

Angiotensin II Type 1 Receptor Autoantibodies in Primary Aldosteronism



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ABSTRACT

Primary aldosteronism (PA) is the most common form of endocrine hypertension. Agonistic autoantibodies against the angiotensin II type 1 receptor (AT₁R-Abs) have been described in transplantation medicine and women with pre-eclampsia and more recently in patients with PA. Any functional role of AT₁R-Abs in either of the two main subtypes of PA (aldosterone-producing adenoma or bilateral adrenal hyperplasia) requires clarification. In this review, we discuss the studies performed to date on AT₁R-Abs in PA.

Introduction

Primary aldosteronism (PA) displays a prevalence of around 5–10% in patients with hypertension increasing up to 20% in patients with treatment-resistant hypertension [1–3]. Patients with PA have an elevated risk of cerebrovascular and cardiovascular events relative to patients with hypertension with matched cardiovascular risk profiles [4–6]. Different subtypes of PA have been defined which affect one or both adrenal glands. The main subtypes are a unilateral aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia (BAH, also called idiopathic hyperaldosteronism). Other sporadic forms include unilateral adrenal hyperplasia and the very rare occurrences of aldosterone-producing carcinoma. Hereditary forms of familial hyperaldosteronism are rare and the genetic basis of the 4 described forms of familial hyperaldosteronism (FH types I–IV) has been identified [7, 8]. A number of somatic mutations, mainly in ion channels and ATPases, have been reported which are

likely to drive the aldosterone excess in the majority of APAs. Increasing interest in autoantibodies led to studies reporting a potential role for autoantibodies against the G-protein-coupled receptor (GPCR) angiotensin II type 1 receptor in PA.

G-Protein-Coupled Receptors

The largest superfamily of receptors in the human genome are GPCRs that are located in the plasma membrane of nearly all cell types [9]. The research of Kobilka and Lefkowitz about the molecular structure and function of GPCRs, especially β 2-adrenergic G-protein-coupled receptors, was rewarded in 2012 by the Nobel prize in chemistry [10]. GPCRs are responsible for signal transduction to regulate numerous essential functions by mediating extracellular signals from hormones, neurotransmitters or environmental stimulants to the intracellular metabolic pathways [9]. Their structure consists of seven transmembrane-spanning helices

bound by intra- and extracellular loops [11]. On the extracellular side, GPCRs are targeted by their ligands but also by autoantibodies, which may induce agonistic receptor stimulation or inhibition dependent on the autoantibody binding site to the first and second or third extracellular loops, respectively [9].

Binding of extracellular agonists activate the receptor by initiating a conformational change that induce further signal transduction pathways [12]. Conformational changes result in the hetero-trimeric G-protein to exchange GDP for GTP at its $G\alpha$ subunit [13]. The $G\alpha$ subunit with GTP and the $G\beta\gamma$ subunit both dissociate from the receptor, resulting in the activation of specific signaling pathways such as, adenylyl cyclase (via cAMP generation) and phospholipase C [via diacylglycerol (DAG) and inositol trisphosphate (IP_3) production] [13]. The $G\alpha$ subunit bound to GTP hydrolyses GTP back to GDP to reassociate with the $G\beta\gamma$ subunit [13]. Receptor signaling determination is mediated by G-protein-coupled receptor kinases (GRK) which phosphorylate the activated receptor to bind to a distinct scaffold protein for subsequent internalization into the cells [13]. These scaffold proteins are arrestins and are known to desensitize GPCRs and induce G-protein independent signaling [14]. Following internalization the receptor is either degraded or recycled back to the plasma membrane [13]. Besides its involvement in GPCR internalization, arrestins are able to interact directly with activated GPCRs resulting in a clear conformational change that could initiate further downstream signaling pathways [13]. There is some evidence for biased agonism of GPCRs towards β -arrestin-mediated signaling [15, 16].

The angiotensin II type 1 and 2 receptors (AT_1R and AT_2R) are GPCRs with opposing functions in blood pressure regulation and sodium excretion [17–19]. The two subtypes share 34% sequence homology and stimulate different signaling pathways to elicit distinct and counter-regulatory biological functions [17]. AT_2R is highly expressed in the fetal state although lower levels are present in the adult brain, heart, kidney and the adrenal [17]. In some diseases, AT_2R is upregulated acting as an anti-inflammatory and repairing factor for wound healing or after cardiac or vascular events [20, 21]. In contrast, the AT_1R is widely distributed, for example, in the adrenal gland, liver, kidney, fat, brain, placenta, spleen, or thyroid, and its physiological role as a component of the renin-angiotensin-aldosterone system (RAAS) is well characterized via binding of its cognate ligand angiotensin II for blood pressure regulation, vasoconstriction, inflammatory response and vascular and cardiac hypertrophy [21].

The Discovery of Autoantibodies Against Angiotensin II Type 1 Receptor

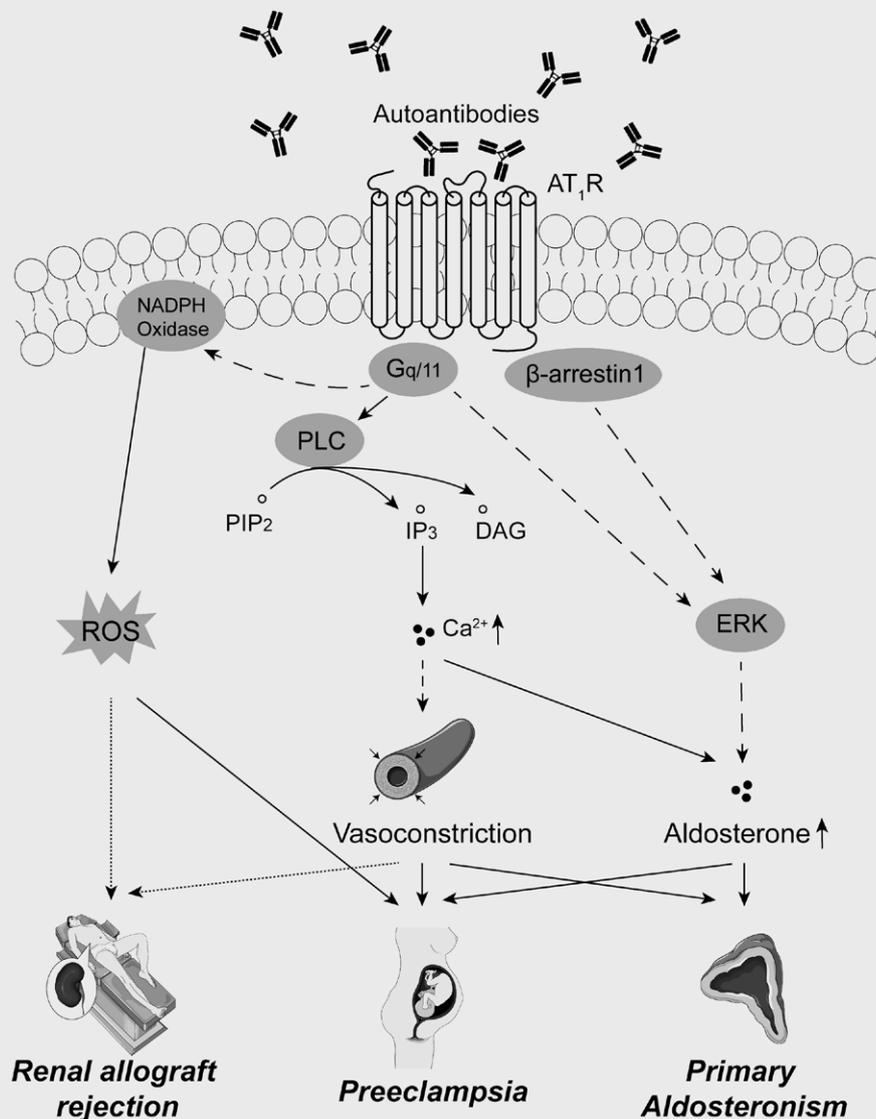
The prevalence of autoimmune diseases in the population is around 2.5% although autoantibodies are also often present in healthy individuals [22, 23]. Impaired B cell tolerance can allow autoantibody-producing B cells with medium or low binding affinity to self-antigens to escape from elimination or further anergy during B cell maturation thus becoming autoantibody-secreting plasma cells [22]. However, the pathogenic role of autoantibodies is mostly unknown.

The role of autoantibodies against the AT_1R (AT_1R -Abs) in hypertension has been investigated over the last decades. In 1999, Wallukat et al. described the presence of AT_1R -Abs in patients with

pre-eclampsia, which is discussed further below [24]. This discovery was extended one year later by Fu and coworkers who observed elevated AT_1R -Ab levels in 33% patients with malignant secondary hypertension, but also found increased levels of AT_1R -Abs in 14% of the control group [25]. Soon afterwards, an association of AT_1R -Abs and renal graft failure was observed by many research groups, especially in patients without donor-specific human leukocyte antigen (HLA)-antibodies [26–29]. Dragun et al. reported a comparable prevalence of kidney rejections associated with either AT_1R -Abs (3.6%) or donor-specific HLA-antibodies (3.2%) in a cohort of 278 kidney transplantations including 119 rejections [29]. High levels of AT_1R -Abs (>17 units) without additional presence of donor-specific antibodies have been ascribed to an increased risk for antibody-mediated kidney rejection [27]. Other studies reported on AT_1R -Ab levels of >9 U/ml or >10 U that have been shown to elevate the risk of graft loss [28, 30]. Notably, in some patients (17–47%) AT_1R -Abs were already present before transplantation [26–28, 30]. A case report of Jobert et al. described a 28-year old male recipient of a well-matched renal allograft lacking anti-HLA-antibodies but with a high AT_1R -Ab level of 14.1 U/ml prior transplantation who developed acute vascular rejection four days post-transplantation [31]. The authors hypothesized that the AT_1R -Abs are the only factor that could have led to the allograft rejection due to the otherwise well-matched HLA-type and virus status [31]. Treatment with anti-thymocyte globulin, methylprednisolone, candesartan and plasma exchange was successful suggesting probably plasma exchange as a considerable option in patients with AT_1R -Abs [31]. However, AT_1R -Abs also appeared after kidney transplantation in prior AT_1R -Ab-negative patients without an associated worse clinical outcome [32]. Of note is that Taniguchi et al. stressed that such studies report an association of AT_1R -Ab levels and allograft failure and do not explain any causal relationship [26]. The occurrence of autoantibodies is widely distributed in transplantation medicine but also in autoimmune diseases such as Huntington, multiple sclerosis or systemic sclerosis [33, 34]. A summary of diseased states widely reported as associated with increased AT_1R -Ab levels is shown in the ► **Fig. 1**.

Comparison of Different Assays for AT_1R -Ab Characterization

AT_1R -Abs in patient serum have been widely measured by enzyme-linked immunosorbent assays (ELISA). For such ELISAs, the target antigens are immobilized on a solid phase, mainly on a microplate to detect binding autoantibodies from serum samples [22]. As antigens serve the human full-length AT_1R or peptides comprising known epitopes (AFHYESQ or ENTNIT) for AT_1R -Abs in the AT_1R second extracellular loop [29]. The bound autoantibodies are then either directly labelled with reporter enzymes (direct ELISA) or by a secondary labelled antibody coupled to a reporter enzyme (indirect ELISA) for quantification. Internal laboratory assays have been developed as well as commercially available kits (► **Table 1**). Piazza et al. compared in a study with patients diagnosed with APA two of the commercially available Sandwich-ELISA kits used for AT_1R -Abs determination [35, 36] by CellTrend and Cusabio [37]. The results of both assays were consistent and both demonstrated higher AT_1R -Ab levels in patients diagnosed with an APA compared with healthy controls [37]. In contrast, the same



► **Fig. 1** Potential pathological effects of agonistic AT₁R autoantibodies: Autoantibodies to the AT₁R have been described in a number of different pathological states as indicated. Continuous lines represent a direct effect, dashed lines indicate an indirect effect, dotted lines denote putative effects. Figure compiled by the authors using elements of <https://smart.servier.com/> (licensed under Creative Commons Attribution 3.0 Unported License).

CellTrend kit and a second kit from ELISA-Creative Diagnostics revealed the detection of contrasting AT₁R-Ab levels depending on the assay used for patients with pre-eclampsia relative to controls [38]. This highlights the inherent drawbacks of assays based on the detection of AT₁R-Ab concentrations and not AT₁R bioactivity. To investigate if AT₁R-Abs activate the AT₁R, assays have been developed, which measure cultured spontaneously beating neonatal rat cardiomyocytes in response to immunoglobulins G (IgGs) and the perfused rat cremaster arteriole assay to measure losartan-sensitive antibody-mediated vasoconstriction [24, 39]. In vitro experiments using genetically engineered cells (for example, Chinese hamster ovary cells (CHO), or human bone osteosarcoma cells (U2OS)) stably transfected with human AT₁R) to measure the functional activation of the AT₁R in response to whole serum or purified

IgGs have also been used [37–40]. Upon receptor activation, the transfected U2OS or CHO cells mediate chemiluminescent or fluorescent signals that can be quantitatively measured. Of note is that many cell-based assays used to quantify AT₁R-Ab agonistic activity measure responses mediated by the β-arrestin signaling pathway. In addition, the production of aldosterone or increased expression of the aldosterone synthase gene (*CYP11B2*) in response to treatment with IgG fractions or whole serum has been measured using human adrenocortical carcinoma cell line (HAC15) [37].

AT₁R-Abs and Pre-Eclampsia

Pre-eclampsia is the sudden appearance of hypertension in pregnant women after 20 weeks' gestation week that is associated with increased risks of long-term hypertension, stroke, cardiovascular

► **Table 1** Angiotensin II type 1 receptor autoantibody measurements in primary aldosteronism.**ELISA****Indirect ELISA using immobilized AT₁R peptides of extracellular loop 2**

Rossitto et al. 2013 [51]	<ul style="list-style-type: none"> – AT₁R-Ab level in patients with PA (n = 46) higher than in NT (n = 45) – AT₁R-Ab level in patients with APA (n = 26) 2-fold higher than IHA (n = 20)
Kem et al. 2014 [39]	– elevated AT ₁ R-Ab levels in 4 of 13 patients with PA (31%)
Li et al. 2015 [40]	– elevated AT ₁ R-Ab levels in 5/12 (42%) of BAH, 3/13 (23%) of APA and 1/15 (7%) of NT

Sandwich-ELISA with full-length AT₁R

Sabbadin et al. 2018 [35]	Human angiotensin II receptor 1 antibody, ATIIR1 Ab ELISA Kit (Cusabio, Wuhan, China) <ul style="list-style-type: none"> – elevated AT₁R-Ab titers in PA (n = 44) than in NT (n = 18) – AT₁R-Ab level comparable between APA and BAH
Piazza et al. 2019 [37]	Human angiotensin II receptor 1 antibody, ATIIR1 Ab ELISA Kit (Cusabio, Wuhan, China) <ul style="list-style-type: none"> – AT₁R-Ab levels in patients with APA (n = 12) compared to NT (n = 7)
Piazza et al. 2019 [37]	CellTrend, Luckenwalde, Germany <ul style="list-style-type: none"> – higher AT₁R-Ab levels in patients with APA (n = 27) compared to NT (n = 7)
Williams et al. 2019 [38]	CellTrend, Luckenwalde, Germany <ul style="list-style-type: none"> – equal levels of AT₁R-Abs in APA (n = 40), BAH (n = 40), PH (n = 40), NT (n = 25)
Williams et al. 2019 [38]	Creative Diagnostics <ul style="list-style-type: none"> – equal levels of AT₁R-Abs in APA (n = 40), BAH (n = 40), PH (n = 40), NT (n = 25)

Functional AT₁R agonist measurements**Cell-based functional assays**

Williams et al. 2019 [38]	SERUM: <ul style="list-style-type: none"> – without losartan: no group differences between APA (n = 40), BAH (n = 40), PH (n = 40), NT (n = 25) – with losartan: AT₁R-bioactivity of APA, PE and NT reduced in comparison to BAH Purified IgG: <ul style="list-style-type: none"> – without losartan: AT₁R-bioactivity of BAH higher than of APA; AT₁R-bioactivity of BAH, PH, PE all higher than NT – with losartan: AT₁R-bioactivity not abolished by losartan
Kem et al. 2014 [39]	SERUM: <ul style="list-style-type: none"> – elevated AT₁R-bioactivity through serum from PA patients compared to NT, mainly suppressed by losartan/candesartan
Li et al. 2015 [40]	SERUM: <ul style="list-style-type: none"> – 15/25 (60%) of patients with PA showed autoantibody-mediated AT₁R-bioactivity: 9/12 (75%) patients with BAH; 6/13 patients with APA (46%) – NT were negative for AT₁R-Ab induced AT₁R-bioactivity – losartan inhibited AT₁R-bioactivity
Piazza et al. 2019 [37]	SERUM: <ul style="list-style-type: none"> – values below threshold; AT₁R-bioactivity from APA patient serum does not differ from NT

Perfused rat cremaster arteriole assay

Kem et al. 2014 [39]	SERUM: <ul style="list-style-type: none"> – higher cremaster arteriole contractility in patients with PA (n = 3) than NT which could be inhibited by losartan
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CYP11B2-mRNA expression in HAC15 cells

Piazza et al. 2019 [37]	Purified IgG: <ul style="list-style-type: none"> – purified IgG from patients with an APA (n = 10) induced a 40% increase in CYP11B2-mRNA expression
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Ab: Antibody; APA: Aldosterone producing adenoma; AT₁R: Angiotensin II type 1 receptor; BAH: Bilateral adrenal hyperplasia; ELISA: Enzyme-linked immunosorbent assay; HAC15: Human adrenocortical cell line; NT: Normotensive controls; PH: Primary hypertension.

morbidity and proteinuria for the mother and uteroplacental dysfunction, preterm birth, fetal distress and fetal death for the unborn child [24, 41]. In 1999, Wallukat et al. were the first who reported the presence of AT₁R-Abs in pregnant women with pre-eclampsia compared to controls [24]. The purified IgG fractions from patients with preeclampsia demonstrated losartan-suppressible AT₁R stimulation and identified the amino acid sequence AFHYESQ in the secondary extracellular loop of the AT₁R as the possible bind-

ing site for these autoantibodies [24]. Peptides against the AFHY-ESQ epitope successfully abolished AT₁R-Ab mediated activation of the AT₁R suggesting a potential target for the treatment of patients with pre-eclampsia [42, 43]. Despite *postpartum* persisting AT₁R-Ab levels in 17% of women with previous pre-eclampsia [44], the drop in AT₁R-Ab levels after giving birth and the correlation of AT₁R-Ab levels with the severity of the disease indicated a role for AT₁R-Abs in pre-eclampsia [24, 45]. Numerous studies subsequent-

ly supported the findings of Wallukat et al. with reports of AT₁R-Abs in pregnant women with pre-eclampsia [43, 46, 47]. Diverse studies have reported a role for AT₁R-Abs in pre-eclampsia in mediating intracellular Ca²⁺ release [43], induction of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and reactive oxygen species (ROS) production leading to the activation of the transcription factor nuclear factor Kappa B (NFκB) activation [46] and initiation of vasoconstriction [47], all of which were inhibited by losartan. Further in vivo experiments ascribed a causative role for AT₁R-Abs in the development of pre-eclampsia. Zhou et al. infused pregnant mice with human total IgG or affinity purified AT₁R-Abs which resulted in the characteristic symptoms of pre-eclampsia including proteinuria, hypertension and abnormal appearance of the placenta [42]. In addition, Wenzel et al. injected angiotensin II, purified rabbit AT₁R-Abs or both in pregnant rats which induced a phenotype resembling pre-eclampsia when a combination of both, angiotensin II and purified AT₁R-Abs were applied [48]. In contrast to the aforementioned findings, in a population of Mexican-Mestizo women diagnosed with pre-eclampsia no AT₁R-Abs were found [49]. Despite an apparently clear role for AT₁R-Abs in pre-eclampsia, a recent retrospective analysis of 485 women with pre-eclampsia concluded that AT₁R-Ab levels alone are not sufficient to predict hypertension in such patients, and additional risk factors for hypertension should be considered [50].

AT₁R-Abs in Primary Aldosteronism

Recently, AT₁R-Abs were also detected in patients diagnosed with PA suggesting a possible role of AT₁R activation in aldosterone production in some patients. Rossitto et al. described for the first time AT₁R-Abs in patients with PA which was subsequently reported by other research groups [39, 40, 51]. In this study, the AT₁R-Ab levels of serum from 46 patients with PA (26 APA, 20 BAH) as well as 62 patients with primary hypertension (PH) was measured by an indirect ELISA assay using an immobilized peptide of the second extracellular loop of AT₁R. Thirteen pregnant women with preeclampsia and 45 normotensive control patients (NT) were used as positive and negative controls, respectively. Patients with PA and PH showed significantly higher AT₁R-Ab levels than NT, with higher levels in patients with PA than in PH [51]. In 92.3 % of patients with APA, AT₁R-Abs were detected comprising a concentration 2-fold higher than of patients diagnosed with BAH or PH, despite comparable blood pressure levels (▶ **Table 1**) [51]. Interestingly, the AT₁R-Ab levels of patients with APA and women with pre-eclampsia were similar (3.43 ± 1.20 vs. 3.66 ± 1.79) [51]. Another indirect ELISA using the peptide AFHYESQ located in the extracellular loop 2 was performed in the studies of Kem et al. and Li et al. [39, 40]. Kem et al. described elevated AT₁R-Ab levels in 4 of 13 patients with PA (31 %) while the latter study focused on the subtypes of PA and measured elevated AT₁R-Ab levels in 42 % of BAH, 23 % of APA and 7 % of NT which contradicts the initial findings of the same group [40].

Using a commercially available ELISA kit with full-length AT₁R as antigen, Sabbadin et al. found higher AT₁R-Ab levels in patients with PA compared with healthy controls which is in accordance with previous findings [35]. However, the authors could not distinguish patients with APA (n = 15) and BAH (n = 29) [35]. In contrast, a larger study comprising a cohort of 80 patients with PA (40 APA, 40 BAH), 40 with PH, 23 with pre-eclampsia and 25 NT observed equal lev-

els of AT₁R-Abs in all groups except for patients with pre-eclampsia using two different commercial available ELISA kits (▶ **Table 1**) [38]. Using the same CellTrend ELISA kit, Piazza et al. described higher AT₁R-Ab levels in patients with APA (n = 27) compared to healthy controls (n = 7) [37]. Overall, it is clear that these studies using ELISA-based assays have yielded highly contrasting results.

AT₁R-Ab levels pre- and post-adrenalectomy were investigated in 14 patients with APA [37] who were biochemically cured following surgery according to the PASO criteria [52]. The authors found no significant decrease in AT₁R-Ab levels at one month after adrenalectomy indicating that the resected adrenal was not the source of antigens stimulating the immune response [37].

When summarizing the results of AT₁R-Ab quantification by ELISA it can be stated that there is a high variability in the AT₁R-Ab levels of patients with PA with studies reporting contrasting AT₁R-Ab levels for the different subtypes of PA [39, 51]. Of note is that elevated AT₁R-Ab levels were also described in healthy individuals without initiating subsequent AT₁R activation [40]. Furthermore, Kem et al. found for instance more frequently elevated AT₁R-bioactivity in patients with PA by a cell-based assay using AT₁R-transfected CHO cells than autoantibodies were quantified by ELISA which is in agreement with a second study from the same group [39, 40]. The authors hypothesized that the use of a linear peptide for the secondary extracellular loop instead of full-length AT₁R could miss other potential binding sites for the autoantibodies in ELISA [39]. This is supported by the findings of multiple binding sites for IgG on the AT₁R [53] and the recommendations to avoid using immobilized peptides in ELISA for GPCR-autoantibody detection [54]. However, using the full-length AT₁R does not guarantee the functional activity of the captured autoantibodies which can be demonstrated in cell based-functional assays using whole serum and/or purified IgG [38–40].

The rat cremaster arteriole assay or AT₁R-transfected CHO cells both demonstrated elevated receptor activation when exposed to serum of patients with PA compared to controls, which was largely normalized by the AT₁R-blockers losartan or candesartan [39]. Similarly, candesartan was able to reduce AT₁R-Ab-stimulated aldosterone production in vitro in HAC15 cells [39]. AT₁R-activity is more frequently observed in patients diagnosed with BAH (75 %) compared to APA patients (46 %), while serum of NT induced no AT₁R-bioactivity [40]. However, one research group failed to measure AT₁R-bioactivity in patients with APA and controls using this assay which might be due to the lack of activating function of the autoantibodies [37]. AT₁R-transfected U2OS cells also showed no group differences when incubated with serum of patients with APA, BAH or pre-eclampsia or NT in the absence of losartan [38]. Notably, the administration of losartan only barely reduced AT₁R-activation in the BAH group in comparison to APA, pre-eclampsia and NT indicating a losartan-independent activation pathway of the receptor [38]. Purified IgGs also caused an increase in AT₁R-bioactivity in the BAH compared to APA group, independent of losartan [38]. This can be explained by different binding sites for IgG and losartan at the AT₁R while angiotensin II and losartan share a common binding site on the AT₁R [53].

Autoantibody levels were not correlated with age, gender, BMI, blood pressure, baseline aldosterone and aldosterone-to-renin ratio (ARR) [35, 51]. Mineralocorticoid receptor antagonist (MRA) treatment of patients with PA was also not associated with AT₁R-

Ab levels [35]. Agonistic AT₁R-Abs displayed vasoconstrictive effects and correlate with the mean arterial pressure [39, 40]. The previously mentioned elevated agonistic activity of AT₁R-Abs in patients diagnosed with BAH and the higher responsiveness to angiotensin II in those patients [55] suggest an allosteric function of the autoantibodies which promotes the binding angiotensin II to its receptor. This is supported by the reduction of aldosterone levels after captopril-challenge in AT₁R-Ab-positive compared with AT₁R-Ab-negative patients with hypertension or PA [51]. In addition, treatment of HAC15 cells with angiotensin II and affinity-purified IgGs increased aldosterone production compared with angiotensin II treatment alone [39].

As previously indicated, upon activation, the AT₁R can initiate two distinct independent signaling pathways. More prominent is the G-protein signaling pathway in which PIP₂ is cleaved to DAG and IP₃ resulting in increased intracellular Ca²⁺ and aldosterone production [15]. Secondly, biased signaling mediated by β-arrestin1 is independent of G-proteins and may occur in parallel to the G-protein signaling pathway [15]. Aldosterone production appears to be stimulated via extracellular signal-regulated kinase (ERK)-mediated activation of steroidogenic acute regulatory protein (StAR) to promote transfer of cholesterol to mitochondria [15]. When AT₁Rs are activated by angiotensin II, both pathways appear to be initiated [15]. This has been demonstrated in vivo in rats with overexpression of β-arrestin1, which showed suppressed aldosterone production only through the administration of candesartan or valsartan and not by losartan or irbesartan despite belonging to the same pharmacological class [56, 57].

In conclusion, some evidence supports a pathological role for autoantibodies against the AT₁R in different diseases. It has not been established if AT₁R-Abs play a causative role or are a consequence of the pathology. Further studies are warranted to address the functional relevance of AT₁R-Abs in PA and the significance of AT₁R biased signaling.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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