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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1725959 since 2020-01-30T16:36:06Z

Published version:

DOI:10.1161/HYPERTENSIONAHA.119.13772

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02 May 2026

American Heart Association.
Hypertension
Manuscript Submission and Peer Review System

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URL: http://hype-submit.aha-journals.org

Title: Renin-angiotensin-aldosterone system triple-A analysis for the screening of primary aldosteronism

Manuscript number: HYPE201913772

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Renin-angiotensin-aldosterone system triple-A analysis for the screening of primary aldosteronism.

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Word count: abstract 250 words, manuscript: 7168 words including tables, references and figure captions. Number of Tables: 2; Number of Figures: 5; Supplemental file: 1.

Short title: AA2R as a screening for primary aldosteronism

Key words: primary aldosteronism, renin-angiotensin system, secondary hypertension, adrenal gland, angiotensin II

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Abstract

Primary aldosteronism (PA) 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 (ARR) during screening process is a major confounder. Renin-angiotensin-aldosterone system (RAAS) Triple-A analysis is a novel liquid chromatography/tandem mass spectrometry diagnostic assay that allows simultaneous quantification of aldosterone, equilibrium angiotensin I (eqAngI) and equilibrium angiotensin II (eqAngII) in a single sample of serum. We performed a comparative evaluation of the diagnostic performance of the Aldosterone-to-AngII-Ratio (AA2R) and four 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 PA and 77 with essential hypertension). All ratios showed comparable areas under the curves ranging between 0,937 and 0,970 without significant differences between each other. The evaluation of the AngII-to-AngI ratio revealed persistent drug intake in some patients as cause for suppressed renin-based diagnostic ratios, while AA2R remained unaffected. The Youden-Index optimal cut-off value for the AA2R was 6.6 [(pmol/L)/(pmol/L)] with a sensitivity of 90% and a specificity of 93%, proving non-inferiority compared with the ARR while suggesting superiority over renin-based ratios in terms of interference with ACE-inhibitor therapy. This study shows for the first time the accuracy and reliability of RAAS triple-A analysis for the screening of PA, that can be applied in clinical routine without the need for drug withdraw periods that impose additional risks to the patients.

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ABBREVIATIONS: PA, Primary Aldosteronism; EH, Essential Hypertension; PRA, Plasma Renin Activity; ARR, Aldosterone to Renin Ratio; AGT, Angiotensinogen; Ang, Angiotensin; RAAS, Renin-Angiotensin-Aldosterone System; ACE, Angiotensin Converting Enzyme; LC-MS/MS (M), Liquid Chromatography-tandem Mass Spectrometry; eqAng I, equilibrium Ang I; eqAng II, equilibrium Ang II; PRA-S, PRA surrogate, as RAAS Triple-A derived marker; ACE-S, ACE surrogate, as RAAS Triple-A derived marker; AA2R, Aldosterone to Ang II Ratio; RIA (R), Radio-Immunoassay; ARR-S, Aldosterone to Renin Ratio using PRA-S as denominator; APA, Aldosterone Producing Adenoma; BAH, Bilateral Adrenal Hyperplasia; AUC, Area Under the Curve.

INTRODUCTION

Primary aldosteronism (PA) is a frequent form of secondary hypertension[1,2] that is caused by excessive secretion of aldosterone from the adrenal glands. Patients with PA undergo more often than patients with essential hypertension (EH) to cardiovascular and metabolic complications, such as stroke, myocardial infarction, heart failure, atrial fibrillation, metabolic syndrome and type 2 diabetes mellitus[3]. 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[4]. Therefore, 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[5].

The suggested screening test is the Aldosterone-to-Renin (measured as direct renin or plasma renin activity, PRA) Ratio (ARR) that should be performed under controlled condition[1]. Ideally, patients should be tested before the beginning of anti-hypertensive 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 anti-hypertensive therapy.

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Alpha$_1$-adrenergic receptor blockers and non-dihydropyridine calcium channel blocker, which displayed 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 anti-hypertensive drugs is considered unsafe, for example in patients with previous myocardial infarction[6].

Angiotensinogen (AGT), the precursor hormone of angiotensins, is primarily secreted by the liver and serves as a pre-hormone for all angiotensin metabolites. Angiotensin I (Ang 1-10, Ang I) is produced from AGT by the enzymatic action of renin, which cleaves the decapeptide Ang I off the N-terminus of AGT in a highly regulated reaction. Both, the concentration of circulating active renin as well as the levels of AGT contribute to the Ang I formation rate, which is also referred to as PRA. Ang I serves as a substrate to multiple plasma enzymes converting it to N- or C-terminally truncated metabolites including angiotensin II (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 biochemical evaluation of the circulating RAAS in clinical samples, being RAAS equilibrium analysis. The underlying principle is based on the condition that all components required for *in vivo* angiotensin metabolite formation (angiotensinogen, renin, angiotensin converting enzyme, or ACE) 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[7-9].

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[10], but also to guide anti-hypertensive therapy in patients with essential hypertension[11,12].

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Here we introduce and validate a novel molecular 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 multi-layer stratification of hypertensive patients in terms of RAAS activity (sum of Ang I and Ang II, PRA-S), ACE activity (Ang II-to-Ang I-Ratio, ACE-S) and PA screening that remains unaffected by ACE inhibition (Aldosterone-to-AngII Ratio, AA2R). RAAS Triple-A testing simultaneously provides molecular information on treatment adherence, RAAS activity and secondary hypertension in patients, which addresses multiple key issues in controlling hypertension in clinical practice.

Methods

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 "RENin and Aldosterone measurements in hypertensive patients in TOrino" -RENATO study[13]. 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[13]. Briefly, patients were screened for PA after withdrawal of interfering medications; when it was not possible to stop all antihypertensive drugs, patients received the α-blocker doxazosin and/or the non-dihydropiridine calcium channel blocker verapamil. The cut-off 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 equal or greater than 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 h) that was carried in seated position[14] 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

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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 h and then been seated for at least 15 min before venipuncture; blood was collected at room temperature into anticoagulant-free tubes, centrifuged (3.000 rpm for 15 min at 27-28°C), frozen at – 20°C and never thawed before analysis..

PRA was determined based on Ang I formation capacity of serum samples in presence of Ang protease inhibitors. Samples were supplied with a mixture of Ang I stabilizing protease inhibitors containing EDTA (ethylenediaminetetraacetic acid) and AEBSF (4-[2-Aminoethyl]-benzenesulfonyl fluoride hydrochloride) in phosphate buffer (pH=7). Following splitting the sample in 2 identical aliquots, one was incubated at 37°C for 1 hour, while the other aliquot was kept on ice during the incubation period. Following incubation, all samples were subjected to quantification of Ang I using LC-MS/MS, as described below. PRA was calculated by subtracting the Ang I value of the non-incubated sample from the Ang I value in the 37°C incubated sample and the Ang I formation rate was expressed in [(ng/ml)/h].

eqAng I and eqAng II were measured following ex vivo equilibration and subsequent stabilization of conditioned serum samples, as previously described[7,8]. Following equilibration and stabilization, samples were spiked with stable isotope-labeled internal standards for angiotensins and aldosterone at concentrations of 200 pg/ml and 500 pg/ml respectively. Following C18-based solid-phase-extraction, 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

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TQ/S, Milford, Massachusetts, USA) in multiple reaction monitoring mode. Internal standards were used to correct for analyte recovery across the sample preparation procedure in each individual 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 lower limits of quantification (LLOQs) for eqAng I, eqAngII and aldosterone as defined as the lowest concentrations tested showing a coefficient of variation (CV) <20% according to FDA criteria, were 5 pmol/L, 2 pmol/L and 14 pmol/L, respectively. At 50 pmol/L, the inter-assay CVs for eqAng I, eqAngII and aldosterone were 10.2%, 6.1%, and 7.9%. The intra-assay CVs for eq Ang I, eqAngII and aldosterone were 8.6%, 4.4%, and 5.2%, respectively.

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 (Angiotensin I, Angiotensin 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)[7] 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: three variants of the classic ARR using PRA as denominator were compared by combining aldosterone and PRA values either determined by radio-immunoassay (RIA, R) 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. ARR (R/R) indicates the ratio initially determined on the basis of RIA 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 RIA 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). Moreover,

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two 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 (Figure 1A).

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

Statistics. Prism 6 was used as software tool for statistical analyses. Data were expressed as median (25th to 75th percentile) and analyzed by Mann–Whitney's. Categorical variables were compared through a χ^2 test (Fisher exact test when sample size was ≤ 5). We compared PRA with Ang II and PRA-S (eqAng I + eqAng II) and ARR with AA2R through correlation analysis (Pearson's 'R' correlation test) and assessment of the regression line. Bland-Altman plot was used to compare the within-patient relationship and to 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 two areas were different. The Younden-Index was used to determine the optimal diagnostic thresholds for PA diagnosis.

RESULTS

Description of the population. 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, PRA-S and eqAng II were significantly lower in patients with PA compared with

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patients with EH (*P*-value < 0.001 for all comparison). ACE-S revealed a significant increase in patients with PA compared with patients with EH. Across all patient sub-cohorts, RIA based measurements resulted in significantly higher median values for PRA as well as aldosterone when compared to data obtained from LC-MS/MS analysis. All five diagnostic ratios investigated (ARR (R/R), ARR (R/M), ARR (M/M), ARR-S and AA2R) were significantly higher in PA patients compared to EH patients ($p < 0.001$). Among patients with PA, 23 were diagnosed with bilateral adrenal hyperplasia (BAH), whereas 10 had an aldosterone producing adenoma (APA). Except for blood pressure, potassium and RIA-based aldosterone levels, none of the molecular markers investigated was significantly different between patients with BAH vs. APA (Supplementary Table 1).

Comparative analysis of PA screening performance for ARR, ARR-S and AA2R (ROC-Analysis). Diagnostic ratios for PA were calculated using 4 different denominators (PRA (RIA), PRA (MS), PRA-S and eqAng II) and two different numerators (Aldosterone (RIA) and Aldosterone (MS)) in five combinations: ARR (R/R), ARR (M/R), ARR (M/M), ARR-S and AA2R, as shown in Figure 1A, together with corresponding units. ROC analysis was performed for each individual ratio considering the final diagnosis obtained in RENATO-I as gold standard. All five diagnostic ratios showed comparable areas under the curves (AUCs) ranging between 0,937 and 0,970 without significant differences between each other (Figure 1 B-F). ARR ratios involving PRA values measured by RIA but differently measured aldosterone levels rapidly reached 100% sensitivity (Figure 1 B-C), while 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 AUCs (Figure 1 D-F). Seeking to get behind the cause of this observation, we decided to investigate the correlations between the ARR (R/R) and the AA2R as well as the correlation between different denominators and numerators in more detail.

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Correlation analysis of different RAAS markers (denominators) and diagnostic ratios. The correlation analysis between ARR and AA2R using RIA (Figure 2A) or LC-MS/MS based values (Figure 2D) for calculation of the ARR calculation revealed a good correlation. When correlating ARR (M/M) with the AA2R, a separate population of patients not fitting the otherwise very solid correlation (Spearman R = 0.93) was observed and highlighted in red (Figure 2D). Similar patients were highlighted in Figure 2A, revealing a similar pattern for AA2R which was relatively increased over the ARR in these patients. In order to reduce potential interferences of differences in RIA and MS aldosterone levels, we further compared the denominators PRA (RIA) and PRA (MS) with eqAng II directly (Figure 2B and 2E), which revealed a reduced recovery of eqAng II from renin in highlighted patient samples. In order to normalize for differences in absolute values ARR and AA2R, calculated individual values for the AA2R were adjusted to obtain a similar median compared to the ARR across the cohort, which allowed for a direct comparison of the two diagnostic ratios using a Bland-Altman analysis. ARR (R/R) showed less correlation between adjusted values for the AA2R when compared to ARR (M/M) (Figure 2C, F), while red labeled patient samples stayed clearly below the line of equality, indicating selective suppression of the ratios using the PRA as denominators. Correlation analysis and Bland-Altman analysis of PRA values (MS vs RIA) and aldosterone values (MS vs RIA) revealed an overestimation of RIA based measurements of 49.3% and 68.2% for PRA and aldosterone, respectively (Supplementary Figure 1A, B, D, E). PRA-S showed a very good correlation (Spearman R=0,71) with PRA (MS) across the whole patient cohort combining EH and PA (Supplementary Figure 1C, F) confirming the similarity between the ROC curves for ARR and ARR-S as shown in Figure 1D and 1E.

ACE inhibition may cause ARR suppression in a subset of patients. Given that the recovery of Ang II from renin was suppressed in a subset of patients, we further investigated the correlation between PRA values and ACE-S, a marker for ACE activity that is obtained by calculating the ratio between eqAng II and eqAng I[7]. We showed that patients dropping out

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the correlation between the ARR and the AA2R displayed a significant suppression of ACE-S, therefore suggesting the presence of ACE inhibition (Figure 3A, C). 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 (UHPLC-MS/MS) based quantification of drug levels[15] further revealed that ramipril was present in these 9 samples at pharmacologically active concentrations ranging between 5.7 and 35 ng/mL, indicating non-compliance with the withdrawal procedure. The one remaining sample did not contain detectable concentrations of ramipril (<0,78 ng/ml = LLOQ). Both, eqAng I and eqAng II were below LLOQ in this sample, causing an artifactual suppression of ACE-S by calculation with indiscrete values eqAng I and eqAng II in this sample, indicating that ACE inhibition was correctly detected in 100% patients with detectable angiotensin levels. When stratifying patients according to their ACE-S value in two groups of 100 and 10 patients respectively, a significant suppression of the ARR compared to the AA2R was observed in samples analyzed by LC-MS/MS (Figure 3D). Although a similar pattern was also observed when investigating the relationship between RIA based ratio and the AA2R, these differences didn't reach significance (Figure 3B), which might be explained by additional technical confounding factors associated with RIA based aldosterone values (Supplementary Figure 1B, E). As a result of these observations, we decided to repeat the ROC analysis after exclusion of these 10 patients with confounded ARR values potentially interfering with ROC analysis.

Exclusion of low ACE patients from ROC evaluation results in improved curve shape for the AA2R. We re-plotted the ROC curves for all five diagnostic ratios following elimination of patients with ACE-S < 1 [pM/pM] (Supplementary Figure 2). Removing low ACE patients resulted in a selective shift of the AA2R back towards the y-axis. The ROC curves for ARR-S and other renin-based ratios remained unaffected. As shown by the overlay graphs in Figure 4 comparing ROC curves before and after excluding low-ACE patients from

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the ROC evaluation, the minor discrepancies observed for the AA2R disappear when excluding low-ACE patients. The optimal diagnostic cut-off values for PA screening were determined for all five ratios based on the Youden-Index analyzed in the cohort excluding low ACE patients, consisting of 71 EH and 29 PA patients (Supplementary Figure 3 A-E). The optimal cutoff for the AA2R was 6.6 [pM/pM], corresponding to a sensitivity of 90% and a specificity of 93% (Youden-Index: 0.83). Table 2 shows sensitivity and specificity for indicated cut-off values and number of false-positives and false-negative screening results are shown for each diagnostic ratio investigated. 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 decrease the cut-off value for the AA2R until to reach 100% sensitivity resulted in a comparable number of false-positives between ARR (R/R) and AA2R (14 vs. 15), while one patient (ID: 80) remained negative with an AA2R clearly below the cut-off (1.4 pM/pM). Of note, all three ratios involving LC-MS/MS based numerators and denominators stayed clearly below the cut-off values, while the aldosterone (RIA) value appeared to more than 5-fold higher compared to the aldosterone (MS) value in this patient (Supplementary Table 2).

ACE suppression potentially causes false-negative screening results when using the ARR. We performed an in-depth analysis of all false-positive and false-negative cases observed for our 5 different diagnostic ratios when using the Youden-Index based cut-off values. Supplementary Table 3 summarizes cut-offs and patient IDs for all false-positive and false-negative test results. Screening results that were divergent to the ARR (R/R) as state-of the art test were highlighted in red. Two major groups of false-positives could be identified. One where all LC-MS/MS based ratios showed screening results that were comparable in terms of their distance to the Youden-Index based cut-offs (Patient IDs: 26, 29, 55), and one where only the AA2R showed a clear relative increase compared to other ratios involving LC-MS/MS based denominators (Patient IDs: 105, 106, 109). In depth analysis revealed that the

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first group showed variations between initially measured RIA values and MS values obtained in re-analysis of samples, which might explain discrepancies in screening outcomes especially for patients being near the cut-off value (Borderline: IDs 29 and 55, Supplementary Table 2). The second group (Patient IDs: 105, 106, 109) showed a selective relative decrease of the AA2R when comparing to other ratios. Of note, these 3 patients were all on ACE inhibitor therapy before withdrawing drugs before screening and were also among the patients we could previously identify to be suppressed in ACE-S. Potentially false-negative screening results using the AA2R occur for borderline PA cases (IDs: 72, 80, 93) in the context of variations between MS and RIA test results for PRA and aldosterone, which could be avoided by reducing the screening cut-off value to 3.9 pM/pM, which still resulted in an acceptable number of false-positives compared to the ARR (R/R).

Re-Evaluation 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, the SIT 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 ACEi independent AA2R for PA screening.

RAAS Triple-A analysis can be used to screen patients for PA while providing insight in the efficacy of the drug withdrawal procedure. The complete cohort of 110 patients was subjected to RAAS Triple-A analysis. Therefore, patients were classified in 4 quartiles based on their PRA-S values (Figure 5A-D). Data were illustrated in dot-plots showing data pairs for AA2R and ACE-S. Cut-Off values for PA (YI-based: 6.6 pM/pM, Sensitivity: 90%, Specificity: 93%) and ACE inhibition (1.0 pM/pM, 95% Sensitivity for ACEi within previous 24h, data not shown) are indicated as dotted lines. Red dots indicate patients with confirmed PA, while black dots indicate patients diagnosed with EH. The majority of PA cases occur in

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the lower 50% of PRA-S values, with a clear dominance of PA cases in the lower quartile for PRA-S being < 44 pM. Five of the 10 patients showing suppressed ACE-S belonged to the lowest PRA-S quartile. Among high renin patients, an accumulation of low AA2R individuals was observed, which could be explained by the presence of ARBs in these individuals, which has to be expected when assuming non-adherence to drug withdrawal to be the underlying cause for ACE inhibition under screening conditions.

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[13]. RAAS Triple-A analysis is based on the quantification of serum equilibrium levels of Ang I, Ang II and aldosterone, followed by calculation of angiotensin derived markers for PRA (PRA-S: eqAng I + eqAng II), ACE activity (ACE-S: eqAng II/eqAng I) and adrenal function (AA2-R: Aldosterone/eqAng II). Our study aimed to investigate the diagnostic performance of the AA2-Ratio in detecting PA cases among hypertensive patients 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 RIA; it is also 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 cardio-, cerebro-vascular and renal complications. 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 anti-hypertensive therapy[5]. Not only physicians, but

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also patients are reluctant to withdraw anti-hypertensive medications as shown in the present study: 9 of the patients 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 (3.9 pM/pM) 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 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, PA patients showed significantly suppressed activity of renin, as indicated by PRA (MS or RIA), PRA-S (eqAng I + eqAng II) and Ang II (Table 1). 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 RIA were significantly higher compared to levels obtained by LC-MS/MS based quantification, which is in line with previous findings[16,17] and underlines that RIA 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.

RIA based measurement of PRA and LC-MS/MS based determination of PRA delivered comparable results. While analysis of PRA by RIA has been performed in freshly collected plasma samples, PRA-S 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 RIA based analysis compared to an LC-MS/MS based median value of 0.5 (ng/ml)/h, previous observations relating to cryo-activation of renin

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induced by freezing and thawing of samples could not be confirmed[18]. 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[19-21].

Using the ARR to screen for autonomous adrenal aldosterone secretion was reasonable so far, as no appropriate methods to evaluate Ang II physiologic activity were available at the time of introducing ARR as a diagnostic value. However, it should be noted that in contrast to Ang II, renin is not directly affecting aldosterone secretion, and in contrast to Ang II, renin is up-regulated by ACE inhibitors, which can result in false-negative screening results for PA[1]. The use of renin as a surrogate marker for Ang II therefore seems to be a technical trade-off, knowing that its correlation with Ang II levels is strongly affected by the therapeutic use of ACE inhibitors.

In the current paper, we show non-inferiority of the AA2R to screen for PA in hypertensive patients 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]. Although ROC analysis did not reveal significant differences between all five 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 pharmacologic 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 ACEi 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

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for the 9 patients on ACE inhibitor (Figure 3). 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 non-responders in hypertension.

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 anti-hypertensive therapy reflected by the recently amended guidelines for treatment of hypertension, recommending dual drug combinations with RAAS blockers as first-line therapies for hypertension[6]. The concept to stratify hypertensive patients on the basis of renin activity aiming to develop more effective treatment schemes for anti-hypertensive patients is not new and has been extensively studied by Laragh and colleagues[22].

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 under-dosing and secondary forms of hypertension, including PA, are the most common[23]. 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

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and high costs[5,24]. The possibility to screen for PA while simultaneously gaining information on pharmacologic drug efficacy on a functional level is a unique feature of RAAS Triple-A testing.

As a consequence of the findings generated in this paper, 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 employing solely plasma renin activity or plasma renin concentration as stratification markers, RAAS Triple-A testing delivers simultaneous read outs on drug dose efficacy and treatment adherence, plasma renin activity and allows for PA screening even in the presence of ACE inhibitor treatment. Although cut-offs for drug efficacy and detection of PA have to be clearly defined in further studies, RAAS Triple-A analysis may be a versatile tool to simplify primary care of hypertension by providing multiple layers of information based on a simple blood test being 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 under-dosing 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 ARB efficacy and compliance can simultaneously be evaluated and on the basis of PRA-S, the discrimination between low and high renin hypertension can be made, together building the basis for an advanced stratification scheme for first-line non-responders in hypertension.

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However, prospective randomized approaches are required to study the overall benefits of RAAS Triple-A profiling in hypertension.

Acknowledgments

We are most grateful to our team of nurses and in particular to Silvano Bertinetti, Cristina Bertone and Sonia Zamburru, for their valuable help in patient management.

Source(s) of Funding

RENATO-I, where samples have been collected and banked, was partially supported by an unrestricted grant from DIASORIN to the Department of Medical Sciences, University of Torino, Italy. The organization, sample logistics and evaluation of measurements results has been supported by funding specifically dedicated to the Department of Medical Sciences from Italian Ministry for Education, University and Research (Ministero dell'Istruzione, dell'Università e della Ricerca - MIUR) under the programme "Dipartimenti di Eccellenza 2018 – 2022", Progetto Strategico di Eccellenza Dipartimentale, DSM, UNITO - Project n. D15D18000410001. Attoquant Diagnostics GmbH is a privately funded company and covered the costs for the analysis of samples in the course of RENATO-II as an in-kind contribution.

Conflict(s) of Interest/Disclosure(s)

Marko Poglitsch is an employee at Attoquant Diagnostics, a company developing angiotensin-based biomarkers for hypertension. Paolo Mulatero received fees for educational speech from DIASORIN. The other authors declared no conflict of interest.

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Novelty and Significance:

What is New?

- We investigated for the first time the AA2R in a cohort of 110 patients with hypertension consisting of 77 patients with EH and 33 with confirmed PA;
- The AA2R remains unaffected in the presence of ACE inhibition, while renin-based ratios were suppressed;
- We defined the cut-off value for the AA2-Ratio 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;

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Figure Legends

Figure 1: ROC analysis for different diagnostic ratios. (A) Numerators and denominators for investigated diagnostic ratios are shown together with corresponding units. (B-F) ROC curves for individual diagnostic ratios are shown (ARR, Aldosterone-to-Renin-Ratio; -S, RAAS Triple-A derived marker; R, RIA; M, LC-MS/MS; AA2-R, Aldosterone-to-Ang II-Ratio; AUC, Area under the curve; CI, Confidence interval).

Figure 2: Correlation analysis of RAAS markers and diagnostic ratios. (A) Correlation analysis of the AA2-Ratio with the ARR (R/R). (D) Correlation analysis of the AA2-Ratio with the ARR (M/M). The correlation analysis between PRA (RIA or MS) and eqAng II, the denominators for ratios compared in panels (A) and (D) are shown in panels (B) and (E). Panels (C) and (F) show Bland-Altman analysis comparing the ARR (R/R) or the ARR (M/M) and the AA2-Ratio as indicated (R/R, Aldosterone and PRA determined by RIA analysis; M/M, Aldosterone and PRA determined by LC-MS/MS analysis). Samples showing a significant deviation between ARR (M/M) and the AA2-Ratio were highlighted in red.

Figure 3: Selective interference of ACE inhibition with the ARR. Dot plots in panels (A) and (C) show the correlation between PRA (RIA or MS) and ACE-S. The dotted line indicates a potential cut-off for suppressed ACE-S (ACE-S, angiotensin-based marker for ACE activity). The ratios between ARR (R/R) or ARR (M/M) and the AA2-Ratio are shown in panels (B) and (D), respectively. Data points highlighted in red are the same as in panels A-D and Figure 2 (R/R, Aldosterone and PRA determined by RIA analysis; M/M, Aldosterone and PRA determined by LC-MS/MS analysis).

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Figure 4: ROC analysis of ARR-S and AA2-R after ACE inhibitor exclusion. (A) Overlay graph of ROC curves for the ARR-S are shown before (filled symbols) and after (open symbols) excluding patients with confirmed presence of ACE inhibition. (B) Overlay graph of ROC curves for the AA2-R are shown before (filled symbols) and after (open symbols) excluding patients with confirmed presence of ACE inhibition (AUC, Area under the curve; CI, Confidence interval).

Figure 5: RAAS Triple-A Analysis. Patients were stratified based on PRA-S and the resulting quartiles were plotted in RAAS Triple-A plots analyzing the AA2-Ratio versus ACE-S (A-D). Dotted lines indicate the cut-off of 1.0 pM/pM used to detect ACE inhibition (ACEi) and the YI-based AA2-R cut-off of 6.6 pM/pM used for PA screening. Data pairs highlighted in red relate to confirmed PA patients. (E) Schematic representation of the RAAS and explanation of angiotensin-based biomarkers used in RAAS Triple-A analysis (ACE, Angiotensin-Converting-Enzyme; AT1R, Angiotensin II type 1 receptor; PRA, Plasma-Renin-Activity; ALDO, Aldosterone; -S, RAAS Triple-A derived marker).

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Table 1: Cohort characteristics

Variable	EH (n=77)	PA (n=33)	P-value
Sex (Male/Female)	43 / 34	17 / 16	0.68
Age [Years]	49.0 (44.0-54.0)	50.0 (45.0-60.0)	0.07
SBP [mmHg]	145 (135-150)	155 (140-170)	<0.001
DBP [mmHg]	90 (85-100)	95 (85-100)	0.17
K [mEq/L]	4.1 (3.9-4.4)	3.6 (3.0-3.8)	<0.001
PRA (RIA) [(ng Ang I/ml)/h]	0.9 (0.4-2.0)	0.1 (0.1-0.3)	<0.001
PRA (MS) [(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) [pM]	165.6 (80.6-328.3)	40.5 (18.2-57.5)	<0.001
eqAng II [pM]	100.9 (56.3-227.0)	24.2 (13.3-42.0)	<0.001
Aldosterone (RIA) [pM]	385.6 (246.9-590.9)	693.6 (507.7-885.0)	<0.001
Aldosterone (MS) [pM]	186.2 (102.7-293.2)	391.5 (331.1-486.3)	<0.001
ACE-S [pM/pM]	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/M) [ng/dl]/[(ng/ml)/h]	12.4 (5.8-23.4)	126.3 (81.2-281.5)	<0.001
ARR-S [(pM)/(pM)]	1.2 (0.5-2.0)	10.7 (7.0-22.9)	<0.001
AA2-Ratio [pM/pM]	1.8 (0.7-3.1)	15.6 (9.5-43.1)	<0.001

Legend to Table 1 – Medians and interquartile ranges (IQR) are given for indicated variables. Corresponding units are shown in as [unit]. Essential hypertensive (N=77) and PA (N=33) patients are statistically compared. *P*-values are shown on the right (ARR, Aldosterone-to-

636 Renin-Ratio; AA2-R, Aldosterone-to-Ang II-Ratio; ACE, Angiotensin-Converting-Enzyme;
637 PRA, Plasma-Renin-Activity; -S, RAAS Triple-A derived marker).
638
639 **Table 2: Diagnostic performance and cut-off values.**

Diagnostic Ratio			Cut-Off	YI	FP	FN	Sens [%]	Spec [%]
ARR (R/R) =	Aldosterone (RIA)	[ng/dl]	62.1	0.82	11	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]	26.0	0.83	12	0	100	83
ARR (M/M) =	Aldosterone (MS)	[ng/dl]	61.1	0.87	3	4	90	97
	PRA (MS)	[(ng/ml)/h]	30.0	0.73	15	2	93	80
ARR-S =	Aldosterone (MS)	[pM]	5.1	0.86	4	4	90	96
	Ang I + Ang II (MS)	[pM]	3.0	0.81	13	2	97	84
AA2-R =	Aldosterone (MS)	[pM]	6.6	0.83	8	4	90	93
	Ang II (MS)	[pM]	3.9	0.81	15	1	97	84

640
641 *Legend to Table 2* – Youden-Indices (YI), number of false-positives (FP), number of false-
642 negatives (FN), sensitivity and specificity for five diagnostic ratios for PA are shown for
643 indicated cut-off values obtained from ROC analysis of patients with ACE-S > 1.0 pM/pM
644 (N= 100). The higher cut-off value for each diagnostic ratio indicates the value corresponding
645 to the maximum of the YI curve shown in Supplementary Figure 3. The lower cut-off value
646 was obtained by minimizing the number of false-negatives for each ratio (ARR, Aldosterone-
647 to-Renin-Ratio; AA2-R, Aldosterone-to-Ang II-Ratio; -S, RAAS Triple-A derived marker).

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