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Diagnostic accuracy of aldosterone and renin measurement by chemiluminescent immunoassay and radioimmunoassay in primary aldosteronism

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(Article begins on next page)

1 **DIAGNOSTIC ACCURACY OF ALDOSTERONE AND RENIN MEASUREMENT BY**
2 **CHEMILUMINESCENT IMMUNOASSAY AND RADIOIMMUNOASSAY IN PRIMARY**
3 **ALDOSTERONISM.**

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13 **Short title:** renin and aldosterone assays in primary aldosteronism

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1 **Abstract**

2 **Objective:** up to 50% of hypertensives should be screened for primary aldosteronism (PA), using
3 the aldosterone to renin (or plasma renin activity, PRA) ratio (AARR and ARR, respectively). Aim
4 of the study was to prospectively compare the diagnostic accuracy of AARR (measured by
5 chemiluminescent immunoassay) and ARR (measured by radioimmunoassay) as screening tests for
6 PA and aldosterone assays (measured by chemiluminescence and radioimmunoassay) during
7 confirmatory testing. **Methods:** 100 patients were screened for PA and 34 underwent confirmatory
8 testing. **The cut-offs for ARR and AARR were 30 ng/dL/ng/mL/h and 3.7 ng/dL/mU/L,**
9 **respectively.** Patients with positive confirmatory test underwent subtype diagnosis. **Results:** 75
10 patients were essential hypertensives, 15 had idiopathic hyperaldosteronism, 5 an aldosterone
11 producing adenoma (APA) and 5 with undefined diagnosis. The AARR displayed a sensitivity of
12 90% and a specificity of 99%, the ARR had a sensitivity of 100% and a specificity of 73%. Of the
13 2/20 PA patients missed by AARR none resulted affected by APA. All PA patients were correctly
14 diagnosed by chemiluminescence at confirmatory testing. In the total sample of 168 measurements
15 both the correlation for PRA with renin and for aldosterone in chemiluminescence and
16 radioimmunoassay were highly significant ($Rho=0.70$, $p<0.001$ and $Rho=0.78$, $p<0.001$,
17 respectively). On ROC curves, the AUC for AARR was 0.989 (95% CI 0.97-1) and 0.934 for ARR
18 (95% CI 0.89-0.98), which were not significantly different. **Conclusions:** the automated aldosterone
19 and renin chemiluminescent assay is a reliable alternative to the radioimmunometric method,
20 especially for APA detection.

21

22 **Condensed Abstract**

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

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

4

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

7

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

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1 **Introduction**

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

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

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

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

19 Case-finding, case-confirmation and subtype differentiation tests in PA management are all
20 dependent on the measurement of AC and/or PRA/DRC⁽⁶⁾ and therefore the accuracy and
21 reproducibility of hormonal assays is fundamental to obtain reliable diagnoses. Liquid
22 chromatography with tandem mass spectrometry detection and chemiluminescence-based
23 methods^(9,13,14) have become available over recent years and in many laboratories these methods are
24 currently used instead of the traditional radioimmunometric assays.

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

7

8 **Study design and Methods**

9 *Patients selection*

10 We prospectively recruited 100 hypertensive patients with suspected PA referred to our
11 hypertension center from April 2014 to November 2014 (figure 1). Of the 100 patients screened for
12 PA, 34 underwent confirmatory testing. We performed 26 intravenous saline loading tests and 8
13 captopril challenge tests. We performed captopril test in patients who were at increased risk of acute
14 volume overload resulting from saline administration. The cut-off levels for a positive AARR were
15 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
16 positive ARR, together with AC \geq 10 ng/dL (277.4 pmol/L). A table with cut-offs for screening and
17 confirmatory tests in traditional and SI units is available in the supplemental file (supplemental
18 table S1). Patients who tested positive to at least one of the two screening tests (AARR with CL, or
19 ARR with RIA) underwent confirmatory testing. The confirmatory saline infusion test consisted of
20 an intravenous saline load (2 L of 0.9% NaCl infused over 4 hours) that was carried in seated
21 position⁽¹⁵⁾ that was considered positive if post-test aldosterone levels were higher than 5 ng/dL
22 (138.7 pmol/L)^(16,17). For patients undergoing captopril test, PA was considered confirmed when the
23 ARR was higher than 30 (ng/dL/ng/mL/h) (832.2 pmol/L/ng/mL/h) 120' after captopril 50 mg and
24 AARR higher than 3.7 (ng/dL/mU/L) (102.6 pmol/L/mU/L).

1 For confirmatory testing, if the aldosterone levels measured by RIA and CL methods resulted in a
2 discordant final diagnosis, we excluded the patients from final analysis as undefined (all captopril
3 tests were concordant) (supplemental table S4). All patients with a confirmed diagnosis of PA
4 underwent subtype diagnosis by adrenal CT scanning and adrenal venous sampling (AVS),
5 according to ES guidelines¹ (figure 1). An expanded method section with detailed diagnostic work-
6 up is described in the supplemental file.

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

11

12 ***Biochemical measurements***

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

15 For PRA, samples were collected into prechilled tubes containing EDTA, immediately centrifuged
16 (3000 rpm, 15 min, 28°C) and the plasma frozen at -20°; for AC (serum) and DRC (plasma EDTA),
17 samples were collected into room temperature tubes, centrifuged (3000 rpm, 15 min, 27-28°C) and
18 frozen at -20°C.

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

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

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

4 DRC was measured with a chemiluminescent immunometric method (LIAISON®, DiaSorin,
5 Saluggia, Italy) applied to a fully automated analyzer. The intra-assay CVs were 5.0% and 4.8% in
6 control plasma samples containing 27.2 mU/L and 96.5 mU/L of DRC, respectively. The functional
7 sensitivity was 0.33 mU/L. The limit of detection was <2.0 mU/L. AC was measured using the fully
8 automated LIAISON® aldosterone chemiluminescent immunoassay (DiaSorin, Saluggia, Italy).

9 This assay has a wide measuring range from 26.9 (analytical sensitivity) up to 2774 pmol/L, with a
10 functional sensitivity of 52 pmol/L. Intra-assay CV% are < 4.2 as well as < 10.1 on samples with
11 mean aldosterone concentrations of 294 and 1101.3 pmol/L, respectively. For both AC

12 measurements by RIA and CL, the procedures do not include a pre-extraction step, which may
13 explain the overestimation of AC with respect to liquid chromatography.

14

15 *Statistical analysis*

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

1 Belgium). To compare the within-patient relationship between aldosterone measured by CL or RIA,
2 we used Bland-Altman plots. We used the Bland-Altman plot to detect systematic error,
3 proportional error or a magnitude dependent bias. To assess the diagnostic accuracy of AARR and
4 ARR for PA diagnosis, we used receiver operator characteristics (ROC) curves. ROC curves were
5 compared by the area under the curve: a value of z above the critical level of 1.96 was used to
6 accept the hypothesis that two areas were different.

7

8 **Results**

9 *Description of the population*

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

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

19

20 *Comparison between DRC and PRA*

21 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
22 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
23 patients) (Table1). To assess the within-patient correlation, we compared DRC by CL with PRA by
24 RIA; both screening test and confirmatory testing data were used for this comparison ($n = 168$);

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

3 The DRC and PRA values showed a significant within-patient correlation ($R = 0.7$; $P < 0.001$).
4 After conversion of the data to natural logarithms to obtain a normal distribution we performed a
5 linear regression ($R^2 = 0.532$), (Figure 2). Subsequently, we repeated the same analysis including
6 only PRA values < 1 ng/mL/h and DRC < 12 mU/L ($n = 129$); the correlation was lower but still
7 significant ($R = 0.3$; $P = 0.001$); a linear regression displayed an increased value dispersion ($R^2 =$
8 0.092). The regression line equations are given in the legend to Figure 2 and in the supplemental
9 table S2. The Bland-Altman plot of the Z score for PRA and DRC is provided in the supplemental
10 figure S1.

11

12 *Comparison between aldosterone concentrations in CL and RIA*

13 In our population the median AC (measured by CL) was 375.9 pmol/L (313.5 pmol/L in EH and
14 558.9 pmol/L in PA patients), whereas median AC measured by RIA was 471.6 pmol/L (416.1
15 pmol/L in EH and 707.3 pmol/L in PA patients). In the overall sample ($n = 168$) the correlation for
16 aldosterone in CL and RIA was highly significant ($R = 0.782$; $P < 0.001$); the linear regression (R^2
17 $= 0.604$) is shown in Figure 3; if only patients with $AC \leq 10$ ng/dL (corresponding to 277.4 pmol/L)
18 ($n = 66$) are considered, the correlation and linear regression are $R = 0.555$ ($P < 0.001$) and $R^2 =$
19 0.279 , respectively; the regression line equations are given in the legend to Figure 3 and in the
20 supplemental table S2.

21 Passing and Bablok regression analysis yielded the following equation: $RIA = -118.41$ (95% CI, -
22 171 to -66.8) + 1.49 (1.34 to 1.67) \times CL, with a significant deviation from linearity and a systematic
23 underestimation by the CL method, although less evident on samples lower than 10 ng/dL. We also
24 performed a Bland-Altman plot (Figure 4): in the overall 168 samples, the mean difference between
25 AC by RIA and AC by CL was 96.8 pmol/L (95% CI $-408.9 - 602.5$), whereas if only patients with

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

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

9

10 ***Diagnostic accuracy of AARR versus ARR***

11 We calculated the aldosterone (measured by CL)-to-DRC (AARR) and the aldosterone (detected by
12 RIA) -to-PRA ratio (ARR) using as cut-off levels for a positive AARR and ARR 3.7 (ng/dL/mU/L)
13 (102.6 pmol/L/mU/L) and 30 (ng/dL/ng/mL/h) (832.2 pmol/L/ng/mL/h), respectively. For PA
14 diagnosis, AARR displayed a sensitivity of 90% and a specificity of 98.7% (positive predictive
15 value 94.7%; negative predictive value 97.4%), whereas the ARR had a sensitivity of 100% and a
16 specificity of 73.3% (positive predictive value 50%; negative predictive value 100%). Of the 2/20
17 PA patients missed by AARR, none resulted to be affected by APA, therefore sensitivity of both
18 methods on APA recognition were 100%.

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

22 In literature different cut-offs are used, tailored on laboratory experience, average sodium intake,
23 assay methods and ethnicity¹⁰. For these reason the guidelines do not suggest a specific AARR and
24 ARR cut-off for PA case detection¹⁰. Sensitivity, specificity, positive and negative predictive values
25 with different cut-offs are given in the supplemental table S3. For case detection, that requires a

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

3

4 ***CL versus RIA performance on confirmatory tests for PA***

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

16

17 **Discussion**

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

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

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

9 In our prospective study comprising 168 samples DRC and PRA values displayed a good overall
10 correlation between these variables. It should be underlined that in our cohort we had a high
11 prevalence of patients with low-renin hypertension (63%), which is attributable to a referral bias to
12 our center: therefore, the correlation curves would be expected to display a higher correlation within
13 the general hypertensive population in which the prevalence of low-renin hypertension is not higher
14 than 30%⁽²⁴⁾. Regression curves demonstrated a weaker correlation for low renin values as
15 expected⁽⁹⁾; this finding may be partly explained by the sensitivity of the RIA assay (lower limit was
16 0.1 ng/mL/h). Moreover, prorenin circulates in significantly higher concentrations (10 to 100-fold)
17 compared to the active enzyme; cross reaction between pro-renin and renin has been previously
18 described⁽²⁵⁾ and it may interfere in DRC measurement: a recent study demonstrated an increase in
19 DRC proportional to the amount of exogenous pro-renin added⁽⁹⁾. For this reason, the interference
20 by pro-renin it is expected to be higher in the lower range concentrations of renin assays.

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

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

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

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

12 The ES guidelines for PA diagnosis and management⁽⁹⁾, recommend both ARR (with PRA) and
13 AARR (with DRC) for patient detection. Several authors have recently compared ARR and AARR
14 using in both cases AC measured by RIA and observed similar diagnostic accuracy^(8,11,28). Our
15 study compared for the first time simultaneously the diagnostic accuracy of ARR, with PRA and
16 AC measured by RIA, and AARR, with DRC and AC measured by CL. We confirmed a similar
17 diagnostic performance in the case detection with ROC curves that were not significantly different
18 as observed by others when the AC was measured with the same method for both ARR and
19 AARR⁽⁸⁾. It should be highlighted that all patients with an APA were detected by both methods at
20 screening and confirmed by AC measurement by both RIA and CL. The AARR missed only 2
21 patients with IHA at screening: these patients displayed a very mild hormonal and clinical
22 phenotype, and therefore the distinction with a status of low-renin EH is not possible.

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

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

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

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

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

24

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2 Medical Sciences of the University of Torino, Italy. We are most grateful to our team of nurses and
3 in particular to Silvano Bertinetti, Cristina Bertone and Sonia Zamburru, for their valuable help in
4 patient management.

5

6 **Legends to figures.**

7 **Legend to figure 1**

8 **Figure 1. Patients selection**

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

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

12

13 **Legend to figure 2**

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

15 PRA, plasma renin activity (expressed in ng/mL/h); DRC, direct renin concentration (expressed in
16 mU/L); CL, chemiluminescence; RIA, radioimmunometric assay; Lg, natural logarithm; EH,
17 essential hypertension; PA, primary aldosteronism; Und, undefined. On X-axis PRA by RIA natural
18 logarithm; on Y-axis DRC by CL natural logarithm; circles: EH; triangles: PA; squares: Und;
19 dashed lines: confidence interval; continuous line: regression curve. $N = 168$; $R^2 = 0.532$; $Y = 2.88$
20 $+ 0.69 * X$.

21

22 **Legend to figure 3**

23 **Figure 3. AC by CL versus RIA regression curve**

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

6

7 **Legend to figure 4**

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

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

15

16 **Legend to figure 5**

17 **Figure 5. ROC curves for AARR and ARR**

18 On X-axis 1 - Specificity; on Y-axis Sensitivity. The analysis was performed using screening test
19 values from the 100 patients included in the study. Dashed line: ROC curve for AARR; AUC was
20 0.989 (95% CI 0.974-1). Continuous line: ROC curve for ARR; the AUC was 0.934 (95% CI 0.885-
21 0.982). AUC values are not significantly different ($P > 0.05$).

22

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Total	EH	PA	<i>P</i> -value EH vs PA
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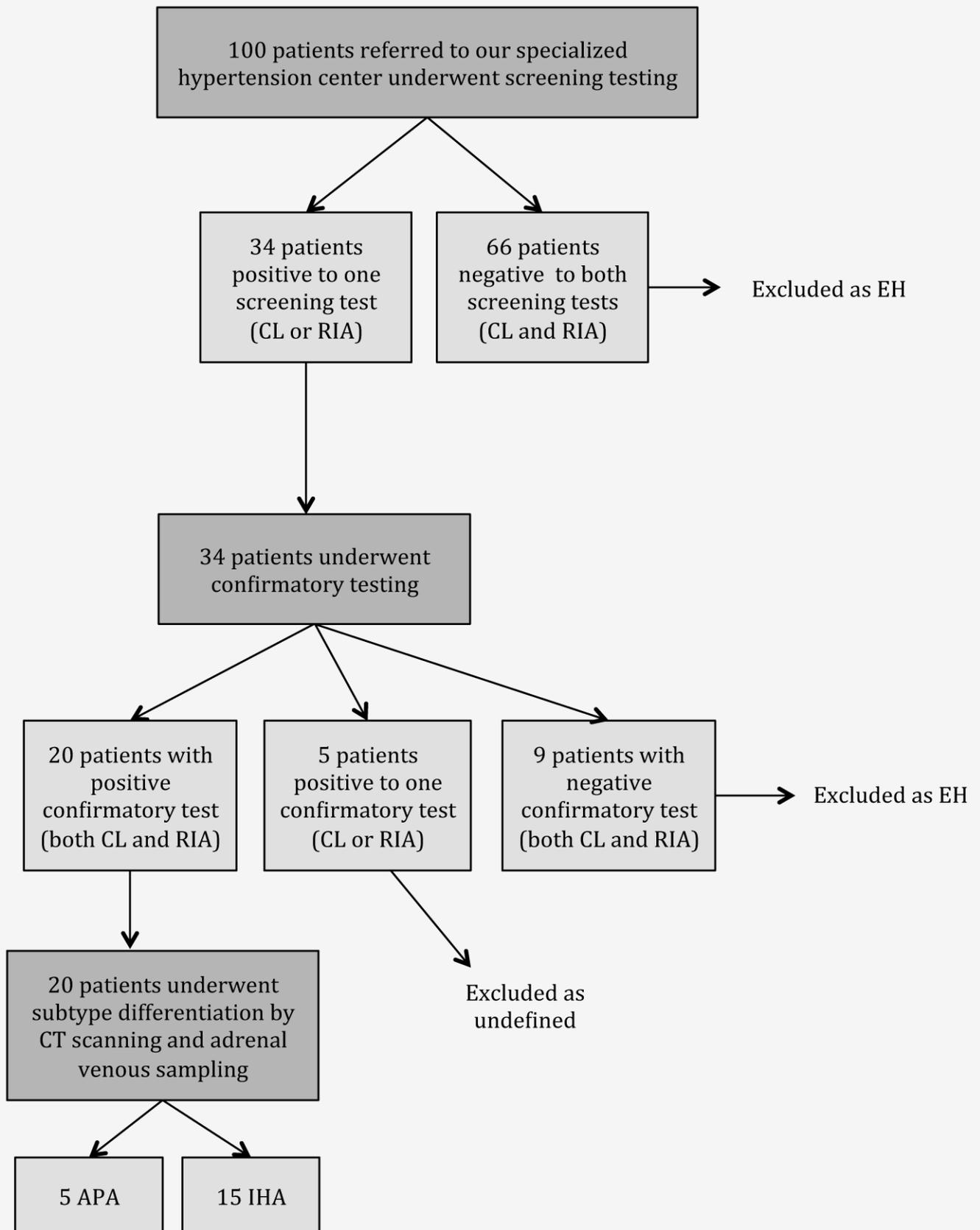
Number of patients	100	75	20	-
Age (years)	49 ± 11	48 ± 11	54 ± 7	0.010
Sex (%) (M/F)	54 / 46	56 / 44	50 / 50	0.239
SBP (mmHg)	147 ± 17	146 ± 17	154 ± 18	0.053
DBP (mmHg)	91 ± 10	92 ± 9	92 ± 11	0.957
K⁺ (mEq/L)	4.0 ± 0.5	4.2 ± 0.4	3.6 ± 0.6	< 0.001
PRA by RIA (ng/mL/h)	0.59 [0.15-1.71]	0.97 [0.32-1.99]	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	-

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3 **Table 1 – Characteristics of the population screened**

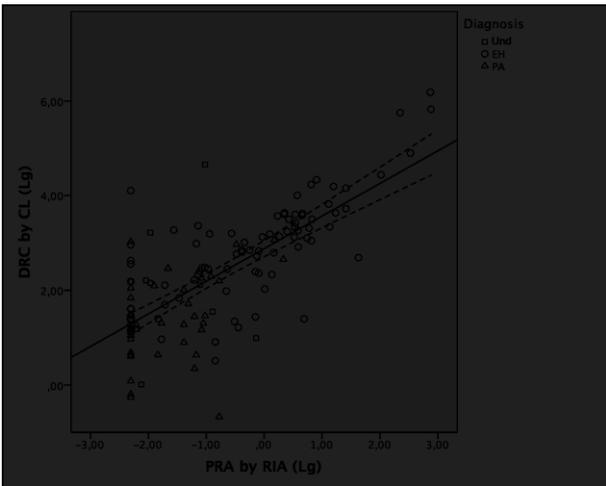
4 EH, essential hypertension; PA, primary aldosteronism; M/F, Male/Female; SBP, systolic blood
5 pressure; DBP, diastolic blood pressure; K⁺, potassium; PRA, plasma renin activity; DRC, direct
6 renin concentration; AC, aldosterone concentration; RIA, radioimmunoassay; CL,
7 chemiluminescence. Age, SBP,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

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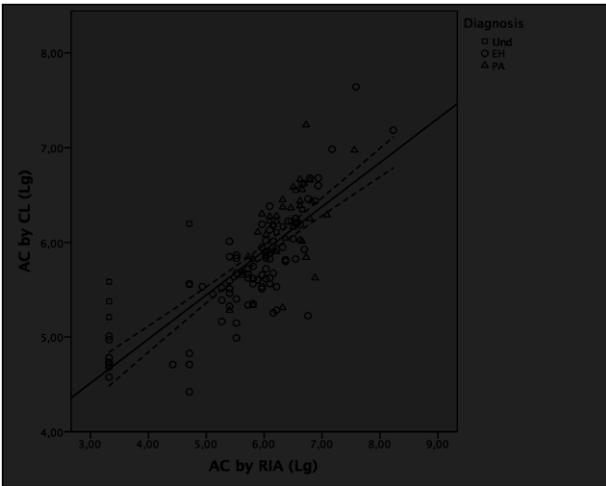
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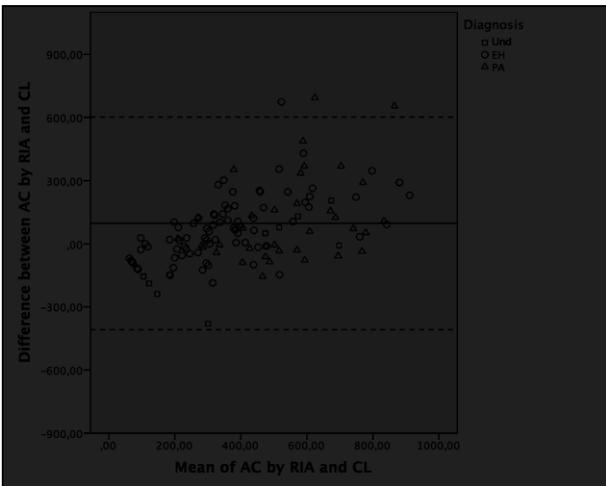
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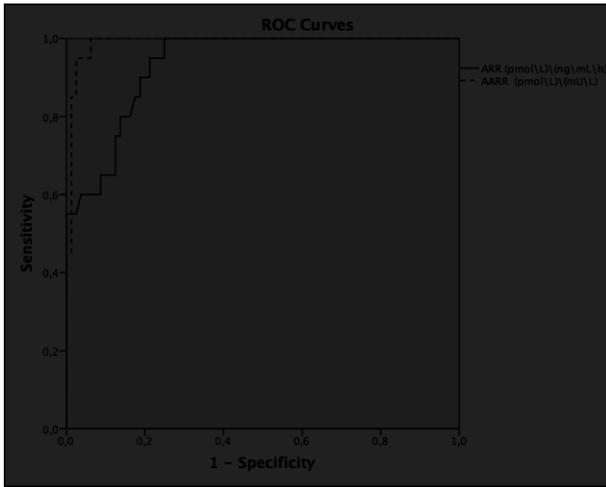
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2 **SUPPLEMENTAL FILE**

3 **DIAGNOSTIC ACCURACY OF ALDOSTERONE AND RENIN MEASUREMENT BY**
 4 **CHEMILUMINESCENT IMMUNOASSAY AND RADIOIMMUNOASSAY IN PRIMARY**
 5 **ALDOSTERONISM.**

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15 **Short title:** renin and aldosterone assays in primary aldosteronism

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5 **Word count:** abstract: 242; condensed abstract: 73; manuscript: 3857 (excluded references and
6 figure captures); 5 figures, 1 table, 1 supplemental file.

1 **Supplemental method section**

2

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

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

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

26 All patients with PA were screened for GRA using a long-PCR technique⁽²⁾.

1 Subtype diagnosis was performed by CT scanning with contrast and fine cuts of the
2 adrenal and subsequent AVS according to ES guidelines⁽⁹⁾ (figure 1). Sampling was
3 considered successful if the adrenal vein/inferior vena cava cortisol gradient was at
4 least 3⁽⁴⁾ and lateralization was defined as an aldosterone/cortisol ratio value (A/C)
5 from one adrenal at least 4 times the ratio from the other adrenal gland, or 3 times the
6 A/C of the contralateral with the A/C in the contralateral less than the A/C in the
7 peripheral vein⁽⁹⁾. A final diagnosis of APA was considered proven, providing that all
8 the following conditions were satisfied: 1) histological demonstration of adenoma, 2)
9 normalization of hypokalemia, 3) cure or improvement of hypertension, and 4)
10 normal ARR and suppression of aldosterone levels under saline load.

11

12 **Supplemental Results**

13 Between-method variability for aldosterone measurement could be observed from
14 *External Quality Assessment (EQA)* reports. According to 2015 final evaluation of
15 Immunocheck Qualimedlab srl (EQAS CNR, Pisa), total CV, taken as an index of
16 between-method agreement, was 27%, 20% and 14% on specimen with mean
17 aldosterone concentrations, calculated as the consensus among different assays, of
18 less than 83.2, between 83.2 to 221.9 and higher than 221.9 pmol/L, respectively.
19 Interestingly, when focusing on some samples, between-method variability may result
20 up to 42% for a mean aldosterone level of 244.1 pmol/L (reported values from 33.3 to
21 457.7 pmol/L) and up 36.1% for a mean aldosterone concentration of 432.7 pmol/L
22 (from 38.8 to 840.5 pmol/L). EQAS reports also indicated CVs ranging from 8.7% to
23 51.4% within the same method. Till now, very few laboratories adopted LC-MS/MS
24 (liquid chromatography associated with tandem mass spectrometry) to measure
25 aldosterone. These preliminary results found values that are slightly lower (-8%) with

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

7

8 The 5 patients with an undefined diagnosis (discordant results in the confirmatory
9 tests) could theoretically be classified as having either all PA or alternatively all EH.

10 The effect of the inclusion of these patients in one or the other groups of patients
11 would affect the diagnostic performance of the ARR and AARR. The sensitivity,
12 specificity and AUC after the inclusion of the patients are described in the
13 supplemental Table S5.

14

15 **References**

16

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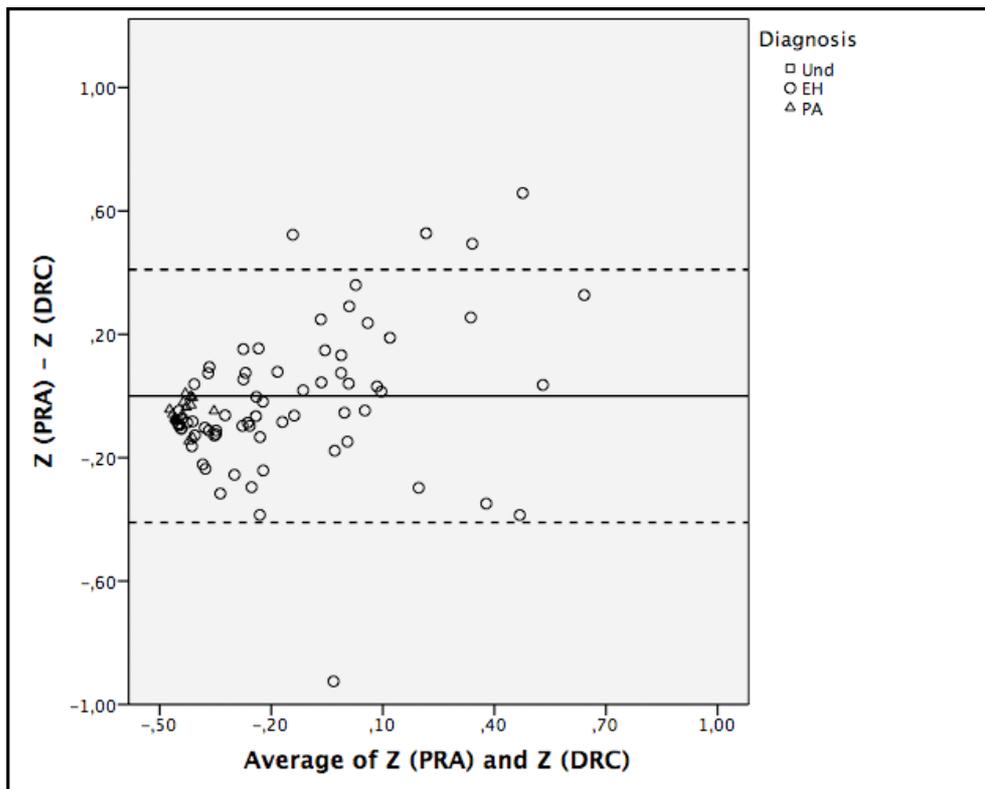
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5

1 **Figure S1.**



3 **Legend to Figure S1**

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

6 Z score is calculated as follows: $Z \text{ score} = (X - M) / SD$; X=value; M=mean; SD=
7 standard deviation.

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

4
 5 AC, aldosterone concentration; ARR, aldosterone to PRA (plasma renin activity)
 6 ratio; AARR, aldosterone to DRC (direct renin concentration) ratio; RIA, radio-
 7 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 \geq 3.7 and AC \geq 15	80.0%	100.0%	100.0%	94.9%
AARR \geq 2.7 and AC \geq 15	85.0%	100.0%	100.0%	96.2%
AARR \geq 1.0 and AC \geq 15	90.0%	92.0%	75.0%	97.2%
AARR \geq 3.7 and AC \geq 10	90.0%	98.7%	94.7%	97.4%
AARR \geq 2.7 and AC \geq 10	95.0%	97.3%	90.5%	98.7%
AARR \geq 1.0 and AC \geq 10	100.0%	77.3%	54.1%	100.0%
ARR \geq 30 and AC \geq 15	100.0%	85.3%	64.5%	100.0%
ARR \geq 18 and AC \geq 15	100.0%	73.3%	50.0%	100.0%
ARR \geq 30 and AC \geq 10	100.0%	73.3%	50.0%	100.0%
ARR \geq 18 and AC \geq 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.

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3 **Table S4. Patients with discordant results at confirmatory testing.**
4

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

5
6 Five patients displayed a negative saline load test with the RIA method and positive results with the CL method. These patients were considered as
7 having an undefined final diagnosis. Three of these patients underwent AVS to exclude unilateral PA and none of them displayed lateralization of
8 aldosterone secretion (i.e. unilateral PA).

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

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

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

3

4 Sensitivity and specificity values are expressed as percentage.

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

6 Und, undefined; PA, primary aldosteronism; EH, essential hypertension; AUC, area under the curve; ARR, aldosterone PRA (plasma renin activity)

7 ratio; AARR, aldosterone DRC (direct renin concentration) ratio.

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