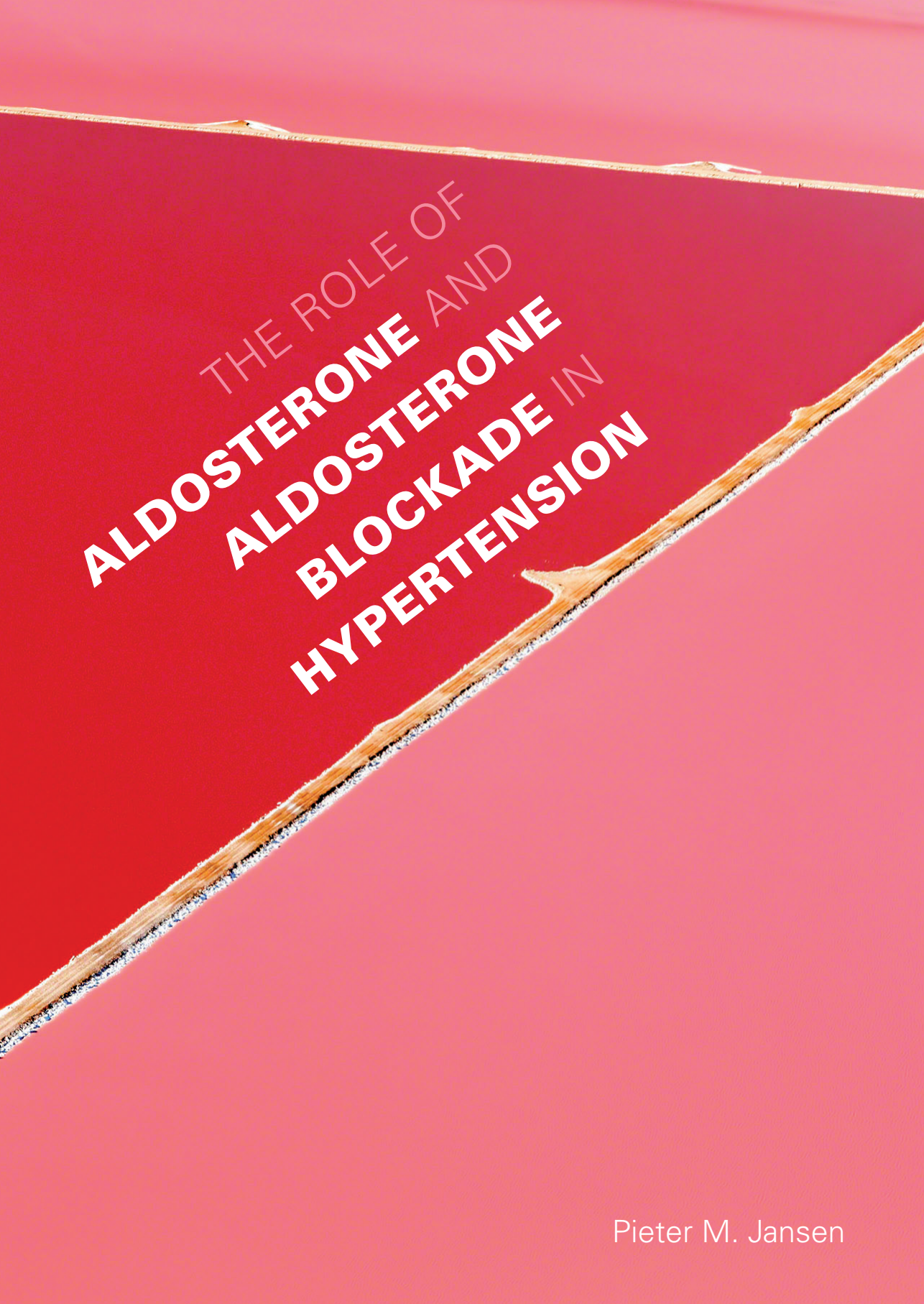
THE ROLE OF
ALDOSTERONE AND
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HYPERTENSION
Pieter M. Jansen

# THE ROLE OF ALDOSTERONE AND ALDOSTERONE BLOCKADE IN HYPERTENSION

Pieter M. Jansen

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# THE ROLE OF ALDOSTERONE AND ALDOSTERONE BLOCKADE IN HYPERTENSION

DE ROL VAN ALDOSTERON
EN ALDOSTERONBLOKKADE
BIJ HYPERTENSIE

**Proefschrift**

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*To Sunny and Norma*

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# Part I

## General introduction

# Chapter 1

## General introduction

## THE ROLE OF ALDOSTERONE IN HEALTH AND DISEASE

Hypertension is the most prevalent and significant modifiable risk factor for cardiovascular disease.[1] It has been estimated that 7.6 million premature deaths and 54% of stroke and 47% of ischemic heart disease worldwide are attributable to elevated blood pressure levels.[2] Blood pressure regulation is a complex process involving several organs, including the heart, brain, kidneys and vasculature. The renin-angiotensin-aldosterone system (RAAS) has a central role in blood pressure regulation. A decrease in renal perfusion leads to the synthesis and release of renin by the juxtaglomerular cells in the afferent arterioles of the kidney. Renin converts angiotensinogen, which is produced in the liver, to angiotensin I (Ang I). This is subsequently converted to angiotensin II (Ang II) by angiotensin-converting enzyme (ACE) in vascular tissues. Ang II increases blood pressure via Ang II type 1 receptor (AT1R)-mediated vasoconstriction, by increasing sympathetic tone and by stimulation of arginine vasopressin release.[3] Ang II also stimulates sodium and water reabsorption by a direct action in the kidney, and indirectly by stimulating aldosterone synthesis and release in the adrenal gland.[3] When blood pressure goes up, the increase in renal perfusion will slow down renin release, thereby providing a negative feedback mechanism regulating RAAS activity to its required level. Figure 1 shows a schematic overview of the RAAS.

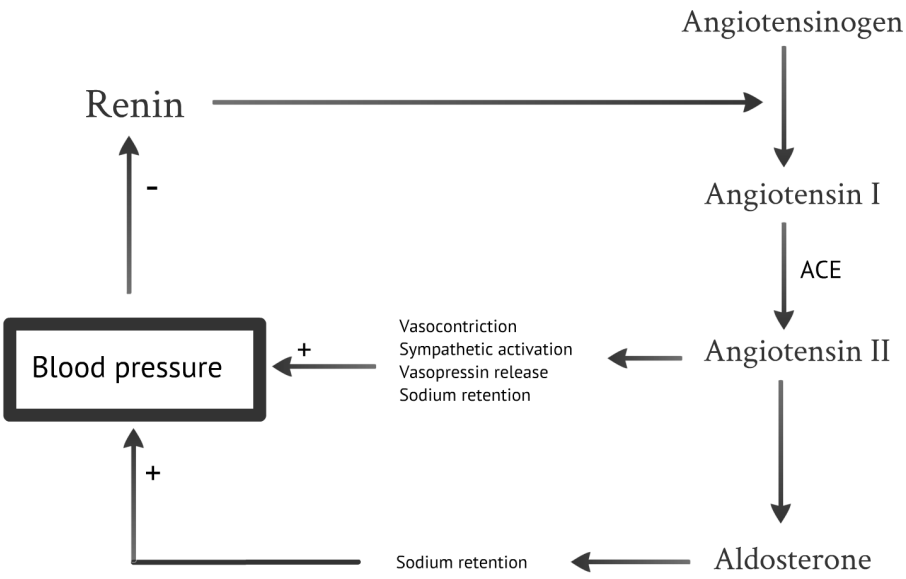


**Figure 1.** Schematic overview of the renin-angiotensin-aldosterone system. ACE, angiotensin-converting enzyme.

Aldosterone is a steroid hormone produced in the zona glomerulosa of the adrenal cortex. Its synthesis and release is mainly determined by the RAAS via activation of the AT1R by Ang II. In addition to this, serum potassium and adrenocorticotropic hormone (ACTH) can stimulate aldosterone synthesis.[4] The regulation of aldosterone synthesis is discussed in detail in Chapter 2. Aldosterone has actions in various parts of the body. Its main actions, however, are in the distal part of the nephron (distal convoluted tubule, connecting tubule and cortical collecting duct), where it has a regulatory role in volume and electrolyte homeostasis.[5] Binding of aldosterone to the intracellular mineralocorticoid receptor (MR) in the principal cells of the cortical collecting duct results in the expression of several intracellular kinases, including serum and glucocorticoid-inducible kinase 1 (SGK1) and Kirsten Ras GTP-binding protein 2A (Ki-RasA). This cascade results in the upregulation and activation of the apical epithelial sodium channel (ENaC) and the basolateral $Na^+/K^+$-ATPase, promoting reabsorption of sodium and water.[4, 5] Sodium reabsorption subsequently drives potassium excretion via Renal Outer Medullary $K^+$ (ROMK) channels located on the apical membrane to maintain electroneutrality.[5] Aldosterone also activates the thiazide-sensitive sodium chloride cotransporter (NCC) in the distal convoluted tubule, further contributing to sodium reabsorption.[6] In addition to this, aldosterone stimulates hydrogen excretion by type A intercalated cells in the distal nephron, indirectly by creating a negative luminal voltage potential, and directly by increasing the activity of $H^+$-ATPases and $Cl^-/HCO_3^-$exchangers.[5] The renal response to aldosterone is not always the same. Hypovolemia and hyperkalemia can both lead to elevated aldosterone levels, but the aim is sodium and water retention in the former and potassium excretion in the latter situation. During hypovolemia, both Ang II and aldosterone levels are elevated. This promotes sodium reabsorption through a synergistic effect of Ang II on the NCC and ENaC. Furthermore, Ang II inhibits ROMK, thereby minimizing potassium wasting. During hyperkalemia, NCC activity is lower, due to the absence of Ang II. As a result, sodium delivery to the cortical collecting duct is higher where it is reabsorbed by eNaC, which is upregulated by aldosterone. This drives potassium excretion as described above.[7]

In addition to the kidney, MR expression is found at several other places, including the vessel wall, heart, brain and blood cells.[4] Aldosterone enhances vascular reactivity to Ang II through an increased expression of the AT1R.[8] In the brain, MR activation may be involved in central blood pressure regulation.[9] Aldosterone classically works in a genomic way, *i.e.* through the induction and modulation of gene transcription. In recent years, several studies have shown rapid, nongenomic effects in several tissues, such as the renal tubule,[10] vascular tissue[11, 12] and the heart.[13] Some of these effects could not be blocked by aldosterone receptor antagonists (ARAs),[13] suggesting the presence of a different, yet unidentified receptor.

Although the RAAS in general, and aldosterone in particular, has an important physiological role in blood pressure regulation, chronic activation is a key factor in the pathophysiology of cardiovascular diseases, including hypertension and heart failure. Elevated aldosterone levels not only contribute to the development of hypertension,[14] chronic MR stimulation also

induces a proinflammatory state which ultimately leads to end-organ damage, such as cardiac and vascular fibrosis.[15] Elevated aldosterone levels have been associated with a higher rate of morbidity and mortality in heart failure.[16] The detrimental effects of aldosterone on end-organ damage is further illustrated by the increased cardiovascular complication rate in patients with primary aldosteronism, which will be discussed in the following paragraph. For these reasons, drugs interfering with the RAAS are now an integral part of the treatment of hypertension and heart failure.

## PRIMARY ALDOSTERONISM

In 1955, Jerome W. Conn described a 34-year old patient presenting with hypertension and muscle weakness. Her biochemical profile was characterized by severe hypokalemia and metabolic alkalosis. Further studies revealed that her condition was caused by mineralocorticoid excess. On surgical exploration, a 4-cm adrenal tumour was found and removed.[17] This was the first description of what later became known as "Conn's Syndrome" or primary aldosteronism (PA). Since its discovery, it has become a well-known cause of hypertension. For a long time, however, PA was considered to be a very rare condition, accounting for approximately 0.05-2% of all hypertensive patients.[18] Its presence was mainly suspected in patients presenting with hypertension and hypokalemia. Since the introduction of the aldosterone-to-renin ratio (ARR) as a screening test for PA,[19] it has become clear that many more patients suffer from this condition and that a large proportion has normal serum potassium levels.[20]

The common clinical and biochemical abnormalities can be explained by the features of the RAAS and the actions of aldosterone (Figure 2). An excessive production of aldosterone from the adrenal gland results in hypertension through retention of sodium and water as described above. The increased renal perfusion pressure will suppress renin production by the juxtaglomerular cells. Aldosterone levels, however, remain elevated because its production is dissociated from the RAAS. The excessive aldosterone levels also stimulate renal potassium and hydrogen loss causing hypokalemia and a metabolic alkalosis. The hypokalemia may result in severe muscle weakness.

In addition to (sometimes severe and refractory) hypertension, patients with PA were shown to have a higher cardiovascular risk than can be explained by the elevated blood pressure levels alone.[21] The chronically elevated aldosterone levels lead to severe target-organ damage, such as myocardial[22, 23] and large artery[24] remodelling and fibrosis. Therefore, early detection and treatment is essential to prevent long-term complications.

In contrast to previous beliefs, only a minority of patients is now thought to have an aldosterone-producing adenoma (APA). The two major subtypes are bilateral adrenal hyperplasia (BAH) and APA.[25] In BAH, aldosterone overproduction is bilateral, and radiological imaging may, but not always, show bilateral enlargement of the adrenal glands. It is estimated that

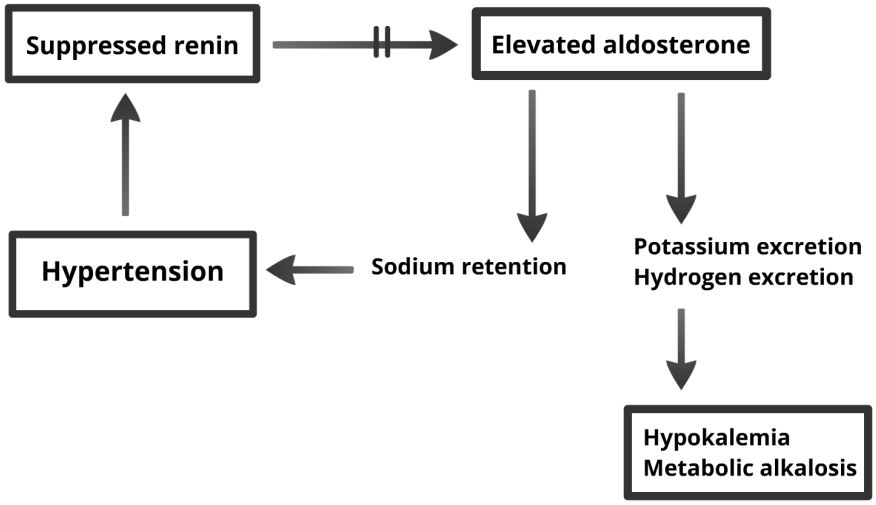


**Figure 2.** The pathophysiology of primary aldosteronism.

around 65% of PA patients has BAH. APA accounts for 30% of PA cases and these patients often present with a more severe phenotype than patients with bilateral disease. Rare subtypes include unilateral adrenal hyperplasia (3%), aldosterone-producing adrenocortical carcinomas (1%) and aldosterone-producing ovarian tumors (<1%).[25] Glucocorticoid-remediable aldosteronism (GRA) or familial hyperaldosteronism type I (FH-1) is a rare genetic variant of PA (accounting for <1 % of cases[25]) in which a crossing-over mutation has produced a hybrid CYP11B1/CYP11B2 gene. This hybrid gene codes for an enzyme with aldosterone synthase activity, but its regulatory domains are derived from CYP11B1, causing its expression to be regulated by ACTH. The inheritance is in an autosomal dominant pattern. Patients with GRA have a high expression of the "hybrid steroids" 18-hydroxycortisol and 18-oxocortisol. In healthy subjects, aldosterone synthase activity is mainly expressed in the zona glomerulosa, whereas in GRA the hybrid gene is expressed in all zones of the adrenal cortex. Aldosterone synthase activity in the zona fasciculata, subsequently, results in the production of 18-hydroxycortisol and 18-oxocortisol by the 18-hydroxylase and 18-oxidase activity of the hybrid gene on cortisol.[26] Some PA patients show a strong family history of PA but have no evidence of the hybrid gene as in GRA. This has been labelled as familial hyperaldosteronism type 2 (FH-2). Its phenotype is heterogenous and indistinguishable from sporadic cases.[26, 27] Mutations in a region on chromosome 7 (locus 7p22) have been linked to FH-2,[28, 29] but its exact genetic basis remains to be determined. In 2008, a third form of familial hyperaldosteronism was described (FH-3).[30] These patients present with severe hypertension in their childhood, have elevated aldosterone levels and enlarged adrenal glands with diffuse hyperplasia of the zona fasciculata and atrophy of the zona glomerulosa. Like patients with FH-1, they have high levels of the hybrid steroids 18-oxocortisol and 18-hydroxycortisol consistent with aldo-

sterone synthase activity in the zona fasciculata. However, unlike in FH-1, aldosterone, and interestingly also cortisol synthesis, is not inhibited by the administration of dexamethasone. This suggests a more general defect in the regulation of adrenal steroid synthesis.[30] Recently, these subjects were shown to have a mutation in the inwardly rectifying potassium channel, subfamily J, member 5 (KCNJ5).[31] In the same article, it was shown that two other mutations in KCNJ5 were frequently found in sporadic APAs. These mutations affect the selectivity filter of the channel resulting in an enhanced sodium influx and subsequent cell depolarization.[31] This is known to stimulate aldosterone synthesis[32, 33] and cell proliferation through activation of voltage-gated calcium channels[31] and KCNJ5 mutations are now thought to have an important role in the pathogenesis of APA and some familial forms of PA.[34]

Treatment of PA can be either medical or surgical, and the choice is based on the subtype of PA and the condition and preference of the patient.[35, 36] Patients with bilateral disease should be treated medically with ARAs, either spironolactone or eplerenone. These agents attenuate the effects of aldosterone on the MR, resulting in a decrease in sodium reabsorption. Alternative drugs are blockers of the epithelial sodium channel, such as amiloride and triamterene, but these are probably less effective than aldosterone-receptor antagonists.[35] Patients with GRA should initially be treated with a glucocorticoid to suppress ACTH secretion, such as dexamethasone. If this does not result in adequate blood pressure control, an aldosterone receptor antagonist can be added.[36]

In patients with unilateral disease, surgical removal by laparoscopic adrenalectomy is the treatment of choice, because this could potentially cure or ameliorate the elevated blood pressure levels[37] and biochemical abnormalities.[38] Adrenalectomy was also shown to reverse myocardial fibrosis,[39] to decrease carotid intima-media thickness and vascular stiffness[40, 41] and to reduce cardiovascular risk in general.[42] Although there appears to be no difference in cardiovascular outcome between surgically and medically treated PA patients,[42] a trial that prospectively compares surgical and medical treatment in patients with an APA would be helpful. However, it has been estimated that adrenalectomy results in a larger cost reduction than long-term medical therapy alone.[43] The condition and comorbidities of the patient are important points of consideration whether to proceed to surgery. Predictors for a favorable surgical response (cure or control of hypertension) are a shorter duration of hypertension before operation,[44-46] well-controlled blood pressure levels[47] and a lower number of antihypertensive drugs[44, 46] preoperatively and a proven response to spironolactone.[46] In addition, the absence of a family history for hypertension[44] and the presence of the CYP11B2 (344 C/T) genotype[46] are also associated with a favorable outcome after adrenalectomy. In patients with an APA who are ineligible for surgery, computed tomography-guided radiofrequency ablation of the adenoma may be considered.[48, 49] Alternatively, these patients can be treated medically, as discussed previously.

## ALDOSTERONE RECEPTOR ANTAGONISTS IN HYPERTENSION

In addition to their central role in the treatment of patients with PA, ARAs are also used in other cardiovascular disorders. Considering the important role of aldosterone as a mediator of end-organ damage, aldosterone receptor antagonism could be beneficial in the prevention of cardiovascular complications. This is supported by the Randomized Aldactone Evaluation Study (RALES)[50] and Eplerenone Post-Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS)[51] which showed that the treatment of heart failure patients with ARAs resulted in a marked reduction in morbidity and mortality that is independent of blood pressure reduction. Another population in which ARAs could be of benefit are patients with resistant hypertension. Resistant hypertension is defined as an elevated blood pressure despite the use of at least 3 antihypertensive drugs, including a diuretic, and prescribed in optimal doses, and is an important clinical problem. The exact prevalence among hypertensive patients is unknown, but it is estimated to be as high as 20-30%.[52] Patients with resistant hypertension have a significantly higher risk of cardiovascular complications than patients who respond well to antihypertensive treatment.[53] Therefore, it is of eminent importance to develop treatment strategies that effectively lower blood pressure in these patients. Elevated aldosterone levels are thought to be a contributing factor in therapy resistance in some patients. First of all, elevated aldosterone levels were negatively associated with treatment success.[53] Furthermore, although plasma aldosterone levels are expected to decrease after treatment with ACE inhibitors (ACE-Is) or angiotensin receptor blockers (ARBs), some patients show elevated levels after prolonged treatment.[54] The reasons for this "aldosterone breakthrough" are incompletely understood, but it may contribute to therapy resistance. ARAs could therefore be a rational treatment choice for patients with resistant hypertension. Chapman *et al.*[55] reported an impressive systolic and diastolic blood pressure reduction of 21.9 and 9.5 mmHg, respectively, when spironolactone was added to the antihypertensive regime in patients with resistant hypertension. The problem with this study, and many other studies on this topic, was its retrospective and uncontrolled design. The effect in a recent randomized, placebo-controlled trial, was substantially smaller.[56] However, this needs further confirmation. In addition, few studies have focused on eplerenone, a more selective ARA, which could be more appropriate when patients experience sex hormone-related side effects to spironolactone.[57]

## AIMS OF THE THESIS

Aldosterone is a key hormone in the physiology of blood pressure regulation and the pathophysiology of cardiovascular disease. Elevated aldosterone levels are an important mediator of end-organ damage in several disease states such as heart failure and primary aldosteron-

ism. Inhibition of the deleterious effects of aldosterone is, therefore, an important therapeutic goal. Although blockade of the effects of aldosterone at the level of the mineralocorticoid receptor has been well established, a better understanding of the mechanisms involved in aldosterone synthesis and inhibition will help to identify new therapeutic targets and point out the patients who will benefit most from this treatment. In addition, a significant proportion of hypertensive patients has primary aldosteronism. It is important to diagnose these patients at an early stage, first, to prevent cardiovascular complications and second, because specific treatment is warranted including the option of surgical cure. This thesis aims to explore the role of aldosterone and aldosterone blockade in hypertension further. More specifically, this thesis describes the regulation of aldosterone synthesis (Part II), the screening of patients with primary aldosteronism (Part III) and the benefits of aldosterone blockade in patients with difficult-to-treat and resistant hypertension (Part IV).

Part II starts with an overview of the regulation of aldosterone synthesis in the adrenal gland (Chapter 2). In recent years, studies have focused on ways to interfere in this pathway to inhibit aldosterone synthesis directly. This may have benefits above blockade of aldosterone at the level of its receptor. The recent developments in this field are briefly discussed. Although the classical pathways of aldosterone synthesis regulation are well defined, other mechanisms may be involved. As mentioned in the introduction, some patients on ACE-I or ARB therapy will develop elevated aldosterone levels, despite the supposed inhibition of Ang II or its effects. The discovery of the (pro)renin receptor ((P)RR), which not only facilitates the non-proteolytic activation of prorenin to display Ang I-generating activity,[58] but which was also shown to have direct cellular effects,[58, 59] raises the question whether renin or prorenin can stimulate aldosterone synthesis directly, particularly because (P)RR-overexpression in rodents was accompanied by elevated aldosterone levels.[60] In two human adrenocortical cell lines, we studied whether the (P)RR was present and whether stimulation of this receptor by renin and prorenin had a stimulatory effect on the production of aldosterone (Chapter 3).

Part III describes the screening of patients with primary aldosteronism. The diagnostic workup for primary aldosteronism is outlined in Chapter 4. Although the ARR is widely used for screening purposes, this test is not undisputed. Some raise doubts on its validity and reproducibility.[61-63] We studied the test characteristics of the ARR in a prospective manner in 178 patients with difficult-to-treat hypertension and related these to the recent Endocrine Society Clinical Practice guideline on this topic[36] (Chapter 5).

Although ARAs have been in use for several decades, there has been a renaissance of these agents in patients with difficult-to-treat and resistant hypertension in recent years. Retrospective and observational studies have reported spectacular improvements in blood pressure values after ARA treatment, but prospective evidence is relatively limited. In addition, most studies have a relatively short follow-up and whether the blood pressure reduction is sustained in the long run has never been studied. Last, efforts to identify patients who will

benefit most from ARA treatment have gained inconclusive results. These aspects are dealt with in Part IV. Chapter 6 reviews the current evidence for the use of ARAs in hypertension, their proposed mechanism of action and side effects. The long-term efficacy of ARAs was studied retrospectively in 123 patients with difficult-to-treat hypertension who were treated with an ARA (Chapter 7). The reduction in office and ambulant blood pressure by a fixed daily dose of eplerenone was evaluated prospectively in 117 patients with difficult-to-treat hypertension. This included regression analyses to assess which clinical and biochemical parameters predicted this response (Chapter 8). The main findings of this thesis are discussed in Chapter 9 and placed in a clinical perspective with suggestions for further studies.

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# Part II

## The regulation of aldosterone synthesis

# Chapter 2

## Aldosterone synthase inhibitors: pharmacological and clinical aspects

Pieter M. Jansen, Anton H. van den Meiracker and A.H. Jan Danser
*Division of Pharmacology, Vascular and Metabolic Diseases, Department of Internal Medicine, Erasmus Medical Centre, Rotterdam, The Netherlands.*

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## ABSTRACT

Aldosterone plays an important pathophysiological role in cardiovascular disease, and aldosterone (mineralocorticoid) receptor antagonism has been used in the treatment of heart failure and hypertension. However, aldosterone receptor antagonism is associated with an increase in plasma aldosterone levels, which may result in non-mineralocorticoid receptor-mediated (non-genomic) adverse effects. Therefore, the inhibition of aldosterone synthesis may be preferable to blockade at the receptor level. Direct inhibitors of aldosterone synthase are currently under development. However, specificity for aldosterone synthase remains a challenge, and the search for more potent and more selective compounds is ongoing. Experimental animal and human studies are required to establish whether aldosterone synthase inhibitors will be successful as therapeutic agents.

## INTRODUCTION

The adrenocortical hormone aldosterone plays a pivotal role in heart failure and hypertension. In heart failure, activation of the renin-angiotensin-aldosterone system leads to elevated aldosterone levels,[1] which have been associated with a higher rate of morbidity and mortality.[2, 3] A role for aldosterone has also been established in hypertension: since the introduction of the aldosterone-to-renin ratio as a screening test, and as its use has become more common, the prevalence of primary aldosteronism (PA) as a cause of hypertension appears to be substantially higher than previously expected. A recent review demonstrated that 4.3% of hypertensive patients in a primary care setting and 9.0% of referred patients had confirmed PA.[4] Aldosterone is also involved in the development of hypertension and disease progression in essential hypertension (EH).[5] In addition, aldosterone at inappropriately high levels contributes to therapy resistance.[1] Furthermore, in a substantial proportion of patients with heart failure treated with an ACE inhibitor (ACE-I), or an angiotensin II (Ang II) type 1 ($AT_1$) receptor blocker (ARB), after an initial decline in aldosterone, prolonged treatment has been demonstrated to lead to a secondary increase in the hormone, to levels greater than those observed prior to treatment.[6] Therapy resistance may be a consequence of such 'aldosterone escape' or 'aldosterone breakthrough'.[6] In addition, aldosterone is an important mediator of end-organ damage, through the promotion of vascular inflammation and myocardial and perivascular fibrosis.[7] As noted, aldosterone contributes to morbidity and mortality in patients with heart failure.[2, 3] Patients with PA have a higher rate of cardiovascular complications compared with patients with EH.[8] Furthermore, left ventricular (LV) mass is greater and arterial wall stiffness is more pronounced in patients with PA or secondary aldosteronism compared with patients with EH.[9]

Thus, inhibiting the effects of aldosterone represents an important target for cardiovascular disease therapy. Until recently, most studies focused on the blockade of aldosterone at the level of the mineralocorticoid receptor (MR; also known as the aldosterone receptor). MR blockade by aldosterone receptor antagonist (ARA) therapy has been demonstrated to be effective in the treatment of cardiovascular disease; the RALES (Randomized Aldactone Evaluation Study) and EPHESUS (Epleronone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study) clinical trials demonstrated a marked reduction in morbidity and mortality in patients with heart failure receiving standard treatment, following the addition of the ARA compounds spironolactone and eplerenone, respectively.[10, 11] Similarly, when patients with PA were treated with spironolactone, the cardiovascular complication rate declined to levels that were comparable to those observed in patients with EH.[12] However, spironolactone lacks specificity for the MR and, because of its anti-androgenic and progestagenic effects, can cause gynecomastia, erectile disorders and menstrual irregularities. Although these side effects are dose-dependent, with relatively low incidences observed at

low doses of the compound,[13] the effects constitute a limitation for the widespread use of spironolactone. This issue has been partly overcome by the development and introduction of the more selective MR antagonist eplerenone, thereby providing more options for high-dose treatment.

However, in addition to the classic, genomic effects (ie, effects that are mediated via intracellular MR, require *de novo* protein synthesis and occur several hours after dosing), aldosterone also exerts rapid, non-genomic effects in various tissues. Such non-genomic effects do not require *de novo* protein synthesis, occur within minutes, and cannot always be blocked by MR antagonists.[14-16] For example, aldosterone rapidly decreases contractility in the atrial and ventricular trabeculae of human hearts and potentiates the contractile response of human coronary arteries to Ang II.[17] Neither spironolactone nor eplerenone block these effects, suggesting the involvement of an alternative, as yet unidentified, receptor. These observations are not unique to aldosterone: non-genomic effects can also be exerted by sex steroids.[18] Because ARA treatment, such as prolonged ACE-I and ARB therapy, results in a compensatory increase in aldosterone levels, the non-genomic, non-MR-mediated effects of aldosterone may partly counteract the beneficial actions of such treatment.[14] Blockade of aldosterone synthesis may therefore be more beneficial compared with MR blockade.

This review provides a brief overview of the normal regulation of aldosterone synthesis, and discusses the development of aldosterone synthase inhibitors and their activity in experimental studies.

## ALDOSTERONE SYNTHESIS

### The synthesis of aldosterone from cholesterol

Aldosterone is produced in the *zona glomerulosa* of the adrenal cortex from its precursor, cholesterol (Figure 1). The first step in aldosterone synthesis is the transport of cholesterol to the inner mitochondrial membrane, mediated by the steroidogenic acute regulatory (StAR) protein. In the mitochrondia, CYP11A1 (cytochrome P450 family 11, subfamily A, polypeptide 1) converts cholesterol to pregnenolone by 20$\alpha$-hydroxylation, 22-hydroxylation and side-chain cleavage between C(20) and C(22) of the cholesterol carboskeleton. In the cytosol, pregnenolone on the smooth endoplasmatic reticulum membrane is converted to progesterone by 3$\beta$-hydroxysteroid dehydrogenase. CYP21A then converts progesterone to 11-deoxycorticosterone (DOC) by 21-hydroxylation. The final steps in the synthesis of aldosterone are mediated by aldosterone synthase (CYP11B2), which is located on the inner mitochondrial membrane and catalyzes the 11$\beta$-hydroxylation of DOC to corticosterone, as

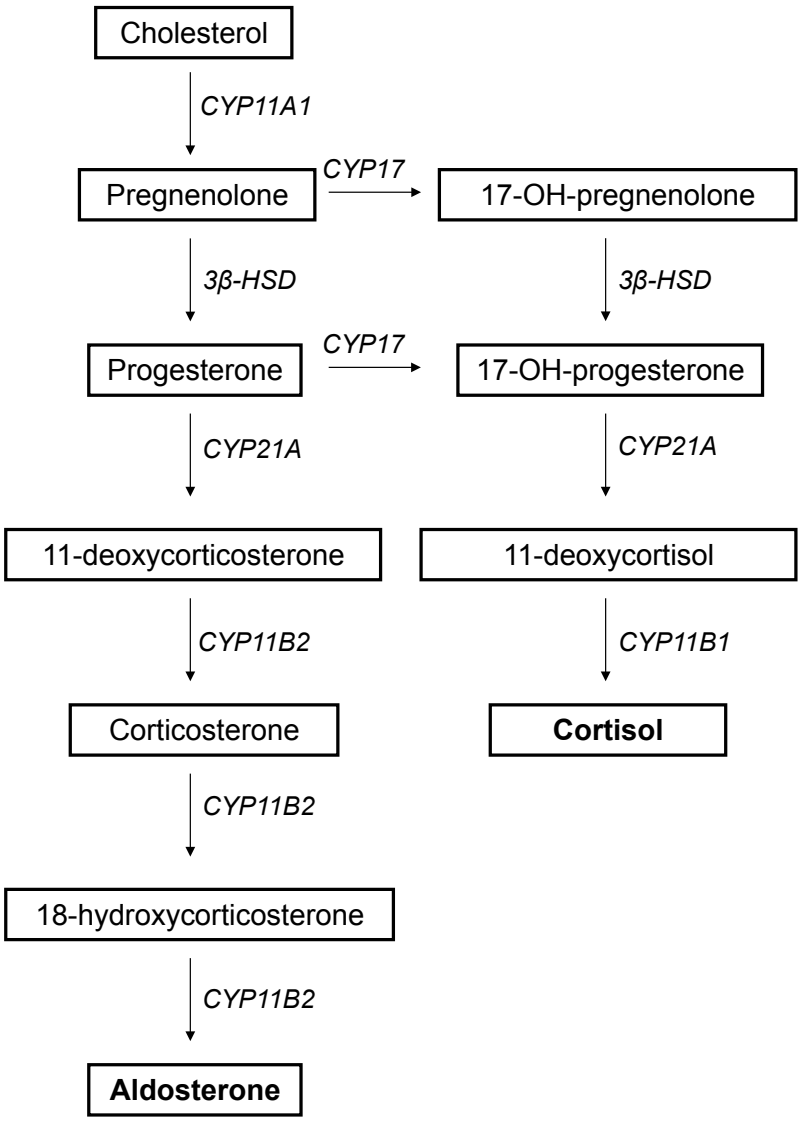


**Figure 1.** The synthesis of aldosterone and cortisol in the adrenal gland. 3β-HSD, 3β-hydroxysteroid dehydrogenase; CYP11A1, cytochrome P450 family 11, subfamily A, polypeptide 1; CYP11B1, cytochrome P450 family 11, subfamily B, polypeptide 1; CYP11B2, cytochrome P450 family 11, subfamily B, polypeptide 2; CYP21A, cytochrome P450 family 21, subfamily A
(*Adapted from Connell and Davies*[19])

well as the subsequent 18-hydroxylation of corticosterone to 18-hydroxycorticosterone and, finally, the 18-methyloxidation of this compound to aldosterone.[19]
The synthesis of aldosterone is closely linked to the synthesis of cortisol (Figure 1). Pregnenolone and progesterone can be hydroxylated to 17-OH-pregnenolone and 17-OH-progesterone, respectively, by CYP17.[19] Following the conversion of 17-OH-progesterone to 11-deoxycortisol by CYP21A, cortisol is formed by the 11β-hydroxylation of 11-deoxycortisol by CYP11B1.[19]

## The regulation of aldosterone synthesis

The main regulators of aldosterone synthesis are Ang II, serum $K^+$ and adrenocorticotropic hormone (ACTH).[19] The acute synthesis of aldosterone is increased by the upregulation of StAR, of which Ang II is an important mediator, whereas the chronic synthesis of aldosterone is regulated at the level of aldosterone synthase, which is encoded by the *CYP11B2* gene.[19]

ACTH has a differential effect on aldosterone synthesis. The hormone increases acute aldosterone production, and inhibits chronic CYP11B2 expression.[20] The main regulators of CYP11B2 expression, however, are Ang II and $K^+$. In part, these factors act through a common intracellular signaling pathway in which calcium plays a crucial role[21] (Figure 2).

An increase in $K^+$ leads to membrane depolarization,[21] resulting in the opening of voltage-gated $Ca^{2+}$ channels and leading to an influx of $Ca^{2+}$. The T-type $Ca^{2+}$ channel appears to be the most important in this process. This is supported by the observation that aldosterone production in the human adrenocortical cell line NCI-H295 was abolished by the T-type-specific $Ca^{2+}$ channel blocker benidipine, but not by L-type-specific $Ca^{2+}$ channel blockers.[22] However, at higher $K^+$ concentrations, T-type $Ca^{2+}$ channels are inactivated and other voltage-gated $Ca^{2+}$ channels may become relevant.[23] Further downstream, signaling appears to be mediated through the binding of $Ca^{2+}$ to calmodulin and the subsequent activation of calmodulin-dependent kinases (CaMKs). The fact that $K^+$-induced expression of CYP11B2 in H295R cells was inhibited by the calmodulin inhibitor calmidazolium, as well as by the CaMK inhibitor KN-93, supports this notion.[24] Further investigation using this cell line revealed that CaMKI, and to a lesser degree CaMKIV, are involved in this pathway.[25]

The Ang II stimulation of CYP11B2 expression begins with the binding of Ang II to the $AT_1$ receptor[26] (Figure 2). Upon binding, activated phospholipase C-β leads to the formation of inositol triphosphate ($IP_3$) and diacylglycerol (DAG) from phosphatidylinositol biphosphate. $IP_3$ induces the release of calcium from intracellular stores, resulting in the activation of the calmodulin/CaMK pathway as described above.[20] The blockade of this pathway only partially abolishes Ang II-induced CYP11B2 expression, however, suggesting that additional

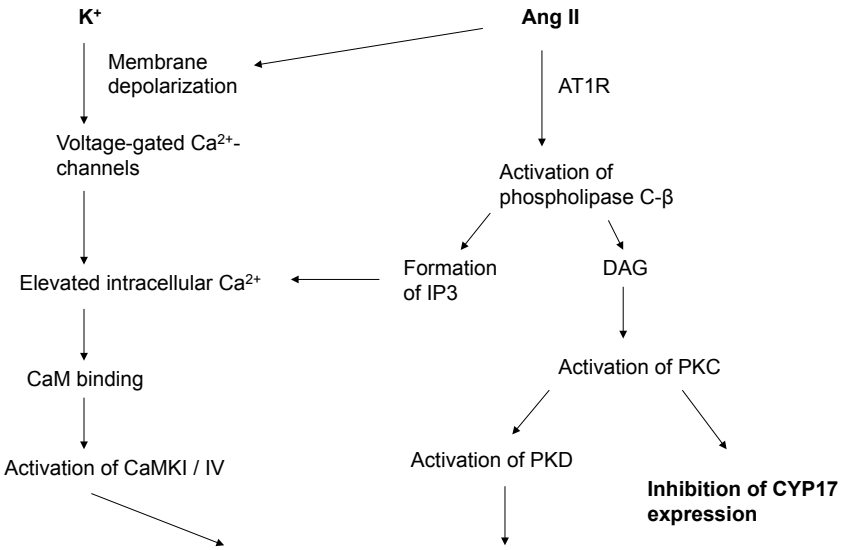


**Figure 2.** The main mechanisms involved in the upregulation of CYP11B2 expression by $K^+$ and angiotensin II. Ang II, angiotensin II; $AT_1R$, angiotensin II type 1 receptor; CaM, calmodulin; CaMKI/IV, calmodulin-dependent kinase I/IV; CYP11B2, cytochrome P450 family 11, subfamily B, polypeptide 2; CYP17, cytochrome P450 subfamily 17; DAG, diacylglycerol; $IP_3$, inositol triphosphate; PKC, protein kinase C; PKD, protein kinase D.

Ang II-induced signalling pathways are also involved.[24] DAG, produced along with $IP_3$, is an activator of protein kinase C (PKC). Because the administration of Ang II or the PKC activator 12-O-tetradecanoylphorbol-13-acetate has been demonstrated to block forskolin-induced CYP17 expression in H295R cells,[27] it has been postulated that PKC serves mainly to divert steroidogenesis toward the production of aldosterone.[20] However, screening for protein kinase expression levels in H295R cells following the administration of Ang II revealed a large increase in the levels of phosphorylated protein kinase D (PKD), and was related to the increased expression of aldosterone synthase.[28] This pathway could be blocked by several PKC inhibitors and was stimulated by the PKC activator phorbol myristate acetate, suggesting that PKC phosphorylates PKD and that the PKC/PKD pathway is an additional pathway involved in Ang II-stimulated aldosterone synthesis.[28] Other signalling pathways, such as the 12-lipoxygenase and the Src tyrosine kinase pathways, may also play a role in Ang II-stimulated aldosterone synthesis.[20]

In addition to acting partly through a common signalling pathway, Ang II and $K^+$ demonstrate interrelated effects in terms of regulating aldosterone synthesis. In addition to the pathways

mentioned previously, Ang II can also induce aldosterone synthesis by an inhibition of leak- and voltage-gated $K^+$ conductances, resulting in membrane depolarization[23] (Figure 2). Moreover, Ang II can sensitize glomerulosa cells to increases in extracellular $K^+$ levels, resulting in enhanced $Ca^{2+}$ influxes.[29] Although a sustained phosphorylation of StAR has been proposed for this enhanced aldosterone secretion,[29] an effect on CYP11B2 expression cannot be excluded.

The regulation of CYP11B2 expression likely occurs via an effect on transcription factors. Bassett *et al.*[20] demonstrated that the transcription factors NGFIB (nerve growth factor-induced clone B) and NURR1 (Nur-related factor 1) activated the *CYP11B2* promoter. The demonstration of $K^+$-induced NGFIB and NURR1 expression, which was partly inhibited by KN-93, indicated that these transcription factors were upregulated by CaMK.[30] The same research group also identified two functional binding sites for these transcription factors on the *CYP11B2* gene promoter: NBRE-1 (NGFIB response element 1) and Ad5 (-129/-114 element), respectively.[30] COUP-TFI (chicken ovalbumin upstream promoter-transcription factor I), with Ubc9 (ubiquitin-like protein SUMO-1 conjugating enzyme) and PIAS1 (protein inhibitor of activated STAT 1) as coactivators, is another transcription factor for the *CYP11B2* gene, and binds to Ad5.[30, 31] ATF1 (activating transcription factor 1) and CREB (cAMP response element-binding protein) are additional transcription factors that have been identified to be likely activated by phosphorylation mediated by CaMK.[20] ATF1 and CREB both bind to CRE, which has also been identified on the *CYP11B2* gene promoter.[32] The transcription factor SF1 (steroidogenic factor 1) is particularly important for steroidogenesis in extra-adrenal tissues;[19] however, SF1 also inhibited the effects of NGFIB and NURR1 on CYP11B2 expression in H295R cells.[30]

## Aldosterone synthase: structure and enzymatic activity

The *CYP11B2* gene encoding aldosterone synthase, located on chromosome 8q21-q22, exhibits 93% homology with the *CYP11B1* gene, which encodes 11β-hydroxylase.[33] Aldosterone synthase is a mitochondrial CYP enzyme, characterized by an heme prosthetic group in the core of its active site; this heme group is essential for the multi-local oxidation of the steroid skeleton that occurs during the final steps of aldosterone biosynthesis.[34] Despite extensive homology with CYP11B1, only aldosterone synthase appears to be able to catalyze these final steps of biosynthesis.[34] Substrate specificity is crucial for enzyme activity. Böttner *et al.*[35] demonstrated that the substitution of three residues distant from the active site converted the catalytic activity of CYP11B2 into the activity of CYP11B1. Based on these experiments, substrate conversion was postulated to depend on the positioning of the substrate above the heme molecule in the active site of the enzyme.[34] By constructing CYP11B1 and CYP11B2 homology models, the steric fit of the steroidal ligands to the enzyme was determined to

be of major importance for substrate specificity.[34] This insight is undoubtedly crucial for the development of CYP11B2-selective inhibitors.



## THE DEVELOPMENT OF SELECTIVE ALDOSTERONE SYNTHASE INHIBITORS

The inhibition of aldosterone synthesis can occur in several ways; a number of drugs can act as aldosterone synthesis inhibitors, including ARBs (by blocking the binding of Ang II to the $AT_1$ receptor), as well as $Ca^{2+}$-channel blockers and digoxin (by interfering with intracellular $Ca^{2+}$ levels).[22, 36] Recent research interest has focused on the direct inhibition of aldosterone synthase.

Aromatase (CYP19A1) inhibitors have been demonstrated to be successful in the treatment of breast cancer, by inhibiting the formation of estrogen. Fadrozole, a CYP19 inhibitor, is an aromatase inhibitor that exerts a strong suppressive effect on aldosterone and cortisol synthesis when administered at a dose that is 10-fold higher than the therapeutic dose.[37] This effect illustrates the advantages and disadvantages of CYP inhibition, namely that these enzymes are potent therapeutic targets, but that their inhibition is associated with a risk of side effects because of a lack of specificity. Thus, to be useful in the clinic, aldosterone synthase/CYP11B2 inhibitors should exhibit high potency in blocking aldosterone synthase, yet their activity should be specific for this enzyme (Table 1). As noted, CYP11B2 is highly homologous to CYP11B1, and designing a drug that blocks aldosterone synthesis without affecting cortisol production is therefore challenging. Furthermore, the pharmacokinetic and pharmacodynamic profile of an aldosterone synthase inhibitor should enable the inhibitor to be present at adequate levels in the blood following oral administration, as well as to be delivered to the target organ efficiently. In addition, the tolerability and toxicity of an inhibitor should be within an acceptable range.

By screening a library of CYP inhibitors, Hartmann *et al.*[38] identified a large number of potential CYP11B2 inhibitors. These compounds were tested according to the following screening procedure: following preselection by using bovine adrenal mitochondria containing CYP18 (18-hydroxylase), the inhibitor activity of the compounds was evaluated in *Schizosaccharomyces pombe* strains expressing the human CYP11B2 enzyme. Strong inhibitors were further

**Table 1.** The ideal properties for aldosterone synthase inhibitors.

| | |
|---|---|
| 1. | High potency toward aldosterone synthase |
| 2. | High specificity, with no effect on other cytochrome P450 enzymes, including CYP11B1 |
| 3. | Good pharmacokinetic and pharmacodynamic profiles, allowing adequate delivery to the target organ following oral administration |
| 4. | An acceptable tolerability and toxicity profile |

CYP11B1, cytochrome P450 family 11, subfamily B, polypeptide 1.

tested for activity and selectivity in VZ79MZ cells transfected with either the *CYP11B2* or *CYP11B1* gene. Potential activity toward other steroidogenic enzymes was then tested using microsomes from human placenta (expressing CYP19), from *Escherichia coli* (expressing recombinant human CYP17) and adrenal cortex mitochondria (expressing CYP11A1).[38-40] The NCI-H295R cell line was identified as a suitable tool for screening for the effects of these compounds on several steroidogenic enzymes.[41]

The identified compounds of interest were aryl-substituted cyclopropatetrahydronaphtalines, aryl- and arylmethyl-substituted tetrahydronaphthalenes and arylmethylene-substituted indanones.[38] Following additional modifications, some highly potent and selective CYP11B2 inhibitors were synthesized, exhibiting $IC_{50}$ values as low as 2 nM in VZ79MZ cells, with selectivity factors (defined as $IC_{50}$ CYP11B1/$IC_{50}$ CYP11B2) of up to 1421.[42-45] For comparison, fadrozole has an $IC_{50}$ value of 1 nM, but a selectivity factor of only 10.[43-45] Some of the identified compounds also caused the marginal inhibition of CYP19 and CYP17, and exhibited promising pharmacokinetic properties in *in vitro* assays.[43, 44] Docking and molecular dynamics studies for some of these compounds demonstrated that the position of the ligand nitrogen relative to the heme group in the catalytic domain was important for binding affinity; an angle close to 90° was optimal, preventing the oxygen activation of the heme group that is necessary for the catalytic process.[46] Steric properties, as well as molecular interactions, determined the positioning and stabilization of the ligand at the active site, thereby also determining the inhibitory properties and substrate specificity.[34, 40, 43-45] Based on these data, models could be constructed for the design of suitable aldosterone synthase inhibitor compounds.[34, 46, 47]

The initial specific aldosterone synthase inhibitors that were developed were observed to be strong inhibitors of the hepatic CYP1A2 enzyme, which is of major importance for enzymatic metabolism within the liver.[42-48] Heim *et al.*[48] succeeded in creating new compounds with marginal CYP1A2 inhibitory activity. These promising compounds should be further tested for their potential use *in vivo*.

## In vivo studies with aldosterone synthase inhibitors

An ideal aldosterone synthase inhibitor has not yet been identified, and the *in vivo* benefits of such a compound remain to be determined. Most *in vivo* studies conducted to date have focused on FAD-286, the dextroenantiomer of the aromatase inhibitor fadrozole.

FAD-286 decreased urinary free aldosterone excretion in spontaneously hypertensive rats on either a low $Na^+$/high $K^+$ or high $Na^+$/low $K^+$ diet.[49] Double transgenic rats (dTGR) expressing the human renin and angiotensinogen genes were used as a model of Ang II/aldosterone-

dependent organ damage to assess the effect of FAD-286 on the target organs.[50] FAD-286 (4 mg/kg/day for 3 weeks) was compared with the ARB losartan (30 mg/kg/day) in this system. Both FAD-286 and losartan markedly reduced serum aldosterone levels. The mortality rate in FAD-286-treated rats was 10%, compared with 40% in dTGR controls. There was a slight reduction in blood pressure in FAD-286-treated rats following 3 weeks of treatment, whereas treatment with losartan resulted in a normalization of blood pressure. Cardiac hypertrophy was reduced by FAD-286, and was normalized by losartan. In addition, both FAD-286 and losartan reduced urinary albumin excretion, the infiltration of inflammatory cells and the deposition of collagen IV and fibronectin in the kidney; losartan exhibited more potency than FAD-286.[50] However, a direct comparison with an aldosterone receptor antagonist was not performed in this model. Such a comparison was conducted in a model of ischemic congestive heart failure, in which rats were treated with either FAD-286 (4 mg/kg/day) or spironolactone (80 mg/kg/day) for 12 weeks.[51] Both FAD-286 and spironolactone improved LV hemodynamics, remodelling and function. FAD-286 was more potent in reducing LV end-diastolic pressure, the relaxation constant and dilatation compared with spironolactone. Following 12 weeks of treatment, FAD-286 reduced myocardial oxidative stress to a greater extent than spironolactone. In addition, only FAD-286 normalized the congestive heart failure-induced reduction in Ang II type 2 receptor protein levels.[51]

The role of locally produced aldosterone can be assessed with aldosterone synthase inhibitors. Although aldosterone in the heart is mainly derived from the circulation,[50, 52, 53] there is also evidence for the local production of aldosterone in the brain,[28, 54] causing the induction of sympathetic hyperactivity and hypertension.[55] Such an induction was observed in Wistar rats following the intracerebroventricular infusion of hypertonic saline. The concomitant intracerebroventricular infusion of FAD-286 prevented the increase in hypothalamic aldosterone levels following hypertonic saline infusion, and also inhibited the increase in sympathetic tone and blood pressure.[55] A similar approach was followed to assess whether locally produced aldosterone in the CNS contributed to sympathetic hyperactivity and LV dysfunction after myocardial infarction.[56] Wistar rats underwent coronary ligation and were administered FAD-286 (icv) for 4 weeks. FAD-286 prevented the increase of aldosterone in the hippocampus and hypothalamus, and attenuated the development of LV dysfunction and remodelling.[56]

Although these results are promising, the studies involved had a short duration. Little is known about the long-term benefits of a compound such as FAD-286, the side effects potentially related to the inhibition of other CYP enzymes, such as CYP19, or the general long-term toxicity. It is therefore crucial that more specific aldosterone synthase inhibitors are subjected to extensive evaluation *in vivo*.

## CONCLUSION

The inhibition of aldosterone at the level of the MR has proven to be useful in the treatment of heart failure,[10, 11] primary aldosteronism[12] and essential hypertension.[5] However, an increase in aldosterone levels following ARA treatment may result in undesirable effects, because of non-genomic, non-MR-mediated activity.[17, 57] The direct inhibition of aldosterone synthesis may therefore be more beneficial compared with MR blockade. Several promising aldosterone synthase inhibitors have been developed; however, selectivity for the enzyme remains a challenge. The synthesis of cortisol, as well as the activation of other CYP450 enzymes including hepatic enzymes, should remain unaffected. *In vitro* testing systems are useful in identifying potent and selective compounds. However, only long-term, *in vivo* experimental studies, followed by clinical trials, can provide sufficient data on the efficacy, tolerability and safety of these drugs. Whether aldosterone synthase inhibitors can have a beneficial effect on primary endpoints when used instead of, or in addition to, ARA treatment, remains to be determined to assess whether these compounds can be used successfully in the treatment of cardiovascular disease. Such information will also provide more insight regarding the clinical relevance of the non-genomic effects of aldosterone.

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# Chapter 3

## Renin and prorenin have no direct effect on aldosterone synthesis in the human adrenocortical cell lines H295R and HAC15

*Pieter M. Jansen[1], *Johannes Hofland[2], Anton H. van den Meiracker[1], Frank H. de Jong[2], A.H. Jan Danser[1]

*Divisions of Pharmacology, Vascular and Metabolic Diseases[1] and Endocrinology[2], Department of Internal Medicine, Erasmus Medical Centre Rotterdam, The Netherlands.*

**both authors contributed equally*

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## ABSTRACT

*Introduction:* Transgenic rats expressing the human (pro)renin receptor (h(P)RR) have elevated plasma aldosterone levels despite unaltered levels, in plasma and adrenal, of renin and angiotensin II.

*Materials and methods:* To investigate whether renin/prorenin-(P)RR interaction underlies these elevated aldosterone levels, the effect of (pro)renin on steroidogenesis was compared with that of angiotensin II in two (P)RR-expressing human adrenocortical cell lines, H295R and HAC15.

*Results:* Angiotensin II rapidly induced extracellular signal-regulated kinase (ERK) phosphorylation and increased the expression of STAR, CYP21A2, CYP11B2 and CYP17A1 at 6 and 24 hours, whereas the expression of CYP11A1 and HSD3B2 remained unaltered. Incubation with renin or prorenin at nanomolar concentrations had no effect on the expression of any of the steroidogenic enzymes tested, nor resulted in ERK phosphorylation. Angiotensin II, but not renin or prorenin, induced aldosterone production.

*Conclusion:* Although the (P)RR is present in adrenocortical cells, renin and prorenin do not elicit ERK phosphorylation nor directly affect steroid production via this receptor at nanomolar concentrations. Thus, direct (pro)renin-(P)RR interaction is unlikely to contribute to the elevated aldosterone levels in human (P)RR transgenic rats. This conclusion also implies that the aldosterone rise that often occurs during prolonged renin-angiotensin system blockade is rather due to the angiotensin II 'escape' during such blockade.

## INTRODUCTION

(Pro)renin receptors ((P)RRs) bind both renin and its inactive precursor, prorenin, and such binding allows prorenin to display angiotensin (Ang) I-generating activity.[1-4] Surprisingly, renin and prorenin also act as agonists for this receptor, inducing effects in an angiotensin-independent manner.[1, 5-7] Transgenic rats with either ubiquitous or vascular smooth muscle-specific expression of the human (P)RR have increased plasma aldosterone levels. Yet, their plasma levels of renin and angiotensin are normal,[8, 9] and elevated adrenal levels of Ang II did not occur in the animals with ubiquitous (P)RR expression.[10] Possibly therefore, renin- and/or prorenin-induced stimulation of this receptor in the adrenal cortex (i.e. independently of angiotensin) is responsible for increased aldosterone production. Recently, the (P)RR was shown to be present in aldosterone-producing adenomas (APAs) as well as in two adrenocortical cell lines (H295R and HAC15) suggesting a role for this receptor in aldosterone synthesis.[11] Indeed, adrenal (P)RR expression in (P)RR transgenic rats correlated with CYP11B2 expression.[10] Moreover, some of the mitogen-activated protein (MAP) kinases linked to aldosterone synthesis and release (extracellular signal regulated 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK)),[12, 13] are known to be activated by renin/prorenin (hereafter referred to as (pro)renin) binding to the (P)RR.[7, 14]

Aldosterone is produced from cholesterol through a series of enzymatic reactions involving cytochrome P450 (CYP) enzymes and a hydroxysteroid dehydrogenase (HSD). After mitochondrial transport by steroid acute regulatory protein (encoded by STAR), cholesterol is converted into pregnenolone by P450scc (CYP11A1). Aldosterone is formed through sequential reactions by 3β-HSD (HSD3B2), 21-hydroxylase (CYP21A2) and aldosterone synthase (CYP11B2). P450c17 (CYP17A1) diverts steroidogenesis from aldosterone towards glucocorticoid and sex steroid synthesis.[15] These enzymes are present in the adrenocortical carcinoma cell lines H295R and HAC15, making them useful and well-characterized models to study regulatory mechanisms for aldosterone synthesis.[16-18]

The purpose of this study was to assess whether there is a direct, Ang II-independent effect, of prorenin and/or renin on the MAP kinase signalling cascade and adrenocortical steroidogenesis in H295R and HAC15 cells. To that end, we studied the effects of (pro)renin on aldosterone production and steroidogenic enzyme expression in these cells.

## MATERIALS AND METHODS

### Cell culture studies

H295R cells (ATCC, Manassas, VA) were maintained in DMEM/F12, containing 5% foetal calf serum (FCS), 400 IU/ml penicillin and 0.4 mg/ml streptomycin (Invitrogen, Carlsbad, CA).

H295R cells were seeded in 24-well plates at a concentration of $1x10^5$ cells/ml for 24 hours. Cells were then serum-starved overnight and subsequently incubated with Ang II (Sigma-Aldrich, St. Louis, MO; 100 nmol/l), recombinant human renin (see below, 1 and 10 nmol/l), or recombinant human prorenin (1 nmol/l). Renin was applied at higher concentrations than prorenin, given its lower affinity for the (P)RR.[2] At 6, 24 and 48 hours, medium was removed and the plates were snap-frozen and stored at -80°C until further analysis.

HAC15 cells (kindly provided by Dr. W.E. Rainey, Medical College of Georgia, Augusta, GA) were maintained in DMEM/F12 containing 10% cosmic calf serum (CCS, Thermo Scientific, Breda, The Netherlands), penicillin and streptomycin. These cells were seeded in 24-well plates at a concentration of $1x10^5$ cells/ml for 24 hours. Cells were put on 0.1% CCS overnight and subsequently incubated with Ang II (100 nmol/l[19-21]), recombinant human renin (10 nmol/l), recombinant human prorenin (2.5 nmol/l), or Ang II in combination with renin or prorenin. At 48 hours, culture media were collected and the plates were snap-frozen and stored at -80°C until further analysis.

To study whether renin or prorenin activates the ERK1/2 phosphorylation pathway, H295R cells were seeded in 6-well plates at a concentration of $4.5x10^5$ cells/ml for 24 hours. Cells were serum-starved overnight and subsequently stimulated with Ang II (100 nmol/l), recombinant human renin (10 nmol/l), or recombinant human prorenin (2.5 nmol/l). After 5 and 30 minutes of incubation, the cells were harvested and lysed with lysis buffer containing protease inhibitor Complete (Roche, Almere, The Netherlands) and phosphatase inhibitor mixture (Sigma, Zwijndrecht, The Netherlands) as described previously.[7] The cell lysates were stored at -20°C until Western blot analysis.

The capacity of H295R cells to generate (pro)renin was assessed by collecting medium following exposure of the cells to 10 µmol/l forskolin (Sigma-Aldrich) or 1 mmol/l cAMP (Sigma-Aldrich) (to maximally stimulate renin release[22]) for 48 hours in the absence or presence of 5% FCS. In order to evaluate their angiotensin-converting enzyme (ACE) content, serum-starved cells were incubated at 37°C with 10 nmol/l Ang I in the absence or presence of 10 µmol/l captopril. Medium samples (50 µl) for Ang I and II measurements were then collected at 30, 60, 180 and 360 minutes and rapidly mixed with angiotensinase inhibitor mix as described previously.[23]

## Recombinant human (pro)renin

Recombinant human prorenin was a kind gift of Dr. S. Matthews (Hoffmann-LaRoche, Basel, Switzerland). It was secreted by CHO cells transfected with a vector containing human prorenin cDNA. It was partially purified, to remove traces of renin, by Cibacron Blue Sepharose affinity chromatography (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). The intrinsic renin activity of the prorenin preparation, prior to proteolytic activation, was less

than 2% of the activity after complete proteolytic activation.[24] After proteolytic activation, the prorenin preparation contained approximately 4 μmol/l renin.

### (P)RR expression

(P)RR expression was investigated in non-stimulated H295R cells and human adrenal tissue, obtained as residual tissue after nephrectomy (n=2), by reverse transcriptase-polymerase chain reaction (RT-PCR). Cells and frozen tissues were homogenized in TRIzol reagent (Invitrogen) with the Polytron PT 3000 (Kinematica AG, Littau-Luzern, Switzerland) and RNA was isolated according to the manufacturer's instructions. Following isolation, 1 μg of RNA was reverse transcribed as described previously.[25] In the PCR reaction, CAT TGT CCA TGG GCT TCT CT was used as forward primer, and 2 different reverse primers (CAA ACT TTT GCA GAG CGT CA and ATC CAG GAT CCA TGT TCC AA) were used, resulting, respectively, in the detection of 603 and 864 bp fragments.[1] A 30 μL mixture containing AmpliTaq mix (Roche Applied Science, Penzberg, Germany), dNTPs, 1250 nmol/l primers and 30 ng cDNA was incubated for 5 minutes at 95°C. Amplification was performed in forty cycles consisting of the following steps: 1 minute at 95°C, 1 minute at 64°C, and 1 minute at 72°C. The amplification was ended with a 10-minute incubation at 72°C. PCR products were run on an agarose 2% gel

### Western Blotting

For the detection of ERK1/2 and phosphorylated ERK1/2, 10 μg of protein was loaded on a 12% polyacrylamide gel. For the detection of (P)RR and β-actin, 20 μg of protein was applied. Cell lysates of Mardin Darby Canine Kidney (MDCK) cells (15 μg protein) were used as a positive control for the presence of the (P)RR. We used polyclonal rabbit antibodies against ERK1/2 and phospho-ERK1/2 (Cell Signaling, Danvers, MA) for the detection of total and phosphorylated ERK, polyclonal rabbit anti-ATP6AP2 antibodies (Sigma-Aldrich) for the detection of the (P)RR, and monoclonal mouse anti-actin (clone C4) antibodies (Millipore, Amsterdam, The Netherlands) for the detection of β-actin. Horseradish peroxidase-conjugated secondary antibodies were purchased from Biorad (Veenendaal, The Netherlands). Blots were developed with chemiluminescence substrate. Quantification of protein levels was performed with Image J (National Institutes of Health, Bethesda, MD).

### RNA isolation and quantitative RT-PCR

RNA was isolated from the H295R cells using TRIzol, followed by reverse transcription. Thereafter, expression of steroidogenic enzyme and housekeeping gene hypoxanthine ribosyl transferase 1 (HPRT1) mRNAs was evaluated using real-time PCR as described previously.[25] Primer and probe sequences have been described previously.[15] cDNA specificity was checked

for each gene product and PCR efficiency exceeded 90% for each probe-primer pair. Expression of mRNAs was calculated relative to HPRT1 using the δCt-method.

### Prorenin, renin, angiotensin and aldosterone measurements

Renin and prorenin in the culture medium were measured by immunoradiometric assay (Cisbio Bioassays, Bagnols-sur-Cèze, France) as described before.[26] The detection limit was 0.024 pmol/l. Ang I and II were measured with sensitive radioimmunoassay.[27] Their detection limits were 80 and 40 pmol/l, respectively. Aldosterone was measured with a radioimmunoassay (Coat-a Count, Siemens-DPC, Los Angeles, CA) with a detection limit of 61 pmol/l.

### Statistical analysis

Levels are expressed as mean ± standard error of the mean (SEM). ERK phosphorylation and log-converted steroidogenic enzyme expression levels were analyzed by one-way analysis of variance (ANOVA), applying post-hoc Dunnett's test for multiple comparisons. Aldosterone levels were analyzed by Kruskal-Wallis test, applying post-hoc Dunn's test for multiple comparisons because of a non-Gaussian distribution even after log-conversion. P-values $<0.05$ were considered statistically significant. Analyses were performed with GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA).

## RESULTS

### Local expression of the (P)RR and RAAS components

Both H295R cells and human adrenal tissue expressed the (P)RR, as evidenced by the appearance of the expected 603 and 864 bp fragments following RT-PCR (data not shown). Western blots on H295R cell lysates confirmed the presence of the (P)RR protein (Figure 1).

Neither renin nor prorenin could be detected in the medium of forskolin- or cAMP-stimulated H295R cells with or without serum (n=3). The cells metabolized Ang I to the same degree without or with captopril (($t_{1/2}$'s 2.1±0.1 and 2.6±0.2 hours, respectively; n=4), and Ang II in the medium remained below the limit of detection under all conditions.

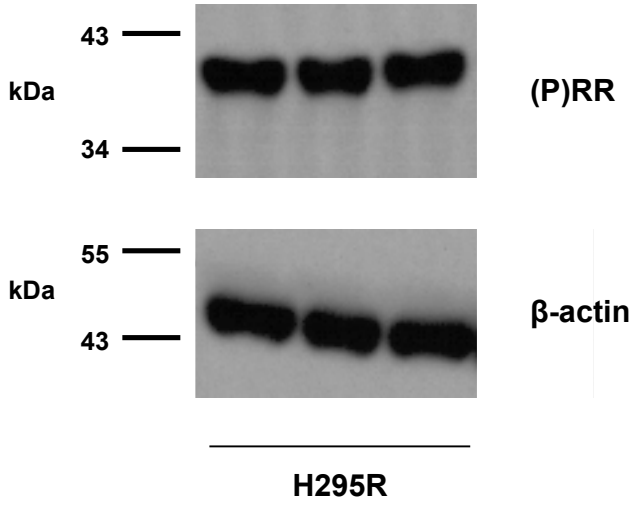


**Figure 1.** Western blot analysis on the presence of the (pro)renin receptor ((P)RR) in the H295R cell line with β-actin serving as reference for protein loading. Mardin Darby Canine Kidney (MDCK) cells were used as positive control for (P)RR expression; they yielded a product of similar size.



## Effect of (pro)renin on adrenocortical cells

Incubation with Ang II rapidly induced phosphorylation of ERK at both 5 and 30 minutes, whereas supraphysiological concentrations of both renin and prorenin did not (Figure 2).

Aldosterone concentrations in the supernatants of unstimulated H295R cells were below the detection limit of the assay. Ang II led to a rise in aldosterone concentration in HAC15 cell culture media after 48 hours of incubation (Figure 3). This was not affected by coincubation with renin or prorenin. Stimulation with renin or prorenin alone did not result in higher aldosterone secretion as compared with unstimulated cells.

Ang II (n=4) significantly increased the expression of STAR, CYP11B2, CYP21A2 and CYP171A1 (Figure 4) in H295R cells at 6 and/or 24 hours, but not at 48 hours. No significant effects were observed for HSD3B2 and CYP11A1 (data not shown). Renin and prorenin (n=4 for all concentrations) had no significant effect on the expression of any of the steroidogenic enzymes studied. After 48 hours of incubation, only Ang II (in the presence or absence of (pro) renin), but not renin or prorenin alone, increased the expression of CYP11B2 and decreased the expression of CYP17A1 in HAC15 cells (n=3 for all conditions; Figure 5). No significant effects were observed for the expression of the other steroidogenic enzymes.

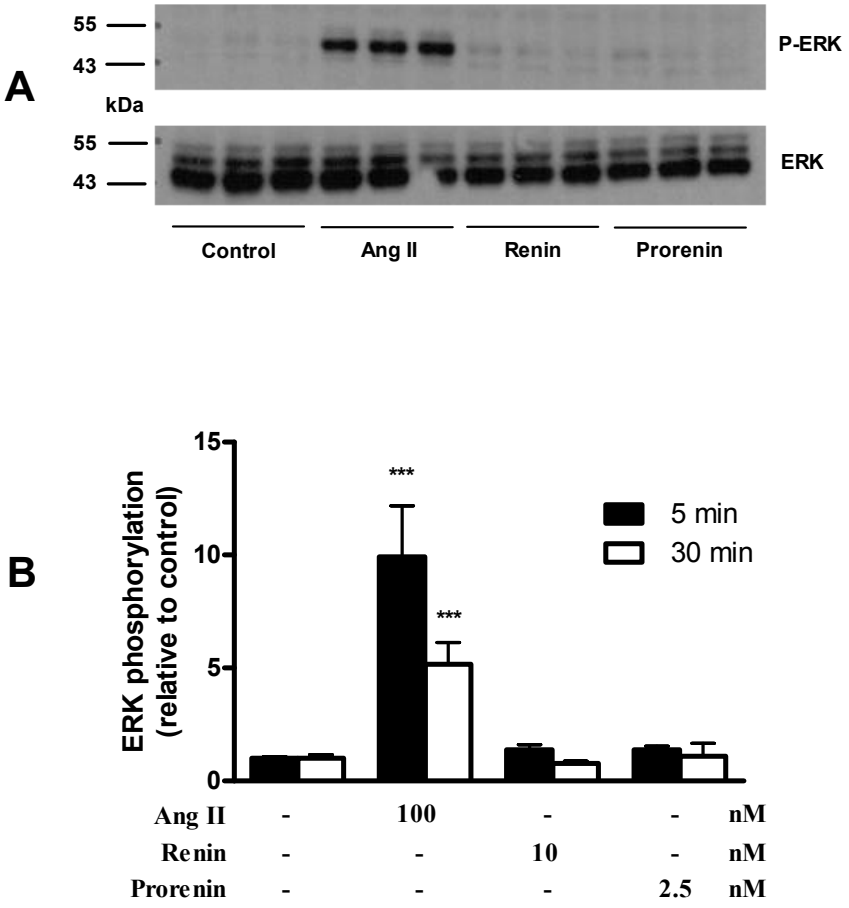


**Figure 2.** Total ERK1/2 (ERK) and phosphorylated ERK1/2 (p-ERK) in H295R cells after a 5-minute incubation with angiotensin II (Ang II, 100 nmol/l), renin (10 nmol/l), and prorenin (2.5 nmol/l) analyzed by Western blot (A). Quantification of ERK phosphorylation relative to unstimulated cells at 5 and 30 minutes stimulation (B). *** $P<0.001$ versus control. Values are mean ± SEM of six experiments except for Ang II at t=5 min and control at t = 30 min (n=5).

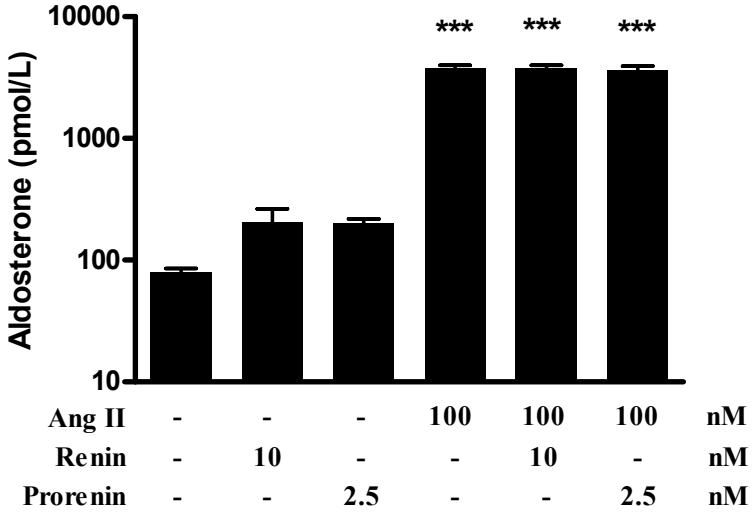


**Figure 3.** Aldosterone concentrations in HAC15 culture media after 48 hours of incubation with renin (10 nmol/l), prorenin (2.5 nmol/l), angiotensin II (Ang II, 100 nmol/l), and Ang II (100 nmol/l) coincubated with renin (10 nmol/l) or prorenin (2.5 nmol/l), compared with unstimulated cells. *** P<0.001 versus control. Values are mean ± SEM of 12 experiments.

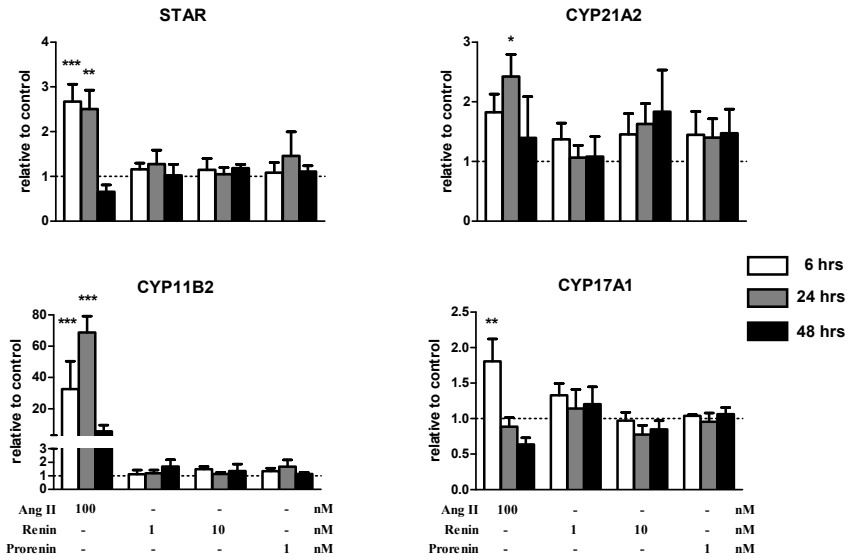


**Figure 4.** Expression of STAR, CYP21A2, CYP11B2 and CYP17A1 mRNA in H295R cells after incubation with angiotensin II (Ang II, 100 nmol/l), renin (1 and 10 nmol/l) and prorenin (1 nmol/l) for 6, 24 and 48 hours. Values were related to the housekeeping gene HPRT1 and depicted relative to unstimulated cells (mean ± SEM of four experiments). *P<0.05, **P<0.01, ***P<0.001 versus control.

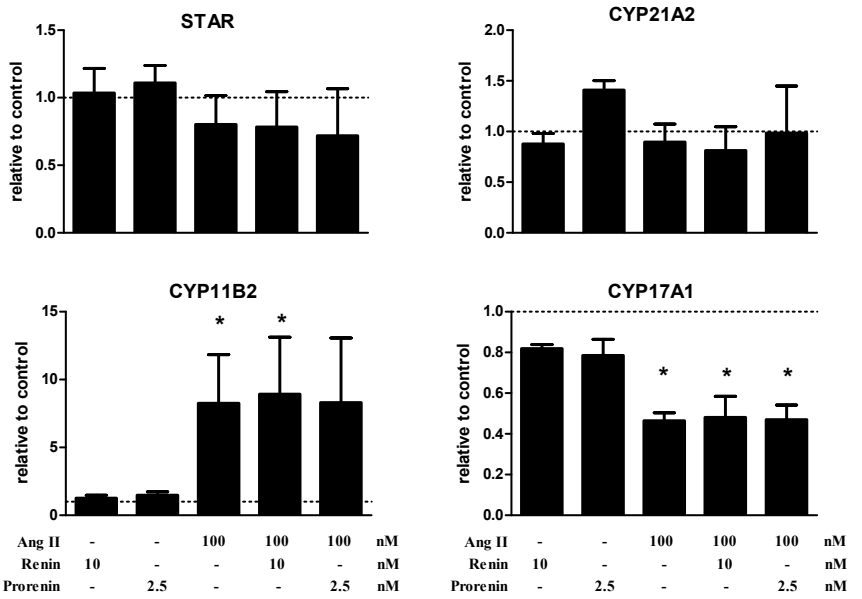


**Figure 5.** Expression of STAR, CYP21A2, CYP11B2 and CYP17A1 mRNA in HAC15 cells after incubation with angiotensin II (Ang II, 100 nmol/l), renin (10 nmol/l), prorenin (2.5 nmol/l) for 48 hours. Values were related to the housekeeping gene HPRT1 and depicted relative to unstimulated cells (mean ± SEM of three experiments). *P<0.05 versus control.

## DISCUSSION

Transgenic rats overexpressing the human (P)RR have increased levels of plasma aldosterone despite normal plasma and tissue levels of renin and Ang II.[8-10] Given the direct effects of both renin and prorenin via (P)RR-mediated activation of ERK1/2 and p38 MAPK,[1, 14] we hypothesized that renin and prorenin might directly stimulate aldosterone synthesis through activation of the adrenocortical (P)RR. This hypothesis was tested in the adrenocortical cell lines H295R and HAC15, making use of nanomolar levels of (pro)renin. These concentrations were chosen based on previous in-vitro studies showing effects of (pro)renin in cardiomyocytes, monocytes and renal cells.[5, 7, 14, 28] It should be realized that such concentrations are three to four orders of magnitude above the physiological levels of (pro)renin.[29] Although (pro) renin levels may rise under pathological conditions and/or during renin-angiotensin system (RAS) blockade, such rises are usually <10-fold,[29-31] i.e., far below the 1000-10.000-fold higher levels applied here. Therefore, at most, such high levels might exist at sites where (pro)renin is produced, e.g. in the kidney and the adrenal gland.

First, (P)RR expression in H295R cells was confirmed both at the mRNA and protein level. Second, we verified whether H295R cells are capable of generating RAS components. In agreement with a previous study,[32] we were unable to detect ACE activity in these cells. In

fact, following the addition of Ang I to the medium, these cells did not generate any Ang II, ruling out Ang II generation by non-ACE pathways as well.

Adrenal cells are thought to express a renin transcript lacking exon 1, encoding for a non-secretory intracellular renin.[33] Immunohistochemistry indeed supported the cytosolic presence of renin in H295R cells.[32] We were unable to detect renin or prorenin in the medium of these cells, even following stimulation of the renin-stimulatory[22] adenylyl cyclase-cAMP pathway. This confirms that renin lacking the transport signal (which is encoded by a signal derived from exon 1 of the renin gene) cannot be secreted.[34] The function of intracellular renin is so far unknown. Although elevated levels of adrenal (cytosolic) renin have been reported to occur in conjunction with elevated plasma levels of aldosterone,[34] it remains to be determined whether intracellular renin displays Ang I-generating activity, and if so, to what degree it has access to angiotensinogen in adrenal cells. Alternatively, such renin might stimulate intracellular (P)RRs in an Ang II-independent manner, and thereby increase aldosterone synthesis. Renin and prorenin, when added to the medium, will accumulate intracellularly,[2, 35, 36] and thus our experimental approach should allow the stimulation of (P)RRs both on the cell surface and in the cells.

The current study, however, does not support aldosterone synthesis by adrenocortical cells following their exposure to nanomolar concentrations of either renin or prorenin. In fact, although present in H295R cells, stimulation of the (P)RR with renin or prorenin did not lead to phosphorylation of ERK1/2. In contrast, Ang II did induce ERK1/2 phosphorylation and resulted in a profound stimulation of steroidogenic enzyme expression, in particular aldosterone synthase (CYP11B2), in full agreement with previous reports.[17, 19] Aldosterone concentrations in culture media of unstimulated H295R cells remained below the detection limit. In order to be able to assess even small effects on aldosterone secretion, additional experiments were performed in HAC15 cells. Ang II, but not renin or prorenin, increased aldosterone secretion in these cells. Clearly, therefore, our findings rule out a direct effect of renin/prorenin on aldosterone synthesis via activation of (P)RR on either the cell surface or in the cells.

One may speculate that, in the intact adrenal gland in vivo, in the presence of sufficient angiotensinogen and ACE, (pro)renin binding to the (P)RR would allow angiotensin generation, thus resulting in aldosterone synthesis in an indirect (Ang II-dependent) manner. However, given the unchanged plasma and tissue angiotensin levels in (P)RR transgenic rats,[8-10] this mechanism cannot underlie the elevated aldosterone levels in these rats.

In conclusion, neither renin nor prorenin stimulates aldosterone synthesis directly via (P)RR in H295R and HAC15 cells, not even when applied at supraphysiological levels. Most likely therefore, the elevated aldosterone levels in (P)RR transgenic rats are the consequence of other mechanisms. Given the recent observation that the (P)RR is indispensable for the integrity of vacuolar $H^+$-ATPase (an enzyme that plays an important role in the acidification of subcellular compartments),[37] one possibility is that intracellular pH changes per se (i.e.,

independently of renin/prorenin) affect aldosterone synthesis. Alternatively, the renal damage in (P)RR transgenic rats may stimulate adrenal aldosterone production via an increase in serum potassium levels.[8, 9] Although (P)RR blockade with the handle region peptide (HRP) prevented renal damage in (P)RR transgenic rats,[38] the effect of such normalization on plasma aldosterone levels or serum potassium levels has not yet been reported. Importantly, the current data rule out that the 'aldosterone escape' occurring during single or dual RAS blockade[39, 40] is due to the renin/prorenin rise that accompanies such blockade. More likely, the Ang II escape that usually also occurs during prolonged RAS blockade[41, 42] contributes to this aldosterone rise.

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# Part III

## The screening for primary aldosteronism

# Chapter 4

## The aldosterone-to-renin ratio as a screening test for primary aldosteronism.

Adapted from: The aldosterone-to-renin ratio as a screening test for primary aldosteronism in patients with therapy-resistant hypertension. The Dutch ARRAT Study

Pieter M. Jansen, Frans Boomsma, Anton H. van den Meiracker, on behalf of the Dutch ARRAT investigators.

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## ABSTRACT

Since the introduction of the aldosterone-to-renin ratio (ARR) as a screening test for primary aldosteronism (PA), there has been a marked increase in the reported prevalence of this condition among hypertensive subjects. A meta-analysis from the literature shows a prevalence of 8 % among hypertensive patients, with a twofold higher prevalence in referred patients as compared with primary care patients (9.5 vs 4.3 %). The diagnostic workup includes screening, confirmation and evaluation of the subtype of PA. Although the ARR is the recommended screening test, the usefulness of the ARR remains subject of debate, because of doubts on its validity, the many factors affecting the ARR, including posture, time of day of blood sampling and use of antihypertensive medication and a poor reproducibility. Additional studies are needed to evaluate whether the ARR really is a reliable screening test for PA.

## BACKGROUND

Primary aldosteronism (PA) has been a well-known cause of hypertension since the 1950s when Jerôme Conn described a disease state characterized by severe hypertension and hypokalemia. The cause turned out to be an aldosterone-producing adrenal tumour.[1] Initially, PA was considered to be extremely rare with an estimated prevalence ranging from 0.05 to 2 %. This was partly due to the lack of reliable screening tests. PA was mainly suspected when patients presented with (resistant) hypertension and hypokalemia.[2] Since not all patients with PA have reduced potassium levels, hypokalemia is an unreliable screening parameter. An elevated plasma aldosterone in itself is also a poor indicator of autonomous aldosterone excess, as levels can be raised secondary to activation of the renin-angiotensin system (RAS), for instance in the context of diuretic use or a renal artery stenosis. Therefore, plasma aldosterone levels should be considered in relation to the activity of the other elements of the RAS. Simultaneous measurement of aldosterone levels and renin levels or activity was shown to enable differentiation of major disorders of the RAS, including PA.[3,4] Since PA is characterized by elevated aldosterone levels and suppressed renin levels, an elevated aldosterone-to-renin ratio (ARR) could be a better indicator of (relatively) autonomous aldosterone production (Figure 1). The ARR was introduced as a screening test for PA in 1981 by Hiramatsu *et al*.[5] Since



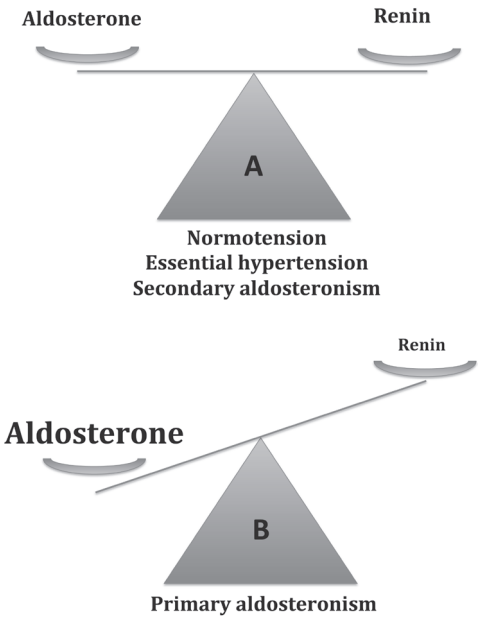


**Figure 1.** Rationale for the aldosterone-to-renin ratio (ARR): primary aldosteronism is associated with an inadequately elevated aldosterone level in relation to renin levels or activity resulting in an elevated ARR.

then, the reported prevalence of PA has increased considerably,[6] and it is now considered to be one of the major causes of secondary hypertension.

## PREVALENCE STUDIES

Since the introduction of the ARR, numerous studies have investigated the prevalence of a raised ARR and PA.[7-35] These studies differ in the population that was subjected to screening, the screening test that was used and the cutoff values to define abnormality. Also, there were differences in the diagnostic workup, as will be discussed later. An overview of these studies is given in table 1, subdivided into primary care patients (table 1A), referred patients with moderate to severe, often poorly controlled hypertension (table 1B) and special subgroups (table 1C).

The prevalence of an elevated ARR in the reported studies ranged from 0 to 41 %, with a weighed mean value of 19.1 %. The prevalence of confirmed PA ranged from 0.7 to 34 % with a weighed mean value of 8.3 %. The chance of having PA is higher in patients with more severe hypertension. A combined analysis of two population-based studies in Germany showed that 7.0% of the hypertensive population has an elevated ARR. In the subgroup with Stage III hypertension, this number was markedly higher.[35] Rossi *et al*.[19] found a mean PA prevalence of 11.2% in a population of referred hypertensive patients, but the prevalence ranged from 6.6 % in patients with grade I to 19 % with grade III hypertension.[19] A similar trend was observed by Mosso *et al*.[16] who found a prevalence of 2.0 % in grade I, up to 13.2% in grade III hypertension. Our meta-analysis shows that the prevalence is twice as high in referred patients as in primary care patients (Figure 2B) which is not surprising because the referred patients are expected to have more severe hypertension. Remarkably, the prevalence of an elevated ARR is almost as high in primary care as in referred patients (Figure 2A), indicating that a higher number of patients will have a false-positive result when the ARR is applied in a primary care setting. Even in normotensive subjects a small subset appears to have PA, with reported prevalences of around 1.5 %.[10, 16]

In contrast to former beliefs, many patients with PA present without hypokalemia, and percentages range from 0 % to 70 %.[7, 10-16, 19, 25, 36] In some studies only normokalemic patients were included.[8, 22, 26] A retrospective evaluation from centres in five continents showed that between 9 and 37 % of patients were hypokalemic.[6] Overall, the percentage of hypokalemic patients among PA cases was higher in referred patients than in primary care patients (Figure 2C). Furthermore, the reported numbers of aldosterone-producing adenomas were higher in this group (Figure 2D). It seems reasonable to conclude that referred patients more frequently have an APA reflected by a more severe phenotype of higher blood pressure levels and lower serum potassium values.

**Table 1A.** Overview of studies on the prevalence of primary aldosteronism based on aldosterone-to-renin ratio and formal confirmation testing in primary care patients.

| Reference | Population | N | Region | Medication protocol | Screening test | Units | Cut-off values | P(ARR) (%) | Confirmation test | P(PA) (%) | Hypokalemia (%) | APA (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gordon *et al.*[7] | Drug trial volunteers with hypertension | 52 | Australia | Cessation of diuretics | PAC/PRA (3x) | PAC: ng/dL<br>PRA: ng/mL per hr | 30 | 12 | FST | 12 | 0 | 33 |
| Loh *et al.*[11] | Primary care clinic hypertensive patients | 350 | Singapore | Unchanged antihypertensive regimen | PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | PAC/PRA>20<br>PAC>15 | 18 | i.v. SLT | 4.6 | 38 | 50 |
| Mosso *et al.*[16] | Primary care clinic hypertensive patients | 609 | Chili | Cessation of betablockers, ACE-I, ARB, diuretics, spironolactone and aspirin | SA/PRA | SA: ng/dL<br>PRA: ng/mL per hr | 25 | 10 | FST | 6.1 | 2.7 | 5.4 |
| Schwartz and Turner[18] | Patients with essential hypertension | 118 | USA | Unchanged antihypertensive regimen | PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | 12.4 (ROC curve) | 32 | Oral SLT | 13 | NA | 0 |
| Williams JS *et al.*[22] | Mild to moderate, normokalaemic hypertensive patients | 347 | USA | Cessation of all antihypertensive medication | SA/PRA + elevated SA | SA: ng/dL<br>PRA: ng/mL per hr | SA/PRA > 25<br>SA > 8 | 7.5 | Oral SLT | 3.2 | 0 (per definition) | NA |
| Westerdahl *et al.*[24] | Primary care hypertensive patients | 200 | Sweden | Cessation of all antihypertensive medication except calciumblockers | SA/PRC | SA: pmol/L<br>PRC: ng/L | 100 | 25 | FST | 8.5 | NA | 6.3 |
| Olivieri *et al.*[27] | Randomly selected, primary care hypertensive patients | 287 | Italy | Cessation of antihypertensive medication except doxazosin and verapamil | PAC/PRC | PAC: pg/mL<br>PRC: pg/mL | 32 | 32 | ND | NA | NA | NA |

**Table 1A.** Overview of studies on the prevalence of primary aldosteronism based on aldosterone-to-renin ratio and formal confirmation testing in primary care patients. *(continued)*

| Reference | Population | N | Region | Medication protocol | Screening test | Units | Cut-off values | P(ARR) (%) | Confirmation test | P(PA) (%) | Hypokalemia (%) | APA (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hood *et al.*[28] | Unselected primary care hypertensive patients | 846 | UK | Unchanged antihypertensive regimen | PAC/PRA | PAC: pmol/L<br>PRA: pmol/mL per hr | 800 | 14 | PAC/PRA > 800 + PAC > 400 **and** adrenal adenoma **or** ΔSBP > 20 mmHg on spironolactone | 0.7 | 17 | 17 |
| Schmiemann *et al.*[34] | Primary care patients with resistant hypertension | 63 | Germany | Cessation of betablockers and/or spironolactone if clincally feasible (n=34) | PAC/PRC | PAC: pg/mL<br>PRC: pg/mL | 45 | 24 | ND | NA | NA | NA |
| **Mean** | | | | | | | | **16.3** | | **4.3** | **12.4** | **14.5** |

ARR, aldosterone-to-renin ratio; PA, primary aldosteronism; P(ARR), prevalence of an elevated ARR; P(PA), prevalence of PA; PAC, plasma aldosterone concentration; SA, serum aldosterone concentration; PRA, plasma renin activity; PRC, plasma renin concentration; FST, fludrocortisone suppression test; SLT, salt loading test; LDF-score, logistic discriminant function – score; ACE-I, angiotension converting enzyme inhibitors; ARB, angiotensin II receptor blocker; NA, not available; ND, not done

PAC: to convert ng/dL to pmol/L multiply by 27,7

* in patients with an elevated ARR

** the LDF-score is explained in Rossi *et al.*[98]

Weighed means for the prevalence of an increased ARR and PA are based on the total number of cases divided by the total number of patients in the reported studies. Mean percentages of hypokalemia and APA are weighed for the total number of PA cases in the reported studies.

**Table 1B.** Overview of studies on the prevalence of primary aldosteronism based on aldosterone-to-renin ratio and formal confirmation testing in referred patients.

| Reference | Population | N | Region | Medication protocol | Screening test | Units | Cut-off values | P(ARR) (%) | Confirmation test | P(PA) (%) | Hypokalemia (%) | APA (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gordon *et al.*[8] | Referred, normokalaemic hypertensive patients | 199 | Australia | None | PAC/PRA (3x) | PAC: ng/dL<br>PRA: ng/mL per hr | 30 | 11 | FST | 8.5 | 0 (per definition) | 29 |
| Lim *et al.*[9] | Unselected hypertension clinic population | 465 | UK | Cessation of antihypertensive treatment if possible (60 %) | PAC/PRA | PAC: pmol/L<br>PRA: ng/mL per hr | 750 | 17 | FST | 9.2 | 4.7 | 12 |
| Fardella *et al.*[10] | Hypertension clinic population | 305 | Chili | No antihypertensive treatment | SA/PRA (2x) | SA: ng/dL<br>PRA: ng/mL per hr | 25 | 14 | FST | 9.5 | 0 | 3.4 |
| Gallay *et al.*[12] | Referred patients with poorly controlled hypertension | 90 | USA | Continuation of antihypertensive treatment | PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | 100 | 17 | ND | NA | 40 * | 67 * |
| Calhoun *et al.*[30] | Consecutive ptients with resistant hypertension | 88 | USA | Cessation of spironolactone, amiloride or triamterene | PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | 20 | 41 | Oral SLT | 20 | 72 | 17 |
| Rossi *et al.*[13] | Referred hypertensive patients | 1065 | Italy | Cessation of antihypertensive treatment except α-blockers | Post-captopril (50 mg) PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | 35 | 13 | i.v. SLT | 6.3 | 39 | 24 |
| Strauch *et al.*[14] | Moderate to severe hypertensive patients | 402 | Czech Rep. | Cessation of antihypertensive treatment except α-blockers | PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | 50 | 22 | i.v. SLT | 19 | 70 | 36 |
| Stowasser *et al.*[15] | Referred hypertensive patients | 300 | Australia | Cessation of diuretics, betablockers, central antihypertensive agents and dihydropyridine calciumblockers | PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | 30 | 20 | FST | 18 | 13 | 31 |

**Table 1B.** Overview of studies on the prevalence of primary aldosteronism based on aldosterone-to-renin ratio and formal confirmation testing in referred patients. *(continued)*

| Reference | Population | N | Region | Medication protocol | Screening test | Units | Cut-off values | P(ARR) (%) | Confirmation test | P(PA) (%) | Hypokalemia (%) | APA (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nishizaka *et al.*[17] | White subjects with resistant hypertension | 150 | USA | Cessation of spironolactone, triamterene, or amiloride | PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | 20 | 32 | Oral SLT | 20 | 15 | NA |
| Nishizaka *et al.*[17] | African Americans with resistant hypertension | 115 | USA | Cessation of spironolactone, triamterene, or amiloride | PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | 20 | 28 | Oral SLT | 24 | | NA |
| Rossi *et al.*[19] | Consecutive referred hypertensive patients | 1125 | Italy | Cessation of antihypertensive medication except calciumblockers and/or doxazosin | SA/PRA | SA: pg/mL<br>PRA: ng/mL per hr | 40 | 19 | ARR baseline ≥40<br>+<br>ARR post captopril ≥ 30<br>and/or<br>LDF score ≥ 50 % (**) | 11 | 30 | 43 |
| Williams *et al.*[20] | Unselected referred hypertensive patients | 122 | UK | Continuation of antihypertensive treatment | PAC/PRA | PAC: pmol/L<br>PRA: ng/mL per hr | 750 | 16 | ND | NA | 25 * | NA |
| Fogari *et al.*[25] | Unselected, consecutive hypertensive patients | 3000 | Italy | Cessation of all antihypertensive medication and other interfering medication | SA/PRA | SA: ng/dL<br>PRA: ng/mL per hr | 25 | 23 | i.v. SLT | 5.9 | 25 | 30 |
| Douma *et al.*[31] | Consecutive ptients with resistant hypertension | 1616 | Greece | Cessation of diuretics, ACEIs, ARBs, betablockers, CCBs and centrally acting antihytpertensives | SA/PRA<br>+ elevated SA | SA: pmol/L<br>PRA: pmol/L per min | SA/PRA>65<br>SA>416 | 21 | i.v. SLT and FST | 11.3 | 46 | |
| Ribeiro *et al.*[32] | Referred hypertensive patients without betablockers and spironolactone | 105 | Brazil | Continuation of antihypertensive treatment | SA/PRA | SA: ng/dL<br>PRA: ng/mL per hr | 25 | 9 | i.v. SLT | 1.0 | 0 | 0 |
| **Mean** | | | | | | | | **19.9** | | **9.5** | **33.1** | **29.8** |

**Table 1C.** Overview of studies on the prevalence of primary aldosteronism based on aldosterone-to-renin ratio and formal confirmation testing in special subgroups.

| Reference | Population | N | Region | Medication protocol | Screening test | Units | Cut-off values | P(ARR) | Confirmation test | P(PA) (%) | Hypokalemia (%) | APA (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fardella *et al.*[10] | Normotensive control subjects | 205 | Chili | No antihypertensive treatment | SA/PRA (2x) | SA: ng/dL<br>PRA: ng/mL per hr | 25 | 1.5 | FST | 1.5 | 0 | 0 |
| Jefic *et al.*[21] | Diabetic patients with hypertension | 61 | USA | Cessation of spironolactone | PAC/PRA | PAC: ng/dL<br>PRA: ng/dL per hr | 30 | 0 | ND | NA | NA | NA |
| Umpierrez *et al.*[23] | Patients with type 2 DM and resistant hypertension | 100 | USA | None | PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | 30 | 34 | Oral SLT (11 %)<br>i.v. SLT (89 %) | 14 | NA | NA |
| Bernini *et al.*[26] | Normokalaemic hypertensive patients with adrenal incidentalomas | 90 | Italy | Cessation of antihypertensive medication | PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | 112 | 8.8 | i.v. SLT captopril suppression test | 5.6 | 0 (per definition) | 40 |
| Pizzolo *et al.*[29] | Patients with residual hypertension after succesful endovascular treatment of renal artery disease | 24 | Italy | None | PAC/PRC | PAC: pg/mL<br>PRC: pg/mL | 23 | 33.3 | i.v. SLT | 27 | NA | 29 |
| Di Murro *et al.*[33] | Consecutive newly diagnosed hypertensive patients with obstructive sleep apnoea | 53 | Italy | Cessation of antihypertensive medication except calciumblockers and/or doxazosin | PAC/PRA | PAC: ng/dL<br>PRA: ng/mL per hr | PAC/PRA>40<br>PAC>15 | NA | i.v. SLT | 34 | NA | 28 |

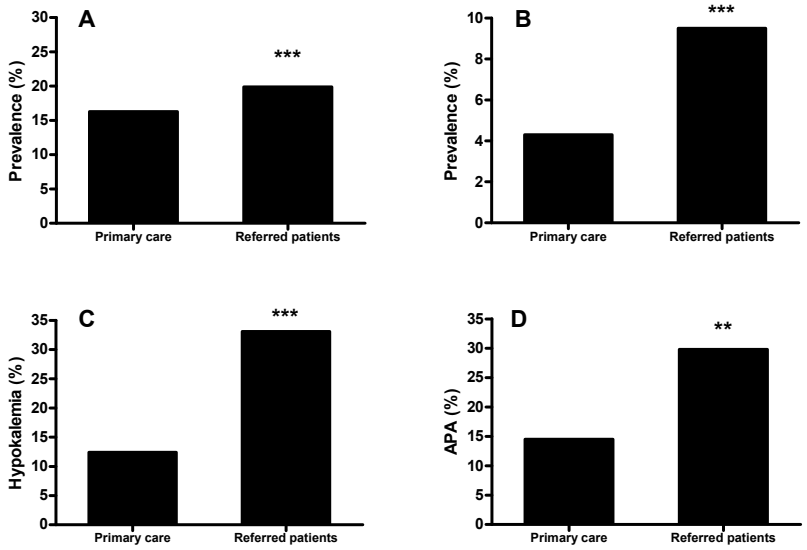


**Figure 2.** Prevalence of A) an elevated aldosterone-to-renin ratio (ARR), B) primary aldosteronism (PA), C) percentage of hypokalemia and D) aldosterone-producing adenomas (APA) among PA cases in primary care and referred patients. Figures are based on weighed mean values for the studies reported in table 1. The differences are tested using $\chi 2$ statistics. ** $p<0.01$; *** $p<0.001$

Whether special subgroups have a higher prevalence of PA has been subject of investigation. These studies include African American patients with resistant hypertension,[17] patients with type 2 diabetes mellitus (DM) and resistant hypertension[23] and hypertensive patients with adrenal incidentalomas.[26] Black subjects generally have lower plasma renin levels than white subjects.[37] However, neither ARR levels nor the prevalence of PA in black and white patients with resistant hypertension were statistically different (24% in African Americans and 20% in white patients).[17, 30] In a group of 100 patients with type 2 DM and poorly controlled hypertension, a 14% prevalence of PA was reported. This was independent of glycemic control. These numbers are similar to those found in other populations.[23] A high prevalence of PA has been described in patients with obstructive sleep apnea[33] and although the exact pathophysiological mechanism is still unclear, increased aldosterone levels appear to increase the risk of sleep apneas contributing to treatment resistance.[38] Patients with adrenal incidentalomas form another group potentially at risk for having PA. Bernini *et al*.[26] screened 90 normokalaemic subjects with an adrenal incidentaloma with hypertension and 35 subjects without hypertension for the presence of PA. Of the subjects with hypertension, 5.6 % had PA, whereas no cases were found in the normotensive subgroup, indicating that an adrenal incidentaloma *per se* should not be an indication for screening, unless hypertension is present.

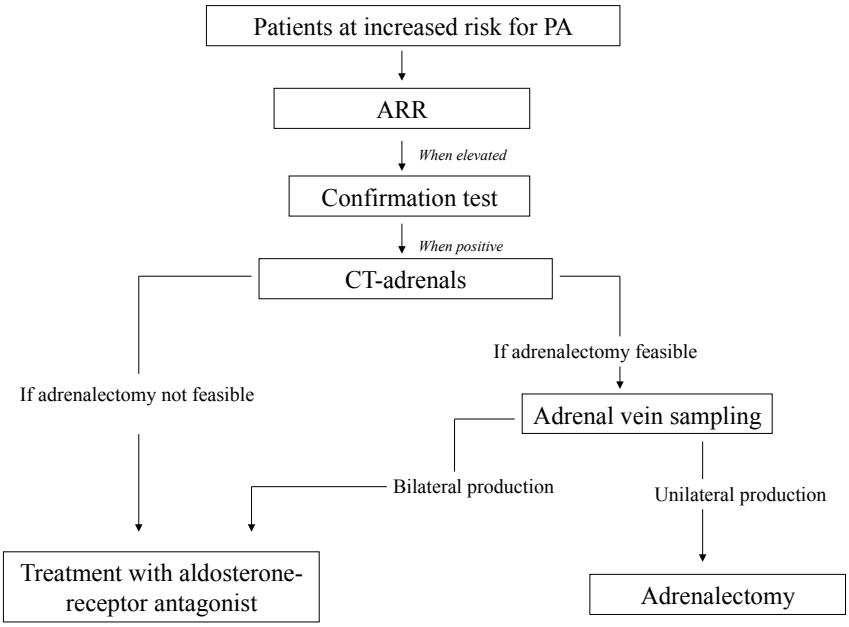


**Figure 3.** Flowchart for the diagnostic workup of primary aldosteronism.
ARR, aldosterone-to-renin ratio; CT, computed tomography; PA, primary aldosteronism. (Adapted from Funder *et al.*[39]).

## DIAGNOSTIC WORKUP FOR PA

In 2008, the Endocrine Society published a clinical practice guideline for case detection, diagnosis and treatment of patients with primary aldosteronism.[39] The diagnostic workup is summarized in Figure 3.

### Screening

The guideline recommends screening of patients with Joint National Commission stage 2, stage 3 or resistant hypertension, hypertension and spontaneous or diuretic-induced hypokalemia, hypertension with an adrenal incidentaloma, or hypertension and a family history of early-onset hypertension, stroke at a young age or a first-degree relative with PA.[39] The ARR is the screening test of choice. Some investigators include an elevated aldosterone level in the screening test, but this is not recommended in the guideline because of an unacceptable risk of false-negative tests.[39] The recommended cutoff values for the most commonly used assays are shown in Table 2.

**Table 2.** The most commonly adopted ARR cutoff values for the most common laboratory assays and units.

| | PRA (ng/ml per hr) | PRA (pmol/l per min) | PRC (mU/l) | PRC (ng/l) |
|---|---|---|---|---|
| **PAC (ng/dl)** | 30 | 2.5 | 3.7 | 5.7 |
| **PAC (pmol/l)** | 750 | 60 | 91 | 144 |

(Adapted from Funder *et al*.[39])

## Confirmation tests

A positive ARR in itself is insufficient to establish the diagnosis of PA and a confirmation test is needed to show that adrenal aldosterone synthesis cannot adequately be suppressed. The most frequently used confirmation tests are the intravenous or oral salt loading test, the fludrocortisone suppression test (FST) and the captopril suppression test.[40] Each test has its own dynamics. Although there is insufficient data to give preference for one test above the other at this point,[39] many authors consider the FST as the gold standard for the diagnosis of PA.[40] However, this test, consisting of the administration of fludrocortisone (100 µg every 6 hours for 4 days) while on a high sodium diet, is time-consuming and impracticable because of the need for hospitalization for close monitoring and replacement of serum potassium levels.[40] The intravenous SLT consists of the infusion of 2 liters of 0.9% sodium chloride solution over 4 hours.[40] Although the reliability of the intravenous SLT has been debated,[41] especially in normokalemic PA patients,[42] it is easier to carry out in an outpatient setting. For this reason, the intravenous SLT is the most frequently used test in The Netherlands.[43] An inadequately high posttest plasma aldosterone level is indicative of PA.[40] Unfortunately, the optimal SLT cutoff value has never been unequivocally defined. The guideline[39] states that a postinfusion PAC above 277 pmol/L (100 pg/mL) makes the diagnosis of PA very likely and below 139 pmol/L (50 pg/mL) unlikely. A postinfusion PAC between these values is indeterminate. However, the literature on this is limited and local protocols have their own cutoff values. A postinfusion PAC of 139 pmol/L was shown to be the optimal cutoff by some[44, 45] with a sensitivity of 88 % compared to the FST.[45] On the other hand, Streeten *et al*.[46] found 235 pmol/L to be the optimal cutoff with 77 % sensitivity compared to the FST although not all patients were subjected to the FST.

## Subtyping

After establishing the diagnosis of PA, additional studies should be performed to assess the subype of PA, as every subtype has its own specific treatment. The most important subgroups of PA are aldosterone-producing adenomas (APA) and idiopathic primary aldosteronism (IPA). Glucocorticoid-remediable aldosteronism (GRA) is a genetic form of PA in which crossing-over of the CYP11B1 and CYP11B2 genes leads to a hybrid gene, coding for aldosterone synthase, but under main regulation by ACTH instead of angiotensin II.[47] Mulatero *et al*.[6] showed that

widespread screening for PA has led to a shift in the proportion of bilateral hyperplasia as a cause of PA, with this subtype now comprising the majority of cases. The prevalence of APAs among PA cases is around 12% in the primary care setting and 33% in referred patients (Figure 2).

Imaging of the adrenal glands by computed tomography (CT) or magnetic resonance imaging (MRI) are frequently used to differentiate between unilateral and bilateral disease. Although the finding of an adrenal adenoma on imaging in a patient with biochemically proven PA is very suggestive of an APA, the presence of a non-functional mass (incidentaloma) cannot be ruled out.[48, 49] On the other hand, imaging techniques may not be sensitive enough to detect adrenal microadenomas.[49, 50] Several studies have shown that a large number of disconcordant results between conventional imaging techniques and adrenal vein sampling (AVS) exists, leading to a misdiagnosis of the subtype in a considerable proportion of patients.[15, 49, 51-53] Although an adrenal CT scan is recommended as the initial study for subtyping to exclude an adrencortical carcinoma and to guide AVS, the latter study is now considered to be the gold standard to differentiate unilateral from bilateral aldosterone overproduction.[39] In AVS, aldosterone and cortisol levels are measured in blood collected from both adrenal veins to assess whether aldosterone is unilaterally overproduced. At the same time, a cortisol sample from the peripheral circulation (either a peripheral vein or the inferior vena cava) is taken to confirm whether the cannulation of the adrenal veins was succesful because cortisol levels in the adrenal veins should be much higher than in the peripheral circulation.[54] Interestingly, the detection rate of APAs appears to be highly dependent on the availability of AVS, with higher proportions found in centres where AVS is available.[6, 19] This supports the notion that AVS is superior for detection of lateralized aldosterone production over CT or MRI. However, uniform diagnostic criteria for the interpretation of AVS results are lacking and the final subtype diagnosis is greatly dependent on the criteria that are used.[54, 55] Nuclear imaging techniques, such as $^{131}$I-19-iodocholesterol, have been developed to obtain a functional, yet noninvasive, way of localization.[56] However, these techniques lack sensitivity and are currently not recommended for this purpose.[39, 57] GRA can be detected by genetic testing and this is recommended in PA patients with family history of PA or strokes at a young age, or the presentation of the disease at a young age.[39]

## DEBATE ON THE ARR AS A SCREENING TEST FOR PA

Despite these guidelines, the validity of the ARR as a screening test for PA remains subject of debate because studies are difficult to compare, many factors affect its results and its test characteristics have incompletely been characterized.[58-60] Furthermore, an elevated ARR may be merely a reflection of low renin levels, therefore not a real indication of autonomous aldosterone secretion *per se*, and could in many cases indicate "regular" low-renin hyperten-

sion.[59, 61-63] For this reason, the clinical relevance of an increased ARR remains unclear.[64] The application of the ARR in an unselected hypertensive population could therefore lead to an enormous increase in costs.[60] The major concerns regarding the ARR as a diagnostic test are considered below.

### Differences in diagnostic protocols

The ARR is widely used for screening purposes, but there are important differences in the diagnostic protocols reported in the literature, making a comparison difficult. These include differences in cutoff values, which also depend on assays and locally established reference values, and the extent to which antihypertensive treatment was changed prior to the test (Table 1). Furthermore, in some studies the ARR had to be raised on more occasions for the test to be positive.[7, 8, 10] Rossi *et al*.[13] assessed the ARR after acute administration of captopril, to raise specificity while other groups included an elevated aldosterone level in the screening test for this purpose.[11, 22] In many of the older studies, renin was assessed as plasma renin activity (PRA),[7-23, 25, 26, 28] while newer studies used plasma renin concentration (PRC).[24, 27, 29] The PRA assay is time-consuming, is dependent on endogenous angiotensinogen levels and difficult to standardize.[65] The PRC assay is easier to standardize allowing for comparison with international reference values. Although the PRC may be less sensitive in the very low range,[65, 66] it was shown to be a reasonable alternative for the PRA.[66-69] The Endocrine Society recommendations for the ARR cutoff values for the different techniques are given in Table 1, but conversion factors may change if renin tests evolve.[39]

### Sensitivity and specificity of the test

Several groups have reported sensitivity and specificity estimates for the ARR[17-19, 30, 59, 70-73] with markedly variable findings. Sensitivities between 66 and 100 % and specificities between 61 and 96 % have been reported. Clearly, these numbers are dependent on the cutoff value that is used. In addition to this, a comparison should be performed with caution because of differences in laboratory assays (PRA versus PRC), the conditions of testing and the confirmation tests used. An important flaw in many studies is the presence of verification bias, which implies that usually only patients with an ARR above the predefined cutoff value were subjected to confirmatory testing. Such an approach is likely to result in an overestimation of diagnostic performance.[74] The same problem may occur in retrospective studies, because of a preselection of the cases and controls that is not independent of the screening test. The area under the curve (AUC) of the receiver operating characteristic (ROC) curve provides an estimation of the test performance independent of cutoff values. Most studies showed AUCs for the ARR between 79-87 %.[17, 18, 75] In studies where the PA cases were preselected, the reported AUCs were much higher (up to 99.6%),[70, 72, 76] possibly for reasons mentioned above.

## Factors affecting the ARR

Many factors are known to influence the ARR, including posture,[77] time of blood sampling,[77, 78] age,[79, 80] salt intake,[80, 81] the presence of hypokalemia,[82] sex[83] and medications including oral anticonceptives,[83-85] nonsteroidal anti-inflammatory drugs[86] and certain selective serotonin reuptake inhibitors.[87] Antihypertensive drugs are an important factor to consider when using the ARR. Beta-adrenergic receptor blockers and centrally-acting antihypertensives can cause falsely elevated ARR levels, because of their suppressive effect on renin.[88-90] On the other hand, angiotensin converting enzyme inhibitors (ACE-Is), angiotensin II type 1 receptor blockers (ARBs) and diuretics cause an increase in renin levels, resulting in a decrease in ARR.[61, 88, 90, 91] Alpha-adrenergic blockers, such as doxazosin, have no effect on the ARR,[88] as well as non-dihydropyridine calcium channel blockers (CCBs).[39] Dihydropyridine CCBs may cause a decrease in ARR via an increase in renin levels through reflex sympathetic mechanisms and a decrease in aldosterone by inhibition of calcium-dependent adrenal aldosterone synthesis[88, 92] but a cross-sectional study showed no effect of dihydropyridine CCBs on the ARR, indicating that this is probably neglible.[90] If these drug effects on the ARR are also clinically relevant in a multidrug regimen, and which drugs in particular, has been addressed in a few papers. Beta-adrenergic blockers, ACE-Is and ARBs were shown to have the largest effect on the ARR in multidrug therapy.[90] However, Schwartz and Turner[18] showed that the AUC under the ROC curve for the ARR did not change when drug therapy was ceased. This implies that the ARR is still valid under multidrug therapy, but may require a different cutoff value.[93]

Serum potassium is an important stimulus for aldosterone synthesis and hypokalemia can inhibit aldosterone secretion.[82] Failure to correct hypokalemia can therefore lead to false-negative ARR values, although it is questionable whether this holds true in the context of severe PA with largely autonomous aldosterone production. In addition this, sodium intake also affects renin and aldosterone levels. A sodium restriction will stimulate renin levels, thereby decreasing the ARR.[94] It is therefore adviced that patients are on an unrestricted dietary salt intake when the ARR is assessed.[39]

Because of the diurnal and postural effects on aldosterone and renin levels, a standardized approach for blood sampling is needed to minimize variation.[77] The Endocrine Society guidelines recommend to perform blood sampling mid-morning after 5-15 minutes in sitting position in a previously ambulant patient. Interfering medication is preferably stopped, and if necessary replaced by an alpha-adrenergic blocker, non-dihydropyridine CCB and/or hydralazine.[39]

## Variability of the ARR

Even under standardized conditions, biological variability is considerable and even patients with a proven PA will occasionally have ARR values within normal ranges due to natural vari-

ability.[95] It was also shown that when the ARR is repeated in the same patient under the same conditions, the difference between the two readings can be as high as 10-fold.[96, 97] This variability will negatively affect the usefulness of the ARR as a screening test and repeated tests may be required to establish or exclude the diagnosis of PA.

### Alternative screening tests

The discussion about the validity of the ARR as a screening test has led to the development of alternative screening methods. Rossi *et al*.[98] constructed a logistic multivariate model in which the probability of PA is calculated based on parameters such as PRA, serum potassium and plasma aldosterone. This approach was tested and further simplified to the (serum aldosterone)$^2$-to-PRA ratio by Seiler *et al*.[99] This study showed that these methods have a higher AUC on ROC analysis, but this approach has not been evaluated prospectively since.

## CONCLUSION

PA is a relatively frequent cause of hypertension, with prevalences ranging up to more than 20%, depending on the population subjected to screening. The recent Endocrine Society clinical practice guideline[39] provides a clear protocol for the diagnostic workup. The ARR is the screening test of choice. However, this test is not undisputed because of the many factors it is influenced by and concerns about its reproducibility. Diagnosing PA as a cause of hypertension is important. First, because patients with PA have more cardiovascular events than patients with essential hypertension, independent of blood pressure, stressing the need for early detection to prevent complications.[100] Second, because specific treatment is available: adrenalectomy in case of an adrenal adenoma and treatment with an aldosterone-receptor antagonist in case of bilateral adrenal hyperplasia.[43, 101] Additional studies are needed to evaluate whether the ARR really is a reliable screening test for PA.

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# Chapter 5

## Test characteristics of the aldosterone-to-renin ratio as a screening test for primary aldosteronism

Pieter M. Jansen[1], Bert-Jan H. van den Born[2], Wijnanda J. Frenkel[2], Emile L.E. de Bruijne[3], Jaap Deinum[4], Michiel N. Kerstens[5], Yvo M. Smulders[6], Arend Jan Woittiez[7], Johanna A.M. Wijbenga[8], Robert Zietse[1], A.H. Jan Danser[1], and Anton H. van den Meiracker[1].

[1] *Erasmus Medical Centre, Department of Internal Medicine, Rotterdam, The Netherlands*
[2] *Academic Medical Centre, Department of Internal Medicine, Amsterdam, The Netherlands*
[3] *Red Cross Hospital, Department of Internal Medicine, Beverwijk, The Netherlands*
[4] *Radboud University Nijmegen Medical Centre, Department of Internal Medicine, Nijmegen, The Netherlands*
[5] *University of Groningen, University Medical Centre Groningen, Department of Endocrinology, Groningen, The Netherlands*
[6] *Vrije Universiteit Medical Centre, Department of Internal Medicine, Amsterdam, The Netherlands*
[7] *Ziekenhuis Groep Twente, Department of Internal Medicine, Almelo, The Netherlands*
[8] *Vlietland Hospital, Department of Internal Medicine, Schiedam, The Netherlands*

Submitted

## ABSTRACT

*Background:* The aldosterone-to-renin ratio (ARR) is a widely used screening test for primary aldosteronism (PA). Current guidelines recommend a cutoff value of 91 pmol/mU. Studies on its sensitivity, specificity, reproducibility and the role of medication have been conflicting. We prospectively assessed the test characteristics of the ARR and the effect of combination antihypertensive treatment.

*Methods:* In 178 patients with persistent hypertension despite the use of at least 2 antihypertensives, plasma renin and aldosterone were assessed twice within an interval of 4 weeks. All patients underwent an intravenous salt loading test. A post-test plasma aldosterone exceeding 235 pmol/L was considered diagnostic for PA. ARR was repeated after 4 weeks of standardized treatment with a calcium channel blocker and/or α-adrenergic-receptor blocker.

*Results:* The prevalence of PA was 15.2 %. The median ARR was 35.0 (interquartile range 16.2-82.0) in PA versus 7.1 (2.2-17.5) pmol/mU in essential hypertensives ($p<0.001$). Under random medication, the ARR had 22.2 % sensitivity and 98.7 % specificity. On standardized treatment, the ARR rose from 9.6 (2.5-24.8) to 21.4 (10.8-52.1) ($p<0.001$). Multivariate regression showed that ACE-inhibitors and angiotensin II-receptor blockers were responsible for the lower ARR during random treatment. The area under the receiver operating characteristic curve was, however, similar under random and standardized treatment (84 vs 86 %, respectively, $p=0.314$). Bland-Altman plots showed an almost 5-fold difference in ARR values taken under the same conditions.

*Conclusion:* ARR sensitivity for PA is low when the recommended cutoff is used. Reproducibility is also poor, stressing the need for alternative screening tests.

## INTRODUCTION

Since the introduction of the aldosterone-to-renin ratio (ARR),[1] the diagnosis of primary aldosteronism (PA) has increased dramatically.[2] PA is now considered to be the most prevalent cause of secondary hypertension with reported prevalences between 3.2 and 20 %.[3-15] The Endocrine Society recommends screening of patients with Joint National Commission stage 2, stage 3 or resistant hypertension, hypertension and spontaneous or diuretic-induced hypokalemia, hypertension with an adrenal incidentaloma, or hypertension and a family history of early-onset hypertension, stroke at a young age or a first-degree relative with PA.[16] The ARR is the screening test of choice with a recommended cutoff value of 91 pmol/mU.[16] However, the diagnostic workup for PA remains a challenge and several factors are known to influence the ARR, including posture,[17] time of blood sampling,[17, 18] age,[19, 20] salt intake,[20, 21] presence of hypokalemia,[22] sex[23] and use of anticonceptives,[23-25] nonsteroidal anti-inflammatory drugs[26] and certain selective serotonin reuptake inhibitors.[27] Most antihypertensive drugs also affect the ARR,[28] and it is generally accepted that β-adrenergic receptor blocking agents (BBs) and centrally-acting antihypertensives, related to their renin suppressive effect, can lead to falsely elevated ARR levels.[28-31] In contrast, angiotensin-converting enzyme inhibitors (ACE-Is) and angiotensin II type 1-receptor blockers (ARBs) lead to a decrease in ARR.[28, 30] Diuretics, including potassium-sparing, are also thought to lower the ARR,[16, 31, 32] although we recently observed a small and borderline significant increase in ARR in essential hypertension (EH) patients treated with eplerenone.[33] The current guidelines recommend to assess the ARR under an α-adrenergic-receptor blocker (AB), hydralazine and/or a calcium channel blocker (CCB), preferably of the non-dihydropyridine subclass, since these drugs have little or no effect on the ARR.[16] The role of dihydropyridine CCBs is unclear. In theory, these agents cause a decrease in ARR via an increase in renin levels through reflex sympathetic mechanisms and a decrease in aldosterone levels through inhibition of calcium-dependent adrenal aldosterone production.[28, 34] These effects are probably negligible under stable treatment and no effect of dihydropyridine CCBs on the ARR was found in a cross-sectional study.[30] Despite the known effects of individual antihypertensives on the ARR, their clinical relevance when used in combination treatment is less clear and whether patients on combination treatment should be switched to a standardized regime before assessment of the ARR is debated.[35] Gallay *et al.*[35] concluded that the ARR remains a valid screening test when antihypertensive medications are continued but their study design did not allow for a proper assessment of sensitivity and specificity. Another study showed that in combination treatment the ARR is mainly affected by BBs and ACE-Is or ARBs.[30] A different cutoff value for the ARR may be needed when taken under antihypertensive treatment.[36]

Although the ARR is widely used, studies regarding its sensitivity and specificity have been inconsistent. Sensitivities from 66% to 100% and specificities from 61% to 100% have been reported.[11, 37-43] These wide ranges can be explained by differences in cutoff values, laboratory

assays, study population and sampling conditions. Also, many studies have methodological limitations.[44]

In addition to a high sensititivity and specificity, a screening test should be reproducible. Unfortunately, there is a wide spontaneous variation in plasma renin (PRC) and aldosterone concentrations (PAC), even in patients with PA.[45] This may imply that a single ARR is inadequate to confirm or exclude the diagnosis of PA.[45]

Our aim was to assess the test characteristics of the ARR in patients with difficult-to-control hypertension and to evaluate the effect of combination antihypertensive treatment.

## METHODS

### Patients

In this multicenter study, patients aged 18 to 65 years with an office systolic BP (SBP) above 140 mmHg and/or diastolic BP (DBP) above 90 mmHg despite the use of at least two antihypertensive drugs were invited to participate. Patients were excluded if a known cause of hypertension was present, if they suffered from cardiac chest pain or heart failure and in case of a cerebro- or cardiovascular event within 6 months before study entry or pregnancy.

### Design

BBs and potassium-sparing diuretics were discontinued at least 4 weeks before start of the study protocol. All other antihypertensive agents were continued. When hypokalaemia was present, oral potassium replacement was started. PRC and PAC were assessed twice within an interval of 2 to 4 weeks. At the second visit, all patients also underwent an intravenous salt loading test (SLT) consisting of an infusion of 2 litres of NaCl 0.9 % in 4 hours. Patients with a post-infusion PAC exceeding 235 pmol/L were considered to have PA.[46] Subsequently, the random antihypertensive medication (RM) regime was replaced by a standardized medication regime (SM) consisting of a combination of a CCB (in most cases amlodipine 5 or 10 mg q.d.) and doxazosin 4 or 8 mg q.d. depending on BP levels. After 4 to 6 weeks on this combination of drugs, PRC and PAC measurements were repeated. Patients with a positive SLT underwent a computed tomography (CT) scan of the adrenals to screen for the presence of an aldosterone-producing adenoma (APA).

The study was approved by the Institutional Review Board and Ethical Committee of the Erasmus MC in Rotterdam and has been registered at www.clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT00407784). All patients gave written informed consent to participate.

## Clinical and biochemical measurements

At baseline, clinical data, height and weight, and number and types of antihypertensive drugs were recorded. Patients were screened for the presence of left ventricular hypertrophy (LVH) using a standard 12-lead electrocardiogram (EKG) based on the Sokolow-Lyon criteria.[47] BP was measured at all visits as 3 subsequent measurements after at least 5 minutes in sitting position with a validated automatic BP measurement device. The mean value of the last two readings was used for the analysis.

At all visits, serum sodium, potassium, urea and creatinine levels were measured using locally available routine laboratory techniques. In addition, 24-hour urinary samples were collected for determination of sodium, potassium and creatinine. PRC and PAC were determined by drawing blood samples in EDTA plasma by venipuncture between 8 and 10 am after 10 minutes in sitting position. The samples were centrifuged at room temperature for 10 minutes at 3000g. The plasma was then collected and stored at -20°C until analysis. PAC was measured with a radioimmunoassay (Coat-a-Count, Diagnostics Product Corporation, LA, CA, USA). This assay has a detection limit of 30.5 pmol/L (11 pg/ml) and a coefficient of variance (CV) of 8.4%. PRC was assessed using an immunoradiometric assay (Renin III, Cisbio, Gif-sur-Yvette, France) with a detection limit of 2.02 mU/L and a CV of 7.2%. All PAC and PRC measurements were performed at the hypertension research laboratory of the Erasmus MC.

## Statistical analysis

Values are expressed as mean ± SD, or as median and interquartile range (IQR) when not normally distributed. Categorical values are reported as percentages.

Medication use was quantified by adding up the total number of different antihypertensive drugs, as well as by assessing the defined daily dose (DDD) per drug and for total drug use according to the World Health Organization Anatomical Therapeutic Chemical (ATC) index.[48] Antihypertensive agents were grouped in the following categories: a. diuretics, b. renin-angiotensin-system (RAS) blockers (*i.e.* ACE-Is, ARBs, and renin inhibitors), c. CCBs, d. BBs, e. ABs, and f. other antihypertensive drugs.

Differences between two groups were tested with the student's t-test or the Wilcoxon-signed rank test when not normally distributed. Sensitivity and 1-specificity for different cutoff values of the ARR were plotted in Receiver Operating Characteristic (ROC) curves. Areas under the curve (AUC) were compared using a nonparametric approach.[49] The optimal cutoff points were identified using Youden's J statistic and by assessing the cutoff value at which 95% sensitivity was reached.

To evaluate the reproducibility of the ARR, the values of the first two visits were subjected to natural logarithmic transformation (LnARR1 and LnARR2). The differences between LnARR1 and LnARR2 were plotted against the mean of these values in Bland-Altman plots. The 95%

limits of agreement of these differences were calculated and expressed as the ratio between ARR1 and ARR2 by reversing the Ln-transformation.

The effect of individual medication groups on the ARR was assessed in a multivariate linear regression model with the relative change in ARR after medication change from RM to SM as dependent variable after natural logarithmic transformation ((Ln($ARR_{SM}$/$ARR_{RM}$)). The main classes of antihypertensive medication, the presence or absence of PA, age and sex were included as covariates.

Analyses were performed in SPSS Statistics 20 for MacOS X (IBM, Armonk, NY, USA) and Graphpad Prism 5.02 for Windows (GraphPad Software Inc., La Jolla, CA, USA). ROC curve analyses were performed in MedCalc 12.3.0.0 for Windows (MedCalc Software, Mariakerke, Belgium).

## RESULTS

### Patients

A total number of 186 patients was included in the study. Six patients were excluded from the analysis because their BB or potassium sparing diuretic was not stopped after inclusion. Two patients were lost to follow-up before a SLT could be performed. In total, data of 178 patients from 13 hospitals was available for analysis. Of these patients, 27 had a positive SLT (PA) and 151 had a negative SLT (EH). This resulted in a 15.2 % prevalence of PA in our population. Thirteen of the 27 PA patients had evidence of an adrenal adenoma on the CT-scan.

Table 1 shows the baseline characteristics of patients with EH and PA. SBP and DBP were similar in both patient groups, as was the median number of antihypertensive drugs. Medication use expressed in DDD tended to be lower in PA patients (p=0.084). The most frequently used treatment combinations consisted of diuretics, RAS blockers and CCBs (40 %), diuretics and RAS blockers (14 %), RAS blockers and CCBs (13 %), and diuretics, RAS blockers, CCBs and ABs (12 %).

There were no differences in age, sex, BMI and percentage of diabetics between EH and PA groups. Also the proportion of patients with previous cardiovascular complications was similar, but the albumin-to-creatinine ratio was higher in patients with PA (2.09 vs 1.19 mg/mmol, p=0.035). Patients with PA had a higher serum $Na^+$ and lower serum $K^+$ than patients with EH. A higher proportion of PA patients was hypokalemic or on potassium replacement. As expected, PRC was lower, and the PAC and ARR were higher in patients with PA compared to EH.

**Table 1.** Baseline characteristics of patients with essential hypertension (EH) and primary aldosteronism (PA).

| | EH (n=151) | PA (n=27) | p-value |
|---|---|---|---|
| **Age (years)** | 49.9 ± 9.7 | 47.6 ± 9.4 | 0.256 |
| **Males (%)** | 53.0 | 55.6 | 0.805 |
| **BMI (kg/m²)** | 28.7 (26.3-31.7) | 29.0 (24.7-32.0) | 0.826 |
| **Caucasians (%)** | 67.5 | 70.4 | 0.772 |
| **Smokers (%)** | 30.2 | 23.1 | 0.461 |
| **History of CVD (%)** | 10.7 | 3.8 | 0.274 |
| **DM (%)** | 11.9 | 7.4 | 0.494 |
| **LVH (%)** | 18.2 | 28.0 | 0.260 |
| **ACR (mg/mmol)** | 1.2 (0.5-2.9) | 2.1 (1.1-5.7) | 0.035 |
| **Duration of HT (months)** | 261 (94-699) | 261 (87-524) | 0.567 |
| **SBP (mmHg)** | 155.8 ± 21.4 | 159.0 ± 15.9 | 0.452 |
| **DBP (mmHg)** | 94.4 ± 12.2 | 97.4 ± 11.4 | 0.241 |
| **pulse (BPM)** | 72.9 ± 11.7 | 68.2 ± 10.9 | 0.054 |
| **$Na^+$ (mmol/L)** | 141.9 ± 2.3 | 142.9 ± 2.2 | 0.030 |
| **$K^+$ (mmol/L)** | 3.9 ± 0.5 | 3.5 ± 0.5 | <0.001 |
| **Serum creatinine (µmol/L)** | 80.0 ± 19.1 | 82.6 ± 22.6 | 0.526 |
| **$Na^+$ excretion (mmol/24 hours)** | 184 (131-231) | 182 (147-219) | 0.947 |
| **Hypokalemia (%)** | 15.9 | 48.1 | <0.001 |
| **K-suppletion (%)** | 2.7 | 23.1 | <0.001 |
| **TTKG** | 6.1 (4.8-7.7) | 7.3 (5.6-9.5) | 0.032 |
| **PRC (mU/L)** | 27.2 (13.6-87.5) | 16.4 (8.6-28.6) | 0.003 |
| **PAC (pmol/L)** | 227 (158-380) | 565 (382-806) | <0.001 |
| **ARR (mmol/mU)** | 7.1 (2.2-17.5) | 35.0 (16.2-82.0) | <0.001 |
| **Nr of antihypertensives** | 3 (3-4) | 3 (2-3) | 0.028 |
| **DDD** | 4.5 (3.3-6.0) | 3.5 (3.0-6.0) | 0.084 |
| **Diuretics (%)** | 80.8 | 66.7 | 0.099 |
| **RAS blockers (%)** | 88.7 | 77.8 | 0.118 |
| **CCB (%)** | 74.8 | 77.8 | 0.744 |
| **BB (%)** | 43.7 | 29.6 | 0.172 |
| **Alpha-adrenergic blockers (%)** | 16.6 | 25.9 | 0.243 |
| **Other α-HT (%)** | 3.3 | 0.0 | 0.338 |

Data presented are mean ± SD or median (interquartile range).

BMI, body mass index; CVD, cardiovascular disease; DM, diabetes mellitus; LVH, left ventricular hypertrophy; ACR, albumin-creatinine ratio; HT, hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; BPM, beats per minute; $Na^+$, serum sodium; $K^+$, serum potassium; TTKG, transtubular potassium gradient; PRC, plasma renin concentration; PAC, plasma aldosterone concentration; ARR, aldosterone-to-renin ratio; DDD, defined daily dose; RAS, renin-angiotensin-system; CCB, calcium channel blocker; BB, beta-adrenergic blocker; α-HT, antihypertensives

## Sensitivity and specificity of the ARR

Median PRC was 27.2 (IQR 13.6-87.5) mU/L in EH and 16.4 (8.6-28.6) mU/L in PA (p=0.003). Median PAC was 227 (158-380) pmol/L) in EH compared to 565 (371-725) pmol/L in PA (p<0.0001). As a consequence, the ARR was higher in PA than in EH patients (35.0 versus 7.1 pmol/mU, p<0.001; figure 1). PA patients with an APA on CT-scan tended to have a lower PRC and higher PAC than patients without an APA, whereas the ARR was significantly higher in patients with an APA (median ARR 53.4 versus 22.4 pmol/mU, p=0.024; figure 2).

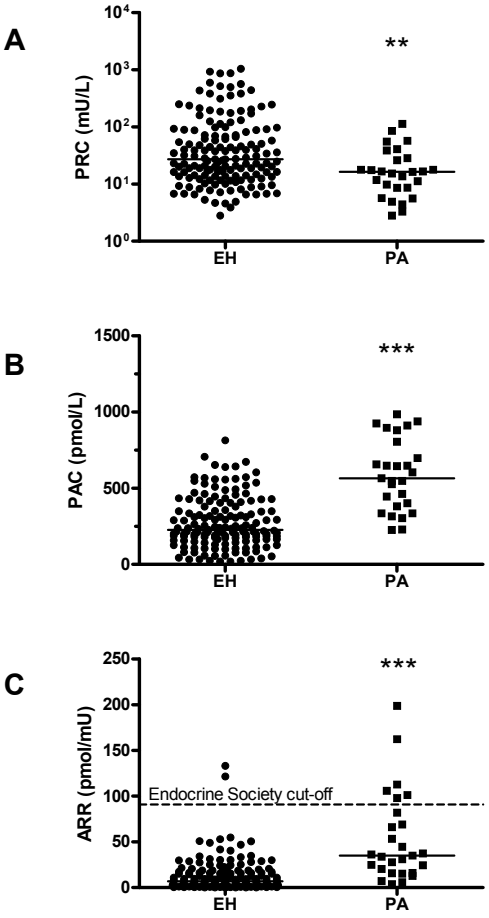


**Figure 1.** Plasma renin concentration (PRC, panel A), plasma aldosterone concentration (PAC, panel B) and aldosterone-to-renin ratio (ARR, panel C) in patients with essential hypertension (EH) and primary aldosteronism (PA) using random medication. The dashed line in panel C represents the Endocrine Society cutoff value of 91 pmol/mU.

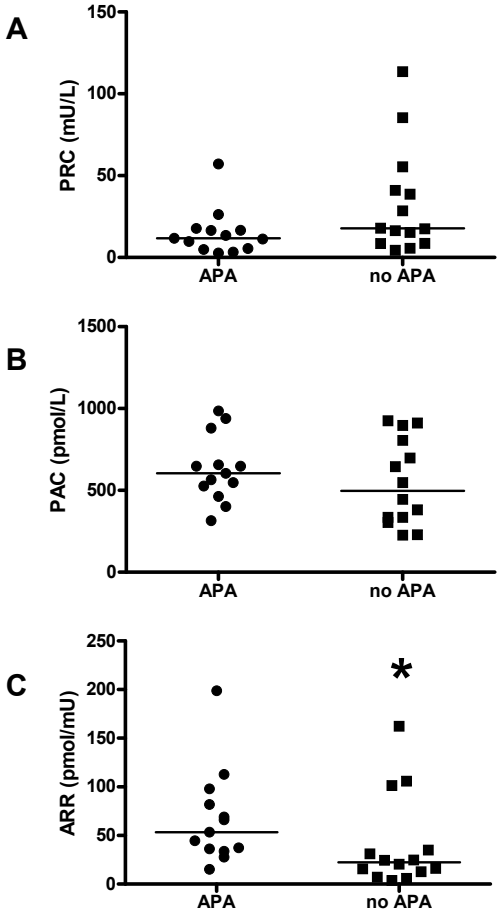


**Figure 2.** Plasma renin concentration (PRC, panel A), plasma aldosterone concentration (PAC, panel B) and aldosterone-to-renin ratio (ARR) in primary aldosterone patients with (APA) or without (no APA) an aldosterone-producing adenoma on computed tomography scanning.

When a cutoff value for the ARR of 91 pmol/mU was used, only 6 patients with PA demonstrated an elevated ARR resulting in a sensitivity of 22.2 %. Two EH patients had a positive ARR resulting in a specificity of 98.7 % (figure 1). The positive predictive value (PPV) of the ARR at this cutoff is 75.0 % and the negative predictive value (NPV) 84.8 % in our population. The positive likelihood ratio (LR) is 17.1 and the negative LR 0.79. Figure 3 shows the ROC curve for the ARR. The area under the curve (AUC) is 0.85 (SE 0.04, $p<0.0001$). The optimal cutoff point according to Youden's J statistic is an ARR of 24.0 (95 % CI 12.4-34.9) pmol/mU, resulting in a sensitivity of 70.4 % and a specificity of 83.4 % (Youden's J Index 0.54 (95% confidence interval (CI) 0.36-0.64)). However, for a screening test a high sensitivity is the most important

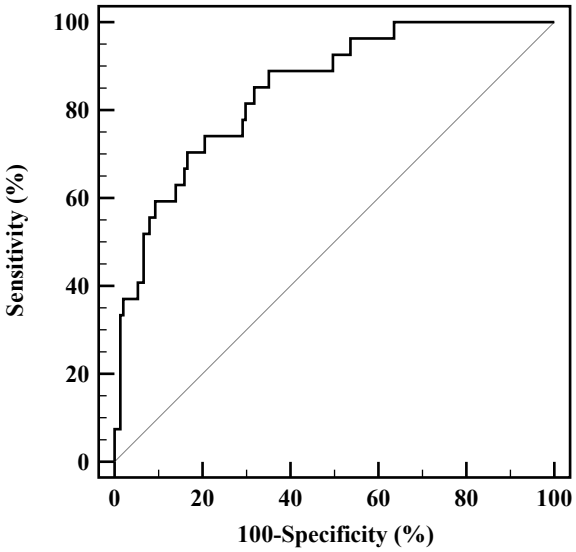


**Figure 3.** Receiver Operating Characteristic curve for the aldosterone-to-renin ratio under a random antihypertensive medication regime. The area under the curve is 0.85 (SE 0.04, p<0.0001).

feature. To achieve 95 % sensitivity the cutoff value of the ARR should be further lowered to 5.6 pmol/mU at the cost of a significant decrease in specificity to 46.9 % (95 % CI 29.9-70.8 %).

Due to the study design, patients could be on any type of antihypertensive medication, with the exception of BBs and potassium-sparing diuretics, during the SLT and this may have affected the outcome of the confirmation test. Nine patients (4 with EH and 5 with PA) were on "ARR-neutral" medication when the SLT was performed. Their clinical and biochemical profile is shown in table 2. Of the 5 patients with PA only 1 had an ARR above 91 pmol/mU. All three patients with an APA had ARR levels below this cutoff value.

## The effect of medication on the ARR

After the diagnostic phase, which included assessment of the ARR and performance of the SLT, the random antihypertensive medication (RM) was changed to a standardized medication (SM) regime consisting of doxazosine and a CCB, in most cases amlodipine. After 4-6 weeks on this antihypertensive regime, the ARR was repeated. Of the total population of 178 patients, this phase could be completed in 145 patients (126 patients with EH and 19 with PA). The main reasons for not completing this phase was that the BP was or became too high to safely change the medication and the occurrence of side effects.

After changing from RM to SM, a significant drop in PRC was seen for the group as whole from 20.9 (11.7-72.1) to 12.4 (7.5-25.0) mU/L (p<0.0001), as well as for EH and PA patients separately (from 22.8 (12.2-88.5) to 13.7 (8.2-25.6) mU/L (p<0.0001) for EH and from 13.6

**Table 2.** Characteristics of patients on ARR-neutral medication during the intravenous salt loading test.

| Age (yrs) | Sex | APA | Antihypertensives | SBP (mmHg) | DBP (mmHg) | Na+ (mmol/L) | K+ (mmol/L) | Creatinine (µmol/L) | Na+-excretion (mmol/24 hours) | PRC (mU/L) | PAC (pmol/L) | ARR (pmol/mU) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Patients with essential hypertension*** | | | | | | | | | | | | |
| 61 | F | N/A | Nifedipine 30 mg<br>Doxazosine 4 mg | 146 | 87 | 142 | 3.8 | 59 | 186 | 9.1 | 219 | 24.0 |
| 52 | F | N/A | Nifedipine 60 mg twice daily<br>Doxazosine 8 mg twice daily | 133 | 87 | 144 | 3.0 | 52 | 196 | 11.7 | 643 | 54.9 |
| 64 | M | N/A | Nifedipine 30 mg<br>Doxazosine 4 mg twice daily | 161 | 95 | 145 | 2.9 | 82 | 171 | 9.3 | 471 | 50.6 |
| 65 | M | N/A | Barnidipine 10 mg | 157 | 116 | 139 | 3.9 | 84 | N/D | 8.9 | 194 | 21.8 |
| ***Patients with primary aldosteronism*** | | | | | | | | | | | | |
| 39 | M | No | Nifedipine 30 mg twice daily<br>Doxazosine 4 mg | 179 | 102 | 144 | 3.7 | 96 | 221 | 28.6 | 698 | 24.4 |
| 41 | F | Yes | Nifedipine 60 mg | 134 | 88 | 144 | 3.3 | 61 | 140 | 16.7 | 565 | 33.8 |
| 40 | F | Yes | Nifedipine 60 mg<br>Doxazosine 8 mg | 137 | 86 | 144 | 3.4 | 56 | 183 | 17.8 | 648 | 36.4 |
| 57 | M | No | Amlodipine 10 mg<br>Doxazosine 8 mg | 174 | 76 | 142 | 3.4 | 96 | 200 | 8.6 | 911 | 106.0 |
| 48 | M | Yes | Amlodipine 10 mg | 144 | 97 | 142 | 3.2 | 72 | 181 | 9.8 | 648 | 66.1 |

APA, aldosterone producing adenoma on computed tomography scan; SBP, systolic blood pressure; DBP, diastolic blood pressure; $Na^+$, serum sodium; $K^+$, serum potassium; PRC, plasma renin concentration; PAC, plasma aldosterone concentration; ARR, aldosterone-to-renin ratio; N/A, not applicable; N/D, not done.

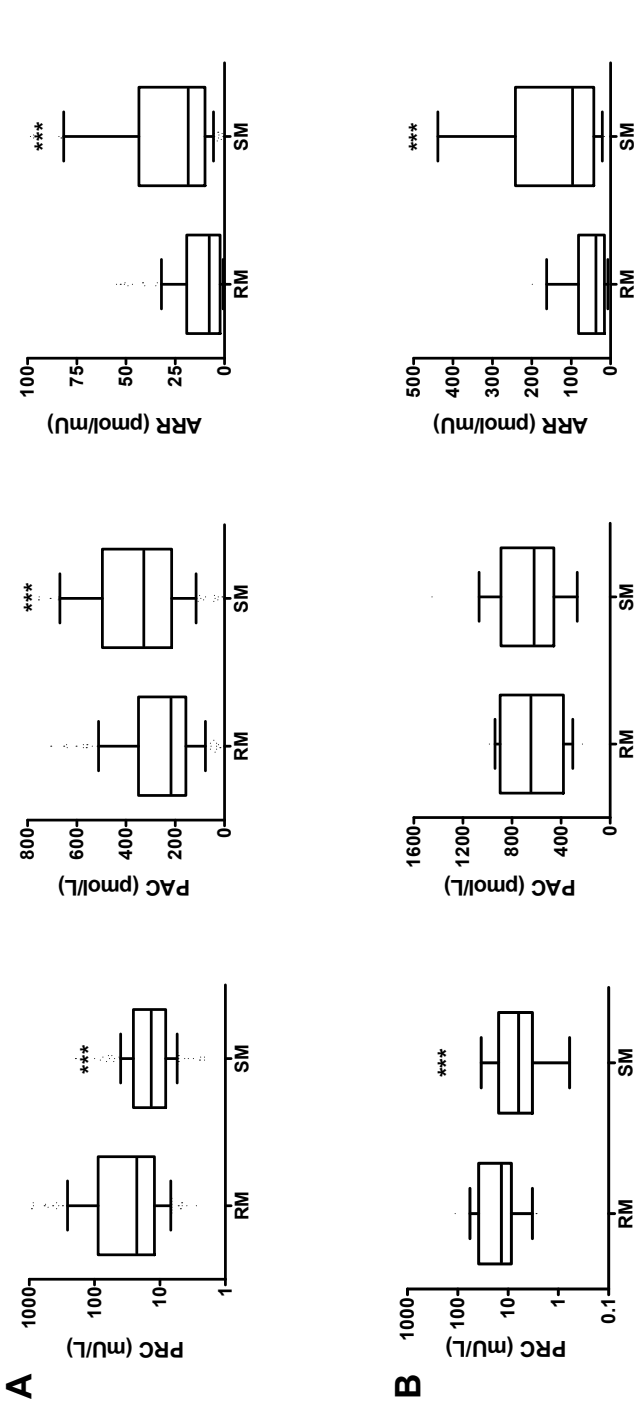


**Figure 4.** Plasma renin concentration (PRC), plasma aldosterone concentration (PAC) and aldosterone-to-renin ratio (ARR) on random medication (RM) and after switching to a standardized medication (SM) regime in patients with essential hypertension (panel A) and primary aldosteronism (panel B). The box-and-whisker plots represent the median, IQR and the 10th–90th percentile.

**Table 3.** Multivariate regression analysis with the natural logarithm of the relative rise in ARR after changing from random medication (RM) to standardized medication (SM) ($Ln(ARR_{SM}/ARR_{RM})$) as dependent variable.

| | Beta coefficient | SE | p-value |
|---|---|---|---|
| Age (years) | -0.018 | 0.009 | 0.045 |
| Sex (0=F, 1=M) | 0.238 | 0.173 | 0.171 |
| PA (0=no, 1=yes) | -0.176 | 0.252 | 0.486 |
| Diuretics | -0.038 | 0.201 | 0.851 |
| RAS blockers | 1.053 | 0.284 | <0.001 |
| CCB | 0.273 | 0.243 | 0.263 |
| Alphablockers | 0.113 | 0.206 | 0.585 |

SE, standard error; PA, primary aldosteronism; RAS, renin-angiotensin-system; CCB, calcium channel blockers.

(8.6-38.7) to 6.2 (3.3-15.6) (p=0.0002) for PA; figure 4). PAC rose significantly in EH (from 217 (157-350) to 328 (215-497) pmol/L, p<0.0001) but remained unchanged in PA patients (645 (382-898) pmol/L on RM and 621 (460-892) pmol/L on SM, p=0.260). The ARR was higher on SM than RM in both EH (increase from 7.8 (2.3-19.2) to 18.5 (10.0-43.6) pmol/mU; p<0.0001) and in PA patients (increase from 37.5 (16.2-82.0) to 97.0 (42.6-242.0) pmol/mU; p<0.0001) (figure 4). The effect of the individual medication classes on the change in ARR was tested in a multivariate regression model with $Ln(ARR_{SM}/ARR_{RM})$ as dependent variable. Of the main medication groups, only RAS-blockers significantly affected the change in ARR after switch-



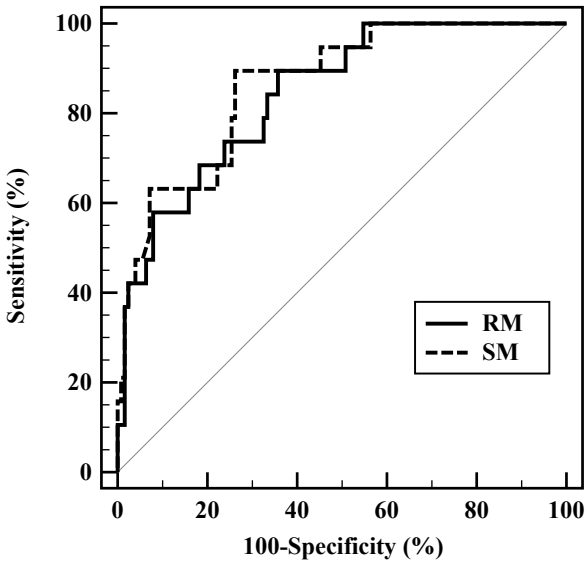


**Figure 5.** Receiver Operating Characteristic curve for the ARR taken under a random antihypertensive medication regime (RM) and under standardized medication (SM) with a calcium channel blocker and doxazosine. The area under the curve (AUC) is 0.84 (SE 0.04) for RM and 0.86 (SE 0.04) for SM (p=0.31).

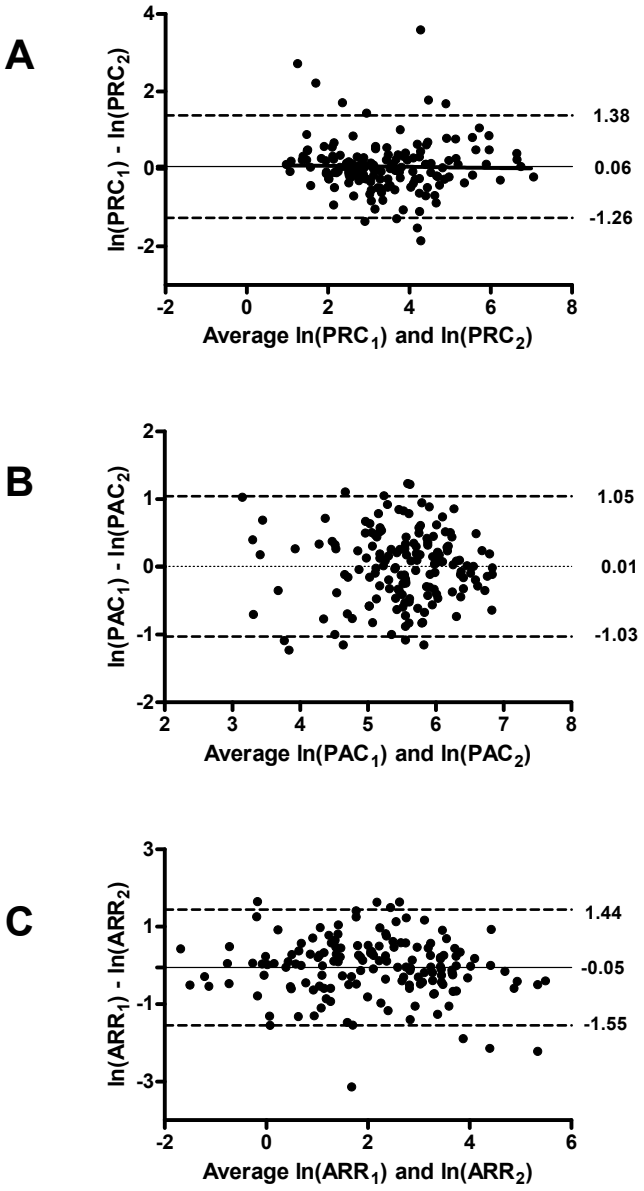


**Figure 6.** Bland-Altman plots for plasma renin concentration (PRC, panel A), plasma aldosterone concentration (PAC, panel B) and aldosterone-to-renin ratio (ARR, panel C) on 2 subsequent visits after natural logarithmic transformation. The dashed lines represent the 95 % limits of agreement.

ing to SM (table 3). Age had a negative association with the rise in ARR in our model. The presence of PA did not affect the change in ARR. Serum potassium had no association with the change in ARR in a univariate analysis (data not shown) and was not included in the model. Figure 5 shows the ROC curves for these 145 patients during both RM and SM. The AUC for both curves was of similar magnitude (84.3 % (SE 4.5 %) for RM versus 86.3 % (SE 4.2 %) for SM (p=0.31)). In this subgroup, the optimal ARR cutoff according to the highest Youden's Index was 15.2 (95 % CI 4.9-20.0) pmol/mU on RM (Youden Index J 0.54 (95 % CI 0.37 – 0.64); sensitivity 89.5 %, specificity 64.3 %) and 40.2 (95 % CI 16.1 – 81.6) pmol/mU on SM (Youden Index J 0.63 (95 % CI 0.44 – 0.75); sensitivity 89.5 %, specificity 73.8 %). When aiming for a sensitivity of 95 % the cutoff value should be lowered to 5.8 pmol/mU on RM (associated specificity 45.2 % (95 % CI 34.9 – 57.3 %)) and 16.3 pmol/mU on SM (associated specificity 43.7 % (95 % CI 32.4 – 57.9 %)).

### Reproducibility

PRC, PAC and the ARR were assessed on two subsequent visits with an interval of 2 to 4 weeks. The antihypertensive medications and the conditions of sampling were the same on both occasions. A total number of 158 patients was available for this analysis (133 EH and 25 PA patients). Six patients (5 PA and 1 EH) had an ARR above 91 pmol/mU at the first visit and 8 patients (7 PA and 1 EH) at the second. Nine patients (7 PA and 2 EH) had at least one elevated ARR. Figure 6 shows Bland-Altman plots for ln(PRC), ln(PAC) and ln(ARR). The 95 % limits of agreement are shown in the figures. The lower limit is -1.55 (95 % CI -1.57 to -1.53) corresponding to an ARR1/ARR2 ratio of 0.21 (0.21-0.22). The upper limit is 1.44 (95 % CI 1.42 to 1.47) corresponding to an ARR1/ARR2 ratio of 4.24 (4.15-4.33). This indicates that the difference between the two readings can range up to almost 5-fold.

## DISCUSSION

The ARR is the standard screening test for PA. However, data on its sensitivity, specificity and reproducibility have been conflicting. Whether the antihypertensive treatment should be standardized before performing the ARR has remained a subject of debate.

The results shown in this study indicate that the sensitivity of the ARR under a random medication regime, but with the exclusion of BBs and/or potassium-sparing diuretics, is low at a cutoff value of 91 pmol/mU as recommended by the Endocrine Society guideline.[16] Lowering the cutoff value improves sensitivity, although at a considerable loss of specificity.

The sensitivity and specificity of the ARR have been studied by others[11, 15, 37, 38, 40-43, 50, 51] yielding markedly variable findings. Sensitivities between 66 and 100 % and specificities between 61

and 96 % have been reported. Comparing the findings of the various studies is confounded by factors such as differences in cutoff values, laboratory assays (PRA versus PRC), the conditions of testing and the confirmation tests used. Many studies suffer from verification bias, as usually only patients with an ARR above the predefined cutoff value were subjected to confirmatory testing. Such an approach is likely to result in an overestimation of diagnostic performance.[44] The same problem may occur in retrospective studies where patients and controls are often selected based on a diagnostic test which at the same time is the subject of evaluation. In our study, both the screening and the confirmation test were performed in all patients with difficult to control hypertension in a prospective manner allowing an unbiased evaluation of the diagnostic performance of the ARR.

For an optimal evaluation of a screening test, the gold standard should be reliable. A limitation in our design is that the SLT was performed during a random medication regime, which could include diuretics and RAS blockers. The Endocrine Society guideline recommends to perform the SLT under ARR-neutral medication.[16] Although it is reasonable to assume that antihypertensives (diuretics and RAS blockers in particular) have an effect on the outcome of the SLT, this has never been systematically studied. Our protocol reflects clinical reality where it is often more feasible and practical to continue ongoing antihypertensive treatment during confirmation testing. In our study, nine patients (5 with PA and 4 with EH) were on a CCB and α-adrenergic receptor blocker when the SLT was performed and their clinical profile and ARR values are reported individually. Although the generalizability of these data is limited, they suggest that even under optimal conditions only a minority of the PA patients has an elevated ARR. Notably, three of these patients had an APA that would have been missed if the ARR cutoff value of 91 pmol/mU had been used.

Another point of debate is the cutoff value for a positive SLT, which was 235 pmol/L in our study.[46] Unfortunately, the optimal SLT cutoff value has never been unequivocally defined. The guideline[16] states that a postinfusion PAC above 277 pmol/L (100 pg/mL) makes the diagnosis of PA very likely and below 139 pmol/L (50 pg/mL) unlikely. A postinfusion PAC between these values is indeterminate. However, the literature on this is limited and local protocols have their own cutoff values.[16] A postinfusion PAC of 139 pmol/L was shown to be the optimal cutoff by some[52, 53] with a sensitivity of 88 % compared to the fludrocortisone suppression test (FST).[53] On the other hand, Streeten *et al.*[46] found 235 pmol/L to be the optimal cutoff with 77 % sensitivity compared to the FST although not all patients were subjected to the FST. Our protocol required a single cutoff value and, although arbitrary, in our view the chosen cutoff provides a fair compromise of all available data and has been used by others.[14, 46, 50] When a post-infusion PAC cutoff of 277 pmol/L would have been used, the prevalence of PA would have been 12.4 % in our population. Six PA patients and 2 EH patients would have had an ARR above 91 pmol/mU resulting in a sensitivity of 27.3 % and a specificity of 98.7 %.

Therefore, a more stringent criterion for the SLT would not have altered the outcome of the study significantly.

It is well-known that antihypertensive medication has a major impact on the ARR. The effects of individual antihypertensive drugs on renin and aldosterone levels have been discussed by others,[28-30, 32] but the relevance of combination treatment has been debated.[35] In our study, the ARR was lower during random combination antihypertensive treatment than during standardized treatment with a CCB and α-adrenergic receptor blocker. A multivariate analysis pointed out that RAS blockers in the treatment regime were responsible for the lower ARR during non-standardized treatment. This is not an unexpected finding since RAS blockers increase the PRC to a greater extent than that they lower the PAC, resulting in a higher ARR. Contrary to the decrease in PRC, PAC did not rise in PA after the medication change, likely reflecting the autonomy of aldosterone production in these patients. It should be noted that BBs and potassium-sparing diuretics were ceased prior to the start of the study protocol. Therefore, no conclusion can be made regarding the contribution of these agents. BBs are known to lower renin levels and to increase the ARR both as monotherapy[28, 29] and as part of combination treatment.[30] With this in mind our results are in agreement with the observations by Seifarth *et al*.,[30] showing that in combination treatment both BBs and ACE-I/ARBs had the largest influence on the ARR. Despite the observed change in ARR, the AUC under the ROC curve remained unchanged, indicating that the standardized treatment regime did not result in an overall improvement of the test performance, which is in line with a previous study.[43] It can therefore be concluded that combination treatment does not need to be stopped but requires a lower ARR cutoff value as has been suggested by others.[36] Many other drugs including NSAIDs,[26] certain SSRIs[27] and oral contraceptives[23-25] can influence the ARR. The use of these drugs was not systematically recorded in this study and could have had a small effect on the test results.

Most of our PA patients had a normal ARR mainly because their PRC was not or only marginally suppressed. This can be explained in part by medication use but even under standardized conditions many of these patients had relatively high PRC levels. Therefore, additional explanations for these higher than expected PRC levels need to be considered. In our study, the ARR was based on PRC and not on PRA. Many of the earlier studies reporting on the ARR have used the PRA. The assessment of the PRA has disadvantages, being time-consuming, dependent on endogenous angiotensinogen levels and difficult to standardize.[54] Use of the PRC allows for standardization of the test procedure and comparison with international reference values. Studies have shown that this can be a reasonable alternative for the PRA,[55-58] although the PRC may be less sensitive in the very low range.[54, 58] The assay used here has a detection limit of 2.02 mU/L, which should enable detection of suppressed levels of PRC. Cryoactivation of prorenin (allowing its detection in a renin assay) can be a cause for falsely elevated PRC levels,[59] particularly at low PRC levels. Incubation of samples at 37°C (like during a PRA measurement) facilitates the return of prorenin to its closed, inactive conformation

so that it can no longer display activity nor will be detected in a renin assay. Theoretically therefore, cryoactivation is expected to affect PRA to a lesser degree than PRC. Nevertheless, in our samples, PRC levels were only rarely close to the detection limit of the assay (Figure 1A). Moreover, to prevent prorenin cryoactivation, samples were always processed at room temperature and the plasma was rapidly frozen until analysis. Furthermore, all PRC as well as PAC measurements were performed in one laboratory with extensive experience in performing these measurements.

Salt intake is a strong determinant of RAS activity and low salt intake can lead to a marked rise in renin.[60] Although salt intake was not actively controlled for, it was generally high in our population and therefore it is highly unlikely that the sometimes high PRC values can be explained by a low salt intake. It has also been suggested that the diagnosis of PA can be masked if hypertensive kidney damage is present, manifested by renal arteriolosclerosis leading to a rise in renin levels.[61] We selected patients with a relatively normal creatinine clearance to exclude the possibility of hypertension caused by renal disease. Nonetheless, subclinical renal damage may have contributed to the relatively high PRC levels in some of our PA patients.

Another factor to take into account are serum potassium levels. Potassium by itself can stimulate aldosterone secretion, while on the other hand hypokalemia can inhibit aldosterone secretion, leading to lower PAC levels.[22] Failure to correct hypokalemia can therefore lead to falsely low ARR levels and an underdiagnosis of PA. Although we aimed to correct hypokalemia as much as possible, this was not successful in all patients. Twelve out of 27 PA patients had a serum potassium level below 3.5 mmol/l at the time of the first ARR measurement and 3 patients below 3.0 mmol/l. To correct hypokalemia completely in patients with severe PA can be a challenge. Since aldosterone production is largely autonomous in PA, it might be questioned whether hypokalemia will affect PAC to an extent that is clinically relevant. Indeed Tanabe *et al*.[45] observed an inverse relation between PAC and serum potassium concentration in PA, supporting the concept that serum potassium levels are determined by PAC in PA and not the other way around.

The question remains whether an elevated ARR is a good reflection of (relatively) autonomous aldosterone production. Montori *et al*.[32] showed that the ARR is mainly driven by renin and is therefore not a good indicator of inappropriately elevated aldosterone levels in relation to renin. On the other hand, it is debatable whether suppressed renin levels are a prerequisite for the diagnosis of PA. Previous studies, supported by the present findings, have shown that a significant number of PA patients has non-suppressed renin levels even in the presence of an APA.[62, 63] In addition to the various possibilities mentioned above, this may be related to individual differences in sensitivity and thresholds for RAS activation. Furthermore, the clinical picture of PA encompasses a broad spectrum with mild biochemical abnormalities on the low end and a florid phenotype with high aldosterone and markedly suppressed

renin levels on the high end. This study confirms that PA patients with an APA had on average lower PRC and higher ARR values than patients without evidence of an APA on their CT-scan.

PRC, PAC and ARR are subject to significant diurnal variations.[18] Sampling at a standardized time helps reducing variability in ARR levels.[17] Blood sampling was performed mid-morning in accordance with recommendations.[16] Nonetheless, there still can be considerable day-to-day variations with negative effects on reproducibility.[45, 64] Our data show that ARR levels can display an almost 5-fold difference when determined under the same conditions. The implication of this is that a single negative test cannot sufficiently rule out PA although it remains to be determined how many tests are needed.

## CONCLUSIONS

PA is the most prevalent cause of secondary hypertension but establishment of this diagnosis can be challenging. The ARR is the standard screening test for PA, but our data show that its sensitivity is poor when the recommended cutoff value is used even under a standardized medication regime. Overall test performance does not improve under a standardized treatment regime but requires a different cutoff value. Reproducibility is also low, stressing the need for multiple tests to establish the diagnosis. Despite these difficulties in screening, it remains clinically important to identify patients with PA, especially with surgically correctable forms. It is recommended to proceed quickly to a confirmation test when clinical suspicion is high.



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## CONFLICTS OF INTEREST

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# Part IV

## Aldosterone-receptor antagonism in hypertension

# Chapter 6

## Aldosterone-receptor antagonism in hypertension

Pieter M. Jansen[a], A.H. Jan Danser[a], Ben P. Imholz[b] and Anton H. van den Meiracker[a].

[a] *Division of Pharmacology, Vascular and Metabolic Diseases, Department of Internal Medicine, Erasmus Medical Centre Rotterdam, The Netherlands*

[b] *Department of Internal Medicine, Twee Steden Hospital, Waalwijk, The Netherlands.*

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## ABSTRACT

The role of the renin-angiotensin-aldosterone system (RAAS) in hypertension has since long been recognized and aldosterone has been acknowledged as one of the key hormones in the pathophysiology, not only in primary aldosteronism but also in essential hypertension and drug-resistant hypertension. Aldosterone-receptor antagonists (ARAs) are increasingly used in patients with resistant hypertension, often with impressive results. However, definitive evidence for the benefit of ARAs in these patients from randomized, controlled trials is lacking. This review gives an overview of the current data on this topic. Future studies should focus on the identification of factors that are able to predict the response to treatment, as to select patients who will benefit most from treatment with ARAs. On the basis of the current knowledge, we recommend prescription of ARAs to patients with primary aldosteronism, resistant hypertension and patients with hypertension and hypokalemia.

## INTRODUCTION

The role of aldosterone in the pathophysiology of hypertension has since long been recognized. This has particularly become clear in patients with primary aldosteronism. Recent studies, however, have shown that aldosterone is also involved in essential hypertension and aldosterone-receptor antagonists (ARAs) are increasingly used in these patients. This review discusses the role of aldosterone in hypertension and summarizes the evidence for the use of ARAs in hypertension, both as monotherapy and as add-on therapy in resistant hypertension.

## THE ROLE OF ALDOSTERONE IN HYPERTENSION

The role of aldosterone in blood pressure (BP) regulation is best illustrated in primary aldosteronism, a state characterized by an excess of aldosterone, with severe, often therapy-refractory, hypertension. Since the introduction of the aldosterone-to-renin ratio (ARR) as a screening test for primary aldosteronism, many groups have studied the prevalence of this condition in different subsets of hypertensive patients.[1-27] We recently performed a meta-analysis on the prevalence of primary aldosteronism. Our meta-analysis showed that 4.3 % of hypertensive patients in a primary care setting and 9.0% of referred patients have confirmed primary aldosteronism.[28] Since many primary aldosteronism patients present without hypokalemia and screening for primary aldosteronism is not performed routinely, a large proportion of primary aldosteronism patients remains undetected.

Evidence for a pivotal role of aldosterone in the pathogenesis and course of essential hypertension has emerged more recently. In nonhypertensive persons, elevated -yet physiological- aldosterone levels increase the risk of developing hypertension.[29] Furthermore, in normotensive young persons with a positive family history of hypertension, suppression of aldosterone on salt loading was inadequate.[30] This may cause a predisposition to fluid overload and risk of developing hypertension.

Aldosterone is also involved in drug-resistant hypertension. In patients with aldosterone-associated hypertension, defined as an elevated ARR and plasma aldosterone level, but no primary aldosteronism based on a captopril suppression test, adequate BP control was reached in a lower fraction of patients and after a longer treatment period as compared with essential hypertensive patients.[31]

Blocking the RAAS with angiotensin-converting enzyme inhibitors (ACE-Is) or angiotensin receptor blockers (ARBs) is often associated with a decrease in plasma aldosterone followed by an increase in aldosterone above pretreatment levels during prolonged treatment.[32, 33]

This so-called aldosterone escape or aldosterone breakthrough may contribute to therapy resistance by counteracting the antihypertensive actions of ACE-Is or ARBs. In a systematic review, Bomback and Klemmer[34] have estimated an incidence of aldosterone breakthrough of 10 – 53% in patients with chronic heart or kidney disease on ACE-I or ARB therapy. In these conditions, aldosterone breakthrough is associated with negative cardiovascular and renal outcomes. The addition of an ARA in this situation could result in an improved outcome through a further and sustained BP reduction

There is also increasing evidence for BP-independent adverse effects of aldosterone on the cardiovascular system. Aldosterone has shown to be a mediator of end-organ damage through the promotion of vascular inflammation and cardiac and perivascular fibrosis.[35] Indeed, a marked reduction in morbidity and mortality with aldosterone blockade, independent of haemodynamic effects, has been shown in the Randomized Aldactone Evaluation Study (RALES) and Eplerenone Post-Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) performed in patients with heart failure.[36, 37] Also unexplained by BP differences, a higher rate of cardiovascular complications has been reported in primary aldosteronism than in essential hypertensive patients.[38, 39] In a retrospective study, Milliez *et al*.[38] found a 4.2 times higher risk of stroke, 6.5 times higher risk of nonfatal myocardial infarction and 12.1 times higher risk of atrial fibrillation in primary aldosteronism compared with age-matched, sex-matched and BP-matched patients with essential hypertension. Patients with primary or secondary aldosteronism have a higher left ventricular mass than those with essential hypertension, after correction for a large number of confounders.[40] Arterial wall

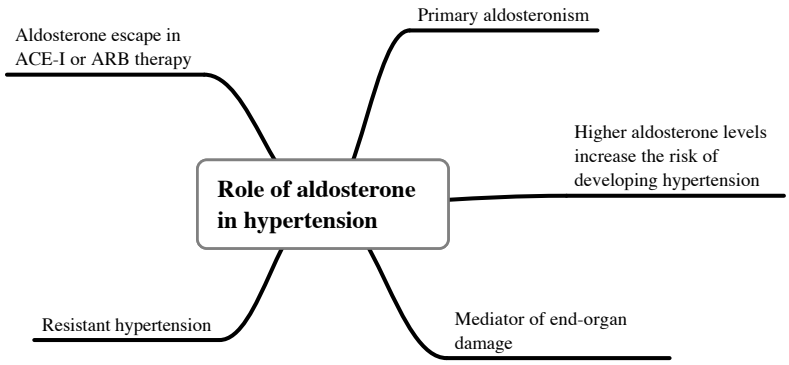


**Figure 1.** The role of aldosterone in hypertension. ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

stiffness is also higher in patients with primary aldosteronism than in those with essential hypertension.[41] Interestingly, in essential hypertension, a positive correlation between the ARR and measures of arterial stiffness has been demonstrated.[42] The mentioned effects of aldosterone that may contribute to the pathogenesis and course of (resistant) hypertension are summarized in Figure 1.

Considering the emerging role of aldosterone in the pathogenesis of hypertension and its deleterious effects on heart, vessels and kidney, there is a rationale for the use of ARAs in the treatment of hypertension. However, the prescription of these drugs has often been limited to specific conditions such as heart failure and primary aldosteronism. In the following paragraphs, the evidence for the use of ARAs in hypertension, both as monotherapy and as add-on therapy in resistant hypertension will be discussed.

## MONOTHERAPY WITH ALDOSTERONE-RECEPTOR ANTAGONISTS IN HYPERTENSION

Treatment of hypertension with monotherapy of spironolactone was described already 30 years ago. When compared with placebo, spironolactone, in a dosage of 100-400 mg daily, was more effective in lowering blood pressure.[43] Spironolactone was shown to be equally effective as propranolol in essential hypertension[43, 44] and high-dose chlorthalidone in low-renin hypertensive patients.[45] Spironolactone efficacy is clearly dose-dependent. However, no additional benefit was shown for doses exceeding 150 mg daily.[46] A single dose is as effective as a divided daily dose.[47] In normotensive persons, a small dose of spironolactone (25 mg) has no BP lowering effect.[48]

The widespread use of spironolactone in hypertension has been limited by the high incidence of side effects, in particular the occurrence of gynaecomastia in men due to the antiandrogenic properties of the drug. However, these adverse effects of aldosterone-receptor blockade have been overcome by the introduction of the more selective ARA, eplerenone.[49]

Several studies have evaluated the antihypertensive potential of eplerenone monotherapy. In a double-blind, randomized, placebo-controlled, parallel group, dose-ranging study, a dose-dependent decrease in office BP was shown for eplerenone, 50-400 mg, in mild-to-moderate hypertensive patients, with a 4.4-15 mmHg decrease in SBP.[50] Long-term safety and efficacy were studied in an open-label, uncontrolled trial by Burgess *et al*.[51] Eplerenone was started at 50 mg and uptitrated to a maximum of 200 mg for a period up to 14 months. If necessary, another antihypertensive drug could be added. Seventy-four percentage of patients on eplerenone achieved BP goals, and 16.8% were excluded because of treatment

failure. In patients with stage 1 - 3 essential hypertension, eplerenone lowered both SBP and DBP in a dose-related manner. Eplerenone lowered BP already at a dose as low as 25 mg. The maximum response of clinic BP was seen at a dose of 100 mg daily with a reduction of 10.4 mmHg in SBP and 6.3 mmHg in DBP.[52]

Eplerenone has also been compared with other antihypertensive drugs. Treatment with eplerenone, 50–200 mg, was as effective as with losartan, 50–100 mg, in white patients with mild-to-moderate hypertension and superior to that with losartan in black patients. The response rate was significantly higher for eplerenone (64.5%) than for losartan (48.3%).[53] In patients with low-renin hypertension, eplerenone reduced BP more effectively than losartan did: the decrease in BP was greater after 8 weeks, response rates were higher and less number of patients needed additional treatment with hydrochlorothiazide.[54] A comparison of eplerenone with enalapril in patients with stage 1 or 2 essential hypertension showed no difference in BP-lowering potential over a 12-month treatment period.[55] In older patients with systolic hypertension, the reduction in SBP and pulse pressure was similar for eplerenone and amlodipine. Reduction in clinic DBP was modestly larger with amlodipine than with eplerenone (6.9 vs. 4.5 mmHg, p=0.014).[56] Pitt *et al*.[57] studied the effects of eplerenone (200 mg), enalapril (40 mg) and eplerenone/enalapril (200/10 mg) combination therapy on left ventricular mass and BP in patients with essential hypertension and left ventricular hypertrophy. Monotherapy with eplerenone had a similar BP-lowering effect as enalapril (23.8/14.4 mmHg vs. 24.7/13.4 mmHg, respectively).

Therefore, monotherapy with eplerenone is an effective antihypertensive treatment, with similar BP-lowering potential as other antihypertensive agents. The potency of eplerenone on a molecular weight basis is 50-75% of that of spironolactone.[50]

## ADD-ON THERAPY WITH ALDOSTERONE-RECEPTOR-ANTAGONISTS

Most antihypertensive agents are used in combination to enhance efficacy and to reduce side effects.[58] Many investigators have studied the effect of ARAs in combination with other antihypertensive drugs or as part of third-line treatment in therapy-resistant hypertension.

An additional antihypertensive effect in combined treatment can be due to a synergistic or an additive effect. The cardiovascular system will react to antihypertensive treatment with counter-regulatory mechanisms. Aldosterone breakthrough can be seen as an example of this. When eplerenone (50–100 mg) was given over an 8-week period to hypertensive patients whose BP was insufficiently controlled despite ACE-I or ARB monotherapy, SBP was significantly lower after addition of eplerenone to ACE-I (-13.4 mmHg) or ARB (-16.0 mmHg) as compared with placebo (-7.5 mmHg in the ACE-I/placebo group and –9.2 mmHg in ARB/

**Table 1.** Overview of studies on the antihypertensive effects of add-on spironolactone treatment in patients with resistant hypertension

| Reference | Population | n | Study design | ARA | Follow-up | Reduction (± SEM) in SBP (mmHg) | Reduction (± SEM) in DBP (mmHg) |
|---|---|---|---|---|---|---|---|
| Ouzan *et al.*[60] | Patients with refractory hypertension despite the use of at least two antihypertensive agents | 25 | Open-label, uncontrolled trial | Spironolactone 1 mg/kg per day | 3 months | -23 (2.0) | -10 (1.4) |
| Nishizaka *et al.*[61] | Patients with uncontrolled hypertension despite the use of at least three antihypertensive agents, including a diuretic and an ACE-I/ARB | 76 | Open-label, uncontrolled trial | Spironolactone 12.5 – 50 mg | 6 months | -25 (2.3) | -12 (1.4) |
| Sharabi *et al.*[62] | Patients with uncontrolled hypertension despite the use of at least two antihypertensive agents | 42 | Retrospective interventional study comparing add-on spironolactone (n=42) with other add-on therapy (n=298) | Spironolactone 12.5 – 25 mg | 3 months | -23.2[a] (vs –7.6 for other add-on therapy) | -12.5[a] (vs –5.8 for other add-on therapy) |
| Lane *et al.*[63] | Patients with resistant hypertension already receiving an ACE-I or ARB in addition to other therapies | 119 | Open –label, observational study | Spironolactone 25 – 50 mg | 3 months | -21.7 (2.2) | -8.5 (1.4) |
| Saha *et al.*[64] | Black hypertensive patients with elevated blood pressure despite treatment including a diuretic and a CCB | 98 | Randomized, placebo-controlled, double-blind, parallel-group trial | Spironolactone 25 mg | 9 weeks | -7.3 (2.3) (compared with placebo) | -3.3 (1.4) (compared with placebo) |
| Chapman *et al.*[65] | Patients with elevated blood pressure despite the use of three antihypertensive agents[b] | 1411 | Retrospective, observational study | Spironolactone (median dose 25 mg) | Median treatment duration 1.3 years | -21.9 (0.6) | -9.5 (0.4) |

[a]SD or SEM for the reduction in SBP and DBP was not reported.

[b]According to the ASCOT-BPLA standardized protocol.

ACE-I, angiotensin-converting enzyme inhibitor; ARA, aldosterone-receptor antagonist; ARB, angiotensin receptor blocker; CCB, calcium channel blocker; DBP, diastolic blood pressure; SBP, systolic blood pressure.

placebo group). DBP was only lowered with eplerenone in the ARB group (-12.7 mmHg for eplerenone vs. –9.3 mmHg for placebo).[59]

Also treatment with the potassium-sparing diuretic amiloride is characterized by a compensatory rise in aldosterone,[48] thereby limiting its antihypertensive effect. Although amiloride (5 mg) and spironolactone (25 mg) had no antihypertensive effect in normotensive persons, the combination significantly lowered SBP (-4.6 mmHg). It is suggested that this is due to a complementary inhibition of the epithelial sodium channel (eNaC).[48]

Several authors have discussed the value of ARAs in resistant hypertension (Table 1).[60-65] Most studies on the efficacy of add-on spironolactone therapy in resistant hypertension are not placebo-controlled. Ouzan *et al*.[60] studied the effect of the addition of spironolactone (1 mg/kg per day) in 25 patients with an elevated BP despite the use of at least two antihypertensive drugs in an open-label, uncontrolled trial. When an ACE-I or ARB was used, these were replaced by spironolactone. Mean 24-h ambulatory BP measurement (ABPM) decreased significantly from 152/86 mmHg to 128/76 mmHg after 1 month of spironolactone. All patients reached target BP after 2 months of treatment. The number of required antihypertensive drugs decreased from 3.2 before to 2.1 during spironolactone use. Another open-label trial studied the BP response to addition of a low-dose of spironolactone (12.5-50 mg) in 76 patients with resistant hypertension. Of these, 34 turned out to have primary aldosteronism. At 6 weeks, SBP and DBP had decreased by 21 mmHg and 14 mmHg, respectively, and this effect was sustained after 6 months. Interestingly, BP reduction was similar in patients with and without primary aldosteronism.[61] A retrospective analysis on the efficacy of add-on treatment with spironolactone in 340 patients referred to a hypertension clinic with ongoing hypertension despite at least two antihypertensive agents, compared with standard add-on therapy, showed a BP reduction of 23.2/12.5 mmHg for spironolactone compared with 7.6/5.8 mmHg for other add-on treatment ($p<0.05$). Clinical judgement appears to have influenced the treatment strategy, as patients put on spironolactone had lower potassium values and higher left ventricular mass at baseline.[62]

Lane *et al*.[63] conducted an open observational study in which general practitioners were advised to add spironolactone 25-50 mg in patients with resistant hypertension who were already using an ARB. Outcome data were available in 119 patients. On an average, SBP decreased by 21.7 and DBP by 8.5 mmHg. The largest study was performed by Chapman *et al*.,[65] who evaluated the BP response of 1411 patients in the Anglo-Scandinavian Cardiac Outcome Trial – Blood Pressure Lowering Arm (ASCOT-BPLA) who received additional spironolactone because of sustained hypertension despite three antihypertensive drugs prescribed according to the study protocol. Spironolactone was given at the investigator's discretion in an open-label, uncontrolled fashion at a median dose of 25 mg. BP decreased by 21.9/9.5 mmHg ($p<0.001$) after spironolactone. Interestingly, BP responses were similar for both pretreat-

ment regimes consisting of amlodipine/perindopril and atenolol/bendroflumethiazide. It should, however, be noticed that the latter group was overrepresented in this population (75 % was pretreated with atenolol/bendroflumethiazide). Clinical judgement could have influenced the choice of spironolactone, selecting those with a larger expected response in both groups.

These studies indicate that there is increasing evidence for the usefulness of spironolactone in the treatment of resistant hypertension, often with impressive results. It should be acknowledged that most of the mentioned studies did not adhere to the current European Society of Hypertension guidelines of resistant hypertension, defined as an elevated BP despite treatment with at least three antihypertensives in optimal dose amounts including a diuretic.[66] Furthermore, although a favourable effect of spironolactone seems plausible from a mechanistic point of view, randomized, double-blind, placebo-controlled trials are needed to provide definite proof for the efficacy of spironolactone as add-on treatment in resistant hypertension. Coming from the same drug class, a similar effect of eplerenone is assumed. To our knowledge, however, no studies on the use of eplerenone in resistant hypertension have been reported.

Amiloride, although not a genuine aldosterone-receptor blocker, should also be discussed in this perspective. In the same way as for spironolactone, Lane and Beevers[67] evaluated in an open observational study the effect of amiloride 10 mg. Although this also resulted in a reduction in BP, the response was less than for spironolactone. This in contrast to a study in black patients with elevated BP on conventional treatment including a diuretic and a calcium channel blocker where addition of spironolactone 25 mg or amiloride 10 mg resulted in a larger BP reduction with amiloride (4.6/1.8 mmHg for spironolactone vs. 9.8/3.4 mmHg for amiloride). The combination exerted an additional BP-lowering effect.[64] In patients with uncontrolled BP who were using hydrochlorothiazide, the reduction in daytime DBP was higher with the addition of enalapril than with the addition of amiloride. Because of uncontrolled BP, more patients randomized for amiloride needed uptitration of drug dose in the fourth week and addition of propranolol in the eigth week of treatment.[68]

There are no studies comparing amiloride and spironolactone as add-on therapy in genuine resistant hypertension. Ramsay *et al*.[69] compared amiloride and spironolactone in thiazide-treated hypertensive patients. Surprisingly, the relative potency to correct hypokalemia was 2.8:1 in favour of amiloride. The presence of a carry-over effect did not allow to make conclusions about possible differences in BP-lowering potential between the two regimens. Therefore, studies evaluating the efficacy of amiloride vs. spironolactone in resistant hypertension are to be welcomed. It should be realized that amiloride only blocks the renal effect of aldosterone. A compensatory rise in plasma aldosterone levels during amiloride administration resulting in adverse cardiovascular effects can therefore not be excluded.

## MECHANISMS FOR THE ANTIHYPERTENSIVE EFFECTS OF ALDOSTERONE-RECEPTOR ANTAGONISTS

ARAs can exert their antihypertensive effect through several mechanisms (Figure 2).

A large part of the antihypertensive effect can be attributed to its diuretic properties. Through competitive antagonism with aldosterone for the mineralocorticoid receptor in the distal convoluted tubule in the kidney, aldosterone-induced upregulation of eNaC and $Na^+$/$K^+$ ATPase is prevented, leading to increased natriuresis, enhanced potassium reabsorption and decrease in circulating volume.

However, in recent years it has become clear that aldosterone has actions at many alternative sites in the body including the heart, blood vessels and brain. A part of the antihypertensive potential of ARAs might be attributable to blockade of mineralocorticoid receptors at these sites. Application of spironolactone, 50 mg twice daily, in eight oligoanuric haemodialysis patients for 2 weeks resulted in a decrease in predialysis SBP from 142 to 131 mmHg. This is an indication that spironolactone can exert antihypertensive effects independent of its diuretic properties.[70]

The extrarenal actions of ARAs can be attributed to intracerebral effects, modulation of sympathetic tone, and direct or indirect vascular effects. Intracerebroventricular infusion of aldosterone caused an increase in BP in rats.[71] Intracerebroventricular administration of a selective mineralocorticoid receptor antagonist in rats with hypertension induced by deoxycorticosterone acetate and salt enhanced urinary sodium and water excretion and decreased BP.[72] A reduction in sympathetic output might have mediated this effect, as the same route of administration reduced sympathetic tone in a rat model of congestive heart failure.[73] In patients with dilated cardiomyopathy, spironolactone reduced sympathetic nerve activity by

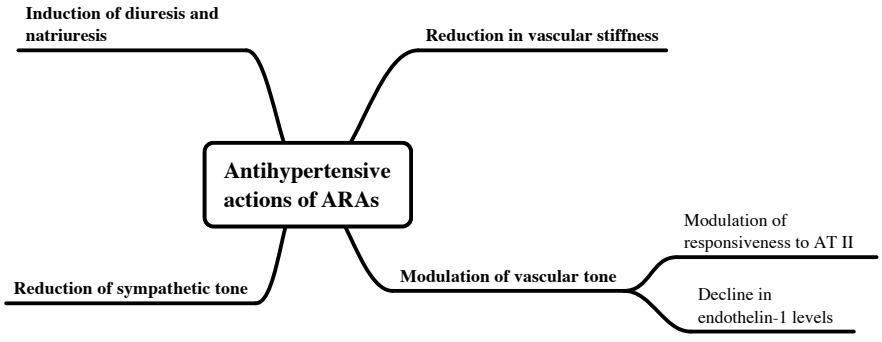


**Figure 2.** Proposed mechanisms for the antihypertensive actions of aldosterone-receptor antagonists. AT II, angiotensin II; ARA, aldosterone-receptor antagonist.

amelioration of aldosterone-induced impairment of noradrenaline reuptake at the synaptic level.[74]

Experimental studies have shown that prolonged exposure to aldosterone enhances vascular reactivity to angiotensin II (AT II) through an increased expression of the angiotensin type 1 receptor (AT1R).[75-79] In human coronary arteries, acute exposure to aldosterone increased the vasoconstrictor response to AT II. Because these effects could not be blocked by classic ARAs[80] the relevance of this phenomenon for the antihypertensive effect of ARAs is uncertain. Treatment with spironolactone has also been shown to be associated with a decline in plasma endothelin-1 levels,[64] posing another potential mechanism for the extrarenal antihypertensive effects of ARAs.

Vascular stiffness is an important determinant of systolic hypertension[81] and an independent predictor of mortality in hypertensive patients.[82] Treatment with spironolactone in patients with essential hypertension reduced pulse wave velocity and augmentation index,[42] thereby on the long-term indirectly contributing to SBP reduction.

The clinical relevance of the aforementioned extrarenal mechanisms of ARAs is unknown. A crossover trial in patients with low-renin hypertension and an elevated ARR and a previous SBP response to spironolactone of at least 20 mmHg showed that even in this selected population, high dose thiazide diuretic treatment (bendroflumethiazide 5 mg) was as effective as 100 mg of spironolactone. Biochemical measures of natriuresis indicated bendroflumethiazide to be less effective than spironolactone.[83] This suggests that natriuresis is the most important mode of action in terms of blood pressure reduction at least in this population.



## ALDOSTERONE-RECEPTOR ANTAGONISTS AND OTHER ENDPOINTS

Apart from lowering BP, beneficial effects on left ventricular hypertrophy, cardiac and perivascular fibrosis, cardiac arrhythmias, and proteinuria, independent of BP lowering, have been reported for ARAs.

### Left ventricular hypertrophy and cardiac fibrosis

Aldosterone-blockade in heart failure patients has been associated with a marked reduction in morbidity and mortality.[36, 37] This has in part been attributed to a (BP independent) reduction in cardiac fibrosis. Patients with primary aldosteronism have a higher left ventricular wall thickness and mass than essential hypertension patients.[40, 84, 85] This is related to changes in myocardial textures suggestive of increased collagen deposition as assessed by videoden-

sitometry.[84] Conversely, treatment with spironolactone in a subgroup of the RALES population was shown to lead to a decrease in serum markers for collagen synthesis and cardiac fibrosis.[86] In patients with LVH, treatment with spironolactone for 6 months decreased left ventricular fibrosis.[87]

In patients with essential hypertension, addition of low-dose spironolactone (25 mg) to ACE inhibition (ACE-I) was followed by a greater decrease in left ventricular mass index (LVMI) than ACE-I alone, as well as a decrease in procollagen type III amino-terminal peptide (PIIINP), a marker for collagen type III synthesis, despite the absence of a BP response to spironolactone.[88] Similar results on LVMI were found after the addition of spironolactone to the ARB candesartan for 1 year in hypertensive patients with concentric LVH, but not in patients with eccentric LVH.[89] Combination therapy with eplerenone and enalapril caused a larger reduction in left ventricular mass than either treatment alone (-27.2 g on combination therapy, compared with −14.5 g for eplerenone and −19.7 g for enalapril).[57] The failure of ACE-I to achieve full regression of left ventricular mass might in part be caused by the previously discussed aldosterone escape, as LVMI did not decrease in patients with aldosterone escape on ACE inhibition as opposed to a decline in LVMI in patients without aldosterone escape.[90]

## Perivascular fibrosis and vascular stiffness

As stated earlier, aldosterone is also an important mediator of perivascular fibrosis and increased vascular stiffness. Aldosterone-blockade was shown to prevent an increase in arterial stiffness and arterial fibrosis in old normotensive rats[91] and aldosterone-salt-treated rats.[92] This effect was mediated by modification of collagen and elastin density within the vascular wall[91] and prevention of aldosterone-induced accumulation of fibronectin.[92, 93] In stroke-prone spontaneously hypertensive rats, high-salt diet increased the media-to-lumen ratio of mesenteric resistance arteries and cardiac collagen content. These effects could be blocked by eplerenone.[94] There is also evidence for a vasculoprotective effect of aldosterone antagonism in humans. A 1-year treatment of hypertensive patients with eplerenone resulted in reduced stiffness in resistance arteries isolated from gluteal subcutaneous biopsies as well as a decreased collagen-to-elastin ratio. This was accompanied by a decrease in circulating inflammatory mediators.[95] The same observation applies for large-vessel disease.

Spironolactone reduced pulse wave velocity (PWV) and augmentation index in previously untreated patients with essential hypertension in a BP-independent manner.[42] A reduction in brachial PWV and PIIINP was also seen in hypertensive diabetic patients treated with spironolactone 50 mg for 4 months.[96] A similar effect on PWV was found by White *et al*.[56] after a 24-week treatment with eplerenone in patients with systolic hypertension, but this reduction was similar in amlodipine-treated patients.

## Cardiac Arrhythmias

Part of the reduction in mortality in the RALES and EPHESUS trials has been attributed to lower sudden death incidence rates under aldosterone blockade.[36, 37] Aldosterone has been linked to ventricular as well as supraventricular arrhythmias. High aldosterone levels on admission in patients presenting with ST-elevation myocardial infarction increased the risk of ventricular tachyarrhythmias, ventricular fibrillation and resuscitated cardiac arrest in a stepwise fashion.[97] In patients with previous atrial fibrillation, the reoccurrence of atrial fibrillation was independently associated with raised plasma aldosterone levels.[98] In patients with primary aldosteronism, the risk of sustained arrhythmias was almost five times higher than in patients with essential hypertension (OR 4.93). Another study showed a prolonged QT interval in patients with primary aldosteronism, with the maximum QT intervals correlating well with ARR and aldosterone levels. However, QT dispersion, a measure of repolarisation inhomogeneity, was small in these patients, thereby protecting them from ventricular arrhythmias.[99] The reason why aldosterone increases the risk of ventricular dysrhythmias in cardiac patients but not in primary aldosteronism patients is unknown, but may involve a larger burden of underlying structural and functional cardiac damage in the first group leading to a greater repolarization inhomogeneity.

Spironolactone has been shown to exert antiarrhythmic actions in experimental[100, 101] as well as clinical conditions.[102, 103] In patients with coronary artery disease, but without cardiac failure, a 3-month period of spironolactone treatment led to a reduction in ventricular extrasystoles and a decrease in QT interval length.[102] Patients with congestive heart failure (CHF) treated with spironolactone for 6 months showed a decrease in 24-h mean heart rate, ventricular and atrial premature beats, and had a lower risk of atrial fibrillation and atrial flutter than placebo-treated patients.[103] Several mechanisms have been proposed for the antiarrhythmic actions of spironolactone. First, as discussed earlier, aldosterone-induced myocardial fibrosis is reduced. Second, aldosterone has a profound effect on electrolyte fluxes such as potassium and magnesium, and spironolactone may alleviate these electrolyte imbalances, thereby reducing the risk of arrhythmias. Indeed, spironolactone was shown to reduce cellular magnesium efflux and loss in CHF patients.[103] On the contrary, maximum QT intervals in patients with primary aldosteronism were correlated with aldosterone but not with potassium levels, suggesting a possible direct effect of aldosterone on myocardial electrophysiology.[99] Finally, conditional cardiac overexpression of the mineralocorticoid receptor in a transgenic mouse model has been reported to result in severe ventricular arrhythmias,[104] suggesting a direct role of cardiac mineralocorticoid receptor in the pathogenesis of cardiac arrhythmia. Interestingly, these arrhythmias occurred in the absence of aldosteronemia.[104] Furthermore, in the isolated rat Langendorff heart mineralocorticoid receptor blockade decreased the risk of ventricular fibrillation after ischemia and reperfusion.[100, 101] As there is hardly any aldosterone

present in these isolated perfused hearts, this effect is most likely due to blockade of mineralocorticoid receptor stimulation by endogenous glucocorticoids.[105]

## Microalbuminuria

Previous studies have shown that treatment of patients with diabetic nephropathy with an ARA results in a considerable reduction of proteinuria,[106-110] even in addition to ACE-I or ARB therapy.[107-110] Similar results are found in hypertensive patients. Both eplerenone (-21.6%) and losartan (-18.2%) reduced urinary albumin-to-creatinine ratio (UACR) compared with placebo (+ 5.2%) in patients with mild-to-moderate hypertension.[53] When eplerenone was compared with amlodipine in patients with systolic hypertension, eplerenone reduced UACR with 21.4 vs. 0.5 % for amlodipine. The reduction in UACR was much larger when microalbuminuria was present at baseline (UACR>30 mg/g) (52.3% for eplerenone vs 10.4% for amlodipine; $p<0.05$).[56] In patients with stage 1 or 2 essential hypertension with normal UACR at baseline, no relevant change in UACR occurred on eplerenone treatment. However, in patients with microalbuminuria at baseline, UACR decreased by 61.5% with 6-month monotherapy with eplerenone (50 to 200 mg), as compared with 25.7 % ($p = 0.01$) with enalapril.[55] In hypertensive patients with LVH, superiority of 200 mg eplerenone above 40 mg enalapril in terms of BP reduction could not be confirmed after 9 months treatment. However, both drugs significantly reduced UACR compared with placebo (24.9% for eplerenone and 37.4% for enalapril). Combination therapy, consisting of 200 mg eplerenone and 10 mg enalapril, further lowered UACR by 52.6%.[57]

The antiproteinuric effect is, at least in part, related to changes in renal haemodynamics after aldosterone blockade. Patients with primary aldosteronism have a higher glomerular filtration rate (GFR) and a state of relative glomerular hyperfiltration as compared with patients with essential hypertension.[111, 112] Treatment of primary aldosteronism patients with either adrenalectomy or spironolactone causes a decline in both GFR and albuminuria, suggesting an interdependence between these two variables.[112] In two of the studies in diabetic nephropathy, changes in GFR and proteinuria were correlated.[107, 110] Although it is likely that BP reduction has contributed to the decrease in proteinuria by lowering glomerular filtration pressure in response to ARA not all antihypertensive drugs lower proteinuria equally, despite similar changes in BP. For instance, Rachmani *et al*.[106] showed that spironolactone was superior to cilazapril in reducing albuminuria independent of BP reduction. Other mechanisms than BP reduction therefore seem to play a role. First, in primary aldosteronism patients aldosterone specifically increases glomerular hydrostatic pressure and adrenalectomy resulted in a significant decline in glomerular pressure by a specific effect on afferent resistance.[111] Second, aldosterone promotes renal inflammation. Renal biopsies from patients with heavy proteinuria showed an increased expression of the mineralocorticoid receptor and glucocorticoid-

regulated kinase 1 (Sgk1), which was related to an enhanced expression of IL-6 and TGF-β1.[113] Third, spironolactone may limit the development of renal fibrosis. Spironolactone treatment in patients with proteinuria resulted in a decrease in the urinary excretion of type IV collagen as a reflection of a lower collagen turnover rate in the glomerular basement membrane and mesangial matrix.[114] Finally, aldosterone blockade by inhibition of the formation of Sgk1 and reducing oxidative stress preserves glomerular barrier function.[115]

## SIDE EFFECTS

The main side effects reported for ARA are sex hormone-related effects and hyperkalemia. Sex hormone-related side effects have extensively been described for spironolactone and include gynaecomastia and erectile disorders and menstrual abnormalities. These side effects can be attributed to the antiandrogenic action of spironolactone. Spironolactone competes with 5-alpha-dihydrotestosterone for the testosterone receptor.[116] Furthermore, spironolactone causes a decrease in plasma testosterone and an increase in estradiol by altering the peripheral metabolism of testosterone.[117] In fact, spironolactone has been shown to be useful in conditions of androgen excess, such as hirsutism,[118] polycystic ovary syndrome[119] and acne vulgaris.[120]

The incidence of sex hormone-related side effects is dose-dependent. In a large, retrospective analysis of patients receiving spironolactone for the treatment of hypertension, the incidence of gynaecomastia in male patients was 13%. At doses of 50 mg or less, the incidence was 6.9%, whereas it was 52% at doses of 150 mg or higher.[46] Although relatively rare for lower dosages, these side effects have importantly limited the use of spironolactone. Side effects can be prevented by using lower doses of spironolactone in combination with other antihypertensive agents.

The introduction of the more specific ARA, eplerenone, have raised possibilities for high-dose treatment with ARAs. Indeed, the incidence rates of sex hormone-related side effects in the studies reported in this review were low, with incidence rates for gynaecomastia ranging from 0 to 2.5%, impotence from 0 to 3.0% and menstrual disturbances from 0 to 2.5%. These side effects were often sporadic and not evidently attributable to the use of eplerenone. In most reported studies, the incidence of side effects in placebo or active control groups was similar.[55-57]

A point of concern is the occurrence of hyperkalemia. Treatment with ARAs is associated with an increase in serum potassium concentration. The incidence rates of hyperkalemia, mostly defined as a serum potassium exceeding 5.5 mmol/l, during use of eplerenone ranged from

0.8 to 4.2%.[50-56] Only Pitt *et al*.[57] reported a relatively high incidence rate of hyperkalemia of 10.9% for 200 mg eplerenone compared to 2.8% for 40 mg enalapril and 4.5% for the combination of 200 mg eplerenone and 20 mg enalapril. However, no clinical symptoms or complications were observed in any of these patients and hyperkalemia persisted in only three patients, necessitating discontinuation or dose modification. Of note, the rise in serum potassium is unrelated to the BP response.[121]

In a case-control study on heart failure patients using spironolactone, higher age, diabetes mellitus, higher baseline potassium levels, lower baseline potassium supplement doses, and use of beta-blockers were identified as risk factors for the development of hyperkalemia, whereas lower baseline body weight, higher baseline serum creatinine and use of other diuretics were related to the development of renal insufficiency.[122]

Case reports have identified a possible association between the use of spironolactone and ulceration and bleeding of the upper gastrointestinal tract. In a case-control study, Verhamme *et al*.[123] found a 2.7-fold increase in the risk of gastrointestinal bleeding. The association was stronger with higher doses of spironolactone. It has been speculated that impaired healing of gastrointestinal erosions by the inhibition of the formation of fibrous tissue underlies the observed association between spironolactone use and the mentioned gastrointestinal problems.[123] With the limited available evidence, we think it is too early at this moment to advice against the use of spironolactone in patients with a history of gastric ulcers or gastrointestinal bleeding, especially in the lower doses used in resistant hypertension.

## TARGETED TREATMENT

Several efforts have been made to select patients who would benefit most from aldosterone receptor blockade. ARAs have been thought to be particularly useful in conditions of aldosterone excess. Several parameters reflecting intravascular volume overload or aldosterone excess have been studied for their predictive value for the BP response to ARAs. One of these parameters is the serum potassium level. In patients with uncontrolled hypertension, add-on therapy with spironolactone was more effective when serum potassium was below 4.0 mmol/l than when it was above 4.0 mmol/l.[62, 124]

Several studies have focused on plasma renin as a predictor for BP response. Whereas propranolol was shown to be more effective in untreated patients with essential hypertension with PRA levels exceeding 2.0 ng/m per hour, spironolactone was more effective when PRA levels were below 1.0 ng/l per hour. Furthermore, the BP response to spironolactone was negatively correlated to baseline PRA.[44] However, in a study comparing enalapril with eplerenone monotherapy in untreated patients with stage 1 or 2 hypertension, the BP reduction was not correlated to baseline renin levels, whereas the response to enalapril was greater at

higher baseline renin levels.[55] A similar result was found in patients with low-renin hypertension in whom eplerenone caused a consistent reduction in BP independently of baseline active renin levels. Losartan, however, was more effective at higher baseline renin levels.[54] Also, in addition to ACE-I or ARB therapy[125] and as add-on treatment in resistant hypertension,[61] the amount of BP reduction could not be predicted by plasma renin levels[125] or PRA.[61] Although most studies indicate that plasma renin is not a determinant of the BP response to ARAs, eplerenone was shown to be more effective in patients with low-renin hypertension than losartan. As black patients are known to have lower renin levels,[126] eplerenone is more effective in these patients than losartan.[53] Recent studies have focused on the ARR as a determinant of the BP response to ARAs. Prisant *et al*.[125] found no predictive value of serum aldosterone or the ARR for the response to ARAs when added to ACE-I or ARB. Mahmud *et al*.,[124] however, demonstrated that the ARR failed to predict the response in patients with uncontrolled hypertension on a multidrug regimen, but proved to be predictive in previously untreated patients. This correlation also existed for low plasma renin activity.[42, 124] It is suggested that this discrepancy is the consequence of the interfering effects of the used antihypertensive drugs on the ARR. This could also be the case for the earlier reported conflicting results for plasma renin. Another explanation is that these biochemical values should be interpreted in relation to salt intake.[125]

In case of proven primary aldosteronism, the indication for treatment with an ARA seems evident when surgery is not considered. Indeed, in patients with an elevated ARR and nonsuppressible aldosterone on salt loading, spironolactone was a highly effective antihypertensive drug, leading the authors to suggest that spironolactone should be prescribed in all patients with an elevated ARR.[127] Nishizaka *et al*.,[61] however, found a similar BP reduction in hypertensive patients with or without primary aldosteronism. When compared with losartan, there was no difference in efficacy of eplerenone in subgroups based on sex, baseline BP values or body mass index.

## CONCLUSION

Aldosterone has a key role in the pathogenesis of hypertension, not only because of its effects on salt and fluid retention but also because of extrarenal effects at many sites in the body. Several studies have shown the antihypertensive potential of ARAs, not only in patients with primary aldosteronism but also in those with essential hypertension. Impressive BP reductions have been reached when used in patients with resistant hypertension, although evidence from randomized, placebo-controlled clinical trials is still lacking. The side effect profile is usually mild. When sex hormone-related side effects occur, the more selective ARA

eplerenone can be prescribed. Hyperkalemia and renal insufficiency can be prevented by proper selection of patients and close monitoring.

The question which patient should be treated with an ARA cannot be answered unanimously. Certainly, primary aldosteronism is an indication for treatment with an ARA when surgery is not considered. There is no evidence that cardiovascular outcome is different in primary aldosteronism patients treated medically or surgically.[39] In essential hypertension, there is insufficient evidence to prescribe an ARA purely on the basis of a high ARR or a low renin level. We suggest treatment with an ARA in patients with resistant hypertension, as well as in patients with hypokalemia, as these patients were shown to be more responsive to ARA therapy and such treatment will contribute to correction of the hypokalemia.

Eplerenone has been shown to be cost-effective in the treatment of heart failure after acute myocardial infarction.[128] Whether this also applies for the treatment of hypertension has not been studied, but selection of patients who in particular will benefit from treatment will definitely result in a decrease in morbidity and mortality and total cost expenditure. As the costs for spironolactone are considerably lower than those for eplerenone, use of the former will considerably contribute to cost-effectiveness, reserving the latter for situations that call for more selective aldosterone blockade. It is evident that confirmation of true resistant hypertension either by 24-h ambulatory BP monitoring or home BP measurements is required for cost-effectiveness, especially in the case of use of eplerenone.

The appropriate daily dose of ARAs in patients with resistant hypertension is unclear. Calhoun[129] has advised to start spironolactone at a dose of 25 mg, which is to be increased to 50 mg when BP is still uncontrolled. In patients with chronic kidney disease, diabetes or higher age, spironolactone should be started at a daily dose of 12.5 mg. If potassium supplementation is used, this should be discontinued or at least substantially reduced. Serum potassium should be monitored 4 weeks after the start of spironolactone in low-risk patients and after 1 week in high-risk patients. In case of side effects, spironolactone should be discontinued and restarted at a lower dose when side effects have subsided or alternatively and dependent on the severity of side effects downtitrated.

Eplerenone is usually started at 50 mg daily (25 mg for higher-risk patients) and titrated to a maximum of 50 mg twice daily.[129]

In conclusion, ARAs have gained a place in the treatment of hypertension. This is especially the case in patients with primary aldosteronism, both in terms of BP reduction as well as the prevention of end-organ damage. For therapy-resistant hypertension, the available studies are promising, but definitive evidence from well controlled trials is awaited.

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# Chapter 7

## Long-term use of aldosterone-receptor antagonists in uncontrolled hypertension: a retrospective analysis

Pieter M. Jansen[1], Koen Verdonk[1], Ben P. Imholz[2], A.H. Jan Danser[1] and Anton H. van den Meiracker[1]

[1] *Division of Pharmacology, Vascular and Metabolic Diseases, Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands.*

[2] *Department of Internal Medicine, Twee Steden Hospital, Waalwijk, The Netherlands.*

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## ABSTRACT

*Background:* The long-term efficacy of aldosterone-receptor antagonists (ARA) as add-on treatment in uncontrolled hypertension has not yet been reported.
*Methods:* Data from 123 patients (21 with primary aldosteronism, 102 with essential hypertension) with difficult-to-treat hypertension who received an ARA between May 2005 and September 2009 were analyzed retrospectively for their blood pressure (BP) and biochemical response at first follow-up after start with ARA and the last follow-up available.
*Results:* Systolic BP decreased by 22 ± 20 and diastolic BP by 9.4 ± 12 mmHg after a median treatment duration of 25 months. In patients that received treatment > 5 years, SBP was 33 ± 20 and DBP 16 ± 13 mmHg lower than at baseline. Multivariate analysis revealed that baseline BP and follow-up duration were positively correlated with BP response.
*Conclusion:* Add-on ARA treatment in difficult-to-treat hypertension results in a profound and sustained BP reduction.

## INTRODUCTION

Aldosterone-receptor antagonists (ARAs) have been shown to be effective in blood pressure (BP) reduction,[1-11] but until recently their use was mainly limited to certain conditions such as liver cirrhosis, heart failure and primary aldosteronism (PA). With the recognition of PA as a common cause of resistant hypertension,[12] a renewed interest in the use of ARAs in hypertension has emerged. However, aldosterone has also shown to be an important factor in other forms of resistant hypertension. In patients with elevated aldosterone-to-renin ratios (ARRs) and plasma aldosterone levels, but without genuine PA based on suppression testing, BP control was harder to achieve than in essential hypertensives (EHs).[13] Furthermore, a proportion of patients treated with angiotensin-converting enzyme inhibitors (ACE-Is) or angiotensin receptor blockers (ARBs) shows aldosterone breakthrough,[14-16] contributing to therapy resistance by partly counteracting the intended blockade of the renin-angiotensin-aldosterone system (RAAS). The use of ARAs in resistant hypertension, therefore, seems rational and several publications have pointed out the potential of aldosterone blockade in difficult-to-treat or resistant hypertension.[17-24] In many of these studies, the addition of spironolactone resulted in an impressive drop in systolic BP (SBP) of up to 25 mmHg and 12 mmHg in diastolic BP (DBP). However, most of these studies were either open-label,[17, 18, 20, 22] or retrospective[19, 21, 23] in design. One randomized, placebo-controlled, double-blind trial was performed comparing spironolactone with amiloride, the combination of both drugs, and placebo in black hypertensive patients with uncontrolled hypertension despite treatment with at least a diuretic and a calcium channel blocker.[24] Interestingly, the BP response was considerably smaller than in the aforementioned studies (-7.3 in SBP and -3.3 mmHg in DBP for spironolactone versus placebo). De Souza *et al*. recently performed an open-label, prospective study on the BP-lowering benefits of spironolactone in patients with resistant hypertension. By using 24-hour ambulatory BP measurements, at least part of the potential white coat and placebo effect could be accounted for. Twenty-four hour SBP and DBP decreased by 16 and 9 mmHg, respectively, after a median treatment duration of 7 months, and in a subgroup, the persistence of this effect was confirmed up to 15 months.[22] So far, longer follow-up periods have not been reported and although a persistence of the effect in the long run is expected, this remains to be confirmed.

Predicting factors for the BP response to ARA treatment have been identified in several studies. Lower serum potassium levels were pointed out by several groups to be associated with a larger decrease in BP.[19, 21, 22, 25] Most studies found no relation between plasma renin concentration or activity and the BP lowering response to ARAs.[5, 9, 17, 26] Also neither plasma aldosterone levels nor ARR levels seem to predict the BP-lowering effect,[22, 26] although this could have been caused by the interfering effects of multidrug antihypertensive regimens on the ARR in these patients.[25] Other factors possibly associated with a better response are

the absence of diabetes,[23] higher waist circumference, lower aortic pulse wave velocity,[22] and a lower baseline high-density lipoprotein (HDL) cholesterol.[21]

ARAs have been prescribed in our clinic to patients with difficult-to-treat hypertension for a long time now, often with good results even after many years. This study aims to retrospectively characterize the long-term response to ARA treatment in patients with difficult-to-treat hypertension and to identify factors associated with this response.

## METHODS

### Patients

All patients who visited the outpatient hypertension clinic of the Erasmus Medical Center in Rotterdam and the Tweesteden Hospital in Waalwijk, the Netherlands, between May 2005 and September 2009 were screened for their eligibility for the study. Patients were selected when they had uncontrolled hypertension (BP > 140/90 mmHg, or >130/80 mmHg for patients with diabetes mellitus (DM) or manifest cardiovascular disease) despite the use of at least two antihypertensive drugs and were put on spironolactone or eplerenone during the study period. Patients who were already using an ARA when referred to our clinic were excluded. Patients of whom insufficient data was available to meet the primary objective (for instance insufficient data on medication use or the absence of a BP measurement at the start of treatment or last follow-up) or patients who were prescribed an ARA for another indication than hypertension were also excluded from the analysis.

### Clinical data

At baseline, patients' sex, height, weight, the time of diagnosis of hypertension, their antihypertensive medication, their family history, and the presence or absence of diabetes at the start of ARA treatment were collected from patient files. Their electrocardiograms (ECGs), when not taken longer than one year before start of treatment, were scored for the presence of left ventricular hypertrophy (LVH) according to the Sokolow-Lyon criteria. The presence or absence of PA was based on the clinical judgement by their physician.

At baseline, at first follow-up (*i.e.*, the first follow-up visit that BP was measured after start of ARA treatment), and at the end of follow-up (*i.e.*, the date that ARA treatment was permanently discontinued or the last visit before the end of data collection), the following parameters were recorded: BP, serum sodium, potassium, urea, creatinine, uric acid, glucose, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and hemoglobin and hematocrit, and plasma renin and aldosterone levels, where available.

BP measurements were taken in triplicate at an interval of five minutes with a semiautomatic BP measuring device after a rest of five minutes in sitting position. The mean of these measurements was used in the analysis.

Biochemical measurements were taken on the visit day or the nearest previous moment.

Plasma renin concentrations (PRCs) were assessed using an immunoradiometric assay (Renin III, Cisbio, Gif-sur-Yvette, France). Plasma aldosterone concentrations (PACs) were measured with a radioimmunoassay (Coat-a Count, Diagnostics Product Corporation, LA, CA. USA). Hyperkalemia was defined as serum potassium levels exceeding 5.5 mmol/L.

### Data analysis

Statistical analyses were performed in SPSS 17.0 for Windows.

Main effects at first follow-up and end of follow-up were calculated. Furthermore, to assess the long-term efficacy of treatment, patients were stratified based on the duration of follow-up into the following categories: < 1 year, 1-5 years, and > 5 years follow-up.

Values are expressed as mean ± SD, or as median and range when not normally distributed. Medication use was quantified by adding up the total number of different drugs, as well as by assessing the defined daily doses (DDDs) per drug and for total drug use according to the World Health Organization Anatomical Therapeutic Chemical (ATC) index.[27] Differences within subjects were tested using paired Student's t-tests for two groups and one-way analysis of variance (ANOVA) for repeated measurements for more groups. Between-subjects differences were tested with unpaired t-tests for two groups and one-way ANOVA for more groups. For values that were abnormally distributed, nonparametric tests were used (Mann-Whitney U test and Wilcoxon Signed Ranks test). Differences in proportions were tested with a chi-square test.

Patients with PA were excluded for regression analysis. A univariate linear regression analysis was performed to identify potential determinants of the BP response. Significant parameters were subsequently tested in a multivariate linear regression analysis. This model was further adjusted for age and sex.

## RESULTS

### Study population

A total of 175 patients was prescribed an ARA during the study period. Fifty-two patients were excluded: 39 because of insufficient data, 5 because our criteria for difficult-to-treat hypertension were not met, 3 because of questionable treatment adherence, 2 because of a follow-up duration less than a month, 1 because baseline BP measurement was not per-

**Table 1.** Baseline characteristics of the study population.

| | Total | EH | PA | p-value |
|---|---|---|---|---|
| **Number** | 123 | 102 | 21 | |
| **Age (years)** | 56.6 ± 10.7 | 56.7 ± 11.2 | 56.5 ± 8.2 | 0.959 |
| **Male (%)** | 60.1 | 56.9 | 76.2 | 0.099 |
| **BMI (kg/m²)** | 29.4 ± 5.0 | 29.3 ± 5.0 | 30.1 ± 5.2 | 0.537 |
| **SBP (mmHg)** | 159.7 ± 19.1 | 158.4 ± 18.3 | 166.0 ± 21.7 | 0.094 |
| **DBP (mmHg)** | 93.3 ± 12.2 | 92.7 ± 12.5 | 96.0 ± 10.8 | 0.268 |
| **Time since diagnosis (years)** | 10.0 (0-50) | 10.0 (0-50) | 7.5 (1.0-34) | 0.319 |
| **Age at diagnosis (years)** | 42.0 ± 13.0 | 41.5 ± 13.3 | 44.7 ± 11.4 | 0.335 |
| **Nr. of antihypertensives** | 3 (2-6) | 3 (2-6) | 3 (2-5) | 0.071 |
| **DDD** | 5.0 (1.25-13.0) | 5.0 (1.25-13.0) | 3.7 (1.5-10.0) | 0.117 |
| **DM (%)** | 22.8 | 23.2 | 21.1 | 0.842 |
| **LVH (%)** | 28.5 | 26.5 | 38.1 | 0.125 |
| **Family history of HT** | 52.0 | 53.9 | 42.9 | 0.355 |
| **Serum sodium (mmol/L)** | 141.5 ± 2.7 | 141.2 ± 2.8 | 143.0 ± 2.14 | 0.008 |
| **Serum potassium (mmol/L)** | 3.9 ± 0.6 | 4.0 ± 0.6 | 3.4 ± 0.5 | <0.001 |
| **Serum creatinine (µmol/L)** | 83.8 ± 20.1 | 83.8 ± 21.1 | 84.1 ± 14.4 | 0.959 |
| **Serum uric acid (mmol/L)** | 0.36 ± 0.08 | 0.37 ± 0.08 | 0.34 ± 0.08 | 0.134 |
| **Haemoglobin (mmol/L)** | 8.9 ± 0.82 | 8.8 ± 0.8 | 9.5 ± 0.6 | 0.001 |
| **Hematocrit (%)** | 0.42 ± 0.04 | 41.3 ± 3.6 | 45.3 ± 2.1 | 0.003 |
| **Cholesterol (mmol/L)** | 5.31 ± 0.96 | 5.27 ± 0.97 | 5.55 ± 0.90 | 0.345 |
| **HDL (mmol/L)** | 1.35 ± 0.42 | 1.37 ± 0.41 | 1.26 ± 0.44 | 0.347 |
| **LDL (mmol/L)** | 3.37 ± 1.02 | 3.41 ± 1.02 | 3.16 ± 1.03 | 0.407 |
| **Glucose (mmol/L)** | 5.5 ± 1.6 | 5.5 ± 1.6 | 5.5 ± 1.8 | 0.943 |
| **ACR (g/mol)** | 2.19 (0.95-12.4) | 2.19 (0.15-453.8) | 1.96 (0.37-592.0) | 0.518 |
| **PAC (pmol/L)** | 282.5 (2.8-4172) | 224.4 (2.8-4172) | 548.5 (199-2282) | p<0.001 |
| **PRC (mU/L)** | 13.9 (1.0-4374) | 19.8 (1.0-4374) | 5.8 (1.8-18.9) | p<0.001 |
| **ARR (pmol/mU)** | 19.4 (0.3-1087) | 9.5 (0.3-781) | 82.7 (17.4-1087) | p<0.001 |

EH, essential hypertension; PA, primary aldosteronism; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; DDD, defined daily dose; DM, diabetes mellitus; LVH, left ventricular hypertrophy; HT, hypertension; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ACR, urinary albumin-to-creatinine ratio; PAC, plasma aldosterone concentration; PRC, plasma renin concentration; ARR, aldosterone-to-renin ratio.

formed with a semiautomatic BP measuring device, and 1 because an ARA was prescribed because of another indication than hypertension. In total, 123 patients were included in the analysis with a mean age of 56.6 ± 10.7 years. The median duration between diagnosis and start of ARA treatment was 10 years (range 0-50 years). The median number of different antihypertensive agents was 3 (total DDD 5.0). Twenty-three percent of patients had DM, and 29 percent had LVH. Twenty-one patients were diagnosed as having PA by their physician. The baseline characteristics of all patients and of the EH and PA subgroups are shown in table 1. Serum potassium levels were lower in patients with PA than with EH (3.4 mmol/L versus 4.0 mmol/L in EH, p<0.001). Serum sodium levels were higher in patients with PA than with EH (143 versus 141 mmol/L, p<0.001).

As expected, PRC was lower in PA than in EH patients (5.8 versus 19.8 mU/L, p<0.001). PAC and ARR were higher in PA patients (548.5 versus 224.4 pmol/L (p<0.001) for PAC, and 82.7 versus 9.5 pmol/mU (p<0.001) for ARR).

Values of haemoglobin and hematocrit were also higher in PA than in EH patients

## Treatment

Ninety-four patients started on spironolactone treatment with a median dose of 50 mg daily (range 12.5-100 mg). Twenty-nine patients started on eplerenone with a median dose of 50 mg (range 25-50 mg). Total starting DDD of ARA was 0.67 (range 0.17-1.33). At the end of follow-up, 91 patients were on spironolactone with a median dose of 25 mg (range 12.5-100 mg) and 32 patients on eplerenone (median dose 50 mg, range 25-100 mg). Median ARA DDD at end of follow-up was 0.67 (range 0.17-2.00). Median treatment duration at first follow-up was 8 weeks (range 1-66 weeks). The median treatment duration at end of follow-up was 25 months (range 1-144 months).

## Main effects of ARA treatment

The BP levels at first follow-up and at the end of follow-up are shown in Figure 1. In EH patients, BP decreased by 13 ± 1.8 mmHg systolically and 6.2 ± 1.0 mmHg diastolically at first follow-up, and by 21 ± 2.1 and 9.7 ± 1.4 mmHg at the end of follow-up. In PA patients, SBP had decreased by 23 ± 4.8 mmHg and DBP by 9.6 ± 2.5 mmHg at first follow-up and by 28 ± 4.9 and 9.7 ± 3.1 mmHg at the end of follow-up. Changes in BP were not significantly different for EH and PA patients at both time points, although a trend existed towards a larger SBP decrease at first follow-up in the PA group (p=0.063).

Serum potassium and creatinine levels increased significantly after start of ARA treatment for both EH and PA patients. Furthermore, in PA patients, serum sodium was significantly lower at first follow-up compared to baseline (Table 2).

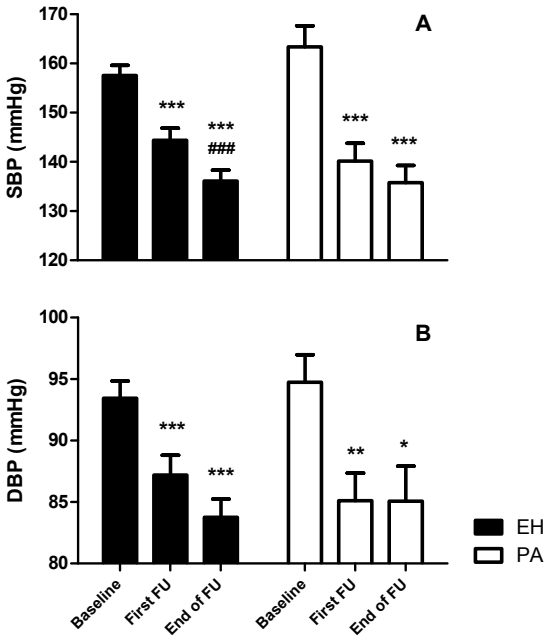


**Figure 1.** Systolic (SBP) and diastolic (DBP) blood pressure before start with an aldosterone-receptor antagonist (baseline), at first follow-up (FU), and at the end of FU in patients with essential hypertension (EH) and primary aldosteronism (PA). Overall trend was tested with one-way ANOVA for repeated measurements ($p<0.001$ for all groups, except for DBP in the PA group ($p=0.001$)). Indicated significance levels are for differences between groups after adjustment for multiple comparisons (*compared to baseline; #compared to first FU).

**Table 2.** Changes in biochemical parameters at first follow-up and end of follow-up after start of treatment with an aldosterone-receptor antagonist for patients with essential hypertension and primary aldosteronism. Values were tested with one-way ANOVA for repeated measurements. Indicated p-values are for differences between baseline and first follow-up (a), first and last follow-up (b), and baseline and last follow-up (c) after Bonferroni adjustment; n represents the number of patients with measurements at all three time points.

| | | n | Baseline | p-value[a] | First FU | p-value[b] | End of FU | p-value[c] |
|---|---|---|---|---|---|---|---|---|
| **EH** | Serum sodium (mmol/L) | 66 | 141.2 ± 2.7 | 1.000 | 140.9 ± 3.0 | 0.927 | 141.3 ± 3.2 | 1.000 |
| | Serum potassium (mmol/L) | 80 | 4.0 ± 0.6 | <0.001 | 4.4 ± 0.6 | 1.000 | 4.4 ± 0.5 | <0.001 |
| | Serum creatinine (µmol/L) | 78 | 84.6 ± 20.8 | <0.001 | 90.8 ± 24.7 | 0.355 | 93.6 ± 26.2 | <0.001 |
| **PA** | Serum sodium (mmol/L) | 17 | 142.9 ± 2.1 | 0.015 | 141.0 ± 3.4 | 0.103 | 142.7 ± 3.4 | 1.000 |
| | Serum potassium (mmol/L) | 19 | 3.4 ± 0.5 | <0.001 | 4.3 ± 0.5 | 1.000 | 4.3 ± 0.5 | <0.001 |
| | Serum creatinine (µmol/L) | 18 | 85.7 ± 14.1 | 0.011 | 96.1 ± 22.6 | 1.000 | 95.7 ± 19.6 | 0.169 |

EH, essential hypertension; FU, follow-up; PA, primary aldosteronism.

At baseline, PA and EH patients used a median number of 3 antihypertensive drugs (range 2-6). At the end of follow-up, the number of drugs had increased to 4 (range 1 to 7, p<0.001). However, when expressed in DDD, the total amount of antihypertensive drugs remained unchanged (5 DDD at baseline versus 4.5 at end of follow-up, p=0.459). Also in the EH subgroup, the number of antihypertensive drugs increased from 3 to 4 (p<0.001), with a nonsignificant decrease in DDD (5 DDD at baseline against 4.6 at end of follow-up, p=0.663). In PA patients, there was no significant change in number of antihypertensive drugs (3 versus 3, p=0.317 or DDD 3.66 versus 3.83, p=0.407).

## Stratification to follow-up duration

Because of the wide variation in follow-up duration and to better assess the long-term efficacy of ARA treatment, patients were stratified according to their treatment follow-up. The following categories were formed: 0-1 year, 1-5 years, and > 5 years. Number of patients in these categories were 33, 49, and 20, respectively, for EH patients, and 5, 8, and 8 for PA patients. In Figure 2, blood pressure reduction is shown for the three categories of follow-up

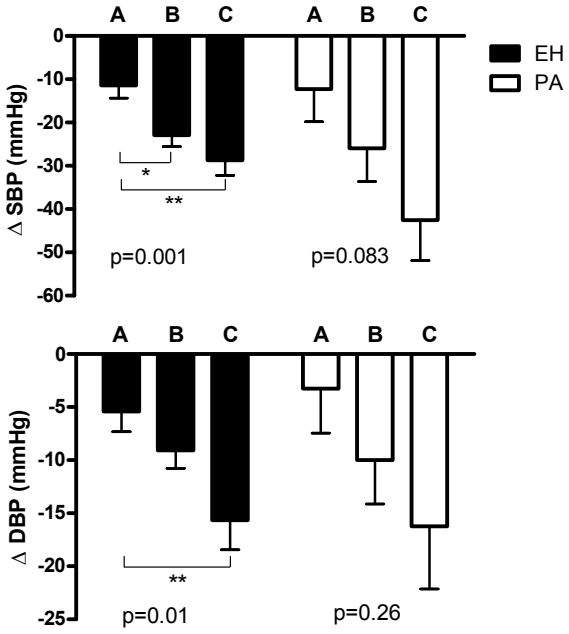


**Figure 2.** Changes in systolic (SBP) and diastolic (DBP) blood pressure at the end of follow-up compared to baseline for three categories of follow-up duration (A < 1 year; B 1-5 years; C > 5 years). P-values are for trend tested with one-way ANOVA; indicated significance levels are for differences between groups after adjustment for multiple comparisons. EH, essential hypertension; PA, primary aldosteronism.

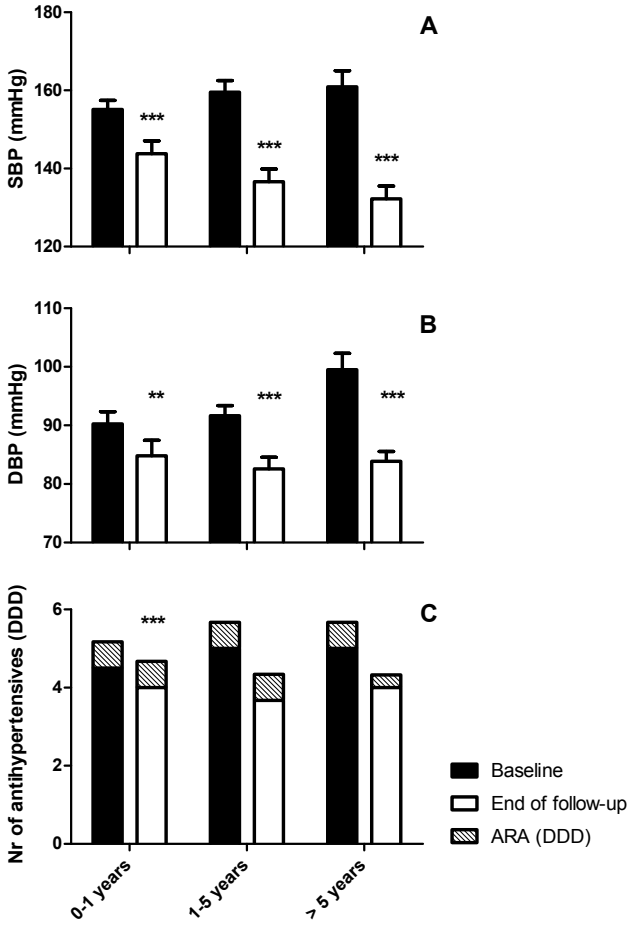


**Figure 3.** Systolic (SBP) (panel A), diastolic (DBP) (panel B) blood pressure and medication use (defined daily dose, DDD) (panel C) at baseline and end of follow-up after stratification for follow-up duration for patients with essential hypertension. Indicated in panel C is the DDD for the aldosterone-receptor antagonist (ARA). For baseline, this is added up to total DDD; at the end of follow-up, this is part of the total DDD since ARA was started at baseline. Differences were tested with paired t-test for SBP and DBP and Wilcoxon Signed Ranks test for DDD (DDD without ARA at baseline versus DDD including ARA at the end of follow-up).

duration. In EH patients larger responses were seen with longer follow-up duration (p=0.001 for ΔSBP and p=0.01 for ΔDBP with one-way ANOVA). In PA patients, a similar trend was seen. The overall trends were not different for EH and PA patients (p=0.467 for ΔSBP and p=0.907 for ΔDBP at two-way ANOVA).

To investigate whether the reduction in BP was merely a result of a greater number of antihypertensive drugs than a specific effect of ARA treatment, baseline and end-of-follow-up

BP is shown in relation to medication use for EH (Figure 3) and PA (Figure 4). The proportion of total DDD that consisted of ARA treatment is separately indicated. These figures show that at longer follow-up, BP further decreased, while the total DDD remained unchanged. In EH patients, the percentage of total DDD consisting of an ARA significantly increased from 9.1 % to 14.2 % (p<0.001) in the 1-5 years follow-up group. In PA patients, the relative contribu-

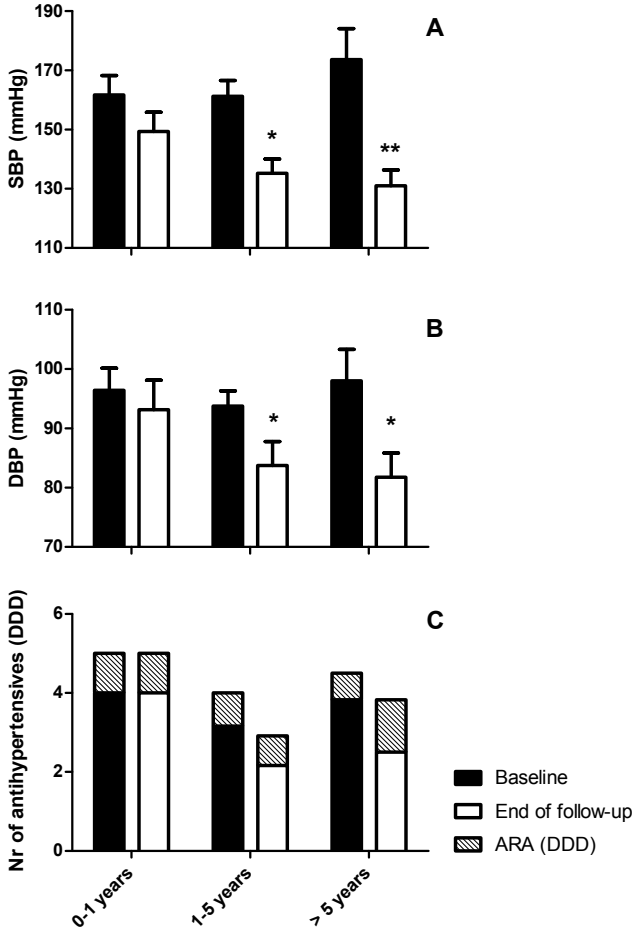


**Figure 4.** Systolic (SBP) (panel A), diastolic (DBP) (panel B) blood pressure and medication use (defined daily dose, DDD) (panel C) at baseline and end of follow-up after stratification for follow-up duration for patients with primary aldosteronism. Indicated in panel C is the DDD for the aldosterone-receptor antagonist (ARA). For baseline, this is added up to total DDD; at the end of follow-up, this is part of the total DDD since ARA was started at baseline. Differences were tested with paired t-test for SBP and DBP and Wilcoxon Signed Ranks test for DDD (DDD without ARA at baseline versus DDD including ARA at the end of follow-up).

**Table 3.** Outcomes of a univariate linear regression analysis with the changes in systolic and diastolic blood pressure at first and last follow-up compared to baseline (ΔSBP1, ΔDBP1, ΔSBP2, and ΔDBP2, respectively) as dependent variables. Included in the table are all independent variables with a significant β-coefficient in one of the ΔBP categories or those assumed to be relevant based on the literature. Also shown are the numbers (n) available for the individual analyses.

| | ΔSBP1 | | | ΔDBP1 | | | ΔSBP2 | | | ΔDBP2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Independent variable** | **β** | **n** | **p-value** | **β** | **n** | **p-value** | **β** | **n** | **p-value** | **β** | **n** | **p-value** |
| **Age (years)** | -0.750 | 83 | 0.660 | 0.030 | 83 | 0.758 | -0.013 | 102 | 0.937 | 0.043 | 102 | 0.689 |
| **Sex (1=female, 2=male)** | 4.199 | 83 | 0.260 | 3.477 | 83 | 0.101 | -4.737 | 102 | 0.207 | -2.789 | 102 | 0.251 |
| **DM** | -5.684 | 78 | 0.202 | -0.746 | 78 | 0.770 | -5.896 | 95 | 0.181 | -0.206 | 95 | 0.943 |
| **LVH** | 2.948 | 63 | 0.526 | 2.875 | 63 | 0.276 | -9.461 | 76 | **0.031** | -2.897 | 76 | 0.343 |
| **$SBP_0$ (mmHg)** | 0.170 | 83 | 0.083 | 0.000 | 83 | 0.994 | -0.388 | 102 | **<0.001** | -0.034 | 102 | 0.608 |
| **$DBP_0$ (mmHg)** | 0.080 | 83 | 0.572 | -0.118 | 83 | 0.143 | -0.378 | 102 | **0.010** | -0.407 | 102 | **<0.001** |
| **$Na^+$(mmol/L)** | -0.742 | 78 | 0.280 | -0.825 | 78 | **0.032** | 0.600 | 95 | 0.393 | -0.028 | 95 | 0.951 |
| **$K^+$(mmol/L)** | 4.206 | 83 | 0.162 | 0.341 | 83 | 0.844 | 0.017 | 102 | 0.996 | -0.344 | 102 | 0.867 |
| **$Na^+/K^+$** | -0.663 | 78 | **0.038** | -0.174 | 78 | 0.343 | -0.123 | 95 | 0.723 | 0.035 | 95 | 0.876 |
| **Hb (mmol/L)** | 6.79 | 52 | **0.023** | 2.632 | 52 | 0.125 | -1.837 | 59 | 0.576 | -1.329 | 59 | 0.529 |
| **Ht (%)** | 162.2 | 30 | 0.084 | 56.75 | 30 | 0.259 | -19.98 | 35 | 0.831 | -23.75 | 35 | 0.707 |
| **TC (mmol/L)** | 5.374 | 61 | **0.025** | 2.609 | 61 | **0.044** | -1.555 | 79 | 0.480 | -1.136 | 79 | 0.424 |
| **HDL (mmol/L)** | 4.880 | 64 | 0.311 | 3.639 | 64 | 0.162 | 0.283 | 81 | 0.956 | 2.826 | 81 | 0.387 |
| **LDL (mmol/L)** | 5.127 | 60 | **0.004** | 2.111 | 60 | **0.031** | 0.585 | 78 | 0.784 | -0.291 | 78 | 0.832 |
| **ACR (g/mol)** | 0.031 | 40 | 0.518 | 0.041 | 40 | 0.170 | 0.078 | 45 | **0.036** | 0.049 | 45 | **0.028** |
| **FU duration (weeks)** | -0.371 | 83 | **0.018** | -0.150 | 83 | 0.098 | -0.039 | 102 | **0.001** | -0.028 | 102 | **<0.001** |
| **Total DDD** | 0.503 | 83 | 0.539 | 0.348 | 83 | 0.458 | -0.649 | 102 | 0.435 | -0.250 | 102 | 0.642 |
| **DDD ARA** | 5.446 | 83 | 0.360 | 6.501 | 83 | 0.054 | -1.130 | 102 | 0.847 | 1.799 | 102 | 0.634 |
| **PAC (pmol/L)** | 0.001 | 46 | 0.788 | 0.003 | 46 | 0.149 | 0.002 | 55 | 0.621 | 0.004 | 55 | 0.117 |
| **PRC (mU/L)** | 0.002 | 47 | 0.553 | 0.002 | 47 | 0.325 | 0.004 | 56 | 0.392 | 0.003 | 56 | 0.225 |
| **ARR (pmol/mU)** | -0.034 | 46 | 0.094 | 0.027 | 46 | **0.020** | -0.027 | 55 | 0.289 | 0.033 | 55 | **0.029** |

DM, diabetes mellitus; LVH, left ventricular hypertrophy; $Na^+$, sodium; $K^+$, potassium; Hb, haemoglobin; Ht, hematocrit; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ACR, urinary albumin-to-creatinine ratio; FU, follow-up; DDD, defined daily dose; ARA, aldosterone-receptor antagonist; PAC, plasma aldosterone concentration; PRC, plasma renin concentration; ARR, aldosterone-to-renin ratio.

tion of ARA to total DDD increased from 14.9% to 22.4 % in the 1-5 years follow-up group (p=0.050) and from 14.9 % to 31.9 % in the > 5 years follow-up group (p=0.018).

## Predictors for the blood pressure response

The main clinical parameters were tested for their potential association with SBP as well as DBP response at first and last follow-up by univariate regression analysis (with the change in BP being negative). Table 3 shows the beta coefficients of all parameters that were significantly associated with BP change in any of the four groups, as well as those considered relevant based on earlier reports. At first follow-up, the sodium/potassium ratio as well as follow-up duration were significantly associated with ΔSBP. The ARR was significantly associated with ΔDBP, yet with a very small and probably irrelevant regression coefficient considering the range in ARR. Interestingly, haemoglobin and hematocrit levels, total cholesterol, and LDL levels were negatively associated with blood pressure change at univariate analysis for ΔSBP, and the latter two also for ΔDBP.

At last follow-up, the change in BP was significantly correlated with baseline BP, urinary albumin-to-creatinine ratio (ACR), LVH, follow-up duration, and, for DBP, the ARR.

To identify independent predictors for BP response, the variables significantly associated in the univariate analyses were included in a multivariate linear regression analysis. In addition, the model was adjusted for age and sex. The regression coefficients and significance levels are shown in Table 4. Unfortunately, haemoglobin, hematocrit, total cholesterol, LDL, LVH, and the ARR could not be included in the analysis because numbers were too small to maintain sufficient statistical power.



**Table 4.** Multivariate linear regression analysis for the change in systolic and diastolic blood pressure at first and last follow-up (ΔSBP1, ΔDBP1, ΔSBP2, and ΔDBP2).

| | ΔSBP1 | | | ΔDBP1 | | | ΔSBP2 | | | ΔDBP2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | β | SEM | p-value | β | SEM | p-value | β | SEM | p-value | β | SEM | p-value |
| **Age (years)** | 0.015 | 0.203 | 0.941 | -0.026 | 0.117 | 0.824 | 0.070 | 0.191 | 0.716 | -0.200 | 0.122 | 0.104 |
| **Sex (1=female, 2 = male)** | 2.669 | 3.990 | 0.506 | 3.387 | 2.291 | 0.144 | -5.776 | 3.799 | 0.132 | -1.125 | 2.429 | 0.644 |
| **$SBP_0$ (mmHg)** | -0.223 | 0.121 | 0.069 | 0.011 | 0.069 | 0.874 | -0.335 | 0.119 | **0.006** | 0.133 | 0.076 | 0.085 |
| **$DBP_0$ (mmHg)** | 0.122 | 0.198 | 0.541 | -0.162 | 0.113 | 0.157 | -0.051 | 0.205 | 0.803 | -0.485 | 0.131 | **<0.001** |
| **FU-duration (weeks)** | -0.251 | 0.160 | 0.121 | -0.099 | 0.092 | 0.283 | -0.031 | 0.013 | **0.016** | -0.018 | 0.008 | **0.030** |
| **$Na^+$ (mmol/L)** | -0.273 | 0.799 | 0.734 | -0.704 | 0.459 | 0.129 | 0.607 | 0.760 | 0.427 | -0.011 | 0.486 | 0.982 |
| **$K^+$ (mmol/L)** | -12.162 | 11.925 | 0.311 | -5.051 | 6.846 | 0.463 | -16.925 | 12.363 | 0.175 | -3.339 | 7.905 | 0.674 |
| **$Na^+/K^+$** | -1.770 | 1.304 | 0.179 | -0.554 | 0.748 | 0.461 | -1.810 | 1.356 | 0.185 | -0.292 | 0.867 | 0.737 |

$SBP_{0,}$ baseline systolic blood pressure; $DBP_{0'}$ baseline diastolic blood pressure; FU, follow-up; $Na^+$, serum sodium concentration; $K^+$, serum potassium concentration.

At first follow-up, only baseline SBP seemed to be an independent predictor (borderline significant) for ΔSBP. For ΔDBP, there were no independent predictors for the response. At the end of follow-up, higher baseline BP and longer FU duration were independently associated with the change in BP.

### Adverse events

ARA treatment was in general well tolerated. In total, 13 adverse events were reported. Five cases of gynaecomastia were reported with spironolactone use resulting in a switch to eplerenone in 1 patient. Two cases of hyperkalemia were seen, and in two patients, a clinically relevant decrease in renal function was observed. Two patients (one on eplerenone and one on spironolactone) reported general discomfort and headache, and one patient experienced gastrointestinal discomfort, although this was probably already present before start of spironolactone. In 1 patient, the nature of the adverse event was not further specified.

## DISCUSSION

This study showed that the addition of aldosterone-receptor antagonists (ARAs) in patients with difficult-to-treat hypertension was highly effective in reducing SBP as well as DBP. This effect was already present at short-term follow-up (median follow-up 8 weeks) and persisted in the long run with a median follow-up of 25 months. The BP reduction in EH and PA patients was comparable, and in both groups ARA treatment resulted in a small rise in serum potassium and creatinine levels.

To assess whether the BP-lowering effect was still present after prolonged treatment, patients were stratified according to their duration of follow-up. We observed larger BP reductions with increasing follow-up, which was highly significant in EH patients. In the subgroup that had a follow-up of more than 5 years, SBP was 29 mmHg and DBP 16 mmHg lower than at baseline. In PA, a similar trend was seen although this failed to reach statistical significance, probably because of the small number of patients in each subgroup. Also in the multivariate regression analysis, we observed a strong correlation between treatment duration and decrease in BP. Although it is appealing to conclude that a longer treatment duration leads to better BP control, for instance by reversing target organ damage, a more likely explanation is some form of effect-bias implicating that patients with a better response are more likely to receive ARA treatment for a longer period. Whether prolonged treatment leads to a better BP control requires a long-term prospective study.

Another explanation for the favourable long-term BP response could be an optimization of the antihypertensive medication or merely the fact that the total amount of medication increased over time. To investigate this further, BP values at baseline and at end of follow-up

were shown in relation to total medication use. Although BP decreased considerably over the study period, the total amount of DDD remained virtually the same. The relative contribution of ARA treatment to total DDD increased over time. The possibility that the improved BP reduction during long-term follow-up is due to an increase in total amount of antihypertensive medication can therefore be excluded.

The BP responses in this study were of similar magnitude as those observed in other retrospective or open-label studies concerning add-on ARA treatment.[17-21, 23] Interestingly, in two prospective trials, BP reductions were considerably smaller than in the aforementioned studies. Saha *et al*.[24] studied the effect of spironolactone in black hypertensive patients with uncontrolled BP despite the use of at least a diuretic and a calcium-channel-blocker in a randomized, placebo-controlled manner and reported a reduction of 7.3 mmHg in SBP and 3.3 mmHg in DBP. In a recent study, De Souza *et al.*[22] assessed the effect of open-label spironolactone treatment in resistant hypertension with 24-hour ambulatory BP measurements, thereby eliminating a white-coat effect and at least in part also a placebo effect. In their study, SBP was reduced by 16 mmHg and DBP by 9 mmHg.

The longest follow-up in all mentioned studies was 15 months. Whether the effect persists over a longer period had not yet been reported. With all the limitations of a retrospective design, our study is the first to show that the BP lowering effect of add-on ARA treatment is profound and persistent even after years of treatment.

Earlier publications have focused on identifying clinical and biochemical predictors for the BP response to ARA treatment. Several studies have shown that neither plasma renin concentration or activity, nor aldosterone or the ARR are good predictors for this response,[5, 9, 17, 22, 26] although this may only hold for patients on multidrug regimens[25] related to the interfering effects of many antihypertensives on renin and aldosterone levels.[28] Low serum potassium levels have consistently been shown to be associated with a better response.[19, 21, 22, 25] Other factors potentially related to a better BP response are higher waist circumference, lower aortic pulse wave velocity,[22] the absence of DM,[23] and a lower baseline HDL cholesterol.[21] In a univariate linear regression analysis, we could not confirm the predictive value of the serum potassium level for BP response. However, the sodium/potassium ratio (as a potential indicator for aldosterone excess) showed a significant correlation with SBP decrease at short follow-up at univariate analysis. In a multivariate analysis, only higher baseline BP and longer follow-up duration independently predicted BP response in the long run. Potential explanations for this have been discussed earlier in this section. In our univariate analysis, also haemoglobin, total cholesterol, and LDL for short-term follow-up and left ventricular hypertrophy and urinary albumin-to-creatinine ratio for long-term follow-up were identified as potential predictors. Unfortunately, because of too many missing values, these variables were not included in the multivariate analysis to maintain enough statistical power. However, these parameters are important candidates for further studies on determinants of BP lowering by ARAs. Plasma renin and aldosterone levels were not associated with BP response, as

has been reported earlier. In our univariate analysis, the ARR was weakly, yet significantly, associated with change in DBP. Considering the median ARR of 9.5 pmol/mU in this patient group, a beta coefficient of 0.027 is probably of little relevance. Also the number of patients with ARR available at baseline was too small to include in the multivariate analysis.

The mechanism that underlies the BP-lowering effect of add-on ARA treatment is most likely induction of natriuresis and diuresis although extrarenal effects of aldosterone blockade may also be of importance, such as a reduction in sympathetic tone and modulation of vascular tone, and in the long run a reduction in vascular stiffness may also play a role (reviewed in Jansen *et al.*[29]). The clinical relevance of these extrarenal mechanisms is unknown. A cross-over trial in patients with low-renin hypertension, an elevated ARR, and a previous favourable BP response to spironolactone showed that even in this selected population, high-dose thiazide diuretic treatment was as effective as 100 mg of spironolactone, strongly suggesting that natriuresis is the most important mode of action.[30] This also underscores the relevance of dietary salt reduction in resistant hypertension as has been shown elsewhere.[31] In general, ARA treatment was well tolerated and side effects were rare. In 13 patients, side effects were reported (10.6 %), most of them presenting with gynaecomastia or hyperkalemia. The occurrence of sex hormone-related side effects with spironolactone is dose-dependent,[32] and in many cases, these side effects can be prevented by using lower doses. When this is also not tolerated, treatment with eplerenone, being a more specific ARA with virtually no sex hormone-related actions in therapeutic doses, can be considered.

Risk factors for hyperkalemia are advanced age, diabetes mellitus, higher baseline potassium levels,[33] and advanced stage 3 nephropathy.[34] The presence of renal function impairment and concomitant use of other diuretics predisposes to the development of renal failure.[33] Frequent monitoring of serum potassium and renal function is warranted in these patients.

Our study has several limitations, the most significant one being its retrospective nature. Because of this, there is an important heterogeneity in patients, treatment and follow-up. To properly assess the long-term efficacy of ARA treatment taking into account the large differences in follow-up, stratification to follow-up duration was made. This makes the analysis prone to bias with overrepresentation of patients with a good response in the group of prolonged follow-up. It would have been more ideal to collect patient data at several time points during the follow-up period, but clinical information in the written files was not always present. Furthermore, biochemical parameters, especially haemoglobin and cholesterol (including HDL and LDL) at baseline, were only available for a limited number of patients, thereby limiting their usefulness for multivariate analysis because of lack of statistical power. Also renin and aldosterone levels were only available for a subset of patients.

This study shows that long-term treatment including an ARA leads to a persistent BP reduction. Whether this is attributable to the ARA itself or to better treatment in general is an important point of consideration. As shown, BP reduction was not accompanied by an

increase in total amount of antihypertensive drugs, thereby making a specific effect of the intervention with an ARA more likely. Last, the distinction between patients with EH and PA was solely based on a clinical diagnosis by the patient's physician. A formal confirmation test for PA was only performed in a proportion of the patients labelled with the diagnosis PA. The recent guidelines for the diagnosis and treatment of PA made by the Endocrine Society advise to perform a confirmation test in patients with an ARR above 91 pmol/mU.[35] From the ranges in ARR reported in Table 1, it could be deduced that some of the EH patients actually had PA and that some of the PA patients had been misdiagnosed. However, considering the substantial differences in renin, aldosterone, and potassium levels between our EH and PA patients, we think that the diagnosis was correct in most of the patients.

With all limitations, our results are in favour of a profound and long-term BP lowering effect of ARA treatment in difficult-to-treat hypertension. To assess the magnitude of the response more accurately, a randomized, placebo-controlled trial is needed. With all evidence available, ARAs at moderate dosages are a welcome treatment option in patients with difficult-to-treat or resistant hypertension.

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# Chapter 8

## Determinants of blood pressure reduction by eplerenone in uncontrolled hypertension

Pieter M. Jansen[1], Wijnanda J. Frenkel[2], Bert-Jan H. van den Born[2], Emile L.E. de Bruijne[3], Jaap Deinum[4], Michiel N. Kerstens[5], Joyce H. A. Arnoldus[6], Arend Jan Woittiez[7], Johanna A.M. Wijbenga[8], Robert Zietse[1], A.H. Jan Danser[1], and Anton H. van den Meiracker[1]

[1] *Erasmus Medical Centre, Department of Internal Medicine, Rotterdam, The Netherlands*
[2] *Academic Medical Centre, Department of Internal Medicine, Amsterdam, The Netherlands*
[3] *Red Cross Hospital, Department of Internal Medicine, Beverwijk, The Netherlands*
[4] *Radboud University Nijmegen Medical Centre, Department of Internal Medicine, Nijmegen, The Netherlands*
[5] *University of Groningen, University Medical Centre Groningen, Department of Endocrinology, Groningen, The Netherlands*
[6] *Vrije Universiteit Medical Centre, Department of Internal Medicine, Amsterdam, The Netherlands*
[7] *Ziekenhuis Groep Twente, Department of Internal Medicine, Almelo, The Netherlands*
[8] *Vlietland Hospital, Department of Internal Medicine, Schiedam, The Netherlands*

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## ABSTRACT

*Background:* Add-on therapy with aldosterone-receptor antagonists has been reported to lower blood pressure (BP) in patients with uncontrolled hypertension. We assessed potential predictors of this response.

*Methods:* In essential hypertensive patients with uncontrolled BP, despite the use of at least two antihypertensives, plasma renin and aldosterone concentrations and the transtubular potassium gradient (TTKG) were measured . Patients were treated with eplerenone 50 mg daily on top of their own medication. The office and ambulatory BP response and biochemical changes were evaluated after 1 week and 3 months of treatment and 6 weeks after discontinuation. Potential predictors for the change in 24-h ambulatory BP were tested in a multivariate regression model.

*Results:* One hundred seventeen patients with a mean age of 50.5±6.6 years were included. Office BP decreased from 149/91 to 142/87 mmHg ($p<0.001$) and ambulatory BP from 141/87 to 132/83 mmHg after 3 months of treatment ($p<0.001$). Six weeks after discontinuation of eplerenone, office and ambulatory BP measurements returned to baseline values. Treatment resulted in a small rise in serum potassium and creatinine, and a small decrease in the TTKG. In a multivariate model, neither renin, aldosterone, or their ratio, nor the TTKG predicted the BP response. Only baseline ambulatory SBP predicted the BP response, whereas the presence of left ventricular hypertrophy was associated with a smaller BP reduction.

*Conclusion:* Add-on therapy with eplerenone effectively lowers BP in patients with difficult-to-treat primary hypertension. This effect is unrelated to circulating renin-angiotensin-aldosterone system activity and renal mineralocorticoid receptor activity as assessed by the TTKG.

## INTRODUCTION

Despite the availability of a large arsenal of antihypertensive agents, blood pressure (BP) remains uncontrolled in a substantial proportion of treated patients.[1] Evidence indicates that these patients are at high risk to develop cardiovascular complications.[2] In patients in whom BP is difficult to control with standard antihypertensive treatment, add-on treatment with an aldosterone receptor antagonist (ARA) has been shown to result in a further BP reduction.[3-16] In retrospective studies, this effect ranged up to 25 mmHg in SBP and 12 mmHg in DBP.[7-9, 11, 14, 15] Few prospective studies with add-on ARA therapy have been performed and although a BP-lowering effect was always present, BP reductions were considerably smaller.[6, 10, 16] For instance, the first randomized, placebo-controlled trial on the effect of add-on treatment with spironolactone in patients with resistant hypertension showed a reduction of 5.4 mmHg in daytime SBP and 1.0 mmHg in daytime DBP after 8 weeks of spironolactone.[16] The lack of a control group is likely the most important cause of the difference in BP-lowering effect between the retrospective and prospective studies. Other contributing factors may be selection bias and the inclusion of patients with subtle forms of primary aldosteronism in retrospective studies.

As is common for antihypertensive regimens in general, the BP response to add-on ARA shows a wide interindividual variation. Several investigators have tried to identify factors that predict a favourable BP response, but data on predictors have been conflicting. Lower serum potassium levels have been reported by several groups to be associated with a better BP response,[7, 8, 10, 17] possibly pointing out inclusion of patients with undetected primary aldosteronism. Although some publications have demonstrated that a low plasma renin level [16, 18] or a higher aldosterone-to-renin ratio (ARR)[16] is associated with a better response, most investigators have found that the fall in BP is unrelated to plasma renin and aldosterone levels or the ARR.[4, 13, 19-21] This absent association might be explained by the variable effects of multidrug antihypertensive regimens on aldosterone and renin levels.[17] Other factors reported to be associated with a favourable response were a high baseline BP,[11, 12, 22] a high BMI,[22] an elevated waist circumference, a low aortic pulse wave velocity[10] and a low high-density lipoprotein cholesterol.[8]

The long-term use of spironolactone is limited by the risk of its antiandrogenic and progestogenic side effects.[23] In this respect, prescription of the more selective ARA eplerenone may have advantages. The potential beneficial effects of eplerenone in patients with resistant hypertension have not been studied extensively. Only Calhoun and White performed an open-label, uncontrolled trial in patients with resistant hypertension showing a significant decrease of 12.2 mmHg in 24-h SBP and of 6 mmHg in 24-h DBP after 12 weeks of eplerenone at a dose of 50-100 mg.[12]

The aim of the present study was to investigate the efficacy of add-on therapy with a fixed dose of the selective ARA eplerenone in patients with persistent hypertension despite the

use of at least two antihypertensive agents and to explore the presence of potential determinants for this response. In all patients, primary aldosteronism was excluded by means of an intravenous salt loading test. In addition, the transtubular potassium gradient (TTKG) was assessed as a measure of kidney mineralocorticoid receptor activity.[24, 25]

## METHODS

### Patients

Male and female patients aged 18-65 years with an office SBP above 140 mmHg and/or an office DBP above 90 mmHg despite the use of at least two antihypertensive drugs were invited to participate. The antihypertensive medication was not necessarily given in the maximal dose. Patients were excluded if a known cause of hypertension was present, in case of cardiac chest pain or heart failure, a cerebrovascular or cardiovascular event within 6 months before inclusion, pregnancy, or a known allergy to eplerenone. Beta-adrenergic blockers (BB) as well as potassium-sparing diuretics were discontinued to allow a proper evaluation for the presence of primary aldosteronism. All patients were screened for the presence of primary aldosteronism by the ARR and an intravenous salt loading test.[26] Only patients with a negative salt loading test (as defined by a posttest plasma aldosterone below 235 pmol/L) were included. A total of 28 patients of 183 patients who were initially screened had a positive salt loading test.

### Design

At baseline, office BP, a 24-h ambulatory BP measurement (ABPM) and routine biochemical tests were taken. In addition, clinical data, height and weight, and number and types of antihypertensive drugs were recorded. Patients were screened for the presence of left ventricular hypertrophy (LVH) using a standard 12-lead electrocardiogram (EKG) based on the Sokolow-Lyon criteria.[27] At this point, eplerenone 50 mg once daily was started. The other antihypertensive drugs were left unchanged. One week after initiation of eplerenone, office BP was measured, as well as routine biochemical parameters. If serum potassium level exceeded 5.6 mmol/l, eplerenone dose was reduced to 25 mg. In other cases, eplerenone was continued at the initial dose. After three months of treatment, office BP, 24-h ABPM and biochemical measurements were repeated. At this point, all antihypertensive medications but eplerenone were continued. After another 6 weeks, office BP, 24-h ABPM and biochemical measurements were again performed.

The study was approved by the Institutional Review Board and Ethical Committee of the Erasmus MC in Rotterdam and has been registered at www.clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT00407784). All patients gave written informed consent to participate.

## Blood pressure measurements

Office BP levels were recorded as three subsequent measurements after at least 5 minutes in sitting position with a validated automatic BP measurement device. The mean value of the last two readings was used for the analysis.

Twenty-four hour ABPM was performed using a locally available validated ABPM monitor. For by far the majority of patients, the Spacelabs 90217 or 90207 ABPM monitor was used (Spacelabs Medical, Issaquah, WA, USA). In one centre, the Mobil-o-Graph ABPM monitor (APC Cardiovascular Ltd., Cheshire, UK) was used. BP was recorded every 20 minutes during daytime and every 30 minutes during night time. The measurement was considered valid in case of at least 40 successful readings.

## Biochemical measurements

At all visits, serum sodium, potassium, urea, creatinine, bicarbonate, uric acid levels and osmolality were measured using locally available routine laboratory techniques. In addition, urine samples were collected for determination of sodium, potassium, creatinine and osmolality. The TTKG was calculated as [(urine $K^+$) * (plasma osmolality)] / [(serum $K^+$) * (urine osmolality)].[24] Only urine samples with a urinary $Na^+$ concentration exceeding 25 mmol/L and an osmolality equal or greater than plasma osmolality were used to calculate the TTKG. In some patients, a 24-h urine sample and in other patients a spot urine sample was available. In patients for whom both were available at baseline, the 24-h sample was used to calculate the TTKG. For serial measurements, only urine samples collected via the same sampling method were used.

In the preceding diagnostic phase and after 3 months of treatment, plasma aldosterone concentration (PAC) and plasma renin concentration (PRC) were determined by drawing blood samples in EDTA plasma by venapuncture between 8 and 10 a.m. after 10 min in sitting position. The antihypertensive drugs allowed at the time of sampling were as described previously, that is without beta-adrenergic blocker and potassium-sparing diuretics. The samples were centrifuged at room temperature for 10 min at 3000g.The plasma was then collected and stored at -20 °C until analysis. PAC was measured with a radioimmunoassay (Coat-a-Count; Diagnostics Product Corporation, Los Angeles, California, USA). PRC was assessed using an immunoradiometric assay (Renin III, Cisbio, Gif-sur-Yvette, France).

### Statistical analysis

Values are expressed as mean ± SD, or as median and interquartile range (IQR) when not normally distributed. Categorical values are reported as percentages.

Medication use was quantified by adding up the total number of different antihypertensive drugs, as well as by assessing the defined daily dose (DDD) per drug and for total drug use according to the World Health Organization Anatomical Therapeutic Chemical (ATC) index.[28] Antihypertensive agents were grouped in the following categories: diuretics; renin-angiotensin-system (RAS) blockers (*i.e.* angiotensin-converting enzyme inhibitors (ACE-Is), angiotensin-receptor blockers (ARBs), and renin inhibitors); calcium-channel blockers (CCBs); beta-adrenergic blockers; alpha-adrenergic blockers; and other antihypertensive drugs.

Upon treatment with eplerenone, changes in BP and biochemical parameters were tested with one-way analysis of variance (ANOVA) for repeated measurements. Post-hoc analysis was performed according to Bonferroni correction for multiple testing. Changes in PAC and PRC were tested with the Wilcoxon signed-rank test because of a skewed distribution.

The differences in 24-h BP at baseline and after 3 months of treatment were grouped in tertiles. Intertertile differences in clinical and biochemical parameters were tested with one-way ANOVA with post-hoc testing according to Bonferroni or the Kruskal-Wallis test for parameters with a nonnormal distribution. To test for differences in proportions, the Pearson $\chi^2$ test was applied.

To identify predictors for the BP response, multivariate regression analysis was performed with the change in 24-h ABPM (ΔASBP and ΔADBP) as dependent variable. Serum potassium, PRC, PAC and the ARR at baseline were selected as covariates based on literature findings as described in the previous section. In addition, variables with an association with the dependent value with a p-value less than 0.20 on univariate regression analysis as well as age and sex were included in the model. All analyses were performed in SPSS Statistics 20 for MacOS X (IBM, Armonk, New York, USA).

## RESULTS

### Study population

A total of 141 patients was included in the study protocol. Twelve patients were excluded before completion of the protocol: four patients because of adverse events, two patients because of a myocardial infarction, one patient because of a hypertensive urgency, and five patients were lost to follow-up. Another 12 patients had a switch in antihypertensive medication other than eplerenone and were excluded from the analysis. In total, 117 patients with a mean age of 51 ± 9.7 years were included in the analysis. The mean office BP was

150/92 (±18/11) mmHg. They used a median number of 3 (IQR 2-3) antihypertensive drugs (total DDD 4.5 (3-5.5)). Diuretics were used by 75 % of patients, RAS blockers by 87 %, CCB by 81 %, alpha-adrenergic blockers by 22 % and other antihypertensive drugs by 1.7 % of patients. Note that beta-adrenergic blockers were discontinued in an earlier phase to allow a

**Table 1.** Baseline characteristics of the total study population (n=117).

| | Mean ± SD or median (IQR) |
|---|---|
| **Age (years)** | 50.5 ± 6.6 |
| **Men (%)** | 56.4 |
| **BMI (kg/m²)** | 28.8 (26.5-32.1) |
| **Whites (%)** | 67.5 |
| **Smokers (%)** | 28.4 |
| **History of CVD (%)** | 11.2 |
| **DM (%)** | 11.1 |
| **LVH (%)** | 19.2 |
| **ACR (mg/mmol)** | 1.1 (0.5-2.5) |
| **Duration of hypertension (months)** | 64 (23-161) |
| **Office SBP (mmHg)** | 150.0 ± 17.8 |
| **Office DBP (mmHg)** | 91.6 ± 10.9 |
| **Office pulse (BPM)** | 77.4 ± 12.7 |
| **24-h SBP (mmHg)** | 140.6 ± 14.9 |
| **24-h DBP (mmHg)** | 87.1 ± 8.6 |
| **$Na^+$ (mmol/l)** | 141.8 ± 2.5 |
| **$K^+$ (mmol/l)** | 4.0 ± 0.5 |
| **Serum creatinine (µmol/l)** | 78.6 ± 17.7 |
| **$Na^+$ excretion (mEq/24 h)** | 174.4 (117.0-221.0) |
| **TTKG** | 6.4 ± 2.1 |
| **PRC (mU/l)** | 23.4 (11.7-72.1) |
| **PAC (pmol/l)** | 227 (152-360) |
| **ARR (mmol/mU)** | 7.0 (2.5-18.9) |
| **No. of antihypertensives** | 3.0 (2-3) |
| **DDD** | 4.5 (3.0-5.5) |
| **Diuretics (%)** | 75.2 |
| **RAS blockers (%)** | 87.2 |
| **CCB (%)** | 81.2 |
| **Beta-adrenergic blockers (%)** | 0.9 |
| **Alpha-adrenergic blockers (%)** | 22.2 |
| **Other antihypertensives (%)** | 1.7 |

ACR, urinary albumin-to-creatinine ratio; ARR, aldosterone-to-renin ratio; BPM, beats per minute; CCB, calcium channel blocker; CVD, cardiovascular disease; DDD, defined daily dose; DM, diabetes mellitus; IQR, interquartile range; $K^+$, serum potassium; LVH, left ventricular hypertrophy; $Na^+$, serum sodium; PAC, plasma aldosterone concentration; PRC, plasma renin concentration; RAS, renin-angiotensin system; TTKG, transtubular potassium gradient.

proper diagnostic workup for primary aldosteronism. However, one patient still used a beta-adrenergic blocker at inclusion. Eleven percent had diabetes mellitus, 28 % smoked (included are patients who quit smoking less than 5 years ago) and 11 % had a history of cardiovascular disease. Patient characteristics are displayed in Table 1.

## Blood pressure response

Figure 1 shows the mean office BP at baseline, after 1 week of treatment with eplerenone 50 mg, after 3 months of treatment, and 6 weeks after discontinuation of treatment. Of the total study population of 117 patients, data of 102 patients were available for this analysis: seven patients were excluded from this analysis because they continued eplerenone after 3 months, and of eight patients, BP values of one of the visits was missing. In four patients, the eplerenone dose was reduced to 25 mg (because of palpitations in one patient and for unknown reasons in three patients). SBP dropped from 149.0 ± 17.8 to 144.1 ± 16.7 mmHg after 1 week, and to 141.8 ± 18.7 mmHg after 3 months. When eplerenone was stopped, SBP

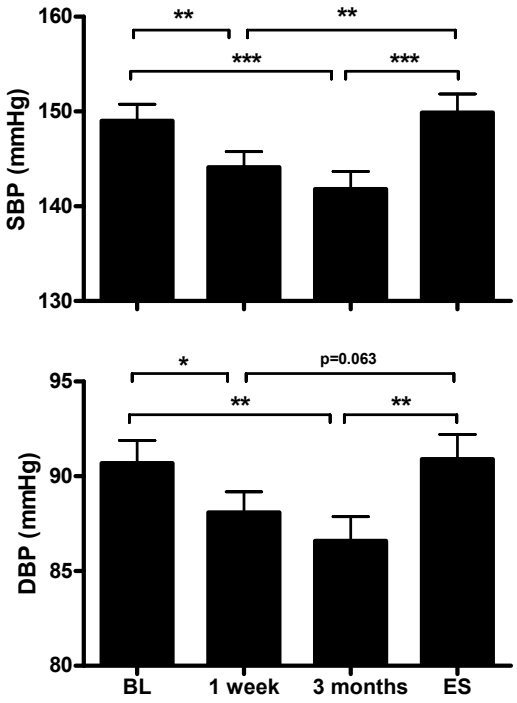


**Figure 1**: Office SBP and DBP at baseline, after 1 week and 3 months of add-on treatment with eplerenone, and 6 weeks after eplerenone was stopped (ES). (Overall p-value<0.001 (one-way analysis of variance, ANOVA for repeated measurements) for both SBP and DBP). BL, baseline.

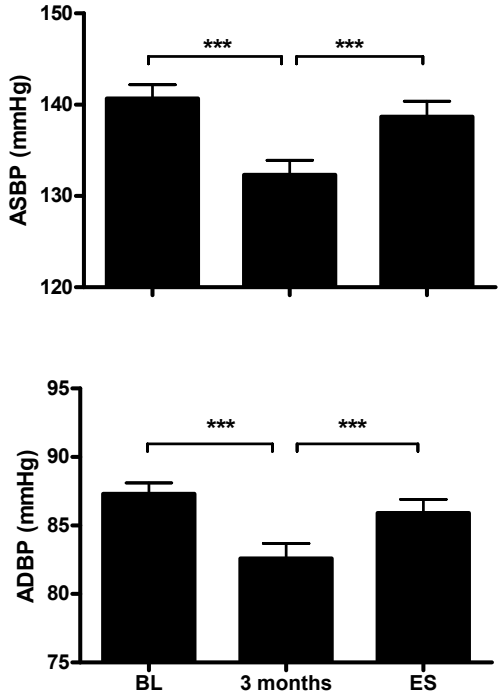


**Figure 2.** Ambulatory SBP (ASBP) and DBP (ADBP) at baseline, after 3 months of eplerenone and 6 weeks after eplerenone was stopped (ES). (Overall p-value<0.001 (one-way analysis of variance, ANOVA for repeated measurements) for both SBP and DBP). BL, baseline.

returned to baseline values (149.9 ± 19.7 mmHg, p<0.001 for total trend). DBP levels were 90.7 ± 11.0, 88.1 ± 10.9, 86.6 ± 12.8 and 90.9 ± 13.3 mmHg at baseline, after 1 week of treatment, after 3 months of treatment and 6 weeks after discontinuation, respectively (P<0.001 for total trend). The pulse rate was similar at all time points.

A similar pattern was observed for 24-h ABPM at baseline, after 3 months of eplerenone treatment and 6 weeks after discontinuation (Figure 2, total number available for analysis was 97 patients). Ambulatory SBP (ASBP) decreased from 140.7 ± 15.1 to 132.3 ± 15.7 mmHg (p<0.001), and ambulatory DBP (ADBP) from 87.3 ± 8.3 to 82.6 ± 10.6 mmHg (p<0.001). After cessation of eplerenone, ASBP rose again to 138.7 ± 16.6 mmHg (p<0.001) and ADBP to 85.9 ± 9.6 mmHg (p<0.001).

## Biochemical changes

There was a small but significant rise in serum potassium and serum creatinine after start with eplerenone (Table 2). This rise was consistent during the total treatment period and values returned to baseline after eplerenone was stopped. Serum sodium and bicarbonate

**Table 2.** Changes in biochemical parameters after 1 week and 3 months of treatment with eplerenone and 6 weeks after its discontinuation.

| | n | Baseline | 1 week | 3 months | Discontinuation | p-value |
|---|---|---|---|---|---|---|
| **$K^+$ (mmol/l)** | 109 | 4.0 ± 0.5 | 4.2 ± 0.4 | 4.2 ± 0.4 | 4.0 ± 0.5 | <0.001 |
| **$Na^+$ (mmol/l)** | 109 | 142.0 ± 2.5 | 141.4 ± 2.3 | 141.1 ± 2.9 | 141.6 ± 2.5 | 0.003 |
| **Serum creatinine (µmol/l)** | 107 | 78.7 ± 17.9 | 80.7 ± 18.2 | 80.6 ± 20.2 | 78.0 ± 19.0 | <0.001 |
| **Serum bicarbonate (mmol/l)** | 91 | 27.6 ± 4.2 | 27.2 ± 2.7 | 26.4 ± 4.1 | 27.5 ± 3.2 | 0.013 |
| **TTKG** | 40 | 7.0 ± 2.0 | 6.3 ± 2.1 | 5.9 ± 1.9 | 6.5 ± 1.8 | 0.035 |
| **PRC (mU/l)** | 93 | 30.2 (12.5-71.9) | | 55.0 (18.7-154.1) | | <0.001 |
| **PAC (pmol/l)** | 93 | 241 (158-388) | | 396 (242-576) | | <0.001 |
| **ARR (pmol/mU)** | 93 | 7.3 (2.8-23.7) | | 7.5 (2.4-16.6) | | 0.071 |

ARR, aldosterone-to-renin ratio; $K^+$, serum potassium; $Na^+$, serum sodium; PAC, plasma aldosterone concentration; PRC, plasma renin concentration; TTKG, transtubular potassium gradient. Data are mean±SD or median and IQR.

showed a marginal lower value with eplerenone. The TTKG was significantly lower after 3 months of treatment compared to baseline (P=0.028; Table 2). Median renin levels increased from 30.2 (IQR 12.5-71.9) mU/l at baseline to 55.0 (18.7-154.1) mU/l (p<0.001) after 3 months. PAC rose from 241 (158-388) to 396 (242-576) pmol/l (p<0.001). There was a trend towards a slightly higher ARR after treatment (p=0.071; Table 2).

## Determinants for the blood pressure response

To identify potential predictors for the BP response to eplerenone, the differences in ASBP (ΔASBP) and ADBP (ΔADBP) between baseline and after 3 months of treatment were arranged in tertiles. Patients with valid ABPM data available at both time points were included in this analysis (n=103). Table 3 shows the clinical characteristics for each tertile. Patients in the highest tertile for ΔASBP were older than those in the lowest tertile (53.8 ± 8.1 compared to 48.0 ± 10.3 years, p=0.039). In addition, a larger proportion of patients with a poor response had LVH. There were no other differences in BP, biochemical profile and medication use between the tertiles.

Subsequently, a multivariate linear regression analysis was performed for ΔASBP and ΔADBP (Table 4). A higher baseline ASBP was associated with a greater reduction in ASBP. The presence of LVH was associated with a smaller response. For ΔADBP, no significant predictors were found, although a trend existed toward a smaller response for patients with LVH. Of note, neither serum potassium levels nor the ARR predicted the BP response. Substitution of the ARR by PRC and PAC showed no significant relation (data not shown). In addition, the 24-h sodium excretion, the change in PRC after eplerenone treatment and the number and

**Table 3.** Characteristics of patients grouped into tertiles of the difference in 24-h ambulatory SBP (ΔASBP) and DBP (ΔADBP) between 3 months of add-on eplerenone treatment and baseline.

| | ΔASBP | | | | ΔADBP | | | |
|---|---|---|---|---|---|---|---|---|
| | 1st tertile | 2nd tertile | 3rd tertile | p-value | 1st tertile | 2nd tertile | 3rd tertile | p-value |
| **ΔASBP (mmHg)** | 4.8 ± 8.5 | -7.4 ± 2.6 | -21.1 ± 9.2 | | | | | |
| **ΔADBP (mmHg)** | | | | | 3.8 ± 4.9 | -4.7 ± 1.2 | -12.7 ± 5.0 | |
| **Age (years)** | 48.0 ± 10.3 | 49.5 ± 10.5 | 53.8 ± 8.1 | 0.037 | 48.4 ± 9.5 | 50.2 ± 10.3 | 52.6 ± 9.9 | 0.286 |
| **Men (%)** | 63.9 | 56.2 | 60 | 0.813 | 66.7 | 55.9 | 57.6 | 0.610 |
| **Duration of HT (months)** | 55.5 (13.3-159) | 61.0 (14.0-197.0) | 84.5 (44.0-172.8) | 0.564 | 61.0 (14.0-176.0) | 64.0 (30.0-189.3) | 75.5 (26.0-168.3) | 0.913 |
| **Smoking (%)** | 40.0 | 21.9 | 20.0 | 0.120 | 27.8 | 33.3 | 21.2 | 0.543 |
| **DM (%)** | 8.3 | 6.2 | 20.0 | 0.160 | 8.3 | 11.8 | 15.2 | 0.678 |
| **BMI (kg/m²)** | 29.0 (25.1-33.0) | 27.5 (25.9-31.5) | 28.9 (27.2-31.4) | 0.644 | 29.2 (25.3-33.5) | 28.5 (26.0-34.2) | 28.2 (27.1-30.6) | 0.518 |
| **Whites (%)** | 69.4 | 62.5 | 74.3 | 0.580 | 63.9 | 67.6 | 75.8 | 0.557 |
| **LVH (%)** | 25.0 | 34.5 | 3.3 | 0.010 | 33.3 | 20.7 | 6.9 | 0.038 |
| **History of CVD (%)** | 11.4 | 9.4 | 14.3 | 0.821 | 5.7 | 17.6 | 12.1 | 0.305 |
| **24-h ASBP (mmHg)** | 138.1 ± 12.6 | 138.4 ± 15.5 | 144.9 ± 16.7 | 0.103 | 139.9 ± 14.4 | 136.7 ± 12.7 | 145.1 ± 17.5 | 0.075 |
| **24-h ADBP (mmHg)** | 88.0 ± 9.1 | 86.3 ± 6.4 | 87.9 ± 9.9 | 0.674 | 88.2 ± 8.4 | 85.2 ± 8.1 | 89.0 ± 9.1 | 0.162 |
| **$Na^+$ (mmol/l)** | 142.3 ± 2.5 | 141.2 ± 2.7 | 142.3 ± 1.9 | 0.104 | 142.2 ± 2.7 | 141.7 ± 2.4 | 141.9 ± 2.1 | 0.722 |
| **$K^+$ (mmol/l)** | 4.1 ± 0.5 | 3.9 ± 0.5 | 4.0 ± 0.5 | 0.576 | 4.0 ± 0.5 | 4.0 ± 0.4 | 4.0 ± 0.5 | 0.878 |
| **Creatinine (μmol/l)** | 78.9 ± 15.0 | 79.3 ± 19.3 | 80.9 ± 18.9 | 0.886 | 81.7 ± 18.4 | 74.9 ± 13.0 | 82.5 ± 20.1 | 0.145 |
| **$Na^+$ excretion (mmol/24 h)** | 177 (81-216) | 154 (125-240) | 178 (123-226) | 0.398 | 171 (82-235) | 178 (141-227) | 176 (120-221) | 0.690 |
| **TTKG** | 6.3 ± 2.2 | 6.7 ± 1.6 | 6.6 ± 2.4 | 0.798 | 6.4 ± 2.0 | 6.4 ± 1.9 | 6.7 ± 2.3 | 0.833 |
| **PRC (mU/l)** | 20.8 (12.6-91.2) | 28.7 (11.8-110.2) | 20.7 (11.0-58.1) | 0.739 | 26.4 (12.6-91.2) | 19.6 (10.2-77.3) | 26.6 (14.5-66.4) | 0.777 |
| **PAC (pmol/l)** | 238.2 (186.3-398.2) | 249.3 (169.7-393.3) | 221.6 (120.5-322.7) | 0.288 | 227.1 (187.7-355.3) | 227.1 (166.2-355.9) | 231.3 (117.0-438.4) | 0.930 |
| **ARR (pmol/mU)** | 8.8 (2.5-22.4) | 7.3 (2.0-22.8) | 5.4 (2.5-16.5) | 0.774 | 5.8 (2.3-20.7) | 7.8 (2.7-24.6) | 5.2 (2.5-12.8) | 0.486 |
| **DDD** | 4.5 (3.6-5.5) | 4.3 (3.0-6.9) | 4.0 (3.0-5.5) | 0.749 | 5.0 (3.6-5.9) | 4.0 (3.4-5.9) | 4.0 (3.0-5.4) | 0.545 |
| **Diuretics (%)** | 72.2 | 87.5 | 74.3 | 0.268 | 80.6 | 79.4 | 72.7 | 0.706 |
| **RAS blockers (%)** | 83.3 | 93.8 | 88.6 | 0.409 | 86.1 | 91.2 | 87.9 | 0.800 |
| **CCB (%)** | 88.9 | 78.1 | 80.0 | 0.450 | 83.3 | 82.4 | 81.8 | 0.986 |
| **Alfa-blockers (%)** | 22.2 | 18.8 | 17.1 | 0.858 | 16.7 | 26.5 | 15.2 | 0.441 |

ARR, aldosterone-to-renin ratio; CCB, calcium channel blocker; CVD, cardiovascular disease; DDD, defined daily dose; DM, diabetes mellitus; HT, hypertension; $K^+$, serum potassium; LVH, left ventricular hypertrophy; $Na^+$, serum sodium; PAC, plasma aldosterone concentration; PRC, plasma renin concentration; RAS, renin-angiotensin system; TTKG, transtubular potassium gradient. Data are mean±SD or median and IQR.

**Table 4.** Multivariate linear regression model for the change in 24-h ambulatory SBP and DBP. Data of 89 patients was available for this analysis.

| | ΔASBP | | | ΔADBP | | |
|---|---|---|---|---|---|---|
| | β | SEM | p-value | β | SEM | p-value |
| **Age** | 0.01 | 0.16 | 0.949 | 0.04 | 0.10 | 0.711 |
| **Sex (0=M, 1=F)** | 0.6 | 2.97 | 0.844 | 0.2 | 1.85 | 0.930 |
| **Ethnicity (0=nonwhite, 1=white)** | 3.8 | 2.95 | 0.205 | 2.9 | 1.86 | 0.125 |
| **Smoking (0/1)** | -5.7 | 2.98 | 0.057 | -2.8 | 1.87 | 0.142 |
| **DM (0/1)** | 7.9 | 4.45 | 0.080 | 3.6 | 2.81 | 0.209 |
| **History of CVD (0/1)** | 4.4 | 4.29 | 0.312 | 3.2 | 2.69 | 0.234 |
| **24-h SBP** | 0.31 | 0.095 | 0.002 | | | |
| **24-h DBP** | | | | 0.14 | 0.097 | 0.171 |
| **$K^+$ (mmol/l)** | -0.96 | 2.79 | 0.731 | -0.81 | 1.76 | 0.646 |
| **LVH (0/1)** | -7.7 | 3.69 | 0.043 | -4.2 | 2.31 | 0.074 |
| **ARR (pmol/mU)** | 0.05 | 0.17 | 0.790 | 0.05 | 0.04 | 0.233 |

ADBP, ambulatory diastolic blood pressure; ARR, aldosterone-to-renin ratio; ASBP, ambulatory systolic blood pressure; CVD, cardiovascular disease; DM, diabetes mellitus; $K^+$, serum potassium; LVH, left ventricular hypertrophy; SEM, standard error of the mean.

subclasses of antihypertensive medication had no association with the BP response at univariate regression analysis and were not included in the multivariate model (data not shown).

### Adverse events

Of the initial study population of 141 patients, 44 patients reported a total of 57 adverse events. Most adverse events were mild and had no suspected relation to treatment. Most reported were dizziness (14%), gastrointestinal complaints (12.3%), tiredness (10.5%), headache (7%) and edema and weight gain (7%). Seven patients left the study protocol because of adverse events: two patients had a myocardial infarction during eplerenone treatment, one patient had a hypertensive urgency, three patients had gastrointestinal complaints and one patient suffered from orthostatic hypotension. One patient developed a mild hyperkalemia (5.9 mmol/l) 1 week after start with eplerenone but serum potassium levels spontaneously normalized to 4.6 mmol/l after 3 months of treatment.

## DISCUSSION

Several studies have demonstrated that treatment of patients with resistant hypertension with ARAs leads to a further, in many cases impressive, BP reduction. However, most of these studies have methodological limitations, being retrospective, lacking objective BP record-

ings or a placebo-controlled design and including patients with primary aldosteronism.[3-5, 7-9, 11, 12, 14, 15] In recent years, a few prospective studies have been reported. In these studies, a reduction in BP during add-on therapy with an ARA was still observed, though substantially smaller than that in the retrospective studies.[6, 10, 16] To our knowledge, only one trial has assessed the effect of the selective ARA eplerenone.[12]

Our study shows that the addition of eplerenone to existing BP-lowering treatment in patients with uncontrolled hypertension, in whom primary aldosteronism was excluded by a salt loading test, results in a significant decrease in both office and 24-h ambulatory BP. After 3 months of treatment, ambulatory BP was reduced by 8.4 mmHg systolic and 4.7 mmHg diastolic. This response is of comparable magnitude as reported in other studies in which 24-h ABPM or a placebo-controlled design was used (Table 5).

The mechanism of the BP-lowering effect of ARAs in resistant hypertension is incompletely understood. It has been suggested that a relative aldosterone excess contributes to therapy resistance. In patients with an elevated ARR and plasma aldosterone level, but a negative confirmation test for primary aldosteronism, BP control was more difficult to achieve than in

**Table 5.** Overview of studies on the antihypertensive effects of add-on treatment with aldosterone receptor antagonists with either a placebo-controlled design or use of 24-h ambulatory blood pressure monitoring.

| Reference | Population | n | Study design | ARA | Follow-up | Reduction (SEM) in SBP (mmHg) | Reduction (SEM) in DBP (mmHg) | Reduction (SEM) in ASBP (mmHg) | Reduction (SEM) in ADBP (mmHg) |
|---|---|---|---|---|---|---|---|---|---|
| Saha *et al*.[6] | Black hypertensive patients with elevated BP despite treatment including a diuretic and a CCB | 98 | Randomized, placebo-controlled trial | Spironolactone 25 mg | 9 weeks | 7.3 (2.3)[a] | 3.3 (1.4)[a] | | |
| De Souza *et al*.[10] | Patients with resistant hypertension | 175 | Open-label, uncontrolled trial | Spironolactone 25-100 mg (median 50 mg) | Median 7 months | 14 (2.3) | 7 (1.3) | 16 (1.3) | 9 (0.8) |
| Václavík *et al*.[16] | Patients with resistant hypertension | 117 | Randomized, placebo-controlled trial | Spironolactone 25 mg | 8 weeks | 6.5 (2.9)[a] | 2.5 (1.3)[a] | 9.8 (2.3)[a] | 1.0 (1.4) [a] (NS) |
| This study | Patients with uncontrolled HT despite at least two antihypertensives | 117 | Open-label, uncontrolled trial | Eplerenone 50 mg | 3 months | 7.2 (1.5) | 4.1 (1.0) | 8.4 (1.3) | 4.7 (0.8) |

ADBP, ambulatory DBP; ARA, aldosterone-receptor antagonist; ASBP, ambulatory SBP; CCB, calcium channel blocker; HT, hypertension; NS, not significant.

[a]compared with placebo.

patients with a low ARR.[29] In addition, a large proportion of patients treated with an ACE-I or ARB shows "aldosterone escape", a situation in which aldosterone levels after an initial decline rise to or even above pretreatment levels during prolonged treatment with these agents.[30, 31] This secondary rise in aldosterone counteracts the BP reduction and possibly contributes to therapy resistance. Moreover, aldosterone excess is thought to be an important mediator of structural cardiovascular damage such as arterial stiffness,[32, 33] which in the long run may further increase BP levels and impair the response to BP-lowering agents. From this perspective, it is reasonable to assume that ARAs are particularly effective in patients with aldosterone excess. However, indices of aldosterone excess such as a low renin level, a high ARR or a low serum potassium level have not uniformly been shown to predict the response to ARAs.[4, 13, 16, 18-21] Likewise, in our study, no relation with any of these variables could be found, although it cannot be ruled out completely that the use of antihypertensive medication at the time of sampling with its associated effects on PRC and PAC may have contributed to this lack of correlation. The TTKG, considered to be a measure of aldosterone activity in the distal convoluted tubule and cortical collecting duct,[24, 25] did not correlate with the eplerenone-induced BP reduction, although a small but significant decrease in the TTKG after treatment with eplerenone was observed. Also the 24-h sodium excretion was not related to the reduction in BP. Inhibition of the extrarenal effects of aldosterone such as a direct regulatory action of vascular tone[34] could play a role as well. Only a higher baseline ambulatory SBP was associated with a larger fall in SBP. However, this is a nonspecific response as it has been observed with different classes of antihypertensive drugs. With all limitations in sensitivity,[35] the presence of LVH on the EKG was associated with a smaller response in BP. The presence of LVH usually implies more severe hypertension and structural vascular changes, which in turn may impair the efficacy of BP-lowering agents.

Antihypertensive drug use had no effect on BP reduction. It should be noted that beta-adrenergic blockers were discontinued before start of the study protocol. Because beta-adrenergic blockers lead to a reduction in PRC, it could be that these agents can hamper the secondary rise in PRC after eplerenone treatment, thereby potentiating its BP-lowering effect. However, we did not observe a relation between the change in PRC and the BP response, making this influence unlikely.

ARAs are generally well tolerated. However, gynecomastia, erectile dysfunction and menstrual disorders are well-known side-effects of spironolactone, especially when used in higher dosages.[23] Eplerenone, being a more selective ARA, has a much lower incidence of sex hormone-related side-effects and can replace spironolactone when this latter compound is not well tolerated.[36] In our study, one patient reported loss of libido, but no cases of gynecomastia or other sex hormone-related side-effects were seen. Although 31 % of patients reported an adverse event during treatment with eplerenone, these were predominantly mild and in most cases probably unrelated to the study medication. Because our study lacked a placebo arm, the true incidence of adverse effects attributable to eplerenone cannot be

assessed. Two patients developed a myocardial infarction and one patient was excluded because of a hypertensive urgency. A relation with the study medication is unlikely and these events probably reflect the high cardiovascular risk profile of the study population.

Another point of concern during treatment with an ARA is the risk of hyperkalemia and deterioration of renal function. Although a significant rise in serum potassium and creatinine levels after eplerenone treatment was observed, this rise was marginal and remained stable over the treatment period of 3 months. Only one patient had a serum potassium level of 5.9 mmol/L after 1 week of treatment, but this normalized without any dose reduction. Pre-existent impairment of renal function,[22] pretreatment elevated potassium levels,[22, 37] higher age, diabetes mellitus, and use of beta-adrenergic blockers[37] have all been identified as risk factors for the occurrence of hyperkalemia, whereas a higher baseline serum creatinine and concomitant use of other diuretics were shown to be associated with the development of renal insufficiency.[37] Therefore, the advice remains to closely monitor serum potassium and creatinine levels, especially when such risk factors are present. We also observed a rise in PAC during add-on treatment with eplerenone. This rise is probably related to a rise in PRC due to volume depletion and the rise in potassium concentration.

As mentioned, most studies concerning the BP-lowering effect of add-on ARA therapy are retrospective. Our study further augments the necessary prospective evidence for their efficacy. The most important limitations of our study are the relatively small numbers and the absence of a blinded, placebo-controlled design. However, the use of 24-h ABPM is known to largely preclude the occurrence of observer bias and placebo-related effects.[38, 39] In addition, the almost complete return of BP to baseline levels after cessation of eplerenone provides strong evidence for a genuine treatment effect. Another limitation is that only patients who completed the full trial with no change in medication other than the study medication were included in the analysis. This could have resulted in selection of patients with a favourable response. However, when including all patients with available data at baseline and 3 months of treatment, the BP response was of similar order of magnitude (office BP decreased from 150.7/92.0 ± 20.2/12.7 to 142.4/87.4 ± 20.1/14.0 ($p<0.001$) making selection bias less likely.

The precise delineation of the place of ARAs in the treatment of patients with difficult-to-treat and resistant hypertension has not been well established. Although both in the European and American hypertension management guidelines, the use of an ARA has been recommended in case of resistant hypertension,[40, 41] their place within the therapeutic arena remains to the judgement of the individual physician. This may lead to underuse, as has recently been shown in a retrospective cohort study evaluating the prescription of BP-lowering agents in patients with resistant hypertension. In this large study, ARAs were prescribed in only 5.9% of patients.[42] Reports on how the efficacy of ARAs relates to other forms of add-on treatment are scarce. When studied in a cross-over design, spironolactone had a greater antihypertensive effect than combination treatment with an ACE-I and ARB.[13] Also in comparison to doxazosin, BP reduction was larger when spironolactone was added to the

therapeutic regimen in patients with resistant hypertension.[9] As also observed in our study, not all patients with resistant hypertension respond favourably to add-on ARA treatment. Patients with hypertension refractory to BP-lowering treatment in general, displayed a smaller response to spironolactone than patients who were responsive to treatment.[43] Segura *et al*.[44] showed that in these patients, the addition of a triple therapy consisting of substitution of the diuretic with chlorthalidone, addition of amlodipine (if not yet prescribed), and the direct renin inhibitor aliskiren on top of the already used treatment can result in a further relevant BP reduction. Future research should focus on identifying the different mechanisms involved in treatment resistance in order to select the best combination of antihypertensive drugs for each individual patient. Also, comparison of ARAs with innovative treatment modalities such as endovascular catheter-based renal sympathetic denervation[45] would be of great interest. Although renal sympathetic denervation has been reported to have a large effect on office BP, the effect on ambulatory BP is comparable to that observed with add-on ARA treatment.[45] Finally, the importance of lifestyle measures, especially a reduction in dietary salt intake, should be emphasized. The median sodium excretion in our population was 174 mmol per day. Probably, the BP of a large proportion of our patients would have benefited from dietary sodium reduction.[46]

## CONCLUSION

In conclusion, eplerenone effectively lowers BP in patients with difficult-to-treat hypertension without evidence of primary aldosteronism. This effect is unrelated to parameters of aldosterone excess such as a high plasma aldosterone, low plasma renin, high ARR or a high TTKG. In our population, the presence of LVH was associated with a smaller BP reduction. Additional studies to compare aldosterone receptor blockade with other treatment modalities are warranted to select the most optimal treatment strategy for the individual patient with difficult-to-treat or resistant hypertension.

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# Part V

## Summary, discussion and perspectives

# Chapter 9

## Summary, discussion and perspectives

## SUMMARY

The adrenocortical hormone aldosterone is a key factor in the regulation of volume homeostasis and blood pressure regulation. However, in pathological circumstances, it is a mediator of elevated blood pressure and end-organ damage, such as cardiac and vascular remodelling and fibrosis. The pathophysiological role of aldosterone in hypertension and aldosterone antagonism as a therapeutic target are further explored in this thesis.

### Regulation of aldosterone synthesis

The classical view is that the renin-angiotensin-aldosterone-system (RAAS) is the main regulator of aldosterone synthesis through the stimulation of angiotensin II type 1 receptors (AT1R) in adrenocortical cells by angiotensin II (Ang II). In addition, potassium and adrenocorticotropic hormone (ACTH) are known inductors of aldosterone synthesis **(Chapter 2)**. However, the regulation of aldosterone synthesis is far more complex and several other factors, including adipose tissue factors, are thought to affect steroid synthesis.[1] Treatment with angiotensin-converting enzyme inhibitors (ACE-Is) or angiotensin receptor blockers (ARBs) normally decreases aldosterone levels by interrupting the RAAS at the level of Ang II production or action. This results in a compensatory rise in renin **(Chapter 1)**. It is known, however, that a significant number of patients shows "aldosterone breakthrough", indicating that after an initial decline, aldosterone levels go up during prolonged treatment.[2, 3] The mechanisms for aldosterone breakthrough are incompletely understood. Binding of prorenin to the (pro) renin receptor ((P)RR) promotes non-proteolytic activation of prorenin to display angiotensin I-generating activity.[4] Moreover, recent studies have shown that binding of renin and prorenin to the (P)RR can induce direct, angiotensin-independent effects through the activation of mitogen-activated protein (MAP) kinases, including extracellular signal regulated kinase 1/2 (ERK1/2).[5, 6] Furthermore, transgenic rats expressing the human (P)RR have elevated aldosterone levels despite normal Ang II levels.[7, 8] Based on these observations, we postulated that (pro)renin could induce adrenal aldosterone synthesis directly via stimulation of the (P) RR. This was investigated *in vitro* in two human adrenocortical cell lines (H295R and HAC15) **(Chapter 3)**. The presence of the (P)RR in human adrenal tissue has been reported before[9] and we confirmed this in isolated adrenal tissues and the adrenocortical cell line H295R. However, stimulation of the (P)RR with renin and prorenin at nanomolar concentrations did not induce any expression of the major enzymes involved in steroid synthesis, phosphorylation of ERK1/2, or aldosterone production. Ang II, in contrast, rapidly induced ERK phosphorylation, the expression of several steroidogenic enzymes and aldosterone production in these cells. These experiments rule out that a direct action of (pro)renin on (P)RRs in adrenocortical cells is the underlying mechanism for aldosterone breakthrough during ACE-I or ARB treatment.

## Screening for primary aldosteronism

Around 8% of the hypertensive population suffers from primary aldosteronism (PA), a condition characterized by (relatively) autonomous overproduction of aldosterone by the adrenal gland. In contrast to previous beliefs, a minority of these patients has an aldosterone-producing adenoma (APA), as originally described by Jerome Conn, and a minority presents with hypokalemia **(Chapter 4)**. It is clinically important to diagnose these patients early, because they often suffer from a hypertension that is difficult to treat, because they have a higher cardiovascular risk[10] due to the deleterious effects of aldosterone on vascular[11] and cardiac[12] tissues and because specific treatment may be available, *i.e.* treatment with aldosterone-receptor antagonists (ARAs) in bilateral disease and adrenalectomy in patients with an APA.[13] The aldosterone-to-renin ratio (ARR)[14] is recommended as the screening test of choice in the Endocrine Society Clinical Practice Guideline for the diagnosis and treatment of PA.[15] However, the validity of the ARR has remained subject of debate, because of concerns about a poor test performance,[16] low reproducibility[17] and the economic costs associated with widespread screening.[16] Interpretation of the test results is further complicated by the many factors that influence the ARR, including antihypertensive medication **(Chapter 4)**. We studied the test characteristics of the ARR in 178 patients with difficult-to-treat hypertension **(Chapter 5)**. Aldosterone and renin levels were assessed twice within 4 weeks on a random antihypertensive treatment regime. Subsequently, all patients were subjected to an intravenous salt loading test (SLT) as the gold standard to diagnose or exclude PA. To study the effect of antihypertensive medication on the ARR, the treatment was then switched to a standardized regime, consisting of a calcium channel blocker and an alpha-adrenergic blocker, which are thought to have minimal effect on renin and aldosterone levels. The ARR was repeated after four to six weeks on standardized treatment. The prevalence of PA in our population was 15%. When the recommended[15] cutoff value for the ARR was used, the sensitivity of the test was only 22%. On standardized treatment, the ARR levels were significantly higher. Multivariate regression analysis showed that ACE-Is and ARBs were responsible for the lower ARR under random treatment. The overall test performance, however, expressed as the area under the receiver operating characteristic curve, remained unchanged (84% during random treatment versus 86% during standardized treatment; p=0.314). The overlap in ARR values between PA and essential hypertensive patients, is considerable, resulting in a very low test specificity if the cutoff is lowered to a level with acceptable sensitivity. The usefulness of the ARR is further compromised by its large within-patient variability: when taken under the same conditions, we observed differences in ARR values that could be as large as 5-fold.

## Aldosterone-receptor antagonism in hypertension

For a long time, ARAs were mainly prescribed in specific conditions such as heart failure and PA. This is surprising, considering the well-recognized role of aldosterone in the pathophysiology of hypertension and cardiovascular disease. Aldosterone may be especially relevant in patients with hypertension that is not easily controlled with one or two agents or that is even resistant to treatment. In the last 10 years, these agents have regained interest as a potent treatment modality in resistant hypertension **(Chapter 6)**. The largest study in this field was by Chapman *et al*.[18] who evaluated the blood pressure response of 1411 patients in the Anglo-Scandinavian Cardiac Outcome Trial – Blood Pressure Lowering Arm (ASCOT-BPLA). These patients received additional spironolactone because of sustained hypertension despite the use of three antihypertensive drugs. After the addition of a relatively low median dose of 25 mg spironolactone, an impressive systolic and diastolic blood pressure reduction of 21.9 and 9.5 mmHg, respectively, was observed. The problem with this study was its retrospective design and the lack of a control group. Other studies showed similar blood pressure reductions, but also had methodological shortcomings, being retrospective, lacking objective BP recordings or lacking a placebo-controlled design. In recent studies, using either 24-hr ambulatory blood pressure monitoring (ABPM)[19] or a placebo-controlled design[20] more modest results were observed. The follow-up in these studies was relatively short (up to a median treatment duration of 1.3 years[18]) and whether the blood pressure reduction is sustained on prolonged treatment is unknown. Furthermore, most studies were done with spironolactone. Whether eplerenone, which may have a better tolerability, exerts the same effect on blood pressure remains to be studied. To answer the first question, we performed a retrospective analysis of 123 patients with difficult-to-treat hypertension, defined as uncontrolled hypertension despite the use of at least two antihypertensive drugs, who received an ARA as part of their blood pressure treatment. Systolic and diastolic blood pressure decreased with 22 and 9 mmHg, respectively, after start of ARA treatment, which is consistent with the aforementioned retrospective and observational studies. However, the follow-up in our study was substantially longer, with a median treatment duration of 25 months. In the subgroup that received ARA treatment for more than 5 years, the blood pressure reduction was even higher (33 and 16 mmHg for systolic and diastolic blood pressure, respectively). This was not attributable to an increase in total druge use (expressed as total defined daily dose, DDD), since DDD did not change significantly over time **(Chapter 7)**.

In a subsequent prospective trial, the effect of 3-month add-on therapy with eplerenone in a fixed daily dose of 50 mg was evaluated in 117 patients with difficult-to-treat hypertension. Office blood pressure decreased from 149/91 to 142/87 mmHg and ambulatory blood pressure from 141/87 to 132/83 mmHg after 3 months of treatment. After discontinuation of eplerenone, office and ambulatory blood pressure measurements returned to baseline

values. In a multivariate model, neither renin, aldosterone, or their ratio predicted the blood pressure response **(Chapter 8)**.

## DISCUSSION

### The regulation of aldosterone synthesis

Aldosterone synthesis is regulated by Ang II, serum potassium and ACTH **(Chapter 2)**. However, steroid synthesis is a complex process and other factors may be relevant. We showed that the (P)RR is present in adrenocortical cells, but direct stimulation of this receptor by supraphysiological concentrations of renin or prorenin did not have any effect on aldosterone synthesis. The question remains what is the role of these locally expressed (P)RRs in adrenocortical cells. We know that binding of prorenin to the (P)RR facilitates non-proteolytic activation allowing it to exert Ang I-generating activity.[4] Therefore, in the presence of sufficient angiotensinogen and ACE in the adrenal gland, this mechanism could contribute to the stimulation of aldosterone synthesis via the classical RAAS pathway. We did not detect any ACE-activity in these cells. It cannot be ruled out that ACE is expressed by other cell types in the intact adrenal gland. However, in transgenic rats expressing the human (P)RR, aldosterone levels were increased despite unchanged plasma and tissue angiotensin levels.[7, 8, 21] This suggest an involvement of the (P)RR in aldosterone synthesis independent of the RAAS. The (P)RR was recently shown to be crucial in vacuolar $H^+$-ATPase assembly, thereby regulating intracellular pH[22] and this could be another mechanism through which the (P)RR exerts its effects on cell functioning.

Our study shows that aldosterone breakthrough is not caused by a direct stimulatory effect of (pro)renin on aldosterone synthesis. The exact causes for aldosterone breakthrough remain uncertain, but may involve Ang II breakthrough via insufficient ACE inhibition, upregulation of ACE under pathophysiological conditions or production of Ang II via ACE-independent pathways. Direct activation of aldosterone synthesis via a rise in serum potassium may also be relevant.[23] A role for endothelin-1 as a stimulatory factor for aldosterone secretion has been postulated as well.[24]

### Screening for primary aldosteronism

**Chapter 5** clearly shows that the ARR has a poor sensitivity for PA if the recommended cutoff value is used. Although many groups have made attempts to define the optimal cutoff value, a comparison of these studies is difficult because protocols and study populations differ considerably **(Chapter 4)**. Many studies have methodological shortcomings, in the sense that only subjects with a positive screening test were subjected to additional testing.[25] In

our protocol, the intravenous SLT was performed in all participants. This allowed for a proper evaluation of the test characteristics.

It is well-known that antihypertensive medication affects the ARR.[26] Whether this is clinically relevant, especially in a multidrug regime, is being debated.[27] We show that the level of the ARR is significantly lowered by ACE-Is and ARBs in a multidrug regime, but that the overall test performance does not change. This implies that the test will perform just as well in a multidrug setting, but requires a lower cutoff value. This is in agreement with observations made by others.[27] It should be emphasized that beta-aderenergic blockers and potassium-sparing diuretics were ceased as part of the study protocol. Therefore, no conclusions can be made about the role of these drugs. It is generally accepted, however, that beta-adrenergic blockers increase the ARR,[26, 28] and this has also been observed in a multidrug regime.[29] The problem remains what the cutoff value should be under random antihypertensive treatment. The change in ARR will be hardly predictable, due to different combinations of ACE-Is, ARBs and/or beta-adrenergic blockers and variations in the prescribed doses and, for this reason, a clear-cut cutoff cannot be defined. This strongly favours the use of standardized treatment.

Ideally, the gold standard should be undisputed. Although the fludrocortisone suppression test is considered the gold standard by some,[30] and the validity of the intravenous SLT has been debated by others,[31, 32] the latter is more practical in an outpatient setting. The Endocrine Society guideline[15] states that there is insufficient evidence to prefer one confirmation test above the other. However, it advices to perform the confirmation test under ARR-neutral medication. This guideline was published after our study had started, and this recommendation was, for this reason, not implemented in the study protocol. However, the effect of medication on the outcome of the SLT has been poorly studied. Only one recent study[33] addressed this issue and found a 34% disconcordance in test outcome (positive or negative) when performed under minimally modified chronic antihypertensive therapy and under neutral therapy (doxazosin and/or verapamil). Although this is considerable, even two subsequent tests under neutral therapy showed a disconcordance of 16%. It should be noted that diuretics were stopped in the modified chronic therapy and that the intravenous SLT was preceded by 3 days of oral salt loading, thereby making this protocol difficult to compare with ours. Additional studies on the effects of medication, diuretics in particular, are warranted to provide a definitive answer on this matter.

Despite this, it can be concluded that the ARR is a poor screening test. The sensitivity is poor when the recommended cutoff value is used, but when the cutoff value is lowered to a level with an acceptible sensitivity of 95 %, the specificity is only 47 % **(Chapter 5)**. The consequence is that 53% of patients without PA will falsely be subjected to a SLT. The test performance is further compromised by a poor within-patient reproducibility.

The attempt to express an inadequate aldosterone production in a single measure likely dismisses the complexity of the RAAS and the large interindividual differences in RAAS regulation. In our study, many PA patients had relatively unsuppressed renin levels. Although

several authors include a suppressed renin level in the diagnostic criteria, by doing so, an elevated ARR as an indication of PA becomes a self-fulfilling prophecy. Relatively high renin levels in patients with proven PA have been reported in the literature before,[34, 35] but were missed in many studies, because only the patients with an elevated ARR were subjected to confirmation testing. An alternative strategy would be to proceed more quickly to a confirmation test when clinical suspicion is high. This concept is not new, since the intravenous SLT was initially considered a screening test.[36]

## Aldosterone-receptor antagonism in hypertension

There is a growing body of evidence that ARAs are a valuable addition to the treatment of patients with difficult hypertension. The blood pressure reduction reported in retrospective and uncontrolled studies is impressive. Our retrospective study **(Chapter 7)** is no exception and the same limitations of the study design apply. However, the follow-up in our study was considerably longer than in the previous trials, providing some insight in the long-term effects of aldosterone blockade. Interestingly, patients who received treatment for a longer duration had a larger reduction in blood pressure at the end of follow-up. As previously described, aldosterone is an important mediator of target-organ dysfunction and damage, and this can contribute to therapy resistance. An explanation could be that ARAs reverse these processes, leading to a better blood pressure control over time. It is very well possible, however, that the duration of treatment is influenced by its effect as well, *i.e.* a clinician is more likely to continue the treatment if it works well. Unfortunately, our study design did not allow for a differentiation between these explanations. The blood pressure reduction could also be merely the result of other interventions related to the follow-up in a referral centre. A control group, either placebo or another "rescue" strategy, would have been helpful, but difficult to achieve in this retrospective design. However, we show that the decrease in blood pressure is not the result of an increase in total drug use *per se*.

The limitations of these uncontrolled studies have been addressed in recent years. De Souza *et al*.[19] evaluated the blood pressure reduction to spironolactone in a median dose of 50 mg in 175 patients with resistant hypertension. Although this was not a genuine placebo-controlled trial, the use of 24-h ABPM is known to partly control for observer bias and placebo-related effects.[37, 38] The first placebo-controlled trial was done by Václavík *et al*.,[20] studying the response to spironolactone 25 mg in patients with resistant hypertension. As said, the blood pressure reductions were substantially smaller in these studies than in the observational ones. Our study, described in **Chapter 8**, was the first to evaluate the effect of eplerenone. Blood pressures were measured with 24-h ABPM. As an additional control, the blood pressure was again assessed after discontinuation of eplerenone. The subsequent rise in blood pressure strongly supports the conclusion that the initial blood pressure reduction was really attributable to mineralocorticoid receptor (MR) blockade by eplerenone.

How ARAs lower blood pressure in difficult-to-treat or resistant hypertension is not fully understood. We found no correlation with plasma renin, aldosterone or the ARR. This is in agreement with most studies on this topic.[39-42] An interfering effect of antihypertensive drugs on aldosterone and renin levels could underly this, as the ARR did predict the blood pressure response in previously untreated patients.[43]

Glucocorticoids such as cortisol can bind the MR with the same affinity as mineralocorticoids.[44] Target tissues for aldosterone, such as renal tubular cells, are normally protected from cortisol-MR interactions by the enzyme 11β-hydroxysteroid dehydrogenase (HSD11B2), which inactivates cortisol by the conversion to cortisone. HSD11B2 polymorphisms, which likely affect the degree of cortisol inactivation, were shown to be associated with hypertension in some patients.[45] This might provide another explanation for the lack of correlation between the response to ARA treatment and aldosterone levels, although a relation with a low renin level would still be expected. Mihailidou *et al*.[46] surprisingly found that spironolactone reduced infarct size in isolated rat hearts, even in the absence of any corticosteroids and a direct protective action through an inverse agonist effect has been suggested. Whether this also applies to renal tubular MRs is unknown.

In theory, ARAs can exert their antihypertensive effects in several ways, including modulation of sympathetic tone, direct or indirect actions on vascular tone and, in the long run, reduction in vascular stiffness **(Chapter 6)**. The decrease we observed after eplerenone treatment in the transtubular potassium gradient, which can be considered as a marker of aldosterone activity in the distal convoluted tubule and cortical collecting duct,[47] suggests that natriuresis was the main mechanism in our study **(Chapter 8)**.

## PERSPECTIVES

PA is a clinically important cause of hypertension. Clinicians should make their best effort to find these patients, especially the ones with an APA, which can be potentially cured by surgical removal. Unfortunately, establishing the diagnosis can be challenging and the ARR is not an ideal screening test. As discussed in the previous section, an alternative would be to proceed more quickly to a confirmation test when the clinical suspicion is high. This has important implications, not only in economic terms, but also in terms of patients' safety, as some patients may be at risk of fluid overload.[30] Alternative ways of screening for PA should be explored. Patients with PA were shown to have higher urinary excretion rates of the hybrid steroids 18-hydroxycortisol and 18-oxocortisol than patients with essential hypertension. Especially urinary 18-hydroxycortisol excretion levels may have additional diagnostic value when combined with the ARR and may alleviate the need for confirmatory testing when very low (strongly arguing against the diagnosis of PA) or high (strongly favouring PA).[48] Another interesting development is the discovery of alternative urinary markers of aldosteronism.

One of them is prostasin, a membrane-bound serine protease that is a modulator of and therefore a potential marker for epithelial sodium channel activity.[49] Recently, Van der Lubbe *et al*.[50] showed that the phosphorylated form of the sodium chloride cotransporter in urinary exosomes, vesicles derived from renal tubular epithelial cells, is increased in states of high aldosterone levels and is potentially a better marker for PA than prostasin. These promising biomarkers need further study to assess their suitability for clinical practice.

Taken together, the evidence that patients with difficult-to-treat and resistant hypertension can benefit from treatment with an ARA is compelling. Although their blood pressure lowering potential is probably not as high as earlier observational studies suggested, these agents deserve a more prominent role in hypertension treatment.

The use of ARAs in resistant hypertension is recommended in European and American hypertension management guidelines.[51, 52] Despite this, ARAs are still infrequently used for this indication.[53] It would be interesting to know how the efficacy of ARAs relates to other forms of add-on treatment in resistant hypertension, but data on this are scarce. Spironolactone was found to have a larger antihypertensive effect than combination treatment with an ACE-I and ARB.[54] Moreover, when compared with doxazosin, the blood pressure reduction was larger when spironolactone was added to the therapeutic regime in patients with resistant hypertension.[55] A comparison with new treatment modalities such as endovascular catheter-based renal sympathetic denervation[56] would be of great interest. Although renal sympathetic denervation has been reported to have a large effect on office blood pressure, the effect on ambulatory blood pressure is of similar magnitude as that observed with add-on ARA treatment.[56] Another promising development is the design of aldosterone synthase inhibitors. The use of ARAs can be compromised by the occurrence of sex hormone-related side effects through its actions on other steroid hormone receptors. Aldosterone synthase inhibitors have the potential of inhibiting the deleterious effects of aldosterone without causing these side effects. And although their clinical relevance is not yet completely understood, not all the non-genomic effects of aldosterone may be blocked by ARAs. A difficulty in the design of aldosterone synthase inhibitors is enzyme specificity. Aldosterone synthase is coded for by cytochrome P450 family 11, subfamily B, polypeptide 2 (CYP11B2) and shows a high homology with CYP11B1, the gene coding for 11-β-hydroxylase. This enzyme catalyzes the final step in cortisol synthesis. Many potential aldosterone synthase inhibitors will, therefore, affect cortisol synthesis to some degree. One compound, FAD-286 (the dextroantiomer of the aromatase inhibitor fadrozole) has recently been subjected to the first clinical studies under the name of LCI699. It was shown to lower blood pressure and plasma aldosterone levels in patients with PA[57] and essential hypertension[58] and to increase plasma 11-deoxycorticosterone concentrations, as is to be expected by its inhibition of aldosterone synthase.[57] Although morning cortisol levels were unaffected by LCI699 treament, ACTH levels increased after treatment.[57] Also, the cortisol response to ACTH stimulation was blunted in a subset of

patients.[57, 58] In a cohort of hypertensive patients, a dose-dependent inhibition of the ACTH-induced cortisol response was confirmed and the maximally tolerated dose for LCI699 based on this aspect of cortisol suppression was 1.30 mg in a once daily dose.[59] In essential hypertension, the reduction in 24-h ABPM was of similar size for LCI699 1 mg daily and eplerenone 50 mg twice daily (-7.7/-5 mmHg versus -10.5/-6.0, respectively; NS).[58] However, in patients with PA, the reduction in 24-h ABPM after 4 weeks of treatment was greater for eplerenone (uptitrated to 100 mg twice daily) than for LCI699 (uptitrated to 1 mg twice daily).[60] Although these results are encouraging, further research is needed to assess its suitability for clinical practice. In particular, the optimal dose of LCI699 should be found, especially in relation to cortisol suppression, and the clinical relevance of the impaired cortisol response to ACTH should be addressed. From a theoretical viewpoint, studies comparing ARAs and aldosterone synthesis inhibitors and possibly a combination of both, could be helpful in unraveling the role of non-MR mediated effects of aldosterone on the one hand, and aldosterone-independent MR activation on the other, in the pathophysiology of hypertension.

The mechanism for treatment resistance is not completely understood, but is multifactorial in most cases.[52] The best treatment strategy, therefore, is likely to be different for every patient. Not all patients with resistant hypertension respond favourably to add-on ARA treatment. Patients with hypertension refractory to blood pressure-lowering treatment in general, displayed a smaller response to spironolactone than patients who were responsive to treatment.[61] Future research should focus on identifying the different mechanisms involved in treatment resistance so that each patient receives treatment that is tailored to his specific needs. An example are obese patients with hypertension, in whom ARAs may be a good treatment choice.[62] An association was found between plasma aldosterone levels and features of the metabolic syndrome, such as dyslipidemia and insulin resistance. Plasma lipoproteins are thought to act as a source of cholesterol for adrenal steroid synthesis, but recent experimental studies indicate that lipoproteins, and very low density lipoproteins in particular, can stimulate aldosterone synthesis directly via upregulation of steroidogenic acute regulatory protein (StAR) and aldosterone synthase (CYP11B2).[63-65] A better understanding of these pathways may help to clarify the complex relation between the metabolic syndrome and hypertension and lead to more effective treatment in obesity-related hypertension.

Finally, the importance of lifestyle measures, especially a reduction in dietary salt intake, should be emphasized. Excessive salt intake has an important contributory role in treatment resistance.[66] It should also be noted that excessive salt intake greatly potentiates the deleterious effects of aldosterone[67] and even in patients with PA, dietary salt restriction can help to restore a normal hemodynamic profile.[68] Therefore, a dietary salt restriction should be the mainstay of the treatment of hypertensive patients.

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# Nederlandse samenvatting

## SAMENVATTING

Het bijnierschorshormoon aldosteron speelt een essentiële rol in volume- en bloeddrukregulatie in het menselijk lichaam. Onder pathologische omstandigheden, echter, is aldosteron een belangrijke factor bij het ontstaan van een verhoogde bloeddruk en eindorgaanschade, zoals remodellering en fibrosering van de hartspier en de vaatwand. De pathofysiologische rol van aldosteron bij het ontstaan van hypertensie en het nut van remming van aldosteron bij hypertensie zijn het onderwerp van dit proefschrift.

### Regulatie van aldosteronsynthese

Het renine-angiotensine-aldosteron-systeem (RAAS) wordt als de belangrijkste regulator van aldosteronsynthese beschouwd via de stimulatie van de angiotensine II (Ang II) type 1 receptoren die op de bijnierschorscellen aanwezig zijn. Daarnaast kan de synthese van aldosteron gestimuleerd worden door het serum kalium en het adrenocorticotroop hormoon (ACTH) **(Hoofdstuk 2)**. De regulatie van aldosteronsynthese is echter veel complexer en verschillende andere factoren spelen waarschijnlijk ook een rol. Wanneer een patiënt wordt behandeld met een angiotensine-converterend enzym remmer (ACE-I) of een angiotensine-receptor blokker (ARB), daalt normaal gesproken de aldosteronspiegel door onderbreking van het RAAS op het niveau van Ang II-produktie of effect. Dit resulteert in een compensatoire stijging in het plasma renine **(Hoofdstuk 1)**. Het is echter bekend dat bij veel patiënten er een "aldosterondoorbraak" kan plaatsvinden. Dit houdt in dat de aldosteronspiegels, na een initiële daling, een stijging laten zien bij langdurige behandeling. Het mechanisme dat ten grondslag ligt aan aldosterondoorbraak is niet volledig bekend. Wanneer prorenine bindt aan de (pro)reninereceptor ((P)RR), faciliteert dit een niet-proteolytische activatie van prorenine waardoor deze de omzetting van angiotensinogeen naar angiotensine I kan katalyseren. Recente studies laten echter zien dat de binding van renine en prorenine aan de (P)RR ook directe, angiotensine-onafhankelijke effecten, tot gevolg kan hebben door de activatie van mitogeen-geactiveerde proteïne kinases, zoals extracellulair signaal-gereguleerde kinase 1/2 (ERK1/2). Daarnaast is gebleken dat transgene ratten, die de humane (P)RR tot expressie brengen, verhoogde aldosteronspiegels hebben, ondanks normale Ang II spiegels. Deze observaties leidden tot de hypothese dat (pro)renine de synthese van aldosteron direct kan stimuleren via activatie van de (P)RR. Dit werd onderzocht in twee humane bijnierschorscellijnen (H295R en HAC15) **(Hoofdstuk 3)**. Al eerder werd aangetoond dat deze cellen de (P)RR tot expressie brengen en dit werd in ons onderzoek bevestigd. Stimulatie van deze receptor met renine en prorenine in nanomolaire concentraties leidde echter niet tot een verhoogde expressie van de enzymen die betrokken zijn bij de produktie van aldosteron, noch tot fosforylering van ERK1/2 of aldosteronproduktie. Dit in tegenstelling tot Ang II dat deze effecten wel liet zien. Deze experimenten laten zien dat aldosterondoorbraak tijdens de

behandeling met een ACE-I of ARB niet het gevolg is van een direct effect van (pro)renine op bijnierschorscellen via de (P)RR.

## Het screenen op primair hyperaldosteronisme

Ongeveer 8% van de mensen met hypertensie heeft primair hyperaldosteronisme (PHA). Dit is een conditie die wordt gekarakteriseerd door een (relatieve) overproduktie van aldosteron door de bijnier. In tegenstelling tot wat vroeger werd gedacht, heeft slechts een minderheid een aldosteron producerend adenoom (APA), zoals oorspronkelijk beschreven door Jerome Conn, en heeft ook slechts een minderheid een verlaagd serum kaliumgehalte. Het is belangrijk om deze patiënten vroeg op te sporen omdat zij vaak een vorm van hypertensie hebben die moeilijk te behandelen is en ook omdat zij een hoger cardiovasculair risico hebben als gevolg van de schadelijke effecten van aldosteron op hart en bloedvaten. Daarnaast is er een specifieke behandeling beschikbaar, behandeling met aldosteron receptor antagonisten (ARAn) bij bilaterale afwijkingen en adrenalectomie in het geval van een APA. De "Endocrine Society" raadt in haar richtlijn de aldosteron-renine ratio (ARR) aan als de beste screeningstest. Desalniettemin is de validiteit van de ARR nog onderwerp van discussie wegens twijfels over de diagnostische waarde, lage reproduceerbaarheid en de economische kosten die gemoeid zijn bij brede screening. De interpretatie van de ARR wordt verder bemoeilijkt door de vele factoren die de test beïnvloeden, waaronder het gebruik van antihypertensieve medicatie **(Hoofdstuk 4)**. In **Hoofdstuk 5** werden de testkarakteristieken van de ARR onderzocht in 178 patiënten met moeilijk te behandelen hypertensie. Aldosteron- en reninespiegels werden tweemaal binnen vier weken bepaald tijdens het gebruik van hun eigen, willekeurige, medicatie. Daarna werd bij alle deelnemers een intraveneuze zoutbelastingstest (ZBT) verricht, als goudstandaard voor de diagnose van PHA. Om het effect van medicatie op de ARR te bestuderen, werd de behandeling vervolgens aangepast naar een gestandaardiseerd regime, bestaande uit een calciumantagonist en een alfa-adrenerge receptor blokker. Van deze middelen weten we dat ze weinig invloed hebben op de spiegels van renine en aldosteron. De ARR werd vervolgens herhaald na vier tot zes weken. De prevalentie van PHA was 15% in onze populatie. Als de aangeraden afkapwaarde werd gebruikt, was de sensitiviteit van de ARR slechts 22%. Onder gestandaardiseerde behandeling was er een significante stijging van de ARR te zien. Een multivariate regressieanalyse liet zien dat het gebruik van een ACE-I en/of ARB de oorzaak was van de lagere ARR onder willekeurige antihypertensieve therapie. Echter, de oppervlakte onder de "receiver operating characteristic" curve was voor beide condities gelijk (84% voor willekeurige versus 86% voor gestandaardiseerde behandeling, p=0.314), hetgeen inhoudt dat de test onder zowel willekeurige als gestandaardiseerde medicatie hetzelfde presteert. De overlap in ARR waardes tussen patiënten met PHA en essentiële hypertensie is aanzienlijk. Dit heeft tot gevolg dat wanneer de afkapwaarde wordt verlaagd naar een niveau met een acceptabele sensitiviteit, de specificiteit fors daalt. De bruikbaarheid

van de ARR wordt verder beperkt door de grote intraindividuele variabiliteit: onder dezelfde condities en in dezelfde patiënt konden twee ARR bepalingen bijna een factor vijf verschillen.

## Het gebruik van aldosteron receptor antagonisten bij hypertensie

Voor lange tijd werden ARAn vooral gebruikt in patiënten met specifieke condities, zoals hartfalen en PHA. Dit is merkwaardig, aangezien veel bekend is over de rol van aldosteron bij de pathofysiologie van hypertensie en hart- en vaatziekten in het algemeen. Aldosteron is waarschijnlijk nog meer van belang in patiënten met hypertensie die niet met een of twee middelen te controleren is of zelfs resistent is voor het effect van behandeling. In de laatste tien jaar zijn ARAn opnieuw in de belangstelling komen te staan als mogelijke behandeling van patiënten met een therapieresistente hypertensie **(Hoofdstuk 6)**. De grootste studie op dit vlak is van Chapman *et al*. die de bloeddrukrespons in kaart bracht van 1411 deelnemers aan de Anglo-Scandinavian Cardiac Outcome Trial – Blood Pressure Lowering Arm (ASCOT-BPLA) die de ARA spironolacton voorgeschreven kregen omdat hun bloeddruk verhoogd bleef ondanks het gebruik van drie antihypertensiva. Na de toevoeging van een relatief lage mediane dosis spironolacton van 25 mg zagen zij een indrukwekkende daling in de systolische en diastolische bloeddruk van respectievelijk 21.9 en 9.5 mmHg. Een belangrijk probleem met deze studie was de retrospectieve opzet en de afwezigheid van een controlegroep. In andere studies werden vergelijkbare bloeddrukdalingen gezien, maar ook deze hadden methodologische tekortkomingen, zoals een retrospectieve opzet, een aan verstoring onderhevige manier van bloeddruk meten of het gebrek aan een placebogroep. In een recente studie waarbij 24-uurs bloeddrukmetingen werden gebruikt en een andere met een placebo-gecontroleerde opzet, werden aanzienlijk kleinere reducties in bloeddruk waargenomen. De volgduur tijdens al deze studies was relatief kort, een maximale mediane behandelduur van 1.3 jaar, en of de bloeddrukdaling duurzaam is tijdens langere behandeling is niet bekend. Daarnaast is het opvallend dat de meeste studies zijn uitgevoerd met spironolacton. Of eplerenon, wat mogelijk minder bijwerkingen heeft, eenzelfde bloeddrukverlagend effect heeft, is nog niet bestudeerd. Om de eerste vraag te beantwoorden, werd een retrospectieve analyse uitgevoerd van 123 patiënten met moeilijk te behandelen hypertensie, gedefinieerd als verhoogde bloeddrukwaarden ondanks het gebruik van tenminste twee antihypertensieve medicijnen, die een ARA voorgeschreven kregen als onderdeel van hun bloeddrukbehandeling. De systolische en diastolische bloeddruk daalde met respectievelijk 22 en 9 mmHg na de start van de ARA, hetgeen overeenkomt met de bovengenoemde retrospectieve studies. De volgduur was echter substantieel langer in onze studie, met een mediane behandelduur van 25 maanden. In de subgroep die meer dan vijf jaar werd behandeld, was de bloeddrukdaling aan het eind van de volgduur zelfs nog groter (33 en 16 mmHg voor respectievelijk de systolische en diastolische bloeddruk). Dit werd

niet veroorzaakt door een toename in het totale aantal gebruikte medicijnen (uitgedrukt in vastgestelde dagelijkse dosis) **(Hoofdstuk 7)**.

In een prospectieve studie werd vervolgens het effect van eplerenon in een vaste dosering van 50 mg als toevoeging aan de bestaande antihypertensieve therapie bestudeerd in 117 patiënten met moeilijk behandelbare hypertensie. De spreekkamerbloeddruk daalde van 149/91 mmHg naar 142/87 mmHg en de ambulante bloeddruk van 141/87 naar 132/83 mmHg na drie maanden. Na het staken van eplerenon steeg de bloeddruk weer naar de uitgangswaarde. Renine, aldosteron, noch de ARR bleek een voorspeller van de bloeddrukrespons in een multivariate analyse **(Hoofdstuk 8)**.

# Abbreviations

## ABBREVIATIONS

| | |
|---|---|
| 3β-HSD | 3 β-hydroxysteroid dehydrogenase |
| AB | Alpha-adrenergic blocker |
| ABPM | Ambulatory blood pressure measurement |
| ACE | Angiotensin-converting enzyme |
| ACE-I | Angiotensin-converting enzyme inhibitor |
| ACR | Urinary albumin-to-creatinine ratio |
| ACTH | Adrenocorticotropic hormone |
| ADBP | Ambulatory diastolic blood pressure |
| Ang | Angiotensin |
| ANOVA | Analysis of variance |
| APA | Aldosterone-producing adenoma |
| ARA | Aldosterone receptor antagonist |
| ARB | Angiotensin II type 1 receptor blocker |
| ARR | Aldosterone-to-renin ratio |
| ASBP | Ambulatory systolic blood pressure |
| ASCOT-BPLA | Anglo-Scandinavian Cardiac Outcome Trial – BP lowering arm |
| AT1R | Angiotensin II type 1 receptor |
| ATC | Anatomical therapeutic chemical |
| ATF1 | Activating transcription factor 1 |
| AUC | Area under the curve |
| AVS | Adrenal vein sampling |
| BAH | Bilateral adrenal hyperplasia |
| BB | Beta-adrenergic blocker |
| BMI | Body mass index |
| BP | Blood pressure |
| BPM | Beats per minute |
| $Ca^{2+}$ | Calcium |
| CaMK | Calmodulin-dependent kinase |
| cAMP | Cyclic adenosine monophosphate |
| CCB | Calcium channel blocker |
| CCS | Cosmic calf serum |
| CI | Confidence interval |
| CNS | Central nervous system |
| COUP-TFI | Chicken ovalbumin upstream promoter-transcription factor I |
| CREB | cAMP response element-binding protein |
| CT | Computed tomography |
| CVD | Cardiovascular disease |

| | |
|---|---|
| CYP11A1 | Cytochrome P450 family 11, subfamily A, polypeptide 1 |
| CYP11B1 | Cytochrome P450 family 11, subfamily B, polypeptide 1 |
| CYP11B2 | Cytochrome P450 family 11, subfamily B, polypeptide 2 |
| CYP17A1 | Cytochrome P450 family 17, subfamily A, polypeptide 1 |
| CYP21A2 | Cytochrome P450 family 21, subfamily A, polypeptide 2 |
| DAG | Diacylglycerol |
| DBP | Diastolic blood pressure |
| DDD | Defined daily dose |
| DM | Diabetes mellitus |
| dTGR | Double transgenic rats |
| EDTA | Ethylene diaminetetraacetic acid |
| EH | Essential hypertension |
| EKG | Electrocardiogram |
| ENaC | Epithelial sodium channel |
| ERK | Extracellular signal-regulated kinase |
| FCS | Fetal calf serum |
| FH-1/2/3 | Familial hyperaldosteronism type 1/2/3 |
| FST | Fludrocortisone suppression test |
| GFR | Glomerular filtration rate |
| GRA | Glucocorticoid-remediable aldosteronism |
| HDL | High-density lipoprotein |
| HPRT1 | Hypoxanthine ribosyl transferase 1 |
| HRP | Handle region peptide |
| HSD11B2 | 11β-hydroxysteroid dehydrogenase |
| HT | Hypertension |
| icv | intracerebroventricular |
| IL-6 | Interleukin-6 |
| $IP_3$ | Inositol triphosphate |
| IPA | Idiopathic primary aldosteronism |
| IQR | Interquartile range |
| $K^+$ | Potassium |
| KCNJ5 | Inwardly rectifying potassium channel, subfamily J, member 5 |
| Ki-RasA | Kirsten Ras GTP-binding protein 2A |
| LDF-score | Logistic discriminant function score |
| LDL | Low-density lipoprotein |
| Ln | Natural logarithm |
| LR | Likelihood ratio |
| LV | Left ventricular |
| LVH | Left ventricular hypertrophy |

| | |
|---|---|
| LVMI | Left ventricular mass index |
| MAPK | p38 mitogen-activated protein kinase |
| MDCK | Mardin Darby Canine Kidney |
| MR | Mineralocorticoid receptor |
| MRI | Magnetic resonance imaging |
| NA | not applicable |
| $Na^+$ | Sodium |
| NBRE-1 | NGFIB response element 1 |
| NCC | Sodium chloride cotransporter |
| ND | Not done |
| NGFIB | Nerve growth factor-induced clone B |
| NPV | Negative predictive value |
| NS | Not significant |
| NSAID | Nonsteroidal anti-inflammatory drug |
| NURR1 | Nur-related factor 1 |
| PA | Primary aldosteronism |
| PAC | Plasma aldosterone concentration |
| PIAS1 | Protein inhibitor of activated Stat1 |
| PIIINP | Procollagen type III amino-terminal peptide |
| PKC | Protein kinase C |
| PKD | Protein kinase D |
| PPV | Positive predictive value |
| PRA | Plasma renin activity |
| PRC | Plasma renin concentration |
| (P)RR | (Pro)renin receptor |
| PWV | Pulse wave velocity |
| RAAS | Renin-angiotensin-aldosterone System |
| RAS | Renin-angiotensin system |
| RM | Random antihypertensive medication |
| ROC | Receiver operating characteristic |
| ROMK | Renal outer medullary potassium channel |
| RT-PCR | Reverse transcriptase-polymerase chain reaction |
| SA | Serum aldosterone |
| SBP | Systolic blood pressure |
| SD | Standard deviation |
| SE | Standard error |
| SEM | Standard error of the mean |
| SGK1 | Glucocorticoid-inducible kinase 1 |
| SLT | Salt loading test |

| | |
|---|---|
| SM | Standardized antihypertensive medication |
| SSRI | Selective serotonin reuptake inhibitor |
| StAR | Steroidogenic acute regulatory protein |
| TGF-β1 | Transforming growth factor β1 |
| TTKG | Transtubular potassium gradient |
| Ubc9 | Ubiquitin-like protein SUMO-1 conjugating enzyme |
| VLDL | Very low-density lipoprotein |
| α -HT | Antihypertensives |

# Curriculum vitae

## CURRICULUM VITAE

Pieter Jansen werd geboren op 12 juni 1977 in Nijmegen. In 1995 deed hij eindexamen aan het Stedelijk Gymnasium Nijmegen. In 2001 behaalde hij het doctoraalexamen in de studie Biomedische Gezondheidswetenschappen (*cum laude*) aan de Katholieke Universiteit Nijmegen (later Radboud Universiteit Nijmegen) met als afstudeerrichting Pathobiologie. In 2005 voltooide hij de studie Geneeskunde aan dezelfde universiteit. In 2006 startte hij als assistent-geneeskundige in opleiding tot klinisch onderzoeker (AGIKO) aan het Erasmus Medisch Centrum Rotterdam waarbij hij onderzoek deed naar de rol van aldosteron en aldosteronblokkade bij hypertensie. In 2009 startte hij met de opleiding tot internist (opleider prof. dr. J.L.C.M. van Saase) in het kader waarvan hij tussen januari 2009 en april 2012 gedurende ruim 2 jaar als arts-assistent verbonden was aan het Maasstadziekenhuis Rotterdam (opleider dr. M.A. van den Dorpel). Momenteel is hij werkzaam als arts-assistent in het Canberra Hospital (Canberra, Australië).

Pieter Jansen was born in Nijmegen, The Netherlands, on June 12, 1977. In 1995, he graduated from grammar school at the "Stedelijk Gymnasium Nijmegen." In 2001, he received his master's degree in Biomedical Health Sciences (*cum laude*) at the University of Nijmegen with a major in Pathobiology, followed by his medical degree at the same university in 2005. In 2006, he started a research project at the Erasmus Medical Centre in Rotterdam, studying the role of aldosterone and aldosterone blockade in hypertension. In 2009, he started his clinical training in Internal Medicine (training director Prof. dr. J.L.C.M. van Saase) and spent over 2 years of registrar training at the Maasstad Hospital in Rotterdam (coordinator dr. MA van den Dorpel). He is currently working as basic physician trainee in The Canberra Hospital (Canberra, Australia).

# Publications

## PUBLICATIONS

**Jansen PM**, Frenkel WJ, van den Born BJ, de Bruijne EL, Deinum J, Kerstens MN, Arnoldus JH, Woittiez AJ, Wijbenga JA, Zietse R, Danser AHJ, van den Meiracker AH. Determinants of blood pressure reduction by eplerenone in uncontrolled hypertension. *J Hypertens* 2013;31:404-413.

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# PhD portfolio

# PHD PORTFOLIO

## The role of aldosterone and aldosterone blockade in hypertension

| | |
|---|---|
| PhD candidate: | P.M. Jansen |
| Erasmus MC Department: | Internal Medicine |
| Research School: | Cardiovascular Research School Erasmus University Rotterdam (COEUR) |
| Promotor: | Prof.dr. A.H.J. Danser |
| Supervisor: | Dr. A.H. van den Meiracker |
| PhD period: | 2006-2010 |

## Courses

| | |
|---|---|
| 2007 | Cardiovascular Medicine (COEUR)<br>Cardiovascular Pharmacology (COEUR)<br>Endocrinology Course Erasmus MC, Noordwijkerhout, The Netherlands (workshop leader "Hypo- and hypertensive syndromes" together with dr. A.H. van den Meiracker) |
| 2008 | Heart Failure Research (COEUR) |
| 2009 | Clinical Pharmacology (Teaching Committee Netherlands Associatian of Internal Medicine) |
| 2010 | Water and Salt (Teaching Committee Netherlands Association of Internal Medicine) |

## Conferences, meetings and symposia

| | |
|---|---|
| 2007 | 17th annual scientific meeting of the European Society of Hypertension, Milan, Italy.<br>Rotterdam Symposium for Internal Medicine, Rotterdam, The Netherlands<br>Annual symposium of the Netherlands Society for Vascular Medicine, Scheveningen, The Netherlands<br>19th annual symposium of the Netherlands Association of Internal Medicine, Maastricht, The Netherlands |

2008 18th annual scientific meeting of the European Society of Hypertension, Berlin, Germany "*The prevalence of primary aldosteronism in primary care and referred hypertensive patients: a meta-analysis*" (***poster presentation***)
Rotterdam Symposium for Internal Medicine, Rotterdam, The Netherlands

2009 21st annual symposium of the Netherlands Association of Internal Medicine, Maastricht, The Netherlands
High Blood Pressure Research Conference of the American Heart Association, Chicago, United States of America "*No direct effect of (pro)renin on aldosterone synthesis in H295R cells*" (**poster presentation**)

2010 20th annual scientific meeting of the European Society of Hypertension, Oslo, Norway "*No direct effect of (pro)renin on steroidogenic enzyme expression in H295R cells*". (**poster presentation**).
Workshop "Endocrine Hypertension" as part of the Radboud Adrenal Symposium, Nijmegen, The Netherlands

2011 Study Meeting SPARTACUS (Subtyping primary aldosteronism: a Randomized Trial comparing Adrenal vein sampling and CompUted tomography Scan) trial, Utrecht, The Netherlands "*The aldosterone-to-renin ratio as a screening test for primary aldosteronism*" (**oral presentation**)
23rd annual symposium of the Netherlands Association of Internal Medicine, Maastricht, The Netherlands. "*Sensitivity, specificity and reproducibility of the aldosterone-to-renin ratio as a screening test for primary aldosteronism*" (**oral presentation**).
21st annual scientific meeting of the European Society of Hypertension, Milan, Italy "*Sensitivity, specificity and reproducibility of the aldosterone-to-renin ratio as a screening test for primary aldosteronism*" (**poster presentation**)
"*Aldosterone-receptor antagonists lead to prolonged blood pressure reduction in uncontrolled hypertension: a retrospective analysis*" (**oral presentation**)

2012 24th scientific meeting of the International Society of Hypertension, Sydney, Australia "*Determinants of blood pressure reduction by eplerenone in patients with uncontrolled hypertension*" (**poster presentation**)
"*Sensitivity, specificity and reproducibility of the aldosterone-to-renin ratio as a screening test for primary aldosteronism*" (**poster presentation**)

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Prof. dr. L.J. Hofland, many thanks for being a member of the reading committee and your assessment of the manuscript. Prof. dr. Y.M. Smulders, a visit to Amsterdam was always a guarantee for a critical, yet constructive, discussion which was often helpful in sharpening my ideas and approach. Thanks for being part of the reading committee. Prof. dr. R. Zietse, dear Bob, your lecturing about the physiology of the different segments of the nephron always sounds incredibly easy, but for some reason very hard to reproduce myself. Thanks for your interest in my studies and your assessment of this thesis as a member of the reading committee.

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Hans, we spent a significant part of our training together and I always enjoyed your clinical, scientific and social skills. Thanks for introducing me to the ins and outs of the steroid hormones. Although it took some time to convince the world of the relevance of our data, our collaboration resulted in an interesting paper.

According to Wikipedia, one of the jobs of the paranymphs is to act as a physical shield in case the debate becomes too heated. Nils, I hope it won't come that far, but it is a safe idea to have someone beside me who knows more about aldosterone than I do. I remember the joy we had making a film for Joost's PhD defence. I trust that you won't do the same to me. Erik, it took me a while to be able to return the favour, and I am honoured to have you as my paranymph. Despite the distance: brothers in arms!

Henk and Margriet, thanks for welcoming me in your family for all those years and your interest in my studies. Mom and dad, thanks for all the support and opportunities I was given

to come to this point. I realise 2012 was a turbulent year in many ways. I am confident that your trip to Australia will go ahead this year, and even more that you're gonna love it.

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# THE ROLE OF **ALDOSTERONE** AND **ALDOSTERONE BLOCKADE** IN **HYPERTENSION**

Pieter M. Jansen