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Evaluation of angiotensin II type-1 receptor antibodies in primary aldosteronism and further considerations about their possible pathogenetic role

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Angiotensin II type-1 receptor autoantibodies (AT1RAb) have been involved in the genesis of primary aldosteronism (PA), both in aldosterone-producing adenoma (APA) and in idiopathic hyperaldosteronism (IHA). In this study, we evaluated the titer of AT1RAb in 44 PA patients (15 with APA and 29 with IHA) compared with 18 normotensive healthy controls who were matched for gender and age. In 17 PA patients (6 APA and 11 IHA) the titer was evaluated under mineralocorticoid receptor (MR) antagonist treatment. We found that PA patients had a significantly higher titer of AT1RAb compared with controls (median values 33 [IQR 15.6] IU/mL vs 17.5 [IQR 10.8] IU/mL, respectively; $P < 0.0001$). No significant difference of the AT1RAb titer was reported among PA patients, subdivided according to the subtypes and the concomitant MR antagonist therapy. No significant correlation was detected between age, gender, BMI, blood pressure values, baseline aldosterone, ARR, and the AT1RAb titer of all patients enrolled. Our data confirm an increased titer of AT1RAb in both subtypes of PA, independently from the concomitant use of MR antagonists and clinical/biochemical characteristics of PA patients. The small sample of patients and the relatively short time of treatment could have influenced these results. Moreover, the ELISA assay fails to evaluate the bioactivity of AT1RAb. Further studies should evaluate if the subtype, the clinical/biochemical recovery of PA, or both, influence the pathogenetic role of AT1RAb. The possible autoimmune pathogenesis and reversal effect with AT1R blocker treatment in PA patients with AT1RAb positivity is intriguing and requires further study.

1 | INTRODUCTION

Angiotensin II type-1 receptor autoantibodies (AT1RAb) have been described in certain conditions characterized by vascular and renal damage, including preeclampsia,[1] malignant hypertension,[2] renal allograft injury.[3] Wallukat et al.[1] first hypothesized a pathogenetic role of AT1RAb in preeclampsia, describing a chronotropic response to immunoglobulin of preeclamptic patients of cultured neonatal rat cardiac myocytes, probably mediated by angiotensin II type-1 receptor (AT1R). Subsequent studies showed that AT1RAb activate AT1R on a variety of cell types, such as trophoblast cells, endothelial cells, mesangial cells, and vascular smooth muscle cells, provoking biological responses that are relevant to the pathophysiology of preeclampsia[4] and some hypertensive condition and that could become future therapeutic targets.[5] Zhou et al.[6] demonstrated that the administration of these autoantibodies into pregnant mice induced a preeclamptic state, while in non-pregnant mice resulted in hypertension. The pathophysiology of gestational hypertension is very complicated, and the role of the renin-angiotensin-aldosterone system (RAAS)

is contradictory; however, it is interesting to note that in normal pregnancy AT1RAb are not increased, and the activation of RAAS is not associated with its biological effects.[7]

In 2012, the Oklahoma group[8] first described the presence of AT1RAb, even in primary aldosteronism (PA). One-year later, Rossitto et al.,[9] measuring these antibodies by an enzyme-linked immunosorbent assay (ELISA), reported a twofold higher titer in patients with an aldosterone-producing adenoma (APA) compared with those with idiopathic hyperaldosteronism (IHA), whose AT1RAb titer was similar to that seen in hypertensive and normotensive patients.

Subsequently, Kem et al.[10] confirmed the presence of AT1RAb in PA patients and their agonistic effect on AT1R. They demonstrated that AT1RAb increased AT1R activation in transfected Chinese hamster ovary (CHO) cells in vitro, produced a contractile effect in perfused rat cremaster arterioles, and stimulated aldosterone production in human adrenocortical carcinoma cell line (HAC15) in vitro. All these effects were blocked by the co-incubation with AT1R blockers, such as losartan and candesartan. These data suggest a likely pathological role of AT1RAb in PA and hypertension. Recently, Li et al.[11] reported that about 60% of PA patients harbor AT1RAb irrespective of their underlying subtype and AT1R blockers uniformly suppressed their activity.

In the present study, we evaluated the titer of AT1RAb in PA patients subdivided according to their subtype and the concomitant therapy with mineralocorticoid receptor (MR) antagonists.

2 | METHODS

2.1 | Study population

We evaluated sera from 44 consecutive patients with PA (15 with APA and 29 with IHA) and compared with 18 normotensive healthy controls (HC) matched for gender and age, and all enrolled at the Endocrinology Unit in the Department of Medicine at the University of Padua.

PA detection was performed with the aldosterone-to-renin ratio (ARR) greater than 750 (pmol/L)/(ng/mL/h). The diagnosis was confirmed by the lack of aldosterone suppression (>138.5 pmol/L) after an iv saline load (2 L of 0.9% saline infused over 4 hours), the persistence of an ARR greater than 750 (pmol/L)/(ng/mL/h) 90 minutes after the administration of 50 mg of captopril orally, or both.[12]

Every patient also underwent an adrenal computed tomography scan and adrenal venous sampling for subtype classification of PA. All patients were evaluated after washout of all interfering antihypertensive treatments 2 to 3 weeks (in case of MR antagonists at least 4 weeks) before the investigation and switched to alpha-blocker, slow-release calcium channel blocker, or both. Female patients under estroprogestinic contraceptives stopped this treatment at least 3 months before the beginning of diagnostic evaluations. Patients with hypokalemia continued with oral potassium supplementation.

At study entry, blood samples were collected from each patient to measure only AT1RAb; hormonal measurements were performed in all patients during diagnostic evaluations. Nine patients with APA and 18 with IHA were still in washout of interfering antihypertensive drugs to complete the related investigations. The remaining 17 PA patients were under MR antagonist treatment after the conclusion of the diagnostic management: 6 with APA waiting for adrenalectomy and 11 with IHA as treatment of choice. Normotensive HC were all healthy volunteers. Exclusion criteria were: hypertension, treatment with medications interfering with the RAAS (antihypertensive drugs, diuretics, glucocorticoids, hormonal contraceptives, abuse of licorice, or laxatives), known autoimmune disorders, liver or renal failure, and pregnancy. HC underwent blood sampling to measure AT1RAb, serum upright aldosterone, and plasma renin activity (PRA). During the same visit, anthropometric (weight, height, and body mass index) and clinical (heart rate and systolic and diastolic blood pressure) measurements were taken for all patients. Personal history of other comorbidities, such as dyslipidemia, diabetes, autoimmune, or inflammatory disorders was collected for every patient. Data regarding serum glucose and creatinine levels of PA patients during diagnostic investigations were also collected. The local ethics committee approved the study, and all the patients gave informed consent.

2.2 | Biochemical evaluation

Serum samples for AT1RAb were stored at −80°C and thawed at room temperature on the day of the assays. The presence of ATR1Ab was assessed using a commercially available ELISA kit (Cusabio, Wuhan, China). Samples were diluted to a concentration of 1:25 using the sample diluent provided. Immunobsorbance was measured at 450 nm and original concentration calculated using a standard curve obtained through serial dilutions of the manufacturer-provided standard of known concentration. The detection threshold of AT1RAb was set at 0.078 IU/mL (intra- and inter-assay variations of less than 8% and less than 10%, respectively). Serum aldosterone levels (intra- and inter-assay variations of 3.5% and 4.12%, respectively) and PRA (6.5% and 8.5%) were measured by RIA using commercially available kits (Radim, Pomezia-Roma, Italy).

2.3 | Statistical analysis

Continuous data are expressed as the median and interquartile range (IQR), the latter calculated as the difference between 75th and 25th percentiles. Comparisons were obtained with ANOVA, and when required, followed by post-hoc analysis with the Tukey-Kramer HSD test. Least-squares linear regression tested any relationships between pairs of variables. The Pearson's correlation coefficient r was used to quantify the strength of the relationships; the statistical significance of r was determined by ANOVA. Receiver operator characteristics (ROC) analysis was applied to evaluate the diagnostic accuracy of the AT1RAb titer through the area under the ROC curve (AUC) and optimal cut-off value (ie, optimal decision threshold) corresponding to the best combination of sensitivity and specificity, was determined. For all the statistical evaluations, a P value less than 0.05 was considered statistically significant (2-tailed).

TABLE 1 Clinical and biochemical features of PA patients (subdivided also as APA and IHA) and normotensive HC. Continuous data are expressed as median and interquartile range (IQR, in parentheses, calculated as the difference between 75th and 25th percentiles)

Parameter	PA (n = 44)	APA (n = 15)	IHA (n = 29)	HC (n = 18)	P[a]
Age (y)	55 (15)	55 (12)	58 (14)	51 (13)	NS
Gender (% Female)	56.8	40.0	65.5	61.1	NS
BMI (kg/m²)	27.7 (6.6)	28.0 (3.7)	25.9 (9.1)	23.8 (3.9)	<0.05
Systolic BP (mm Hg)	152 (17)	155 (10)	150 (21)	120 (10)	<0.0001
Diastolic BP (mm Hg)	100 (10.0)	100 (11.5)	95 (10.5)	80 (1.2)	<0.0001
Serum aldosterone (pmol/L)	537 (547)	798 (555)	438 (348)	240 (62)	<0.005
ARR (pmol/L per ng/mL/h)	2062 (4062)	5005 (5850)	1570 (2426)	99 (65)	<0.0001
AT1RAb (IU/mL)	33 (15.6)	23.6 (16.4)	34.9 (15.7)	17.5 (10.8)	<0.0001
Fasting plasma glucose (mmol/L)	5.1 (0.9)	5.2 (0.9)	5.1 (0.9)	n.d.	—
Serum creatinine (μmol/L)	74 (17)	70 (17)	75 (16)	n.d.	—

APA, aldosterone-producing adenoma; ARR, aldosterone-to-renin ratio; BMI, body mass index; BP, blood pressure; HC, healthy controls; IHA, idiopathic hyperaldosteronism; n.d., not determined; PA, primary aldosteronism.
[a]Comparison PA vs HC.

3 | RESULTS

Clinical and biochemical features of all patients enrolled are listed in Table 1. Compared with normotensive HC, PA patients had higher BMI, systolic and diastolic blood pressure values, serum aldosterone, and ARR levels. Four PA patients and 2 HC had dyslipidemia treated with statins, and 3 PA patients had diabetes mellitus that was well treated with oral anti-diabetic drugs. None of the patients had autoimmune disorders. Among PA patients, the median number of anti-hypertensive drugs was one (with IQR one; lowest zero, highest four drugs).

The titer of AT1RAb was significantly higher in PA than in HC (33 [IQR 15.6] IU/mL vs 17.5 [IQR 10.8] IU/mL, respectively; $P < 0.0001$; Figure 1). On ROC analysis a cut-off value of 19.8 IU/mL was optimal to distinguish PA from HC (sensitivity 84%, specificity 72%, AUC 0.85).

Comparing PA patients with the antibody titer similar to that of HC (ie, values of AT1RAb less than 22 IU/mL, corresponding to 75th percentile) and those with an increased titer (greater than 22 IU/mL), no difference was found in age, gender, blood pressure values, BMI, baseline serum aldosterone, and ARR levels.

After subdividing PA patients according to their subtype, no significant differences were found among clinical and biochemical features (Table 1).

Even the AT1RAb titer was comparable between APA and IHA (23.6 [IQR 16.4] IU/mL and 34.9 [IQR 15.7] IU/mL, respectively) and

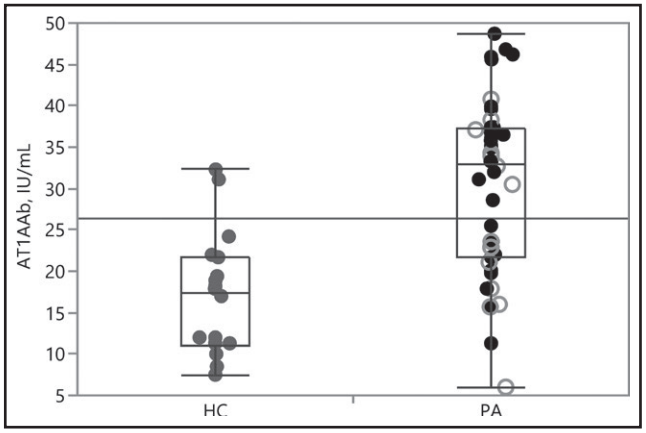

FIGURE 1 Box-plot showing the distribution of the AT1RAb titer on ELISA assay in PA patients and normotensive HC. HC, normotensive patients; ○ APA, aldosterone-producing adenoma; ● IHA, idiopathic hyperaldosteronism. Edges of the box indicate the 25th and 75th quantiles, including the middle 50% of the data. The horizontal line through each box represents the median. Whiskers show the range of data, calculated as (upper quartile +1.5 [interquartile range]) and (lower quartile −1.5 [interquartile range]). The continuous horizontal line through the graph indicates the overall mean of the AT1RAb titer

significantly higher than HC (17.5 [IQR 10.8] IU/mL; $P < 0.001$ vs IHA and $P < 0.02$ vs APA). Considering PA patients under MR antagonist treatment, the AT1RAb titer was comparable both in APA (32.7 [IQR

17.3] IU/mL in washout of interfering drugs and 22.0 [IQR 13] IU/mL under treatment), and in IHA (35.95 [IQR 11.85] IU/mL in washout and 32 [IQR 17.3] IU/mL under treatment; Figure 2).

AT1RAb titer was not correlated with the age, gender, blood pressure, and BMI values of all PA patients, with or without MR antagonist treatment and HC. No significant correlation was detected even between the AT1RAb titer and baseline serum aldosterone, as well as for ARR levels, of all patients enrolled (Figures 3A and 4A), also when PA patients were subdivided according to their subtype (Figures 3B-C and 4B-C) or the concomitant use of MR antagonists.

4 | DISCUSSION

In the last few years, many studies have identified recurrent somatic mutations in genes coding for ion channels and ATPases regulating intracellular ionic homeostasis and cell membrane potential, involved in the pathophysiology of about 50% of APA.[13] However, other potential mechanisms could be involved and this study represents an attempt to bring further considerations about a possible pathogenetic role of AT1RAb in PA. In contrast to the previous results of Rossitto et al.,[9] and in accordance with the previous study of Li et al.,[11] the current study reports a higher titer of AT1RAb both in

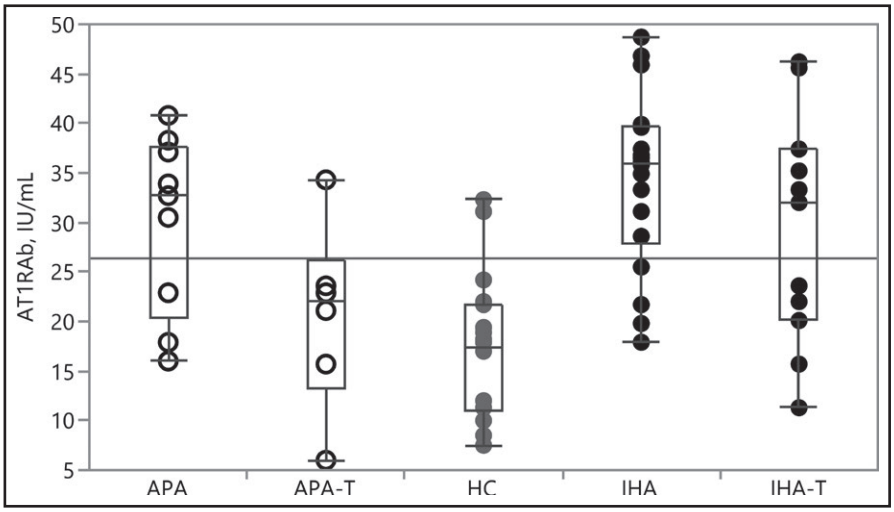

FIGURE 2 Box-plot showing the distribution of the AT1RAb titer on ELISA assay in PA patients, subdivided according to their subtype and the concomitant MR antagonist therapy, and compared with normotensive HC. HC, normotensive patients; APA, aldosterone-producing adenoma; APA-T, aldosterone-producing adenoma under treatment; IHA, idiopathic hyperaldosteronism; IHA-T, idiopathic hyperaldosteronism under treatment. Edges of the box indicate the 25th and 75th quantiles, including the middle 50% of the data. The horizontal line through each box represents the median. Whiskers show the range of data, calculated as (upper quartile + 1.5 [interquartile range]) and (lower quartile −1.5 [interquartile range]). The continuous horizontal line through the graph indicates the overall mean of the AT1RAb titer

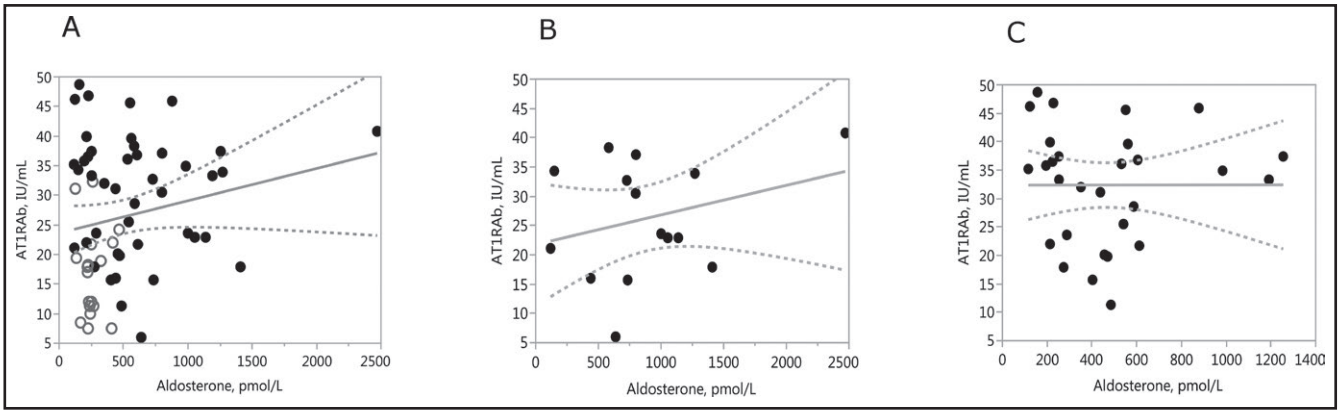

FIGURE 3 Scatterplot of AT1RAb titer vs serum aldosterone concentrations. A, Data for all patients enrolled (○ normotensive patients; ● Primary aldosteronism patients); linear regression analysis by least squares method indicated absence of a significant correlation between the two variables (n = 62, $r = 0.1992$, $P = 0.1204$). Dashed lines indicate confidence curves of the linear fit. B, Data for APA patients only (n = 15, $r = 0.2879$, $P = 0.2980$). C, Data for IHA patients only (n = 29, $r = 0.0013$, $P = 0.9946$). Also for subgroups, linear regression analysis indicated absence of a significant correlation between the two variables

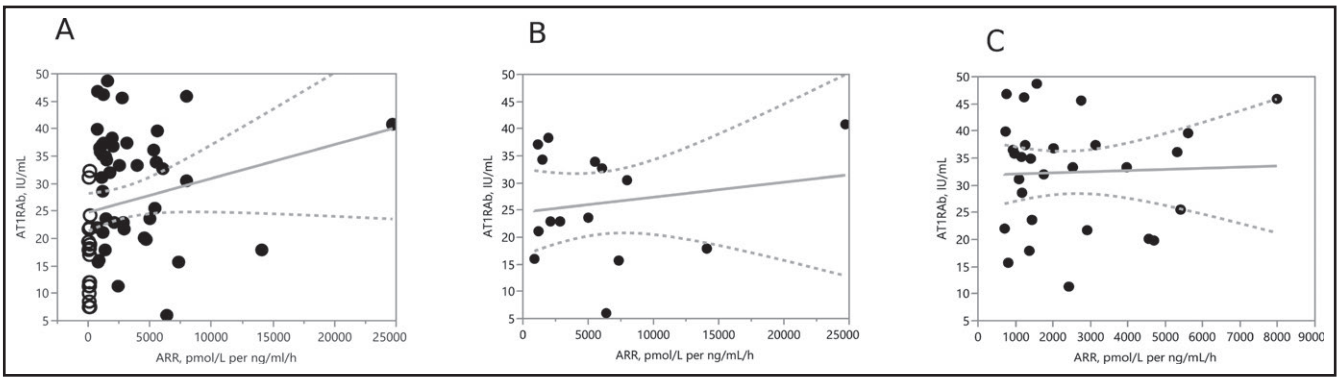

FIGURE 4 Scatterplot of AT1RAb titer vs ARR. A, Data for all patients enrolled (o normotensive patients; ● Primary aldosteronism patients); linear regression analysis by least squares method indicated absence of a significant correlation between the two variables (n = 62, r = 0.2121, P = 0.0980). Dashed lines indicate confidence curves of the linear fit. B, Data for APA patients only (n = 15, r = 0.1732, P = 0.5369). C, Data for IHA patients only (n = 29, r = 0.0396, P = 0.8382). Also for subgroups, linear regression analysis indicated absence of a significant correlation between the two variables

APA and in IHA compared with normotensive HC. No difference was found in age, gender, blood pressure values, BMI, baseline serum aldosterone, and ARR levels even when comparing PA patients with the antibody titer similar to HC and those with an increased titer. In common with the study of Rossitto et al., we found no correlations between the AT1RAA titer and age, gender, BMI, blood pressure values, baseline aldosterone, and ARR levels.[9]

The underlying causes for the production of these antibodies are not known. Hyperaldosteronism or an increased activity of aldosterone may cause a proinflammatory systemic state, inducing the formation of AT1RAb.[14] Since no previous studies evaluated differences of the antibody titer under MR antagonist treatment, we performed the measurement of AT1RAb in a group of treated PA patients, but no significant difference of the titer was found between patients treated or not with MR antagonists. Only in treated APA patients, the antibody titer seems to be close to the titer of HC, suggesting a possible relevant role in APA. However, the small sample of patients and the relatively short time under MR antagonist treatment (from 6 to 24 months) could have influenced these results. Future research may evaluate if the clinical or biochemical recovery of PA, or both (eg, after adrenalectomy in APA patients or after long-term treatment with MR antagonists in IHA patients), could influence the titer and the activity of AT1RAb compared with baseline parameters.

Since the ELISA assay can only detect these antibodies, this study fails to evaluate the avidity or overall biological activity of AT1RAb. Previous studies reported that patients with elevated ELISA values more frequently had increased antibody activity; however, even those with low ELISA values showed increased bioactivity.[11] AT1RAb were previously shown to have an agonistic effect on AT1R and a possible pathogenetic role in PA and hypertension.[1,4,10] Since both the AT1R and the thyroid stimulating hormone receptor are G protein-coupled receptors, AT1RAb act like Thyroid-Stimulating autoantibodies (TSAb) in Graves' disease. AT1RAb have an agonistic effect directed to a specific epitope on the second extracellular loop of the AT1R[1] and act as allosteric agonists, activating the AT1R and facilitating angiotensin II-mediated signal.[10] These mechanisms could stimulate arteriole contractility, explaining residual hypertension following surgery in up to 50% of APA patients and could lead to an autonomous increased aldosterone production, explaining the persistent responsiveness to angiotensin II and aldosterone in IHA. Previous in vitro assays demonstrated that all these mechanisms were suppressed by the coincubation with AT1R blockers, but not with converting enzyme inhibitors.[10,11] Kem et al.[10] reported significant improvement in hypertension and aldosterone levels in two PA patients treated with losartan. A recent retrospective study on kidney transplant recipients reported that perioperative candesartan might alter the risk of rejection in patients with increased AT1RAb titer, reducing the AT1R-mediated inflammatory response.[15] All these findings suggest a potential therapeutic role of AT1R blockers and further studies should investigate in those patients with AT1RAb positivity the effects of AT1R blocker therapy not only on the blood pressure but also on the aldosterone levels.

AT1RAb such as TSAb may be associated with a picture characterized by initial hypertrophy and subsequent micronodularity of the target glands; previous studies have demonstrated that adrenals with APA show hyperplasia of the zona glomerulosa and remodeling similar to IHA that may precede APA development.[16]

However, the possible involvement of AT1RAb in adrenal remodeling is still not known.

Interestingly, the finding of lymphocytic infiltration of adrenal tissue in patients with APA[17] may represent another sign of an organ-specific inflammation, autoimmunity, or both. Autoimmune diseases sometimes tend to aggregate in a patient to combine an autoimmune polyendocrine syndrome (APS).[18] PA has been recently associated with an increased prevalence of Hashimoto's thyroiditis[19] and could represent a new subtype of APS type 3.

AT1RAb could also induce a proinflammatory status, activating NADPH-oxidase, tumor necrosis factor-α, and nuclear factor-kB, which could induce vascular damage, autoimmune disorders, and somatic mutations in nearby cells.[20] About 60% of PA patients have been demonstrated to harbor AT1RAb,[10] and a similar rate has been associated with the presence of genetic abnormalities involved in

the pathophysiology of APA.[13] Recent studies have also reported somatic mutations similar to those described in APA in 35% of aldosterone-producing cell clusters (APCC) from normal adrenals.[21] We did not evaluate the prevalence of somatic mutations in our patients, and this could be the topic of future research.

For all these reasons we think that there are some data suggesting possible autoimmune pathogenesis, underlying some forms of PA according to the criteria proposed by Witebsky in 1957,[22] and subsequently modified by Rose in 1993.[23]

This work presents some strengths, as the enrollment of a sample of PA patients from a single center and the use of strict diagnostic criteria based on adrenal vein sampling for the subtype classification. However, the following limitations have to be considered: the reduced number of patients, especially if subdivided according to the concomitant therapy with MR antagonists, the short time under MR antagonist treatment, the lack of a sample of APA patients after adrenalectomy, and the failure of ELISA assay to provide an assessment of the biological activity and pathophysiological significance of AT1RAb.

In conclusion, further studies should evaluate through in vivo experimental approaches if the subtype, the clinical/biochemical recovery of PA, or both influence the pathogenetic role of AT1RAb. The possible autoimmune pathogenesis and reversal effect with AT1R blocker treatment in PA patients with AT1RAb positivity is intriguing and requires further study.

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CONFLICT OF INTEREST

The authors report no conflicts of interests.

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