Elevation of Angiotensin-II Type-1-Receptor Autoantibodies Titer in Primary Aldosteronism as a Result of Aldosterone-Producing Adenoma

Giacomo Rossitto,* Giuseppe Regolisti,* Ermanno Rossi, Aurelio Negro, Davide Nicoli, Bruno Casali, Antonio Toniato, Brasilina Caroccia, Teresa Maria Seccia, Thomas Walther, Gian Paolo Rossi

Abstract—The mechanisms of excess aldosterone secretion in primary aldosteronism (PA) remain poorly understood, although a role for circulating factors has been hypothesized for decades. Agonistic autoantibodies against type-1 angiotensin-II receptor (AT1AA) are detectable in malignant hypertension and preeclampsia and might play a role in PA. Moreover, if they were elevated in aldosterone-producing adenoma (APA) and not in idiopathic hyperaldosteronism (IHA), they might be useful for discriminating between these conditions. To test these hypotheses, we measured the titer of AT1AA in serum of 46 patients with PA (26 with APA, 20 with IHA), 62 with primary hypertension (PH), 13 preeclamptic women, and 45 healthy normotensive blood donors. We found that the AT1AA titer was higher (P<0.05) in both PA and PH patients (2.65±1.55 and 1.86±0.63, respectively) than in normotensive subjects (1.00±0.20). In APA, it was 2-fold higher than in IHA patients (3.43±1.20 versus 1.64±1.39, respectively, P<0.001), despite similar blood pressure values. Of note, it allowed effective discrimination of APA from either PH or IHA, as shown by Receiver Operator Characteristics curve analysis. Moreover, after captopril challenge, plasma aldosterone concentration fell more in AT1AA-positive than in AT1AA-negative PA patients (−32.4% [21.1–42.9] versus 0.0% [0.0–22.6], P=0.015), suggesting an agonistic role for these autoantibodies. Thus, a higher serum AT1AA titer in patients with APA than in IHA and PH patients can be useful in differentiating APA patients from either PH or IHA, and thus in selecting PA patients to be submitted to adrenal vein sampling. (Hypertension. 2013;61:00-00.)

Key Words: angiotensin-II ■ antibodies ■ AT1 receptor ■ autoimmunity ■ primary aldosteronism

A large prospective study showed that primary aldosteronism (PA) is the most prevalent form of endocrine hypertension among hypertensive patients referred to specialized hypertension centers. The discrimination between its main subtypes—aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia (BAH, also referred to as idiopathic hyperaldosteronism, IHA)—is crucial, as the former is surgically curable, whereas the latter requires lifelong medical treatment. This distinction is, however, challenging because of the biochemical and pathological overlapping of APA and BAH, and the lack of well-defined functional and morphological criteria. The tissue surrounding the APA often contains multiple nodules and shows paradoxical hyperplastic changes. Furthermore, cases of multinodular adrenal hyperplasia have been reported, even though their distinctive criteria from APA remain vague.

Thus, a pathological continuum between APA and BAH has been proposed, which suggests that pathogenic mechanisms common to these conditions exist. However, the stimuli driving this transition eluded identification thus far notwithstanding a long quest. For example, even though angiotensin-II type-1 (AT1) receptors were detected in both the normal adrenocortical zona glomerulosa and in APA, angiotensin-II (Ang-II), one of the known secretagogues of aldosterone, is barely detectable in PA patients.

Of interest, circulating autoantibodies against a specific epitope of the AT1 receptor (AT1AA) with specific agonistic activity have been suggested to play a pathogenic role in diseases characterized by vascular and renal damage, such as preeclampsia and malignant hypertension. These agonistic autoantibodies against type-I angiotensin-II receptor (AT1AA) could stimulate aldosterone secretion and trigger the development of hyperplastic changes in the zona glomerulosa in PA patients. We therefore sought for AT1AA in serum of patients with PA and investigated whether their titer was higher.
than in healthy normotensive (NT) blood donors, patients with essential hypertension, and pregnant women with or without preeclampsia.

Materials and Methods
The biochemical and hormonal variables and the titer of AT1AA were measured in serum of 46 patients with PA (26 with APA, 20 with IHA), 62 with primary hypertension (PH) recruited at 2 referral centers, 13 preeclamptic women, and 45 healthy NT blood donors. APA patients not fulfilling all of these criteria were held to have BAH. PH was diagnosed after exclusion of all the main secondary forms of hypertension. The AT1AA titer was measured with an ELISA assay, according to the method of Liao et al., using a peptide corresponding to the second extracellular loop of the human AT1 receptor.

One-way ANOVA analysis followed by Bonferroni post hoc test, Mann–Whitney test, χ² analysis, or Fisher exact test were used for comparing variables across groups, as appropriate. A correlation (by Spearman ρ) and regression analysis were performed. The diagnostic accuracy of the AT1AA titer was assessed by the Receiver Operator Characteristics curves (area under the Receiver Operator Characteristics curve [AUC]) and compared with different tests, and the Youden index, corresponding to the highest average of sensitivity and specificity, was used to determine the optimal cutoff. Expanded materials and methods and associated references are available in the online-only Data Supplement.

Results

Clinical Data
Because of uncertainties on the subtype of PA, or the unwillingness to undergo adrenal vein sampling or surgery, 6 PA patients were excluded from the analysis. Compared with PH patients, PA patients had higher plasma aldosterone concentration (PAC; 23.5 [14.4–42.4] versus 9.5 [6.5–13.0] ng/dL basal and 17.5 [10.1–24.6] versus 6.0 [4.5–9.5] ng/dL postcaptopril), aldosterone-renin ratio (ARR; 78.6 [51.4–90.0] versus 7.1 [4.7–11.3] baseline and 40 [21.6–76.3] versus 2.7 [1.4–5.6] postcaptopril; P<0.001 for all comparisons), serum Na⁺ (141±2 versus 138±3 mmol/L; P<0.001), and lower K⁺ (3.4±0.5 versus 4.1±0.3 mmol/L; P<0.001) and plasma renin activity (0.35 [0.12–0.56] versus 1.40 [0.75–2.10] ng/mL h⁻¹ baseline and 0.60 [0.15–0.95] versus 2.60 [1.23–4.08] ng/mL h⁻¹ postcaptopril; P<0.001 for all). They were also older (51.4±11.1 versus 42.3±11.0 years; P<0.001) and heavier (27.1±4.2 versus 25.0±3.1 kg/m²; P=0.007), but similar for gender and systolic and diastolic blood pressure (BP) values.

The main data of the PA patients split into APA and IHA are shown in Table 1: the IHA differed from the APA patients only for a higher serum K⁺ level (3.7±0.3 versus 3.3±0.5 mmol/L; P=0.004). Compared with NT controls, the PA and PH patients had higher body mass index (27.0±4.2 and 25.0±3.1 versus 22.3±3.8 kg/m²; P<0.001 and P=0.013, respectively), whereas the men-to-women ratio was similar.

Serum AT1AA Titer in the Different Groups
The titer AT1AA was higher in PA than in PH patients (2.65±1.55 versus 1.86±0.63 positive/negative ratio; P<0.05), and in both PA and PH patients than in NT controls (1.0±0.2, P<0.001 for both; Figure S1 in the online-only Data Supplement). In the PA, it was about 2-fold higher than in the IHA patients (3.43±1.20 versus 1.64±1.39, respectively, P<0.001; Figures 1 and 2) and the PH patients (1.84±0.63, P<0.001; Figures 1 and 2), despite similar BP values. In the APA patients, the AT1AA titer was comparable with that found in preeclamptic women (3.66±1.79; Figure 1), who had a titer much higher than the

### Table 1. Clinical and Biochemical Features of the PA Patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>APA (n=26)</th>
<th>IHA (n=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>48.5±11.2</td>
<td>55.0±10.0</td>
<td>ns</td>
</tr>
<tr>
<td>Sex M/F, %</td>
<td>53.9/41.6</td>
<td>75/25</td>
<td>ns</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.2±4.3</td>
<td>27.8±4.1</td>
<td>ns</td>
</tr>
<tr>
<td>Serum K⁺, mmol/L</td>
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<td>3.7±0.3</td>
<td>0.004</td>
</tr>
<tr>
<td>Serum Na⁺, mmol/L</td>
<td>141±2</td>
<td>142±2</td>
<td>ns</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>83±18</td>
<td>82±16</td>
<td>ns</td>
</tr>
<tr>
<td>Urinary K⁺ excretion, mmol/24 hours</td>
<td>68±39</td>
<td>65±22</td>
<td>ns</td>
</tr>
<tr>
<td>Urinary Na⁺ excretion, mmol/24 hours</td>
<td>117±67</td>
<td>115±41</td>
<td>ns</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>156±14</td>
<td>160±13</td>
<td>ns</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>97±8</td>
<td>99±9</td>
<td>ns</td>
</tr>
<tr>
<td>Baseline PRA, ng/mL h⁻¹</td>
<td>0.40 (0.13–0.65)</td>
<td>0.35 (0.1–0.51)</td>
<td>ns</td>
</tr>
<tr>
<td>Postcaptopril PRA, ng/mL h⁻¹</td>
<td>0.47 (0.10–0.85)</td>
<td>0.65 (0.18–0.95)</td>
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<tr>
<td>Baseline PAC, ng/dL</td>
<td>28.0 (21.3–50.0)</td>
<td>19.7 (13.3–26.3)</td>
<td>ns</td>
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<tr>
<td>Postcaptopril PAC, ng/dL</td>
<td>17.4 (14.8–24.6)</td>
<td>17.7 (8.3–24.0)</td>
<td>ns</td>
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<tr>
<td>Baseline ARR, ng/dL/ng/mL h⁻¹</td>
<td>82.9 (57.9–226.2)</td>
<td>67.8 (40.6–81.3)</td>
<td>ns</td>
</tr>
<tr>
<td>Postcaptopril ARR, ng/dL/ng/mL h⁻¹</td>
<td>44.5 (22.2–157.5)</td>
<td>26.1 (16.2–76.3)</td>
<td>ns</td>
</tr>
</tbody>
</table>

PA indicates primary aldosteronism; APA, aldosterone-producing adenoma; IHA, idiopathic hyperaldosteronism; BMI, body mass index; K⁺, potassium; Na⁺, sodium; Creatinine, serum creatinine; Systolic BP, systolic blood pressure; Diastolic BP, diastolic blood pressure; PRA, plasma renin activity; PAC, plasma aldosterone concentration; and ARR, aldosterone-to-renin ratio.

The cutoff value of the ARR for the centers was 26.8. Values are expressed as mean±SD or median (interquartile range), as appropriate.
patients, we wondered whether this titer could be useful for pinpointing the APA patients from the vast array of hypertensive patients without this surgically curable subtype of PA. To test this hypothesis, we performed a Receiver Operator Characteristics curve analysis and found that the titer of AT1AA was useful in distinguishing APA from PH, the accuracy (estimated from the AUC) being higher than that under the identity line (AUC 0.890 versus 0.500, P<0.001; Youden Index=2.37; Figure 2), and from IHA (AUC 0.843, P<0.001; Youden Index=2.06; Figure 2).

The AT1AA titer performed worse than the baseline and postcaptopril ARR, two established indexes for the screening of PA, in discriminating PA from PH or APA from PH (AUC of baseline ARR 0.993, AT1AA 0.573, P<0.001 and AUC of postcaptopril ARR 0.997, AT1AA 0.860, P=0.03, respectively). This higher performance of the ARR could be anticipated given the following: (1) IHA patients had an AT1AA titer similar to PH patients and (2) ARR had been used beforehand for PA screening, thus providing PA population already selected for a high ARR. Noteworthy, however, the AT1AA titer performed as well as the baseline ARR for distinguishing APA from the hypertensive patients without APA, including IHA and PH (AUC ARR at baseline 0.923, AT1AA 0.848, P=0.305; Figure 3). Moreover, it was better than the baseline ARR for discriminating APA from IHA (AUC of ARR at baseline 0.660 [0.481 to 0.811] vs AUC of AT1AA 0.859 [0.700 to 0.953], P=0.023).

AT1AA-Positive Versus Negative Patients

To identify the distinctive features of patients with raised AT1AA titer, our PA patients were divided into AT1AA-positive and AT1AA-negative, using a positive/negative ratio value of 2.1, a value corresponding to the optimal cutoff for discriminating APA from IHA patients (sensitivity=92.3%, specificity=80%, positive predictive value=85.7%, negative predictive value=88%), as determined by the Youden Index. All NT subjects were below this cutoff value. When the positive and negative PA patients were compared, the former were younger and had lower postcaptopril PAC than the latter (15.1 [7.3–19.6] versus 20.0 [14.0–41.0]; P=0.015). No differences were seen in the distribution of all other clinical or biochemical variables (Table 2). Of interest, the postcaptopril PAC fall was greater in AT1AA-positive than in AT1AA-negative patients, both in hypertensive and in PA patients (−29.0% [23.5–43.0] versus −21.4% [0.0–38.8], P=0.019; –32.4% [21.1–42.9] versus −0.0% [0.0–22.6], P=0.015, respectively; Figure 4).

No differences in AT1AA titers or rate of positive cases were found among tertiles of systolic or diastolic BP in either the whole hypertensive cohort or in PA patients alone (data not shown).

Discussion

This study shows that AT1AA are detectable in serum in 92.3% of the patients with an APA at a titer significantly raised, as compared with both patients with IHA and to PH patients, with similar degree of BP elevation. These findings are novel in that previous studies investigating autoantibodies against the AT1 receptor in hypertensive patients only focused on preeclampsia and malignant hypertension. Wallukat et al first described a chronotropic response to serum of preeclamptic

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**Determinants of AT1AA Titer**

We used a regression analysis to identify the predictors of AT1AA titer. In the whole hypertensive cohort and in all subpopulations (PH, IHA, APA), there was an inverse correlation between postcaptopril PAC and AT1AA (ρ=0.261, P=0.008; Table S1 in the online-only Data Supplement). No correlations between serum AT1AA titer and age, body mass index, systolic or diastolic BP, baseline PAC, plasma renin activity or ARR, or postcaptopril plasma renin activity or ARR were seen.

In the APA, there was a direct correlation of AT1AA with postcaptopril plasma renin activity (ρ=0.485; P=0.033), and an inverse correlation with baseline and postcaptopril ARR (ρ=−0.585, P=0.007 and ρ=−0.819, P=0.001). In hypertensive patients, the AT1AA titer correlated directly also with the fall of aldosterone after captopril (Spearman ρ=0.245, P=0.024).

**AT1AA as a Diagnostic Tool**

Considering the different AT1AA titer in APA and PH or IHA patients, we wondered whether this titer could be useful for...
women of cultured neonatal rat cardiomyocytes, which was attributed to autoantibodies-mediated stimulation of the AT1-receptor. Such autoantibodies were not found in pregnancies with high BP, but without preeclampsia.9 Thereafter, studies both in vivo and in vitro showed that AT1AA have biological effects, including Ca2+ release in vascular smooth muscle cells, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase stimulation, nuclear factor-κB, and JAK-STAT (Janus Kinase and Signal Transducer and Activator of Transcription pathway) activation, and induction of a preeclamptic-like syndrome in pregnant mice.19–24 Circulating autoantibodies against the AT1 were detected in one third of patients with malignant hypertension secondary to renovascular disease,10 and also in patients with acute renal transplant rejection and malignant hypertension.12 Altogether these findings suggested that they could be a marker for vascular damage, rather than high BP per se.

To circumvent the practical unfeasibility of using the chronotropic response of cultured neonatal cardiomyocytes for routine detection of AT1AA, and to overcome the difficulties inherent with the lack of an accepted reference standard for measuring AT1AA, Liao et al developed an ELISA assay and proposed to express the AT1AA titer as positive or negative ratio.11 By using this approach, we found a prevalence of AT1AA positives among the essential hypertensives of 29.1% similar to that (26.8%) found in the general hypertensive population.

Figure 2. Box and whisker, and Receiver Operator Characteristics curves plots showing the agonistic autoantibodies against type-1 angiotensin-II receptor (AT1AA) positive/negative ratio in the primary hypertension (PH) and aldosterone-producing adenoma (APA; A and B), and in the idiopathic hyperaldosteronism (IHA) and APA (C and D). The dashed line shows the Youden Index (YI), for example, the cutoff value furnishing the best tradeoff of sensitivity and specificity, as reported in A and C. B and D show the corresponding Receiver Operator Characteristics curve with the YI (dot). The area under the Receiver Operator Characteristics curve (AUC) is an estimate of overall accuracy, Pos Pred Val indicates positive predictive value; and Neg Pred Val, negative predictive value.

Figure 3. Receiver Operator Characteristics curves for the agonistic autoantibodies against type-1 angiotensin-II receptor (AT1AA) titer and the aldosterone-to-renin ratio (ARR) at baseline for discrimination of aldosterone-producing adenoma (APA) from non-APA (left) and from idiopathic hyperaldosteronism (IHA) (right). The area under the curve (AUC), which provides an overall estimate of diagnostic accuracy, was compared between tests by the method of Hanley and McNeil. While this comparison showed no significant differences between ARRB and AT1AA for the identification of APA in hypertensive patients (left), it showed a significantly higher AUC for AT1AA than for ARRB for the distinction of APA from IHA (right).
Of further importance, we found that the titer of AT1AA is raised in the patients with PA because of an APA (Figure 1). These autoantibodies were previously shown to have agonistic activity and are directed against the second loop (165–191) of the AT1 receptor, which encompasses a sequence of 7 amino acids (AFHYESQ). The binding of AT1AA to this peptide epitope is crucial in mediating the agonistic activity of AT1AA, as AT1AA-induced stimulation of cardiomyocyte contraction rate was antagonized by this epitope in the cardiomyocyte assay. As PA carries a more prominent cardiovascular damage than essential hypertension, this finding might be related to the hyperaldosteronism, the high BP, and their joint detrimental effects on the vasculature. In keeping with this contention, Liao et al found that the prevalence of AT1AA was 4-fold higher in patients with refractory hypertension, some of which could have undetected PA, than in those with nonrefractory hypertension (42% versus 10.4%, respectively). It could therefore be speculated that in APA patients, the more severe vascular damage may alter, or increase, exposure of AT1 receptor on the plasma membrane of vascular smooth muscle cells, thereby triggering autoantibodies formation. A direct involvement of aldosterone excess in this process could also be hypothesized, given the cross-talk between aldosterone and AT1 receptors. Hence, excess aldosterone per se could alter the expression or induce conformational changes of AT1 receptors, thereby increasing their immunogenicity. It is worth underscoring, however, that in our study, in spite of similarly raised plasma aldosterone concentrations, the AT1AA titer was higher in APA than in IHA patients. Moreover, it did not correlate with aldosterone, either in the whole hypertensive population or within the subgroups of APA, IHA, or PH, thus lending no support to this hypothesis. Furthermore, the detection of AT1AA also in PH would suggest a role in AT1AA induction for BP-induced vascular damage. At variance with this contention, no clear-cut differences in AT1AA titers or rate of AT1AA-positive cases across tertiles of systolic or
diastolic BP could be detected. Hence, overall, these findings suggest 2 different (albeit not necessarily alternative) hypotheses: (a) IHA and APA represent different stages of the same disease and (b) they do not belong to a continuum, but rather entail different entities. The latter contention is supported by accumulating findings: for example, the recently identified mutations in the selectivity filter of the KCNJ5 K+ channel were detected in about one third of APA and never in BAH (causing IHA), with the exception of the very rare cases carrying germline mutations.77,78 Likewise, the recently reported increase of parathyroid hormone in PA patients was found in APA patients and not IHA patients.29,30

Thus being clinically helpful in the differential diagnosis between these conditions.31 Furthermore, Sato et al recently reported that 18-OH cortisol and 18-oxo cortisol were raised in patients with APA, and not in those with IHA.32 Hence, the present result is not unique in showing biomarker differences between the APA and IHA patients.

The peculiar increase of AT1AA in APA patients deserves some further comments, as these tumors were found to express functional AT1 receptors,6–8 and a considerable proportion of them are responsive to Ang-II.33,34 Hence, it might be that altered epitopes in these receptors in the adenoma cells trigger production of autoantibodies. Available data, however, do not support this contention as single-stranded conformational polymorphism PCR, and direct DNA sequencing did not reveal mutations in APA tissue.35–37 Yet, the possibility of posttranslational modifications remains to be dismissed at this stage. A tumor site itself could also trigger abnormal immune responses by the so-called epitope-spreading, a process whereby epitopes distinct from, and non-cross-reactive with, an inducing epitope become targets of an evolving antitumor immune response because of cross-presentation.38 The possibility of presentation of additional epitopes by antigen-presenting cells, secondary to the activation of T-cells against malignant cells in vivo, is well testified by the occurrence of autoimmunity in many paraneoplastic syndromes. Hence, further investigation is worth to clarify whether in APA this epitope might entail the AT1 receptor, and whether this mechanism could account for the high titer of AT1AA documented in this study.39

Although it remains contentious whether the occurrence of a raised titer of AT1AA mark the transition from hyperplasia (IHA) to adenoma, the increase of AT1AA titer in APA was clear-cut (Figure 2) and suggested that it might help differentiating between PA patients with and without a tumor. Our Receiver Operator Characteristics curves analysis supports this hypothesis: the AT1AA titer provided an accurate identification of APA patients among the hypertensive patients, and also a distinction between APA and IHA patients referred to the 2 centers. The latter distinction normally requires adrenal vein sampling or PET-CT (positron emission tomography-computed tomography) with metomidate,40 both of which are expensive and not readily available. Therefore the diagnostic discrimination provided by the AT1AA assay could be important from the clinical standpoint in that it might allow selecting a cohort of PA patients to be submitted to either test, owing to a higher risk of carrying a unilateral surgically correctable cause of PA.

Limitations and Strengths

The following limitations of this study need to be mentioned. First, it remains to be investigated whether the AT1AA titer falls after adrenalectomy in APA patients. Second, albeit sizeable, the study was relatively small and entailed only patients of white ethnicity. Therefore, confirmation in larger studies entailing PA patients of different ethnicities is necessary. Third, our present data do not directly demonstrate an agonistic activity of AT1AA on AT1 receptors; however, previous studies indicated that increased levels of AT1AAs, as detected by this ELISA assay, cause vascular constriction and other biological effects that are held to occur through activation of AT1 receptor.21,24

The following strengths are, however, also to be noted: this study was carried out according to the STARD Committee recommendations,40 in that strict diagnostic criteria based on adrenal vein sampling, pathology, and follow-up information were used for diagnosing APA; moreover, different positive and negative control populations were recruited, and a state-of-the-art statistical analysis was exploited to assess diagnostic accuracy and compare different tests.

Thus, the finding that in a sizeable group of patients with unequivocally diagnosed APA, the titer of circulating autoantibodies against the AT1 receptor is higher than in patients with primary (essential) hypertension or with the idiopathic form of PA is novel and stands on solid evidence. Moreover, it suggests that the titer of these autoantibodies is useful for differentiating between the surgically curable APA and the medically treatable IHA.

Perspectives

Interest has been rising in recent years on the involvement of the immune system in cardiovascular disease and hypertension.41–43 Based on the present findings, future research should be devoted to determine whether these autoantibodies play a mechanistic role in the pathogenesis of APA. In this regard, the inverse correlation of AT1AA with postcaptopril plasma aldosterone concentration, along with the direct correlation with its fall after angiotensin-converting enzyme inhibition, suggests that AT1AA sensitize adrenocortical aldosterone-producing cells to endogenous Ang-II. A larger drop in PAC can therefore be expected on Ang-II deprivation in Ang-II–dependent APA. This is consistent with the demonstration of a sensitizing effect of AT1AA to Ang-II in inducing a preeclampsia-like syndrome, in pregnant rats.44 Moreover, AT1AA have been reported to stimulate NADPH-oxidase, with ensuing reactive oxygen species production and activation of nuclear factor-kB in vascular smooth muscle cells,45 and to release PAI-1 (plasminogen activator inhibitor-1) and interleukin-6 in cultured human mesangial cells,46 all of which may cause inflammation and contribute to aldosterone-induced vascular damage.

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Disclosures
None.

References
27. Rossitto et al. Autoantibodies in Primary Aldosteronism 7

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What Is New?
• The finding of a raised titer of autoantibodies against the angiotensin-II type-1 (AT1) receptor in serum of primary aldosteronism (PA) patients with an aldosterone-producing adenoma (APA), and not with bilateral adrenal hyperplasia.

What Is Relevant?
• These autoantibodies could exert an agonistic effect on the AT1 receptor and thereby contribute to maintaining the hyperaldosteronism, despite the suppression of the renin-angiotensin system.

Guidelines recommend that adrenal vein sampling (AVS) be offered to all patients who are candidates for adrenalectomy, but AVS is expensive and not available for all patients in most centers.
• Hence, the titer of these autoantibodies could be useful in the differential diagnosis between the surgically curable and incurable subtypes of PA, and therefore for selecting the patients for AVS.

Summary
An elevated serum titer of AT1 receptor autoantibodies could be useful for the subtyping of PA.

Novelty and Significance

Elevation of Angiotensin-II Type-1-Receptor Autoantibodies Titer in Primary Aldosteronism as a Result of Aldosterone-Producing Adenoma

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ONLINE SUPPLEMENT

Elevation Of AT1-Receptor Autoantibodies Titer In Primary Aldosteronism Due To Aldosterone-Producing Adenoma

Giacomo Rossitto1§, Giuseppe Regolisti2§, Ermanno Negro2, Davide Nicoli2, Bruno Casali2, Antonio Toniato1, Brasilina Caroccia1, Teresa Maria Seccia1, Thomas Walther4 and Gian Paolo Rossi1

1Department of Medicine – DIMED, Internal Medicine 4, Padua University - School of Medicine; 2Department of Internal Medicine, Santa Maria Nuova Hospital, Reggio Emilia, Italy
3 Surgical Pathology Unit, Department of Medical and Surgical Sciences, Padua University,
4Hull York Medical School, Hull University, UK
§these authors equally contributed to this manuscript

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Corresponding author:
Prof. Gian Paolo Rossi, MD. FACC, FAHA
DMCS – Internal Medicine 4
University Hospital
via Giustiniani, 2
35126 Padova, Italy
Phone: 39-049-821-7821 or 2279
Fax: 39-49-8754179
E-mail: gianpaolo.rossi@unipd.it
Expanded Materials And Methods

Patients

We recruited for this study 52 consecutive patients with PA and 62 primary (essential) hypertensive (PH) patients studied at two referral centers (Divisione di Medicina Interna II, Reggio Emilia, and Clinica Medica IV, University of Padua, Italy) from January 1, 2006 to February 15, 2007. The case detection of PA was performed with the aldosterone-to-renin ratio (ARR) as described.\textsuperscript{1, 2} As a confirmatory test, the captopril challenging was performed,\textsuperscript{3} and interpreted using cutoffs determined with ROC curves analysis and calculation of Youden index,\textsuperscript{4} the value on the ROC curve that captures the best combination of sensitivity and specificity.

At the time of measurement of plasma renin activity (PRA), plasma aldosterone concentration (PAC), and of determination of serum titer of AT1-AA, patients were treated only with calcium channel blockers and/or doxazosin. Other antihypertensive agents, including diuretics, beta-blockers, angiotensin-converting enzyme inhibitors, and angiotensin II type 1 (AT-1) receptor antagonists, had been withdrawn for at least 3 weeks, and mineralocorticoid receptor antagonists for at least 6 weeks. Medications withdrawal was considered unsafe for two patients with PA and three with PH who had resistant hypertension and/or evidence of target organ damage and/or previous cardiovascular events. Hence, the ARR was determined under treatment and the diagnosis was made based on knowledge of the effect of the different drugs on the ARR,\textsuperscript{3} and on the outcome of blood pressure values and the demonstration of normalization of the ARR after adrenalectomy. Due to the confounding effect of the drug treatment their PRA, PAC and ARR were not included in the statistical analysis.

Familial (glucocorticoid-remediable) hyperaldosteronism type I was excluded in all cases by long-PCR for the chimeric gene. APA were diagnosed using the “four corners’ criteria”, which entail: 1) biochemical evidence of primary aldosteronism; 2) lateralized aldosterone excess at adrenal vein sampling or, if unavailable, at adrenocortical NP59 scintigraphy; 3) identification of APA at
surgery, pathology, or both; 4) demonstration of correction of the hyperaldosteronism and cure, or marked improvement of the hypertension after adrenalectomy. The PA patients not fulfilling all of these criteria were held to have idiopathic hyperaldosteronism (IHA).

The diagnosis of PH was established after exclusion of all the main secondary forms of hypertension with extensive work-up.

Since pre-eclamptic women are presumed to have a high serum titer of AT1-AA, thirteen such patients (diagnosed by the criteria of the National Institute for Health and Clinical Excellence (NICE) guidelines on the management of hypertensive disorders during pregnancy) were studied as “positive controls”.

Forty-five normotensive healthy blood donors (NT) served as a control group. As further negative control, serum AT1-AA levels were measured in 30 women with uncomplicated pregnancy at gestational week 32-38.

Informed consent was obtained from all patients and the Ethic Committees at both institutions approved the study.

**Immuno-enzymatic assay for AT1-AA**

The AT1-AA titer was measured with an ELISA assay according to the method of Liao et al. A peptide corresponding to the second extracellular loop of the human AT1-R positions 165-191 (I-H-R-N-V-F-F-I-E-N-T-N-I-T-V-C-A-F-H-Y-E-S-Q-N-S-T-L) was selected and synthesized from Invitrogen Corp. (Carlsbad, CA, USA). The peptide (10 μg/ml) in a 100 mM Na2CO3 solution (pH 9.6) was coated on microtiter plates overnight. The wells were then saturated with phosphate-buffered saline (pH 7.4, 0.01 mmol/L PBS-Tween 20). To reduce background interference and improve assay sensitivity, after testing several different blocking agents (Non-Fat Dry Milk, Fish Gelatin, BSA) and concentration (1 to 3%), we selected a commercial solution of purified bovine serum albumin (Thermo Scientific) diluted 10-fold. Optimal dilutions for sera and anti-human IgG antibodies to achieve a zero order kinetic in the relation between AT1AA titer and absorbance and
avoid saturation were assessed by means of specific experiments. After washing three times with PBS-Tween 20, the 1:100 diluted sera were added to the microtiter plates for 1 h at 37°C. After three additional washings, horseradish peroxidase-conjugated anti-human IgG antibodies 1:2000 (Sigma-Aldrich Corporate, St. Louis, MO) were added for 1 h at 37 °C, and the plates then washed again three more times. The substrate (0.01% H2O2 and 0.1% 3′-3′-5′-5′-tetramethyl benzidine) was added for 5 minutes and the reaction was terminated with the appropriate stop solution (S5814, Sigma-Aldrich Corporate, St. Louis, MO). Optical density (OD) was read at 450 nm in a micro plate reader. Due to the unavailability of standard curves, the AT1AA titer was expressed as positive/negative (P/N) ratio [(the OD of sample - the OD of empty control)/(the OD of negative control - the OD of empty control)] for data standardization, according to Liao et al. Intra-assay coefficient of variation was 2.2%, and inter-assay coefficient of variation was 5.7%.

**Biochemical measurements**

Serum creatinine, serum and urine Na⁺ and K⁺ levels, PRA, PAC, and PCC were measured as described. Normal ranges, intra-assay, and inter-assay coefficient of variation and antibody cross-reactivity for the hormonal measurements were previously reported.

**Statistics**

PRA, PAC, ARR, and AT1AA titer values were analyzed after log transformation due to their skewed distribution. One-way ANOVA analysis followed by Bonferroni post-hoc test was used to compare quantitative variables across groups (PA as a whole or as APA and IHA, PH patients, NT subjects and women with preeclampsia or normal pregnancy). Distribution of categorical variables was investigated by χ² analysis or Fisher’s exact test, as appropriate. The relationship between hormones, blood pressure, biochemical and anthropometric variables and serum AT1-AA titer was further analyzed by Spearman’s rho and Regression analysis. Mann-Whitney U test was used to compare PAC change after captopril challenging across populations.
The accuracy of the AT1AA titer for identifying PA, APA and IHA among hypertensive patients was assessed by the area under the receiver operator characteristics (ROC) curves (AUC) and compared between the different tests, according to the method of Hanley and McNeil.\textsuperscript{1, 12} The Youden index (J), defined as $J = \max (c) [\text{sensitivity}(c) + \text{specificity}(c) - 1]$, which corresponds to the value of the ROC curve farthest from the identity line, was employed to determine the optimal cutoff ($c^*$), defined as the value that corresponds to the highest average of sensitivity and specificity.\textsuperscript{13, 14} The positive and negative predictive values were also calculated to obtain information on the performance of each test.

Data are expressed as mean ± SD or median (interquartile range), as appropriate; an alpha level <0.05 was considered statistically significant. All $p$ values are two-sided. Analysis was performed with SPSS 18.0 for Windows (SPSS Inc., 18.0) except for ROC analysis and Youden index calculations for which MedCalc\textsuperscript{TM} Software (Version 12.2.1, Mariakerke, Belgium) was used.
References for expanded materials and methods


Table S1: Correlation of AT1-AA with clinical-biochemical variables.

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Figure S1

AT1AA titers in the cohorts of hypertensive patients and normotensive subjects. AT1AA titer on ELISA assay, expressed as as positive/negative (P/N) ratio [(the OD of sample - the OD of empty control)/(the OD of negative control - the OD of empty control)]; • single cases. AT1AA titer was significantly higher in PA than in PH patients (2.65 ± 1.55 vs 1.86 ± 0.63) and higher than in normotensive controls (1 ± 0.2, respectively) both in PA and PH patients.