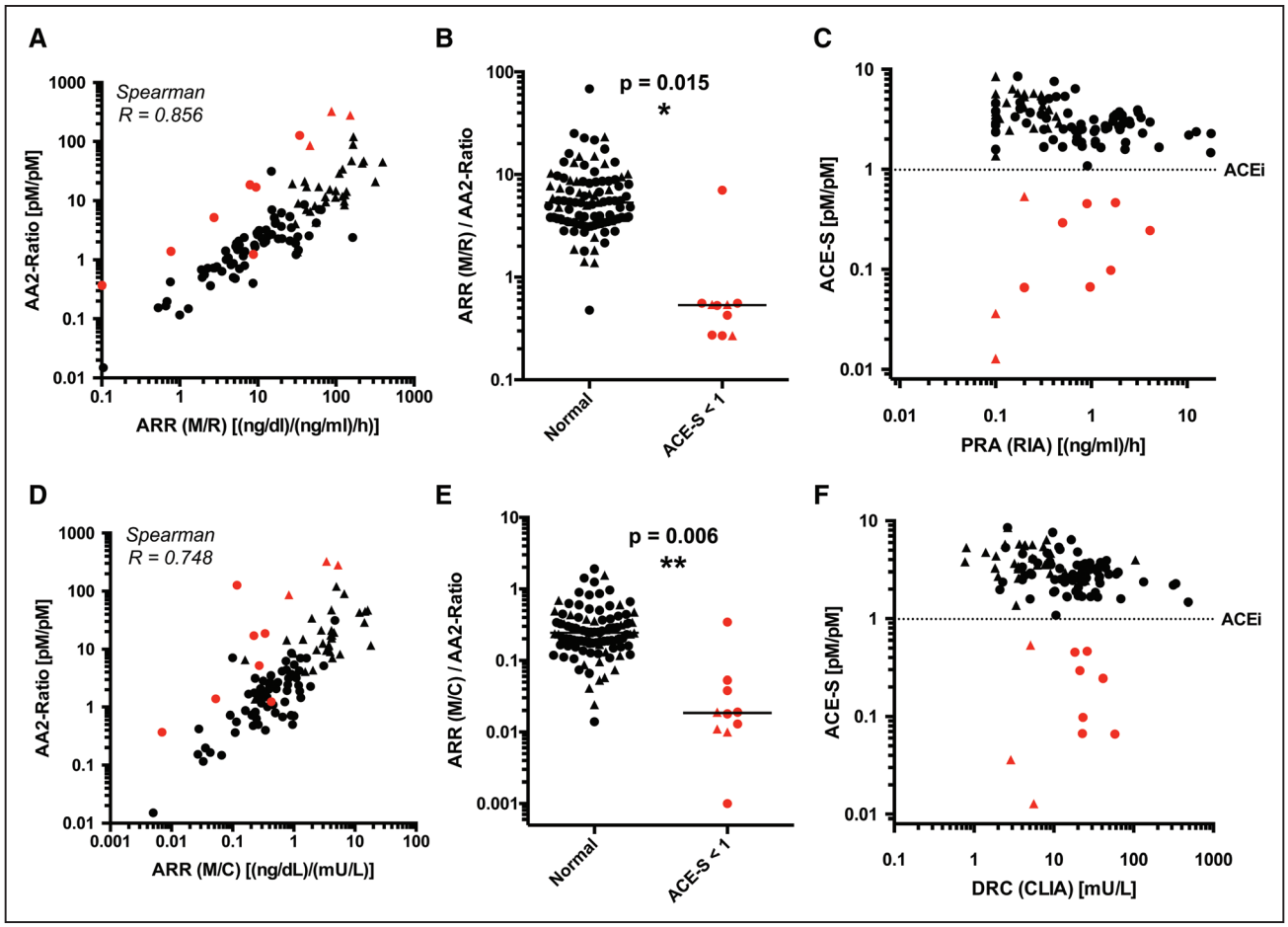

Figure 2. Selective interference of ACE (angiotensin-converting enzyme) inhibition with the aldosterone-to-renin ratio (ARR). **A**, Correlation analysis of the aldosterone-to-Ang II ratio (AA2R) with the ARR (M/R). **B**, Ratios between ARR (M/R) and the AA2R in patient with or without ACE inhibition. **D**, Correlation analysis of the AA2R with the ARR (M/C). **E**, Ratios between ARR (M/C) and the AA2R in patient with or without ACE inhibition. Dot plots in **C** and **F** show the correlation between plasma renin activity (PRA; radioimmunoassay [RIA] or chemiluminescence [CL]) and ACE-S. The dotted line indicates a potential cutoff for suppressed ACE-S (ACE-S, angiotensin-based marker for ACE activity). Patients with primary aldosteronism (PA) or essential hypertension (EH) are respectively represented as triangles or circles. Samples showing suppressed ACE activity were highlighted in red.

(pmol/L), resulting in a sensitivity of 97% at a specificity of 84%. Finally, using aldosterone only for PA screening in the given cohort would result in an optimal Youden index-based cutoff value of 9.8 ng/dL aldosterone, resulting in a sensitivity of 90% at a specificity of 71%.

Reevaluation of AA2R Positive and ARR (R/R) Negative Patients Revealed PA Case That Was Missed Due to In-Effective Drug Withdrawal Before Screening

Following the identification of patients with a clearly elevated AA2R in the presence of ACE inhibition (see Results in the online-only Data Supplement), the saline infusion test was repeated for 2 patients that agreed to re-assessing confirmation testing (IDs: 105 and 106). Patient 105 indeed showed a positive SIT upon re-assessment, which identified him as false negative under the gold-standard screening procedure, while being clearly positive using the ACE inhibitor independent AA2R for PA screening.

Discussion

In the current study, RAAS triple-A testing was performed in a cohort of 110 patients, of which 33 have been previously diagnosed with PA.[14] Our study aimed to investigate the diagnostic performance of the AA2R in detecting PA cases among patients with hypertension as one aspect of RAAS triple-A analysis. We demonstrated a similar accuracy of this new analysis for the screening test for PA in patients with hypertension, compared with the reference analysis in which the diagnosis was performed by radioimmunoassay; it is important to underline that all patients with unilateral PA were correctly detected by a positive screening with the AA2R. This is of particular interest since this method applies LC-MS/MS technology that is considered the gold standard for steroid hormones and peptides measurement. PA is a frequent condition in patients with hypertension and is associated with high risk of cardiovascular, cerebrovascular, and renal complications.[2–4] The detection of this condition is reduced by the difficulty of testing patients with hypertension when they are already treated with medications with a potential interference with the RAAS activity. In a recent study, only 3% of the patients with hypertension underwent RAAS assessment before the beginning of the antihypertensive therapy.[6] Not only physicians but also patients are reluctant to withdraw antihypertensive medications as shown in the present study: 9 of the patients

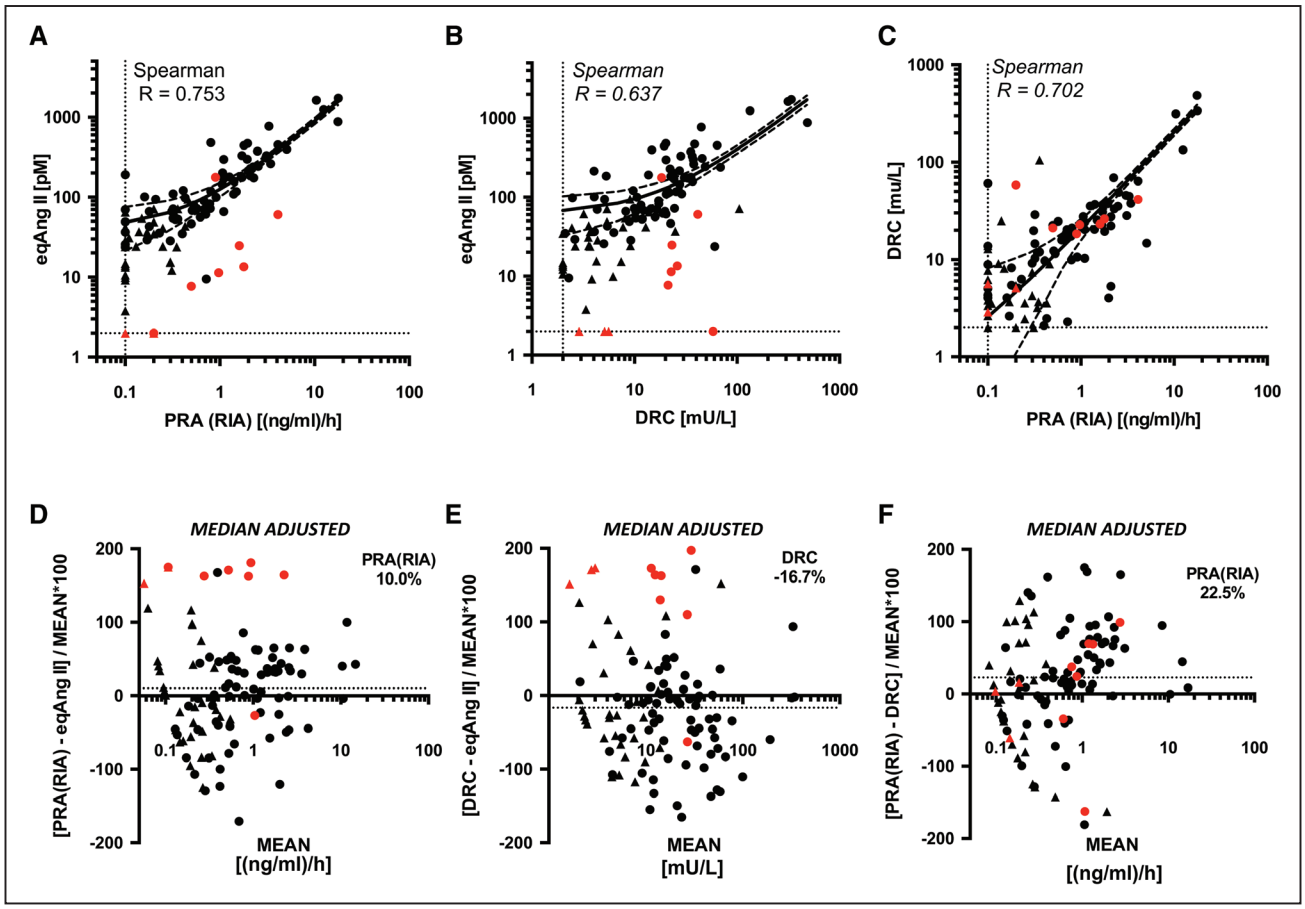

Figure 3. Denominator correlation and Bland-Altman analysis. **A**–**C**, Correlation analyses of plasma renin activity (PRA; radioimmunoassay [RIA]) vs equilibrium angiotensin II (eqAng II) (**A**), direct renin concentration (DRC; chemiluminescence-based immunoassay [CLIA]) vs eqAng II (**B**), and PRA (RIA) vs DRC (CLIA) (**C**). Regression line and 95% CI (dotted lines) are shown for each comparison. **D**–**F**, Bland-Altmann analyses (using adjusted medians) for different denominators: PRA (RIA) vs eqAng II (**D**), DRC (CLIA) vs eqAng II (**E**), and PRA (RIA) vs DRC (CLIA) (**F**). Patients with primary aldosteronism (PA) or essential hypertension (EH) are respectively represented as triangles or circles. Samples showing suppressed ACE (angiotensin-converting enzyme) activity were highlighted in red.

in which ramipril was stopped had still suppressed ACE activity and measurable quantity of the drug in plasma, displaying low compliance to drug withdrawal probably for concerns for potential risk of hypertensive crisis. In the next future, it is expected that the demand of robust and accurate methods to screen for PA will increase to involve up to 50% of the hypertensive population.[1] We determined herein, the AA2R (3.4 [pmol/L]/[pmol/L]) with the highest sensitivity and acceptable specificity to be tested in future prospective confirmatory studies. Moreover, PRA-S and ACE-S were determined and statistically analyzed, revealing a strong correlation between classical PRA and DRC assays and the novel angiotensin-based renin activity marker PRA-S, and unmasking a portion of patients showing suppressed ACE activity despite previous instructions to withdraw their ACE inhibitor therapy to assure compatibility with classical PA screening procedures.

As expected, patients with PA showed significantly suppressed activity and concentration of renin, as indicated by PRA (MS or radioimmunoassay), DRC (CLIA), PRA-S (eqAng I + eqAng II), and Ang II (Table 1). Especially in states of suppressed RAAS activity like PA, a high analytic sensitivity is required for denominator assays to allow for calculation of discrete ratios. Comparing different denominator assays regarding the number of samples where no discrete values could be reported due to the assay sensitivity revealed that LC-MS/MS-based denominator assays clearly outperform radioimmunoassay-based PRA measurements, where up to 18% of samples were below the functional sensitivity of the assay. In contrast, eqAng II was below lower limit of quantification in only 3.6% of samples, while DRC could not be quantified in 4.5% of samples (Table S5). As a consequence, all investigated diagnostic ratios for PA, relating aldosterone secretion to RAAS activity, were significantly increased in PA patients. Aldosterone levels determined by radioimmunoassay were significantly higher compared with levels obtained by LC-MS/MS-based quantification, which is in line with previous findings[17,18] and underlines that radioimmunoassay-based quantification of aldosterone is prone to artifacts and typically delivers much higher absolute quantification results compared to direct and highly specific quantification methods like LC-MS/MS.

Radioimmunoassay-based measurement of PRA, together with CLIA measurement of DRC, and LC-MS/MS-based determination of PRA delivered comparable results. It should be noted that while analysis of PRA by radioimmunoassay has been performed in freshly collected plasma samples, PRA-S

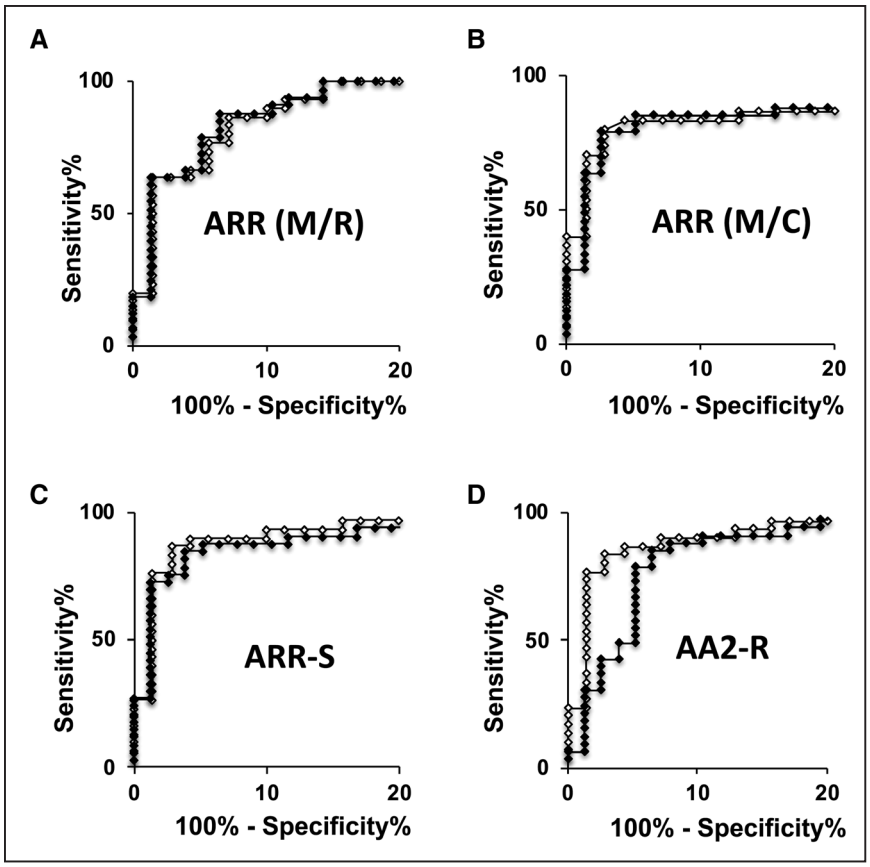

Figure 4. Receiver operator characteristics (ROC) analysis of aldosterone-to-renin ratio (ARR) and aldosterone-to-Ang II ratio (AA2R) after ACE (angiotensin-converting enzyme) inhibitor exclusion. Overlay graphs of ROC curves for different diagnostic ratios are shown before (filled symbols) and after (open symbols) excluding patients with confirmed presence of ACE inhibition. **A**, ARR (M/R); (**B**) ARR (M/C); (**C**) ARR-S; and (**D**) AA2R. C indicates chemiluminescence-based immunoassay; M, liquid chromatography-tandem mass spectrometry; R, radioimmunoassay; RAAS, renin-angiotensin-aldosterone system; and -S, RAAS triple-A derived marker.

has been determined in samples undergoing one freeze/thaw cycle. However, no significant difference was detected between differently obtained PRA values. With a median PRA value of 0.9 (ng/mL)/h in radioimmunoassay-based analysis compared to an LC-MS/MS-based median value of 0.5 (ng/mL)/h, previous observations relating to cryoactivation of renin induced by freezing and thawing of samples could not be confirmed.[19] This could be explained by the very short time (<1 hour) the thawed samples have been exposed to low temperatures in a thawed state before analysis, which seems to be a prerequisite for cryoactivation, although the effect has been controversially discussed in the literature.[20–22]

Using the ARR to screen for autonomous adrenal aldosterone secretion was reasonable so far, as no appropriate methods to evaluate Ang II physiological activity were available at the time of introducing ARR as a diagnostic value. However, in contrast to Ang II, renin is not directly affecting aldosterone secretion, and in contrast to Ang II, renin is upregulated by ACE inhibitors, which can result in false-negative screening results for PA.[1]

In the current article, we show noninferiority of the AA2R to screen for PA in patients with hypertension being off interfering therapies. We observed potential indicators of superiority of the AA2R when screening patients on ACE inhibitor therapy that require to be confirmed in larger prospective studies. Under these conditions, the use of renin-based diagnostic ratios may lead to false-negative test results in the presence of ACE inhibition,[1] which appears reasonable considering the regulation of Ang II formation and aldosterone secretion. Pharmacological interference upstream of Ang II by ACE inhibition is expected to suppress Ang II and aldosterone, while renin undergoes compensatory upregulation by reduced Ang II receptor type 1 signaling in the kidney (Figure S7E). Although ROC analysis did not reveal significant differences between all 6 diagnostic ratios investigated, correlation analysis between AA2R and renin-based ratios revealed a subset of samples showing an underestimation of renin-based ratios compared to the AA2R (Figure 2, red dots).

Further analysis revealed that all patients with a suppressed ARR also had a suppressed Ang II/Ang I ratio (ACE-S), indicating the presence of pharmacological ACE inhibition, which could later be confirmed by quantification of drug levels. Taken together, despite intended withdrawal of ACE inhibitors, 9 of 110 patients were still on ACE inhibitor therapy at the time of PA screening, which resulted in suppression of the ARR and subsequently in one false-negative test result, while a significant suppression of the ARR compared to the AA2R was observed for the 9 patients on ACE inhibitor (Figure 2). Therefore, the distortion of the correlation between the ARR and the AA2R as well as observed slight differences between the AA2R and renin-based ratios in terms of diagnostic performance (Figure 4) appear to be caused by unexpected ACE inhibition in a subset of patients of the investigated cohort.

The AA2R remained elevated with a clearly positive test result for one of the patients being on ACE inhibitor therapy. PA could be confirmed in this patient following re-assessment and confirmation testing by saline infusion. Although ACE inhibition only affected a minor portion of the RENATO-II cohort at the time of PA screening, our findings implicate that screening hypertensive patients while being on ACE inhibitor

therapy could be effective using the AA2R instead of renin-based diagnostic ratios. ACE-S may further be used to monitor the in vivo efficacy of ACE inhibition (compliance, dosing) simultaneously with PA screening, making the approach particularly attractive for stratification of first-line nonresponders in hypertension.

ACE-S was significantly increased in patients with PA compared to patients with EH. A dose-dependent stimulation of ACE gene expression by aldosterone has been described in vitro for murine endothelial cells and ventricular cardiomyocytes.[23,24] These data are consistent with our finding and suggest a pathophysiological role of the aldosterone as a regulator of the RAAS, constituting a positive feedback loop possibly involved in the development of endothelial dysfunction and cardiovascular damage in PA patients.

The RAAS plays a central role in the therapeutic management as well as the diagnostic evaluation of hypertension in clinical practice. RAAS blockade has been evolved to a hallmark of antihypertensive therapy reflected by the recently amended guidelines for treatment of hypertension, recommending dual drug combinations with RAAS blockers as first-line therapies for hypertension.[7] The concept to stratify patients with hypertension on the basis of renin activity aiming to develop more effective treatment schemes for antihypertensive patients is not new and has been extensively studied by Laragh et al.[25]

The determination of the causes of uncontrolled hypertension, which accounts for 50% of pharmacologically treated patients, are critical targets for improving treatment efficacy in hypertension. Among causes of uncontrolled hypertension, lack of treatment adherence, drug underdosing, and secondary forms of hypertension, including PA, are the most common.[26] Although diagnostic tools to monitor patient compliance and to screen for PA are available, technologies are hardly used in primary care due to their complex interpretation under therapy and high costs.[6,27] The possibility to screen for PA while simultaneously gaining information on pharmacological drug efficacy on a functional level is a unique feature of RAAS triple-A testing.

As a consequence of the findings generated in this article, a comprehensive approach of using RAAS triple-A testing for molecular stratification of hypertension to improve treatment efficacy appears reasonable. In contrast to previous stratification approaches using solely plasma renin activity or plasma renin concentration as stratification markers, RAAS triple-A testing may provide simultaneous read outs on drug dose efficacy and treatment adherence, plasma renin activity and could allow for PA screening even in the presence of ACE inhibitor treatment. Although cutoffs for drug efficacy and detection of PA have to be clearly defined in further studies, RAAS triple-A analysis may be a versatile tool compatible for a broad implementation in primary care of hypertension.

Perspectives

Uncontrolled hypertension currently affects up to 50% of patients being on therapy and remains a major challenge for physicians to improve this poor treatment performance, as underlying causes are manifold. Patient adherence, drug underdosing, and secondary hypertension, including PA, are the most frequent causes for inefficient first-line treatment of hypertension and remain difficult to be addressed as convenient diagnostic tools to clearly identify these causes have not been available so far. PA screening in the presence of ACE inhibitor therapy is one aspect of RAAS triple-A testing that has the potential to widely implement PA screening in hypertension care. Importantly, ACE inhibitor and angiotensin receptor blocker efficacy and compliance can simultaneously be evaluated and on the basis of PRA-S, the discrimination between low- and high-renin hypertension could be made, building the basis for an advanced stratification scheme for first-line nonresponders in hypertension. However, prospective randomized approaches are required to study the overall benefits and applications of RAAS triple-A profiling in hypertension.

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Disclosures

M. Poglitsch is an employee at Attoquant Diagnostics, a company developing angiotensin-based biomarkers for hypertension. P. Mulatero received fees for educational speech from DIASORIN. The other authors report no conflicts.

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Novelty and Significance

What Is New?

- We investigated for the first time the aldosterone-to-Ang II ratio (AA2R) in a cohort of 110 patients with hypertension consisting of 77 patients with essential hypertension and 33 with confirmed primary aldosteronism (PA).
- The AA2R remains unaffected in the presence of ACE (angiotensin-converting enzyme) inhibition, while renin-based ratios were suppressed.
- We defined the cutoff value for the AA2R in PA screening.

What Is Relevant?

- The AA2R showed similar diagnostic performance to the reference ARR screening tests.
- The AA2R-based screening for PA might be possible in the presence of ACE inhibitor therapy, providing a major advantage over currently available screening tests.
- Triple-A analysis provides information on therapy compliance.
- Combining drug efficacy or compliance monitoring with PA screening might have a significant impact on the overall performance of PA screening procedures.

Summary

Triple-A analysis is a novel and reliable method to screen hypertensive patients for PA, even in the presence of therapy with ACE inhibitors. This method may also allow evaluation of therapy compliance and efficacy.