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DIAGNOSTIC ACCURACY OF ALDOSTERONE AND RENIN MEASUREMENT BY CHEMILUMINESCENT IMMUNOASSAY AND RADIOIMMUNOASSAY IN PRIMARY ALDOSTERONISM.

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Short title: renin and aldosterone assays in primary aldosteronism

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Abstract

Objective: up to 50% of hypertensives should be screened for primary aldosteronism (PA), using the aldosterone to renin (or plasma renin activity, PRA) ratio (AARR and ARR, respectively). Aim of the study was to prospectively compare the diagnostic accuracy of AARR (measured by chemiluminescent immunoassay) and ARR (measured by radioimmunoassay) as screening tests for PA and aldosterone assays (measured by chemiluminescence and radioimmunoassay) during confirmatory testing. **Methods**: 100 patients were screened for PA and 34 underwent confirmatory testing. The cut-offs for ARR and AARR were 30 ng/dL/ng/mL/h and 3.7 ng/dL/mU/L, respectively. Patients with positive confirmatory test underwent subtype diagnosis. **Results**: 75 patients were essential hypertensives, 15 had idiopathic hyperaldosteronism, 5 an aldosterone producing adenoma (APA) and 5 with undefined diagnosis. The AARR displayed a sensitivity of 90% and a specificity of 99%, the ARR had a sensitivity of 100% and a specificity of 73%. Of the 2/20 PA patients missed by AARR none resulted affected by APA. All PA patients were correctly diagnosed by chemiluminescence at confirmatory testing. In the total sample of 168 measurements both the correlation for PRA with renin and for aldosterone in chemiluminescence and radioimmunoassay were highly significant (Rho=0.70, $p<0.001$ and Rho=0.78, $p<0.001$, respectively). On ROC curves, the AUC for AARR was 0.989 (95% CI 0.97-1) and 0.934 for ARR (95% CI 0.89-0.98), which were not significantly different. **Conclusions**: the automated aldosterone and renin chemiluminescent assay is a reliable alternative to the radioimmunometric method, especially for APA detection.

Condensed Abstract

We measured the aldosterone to renin ratio and compared the diagnostic accuracy of AARR (by chemiluminescent immunoassay) and ARR (by radioimmunoassay) for diagnosis of primary aldosteronism. Both the correlation for the PRA with renin and for aldosterone in

chemiluminescence and radioimmunoassay were highly significant. On ROC curves, the AUC for AARR and for ARR were not significantly different. The automated aldosterone and renin chemiluminescent assay is a reliable alternative to the radioimmunometric method.

Key words: primary aldosteronism, aldosterone, renin, plasma renin activity, aldosterone-producing adenoma

Abbreviation definition list: AARR= aldosterone to active renin ratio; ARR= aldosterone to plasma renin activity ratio; CL= chemiluminescence; RIA radioimmunoassay; PRA= plasma renin activity; PA= primary aldosteronism; AC= aldosterone concentration; DRC= direct renin concentration; ES= Endocrine Society; AVS= adrenal venous sampling; CT= computed tomography; GRA= glucocorticoid-remediable aldosteronism; A/C= aldosterone/cortisol ratio; RENATO = RENin and Aldosterone measurements in hypertensives patients in Torino; APA= aldosterone-producing adenoma; IHA= idiopathic hyperaldosteronism; EH= essential hypertension; EQA= External Quality Assessment; CV= coefficient of variation; LC-MS/MS= liquid chromatography associated with tandem mass spectrometry

Introduction

Primary aldosteronism (PA) is the most common cause of secondary hypertension[1]. The diagnosis of PA is important because it is associated with an increased cardio- and cerebro-vascular risk [2-4] compared to essential hypertension that can be reversed with targeted therapy [5].

According to Endocrine Society (ES) guidelines, up to 50% of hypertensive patients should be screened for PA[1]; nowadays, the most reliable screening test is the aldosterone-to-renin ratio (ARR)[1], using the aldosterone concentration (AC) and plasma renin activity (PRA), even though both measurements are affected by several confounding factors[1,6] such as pharmacological therapies[7].

The traditional PRA radioimmunoassay involves the measurement of angiotensin I generated from angiotensinogen and plasma renin activity is thus calculated as the amount of angiotensin I produced as a function of time. Whilst this procedure is very sensitive, it has the disadvantage of being manual, time-consuming and produces radioactive waste.

Several studies[8-11] have demonstrated that it is also possible to use the direct renin concentration (DRC) to calculate the aldosterone-to-active renin ratio (AARR) instead of the PRA as a screening test for PA. AARR and ARR are both accurate and reproducible, if performed under standardized conditions[1,8,12]; moreover DRC can be measured directly on automated platforms and it is simpler and less time-consuming than a PRA assay.

Case-finding, case-confirmation and subtype differentiation tests in PA management are all dependent on the measurement of AC and/or PRA/DRC[1] and therefore the accuracy and reproducibility of hormonal assays is fundamental to obtain reliable diagnoses. Liquid chromatography with tandem mass spectrometry detection and chemiluminescence-based methods[9,13,14] have become available over recent years and in many laboratories these methods are currently used instead of the traditional radioimmunometric assays.

The aim of this study (RENin and Aldosterone measurements in hypertensives patients in TOrino, the RENATO study) was to prospectively compare the diagnostic accuracy of AARR (calculated through AC and DRC, measured with chemiluminescent assay, CL) and ARR (calculated through AC and PRA, measured with classical radioimmunometric assay, RIA) as screening tests for PA and the RIA and CL aldosterone assays also during confirmatory test in patients with a positive screening test.

Study design and Methods

Patients selection

We prospectively recruited 100 hypertensive patients with suspected PA referred to our hypertension center from April 2014 to November 2014 (figure 1). Of the 100 patients screened for PA, 34 underwent confirmatory testing. We performed 26 intravenous saline loading tests and 8 captopril challenge tests. We performed captopril test in patients who were at increased risk of acute volume overload resulting from saline administration. The cut-off levels for a positive AARR were 3.7 (ng/dL/mU/L) (102.6 pmol/L/mU/L) and 30 (ng/dL/ng/mL/h) (832.2 pmol/L/ng/mL/h) for a positive ARR, together with AC ≥ 10 ng/dL (277.4 pmol/L). A table with cut-offs for screening and confirmatory tests in traditional and SI units is available in the supplemental file (supplemental table S1). Patients who tested positive to at least one of the two screening tests (AARR with CL, or ARR with RIA) underwent confirmatory testing. The confirmatory saline infusion test consisted of an intravenous saline load (2 L of 0.9% NaCl infused over 4 hours) that was carried in seated position[15] that was considered positive if post-test aldosterone levels were higher than 5 ng/dL (138.7 pmol/L) [16,17]. For patients undergoing captopril test, 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 and AARR higher than 3.7 (ng/dL/mU/L) (102.6 pmol/L/mU/L).

For confirmatory testing, if the aldosterone levels measured by RIA and CL methods resulted in a discordant final diagnosis, we excluded the patients from final analysis as undefined (all captopril tests were concordant) (supplemental table S4). All patients with a confirmed diagnosis of PA underwent subtype diagnosis by adrenal CT scanning and adrenal venous sampling (AVS), according to ES guidelines[1] (figure 1). An expanded method section with detailed diagnostic work-up is described in the supplemental file.

Overall (including both screening and confirmatory tests) we prospectively analyzed 168 samples: on each sample we measured AC, PRA and DRC. The approval for the RENATO study was obtained by the local Ethics Committees and fully informed written consent was signed by all patients.

Biochemical measurements

Samples were collected in the morning after patients had been out of bed for at least 2h and then been seated for at least 15 min before venepuncture.

For PRA, samples were collected into prechilled tubes containing EDTA, immediately centrifuged (3000 rpm, 15 min, 28°C) and the plasma frozen at -20°; for AC (serum) and DRC (plasma EDTA), samples were collected into room temperature tubes, centrifuged (3000 rpm, 15 min, 27-28°C) and frozen at -20°C.

AC by RIA was assessed by solid-phase radioimmunoassay ALDOCTK-2 (DiaSorin, Saluggia, Italy). Within-run precision tests yielded coefficient of variation (CV) of 12.0% and 9.8% on samples with mean aldosterone values of 283 and 1040.3 pmol/L, respectively.

PRA was measured using the RENCTK RIA kit (DiaSorin, Saluggia, Italy) according to the manufacturer's instructions. Two aliquots of each sample (one kept at 4°C and the other at 37°C during incubation) were assayed for angiotensin I, and PRA was calculated by subtracting the value of angiotensin I measured at 4°C from that determined at 37°C. Within-run precision tests yielded

CV of 9.1%, and 7.8% on samples with mean PRA values of 1.30 and 7.25 ng/mL/h, respectively. The analytical sensitivity was 0.1 ng/mL/h. Samples with values below the analytical sensitivity were re-assayed after 18 hours of incubation.

DRC was measured with a chemiluminescent immunometric method (LIAISON®, DiaSorin, Saluggia, Italy) applied to a fully automated analyzer. The intra-assay CVs were 5.0% and 4.8% in control plasma samples containing 27.2 mU/L and 96.5 mU/L of DRC, respectively. The functional sensitivity was 0.33 mU/L. The limit of detection was <2.0 mU/L. AC was measured using the fully automated LIAISON® aldosterone chemiluminescent immunoassay (DiaSorin, Saluggia, Italy). This assay has a wide measuring range from 26.9 (analytical sensitivity) up to 2774 pmol/L, with a functional sensitivity of 52 pmol/L. Intra-assay CV% are < 4.2 as well as < 10.1 on samples with mean aldosterone concentrations of 294 and 1101.3 pmol/L, respectively. For both AC measurements by RIA and CL, the procedures do not include a pre-extraction step, which may explain the overestimation of AC with respect to liquid chromatography.

Statistical analysis

IBM SPSS Statistics 22 was used for statistical analyses. Data were analyzed with the Kolmogorov-Smirnov and Shapiro-Wilk test to determine their distributions. Normally distributed variables (age, SBP, DBP and K^+) are expressed as mean ± SD; non-normally distributed variables (PRA, DRC and AC) are expressed as median (25th to 75th percentile). DRC, PRA and AC were analysed after achievement of a normal distribution by natural logarithm transformation. ANOVA analysis of variance followed by Bonferroni's post-hoc test was used to compare variables with a normal distribution, whereas Mann-Whitney's and Kruskal-Wallis's tests were used for non parametric variables. We compare DRC measured by CL versus PRA measured by RIA and aldosterone measured by CL versus RIA with correlation analysis (Pearson's "R" correlation test), linear regression and Passing and Bablok regression (performed using MedCalc Software, Ostende,

Belgium). To compare the within-patient relationship between aldosterone measured by CL or RIA, we used Bland-Altman plots. We used the Bland-Altman plot to detect systematic error, proportional error or a magnitude dependent bias. To assess the diagnostic accuracy of AARR and ARR 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.

Results

Description of the population

Clinical characteristics of the patients included in the study are summarized in Table 1. Our prospective cohort comprises 100 patients, 23 untreated and the remaining receiving non-interfering therapy. The final diagnosis was essential hypertension (EH) in 75 patients and PA in 20 patients and 5 patients with undefined diagnosis. The PA patients comprised 15 with bilateral adrenal hyperplasia (also called idiopathic hyperaldosteronism, IHA) and 5 with aldosterone producing adenoma (APA).

According with the typical phenotype of PA patients, the main clinical and demographic characteristics were higher serum aldosterone levels, lower PRA/DRC and lower potassium levels in PA patients compared to EH ($P < 0.001$ for all comparisons).

Comparison between DRC and PRA

In our cohort of patients median DRC was 14.3 mU/L (19.8 mU/L in EH and 3.3 mU/L in PA patients), whereas median PRA was 0.59 ng/mL/h (0.97 ng/mL/h in EH and 0.11 ng/mL/h in PA patients) (Table1). To assess the within-patient correlation, we compared DRC by CL with PRA by RIA; both screening test and confirmatory testing data were used for this comparison (n = 168);

results below PRA assay sensitivity were assigned the arbitrary value of 0.1 ng/mL/h (in 7 cases the measurement of PRA after 18 h incubation resulted in values below 0.15).

The DRC and PRA values showed a significant within-patient correlation (R = 0.7; $P < 0.001$). After conversion of the data to natural logarithms to obtain a normal distribution we performed a linear regression ($R^2 = 0.532$), (Figure 2). Subsequently, we repeated the same analysis including only PRA values < 1 ng/mL/h and DRC < 12 mU/L (n = 129); the correlation was lower but still significant (R = 0.3; $P = 0.001$); a linear regression displayed an increased value dispersion ($R^2 = 0.092$). The regression line equations are given in the legend to Figure 2 and in the supplemental table S2. The Bland-Altman plot of the *Z* score for PRA and DRC is provided in the supplemental figure S1.

Comparison between aldosterone concentrations in CL and RIA

In our population the median AC (measured by CL) was 375.9 pmol/L (313.5 pmol/L in EH and 558.9 pmol/L in PA patients), whereas median AC measured by RIA was 471.6 pmol/L (416.1 pmol/L in EH and 707.3 pmol/L in PA patients). In the overall sample (n = 168) the correlation for aldosterone in CL and RIA was highly significant (R = 0.782; $P < 0.001$); the linear regression ($R^2 = 0.604$) is shown in Figure 3; if only patients with AC ≤ 10 ng/dL (corresponding to 277.4 pmol/L) (n = 66) are considered, the correlation and linear regression are R = 0.555 ($P < 0.001$) and $R^2 = 0.279$, respectively; the regression line equations are given in the legend to Figure 3 and in the supplemental table S2.

Passing and Bablok regression analysis yielded the following equation: RIA = -118.41 (95% CI, -171 to -66.8) + 1.49 (1.34 to 1.67) x CL, with a significant deviation from linearity and a systematic underestimation by the CL method, although less evident on samples lower than 10 ng/dL. We also performed a Bland-Altman plot (Figure 4): in the overall 168 samples, the mean difference between AC by RIA and AC by CL was 96.8 pmol/L (95% CI -408.9 – 602.5), whereas if only patients with

AC ≤ 10 ng/dL (corresponding to 277.4 pmol/L) are considered, the mean difference displays a negative trend (-46.6; 95% CI -196 – 102.8). There is a mean 7.9% overestimation by RIA on the whole range of values, whereas CL provided higher results on samples with aldosterone concentrations ≤ 277.4 pmol/L.

Data from External Quality Assessment (EQA) reports show wide variability of AC depending on the assay used and also within the same method of measurement. In general all RIA and CL methods tend to overestimate AC with respect to the liquid chromatography/mass spectrometry method. Further information about EQA are given in the supplemental result section.

Diagnostic accuracy of AARR versus ARR

We calculated the aldosterone (measured by CL)-to-DRC (AARR) and the aldosterone (detected by RIA) -to-PRA ratio (ARR) using as cut-off levels for a positive AARR and ARR 3.7 (ng/dL/mU/L) (102.6 pmol/L/mU/L) and 30 (ng/dL/ng/mL/h) (832.2 pmol/L/ng/mL/h), respectively. For PA diagnosis, AARR displayed a sensitivity of 90% and a specificity of 98.7% (positive predictive value 94.7%; negative predictive value 97.4%), whereas the ARR had a sensitivity of 100% and a specificity of 73.3% (positive predictive value 50%; negative predictive value 100%). Of the 2/20 PA patients missed by AARR, none resulted to be affected by APA, therefore sensitivity of both methods on APA recognition were 100%.

To assess the diagnostic accuracy of the two assay we used receiver operator characteristics (ROC) curves (Figure 5); the area under the curve (AUC) for AARR was 0.989 (95% CI 0.974-1) and for ARR 0.934 (95% CI 0.885-0.982); AUC values were not significantly different.

In literature different cut-offs are used, tailored on laboratory experience, average sodium intake, assay methods and ethnicity[1]. For these reason the guidelines do not suggest a specific AARR and ARR cut-off for PA case detection[1]. Sensitivity, specificity, positive and negative predictive values with different cut-offs are given in the supplemental table S3. For case detection, that requires a

high sensitivity, we suggest an AARR between 1-2.7 ng/dL/mU/L (corresponding to 27.7-74.9 pmol/L/mU/L) and for the ARR of 30 ng/dL/ng/mL/h (corresponding to 832.2 pmol/L/ng/mL/h).

CL versus RIA performance on confirmatory tests for PA

In our analysis we investigated 34 patients who underwent confirmatory testing; in 85.3% of cases (n = 29) the results by CL and RIA assay were in agreement (either both positive or negative), whereas in 14.7% of cases (n = 5) were discordant; 20 patients (58.8%) had a positive result to both confirmatory tests (CL and RIA), whereas 9 patients (26.5%) had a negative result to both confirmatory tests. The 5 patients with discordant results all had a positive result with CL assay and negative with RIA; these patients were excluded from further analysis (Figure 1). Hormonal data of these 5 patients are given in the supplemental table S4. The effect of inclusion of these patients in the group with PA or EH in the diagnostic performance of the tests is discussed in the supplemental file and supplemental table S5. Overall, 20 patients underwent subtype differentiation by CT scanning and adrenal venous sampling: 5 patients were diagnosed with unilateral PA due to APA and 15 with IHA.

Discussion

According to the ES guidelines[1] the diagnosis of PA is a three step process, comprising screening, confirmatory testing and subtype differentiation. Screening for PA is recommended in up to 50% of hypertensive patients, including all hypertensives with grade 2-3 and resistant hypertension and hypertensives with hypokalemia independent of blood pressure levels[1,18].

In light of the high prevalence of PA and the associated increase in cardiovascular risk, it is of fundamental importance to have sensitive, simple and highly reproducible assays to diagnose this condition and start a timely targeted therapy. The most reliable means to screen for PA is currently the ARR, which is highly affected by anti-hypertensive medications and testing conditions[1].

Aldosterone and PRA have been traditionally measured by RIA[19,20], which displays many critical issues, such as long incubation time, production of radioactive waste, need to analyze several samples together to minimize the cost and employment of dedicated laboratory staff. More recently, other competitive immunoassay methods have been set up for AC and renin measurements[21,22]: gas-chromatography or liquid chromatography with mass spectrometry detection techniques have excellent sensitivity and specificity, nevertheless they require a specific sample preparation and specialized staff and they are more complex, expensive and time consuming[13,23]. For these reasons these techniques are not widely employed in clinical practice.

In our prospective study comprising 168 samples DRC and PRA values displayed a good overall correlation between these variables. It should be underlined that in our cohort we had a high prevalence of patients with low-renin hypertension (63%), which is attributable to a referral bias to our center: therefore, the correlation curves would be expected to display a higher correlation within the general hypertensive population in which the prevalence of low-renin hypertension is not higher than 30%[24]. Regression curves demonstrated a weaker correlation for low renin values as expected[9]; this finding may be partly explained by the sensitivity of the RIA assay (lower limit was 0.1 ng/mL/h). Moreover, prorenin circulates in significantly higher concentrations (10 to 100-fold) compared to the active enzyme; cross reaction between pro-renin and renin has been previously described[25] and it may interfere in DRC measurement: a recent study demonstrated an increase in DRC proportional to the amount of exogenous pro-renin added[9]. For this reason, the interference by pro-renin it is expected to be higher in the lower range concentrations of renin assays.

For both PRA and DRC measurements, cryoactivation has the potential of causing error in the assay; at a temperature of 4-6 degrees, pro-renin undergoes a reversible conformational change involving the exposition of the active site that results in increased renin activity and overestimation of PRA and DRC values[26]. In the current study, temperatures for potential cryoactivation or intrinsic activity of the enzyme, were carefully avoided. Another confounding factor when

comparing PRA with DRC, is that the level of angiotensinogen is not identical in all patients. This will affect PRA and therefore potentially reduce the correlation between the two assays.

However, even considering all these limitations, the automated CL method for DRC measurement displayed a satisfactory accuracy in the detection of PA patients and therefore it can be successfully used in clinical laboratories that want to replace the RIA method.

Also the correlation between AC by RIA and by CL displayed a good correlation, as reported in literature[27], with a dispersion of values only for very low or very high levels of AC. When measured by CL, AC mean values are lower than those in RIA, as already observed by others[20]; however, for very low AC values this trend reversed with higher AC values by the CL method. This should be taken into account since it could be associated with an underestimation of the AARR during screening testing and overestimation of the AC during confirmatory testing with CL.

The ES guidelines for PA diagnosis and management[1], recommend both ARR (with PRA) and AARR (with DRC) for patient detection. Several authors have recently compared ARR and AARR using in both cases AC measured by RIA and observed similar diagnostic accuracy[8,11,28]. Our study compared for the first time simultaneously the diagnostic accuracy of ARR, with PRA and AC measured by RIA, and AARR, with DRC and AC measured by CL. We confirmed a similar diagnostic performance in the case detection with ROC curves that were not significantly different as observed by others when the AC was measured with the same method for both ARR and AARR[8]. It should be highlighted that all patients with an APA were detected by both methods at screening and confirmed by AC measurement by both RIA and CL. The AARR missed only 2 patients with IHA at screening: these patients displayed a very mild hormonal and clinical phenotype, and therefore the distinction with a status of low-renin EH is not possible.

To confirm or exclude PA diagnosis we performed the intravenous saline load test in seated position or the captopril test[1,15]. PA patients with a discordant diagnosis of PA versus low-renin

essential hypertension with one of the methods all belong all to a group with a mild phenotype which could be considered a grey zone for the overlapping of the two conditions.

The strengths of our study are: in addition to screening patients for PA we have also confirmed/excluded the diagnosis with a confirmatory test (in most cases seated intravenous saline load) and in all confirmed PA we performed AVS to differentiate PA subtypes. We have studied a large sample of patients (n=100) referred to a single Hypertension Unit and therefore all samples were handled in the same way thereby excluding potential pre-analytical variation intrinsic in the multicenter studies; previous studies have analyzed the performance of the aldosterone assay by RIA and CL or compared the diagnostic accuracy of the ARR and AARR using the measurement of the PRA by RIA and DRC by CL, respectively, together with the AC by RIA: in our study we simultaneously compared the accuracy of the AARR with both DRC and AC measured by CL and the ARR with both PRA and AC measured by RIA; we also compared the AC after confirmatory testing in 34 patients who tested positive with one of the two screening test; all samples were carefully handled to avoid pre-analytical errors and were measured with a maximum delay of 1 week (AC by RIA or CL; DRC and PRA).

Potential limitations of our study are: a relatively low number of APA patients (5%) which are the only robust diagnosis of PA since the limit between IHA and low-renin essential hypertension comprises a indefinite grey zone.

Our study demonstrated a robust and comparable diagnostic performance of the AARR measured by a chemiluminescence method in comparison with the classical radioimmunometric method used for case detection and confirmation of PA; this is of particular importance for the progressively increasing widespread use of the aldosterone and renin measurement requiring automated, reliable and non-radioactive methods.

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Legends to figures.

Legend to figure 1

Figure 1. Patients selection

For description of the diagnostic work-up, see text.

EH, essential hypertension; IHA, idiopatic hyperaldosteronism; APA, aldosterone-producing adenoma; CL, chemiluminescence; RIA, radioimmunoassay.

Legend to figure 2

Figure 2. DRC by CL versus PRA by RIA regression curve

PRA, plasma renin activity (expressed in ng/mL/h); DRC, direct renin concentration (expressed in mU/L); CL, chemiluminescence; RIA, radioimmunometric assay; Lg, natural logarithm; EH, essential hypertension; PA, primary aldosteronism; Und, undefined. On *X*-axis PRA by RIA natural logarithm; on *Y*-axis DRC by CL natural logarithm; circles: EH; triangles: PA; squares: Und; dashed lines: confidence interval; continuous line: regression curve. N = 168; R^2 = 0.532; Y = 2.88 + 0.69*X.

Legend to figure 3

Figure 3. AC by CL versus RIA regression curve

AC, aldosterone concentration (expressed in pmol/L); CL, chemiluminescence; RIA, radio-immunometric assay; Lg, natural logarithm; EH, essential hypertension; PA, primary aldosteronism; Und, undefined. On *X*-axis AC by RIA natural logarithm; on *Y*-axis AC by CL natural logarithm; circles: EH; triangles: PA; squares: Und; dashed lines: confidence interval; continuous line: regression curve. N = 168; $R^2 = 0.604$; Y = 3.11 + 0.47*X.

Legend to figure 4

Figure 4. AC by CL versus RIA Bland-Altman plot

AC, aldosterone concentration (expressed in pmol/L); CL, chemiluminescence; RIA, radio-immunometric assay; Lg, natural logarithm; EH, essential hypertension; PA, primary aldosteronism; Und, undefined. On *X*-axis mean of AC measurement by CL and RIA assays; on *Y*-axis difference between AC measurement by CL and RIA assays. Circles: EH; triangles: PA; squares: Und. Continuous line indicates mean difference between AC measurement by CL and RIA; dashed lines indicate difference mean value ± 1.96 standard deviations (IC 95%).

Legend to figure 5

Figure 5. ROC curves for AARR and ARR

On *X*-axis 1 - Specificity; on *Y*-axis Sensitivity. The analysis was performed using screening test values from the 100 patients included in the study. Dashed line: ROC curve for AARR; AUC was 0.989 (95% CI 0.974-1). Continuous line: ROC curve for ARR; the AUC was 0.934 (95% CI 0.885-0.982). AUC values are not significantly different ($P > 0.05$).

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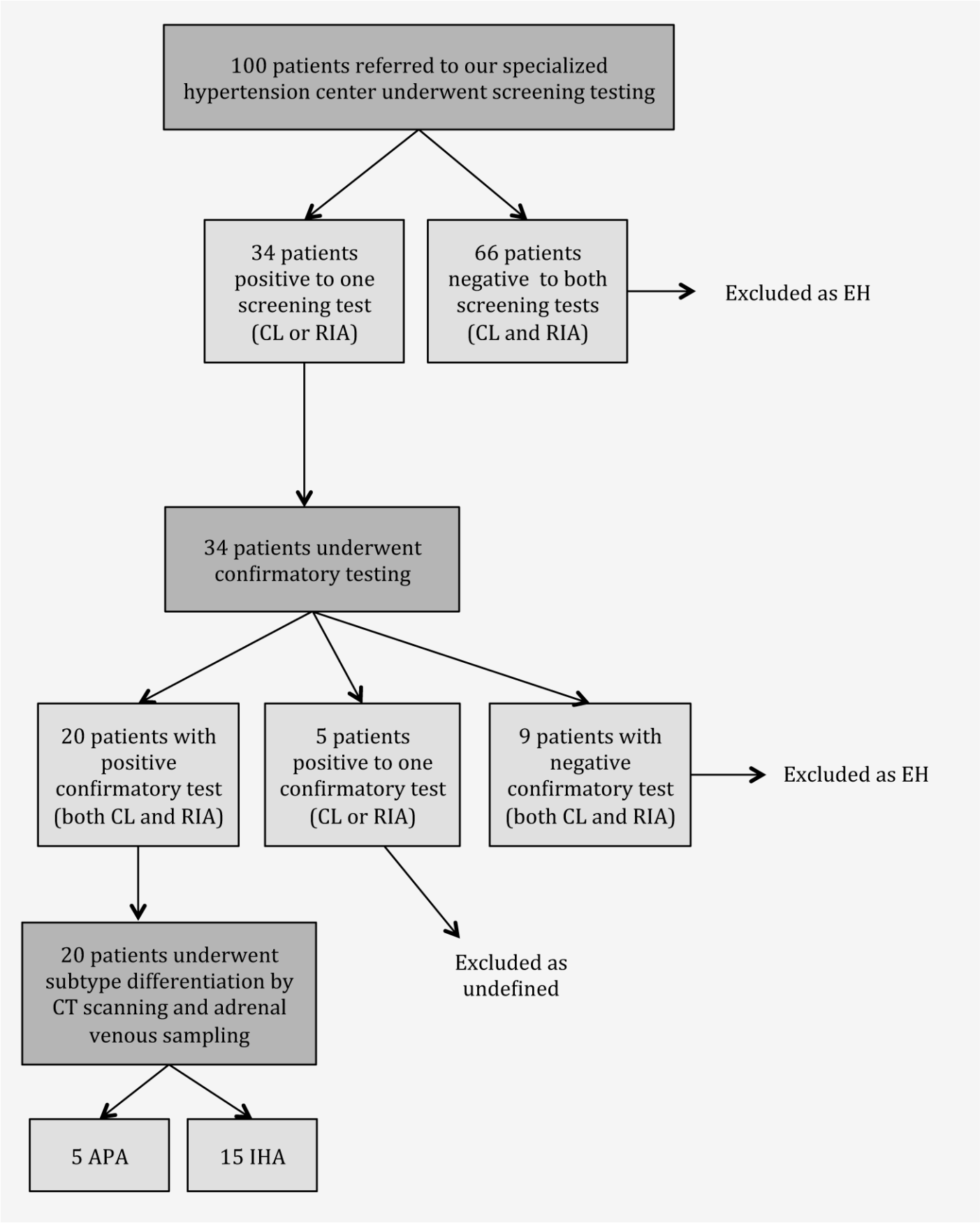
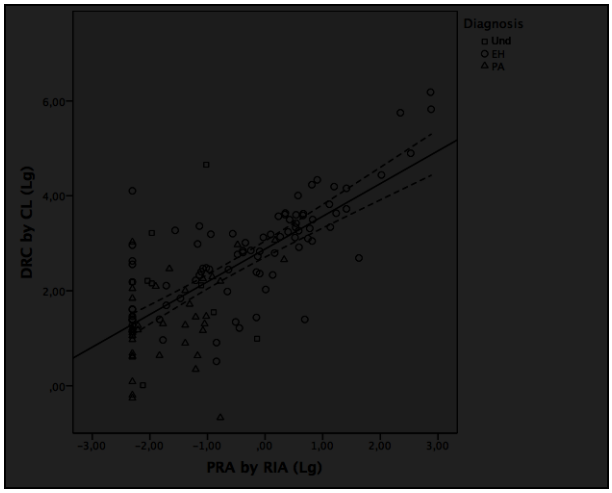
Total	**EH**	**PA**	***P*-value EH vs PA**

Number of patients	100	75	20	-
Age (years)	49 ± 11	48 ± 11	54 ± 7	0.010
Sex (%) (M/F)	54 / 46	56 / 44	50 / 50	0.239
SBP (mmHg)	147 ± 17	146 ± 17	154 ± 18	0.053
DBP (mmHg)	91 ± 10	92 ± 9	92 ± 11	0.957
K^+ (mEq/L)	4.0 ± 0.5	4.2 ± 0.4	3.6 ± 0.6	< 0.001
PRA by RIA (ng/mL/h)	0.59 [0.15-1.71]	0.97 [0.32-1.99]	0.11 [0.10-0.28]	< 0.001
DRC by CL (mU/L)	14.3 [4.3-28.2]	19.8 [10.3-35.5]	3.3 [2.7-4.1]	< 0.001
AC by RIA (pmol/L)	471.6 [332.9-714.3]	416.1 [249.7-610.3]	707.3 [513.2-873.8]	< 0.001
AC by CL (pmol/L)	375.9 [269.8-529.1]	313.5 [249.7-460.5]	558.9 [476.4-630.4]	< 0.001
Pharmacological Wash-out (%)	23	25.3	20.0	-

Table 1 – Characteristics of the population screened

EH, essential hypertension; PA, primary aldosteronism; M/F, Male/Female; SBP, systolic blood pressure; DBP, diastolic blood pressure; K^+, potassium; PRA, plasma renin activity; DRC, direct renin concentration; AC, aldosterone concentration; RIA, radioimmunoassay; CL, chemiluminescence. Age, SBP, DBP, K+ are expressed as mean ± standard deviation; PRA, DRC and AC are expressed as median [25th-75th percentiles]. Gender and patients in pharmacological wash-out are expressed as percentage values.


100 patients referred to our specialized hypertension center underwent screening testing
34 patients positive to one screening test (CL or RIA)
66 patients negative to both screening tests (CL and RIA)
Excluded as EH
34 patients underwent confirmatory testing
20 patients with positive confirmatory test (both CL and RIA)
5 patients positive to one confirmatory test (CL or RIA)
9 patients with negative confirmatory test (both CL and RIA)
Excluded as EH
Excluded as undefined
20 patients underwent subtype differentiation by CT scanning and adrenal venous sampling
5 APA
15 IHA


Diagnosis
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EH
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DRC by CL (Lg)
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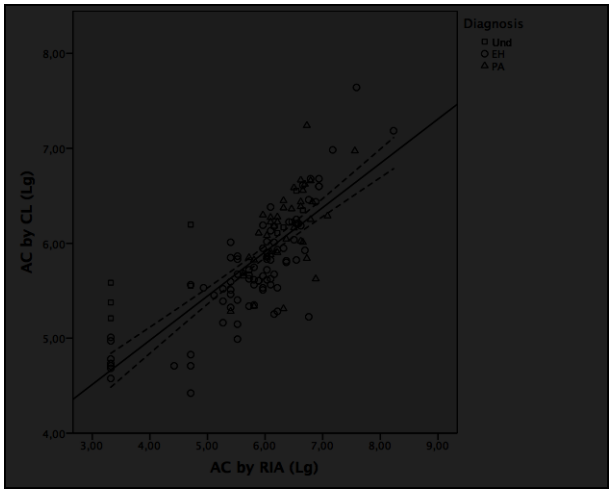
PRA by RIA (Lg)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EH
PA
AC by CL (Lg)
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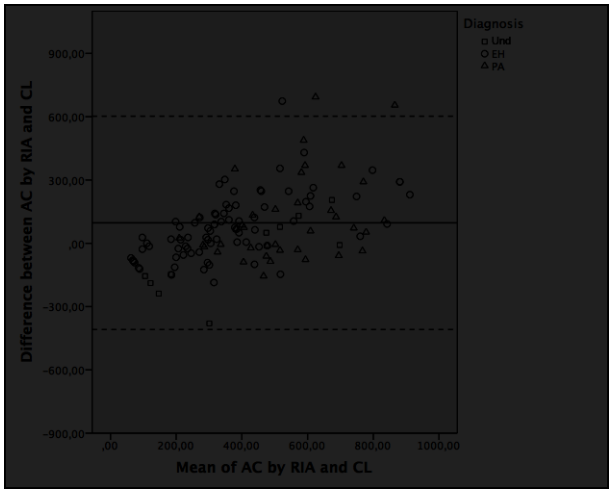
AC by RIA (Lg)


Diagnosis
Und
EH
PA
Difference between AC by RIA and CL
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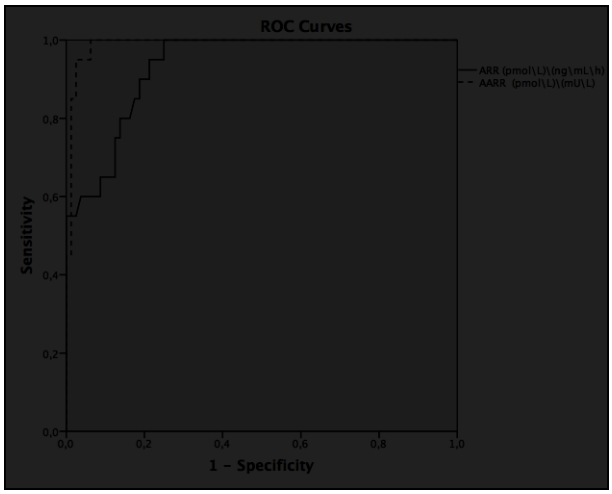
Mean of AC by RIA and CL



SUPPLEMENTAL FILE

DIAGNOSTIC ACCURACY OF ALDOSTERONE AND RENIN MEASUREMENT BY CHEMILUMINESCENT IMMUNOASSAY AND RADIOIMMUNOASSAY IN PRIMARY ALDOSTERONISM.

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Short title: renin and aldosterone assays in primary aldosteronism

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Supplemental method section

We prospectively recruited 100 hypertensive patients with suspected PA referred to our hypertension centre from April 2014 to November 2014 (figure 1). Of the 100 patients screened for PA, 34 underwent confirmatory testing.

Patients were screened for PA after withdrawal of interfering medications; patients remained under the same therapy during the entire diagnostic work-up (from screening to AVS). Patients were left to have a liberal sodium intake to avoid activation of the renin-angiotensin system and to have a better accuracy of the captopril test when necessary, as demonstrated previously in the PAPY study, where it performed similarly to the intravenous saline load test[1]. When possible, all antihypertensive drugs were stopped at least 3 weeks before the aldosterone and DRC/PRA measurements; diuretics and spironolactone were stopped at least 6 and 8 weeks before measurements, respectively. Patients who could not remain untreated received the α-blocker doxazosin and/or the non-dihydropiridine calcium channel blocker verapamil. Potassium levels were measured before the screening test and for hypokalemic patients (n=12, potassium levels below 3.6 mEq/L), potassium supplementation was provided and potassium levels checked again. After potassium supplementation, only 1 patient still displayed low potassium levels, who was subsequently diagnosed as having an APA. Therefore, we are confident that hypokalemia did not interfere with the diagnostic procedure of patients included in the present study.

For confirmatory testing, if the aldosterone levels measured by RIA and CL methods resulted in a discordant final diagnosis, we excluded the patients from final analysis as undefined (all captopril tests were concordant) (supplemental table S4).

All patients with PA were screened for GRA using a long-PCR technique[2].

Subtype diagnosis was performed by CT scanning with contrast and fine cuts of the adrenal and subsequent AVS according to ES guidelines[3] (figure 1). Sampling was considered successful if the adrenal vein/inferior vena cava cortisol gradient was at least 3[4] and lateralization was defined as an aldosterone/cortisol ratio value (A/C) from one adrenal at least 4 times the ratio from the other adrenal gland, or 3 times the A/C of the contralateral with the A/C in the contralateral less than the A/C in the peripheral vein[4]. A final diagnosis of APA was considered proven, providing that all the following conditions were satisfied: 1) histological demonstration of adenoma, 2) normalization of hypokalemia, 3) cure or improvement of hypertension, and 4) normal ARR and suppression of aldosterone levels under saline load.

Supplemental Results

Between-method variability for aldosterone measurement could be observed from *External Quality Assessment (EQA)* reports. According to 2015 final evaluation of Immunocheck Qualimedlab srl (EQAS CNR, Pisa), total CV, taken as an index of between-method agreement, was 27%, 20% and 14% on specimen with mean aldosterone concentrations, calculated as the consensus among different assays, of less than 83.2, between 83.2 to 221.9 and higher than 221.9 pmol/L, respectively. Interestingly, when focusing on some samples, between-method variability may result up to 42% for a mean aldosterone level of 244.1 pmol/L (reported values from 33.3 to 457.7 pmol/L) and up 36.1% for a mean aldosterone concentration of 432.7 pmol/L (from 38.8 to 840.5 pmol/L). EQAS reports also indicated CVs ranging from 8.7% to 51.4% within the same method. Till now, very few laboratories adopted LC-MS/MS (liquid chromatography associated with tandem mass spectrometry) to measure aldosterone. These preliminary results found values that are slightly lower (-8%) with

respect to consensus mean calculated in EQAS reports on samples with aldosterone concentrations between 221.9 and 443.8 pmol/L, whereas at concentrations > 554.8 pmol/L LC-MS/MS data appeared quite similar to those obtained with the other methods. Informations about variability for this approach are still lacking due to the low number of participants, with some exercises reporting CV from 4.3% to 18.7% when using chromatographic assays.

The 5 patients with an undefined diagnosis (discordant results in the confirmatory tests) could theoretically be classified as having either all PA or alternatively all EH. The effect of the inclusion of these patients in one or the other groups of patients would affect the diagnostic performance of the ARR and AARR. The sensitivity, specificity and AUC after the inclusion of the patients are described in the supplemental Table S5.

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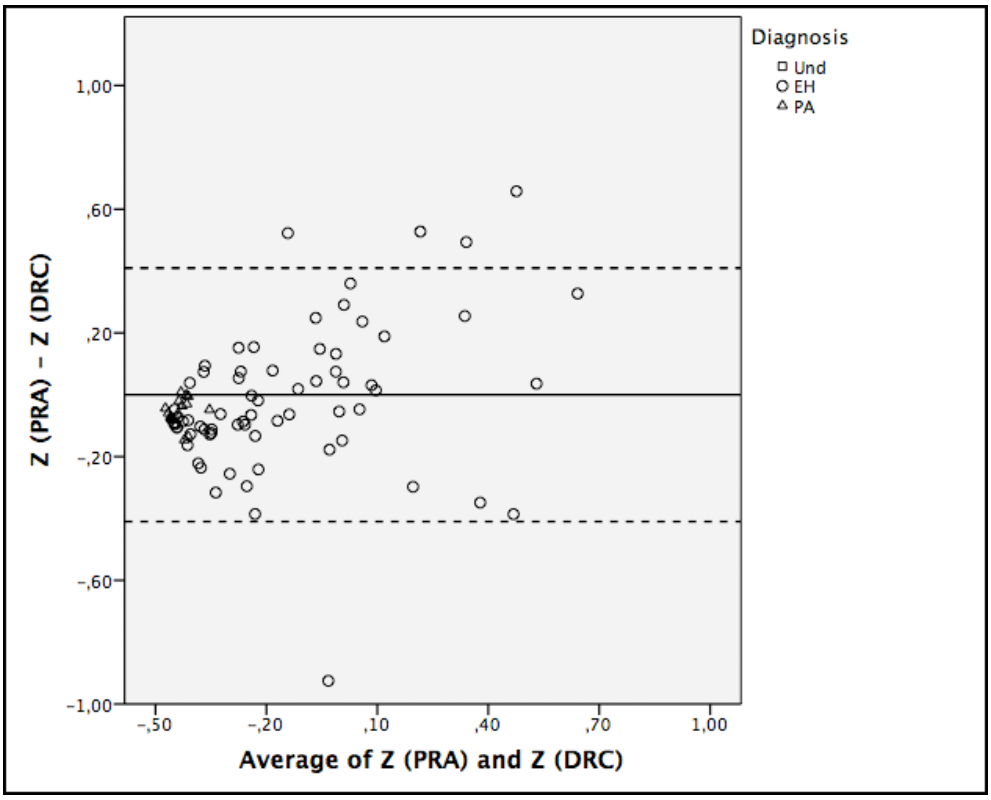
Figure S1.



Legend to Figure S1

Bland-Altman plot of the Z score for DRC and PRA. The mean value of the difference between the DRC and PRA was near zero (0.00001586).

Z score is calculated as follows: *Z* score= (X-M)/SD; X=value; M=mean; SD= standard deviation.

Table S1 – Cut-Offs for AARR, ARR and AC at screening and confirmatory testing.

		AC [ng/dL] ARR [ng/dL / ng/mL/h] AARR [ng/dL / mU/L]	AC [pmol/L] ARR [pmol/L / ng/mL/h] AARR [pmol/L / mU/L]
Screening Test (RIA)	AC	≥ 10	≥ 277.4
	ARR	≥ 30	≥ 832.2
Screening Test (CL)	AC	≥ 10	≥ 277.4
	AARR	≥ 3.7	≥ 102.6
Confirmation Test (RIA)	AC	≥ 5	≥ 138.7
	ARR	≥ 30	≥ 832.2
Confirmation Test (CL)	AC	≥ 5	≥ 138.7
	AARR	≥ 3.7	≥ 102.6

AC, aldosterone concentration; ARR, aldosterone to PRA (plasma renin activity) ratio; AARR, aldosterone to DRC (direct renin concentration) ratio; RIA, radio-immuno assay; CL, chemiluminescence.

Table S2. Regression line equations for PRA vs DRC and AC by RIA vs AC by CL

	PRA by RIA versus DRC by CL				AC by RIA versus AC by CL			
	Regression Line	**R^2 Coeff**	**Pearson's Coeff**	***P*-value**	**Regression Line**	**R^2 Coeff**	**Pearson's Coeff**	***P*-value**
All samples (N = 168)	Y = 2.88 + 0.69*X	0.532	0.700	< 0.001	Y = 3.11 + 0.47*X	0.604	0.782	< 0.001
Low Renin *[PRA < 1 ng/mL/h or DRC < 12 mU/L]* (N = 129)	Y = 2.33 + 0.36*X	0.092	0.300	0.001				
Low Aldosterone [AC ≤ 277.4 pmol/L] [N = 66]					Y = 4.32 + 0.2*X	0.279	0.555	< 0.001
Screening Test (N = 100)	Y = 2.96 + 0.67*X	0.573	0.757	< 0.001	Y = 2.56 + 0.55*X	0.627	0.792	< 0.001
Confirmation Test (N = 68)	Y = 2.44 + 0.53*X	0.183	0.428	< 0.001	Y = 3.36 + 0.42*X	0.583	0.763	< 0.001
Confirmation Test Pre–test (N = 34)	Y = 2.51 + 0.52*X	0.184	0.427	0.012	Y = 2.23 + 0.60*X	0.700	0.837	< 0.001
Confirmation Test Post–test (N = 34)	Y = 2.35 + 0.51*X	0.172	0.415	0.015	Y = 3.61 + 0.37*X	0.511	0.715	< 0.001

PRA, plasma renin activity; DRC, direct renin concentration; AC, aldosterone concentration; RIA, radio-immuno assay; CL, chemiluminescence; Coeff, coefficient; N, number of samples.

Table S3. Sensitivity, specificity, PPV and NPV with different cut-offs

Cut-off	Sensitivity	Specificity	PPV	NPV
AARR ≥ 3.7 and AC ≥ 15	80.0%	100.0%	100.0%	94.9%
AARR ≥ 2.7 and AC ≥ 15	85.0%	100.0%	100.0%	96.2%
AARR ≥ 1.0 and AC ≥ 15	90.0%	92.0%	75.0%	97.2%
AARR ≥ 3.7 and AC ≥ 10	90.0%	98.7%	94.7%	97.4%
AARR ≥ 2.7 and AC ≥ 10	95.0%	97.3%	90.5%	98.7%
AARR ≥ 1.0 and AC ≥ 10	100.0%	77.3%	54.1%	100.0%
ARR ≥ 30 and AC ≥ 15	100.0%	85.3%	64.5%	100.0%
ARR ≥ 18 and AC ≥ 15	100.0%	73.3%	50.0%	100.0%
ARR ≥ 30 and AC ≥ 10	100.0%	73.3%	50.0%	100.0%
ARR ≥ 18 and AC ≥ 10	100.0%	58.7%	39.2%	100.0%

AC, aldosterone concentration; ARR, aldosterone to PRA (plasma renin activity) ratio; AARR, aldosterone to DRC (direct renin concentration) ratio; PPV, positive predictive value; NPV, negative predictive value.

AARR is expressed in [ng/dL / mU/L]; AC is expressed in [ng/dL]; ARR is expressed in [ng/dL / ng/mL/h]. To convert in SI units multiply by 27.74 (see Table S1).

Values in grey are used in the present study.

Table S4. Patients with discordant results at confirmatory testing.

Gender (Male/ Female)	**Age** (Years)	**SBP/ DBP** (mmHg)	**K^+** (mmol/L)	**PRA (RIA) - pre** (ng/mL/h)	**AC (RIA) - pre** (pmol/L)	**DRC (CL) - pre** (mU/L)	**AC (CL) - pre** (pmol/L)	**PRA (RIA) - post** (ng/mL/h)	**AC (RIA) - post** (pmol/L)	**DRC (CL) - post** (mU/L)	**AC (CL)- post** (pmol/L)
F	56	160/100	4.0	0.9	305.1	20.1	499.6	0.1	111.0	8.9	491.0
F	53	135/85	3.9	0.1	111.0	8.9	259.9	0.1	111.0	8.9	258.0
M	55	150/95	4.3	0.2	289.2	11.3	297.5	0.3	27.7	8.3	266.3
F	37	155/100	4.9	0.7	499.3	2.3	391.1	0.1	27.7	2.0	216.4
M	43	140/95	3.7	0.8	249.7	12.3	255.2	0.4	27.7	4.7	183.1

Five patients displayed a negative saline load test with the RIA method and positive results with the CL method. These patients were considered as having an undefined final diagnosis. Three of these patients underwent AVS to exclude unilateral PA and none of them displayed lateralization of aldosterone secretion (i.e. unilateral PA).

SBP, systolic blood pressure; DBP, diastolic blood pressure; K^+, potassium; PRA, plasma renin activity; DRC, direct renin concentration; AC, aldosterone concentration; RIA, radio-immuno assay; CL, chemiluminescence; M/F, Male/Female.

Table S5. Effect of the inclusion of the patients with undefined diagnosis in the PA or EH group

		PA / EH (N=95)	**(PA + Und) / EH** (N=100)	**PA / (EH + Und)** (N=100)
Sensitivity	*ARR*	100	96	100
	AARR	90	80	95
Specificity	*ARR*	73.3	88	83.8
	AARR	98.7	100	98.8
AUC	*ARR*	0.934	0.928	0.949
	AARR	0.989	0.973	0.996

Sensitivity and specificity values are expressed as percentage.

AUC values are not significantly different between the three patients' group subdivision ($P > 0.05$)

Und, undefined; PA, primary aldosteronism; EH, essential hypertension; AUC, area under the curve; ARR, aldosterone PRA (plasma renin activity) ratio; AARR, aldosterone DRC (direct renin concentration) ratio.