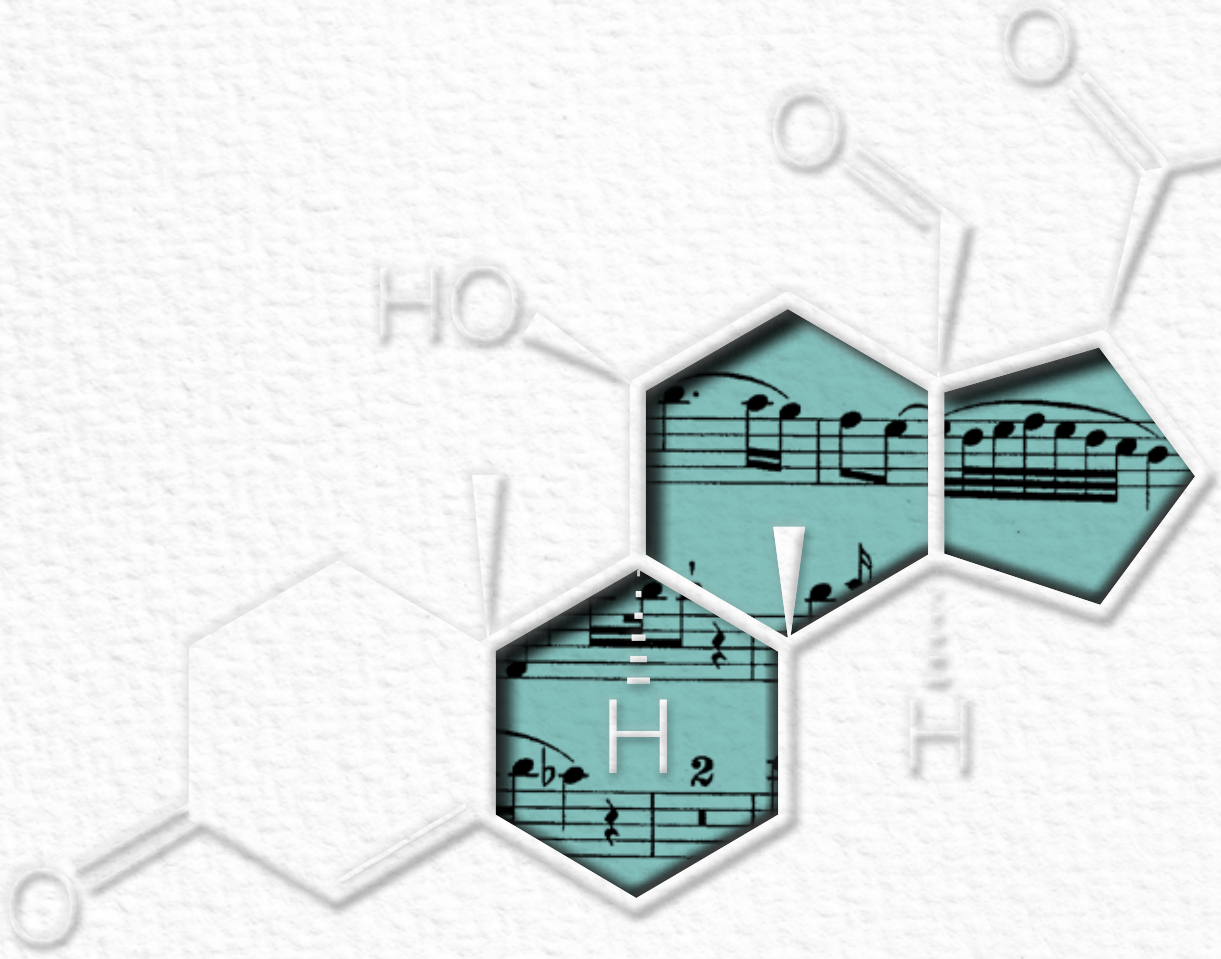
THE ROLE OF **ALDOSTERONE** AND **MINERALOCORTICOID RECEPTOR ANTAGONISTS** IN CARDIOVASCULAR DAMAGE



DANIËLLE VAN DEN BERG

THE ROLE OF ALDOSTERONE AND MINERALOCORTICOID RECEPTOR ANTAGONISTS IN CARDIOVASCULAR DAMAGE

Daniëlle van den Berg

COLOFON

Author:	Daniëlle van den Berg
Cover design en lay-out:	Miranda Dood, Mirakels Ontwerp
Printing:	Gildeprint - The Netherlands
ISBN:	978-90-9031408-2

The work presented in this thesis was carried out within the Radboud Institute for Health Sciences

THE ROLE OF ALDOSTERONE AND MINERALOCORTICOID RECEPTOR ANTAGONISTS IN CARDIOVASCULAR DAMAGE

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op vrijdag 25 januari 2019
om 12.30 uur precies

door

Theodora Nicolasina Antonia van den Berg

geboren op 1 maart 1985
te Berlicum

PROMOTOREN:

Prof. dr. N.P. Riksen

Prof. dr. G.A.P.J.M. Rongen

COPROMOTOR:

Dr. J. Deinum

MANUSCRIPTCOMISSIE:

Prof. dr. A.R.M.M. Hermus (voorzitter)

Prof. dr. N. van Royen

Prof. dr. A.H.J. Danser (Erasmus MC)

TABLE OF CONTENTS


30
f
38
9
55
1
f
69
75
1
85
90
8
p
106
f

CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

1

BACKGROUND

Aldosterone was discovered only 65 years ago. Sylvia Simpson Tait, her husband James Tait and colleague Helen Grundy discovered the steroidal hormone in fractioned bovine adrenal extract and published their empirical work in Nature in 1952. They first described aldosterone as electrocortin, because of its strong sodium-retaining properties. [1]

Indeed, aldosterone plays a major role in salt and body fluid handling, completing the renin-angiotensin-aldosterone system (RAAS) (Figure 1). Aldosterone is mainly produced in the adrenal cortex, with angiotensin II (Ang II) and plasma potassium levels being the most important regulators of its production. Binding of aldosterone to the mineralocorticoid receptor (MR) in the distal convoluted tubule of the nephron results in sodium reabsorption and potassium excretion, thereby increasing blood pressure levels.

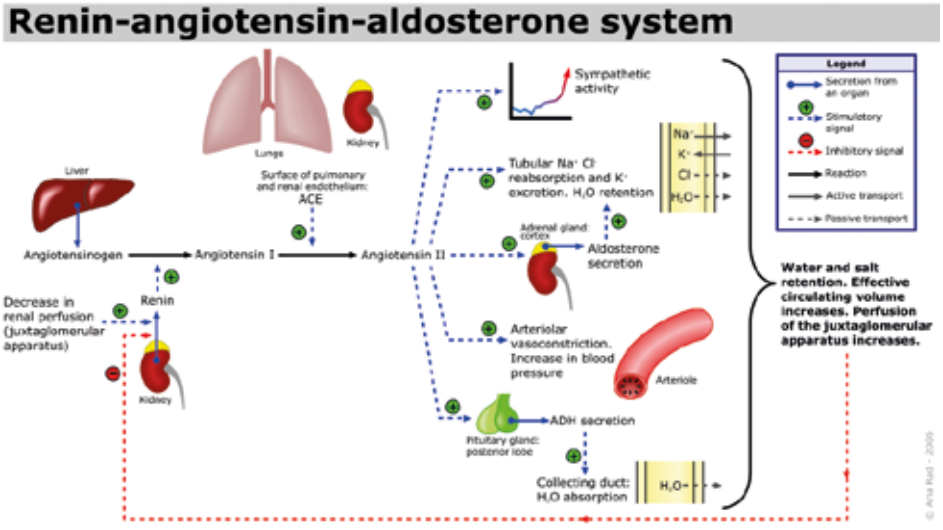


FIGURE 1. The regulatory effects of the RAAS on blood pressure

From Wikimedia Commons, the free media repository

PRIMARY ALDOSTERONISM

Jerome W. Conn was the first who characterized the pathophysiological condition of autonomous adrenal aldosterone overproduction. Conn's syndrome refers to the condition of aldosterone excess due to a (unilateral) aldosterone producing adenoma (APA). Bilateral adrenal hyperplasia (BAH) represents the other major subtype of primary aldosteronism (PA). Most patients with PA present with hypertension and/or hypokalemia, due to the effects of aldosterone in the kidney.

Importantly, patients with PA have a higher risk of future cardiovascular events including myocardial infarction, stroke and atrial fibrillation, in comparison to age- and sex-matched patients with essential hypertension (EHT) and similar blood pressure levels. [2] Similarly, left ventricular hypertrophy (LVH) and heart failure are also more prevalent in patients with PA than in patients with EHT. [2] The increased susceptibility to these cardiovascular complications is beyond the risk that is imposed by the high blood pressure itself and suggests direct toxic effects of aldosterone. Indeed, preclinical data demonstrate that aldosterone has detrimental effects on the cardiovascular system, such as promoting oxidative stress, fibrosis and apoptosis, and increasing infarct size (IS) in animal models of ischemia and reperfusion (IR). [3-8]

The increased cardiovascular risk in patients with PA highlights the importance of early diagnosis and treatment of PA. It is important to realize though, that there is a reported delay in the diagnosis of PA of approximately 8 years. [9] This delay can be explained by several factors:

Firstly, patients with a newly diagnosed hypertension are not routinely screened for PA in primary care. Case detection of PA is advised in selected subgroups of patients. [10] Screening for PA is recommended in therapy resistant hypertension, which is defined by an uncontrolled blood pressure despite 3 antihypertensive drugs including 1 diuretic, or in the presence of hypokalemia. [10, 11] In patients with a true resistant hypertension, the time needed to optimize medical treatment delays screening for secondary causes of hypertension.

Secondly, hypokalemia is present in only 37-56% of the patients with PA. [12, 13] The absence of biochemical features leads to a large proportion of unidentified PA cases. Furthermore, hypokalemia may be masked when medical treatment contains

an MR antagonist, which is currently the add-on drug of choice in therapy resistant hypertension. [11]

Lastly, we speculate that unawareness of the disease contributes to the delay in diagnosis of PA. [14] PA was previously considered to be a rare disease. In contrast, PA is now recognized as the most common cause of secondary hypertension. In primary care hypertensive population prevalence rates of PA of 3-12 % are reported, versus 1-30 % in referral centres. [15, 16] Prevalence rates not only depend on the health care centre (primary care versus referral centres) studied, but also on the population studied: PA is more prevalent in specific patient groups, i.e. patients with severe or resistant hypertension and patients with sleep apnea and hypertension. [10]

The delay in diagnosis automatically leads to a delay in the specific treatment of PA, which differs from the standard antihypertensive treatment in EHT patients. The first choice of treatment in patients with unilateral aldosterone overproduction is laparoscopic removal of the culprit adrenal gland, whereas patients with bilateral aldosterone overproduction or patients who cannot undergo or do not desire surgery are pharmacologically treated with the MR antagonists spironolactone or eplerenone.

In patients with PA, both adrenalectomy and medical treatment with MR antagonists reduce the blood pressure to a level observed in patients with EHT who are treated with standard antihypertensive treatment. [17] A longer duration of hypertension however, lowers the chance of blood pressure normalization after adrenalectomy. [18, 19] Hence, early diagnosis and treatment of PA are essential.

In recent years, more attention has been paid to the quality of life in patients with PA. Patients with PA have an impaired quality of life and compared to patients with EHT, they are more prone to psychological problems. [20-22] After surgical or pharmacological treatment, the quality of life scores in patients with PA improve. [20] These findings again subscribe the relevance of early diagnoses and treatment of PA.

DIAGNOSIS OF PRIMARY ALDOSTERONISM

The Radboud university medical center (Radboudumc) serves as a tertiary referral centre for patients with PA. Concordant with the current international guideline, the level of circulating aldosterone and the aldosterone-to-renin-ratio (ARR) are used to detect possible cases of PA. [10] When the baseline aldosterone concentration is <0.42

nmol/L and the ARR <0.09 nmol/mU, PA is currently considered to be excluded, according to the reference values of the clinical chemistry laboratory. Patients with a plasma aldosterone concentration of >0.42 nmol/L and ARR >0.09 nmol/mU at baseline undergo subsequent testing to confirm or exclude PA.
To date, no confirmatory test is identified as the gold standard. [10] In our centre, we perform a salt loading test (SLT) by the intravenous infusion of 2 L saline in 4 hours. This test relies on the principle that expansion of the effective circulating volume suppresses the RAAS system. A lack of aldosterone suppression in response to salt loading is thus indicative of autonomous aldosterone overproduction, that escapes normal feedback mechanisms. The diagnosis of PA is confirmed when the aldosterone concentration exceeds 0.28 nmol/L after salt infusion. The SLT is considered negative when the aldosterone concentration is suppressed to <0.14 nmol/L after salt infusion. Patients with an aldosterone concentration of 0.14 to 0.28 nmol/L after SLT are discussed in our local adrenal working group that assigns such patients to either a PA or EHT group, based on a number of clinical characteristics, including hypokalemia, plasma renin level and use of potassium suppletion.

Several conditions and drugs affect the interpretation of ARR results. [10] For instance, hypokalemia may lead to a false negative ARR since it suppresses aldosterone production, whereas renal dysfunction may lead to a false positive ARR result. It is important to correct for hypokalemia and withdraw drugs that interfere with the RAAS before evaluation of PA, since these drugs may either falsely elevate ARR results or mask PA. [10] Complete cessation of antihypertensive drugs is often undesirable in patients evaluated for PA and high blood pressure levels. Concordant with international guidelines and our local protocol, treatment with the calcium-channel blockers (preferably the non-dihydropyridine calcium channel blockers diltiazem and verapamil), doxazosin and hydralazine is allowed during evaluation for PA, since these drugs have a minimal effect on aldosterone and renin measurement. [10]

When the diagnosis of PA is established, the current guideline recommends adrenal imaging with sequential adrenal venous sampling (AVS) in patients with PA who are candidates for surgical treatment. [10] Radiologists use computed tomography (CT) to visualize adrenal masses (uni- or bilateral) and to distinguish between benign and

malignant tumors. When AVS is followed, CT is helpful to localize the right adrenal vein which may facilitate cannulation during AVS.

AVS of the right and left adrenal vein is performed during cosyntropin infusion. AVS is determined successful when the adrenal vein to peripheral vein cortisol ratio exceeds 3. Criteria for a unilateral aldosterone overproduction are met when the left versus right (or vice versa) aldosterone-cortisol-ratio is ≥4.0 and the ratio of the contralateral site is ≤1.0, as an indication for contralateral suppression.

Although the current guideline considers AVS to be the gold standard to dinstinguish between uni- and bilateral autonomous aldosterone overproduction, recent observations from a prospective diagnostic management study performed in our centre, the SPARTACUS study, do not necessarily advocate AVS. [10, 23] In this study, there were no differences in the intensity of the antihypertensive treatment, the number of patients in whom target blood pressure was reached, biochemical cure and the quality of life between the patients who were treated based on AVS, compared to treatment based on CT, after a follow up period of 1 year. [23]

ADRENALECTOMY VERSUS MINERALOCORTICOID RECEPTOR ANTAGONISTS

As described above, both adrenalectomy and pharmacological treatment with MR antagonists effectively reduce blood pressure levels in patients with PA to a similar level observed in medically treated patients with EHT. [17] A recent review showed a superior role of adrenalectomy over medical treatment concerning blood pressure lowering and restoration of plasma potassium levels in PA. [24] It is important to realize though that randomized studies comparing adrenalectomy with medical treatment in PA patients with an APA are lacking. Also, medical treatment is the preferred treatment option in PA patients with BAH since bilateral adrenalectomy results in life-long dependency on hormonal substitution and risk of Addison's crisis.

In 33-86 % of the adrenalectomized patients, the target blood pressure of <140/<90 mmHg is reached without the use of any antihypertensive medication. [17, 21, 25, 26] The elimination of chronic antihypertensive drug use in approximately half of the adrenalectomized patients makes adrenalectomy a cost-saving option in PA patients. [27] Laparoscopic adrenalectomy is a safe and minimally invasive procedure, whilst MR

antagonists can have undesirable side effects. [28, 29] Spironolactone and eplerenone are steroidal MR antagonists that competitively inhibit aldosterone at the level of the MR. Spironolactone also binds to other steroid receptors leading to mastodynia or gynecomastia, impotence, loss of libido, or menstrual irregularities. The selective MR antagonist eplerenone displays lower affinity for the progesterone and androgen receptor and therefore, it has less progestagenic or antiandrogenic side effects. However, eplerenone is less potent than spironolactone regarding blood pressure lowering, and should be taken twice daily because of its shorter half-life.

The increased risk of cardiovascular complications in patients with PA, in comparison to patients with EHT with similar blood pressure levels, implies treatment goals in PA beyond blood pressure normalization. One might assume favourable effects of adrenalectomy over and above pharmacological treatment regarding target organ damage and quality of life, since adrenalectomy normalizes aldosterone secretion. Furthermore, MR antagonists do not counteract potential MR-independent effects of aldosterone on the cardiovascular system. [30, 31]
Indeed, although both adrenalectomy and treatment with MR antagonists significantly improve the quality of life scores in patients with PA, the improvement during medical treatment is slower and to a lesser extent than the improvement after surgical treatment. [20] The superiority of adrenalectomy over medical treatment concerning improvement of quality of life in patients with PA has been confirmed in a post-hoc analysis of the SPARTACUS study, that was recently performed in our centre. [23, 32]
Regarding cardiovascular damage however, adrenalectomy and medical treatment with MR antagonists reduce LV mass and the prevalence of LVH to a similar extent after an averaged follow up period of 4 years. [33] After a 1 year follow up however, significant reductions in LV mass are achieved in adrenalectomized patients, but not in medically treated PA patients. [34, 35] In patients with PA who are cured after adrenalectomy versus patients with PA who are medically treated, there is no difference in cardiovascular outcomes compromising myocardial infarction, stroke, any type of revascularization procedure, and sustained arrhythmias, after a mean follow up period of 7.4 years. [36]

It is an interesting observation that in patients with PA, treatment with competitive MR antagonists is non-inferior to adrenalectomy regarding LV mass regression and

cardiovascular outcomes after long-term (4 years) but not after short-term (1 year) follow up.

MR-dependent effects of aldosterone on LVH and other cardiovascular outcome measures are likely to benefit from treatment with MR antagonists also after short-term follow up. Therefore, one might assume that the toxic effects of aldosterone on the above mentioned cardiovascular outcomes are not fully blocked by MR antagonists, or that these effects are MR-independent.

In addition, in preclinical models MR antagonists confer cardioprotection even when there is no endogenous aldosterone present, suggesting inverse agonism of these ligands on MR-receptors or beneficial pleiotropic actions of these drugs. [8] In Langendorff isolated perfused heart models and murine models using adrenalectomized animals, administration of MR antagonists reduces IS in the setting of simulated IR. [37-41] In large studies in patients with systolic heart failure, spironolactone and eplerenone reduce morbidity and mortality, but the underlying mechanism is not fully understood. [42-44]

OUTLINE OF THE THESIS

It follows that several important knowledge gaps remain, which prevent optimal diagnosis and treatment of patients with PA.

Firstly, it is known that with the current diagnostic strategy, patients with PA are at higher risk of cardiovascular events than patients with EHT. To develop novel diagnostic strategies leading to earlier diagnosis, it is essential to know whether there is more profound cardiovascular damage compared to patients with EHT already at an early stage of their disease. We screened patients with a newly diagnosed hypertension from a Dutch primary care cohort for PA. Subsequently, we assessed cardiovascular and renal damage in the patients diagnosed with PA in comparison to matched controls diagnosed with EHT. We described the results of this explorative study in chapter 2.1.

Secondly, patients with PA are at an increased risk for cardiovascular complications such as myocardial infarction and stroke. In preclinical models, aldosterone increases

myocardial IR injury and administration of MR antagonists protects against IR injury. It is currently unknown whether patients with PA are more susceptible to IR than patients with EHT, which would provide yet another mechanism that explains the higher cardiovascular risk in these patients. In chapter 2.2, we hypothesized that patients with PA are more susceptible to endothelial IR than control patients with EHT. To test this hypothesis, we used brachial flow mediated dilation (FMD) before and after simulated forearm IR as a well-validated read out for IR injury. [45, 46]

Thirdly, we aimed to identify novel mechanisms that are active in patients with PA which contribute to the increased cardiovascular risk. We first aimed to translate preclinical findings of recently proposed mechanisms of direct adverse effects of aldosterone on the cardiovascular system to humans. Preclinical studies have reported that aldosterone can stimulate galectin-3 expression and secretion in various cell types relevant for the cardiovascular system via MR activation, and that Gal-3 mediates the pro-fibrotic effects of aldosterone. [47-50] We hypothesized that plasma Gal-3 concentrations are higher in patients with PA in comparison to patients with EHT, and that Gal-3 levels return to normal after adrenalectomy. The results of this study are described in chapter 2.3. Subsequently, we aimed to study the endogenous purine nucleotide adenosine in patients with PA. The adenosine concentration rapidly increases in situations of impending tissue danger, such as during ischemia, and adenosine receptor stimulation elicits various protective cardiovascular effects. In chapter 2.2, we describe the circulating adenosine concentration in patients with PA and EHT.

In the final part, we focussed on the protective effects of MR antagonists. First, we provided an overview of the literature on the cardioprotective effects of MR antagonists in chapter 3.1. MR antagonists consistently reduce IS in animal models of IR and attenuate cardiac remodeling in animal models with permanent arterial occlusion. Cardioprotection is also conferred in the absence of endogenous aldosterone. [8] These findings suggest inhibition of spontaneously active MRs (inverse agonism) or beneficial off-target effects of MR antagonists (pleiotropic actions) and these may contribute to the well documented benefits of spironolactone and eplerenone in humans *in vivo*: treatment with these MR antagonists improves morbidity and mortality in patients with systolic heart failure, and treatment with MR antagonists is non-inferior to permanent removal of aldosterone excess by adrenalectomy concerning target organ

damage. [34, 36, 42-44, 51] These observations indicate that it is highly relevant to unravel underlying pathways of the cardioprotective effects of MR antagonists, apart from aldosterone antagonism. In chapter 3.2, we hypothesized that eplerenone treatment increases extracellular levels of adenosine, since endogenous adenosine has been shown to be crucial for the cardioprotective effects of MR antagonists in preclinical models of myocardial infarction. [40] Finally, we investigated for the first time in human myocardial tissue whether eplerenone limits IR injury. We described the results of our study in chapter 3.3.

METHODS AND TECHNIQUES APPLIED

IN-VIVO TECHNIQUES

PULSE WAVE ANALYSIS AND PULSE WAVE VELOCITY

We analyzed the pulse wave of the right radial artery using applanation tonometry (SphygmoCor, AtCor Medical, Australia). The SphygmoCor software automatically generates the central aortic blood pressure and other central hemodynamic indices, like the augmentation index (AIx), from a 10 second recording of the radial pulse, after calibration for peripheral blood pressure (Figure 2). Although current guidelines on the diagnosis and treatment of hypertension are based on brachial blood pressure levels, assessment of central blood pressure may be superior in the identification and management of patients with an increased cardiovascular risk. [52] Indeed, target organs such as the heart and large vessels, are directly exposed to central blood pressure and not brachial blood pressure. When compared to brachial blood pressure, central blood pressure is more strongly associated with target organ damage and cardiovascular mortality. [53-55] AIx is a good predictor of future cardiovascular events and all-cause mortality. [56]

Pulse wave velocity (PWV) is the velocity at which the pulse wave, generated during each cardiac cycle, propagates through the arterial tree. PWV depends on the compliance of the arterial system and it is therefore a marker of arterial stiffness. Stiffening of the arteries is reflected in higher PWV's with a PWV of >10 m/s being considered as an indicator of subclinical target organ damage. [57] Arterial stiffness, as a reflection of structural changes in aortic media and adventitia resulting from arteriolosclerosis, is a strong predictor for future cardiovascular events, independent from traditional cardiovascular risk factors. [58-60]

For the assessment of the aortic PWV, we measured the pressure waves at the sites of the right carotid artery and the right femoral artery by applanation tonometry (SphygmoCor, AtCor Medical, Australia). The SphygmoCor software automatically calculates the transit time as the delay between the R-spike in the electrocardiogram and the arrival of the pressure waves at the recording sites. We estimated the travel distance by subtracting the distance from the carotid tonometer location to the sternal notch from the distance between the sternal notch to the femoral tonometer location. [61]

FLOW-MEDIATED DILATION

Ultrasound measurement of the brachial artery flow-mediated dilation (FMD) is a non-invasive validated measure to indirectly assess endothelial function. Brachial artery FMD predicts future cardiovascular events: it has been estimated that per 1 % increase in brachial FMD the risk for future cardiovascular events decreases by 13% (RR 0.87 (0.83-0.91 95% CI)). [62] In healthy children and adolescents, the FMD is 7-9 % and this percentage declines with age to approximately 3.5 % in older male patients (68-79 years). [63, 64]

We imaged the brachial artery in a darkened, temperature-controlled room using a 10-MHz multifrequency linear-array probe attached to a high-resolution ultrasound machine (Terason T3000, Burlington, MA) according to recent international guidelines. [65] After 1 minute of baseline recordings of diameter and blood flow velocity, a blood pressure cuff, positioned around the forearm, was inflated during 5 minutes at a pressure of 200 mmHg. We captured changes in brachial artery diameter and blood flow velocity until 3 minutes post-deflation and analyzed the recordings using computer-assisted software, utilizing edge-detection and wall-tracking.

The endothelial function is quantified by the percentage of change in brachial artery diameter after the increase in shear stress. This FMD is a read-out for nitric oxide production. [66] The increase in shear stress is the result of reactive hyperaemia, induced by the 5 minute occlusion of the forearm circulation by a blood pressure cuff applied distal from the site of measurement. The experimental set up of the FMD measurement is depicted in Figure 2.

To assess endothelial IR injury, the measurement of FMD was repeated after 20 minutes of ischemia and 20 minutes of reperfusion of the forearm, induced by a blood pressure cuff positioned around the upper arm. The reduction in brachial FMD after IR compared to the baseline FMD is a well-validated measure to assess endothelial IR injury. [45, 46]

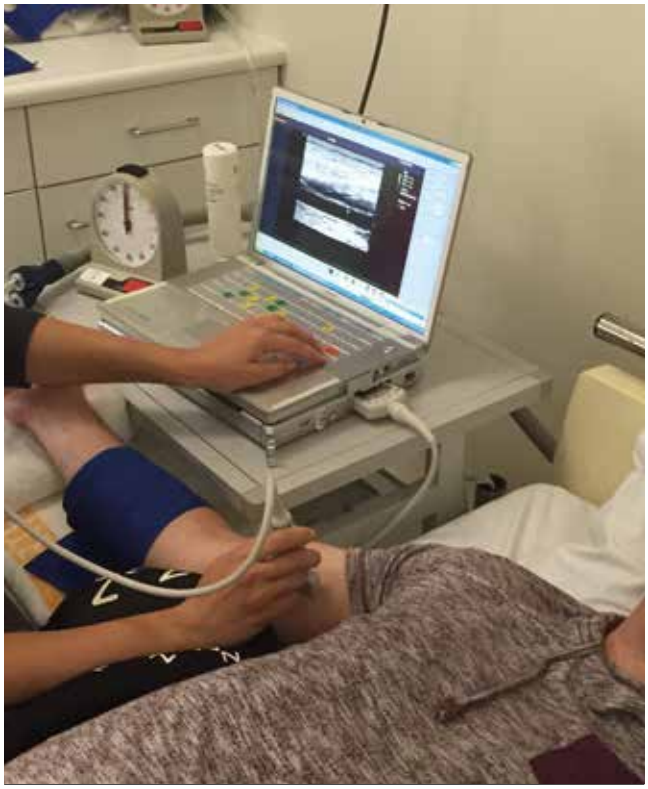


FIGURE 2. Experimental set up of the measurement of brachial FMD measurement

CAROTID INTIMA MEDIA THICKNESS AND CAROTID PLAQUE DETECTION

The early process of atherosclerosis starts with the infiltration of the arterial intima-media complex by lipids and inflammatory cells. Carotid intima-media thickness (cIMT) is a widely accepted non-invasive marker of generalized subclinical atherosclerosis, since it consistently predicts future myocardial infarction and stroke. [67, 68] The added value of cIMT measurement to cardiovascular risk scores however, remains questionable. [57, 69, 70] In contrast to a recent systematic review in which measurement of CIMT was of added value in those at intermediate risk for cardiovascular disease, cIMT measurement did not improve on the Framingham Risk Score for prediction of cardiovascular disease in a large study comparing the added value of risk markers in >6800 patients at intermediate risk. [67, 71]

We measured the intima-media thickness at the common carotid artery far wall over a 1 cm segment caudally from the carotid bulb, in 3 different angles of 90, 120 and 180 °, by high resolution B-mode ultrasound (Esaote Medical Systems, Italy). The integrated software of the Esaote platform uses radio-frequency technology to provide 6 measures, calculated as means from real-time values, obtained during 6 cardiac cycles. During carotid ultrasonography, the extracranial carotid arteries were scanned for the presence of plaques.

VENOUS OCCLUSION PLETHYSMOGRAPHY

Venous occlusion plethysmography is a well-validated technique used to measure the forearm blood flow (FBF) and the vasodilator response to drugs that are infused into the brachial artery. [72, 73] The major advantage of this approach is that the concentration of the tested drug in the forearm vascular bed is sufficiently high to induce local (vascular) responses, while systemic concentrations are sufficiently low to avoid disturbances on systemic haemodynamics.

We measured the FBF in a temperature-controlled room with the healthy volunteers in the supine position. By repetitively in- and deflating an upper arm cuff to a pressure of approximately 40 mmHg, which is above the venous pressure but below arterial pressure, forearm volume will change: arterial inflow does not alter while venous return

is hindered during inflation, leading to substantial increases in forearm volume. The rate of increase in forearm volume is detected by mercury-filled strain gauges, positioned around the proximal part of the forearm. By excluding the hand circulation during the measurements, the increase in FBF during inflation of the upper arm cuff is linear and proportional to the arterial inflow of the arm. The experimental set up is shown in Figure 3.

FIGURE 3. Experimental set up of venous occlusion plethysmography

In chapter 3.2, we used venous occlusion plethysmography during dipyridamole administration into the brachial artery to indirectly assess extracellular adenosine formation.

Adenosine is an endogenous purine nucleoside, which is formed by intra-, and extracellular degradation of adenosine monophosphate by the enzyme 5'-nucleotidase (CD73). Degradation of adenosine only occurs in the intracellular compartment. As a consequence, facilitated diffusion of adenosine over the cellular membrane by the

equilibrative nucleoside transporter (ENT) is normally directed inwards. Stimulation of membrane-bound adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) induces various effects, including vasodilation, inhibition of inflammation, and protection against IR-injury.

Measurement of circulating endogenous adenosine is extremely difficult, because the half life of adenosine in blood is very short due to rapid uptake and degradation by circulating erythrocytes and endothelial cells. [74, 75] This uptake occurs through facilitated diffusion by equilibrative nucleoside transporters (ENT). This facilitated diffusion is strongly inhibited by ENT-inhibitors such as dipyridamole. In the presence of this drug, endogenous adenosine formation is sufficient to induce vasodilation. Therefore, we used the vasodilator effect of the ENT inhibitor dipyridamole as a read-out for endogenous extracellular adenosine formation by the enzyme CD73, as previously described by our group and depicted in Figure 4. [76, 77]

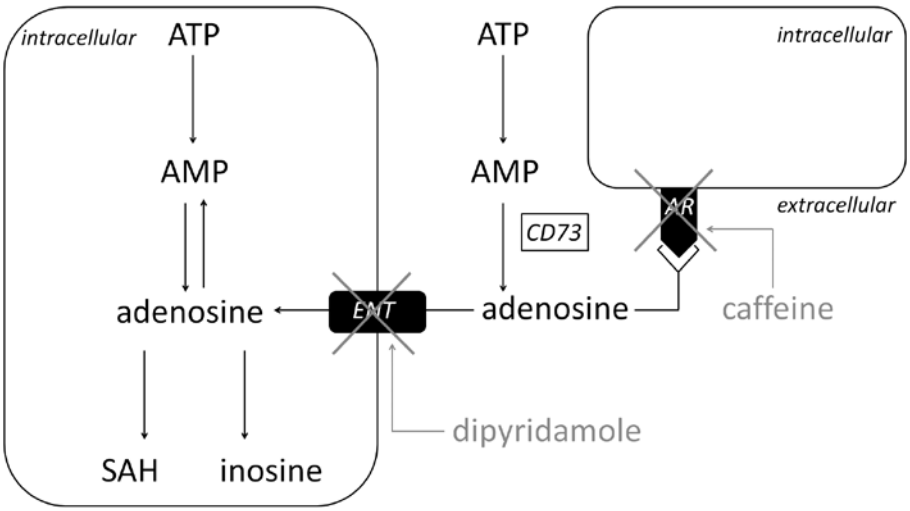


FIGURE 4. Simplified schematic overview of the extracellular formation and transport of adenosine. Dipyridamole increases the extracellular adenosine concentration by inhibition of the ENT, resulting in local vasodilation and increased FBF.

Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; AR, adenosine receptor; CD73 or ecto-5'-nucleotidase; ENT, equilibrative nucleoside transporter; SAH, S-adenosyl-L-homocysteine

EX-VIVO TECHNIQUES

CONTRACTILE FORCE OF ATRIAL TISSUE

We used human atrial trabeculae from patients undergoing cardiac surgery to study the effect of MR antagonists on IR injury. During cardiac surgery with extracorporeal circulation, the cardiothoracic surgeon incises the right atrial appendage to insert the cannula of the extracorporeal circulation. In patients who provided written informed consent, the right atrial appendage was dissected immediately after the introduction of this cannula and placed in a cold buffer solution. We used the experimental set up, as described by Speechly-Dick. [78, 79] (Figure 5)

From each atrial appendage, we isolated 2 trabeculae and suspended these vertically in an organ bath. We performed electrical field stimulation on both sides of the trabeculae and measured contractile force, calculated from the maximal tension during contraction and the minimal tension during relaxation. We exposed the trabeculae to IR and used the recovery of contractile force as a read-out parameter for IR injury.

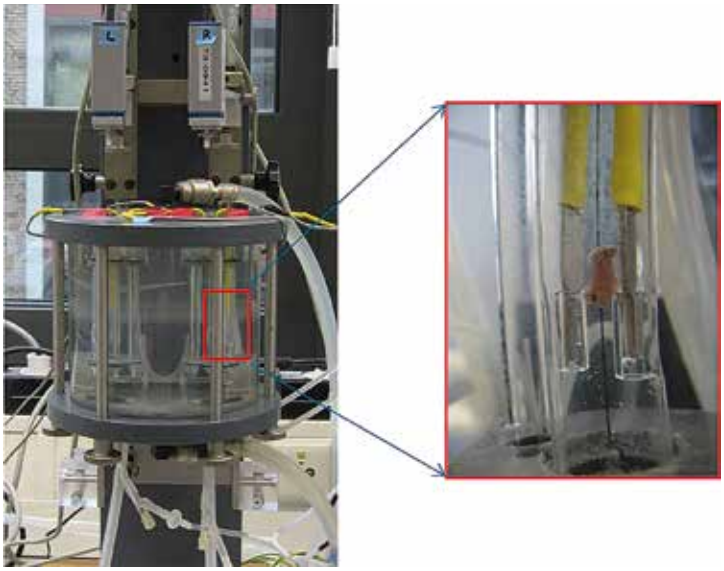


FIGURE 5. Experimental set up (organ bath) of the measurement of contractile force in human atrial tissue

Both pharmacological and ischemic preconditioning have been shown to increase post-ischemic recovery of contractile force in this *ex vivo* model. [78-80] We studied the effect of the MR antagonist eplerenone on IR injury in a paired fashion: per atrial appendage one trabecula was randomly assigned to superfusion with eplerenone and the other to vehicle.

REFERENCES

1. Grundy HM, Simpson SA, Tait JF. Isolation of a highly active mineralocorticoid from beef adrenal extract. *Nature.* 1952;169(4306):795-6.
2. Monticone S, D'Ascenzo F, Moretti C, Williams TA, Veglio F, Gaita F, et al. Cardiovascular events and target organ damage in primary aldosteronism compared with essential hypertension: a systematic review and meta-analysis. *The lancet Diabetes & endocrinology.* 2018;6(1):41-50.
3. Park YM, Park MY, Suh YL, Park JB. NAD(P)H oxidase inhibitor prevents blood pressure elevation and cardiovascular hypertrophy in aldosterone-infused rats. *Biochemical and biophysical research communications.* 2004;313(3):812-7.
4. Pu Q, Neves MF, Virdis A, Touyz RM, Schiffrin EL. Endothelin antagonism on aldosterone-induced oxidative stress and vascular remodeling. *Hypertension.* 2003;42(1):49-55.
5. Azibani F, Fazal L, Chatziantoniou C, Samuel JL, Delcayre C. Aldosterone mediates cardiac fibrosis in the setting of hypertension. *Current hypertension reports.* 2013;15(4):395-400.
6. De Angelis N, Fiordaliso F, Latini R, Calvillo L, Funicello M, Gobbi M, et al. Appraisal of the role of angiotensin II and aldosterone in ventricular myocyte apoptosis in adult normotensive rat. *Journal of molecular and cellular cardiology.* 2002;34(12):1655-65.
7. Fujita M, Minamino T, Asanuma H, Sanada S, Hirata A, Wakeno M, et al. Aldosterone nongenomically worsens ischemia via protein kinase C-dependent pathways in hypoperfused canine hearts. *Hypertension.* 2005;46(1):113-7.
8. van den Berg TN, Rongen GA, Frohlich GM, Deinum J, Hausenloy DJ, Riksen NP. The cardioprotective effects of mineralocorticoid receptor antagonists. *Pharmacology & therapeutics.* 2014;142(1):72-87.
9. Dhanjal TS, Beevers DG. Delay in the diagnosis of Conn's syndrome: a single-center experience over 30 years. *Hypertension.* 2008;52(3):e22; author reply e3.
10. Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, et al. The Management of Primary Aldosteronism: Case Detection, Diagnosis, and Treatment: An Endocrine Society Clinical Practice Guideline. *The Journal of clinical endocrinology and metabolism.* 2016;101(5):1889-916.
11. Nederlands Huisartsen Genootschap. Cardiovascular risicomanagement 2012 (tweede herziening) [Available from: www.nhg.org/standaarden/volledig/cardiovasculair-risicomanagement.

12. Mulatero P, Stowasser M, Loh KC, Fardella CE, Gordon RD, Mosso L, et al. Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. *The Journal of clinical endocrinology and metabolism.* 2004;89(3):1045-50.
13. Born-Frontsberg E, Reincke M, Rump LC, Hahner S, Diederich S, Lorenz R, et al. Cardiovascular and cerebrovascular comorbidities of hypokalemic and normokalemic primary aldosteronism: results of the German Conn's Registry. *The Journal of clinical endocrinology and metabolism.* 2009;94(4):1125-30.
14. Mulatero P, Monticone S, Burrello J, Veglio F, Williams TA, Funder J. Guidelines for primary aldosteronism: uptake by primary care physicians in Europe. *Journal of hypertension.* 2016;34(11):2253-7.
15. Kayser SC, Dekkers T, Groenewoud HJ, van der Wilt GJ, Carel Bakx J, van der Wel MC, et al. Study Heterogeneity and Estimation of Prevalence of Primary Aldosteronism: a Systematic Review and Meta-regression Analysis. *The Journal of clinical endocrinology and metabolism.* 2016:jc20161472.
16. Hannemann A, Wallaschofski H. Prevalence of primary aldosteronism in patient's cohorts and in population-based studies--a review of the current literature. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme.* 2012;44(3):157-62.
17. Rossi GP, Cesari M, Cuspidi C, Maiolino G, Cicala MV, Bisogni V, et al. Long-term control of arterial hypertension and regression of left ventricular hypertrophy with treatment of primary aldosteronism. *Hypertension.* 2013;62(1):62-9.
18. Rossi GP, Bolognesi M, Rizzoni D, Seccia TM, Piva A, Porteri E, et al. Vascular remodeling and duration of hypertension predict outcome of adrenalectomy in primary aldosteronism patients. *Hypertension.* 2008;51(5):1366-71.
19. Wang W, Hu W, Zhang X, Wang B, Bin C, Huang H. Predictors of successful outcome after adrenalectomy for primary aldosteronism. *International surgery.* 2012;97(2):104-11.
20. Ahmed AH, Gordon RD, Sukor N, Pimenta E, Stowasser M. Quality of life in patients with bilateral primary aldosteronism before and during treatment with spironolactone and/or amiloride, including a comparison with our previously published results in those with unilateral disease treated surgically. *The Journal of clinical endocrinology and metabolism.* 2011;96(9):2904-11.
21. Sukor N, Kogovsek C, Gordon RD, Robson D, Stowasser M. Improved quality of life, blood pressure, and biochemical status following laparoscopic adrenalectomy for unilateral primary aldosteronism. *The Journal of clinical endocrinology and metabolism.* 2010;95(3):1360-4.

22. Sonino N, Tomba E, Genesia ML, Bertello C, Mulatero P, Veglio F, et al. Psychological assessment of primary aldosteronism: a controlled study. *The Journal of clinical endocrinology and metabolism.* 2011;96(6):E878-83.
23. Dekkers T, Prejbisz A, Kool LJ, Groenewoud HJ, Velema M, Spiering W, et al. Adrenal vein sampling versus CT scan to determine treatment in primary aldosteronism: an outcome-based randomised diagnostic trial. *The lancet Diabetes & endocrinology.* 2016.
24. Muth A, Ragnarsson O, Johannsson G, Wangberg B. Systematic review of surgery and outcomes in patients with primary aldosteronism. *The British journal of surgery.* 2015;102(4):307-17.
25. Meyer A, Brabant G, Behrend M. Long-term follow-up after adrenalectomy for primary aldosteronism. *World journal of surgery.* 2005;29(2):155-9.
26. Blumenfeld JD, Sealey JE, Schlussel Y, Vaughan ED, Jr., Sos TA, Atlas SA, et al. Diagnosis and treatment of primary hyperaldosteronism. *Annals of internal medicine.* 1994;121(11):877-85.
27. Reimel B, Zanocco K, Russo MJ, Zarnegar R, Clark OH, Allendorf JD, et al. The management of aldosterone-producing adrenal adenomas--does adrenalectomy increase costs? *Surgery.* 2010;148(6):1178-85; discussion 85.
28. Lin Y, Li L, Zhu J, Qiang W, Makiyama K, Kubota Y. Experience of retroperitoneoscopic adrenalectomy in 195 patients with primary aldosteronism. *International journal of urology : official journal of the Japanese Urological Association.* 2007;14(10):910-3.
29. Walz MK, Gwosdz R, Levin SL, Alesina PF, Suttorp AC, Metz KA, et al. Retroperitoneoscopic adrenalectomy in Conn's syndrome caused by adrenal adenomas or nodular hyperplasia. *World journal of surgery.* 2008;32(5):847-53.
30. Alexandre J, Hof T, Puddu PE, Rouet R, Guinamard R, Manrique A, et al. Rapid and MR-Independent IK1 Activation by Aldosterone during Ischemia-Reperfusion. *PloS one.* 2015;10(7):e0132592.
31. Ruhs S, Nolze A, Hubschmann R, Grossmann C. 30 YEARS OF THE MINERALOCORTICOID RECEPTOR: Nongenomic effects via the mineralocorticoid receptor. *The Journal of endocrinology.* 2017;234(1):T107-t24.
32. Velema M, Dekkers T, Hermus A, Timmers H, Lenders J, Groenewoud H, et al. Quality of Life in Primary Aldosteronism: A Comparative Effectiveness Study of Adrenalectomy and Medical Treatment. *The Journal of clinical endocrinology and metabolism.* 2018;103(1):16-24.
33. Marzano L, Colussi G, Sechi LA, Catena C. Adrenalectomy is comparable with medical treatment for reduction of left ventricular mass in primary aldosteronism: meta-analysis of long-term studies. *American journal of hypertension.* 2015;28(3):312-8.

34. Catena C, Colussi G, Lapenna R, Nadalini E, Chiuch A, Gianfagna P, et al. Long-term cardiac effects of adrenalectomy or mineralocorticoid antagonists in patients with primary aldosteronism. *Hypertension.* 2007;50(5):911-8.
35. Rossi GP, Sacchetto A, Visentin P, Canali C, Graniero GR, Palatini P, et al. Changes in left ventricular anatomy and function in hypertension and primary aldosteronism. *Hypertension.* 1996;27(5):1039-45.
36. Catena C, Colussi G, Nadalini E, Chiuch A, Baroselli S, Lapenna R, et al. Cardiovascular outcomes in patients with primary aldosteronism after treatment. *Archives of internal medicine.* 2008;168(1):80-5.
37. Chai W, Garrelds IM, Arulmani U, Schoemaker RG, Lamers JM, Danser AH. Genomic and nongenomic effects of aldosterone in the rat heart: why is spironolactone cardioprotective? *British journal of pharmacology.* 2005;145(5):664-71.
38. Chai W, Garrelds IM, de Vries R, Danser AH. Cardioprotective effects of eplerenone in the rat heart: interaction with locally synthesized or blood-derived aldosterone? *Hypertension.* 2006;47(4):665-70.
39. Mihailidou AS, Loan Le TY, Mardini M, Funder JW. Glucocorticoids activate cardiac mineralocorticoid receptors during experimental myocardial infarction. *Hypertension.* 2009;54(6):1306-12.
40. Schmidt K, Tissier R, Ghaleb B, Drogies T, Felix SB, Krieg T. Cardioprotective effects of mineralocorticoid receptor antagonists at reperfusion. *European heart journal.* 2010;31(13):1655-62.
41. Loan Le TY, Mardini M, Howell VM, Funder JW, Ashton AW, Mihailidou AS. Low-dose spironolactone prevents apoptosis repressor with caspase recruitment domain degradation during myocardial infarction. *Hypertension.* 2012;59(6):1164-9.
42. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *The New England journal of medicine.* 1999;341(10):709-17.
43. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. The *New England journal of medicine.* 2003;348(14):1309-21.
44. Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, et al. Eplerenone in patients with systolic heart failure and mild symptoms. *The New England journal of medicine.* 2011;364(1):11-21.

45. Kharbanda RK, Peters M, Walton B, Kattenhorn M, Mullen M, Klein N, et al. Ischemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischemia-reperfusion in humans in vivo. *Circulation.* 2001;103(12):1624-30.
46. Loukogeorgakis SP, van den Berg MJ, Sofat R, Nitsch D, Charakida M, Haiyee B, et al. Role of NADPH oxidase in endothelial ischemia/reperfusion injury in humans. *Circulation.* 2010;121(21):2310-6.
47. Calvier L, Martinez-Martinez E, Miana M, Cachofeiro V, Rousseau E, Sadaba JR, et al. The impact of galectin-3 inhibition on aldosterone-induced cardiac and renal injuries. *JACC Heart failure.* 2015;3(1):59-67.
48. Calvier L, Miana M, Reboul P, Cachofeiro V, Martinez-Martinez E, de Boer RA, et al. Galectin-3 mediates aldosterone-induced vascular fibrosis. *Arteriosclerosis, thrombosis, and vascular biology.* 2013;33(1):67-75.
49. Martinez-Martinez E, Calvier L, Fernandez-Celis A, Rousseau E, Jurado-Lopez R, Rossoni LV, et al. Galectin-3 Blockade Inhibits Cardiac Inflammation and Fibrosis in Experimental Hyperaldosteronism and Hypertension. *Hypertension.* 2015.
50. Lin YH, Chou CH, Wu XM, Chang YY, Hung CS, Chen YH, et al. Aldosterone induced galectin-3 secretion in vitro and in vivo: from cells to humans. *PloS one.* 2014;9(9):e95254.
51. Milliez P, Girerd X, Plouin PF, Blacher J, Safar ME, Mourad JJ. Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. *Journal of the American College of Cardiology.* 2005;45(8):1243-8.
52. McEniery CM, Yasmin, McDonnell B, Munnery M, Wallace SM, Rowe CV, et al. Central pressure: variability and impact of cardiovascular risk factors: the Anglo-Cardiff Collaborative Trial II. *Hypertension.* 2008;51(6):1476-82.
53. Roman MJ, Devereux RB, Kizer JR, Lee ET, Galloway JM, Ali T, et al. Central pressure more strongly relates to vascular disease and outcome than does brachial pressure: the Strong Heart Study. *Hypertension.* 2007;50(1):197-203.
54. Wang KL, Cheng HM, Chuang SY, Spurgeon HA, Ting CT, Lakatta EG, et al. Central or peripheral systolic or pulse pressure: which best relates to target organs and future mortality? *Journal of hypertension.* 2009;27(3):461-7.
55. Kollias A, Lagou S, Zeniodi ME, Boubouchairopoulou N, Stergiou GS. Association of Central Versus Brachial Blood Pressure With Target-Organ Damage: Systematic Review and Meta-Analysis. *Hypertension.* 2016;67(1):183-90.

56. Vlachopoulos C, Aznaouridis K, O'Rourke MF, Safar ME, Baou K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with central haemodynamics: a systematic review and meta-analysis. *European heart journal.* 2010;31(15):1865-71.
57. 2013 Practice guidelines for the management of arterial hypertension of the European Society of Hypertension (ESH) and the European Society of Cardiology (ESC): ESH/ESC Task Force for the Management of Arterial Hypertension. *Journal of hypertension.* 2013;31(10):1925-38.
58. Vlachopoulos C, Xaplanteris P, Aboyans V, Brodmann M, Cifkova R, Cosentino F, et al. The role of vascular biomarkers for primary and secondary prevention. A position paper from the European Society of Cardiology Working Group on peripheral circulation: Endorsed by the Association for Research into Arterial Structure and Physiology (ARTERY) Society. *Atherosclerosis.* 2015;241(2):507-32.
59. Laurent S, Alivon M, Beaussier H, Boutouyrie P. Aortic stiffness as a tissue biomarker for predicting future cardiovascular events in asymptomatic hypertensive subjects. *Annals of medicine.* 2012;44 Suppl 1:S93-7.
60. Laurent S, Boutouyrie P, Asmar R, Gautier I, Laloux B, Guize L, et al. Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension.* 2001;37(5):1236-41.
61. Weber T, Ammer M, Rammer M, Adji A, O'Rourke MF, Wassertheurer S, et al. Noninvasive determination of carotid-femoral pulse wave velocity depends critically on assessment of travel distance: a comparison with invasive measurement. Journal of hypertension. 2009;27(8):1624-30.
62. Inaba Y, Chen JA, Bergmann SR. Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: a meta-analysis. *The international journal of cardiovascular imaging.* 2010;26(6):631-40.
63. Hopkins ND, Dengel DR, Stratton G, Kelly AS, Steinberger J, Zavala H, et al. Age and sex relationship with flow-mediated dilation in healthy children and adolescents. *Journal of applied physiology* (Bethesda, Md : 1985). 2015;119(8):926-33.
64. van den Munckhof I, Riksen N, Seeger JP, Schreuder TH, Borm GF, Eijsvogels TM, et al. Aging attenuates the protective effect of ischemic preconditioning against endothelial ischemia-reperfusion injury in humans. *American journal of physiology Heart and circulatory physiology.* 2013;304(12):H1727-32.
65. Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, et al. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *American journal of physiology Heart and circulatory physiology.* 2011;300(1):H2-12.

66. Mullen MJ, Kharbanda RK, Cross J, Donald AE, Taylor M, Vallance P, et al. Heterogenous nature of flow-mediated dilatation in human conduit arteries in vivo: relevance to endothelial dysfunction in hypercholesterolemia. *Circulation research.* 2001;88(2):145-51.

67. Peters SA, den Ruijter HM, Bots ML, Moons KG. Improvements in risk stratification for the occurrence of cardiovascular disease by imaging subclinical atherosclerosis: a systematic review. *Heart (British Cardiac Society).* 2012;98(3):177-84.

68. Greenland P, Alpert JS, Beller GA, Benjamin EJ, Budoff MJ, Fayad ZA, et al. 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Journal of the American College of Cardiology.* 2010;56(25):e50-103.

69. Bots ML, Groenewegen KA, Anderson TJ, Britton AR, Dekker JM, Engstrom G, et al. Common carotid intima-media thickness measurements do not improve cardiovascular risk prediction in individuals with elevated blood pressure: the USE-IMT collaboration. *Hypertension.* 2014;63(6):1173-81.

70. Goff DC, Jr., Lloyd-Jones DM, Bennett G, Coady S, D'Agostino RB, Gibbons R, et al. 2013 ACC/AHA guideline on the assessment of cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Circulation.* 2014;129(25 Suppl 2):S49-73.

71. Yeboah J, McClelland RL, Polonsky TS, Burke GL, Sibley CT, O'Leary D, et al. Comparison of novel risk markers for improvement in cardiovascular risk assessment in intermediate-risk individuals. *JAMA : the journal of the American Medical Association.* 2012;308(8):788-95.

72. Benjamin N, Calver A, Collier J, Robinson B, Vallance P, Webb D. Measuring forearm blood flow and interpreting the responses to drugs and mediators. *Hypertension.* 1995;25(5):918-23.

73. Wilkinson IB, Webb DJ. Venous occlusion plethysmography in cardiovascular research: methodology and clinical applications. *British journal of clinical pharmacology.* 2001;52(6):631-46.

74. Moser GH, Schrader J, Deussen A. Turnover of adenosine in plasma of human and dog blood. *The American journal of physiology.* 1989;256(4 Pt 1):C799-806.

75. Ramakers BP, Pickkers P, Deussen A, Rongen GA, van den Broek P, van der Hoeven JG, et al. Measurement of the endogenous adenosine concentration in humans in vivo: methodological considerations. *Current drug metabolism.* 2008;9(8):679-85.

76. Riksen NP, Rongen GA, Yellon D, Smits P. Human in vivo research on the vascular effects of adenosine. *European journal of pharmacology.* 2008;585(2-3):220-7.

77. Meijer P, Oyen WJ, Dekker D, van den Broek PH, Wouters CW, Boerman OC, et al. Rosuvastatin increases extracellular adenosine formation in humans in vivo: a new perspective on cardiovascular protection. *Arteriosclerosis, thrombosis, and vascular biology*. 2009;29(6):963-8.

78. Speechly-Dick ME, Grover GJ, Yellon DM. Does ischemic preconditioning in the human involve protein kinase C and the ATP-dependent K+ channel? Studies of contractile function after simulated ischemia in an atrial in vitro model. *Circulation research*. 1995;77(5):1030-5.

79. Riksen NP, Zhou Z, Oyen WJ, Jaspers R, Ramakers BP, Brouwer RM, et al. Caffeine prevents protection in two human models of ischemic preconditioning. *Journal of the American College of Cardiology*. 2006;48(4):700-7.

80. Morris SD, Yellon DM. Angiotensin-converting enzyme inhibitors potentiate preconditioning through bradykinin B2 receptor activation in human heart. *Journal of the American College of Cardiology*. 1997;29(7):1599-606.


157
fp
fp
173
p
191
197
204
213
219
G.P.
p
f
229
f

CHAPTER 2

THE CARDIOVASCULAR EFFECTS OF ALDOSTERONE BEYOND BLOOD PRESSURE REGULATION IN HUMANS *IN VIVO*


tr
tr
tr
p
8
do
f
17
f
24
tr
tr
31
cre - scen - do
Adagio
Solo
11
19
2

CHAPTER 2.1

DO PATIENTS WITH PRIMARY ALDOSTERONISM HAVE CARDIOVASCULAR DAMAGE AT TIME OF DIAGNOSING HYPERTENSION IN PRIMARY CARE?

T.N.A. (Daniëlle) van den Berg [a,b] | Sabine C. Käyser [c]
Anke C.C.M. van Mil [d] | Niels P. Riksen [a] | Tjard R. Schermer [c,e]
Bianca W.M. Schalk [c] | Dick H.J. Thijssen [d] | Gerard A. Rongen [a,b]
Wim DE Grauw [c] | Jacques W.M. Lenders [a,f]
Marion C.J. Biermans [c] | Jaap Deinum [a,]

[a] Department of Internal Medicine,[b] Department of Pharmacology and Toxicology,
[c] Department of Primary and Community Care, [d] Department of Physiology,
Radboud university medical center, Nijmegen, The Netherlands
[e] Netherlands institute for Health Services Research (Nivel), Utrecht, the Netherlands
[f] Department of Internal Medicine III, University Hospital Carl Gustav Carus,
Technische Universität Dresden, Dresden, Germany

Submitted

ABSTRACT

Background Patients with primary aldosteronism (PA) have a higher risk of cardiovascular complications compared to patients with essential hypertension (EHT) and similar blood pressure levels. It is unclear whether cardiovascular damage is already present at the time of diagnosing hypertension.

Objective The aim of this exploratory study was to assess cardiovascular organ damage in patients who were screened for PA at the time of diagnosing hypertension in primary care.

Methods We prospectively assessed cardiovascular damage in six patients with newly diagnosed PA and 24 matched patients with EHT at the time when hypertension was diagnosed in the primary care. We performed detailed cardiovascular assessment, including ankle-brachial index, echocardiography, flow-mediated vasodilation, carotid ultrasonography, central aortic blood pressure, pulse wave velocity, and urinary albumin-to-creatinine ratio measurement.

Results Two of the six patients with PA versus none of the patients with EHT (p=0.04) fulfilled the criteria of concentric LVH (>115 g/m2 in men and >95 g/m2 in women). After adjustment for gender, age and blood pressure, left ventricular mass index was higher in the patients with PA than in the patients with EHT. We did not observe differences in the other outcome measures between the patient groups.

Conclusion At the time of the diagnosis of hypertension, patients with PA have a higher frequency of LVH than patients with EHT. If confirmed in larger studies, this finding suggests that early biochemical testing for PA, and specific treatment of PA, might contribute to the prevention of further progression of cardiovascular damage due to inadequately treated PA.

INTRODUCTION

Primary aldosteronism (PA) is characterized by unilateral or bilateral autonomous overproduction of aldosterone in the adrenal cortex. PA is the most common cause of secondary hypertension, with prevalence rates varying from 3-12% in primary care versus 1-30 % in referral centres. [1, 2]

According to the Endocrine Society guideline, the diagnosis of PA should be considered in specific patients with hypertension. [3] However, in daily clinical primary care practice, this recommendation on testing for PA is commonly omitted, as general practitioners are generally not aware of this guideline. [4] To complicate matters further, hypokalemia, previously considered to be a prerequisite for a diagnosis of PA, is present in only less than 40% of the PA patients. So, this biomarker has limited utility to incite appropriately testing for PA. [5] For these reasons the diagnosis of PA has been reported to be delayed for up to eight years. [6]

The delay in a timely diagnosis of PA is potentially harmful for patients for at least two reasons. First, treatment of PA differs from usual antihypertensive treatment in patients with essential hypertension (EHT). Patients with PA require specific treatment: those with a unilateral aldosterone-producing adenoma are advised to undergo adrenalectomy, whereas those with bilateral aldosterone overproduction are treated with a mineralocorticoid receptor (MR) antagonist, such as spironolactone. [3] Second, patients with PA have a higher risk of cardiovascular complications in comparison to patients with EHT with similar blood pressure levels. [7] This may be explained by a direct effect of aldosterone unopposed by appropriate treatment during the long pre-diagnostic phase, inducing cardiovascular organ damage well before diagnosis. The higher risk of cardiovascular complications in patients with PA suggests the need for timely biochemical testing for PA in (newly diagnosed) hypertensive patients to prevent further development of cardiovascular organ damage.

In this explorative study in the primary care setting, we prospectively assessed cardiovascular and renal damage in patients in whom PA was detected at the time when

hypertension was diagnosed for the first time. A group of newly diagnosed patients with EHT, matched for gender, age and blood pressure, served as a control group.

METHODS

PATIENTS

We included all patients over 18 years with newly diagnosed never treated hypertension from 55 primary care centres in the Netherlands from August 1st 2013 to December 31st 2015. In the context of a previous study on the prevalence of PA, the participating patients had plasma aldosterone and renin measured at the time of diagnosing hypertension, and before starting antihypertensive treatment. This study has been described in detail elsewhere. [8]

The diagnosis of hypertension was made according to the current guideline by the European Society of Hypertension. [9] In brief, hypertension was diagnosed: 1) if the average office blood pressure of at least two blood pressure measurements per day was ≥140/90 mmHg on two or more different visits within six months, or 2) if 24-hour ambulatory blood pressure measurement (ABPM) was ≥130/80 mmHg.

Patients with newly diagnosed hypertension were screened for PA by measurement of plasma aldosterone and renin. In case of an aldosterone-to-renin ratio (ARR) of >40 pmol/mU and a plasma aldosterone concentration of >400 pmol/L, an intravenous saline loading test (SLT; two litres NaCl 0.9% in four hours) was performed. Diagnosis of PA was made when the aldosterone concentration exceeded 280 pmol/L after saline loading. Patients were considered to have EHT if ARR values were ≤40 pmol/mU with a concomitant low baseline aldosterone level of ≤400 pmol/L (ARR_{neg}), or an aldosterone value of ≤280 pmol/L after SLT ($ARR_{pos}SLT_{neg}$). During biochemical testing, patients did not use medication that interfered with aldosterone and renin levels. [3]

We included all patients diagnosed with PA and applied the following exclusion criteria: age <18 years, hypertensive crisis, heart failure classes II-IV (defined by the New York Heart Association) [10], diabetes mellitus, estimated glomerular filtration rate of <45 ml/min/1.73m^2, pregnancy or breast feeding, and severe comorbidity that would seriously interfere with study procedures. We applied similar exclusion criteria for the patients with EHT. We matched the patients with PA with the patients with EHT for gender, age and baseline blood pressure. For every patient with PA, we included four control patients with EHT: one $ARR_{pos}SLT_{neg}$ patient, and three ARR_{neg} patients. This numeric relation corresponds to the ratio ARR_{pos}/ARR_{neg} that was found among patients with newly diagnosed EHT in primary care. [8]

This study was approved by the Ethics Committee of the Radboud university medical center. All patients provided written informed consent before enrollment. The study was conducted in accordance with Good Clinical Practices, and the Declaration of Helsinki, and was prospectively registered at ClinicalTrials.gov by number NCT01728493.

CLINICAL DATA

We collected the following clinical data: body mass index (BMI), medication use, smoking status (pack years (PY)), previous and family history of cardiovascular disease, alcohol intake per day (units of 10 g), physical exercise (standard defined as 30 minutes/day during five or more days/week), snoring (defined as light or heavy according to the patients' judgement) or diagnosis of obstructive sleep apnea syndrome (OSAS) and history of gestational hypertension. We expressed the antihypertensive medication in daily defined doses (DDD), as defined by the World Health Organisation. [11] From August 1st 2013 to December 14th 2014, plasma aldosterone was measured using the Coat-A-Count aldosterone radioimmunoassay (RIA) from Siemens Medical Solutions Diagnostics (United States of America). From December 15th 2014 to December 31st 2015, plasma aldosterone was measured by the Active Aldosterone RIA kit from Beckman Coulter (Czech Republic). Plasma renin concentration was measured using the DSL-25100 active renin immunoradiometric assay (IRMA) from Diagnostic Systems Laboratories (United States of America). Baseline plasma concentrations of creatinine, potassium, cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and triglycerides were determined using standard assays in a central laboratory (SHO, Velp, the Netherlands).

STUDY PROTOCOL

In patients with PA and $ARR_{pos}SLT_{neg}$ patients with EHT, ankle brachial index (ABI) and urinary albumin-to-creatinine ratio (uACR) were performed during their admission for SLT. Echocardiography was carried out shortly after this. We performed measurements of the other cardiovascular risk markers during a separate visit. In ARR_{neg} patients with EHT, all measures were obtained during a single visit. We measured the brachial blood pressure in the supine position using a manual sphygmomanometer (Welch Allyn, Leiden, the Netherlands), in a quiet room after a period of five minutes rest according to the guideline. [12] All patients abstained from caffeine, alcohol and products rich on vitamin C and/or flavonoids 24 hours before the measurements. We performed the measurements at least six hours after fasting. We asked the patients not to smoke six hours before the experiments and to refrain from exercise during 24 hours before the measurements. Patients took their medication after finishing all vascular measurements on the day of the experiments. [13, 14] We assessed the following seven primary outcomes:

ANKLE-BRACHIAL INDEX (ABI)

For the measurement of the ABI we used the standardized technique as described by the American Heart Association. [15] In brief, we performed limb pressure measurements after at least five minutes of rest in the supine position. All limb pressure measurements were done by Doppler (Dopplex D900, Huntleigh Healthcare Ltd, Cardiff, UK) in the following sequence: right brachial artery, right tibial posterior artery, right dorsal pedal artery, left tibial posterior artery, left dorsal pedal artery, and left brachial artery. When the pressure between both brachial arteries exceeded 10 mmHg, we performed a second measurement of the right brachial artery and discarded the first measurement. We expressed the ABI as the highest lower-extremity blood pressure, divided by the highest blood pressure in both arms.

ECHOCARDIOGRAPHY

Standard echocardiographic examinations were carried out with subjects in the partial left decubitus position using a commercially available instrument (GE Vivid E9, General Electric, Horten, Norway), equipped with the multifrequency 1.5-4.0 MHz M5S transducer. End-diastolic and end-systolic left ventricular internal diameters (LVIDd, LVIDs), interventricular septum and posterior wall thicknesses (PWT) were measured

from two dimensional parasternal long axis view, from which left ventricular mass index (LVMI) was calculated according to the American Society of Echocardiography guidelines and normalized by body surface area. [16] Relative wall thickness (RWT) was calculated as 2 × PWT/LVIDd. A normal LVMI was defined as ≤115 g/m^2 in men, and ≤95 g/m^2 in women. We defined eccentric hypertrophy as an increased LVMI (>115 g/m^2 in men, and >95 g/m^2 in women) with a RWT <0.42, and concentric hypertrophy as an increased LVMI with a RWT >0.42. Furthermore, concentric remodeling was defined as a normal LVMI, but increased RWT (>0.42). [16]

Left ventricular (LV) filling, in casu diastolic LV function was assessed by the standard pulsed and tissue Doppler technique. [17] The following parameters were considered: the early diastolic mitral peak flow velocity (E), the late diastolic mitral peak flow velocity (A), their ratio (E/A ratio), and the average of both maximal early diastolic tissue velocity of the medial and lateral mitral annulus (E') and the average E/E'.

FLOW-MEDIATED DILATION (FMD)

An experienced researcher of the Department of Physiology of the Radboud university medical center measured brachial FMD in a darkened, temperature-controlled room of 22.1 ± 0.4 °C using a 10-MHz multifrequency linear-array probe attached to a high-resolution ultrasound machine (Terason T3000, Burlington, USA) according to the guideline of Thijssen *et al.* [14] The researcher was blinded for the diagnosis. Briefly, a sphygmomanometer blood pressure cuff was positioned around the forearm and the brachial artery was imaged proximally of the antecubital fossa. After one minute of baseline recordings of diameter and blood flow velocity, the cuff was inflated for five minutes, at a pressure of 200 mmHg or at least 50 mmHg above systolic blood pressure (SBP). We captured changes in brachial artery diameter and blood flow velocity 30 seconds before cuff deflation until three minutes post-deflation, and analyzed the recordings offline in a blinded fashion using computer-assisted software, utilizing edge-detection and wall-tracking. We expressed the FMD as the % change in diameter ((peak diameter after deflation minus baseline diameter)/baseline diameter x 100%).

CAROTID INTIMA-MEDIA THICKNESS (CIMT)

The cIMT was measured by high resolution B-mode ultrasound with a 7.5-MHz linear-array transducer (Esaote Biomedica, Genoa, Italy). We measured the intima and media

of the left and right common carotid artery far wall over a 1 cm segment caudally from the carotid bulb, in three different angles of 90, 120 and 180°. The integrated software of the Esaote platform uses radio-frequency technology to provide six measures, calculated as means from real-time values, obtained during six cardiac cycles. The standard deviation (SD) of these six mean measures was directly visible and the data were accepted if the SD did not exceed 20 μm. We calculated the mean diameter and cIMT from 18 measures (six means times three angles) of every patient for the left and right carotid artery. We thoroughly scanned the extracranial carotid arteries for the presence of plaques. A plaque was defined as a focal wall thickening of ≥50% compared to the surrounding vessel wall, or a local cIMT greater than 1.5 mm, according to the consensus statement from the American Society of Echocardiography. [18]

CENTRAL AORTIC BLOOD PRESSURE AND PULSE WAVE VELOCITY (PWV)

With the patient in the supine position, we performed pulse wave analysis of the right radial artery using applanation tonometry (SphygmoCor, AtCor Medical, Australia). The SphygmoCor software generates the central aortic blood pressure and AIx from a ten second recording after calibration for peripheral blood pressure. We discarded measurements that did not meet the quality control criteria of the software. We recorded the median of three valid central aortal blood pressure measurements. For the assessment of the aortic PWV, we measured the pressure waves at the sites of the right carotid artery and the right femoral artery. The SphygmoCor software automatically calculates the transit time as the delay between the R-spike in the electrocardiogram and the arrival of the pressure waves at the recording sites. We estimated the travel distance by subtracting the distance from the carotid tonometer location to the sternal notch from the distance between the sternal notch to the femoral tonometer location. [19] In all patients we performed three measurements and recorded the median PWV. When the difference between the first and second PWV was ≤0.5 m/s, we did not perform a third measurement and recorded the mean PWV of these two measurements. [20] The quality of the pressure wave was directly analyzed by the Sphygmocor software. If the SD was ≥10% of the PWV measurement, we discarded the measurement and replaced it by a novel measurement, up to a maximum of six attempts.

URINARY ALBUMIN-TO-CREATININE RATIO

The uACR was measured in a single urine sample by the Department of Clinical Chemistry of the Radboud university medical center. Urinary albumin was measured using a nephelometric technology (BN II analyzer, Siemens, The Netherlands). Urinary creatinine was analyzed by Cobas 8000, (Roche Diagnostics, The Netherlands).

STATISTICAL ANALYSIS

For the analysis of the data, we used IBM SPSS Statistics 22. We expressed all values as mean ± SD. We considered a significance value of <0.05 (two-sided). Differences between patients with PA and patients with EHT were compared using an independent t-test. Because of the small sample size, we checked the robustness of the independent t-test with bootstrapping. Differences in proportions were compared using the Fisher's exact test (two-sided). For comparisons between more than two groups, we used a one-way ANOVA. We compared each of the single outcome measures between the patients with PA and EHT using a general linear model with correction for gender, age and blood pressure. Central aortic blood pressures were corrected only for gender and age, because the Sphygmocor software calculates the central blood pressure and AIx from the brachial blood pressure.

RESULTS

2.1

PATIENTS AND CLINICAL CHARACTERISTICS

Of the patients with newly diagnosed hypertension, nine were diagnosed with PA.[8] Of these patients, three patients declined to participate in the present study. Therefore, we included six patients with PA and 24 matched control patients with EHT in our study. For an overview of the selection of our study population see Figure 1.

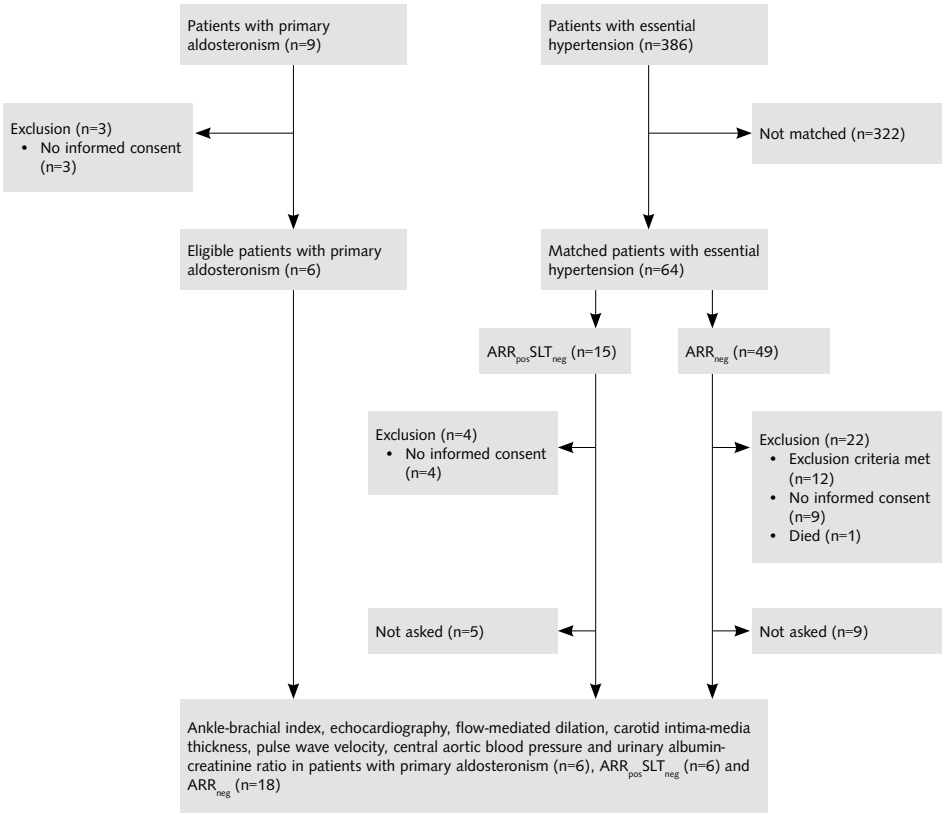


FIGURE 1. Overview of the selection process of patients

In this total group of 30 patients, the diagnosis of hypertension was based on office blood pressure measurements in 26 patients and on APBM in four patients. [9] Four patients with PA had bilateral aldosterone overproduction and one had a unilateral aldosterone-producing adenoma. In one patient laterality could not be determined, as this patient refused adrenal venous sampling and computed tomography. One of the six patients with PA dropped out, because of recently diagnosed breast cancer. We did not perform FMD, PWV and central aortic blood pressure in this patient. Due to technical problems, we excluded the results of FMD in one patient with EHT. In two patients with EHT we did not obtain a valid PWV (SD >10% of the mean PWV) due to obesity.

There were no differences in baseline characteristics between the patients with PA and EHT regarding cardiovascular risk factors and laboratory screening, except for differences in plasma aldosterone, ARR, and potassium values (Table 1). None of the patients had hypokalemia.

In the PA and the $ARR_{pos}SLT_{neg}$ patients, ABI and uACR were assessed after a mean period of one month after the diagnosis of hypertension (Table 2). The patients with PA did not use any antihypertensive medication at that time and their SBP was significantly higher compared to ARR_{neg} patients (Table 3A). During echocardiography, two patients with PA used antihypertensive medication. In the other four patients with PA, antihypertensive treatment started after echocardiography. Echocardiography was performed 3.7 ± 2.9, 6.3 ± 9.2 and 19.1 ± 7.2 months after the diagnosis of hypertension in PA, $ARR_{pos}SLT_{neg}$ patients (with EHT) and ARR_{neg} patients (with EHT) respectively (p<0.01). During assessment of FMD, cIMT, PWV, and central aortic blood pressure, blood pressure levels and DDD of antihypertensive drugs were comparable between patients with PA and patients with EHT (Table 3B). The general practitioner had started antihypertensive drugs in 12 of the 24 patients with EHT without reaching target blood pressure levels of <140/90 mmHg (RR 151 ± 15/93 ± 10 mmHg). [9] The remaining 12 patients did not use antihypertensive agents and their blood pressure was 164 ± 19/89 ± 6 mmHg.

TABLE 1. Baseline characteristics

	PA (n=6)	EHT (n=24)	*p*-value
Demographics			
Male (%)	3 (50)	12 (50)	1.00
Age (mean ± SD)	55.8 ± 9.1	56.6 ± 8.3	0.85
Cardiovascular risk factors			
BMI in kg/m^2 (mean ± SD)	26.6 ± 2.9	28.4 ± 3.4	0.23
Units alcohol/day (mean ± SD)	1.0 ± 1.2	1.2 ± 1.8	0.73
Smoking			0.12
Current smoker (%)	1 (16.7)	0	
Former smoker (%)	1 (16.7)	12 (50)	
PY (mean ± SD)*	23 ± 30	21 ± 19	0.96
Daily exercise			
less than standard (%)	2 (33.3)	6 (25.0)	0.21
1st degree family history			
CVD (%)	2 (33.3)	17 (70.8)	0.33
Unknown (%)	0	1 (4.2)	
2nd degree family history			0.35
CVD (%)	0	9 (37.5)	
Unknown (%)	0	2 (8.3)	
Gestational hypertension (%)	1 (33.3)	6 (50.0)	1.00
Snoring			0.08
Light (%)	1	15	
Heavy (%)	2	2	
OSA syndrome (%)	1	0	0.20
Diagnosis			
Reason of visit			0.19
Complaints (%)	4 (66.7)	7 (29.2)	
High blood pressure at screening test (%)	2 (33.3)	8 ((33.3)	
At clinic for other reason (%)	0	9 (37.5)	
SBP in mmHg (mean ± SD)	169 ± 9	164 ± 11	0.24
DBP in mmHg (mean ± SD)	104 ± 7	97 ± 9	0.07
Laboratory screening (mean ± SD)			
Aldosterone in pmol/L	721 ± 90	338 ± 218	<0.01
Renin in pmol/L	0.6 ± 0.3	2.1 ± 3.5	0.05

ARR in pmol/mU	104.9 ± 57.6	26.8 ± 22.7	0.02
Sodium in mmol/L	141 ± 3	142 ± 2	0.36
Potassium in mmol/L	4.1 ± 0.3	4.5 ± 0.3	0.03
MDRD in mL/min	78 ± 8	74 ± 13	0.29
Fasting glucose in mmol/L	5.4 ± 0.6	5.5 ± 0.7	0.88
Cholesterol in mmol/L	6.8 ± 1.2	5.9 ± 1.0	0.15
HDL in mmol/L	1.5 ± 0.5	1.5 ± 0.4	0.98
Triglycerides in mmol/L	2.8 ± 2.6	1.3 ± 0.6	0.22
LDL in mmol/L	4.6 ± 0.8	4.1 ± 0.9	0.23

˚ For current and former smokers

Abbrevations: ARR, aldosterone-to-renin ratio; CVD, cardiovascular diseases; BMI, body mass index; EHT, essential hypertension; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OSA syndrome, obstructive sleep apnea syndrome; PA, primary aldosteronism

TABLE 2. Duration in months from diagnosis to measurement of the primary outcomes

	PA	**EHT**	
	(n=6)	**$ARR_{pos}SLT_{neg}$ (n=6)**	**ARR_{neg} (n=18)**
ABI, uACR	1	1	18
Echocardiography	4	6	19
FMD, cIMT, central aortic blood pressure and PWV	19*	12	18

** n=5*

Abbrevations: ABI, ankle-brachial index; ARR, aldosterone-to-renin ratio; cIMT, carotid intima media thickness; EHT, essential hypertension; FMD, flow-mediated dilation; PA, primary aldosteronism; PWV, pulse wave velocity; SLT, salt loading test; uACR, urinary albumin-to-creatinine ratio

TABLE 3. Blood pressure and antihypertensive treatment during assessment of ABI, uACR and shortly before echocardiography **(A)**, and during FMD, cIMT, PWV and central aortic blood pressure **(B)**

A

	PA	EHT		*p*-value
	(n=6)	$ARR_{pos}SLT_{neg}$ (n=6)	ARR_{neg} (n=18)	
Peripheral blood pressure (mean ± SD)				
Brachial SBP in mmHg	172 ± 15	172 ± 23	154 ± 10	0.01
Brachial DBP in mmHg	100 ± 10	91 ± 6	92 ± 7	0.05
Duration of hypertension in months (mean ± SD)	1.0 ± 0.9	1.0 ± 1.1	18.0 ± 7.1	<0.01
Medication use				
Number of patients taking antihypertensive drugs (%)	0	1 (16.7)	11 (61.1)	0.01
Thiazides (%)	0	0	3 (16.7)	1.00
ACE inhibitors (%)	0	0	6 (33.3)	0.30
Angiotensin II receptor antagonists (%)	0	0	1 (5.6)	1.00
Calcium channel blockers (%)	0	1 (16.7)	2 (11.1)	1.00
B-blockers (%)	0	0	0	
MR antagonists (%)	0	0	0	
Statin use (%)	0	0	2 (11.1)	1.00

B

	PA	EHT		*p*-value
	(n=6)	$ARR_{pos}SLT_{neg}$ (n=6)	ARR_{neg} (n=18)	
Peripheral blood pressure (mean ± SD)				
Brachial SBP in mmHg	139 ± 17.6	156 ± 26	154 ± 10	0.14
Brachial DBP in mmHg	86 ± 14	88 ± 10	92 ± 7	0.41
Duration of hypertension in months (mean ± SD)	18.8 ± 7.4	12.3 ± 6.0	18.0 ± 7.1	0.20
Medication use				
Number of patients taking antihypertensive drugs (%)	5 (83.3)	2 (33.3)	11 (61.1)	0.09
Thiazides (%)	0	1 (16.7)	3 (16.7)	1.00
ACE inhibitors (%)	0	1 (16.7)	6 (33.3)	0.30

Angiotensin II receptor antagonists (%)	0	1 (16.7)	1 (5.6)	1.00
Calcium channel blockers (%)	2 (40.0)	2 (33.3)	2 (11.1)	0.27
B-blockers (%)	0	1 (16.7)	0	1.00
MR antagonists (%)	4 (80.0)	0	0	0.00
Statin use (%)	2 (40.0)	0	2 (11.1)	0.17

Abbrevations: ACE inhibitors, angiotensin converting enzyme inhibitors; ARR, aldosterone-to-renin ratio; EHT, essential hypertension; MR, mineralocorticoid receptor; PA, primary aldosteronism; SLT, salt loading test

PRIMARY OUTCOMES

The unadjusted primary outcome measures are presented in Table 4A. None of the patients had eccentric LVH, whereas two female patients with PA had concentric LVH on echocardiography (p=0.03). Concentric remodelling was present in two patients with PA (33.3%) and five patients with EHT (20.8%; p=0.60). LVMI was higher among patients with PA, but the difference with patients with EHT was not significant in the unadjusted analysis. After correction for blood pressure, gender and age, LVMI was significantly higher in patients with PA compared to patients with EHT (90.50 ± 7.73 versus 70.70 ± 3.61 g/m^2, respectively; p=0.04). There was no increased frequency of diastolic dysfunction, atrial dilation and carotid plaques in patients with PA compared to control patients with EHT.

The adjusted mean values of the single outcome measures with correction for gender, age and baseline blood pressure are depicted in Table 4B. We did not observe differences in ABI, cIMT, FMD, central blood pressure, AIx, PWV, and uACR between patients with PA compared to patients with EHT (Table 4).

TABLE 4. Unadjusted values for each of the primary outcome measures **(A)** and primary outcome measures adjusted for gender, age, and systolic blood pressure **(B)**

A

	PA	EHT	*p*-value
ABI (mean ± SD)			
Left ABI	1.1 ± 0.0	1.1 ± 0.1	0.76
Right ABI	1.1 ± 0.1	1.1 ± 0.1	0.53
Echocardiography			
Concentric hypertrophy (%)	2 (33.3)	0 (0)	0.03
Concentric remodeling (%)	2 (33.3)	5 (20.8)	0.60
LVMI (mean ± SD) in g/m^2	83.48 ± 16.72	72.48 ± 16.92	0.19
Diastolic dysfunction (%)	3 (50.0)	11 (45.8)	1.00
Atrial dilation (%)	2 (33.3)	5 (20.8)	0.60
FMD (mean ± SD)*			
Baseline diameter in cm	0.439 ± 0.114	0.407 ± 0.057	0.57
% change in diameter	4.3 ± 3.3	4.6 ± 3.0	0.88
Time to peak in seconds	54.4 ± 33.5	73.7 ± 47.9	0.32
cIMT (mean ± SD)**			
Carotid plaques (%)	1 (20.0)	8 (33.3)	1.00
Left cIMT in µm	733.8 ± 188.8	747.0 ± 150.5	0.89
Right cIMT in µm	732.7 ± 177.5	727.6 ± 135.2	0.96
Central aortic blood pressure (mean ± SD)**			
Central SBP in mmHg	130 ± 18	145 ± 15	0.15
Central DBP in mmHg	87 ± 14	92 ± 8	0.49
Central AIx in %	28.4 ± 7.8	30.7 ± 8.0	0.55
PWV in m/s ((mean ± SD)***	8.7 ± 1.3	9.6 ± 1.7	0.21
uACR in mg/mmol (mean ± SD)	3.4 ± 3.6	3.9 ± 13.0	0.89

** in PA (n=5) and EHT (n=23)*
*** in PA (n=5)*
**** in PA (n=5) and EHT (n=22)*

B

	PA	EHT	*p*-value
ABI (mean ± SD)			
Left ABI	1.1 ± 0.0	1.1 ± 0.0	0.70
Right ABI	1.1 ± 0.0	1.1 ± 0.0	0.56
Echocardiography (mean ± SD)			
LVMI in g/m²	90.50 ± 7.73	70.70 ± 3.61	0.04
FMD (mean ± SD)*			
% change in diameter	4.47 ± 1.48	4.63 ± 0.66	0.92
Time to peak in seconds	67.0 ± 21.9	71.6 ± 9.7	0.85
cIMT (mean ± SD)**			
Left cIMT in µm	748.8 ± 62.0	743.4 ± 26.8	0.94
Right cIMT in µm	741.4 ± 62.9	725.3 ± 27.3	0.82
Central aortic blood pressure (mean ± SD)**			
Central SBP in mmHg	131 ± 7	145 ± 3	0.07
Central DBP in mmHg	87 ± 4	92 ± 2	0.25
Central AIx in %	29.0 ± 3.5	30.7 ± 1.6	0.67
PWV in m/s (mean ± SD)***	9.0 ± 0.7	9.5 ± 0.3	0.55
uACR in mg/mmol (mean ± SD)	5.1 ± 5.6	3.4 ± 2.6	0.80

** in PA (n=5) and EHT (n=23)*
*** in PA (n=5)*
**** in PA (n=5) and EHT (n=22)*
Abbrevations: ABI, ankle-brachial index; AIx, augmentation index; ARR, aldosterone-to-renin ratio; cIMT, carotid intima media thickness; EHT, essential hypertension; FMD, flow-mediated dilation; LVH, left ventricular hypertrophy; LVMI, left ventricular mass index; PA, primary aldosteronism; PWV, pulse wave velocity; SLT, salt loading test; uACR, urinary albumin-to-creatinine ratio

DISCUSSION

In a Dutch primary care population we screened patients with newly diagnosed hypertension for PA. [8] We demonstrated that the proportion of patients with LVH was higher in patients with PA as compared to patients with EHT. There were no differences in ABI, FMD, cIMT, central aortic blood pressure, PWV, and uACR between patients with PA and EHT.

The prevalence of LVH in patients with PA is in the range of 20-60% in referral centres. [7, 21-23] This high proportion of LVH in these studies may be due to persistent exposure to high circulating aldosterone levels, since the diagnosis and treatment of PA are delayed. [6] In our study, echocardiography was performed in patients with newly diagnosed hypertension, with hardly any delay in the diagnosis of PA. Our findings therefore suggest that in patients with PA, LVH is already present at the time of diagnosing hypertension.

In addition to the increased prevalence of LVH, LVMI was higher in patients with PA compared to patients with EHT after correction for gender, age and baseline blood pressure (Table 4). LVMI is a strong and independent predictor of future cardiovascular events. [24] It has been shown that every g/m2 increase in LVMI results in a hazard ratio of 1.013-1.015 for the risk of cardiovascular events in the general population. [24]

To prevent progressive cardiovascular damage, it might be important to screen for PA as early as possible. However, it remains challenging when and who to screen. In our study there were no differences in baseline characteristics between patients with PA and patients with EHT. Moreover, none of the patients with PA presented with hypokalemia, which is one of criteria to screen for PA according to the Endocrine Society guideline. This guideline recommends screening also in case of sustained blood pressure >150/100 mmHg. [3] In our cohort, 83% of the patients with PA had a blood pressure above 150/100 mmHg on separate visits at the time of diagnosing hypertension, which highlights the relevance of this clinical clue in the primary care setting.

Our findings of an increased risk of LVH in patients with PA, but lack of differences in other surrogate endpoints of target organ damage between patients with PA and EHT, suggests that the toxic effects of aldosterone affect mainly the heart but not other organ systems. However, others have shown that patients with PA have more morphological and functional vascular damage and an impaired endothelial function, when compared to matched patients with EHT. [25-29] Importantly, the patients in these studies were not included at the time hypertension was diagnosed for the first time. Therefore, we speculate that aldosterone-mediated effects on the vascular system may become manifest later on, and thus might be prevented by early diagnosis and adequate treatment.

The strength of our study is that the diagnosis of PA was made without a substantial delay. Furthermore, the diagnoses of PA and EHT were based on stringent criteria, according to the international guideline of the Endocrine Society. [3] Another strength of our study is that we assessed cardiovascular damage using the combination of seven different cardiovascular risk markers. The results of our study might be helpful in the design and sample size calculation of future prospective trials.

A major limitation of the study is the limited power to detect differences in outcomes between the study groups, except for LVH. During the two year inclusion period, an ARR was measured in less than 10% of the patients with newly diagnosed hypertension. [8] Only nine patients were diagnosed with PA, which resulted in a lower power than anticipated for the current study. Our study was designed as a prospective cohort study, but given the lagging recruitment it has a explorative character.

Other limitations are the timing of the vascular investigations due to logistic difficulties, and the ensuing difference in medication use across the groups. We assessed ABI, uACR and echocardiography in the patients with PA and ARRposSLTneg patients with EHT shortly after their diagnosis. In patients with EHT, these outcomes were assessed approximately 18 months after the diagnosis (Table 1). Fifty % of these patients used antihypertensive drugs during a mean period of 16 ± 8 months at the time of the vascular investigations. One might argue that therapy-related improvements in the EHT group may have accounted for the differences in LVH and LVMI between patients with PA and patients with EHT. However, half of the patients with EHT were not treated despite being hypertensive with, in some cases, a stage 2 hypertension (systolic blood

pressure ≥160 mmHg and/or diastolic blood pressure ≥ 100 mmHg). Moreover, in the treated patients, blood pressure control appeared to be poor. Therefore, the longer duration of hypertension and the inadequate treatment in 50 % of the patients with EHT, may have stimulated LVH in the control group. The fact that we still found an increased prevalence of LVH in patients with PA argues for aldosterone being the culprit of LVH.

During FMD, carotid ultrasonography, PWV and central aortic blood pressure, blood pressure levels did not differ between patients with PA and patients with EHT, as shown in Table 3B. However, both patient groups used antihypertensive medications that may have altered the outcomes of these vascular risk markers (Table 3B). [30]

In conclusion, we found a higher prevalence of LVH in newly diagnosed hypertensive patients with PA compared to newly diagnosed patients with EHT in whom PA was excluded. This finding suggests that screening for PA with subsequent treatment at the time of diagnosing hypertension might be useful to prevent further progression of cardiovascular damage.

ACKNOWLEDGEMENTS

We would like to thank all general practitioners and patients in our study. We would like to thank Ms. I. van den Munckhof and the vascular echographers of the Department of Vascular Surgery of the Radboud university medical center for their training in the exection of vascular experiments and the technique of ultrasonographic imaging. Furthermore, we would like to thank M.F. Maessen for his support in the analysis of the FMD recordings. Special thanks to Carel Bakx (†) and Mark van der Wel who were involved in the starting phase of the study.

REFERENCES

1. Kayser SC, Dekkers T, Groenewoud HJ, van der Wilt GJ, Carel Bakx J, van der Wel MC, et al. Study Heterogeneity and Estimation of Prevalence of Primary Aldosteronism: a Systematic Review and Meta-regression Analysis. *The Journal of clinical endocrinology and metabolism.* 2016:jc20161472.
2. Stowasser M, Gordon RD. Primary Aldosteronism: Changing Definitions and New Concepts of Physiology and Pathophysiology Both Inside and Outside the Kidney. *Physiological reviews.* 2016;96(4):1327-84.
3. Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, et al. The Management of Primary Aldosteronism: Case Detection, Diagnosis, and Treatment: An Endocrine Society Clinical Practice Guideline. *The Journal of clinical endocrinology and metabolism.* 2016;101(5):1889-916.
4. Mulatero P, Monticone S, Burrello J, Veglio F, Williams TA, Funder J. Guidelines for primary aldosteronism: uptake by primary care physicians in Europe. *Journal of hypertension.* 2016;34(11):2253-7.
5. Mulatero P, Stowasser M, Loh KC, Fardella CE, Gordon RD, Mosso L, et al. Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. *The Journal of clinical endocrinology and metabolism.* 2004;89(3):1045-50.
6. Dhanjal TS, Beevers DG. Delay in the diagnosis of Conn's syndrome: a single-center experience over 30 years. *Hypertension.* 2008;52(3):e22; author reply e3.
7. Milliez P, Girerd X, Plouin PF, Blacher J, Safar ME, Mourad JJ. Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. *Journal of the American College of Cardiology.* 2005;45(8):1243-8.
8. Kayser SC, Deinum J, de Grauw WJ, Schalk BW, Bor HJ, Lenders JW, et al. Prevalence of primary aldosteronism in primary care: a cross-sectional study. *The British journal of general practice : the journal of the Royal College of General Practitioners.* 2018;68(667):e114-e22.
9. 2013 Practice guidelines for the management of arterial hypertension of the European Society of Hypertension (ESH) and the European Society of Cardiology (ESC): ESH/ESC Task Force for the Management of Arterial Hypertension. *Journal of hypertension.* 2013;31(10):1925-38.
10. Association CCotNYH. Nomenclature and criteria for diagnosis of diseases of the heart and great vessels: Little, Brown & Co; 1994.

11. WHO Collaborating Centre for Drug Statistics Methodology. ATC/DDD Index 2016 [Available from: www.whocc.no/atc_ddd_index/.
12. Mancia G, Fagard R, Narkiewicz K, Redon J, Zanchetti A, Bohm M, et al. 2013 ESH/ESC Practice Guidelines for the Management of Arterial Hypertension. *Blood pressure.* 2014;23(1):3-16.
13. Laurent S, Cockcroft J, Van Bortel L, Boutouyrie P, Giannattasio C, Hayoz D, et al. Expert consensus document on arterial stiffness: methodological issues and clinical applications. *European heart journal.* 2006;27(21):2588-605.
14. Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, et al. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *American journal of physiology Heart and circulatory physiology.* 2011;300(1):H2-12.
15. Aboyans V, Criqui MH, Abraham P, Allison MA, Creager MA, Diehm C, et al. Measurement and interpretation of the ankle-brachial index: a scientific statement from the American Heart Association. *Circulation.* 2012;126(24):2890-909.
16. Lang RM, Badano LP, Mor-Avi V, Afilalo J, Armstrong A, Ernande L, et al. Recommendations for cardiac chamber quantification by echocardiography in adults: an update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *Journal of the American Society of Echocardiography : official publication of the American Society of Echocardiography.* 2015;28(1):1-39.e14.
17. Nagueh SF, Smiseth OA, Appleton CP, Byrd BF, 3rd, Dokainish H, Edvardsen T, et al. Recommendations for the Evaluation of Left Ventricular Diastolic Function by Echocardiography: An Update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *European heart journal cardiovascular Imaging.* 2016;17(12):1321-60.
18. Stein JH, Korcarz CE, Hurst RT, Lonn E, Kendall CB, Mohler ER, et al. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force. Endorsed by the Society for Vascular Medicine. *Journal of the American Society of Echocardiography : official publication of the American Society of Echocardiography.* 2008;21(2):93-111; quiz 89-90.
19. Weber T, Ammer M, Rammer M, Adji A, O'Rourke MF, Wassertheurer S, et al. Noninvasive determination of carotid-femoral pulse wave velocity depends critically on assessment of travel distance: a comparison with invasive measurement. *Journal of hypertension.* 2009;27(8):1624-30.

20. Townsend RR, Wilkinson IB, Schiffrin EL, Avolio AP, Chirinos JA, Cockcroft JR, et al. Recommendations for Improving and Standardizing Vascular Research on Arterial Stiffness: A Scientific Statement From the American Heart Association. *Hypertension.* 2015;66(3):698-722.
21. Cesari M, Letizia C, Angeli P, Sciomer S, Rosi S, Rossi GP. Cardiac Remodeling in Patients With Primary and Secondary Aldosteronism: A Tissue Doppler Study. *Circulation Cardiovascular imaging.* 2016;9(6).
22. Curione M, Petramala L, Savoriti C, Verrenti M, Baiocco E, Salvatore S, et al. Electrical and Myocardial Remodeling in Primary Aldosteronism. *Frontiers in cardiovascular medicine.* 2014;1:7.
23. Savard S, Amar L, Plouin PF, Steichen O. Cardiovascular complications associated with primary aldosteronism: a controlled cross-sectional study. *Hypertension.* 2013;62(2):331-6.
24. Cuspidi C, Facchetti R, Bombelli M, Sala C, Grassi G, Mancia G. Differential value of left ventricular mass index and wall thickness in predicting cardiovascular prognosis: data from the PAMELA population. *American journal of hypertension.* 2014;27(8):1079-86.
25. Bernini G, Galetta F, Franzoni F, Bardini M, Taurino C, Bernardini M, et al. Arterial stiffness, intima-media thickness and carotid artery fibrosis in patients with primary aldosteronism. *Journal of hypertension.* 2008;26(12):2399-405.
26. Holaj R, Zelinka T, Wichterle D, Petrak O, Strauch B, Widimsky J, Jr. Increased intima-media thickness of the common carotid artery in primary aldosteronism in comparison with essential hypertension. *Journal of hypertension.* 2007;25(7):1451-7.
27. Nishizaka MK, Zaman MA, Green SA, Renfroe KY, Calhoun DA. Impaired endothelium-dependent flow-mediated vasodilation in hypertensive subjects with hyperaldosteronism. *Circulation.* 2004;109(23):2857-61.
28. Chou CH, Chen YH, Hung CS, Chang YY, Tzeng YL, Wu XM, et al. Aldosterone Impairs Vascular Smooth Muscle Function: From Clinical to Bench Research. *The Journal of clinical endocrinology and metabolism.* 2015;100(11):4339-47.
29. Matsumoto T, Oki K, Kajikawa M, Nakashima A, Maruhashi T, Iwamoto Y, et al. Effect of aldosterone-producing adenoma on endothelial function and Rho-associated kinase activity in patients with primary aldosteronism. *Hypertension.* 2015;65(4):841-8.
30. Vlachopoulos C, Xaplanteris P, Aboyans V, Brodmann M, Cifkova R, Cosentino F, et al. The role of vascular biomarkers for primary and secondary prevention. A position paper from the European Society of Cardiology Working Group on peripheral circulation: Endorsed by the Association for Research into Arterial Structure and Physiology (ARTERY) Society. *Atherosclerosis.* 2015;241(2):507-32.


9
f
17
fp
fp
1
25
Trio I
4
1
f
16
Trio II
p
9
p
16

CHAPTER 2.2

PLASMA LEVELS OF THE CARDIOVASCULAR PROTECTIVE ENDOGENOUS NUCLEOSIDE ADENOSINE ARE REDUCED IN PATIENTS WITH PRIMARY ALDOSTERONISM

T.N.A. (Daniëlle) van den Berg [a,b] | Dick H.J. Thijssen [c]
Anke C.C.M. van Mil [c] | Petra H. van den Broek [a]
Gerard A. Rongen [a,b] | Houshang Monajemi [d]
Jaap Deinum [b,e] | Niels P. Riksen [b]

[a] Department of Pharmacology-Toxicology, [b] Department of Internal Medicine and [c] Department of Physiology, Radboud university medical center, Nijmegen, The Netherlands
[d] Department of Internal Medicine, Rijnstate Ziekenhuis, Arnhem, The Netherlands
[e] Department of Internal Medicine III, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany

Submitted

ABSTRACT

Background Patients with primary aldosteronism (PA) experience more cardiovascular events compared to patients with essential hypertension (EHT), independent from blood pressure levels. In animals, mineralocorticoid receptor antagonists limit ischemia-reperfusion (IR) injury by increasing extracellular adenosine formation and adenosine receptor stimulation. Adenosine is an endogenous compound with profound cardiovascular protective effects.

Objective We hypothesized that patients with PA have lower circulating adenosine levels which might contribute to the observed increased cardiovascular risk. Secondly, we hypothesized that by this mechanism, patients with PA are more susceptible to IR compared to patients with EHT.

Methods In our prospective study in 20 patients with PA and 20 patients with EHT, circulating adenosine was measured using a pharmacological blocker solution that halts adenosine metabolism after blood drawing. Brachial artery flow-mediated dilation (FMD) before and after forearm IR was used as a well-established method to study IR injury.

Results Patients with PA had a 33% lower adenosine level compared to patients with EHT (15.3 (13.3-20.4) versus 22.7 (19.4-36.8) nmol/l respectively, $p<0.01$). The reduction in FMD after IR however, did not differ between patients with PA and patients with EHT (-1.0 ± 2.9 % versus -1.6 ± 1.6 % respectively, $p=0.52$).

Conclusions As adenosine receptor stimulation induces various powerful protective cardiovascular effects, its lower concentration in patients with PA might be an important novel mechanism that contributes to their increased cardiovascular risk. We suggest that modulation of the adenosine metabolism is an exciting novel pharmacological opportunity to limit cardiovascular risk in patients with PA that needs further exploration.

INTRODUCTION

Primary aldosteronism (PA) is the most common cause of secondary hypertension, with an estimated prevalence of 10% in the hypertensive population. [1] Importantly, patients with PA experience more cardiovascular events, including stroke and myocardial infarction, compared to patients with essential hypertension (EHT), independent from the blood pressure level. [2] Also, in patients without PA, a high plasma aldosterone level is associated with an increased risk of cardiovascular events. [3] In patients with heart failure, plasma aldosterone is increased and treatment with mineralocorticoid receptor (MR) antagonists improve outcome. [4, 5] These observations suggest that aldosterone has direct adverse cardiovascular effect, over and above the detrimental effect of blood pressure elevation.

Indeed, preclinical studies have shown that aldosterone has direct adverse cardiovascular effects: aldosterone increases atherosclerosis and promotes plaque formation via the MR [6, 7], aldosterone reduces coronary blood flow [8], and aldosterone increases infarct size in animal models of myocardial infarction [9], although this latter result has not been reported in other studies. [10, 11] In addition, the administration of MR antagonists consistently reduces myocardial infarct size in these animal models. [12]

Schmidt *et al* recently proposed that the endogenous nucleoside adenosine might be involved in these detrimental effects, by showing that the cardioprotective effects of MR antagonists are fully dependent on adenosine receptor signaling. [10] Adenosine is formed by intra-, and extracellular degradation of adenosine monophosphate by the enzyme ecto-5'-nucleotidase (CD73). Stimulation of membrane-bound adenosine receptors induces various protective effects, including vasodilation, inhibition of inflammation and fibrosis, prevention of atherosclerosis, and protection against IR-injury. [13] Endogenous adenosine is considered a 'retaliatory metabolite' which protects the cardiovascular system in situations of impending danger, and acts as a key mediator of the infarct size-limiting effect of several pharmacological and non-pharmacological strategies. [13]

We now hypothesized that patients with PA have lower adenosine levels and that this contributes to their increased cardiovascular risk compared to patients with EHT. Secondly, we hypothesized that a lower adenosine concentration is associated with increased susceptibility to IR. We measured circulating adenosine concentrations and the activity of the main adenosine-producing enzyme CD73 on isolated mononuclear cells. To study IR in humans *in vivo*, a safe and well-validated method is examining brachial artery flow-mediated dilation (FMD) before and after forearm IR. [14] This protocol of IR results in an immediate decrease in brachial artery FMD, which reflects IR-induced endothelial dysfunction. [15, 16]

PATIENTS AND METHODS

The Radboud university medical center serves as a tertiary referral centre for PA in The Netherlands. The diagnosis of PA is made according to the current international guideline, by aldosterone and renin measurement, followed by a confirmation test. [17] For the inclusion of control patients with EHT, we asked patients from the outpatient clinic of the Radboud university medical center and the Rijnstate Hospital, Arnhem, The Netherlands.

All volunteers were 18-75 years of age and provided written informed consent. Exclusion criteria were: a history of atherosclerotic disease, cardiac failure, diabetes mellitus or severe renal dysfunction (MDRD <30 mL/min), current smoking, a 2nd or 3rd degree atrioventricular block on electrocardiography and the usage of drugs that influence adenosine formation: non-steroidal anti-inflammatory drugs, theophylline, or dipyridamole. An overview of the patient selection and inclusion process is depicted in Figure 1.

The study was approved by the Institutional Review Board of our centre and conducted in accordance with Good Clinical Practices and the Declaration of Helsinki. We prospectively registered our study at ClinicalTrials.gov by number NCT 01978132.

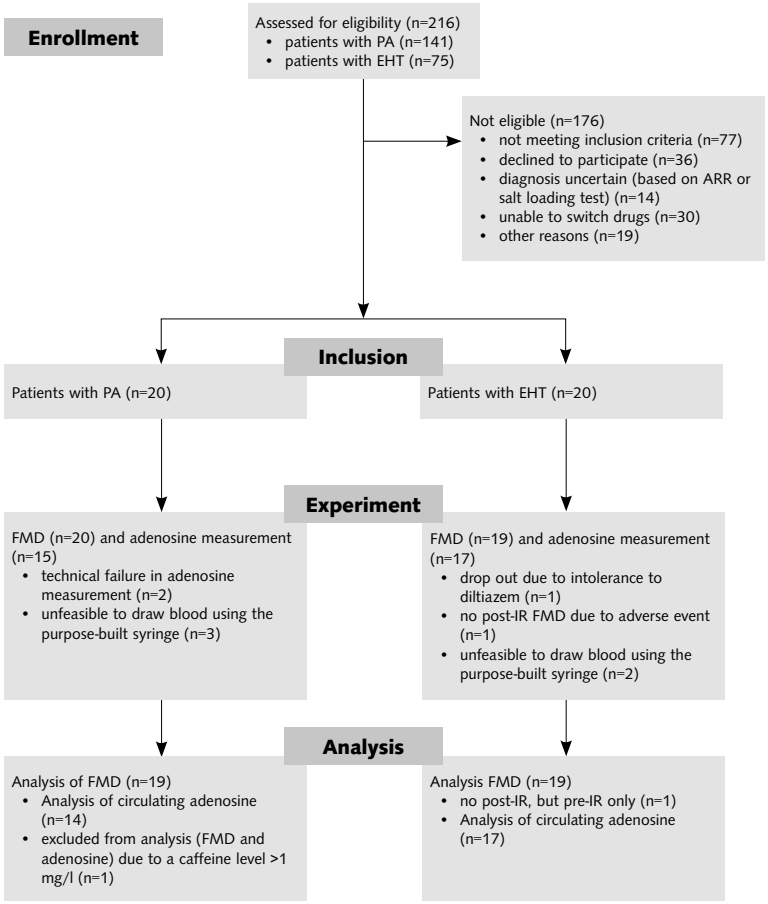


FIGURE 1. Overview of the selection process of patients

DIAGNOSIS OF PA AND EHT

Concordant with the guideline of the Endocrine society, no antihypertensive drugs other than calcium channel blockers, doxazosin and/or hydralazin were taken in the 4 (for spironolactone, eplerenone, amiloride, triamterene or aliskiren) or 2 (for all other antihypertensive drugs) weeks prior to aldosterone and renin measurements. [17]

In the Radboud university medical center, plasma active renin concentration was measured by an immunoradiometric assay (RENIN III generation, CIS Bio International). Serum aldosterone concentration was measured after extraction and paper chromatography

with recovery correction, as described earlier. [18] In patients from the Rijnstate Hospital, plasma aldosterone and plasma renin acticity were measured by radioimmunoassays (Labor Stein, Mönchengladbach, Germany and IJsselland Hospital, Capelle a/d IJssel, The Netherlands respectively).

Patients with PA had a baseline aldosterone level of >0.42 nmol/l and ARR level of >0.09 nmol/mU. In all patients with PA the diagnosis was confirmed by a salt loading test (aldosterone concentration > 0.28 nmol/l after infusion of 2 L of saline in 4 hours). Patients with confirmed PA underwent sequential adrenal venous sampling of the right and left adrenal vein during cosyntropin infusion to assess uni- or bilateral aldosterone overproduction. Criteria for a unilateral aldosterone overproduction were met when the left versus right (or vice versa) aldosterone-cortisol-ratio was ≥4.0 and the ratio of the contralateral site was ≤1.0, as an indication for contralateral suppression. Of the patients with unilateral aldosterone overproduction who underwent adrenalectomy, we screened the pathology reports.

In all patients with EHT, PA was excluded by a baseline aldosterone concentration of <0.42 nmol/l and ARR value of <0.09 nmol/mU or <0.65 nmol/l per ng/ml/hr.

EXPERIMENTAL DESIGN

We performed the experiments shortly after the diagnosis of PA was confirmed. Upon screening, most patients with EHT used various antihypertensive drugs. In both patients with PA and EHT, we changed the antihypertensive medication into diltiazem, with or without doxazosine or hydralazin, to minimize variation in medication and to exclude effects on the experiments. [19] At least one week after the change in medication, we draw blood to determine the adenosine concentration and performed the FMD experiment (see below). Since statins are known to upregulate CD73, these drugs had to be temporarily withdrawn during at least one week before the experiments. [20] In addition, we aimed to avoid hypokalemia during the FMD experiment by potassium suppletion, if needed.

On the experimental day, patients took their medication, except for potassium suppletion, after brachial FMD measurement, to avoid interference of these drugs in FMD assessment. [21]

CIRCULATING ADENOSINE CONCENTRATION

It is notoriously difficult to measure circulating adenosine, because of the extremely short half life, necessitating immediate pharmacological blockade of adenosine metabolism as soon as blood is withdrawn. We used a state-of-the-art technique, which we validated previously. [22] Using a purpose-built syringe, the blood mixes immediately at the end of the needle with a solution containing pharmacological blockers of the proteins involved in adenosine formation, transport, and degradation.

In more detail, we drew 2.5 mL of blood before the start of the experiment that was immediately mixed with a 2.5 mL solution containing 40 μmol/L dipyridamole (Sigma), 10 μmol/L erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (Sigma), 10 μmol/L 5-iodotubercidin (Biomol), 11.5 μmol/L forskolin (Fluka) and 115 μmol/L IBMX (Sigma), buffered in 13.2 mmol/L Na_2EDTA, 118 mmol/L NaCl, and 5 mmol/L KCl; pH 7.4.

The hemocrit of this solution was measured in order to correct for dilution. We directly centrifugated the blood mixed with blockers for 10 minutes at 1000 g, at 4 °C. The plasma was then stored at -80 °C until analysis.

After derivatization with chloroacetaldehyde, the formed $1,N^6$-ethenoadenosine concentration was analyzed using reversed-phase high performance liquid chromatography and fluorescence detection with excitation and emission wavelength set at 280 nm and 420 nm. Separation took place on a Polaris column (Varian, Polaris 3 μm C18-A 150 x 4.6 mm) with a mobile phase containing 50 mmol/L $NH_4H_2PO_4$ 5 mmol/l of hexane sulfonic acid (pH 3.0). Acetonitril was used as the organic modifier.

CD73 ACTIVITY ON MONONUCLEAR CELLS

Before start of the FMD experiment, we drew blood for the isolation of mononuclear cells. We used Cell Preparation Tubes (CPT 8 mL, BD Vacutainer) for the separation of mononuclear cells from whole blood. Within 2 hours after blood collection, we centrifuged the tubes for 20 minutes at 1600 g, at 20 °C without brake. We transferred the layer of mononuclear cells and washed the cells twice using phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4; pH 7.4). Subsequently, we resuspended the mononuclear cells in 0.5 mL Hank's balanced salt solution (HBSS, Gibco by Life Technologies) at room temperature. We determined the activity of CD73 exposed on the surface of these intact mononuclear cells measuring

the conversion of 1,N^6-ethenoadenosine 5'-monophosphate to 1,N^6-ethenoadenosine with HPLC as previously described. [20]

ADDITIONAL BLOOD DRAWING

Before start of the experiment we drew blood to determine plasma potassium levels and caffeine concentrations, as described previously. [23] Subjects with a circulating caffeine concentration >1.0 mg/l were excluded from analysis, since caffeine is a potent adenosine receptor antagonist. [24]

FLOW-MEDIATED DILATION (FMD)

All FMD experiments were performed in the morning, after an overnight fast and after 24 hours of alcohol and caffeine abstinence.

We measured the brachial blood pressure in the supine position using a manual sphygmomanometer, in a quiet room after a period of five minutes rest. We measured the blood pressure 3 times and reported the mean of the second and third blood pressure measurement.

An experienced sonographer, who was blinded for the diagnosis, examined brachial FMD in a darkened, temperature-controlled room of 22.3±0.5 °C, after a minimum time of rest of 15 minutes after venipuncture. We used a 10-MHz multifrequency linear-array probe attached to a high-resolution ultrasound machine (Terason T3000, Burlington, MA), according to the guideline of Thijssen *et al.* [21]

The patients rested in a supine position with both arms extended and immobilized, supported at an angle of ~80° abduction from the torso. For the assessment of FMD, we positioned a rapid inflation/deflation pneumatic cuff distal to the olecranon process to provide an ischemic stimulus distal from the brachial artery, leading to reactive hyperemia and subsequent shear stress. We imaged the brachial artery in the distal third of the upper arm. We recorded baseline diameter and blood flow velocity during 1 minute. This was followed by inflation of a pneumatic cuff around the forearm for 5 minutes to a pressure of 200 mmHg. We captured changes in brachial artery diameter, blood flow velocity and shear rate 30 seconds before cuff deflation until 3 minutes post-deflation continuously.

For the assessment of endothelial IR injury, we positioned the rapid inflation/deflation cuff around the upper arm and inflated the cuff to a pressure of 200 mmHg (or

50 mmHg above systolic blood pressure (SBP)) during 20 minutes, followed by 20 minutes of reperfusion. After this period of IR, we repeated the FMD measurements as described above.

Analysis of the brachial artery diameter was performed offline, in a blinded fashion, using custom-designed edge-detection and wall-tracking software, which is independent of investigator bias. [25] Baseline data were calculated across the 1 minute preceding cuff inflation. We used the automatically detected peak diameter after cuff deflation to express the FMD as the % change in diameter ((peak diameter after deflation - baseline diameter)/baseline diameter x 100 %). Other outcome measures that were assessed include time to peak (sec) and shear rate (area under the curve).

STATISTICAL ANALYSIS

In a recent study with a similar experimental design, we observed a reduction in FMD by forearm IR injury from 6.4 % to 4.4 %. [26] The average reduction in FMD by IR was 2.0 % (standard deviation (SD) 2.4 %). To demonstrate a two-fold increase in IR-damage with an alpha of 0.1 and a power of 80%, a sample size of 18 patients per group was required. To account for drop outs, we included 20 patients in both groups.

We used IBM SPSS Statistics 22 for the analysis of the data. We expressed normally distributed variables as mean ± SD and non-normally distributed variables as median (interquartile range). The baseline characteristics were compared using an independent *t*-test for normally distributed values and a Mann-Whitney Test for non-normally distributed variables. We assessed differences between proportions with the Pearson chi-square test or Fisher's exact for smaller proportions. We assumed a significance level of ≤ 0.05.

RESULTS

PATIENTS

We included 5 patients with EHT from the Rijnstate Hospital. All other patients (20 patients with PA and 15 patients with EHT) were recruited from the Radboud university medical center.

As expected, baseline aldosterone, ARR and plasma potassium levels differed significantly between patients with PA and patients with EHT. There were no differences regarding other baseline characteristics (Table 1).

Of the 20 patients with PA, 13 patients had unilateral aldosterone overproduction and 7 patients had bilateral aldosterone overproduction.

One patient with EHT dropped out because of intolerance to diltiazem. In one patient with EHT we ended the experiment during the period of upper arm ischemia, because of sudden appearance of petechiae of the ischemic arm. Most likely this was caused by a rise in SBP during upper arm occlusion, leading to venous congestion in the arm. During follow up, this patient recovered without any symptoms or complaints. We had to exclude one patient with PA from the analysis, because of a circulating caffeine concentration >1.0 mg/l.

On the experimental day, blood pressure and the daily defined dosage of antihypertensive drugs did not differ between patients with PA and patients with EHT (Table 2). Among the 19 patients with PA, there was no need for antihypertensive therapy in 6 patients. Eleven patients used diltiazem, one patient used diltiazem plus doxazosin and one patient used diltiazem, doxazosin and hydralazin. Of the 19 patients with EHT, 9 patients did not use any antihypertensive drug before FMD measurement, 8 used diltiazem only and 2 patients used diltiazem plus doxazosin.

Despite the usage of potassium suppletion in 17 of 19 patients with PA, plasma potassium values were slightly lower than in patients with EHT (Table 2).

Table 1. Baseline characteristics

	PA (n=20)	EHT (n=20)	*p*-value
Demographics			
Male (%)	12 (60)	13 (65)	1.00
Mean age (SD)	50.9 (12.2)	48.4 (14.6)	0.21
Screening			
Mean SBP (SD)	155 (19)	155 (25)	1.00
Mean DBP (SD)	91 (14)	91 (12)	0.88
Mean heart rate (SD)	71 (17)	70 (13)	0.86
Median duration of known hypertension in years (IQR)	7.5 (2.6-12.5)	6.0 (4.0-11.5)	0.84
Median baseline aldosterone in nmol/l (IQR)	0.81 (0.61-0.93)	0.22 (0.11-0.28)	**<0.01**
Median baseline ARR in nmol/mU (IQR) (n=15)	0.22 (0.17-0.25)	0.01 (0.01-0.02)	**<0.01**
Median baseline ARR in nmol/l per ng/ml/hr (IQR) (n=5)	-	0.41 (0.15-0.49)	-
Mean plasma sodium in mmol/l (SD)	141.9 (2.7)	141.0 (1.7)	0.14
Median plasma potassium (IQR)	3.8 (3.6-4.0)	4.0 (3.8-4.1)	**0.03**
Median plasma creatinine in µmol/l (IQR)	75.0 (68.0-84.0)	84.5 (71.5-89.5)	0.13
Median kidney function (MDRD) in ml/min (IQR)	84 (76-91)	79 (75-91)	0.57
Mean total plasma cholesterol in mmol/l (non-fasting) (SD)	5.2 (1.0) n=19*	4.9 (1.0) n=19*	0.33
Median plasma glucose in mmol/l (non-fasting) (IQR)	5.3 (4.9-5.9) n=20	5.2 (4.8-5.8) n=18†	0.59
Risk factors			
History of smoking (%)	8 (40)	10 (50)	0.75
Median units of alcohol per week (IQR)	3.5 (0-7.0)	2.0 (0-10.0)	0.67
Mean BMI (SD)	27.5 (5.6)	27.4 (4.3)	0.96
Dyslipidemia (%)	4 (20)	6 (30)	0.72
1st grade family history of hypertension or CVD (%)	12 (60)	16 (80)	0.30

** in 1 patient a value of a fasting (versus non-fasting) cholesterol was available*

† in 2 patients a value of a fasting (versus non-fasting) glucose value was available

Abbreviations: BMI, body mass index; CVD, cardiovasculair disease; DBP, diastolic blood pressure; IQR, interquartile range; MDRD, modification of diet in renal disease; PY, pack years; SBP, systolic blood pressure

Table 2. Clinical parameters at the moment of FMD measurement

	PA (n=19)	EHT (n=19)	*p*-value
Mean potassium value in mmol/l (SD)*	3.5 (0.3)	3.9 (0.4)	**<0.01**
Mean SBP in mmHg (SD)	158 (19)	155 (21)	0.66
Mean DBP in mmHg (SD)	96 (9)	93 (10)	0.37
Median heart rate in x/min (IQR)	64 (60-68)	60 (60-68)	0.47
Median number of antihypertensive drugs (IQR)	1 (0-1)	1 (0-1)	0.31
Median DDD antihypertensive drugs (IQR)	0.83 (0.00-1.00)	0.75 (0.00-0.83)	0.20
Mean duration in min between reperfusion and post-FMD measurement (SD) †	21.7 (1.9)	22.7 (2.7)	0.20

** n=18 in both patient groups*

† no post-IR FMD measurement is available in 1 patient with EHT

Abbrevations: DBP, diastolic blood pressure; DDD, daily defined dosage; IQR, interquartile range; SBP, systolic blood pressure

CIRCULATING ADENOSINE LEVELS

Levels of circulating adenosine were measured in 17 patients with EHT (11 male) and 14 patients with PA (9 male). Next to the earlier mentioned 2 drop outs, we were not able to draw blood using the purpose-built syringe in n=5. In 2 other patients, we did not obtain a circulating adenosine concentration due to technical failures.

The sex distribution, age, and blood pressure did not differ significantly between the 14 patients with PA and 17 patients with EHT (data not shown).

As depicted in Figure 2, the concentration of circulating adenosine was 15.3 (13.3-20.4) nmol/l in patients with PA and 22.7 (19.4-36.8) nmol/l in patients with EHT (p=0.008).

CD73 ACTIVITY

There was no significant difference in CD73 activity of intact mononuclear cells of patients with PA versus patients with EHT. Patients with PA had a CD73 activity of 0.43 (0.19-0.55) versus 0.54 (0.33-0.80) nmol/min per mg protein in patients with EHT; p=0.21.

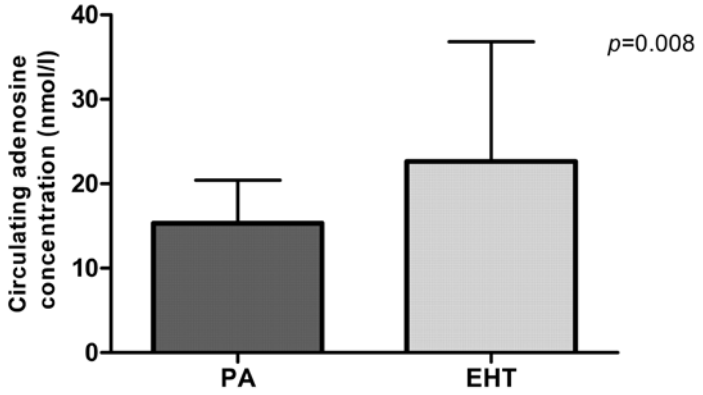


FIGURE 2. Circulating adenosine concentrations in nmol/l

Table 3. Brachial artery characteristics before and after IR

	Pre-IR		Post-IR		*p*-values	
	PA (n=19)	EHT (n=19)	PA (n=19)	EHT (n=18)*	Pre-IR	Post-IR
Mean brachial artery diameters in mm (SD)	0.422 (0.074)	0.418 (0.084)	0.469 (0.084)	0.431 (0.081)	0.86	0.18
Mean FMD in mm (SD)	0.441 (0.076)	0.437 (0.083)	0.483 (0.081)	0.445 (0.080)	0.90	0.16
Mean FMD in % (SD)	4.4 (2.1)	4.9 (1.9)	3.3 (2.3)	3.3 (2.0)	0.47	1.00
Median time to peak in sec (SD)	54 (38-89)	50 (40-74)	63 (32-75)	50 (31-77)	0.58	0.73
Mean shear rate AUC (SD)	21945 (10103)	19968 (8572)	17623 (11737)	19293 (11910)	0.52	0.67

** no post-IR FMD measurement is available in 1 patient with EHT*

FMD MEASUREMENT

Brachial artery characteristics before and after IR are shown in Table 3. FMD (peak diameter after cuff deflation), the % FMD, time to peak and shear rate did not differ significantly between patients with PA and patients with EHT before and after IR. In patients with EHT, % FMD decreased significantly after IR (4.9 ± 1.9 to 3.3 ± 2.0 %, p=0.001). The decrease in % FMD was not significant within the group of patients with PA (4.4 ± 2.1 to 3.3 ± 2.7, p=0.14).

Post-IR % FMD minus pre-IR % FMD did not differ between patients with PA and patients with EHT (-1.0 ± 2.9 % versus -1.6 ± 1.6 % respectively, p=0.52).

DISCUSSION

In the present study, we show for the first time that patients with PA have lower levels of circulating adenosine compared to patients with EHT. Since adenosine has potent cardiovascular protective properties, this mechanism could, at least in part, contribute to the increased risk of cardiovascular complications in patients with PA compared to patients with EHT, and might offer novel potential targets for drug treatment.

Adenosine is an endogenous purine nucleoside with several beneficial effects on the cardiovascular system, including vasodilation, anti-atherosclerotic effects, inhibition of inflammation, fibrosis, and limitation of IR-injury. [27] The importance of these effects is highlighted by previous studies reporting that genetic variants in the adenosine metabolism leading to increased endogenous adenosine formation improve cardiovascular survival in patients with coronary artery disease. [28]
Given these beneficial effects of adenosine receptor stimulation, it is logical to assume that a reduction in circulating adenosine impairs cardiovascular function. In line with this, we previously reported that patients with severe hyperhomocysteinemia, in whom the risk of cardiovascular events is strongly increased, adenosine-induced vasodilation is impaired due to an increased uptake of adenosine into the intracellular compartment, limiting adenosine receptor stimulation. [29]

In our study, we have now shown that also in patients with PA the endogenous adenosine concentration is reduced. We therefore propose that modulation of the adenosine metabolism might prove to be an exciting and novel pharmacological approach to reduce the excess risk of cardiovascular events in patients with PA. We previously showed that treatment with MR antagonists does not increase extracellular adenosine formation in healthy humans *in vivo*. [30] It is of great interest to explore the effects

of drugs known to increase extracellular adenosine levels, including dipyridamole or statins, in patients with PA. [31, 32]

The circulating concentration of adenosine is the sum of adenosine production, cellular uptake, and intracellular degradation. Activity of the enzyme CD73, which catalyzes the extracellular formation of adenosine from adenosine monophosphate, did not differ between the patients with PA and EHT. Therefore, increased cellular uptake and degradation of adenosine most probably explains the lower adenosine concentration, comparable to patients with hyperhomocysteinemia. [29] Future studies should focus on unravelling the metabolic changes driving lower adenosine levels, to be able to predict how these levels can be increased pharmacologically.

Since adenosine is known to limit IR injury, we subsequently hypothesized that the susceptibility to IR is higher in patients with PA. However, in our study, lower levels of circulating adenosine were not associated with increased susceptibility to endothelial IR. There are several potential explanations for this finding. First, the beneficial effect of adenosine on IR injury is controversial, at least in humans *in vivo*. Whilst administration of adenosine before reperfusion diminished IS in patients with an anterior wall MI [33, 34], several preclinical studies [35-37] and clinical studies [38, 39] failed to show an effect of exogenous adenosine on IR injury. In addition, even if enhanced adenosine receptor stimulation might limit IR injury, this does not necessarily mean that a reduction in adenosine receptor stimulation would augment IR injury, particularly considering the fact that many endogenous substances regulate IR susceptibility. For example, adenosine receptor antagonists did not increase infarct size itself in preclinical models of IR injury [10], although these antagonists did significantly prevent the beneficial effects of ischemic pre-conditioning and post-conditioning. [40, 41]

Future studies in patients with PA should therefore not focus on IR injury, but on alternative determinants of cardiovascular damage. An attractive topic to study is atherosclerosis for several reasons. First, patients with PA have an increased risk of atherosclerotic complications, including myocardial infarction and stroke, compared to patients with EHT. [2] Second, in animal models aldosterone increases atherosclerosis and promotes plaque formation via the MR. [6, 7] Third, adenosine has anti-atherosclerotic properties [13] and in genetic deletion models, inactivation of the adenosine metabolism leads to progression of atherosclerosis [42]. We therefore propose that the reduced

circulating adenosine levels in patients with PA may, at least in part, contribute to progression of atherosclerosis.

Our study benefits from the use of stringent criteria for the diagnosis of PA and EHT, detailed clinical characterisation of the patients, and much care to avoid the use of interfering antihypertensive and cholesterol lowering drugs. In addition, having a long tradition in human *in vivo* research on adenosine, we used optimal and well-validated methods to detect circulating adenosine. Finally, FMD was measured according to expert consensus guidelines that were developed by one of the authors. [21]

Nevertheless, some aspects of our methods and results merit critical discussion. First, we studied endothelial IR injury in the forearm vasculature and not directly in myocardial tissue. Despite important differences between brachial and coronary arteries however, brachial FMD accurately reflects coronary endothelial function [21, 43], and brachial FMD is a good predictor of future cardiovascular events. [44] The reduction in FMD immediately after a period of forearm ischemia has been well-validated in the literature to reflect endothelial IR-injury, which can be prevented by strategies that are known to also limit histological myocardial infarct size in animal models. [14, 45]

Secondly, we did not observe a difference in baseline FMD between patients with PA and patients with EHT. This is in contrast to previous clinical studies in these patients. [46-48] Several explanations can be found for the discrepancies between our study and these studies. In contrast to the study of Nishizaka *et al*, we used stringent diagnostic criteria for PA and EHT, concordant to international guidelines. [46] Importantly, we standardized antihypertensive treatment to diltiazem with or without hydralazin and/or doxazosin. In previous studies [46-48], different antihypertensive drugs may have modulated endothelial function and therefore, their results have to be interpreted with caution. [49] Furthermore, the above mentioned studies do not describe any dietary restrictions before FMD measurement. [46-48] In our study, patients were 24 hours free of alcohol and caffeine before FMD measurement, as recommended in the expert guideline. [21] Next, we excluded patients with an history of cardiac failure, atherosclerotic disease, severe renal dysfunction, diabetes mellitus and/or current smoking. Chou and colleagues do not describe any of these baseline characteristics. [48] It is therefore unclear whether the presence of co-morbidities may have led to the observed difference in baseline FMD between patients with PA and EHT. [48] Finally, in the study by Matsumoto *et*

al, a reduction in % FMD was seen only in those patients with PA who suffered from aldosterone producing adenomas, and not idiopathic aldosteronism.[47] Likewise, of the 35 patients with PA in the study by Chou *et al*, 91 % had an aldosterone producing adenoma.[48] The percentage of patients with a histologically proven unilateral aldosterone producing adenoma in our study was smaller, namely 53 %. Nevertheless, we did not observe differences in baseline FMD between the patients with unilateral aldosterone producing adenoma and bilateral aldosterone overproduction (4.8 ± 1.9 % versus 4.3 ± 2.5 % respectively; p=0.67). Interestingly, we did observe a trend towards an increased susceptibility to IR in the subset of patients with a unilateral aldosterone producing adenoma. FMD decreased from 4.8 ± 1.9 % to 2.6 ± 2.2 % in patients with a histologically proven unilateral producing adenoma compared to 4.3 ± 2.5 % to 3.8 ± 1.7 in patients with bilateral aldosterone overproduction; p=0.13. Circulating adenosine concentrations did not differ between the patients with a histologically proven unilateral producing adenoma and the patients with bilateral aldosterone overproduction (data not shown).

In conclusion, patients with PA have lower levels of circulating adenosine compared to patients with similar blood pressure levels due to EHT. This mechanism provides a novel and exciting explanation for the increased risk of cardiovascular events in patients with PA, compared to patients with EHT. Drugs beneficially affecting the adenosine metabolism could therefore potentially reduce the risk of future cardiovascular events in patients with PA.

ACKNOWLEDGEMENTS

We would like to thank all patients in our study. Furthermore, we would like to thank M.F. Maessen for his support in the analysis of the FMD recordings.

REFERENCES

1. Piaditis G, Markou A, Papanastasiou L, Androulakis, II, Kaltsas G. Progress in aldosteronism: a review of the prevalence of primary aldosteronism in pre-hypertension and hypertension. *European journal of endocrinology / European Federation of Endocrine Societies*. 2015;172(5):R191-203.
2. Monticone S, D'Ascenzo F, Moretti C, Williams TA, Veglio F, Gaita F, et al. Cardiovascular events and target organ damage in primary aldosteronism compared with essential hypertension: a systematic review and meta-analysis. *The lancet Diabetes & endocrinology*. 2018;6(1):41-50.
3. Kisaka T, Ozono R, Ishida T, Higashi Y, Oshima T, Kihara Y. Association of elevated plasma aldosterone-to-renin ratio with future cardiovascular events in patients with essential hypertension. *Journal of hypertension*. 2012;30(12):2322-30.
4. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *The New England journal of medicine*. 1999;341(10):709-17.
5. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *The New England journal of medicine*. 2003;348(14):1309-21.
6. Moss ME, Jaffe IZ. Mineralocorticoid Receptors in the Pathophysiology of Vascular Inflammation and Atherosclerosis. *Frontiers in endocrinology*. 2015;6:153.
7. van der Heijden C, Deinum J, Joosten LAB, Netea MG, Riksen NP. The mineralocorticoid receptor as a modulator of innate immunity and atherosclerosis. *Cardiovascular research*. 2018;114(7):944-53.
8. Fujita M, Minamino T, Asanuma H, Sanada S, Hirata A, Wakeno M, et al. Aldosterone nongenomically worsens ischemia via protein kinase C-dependent pathways in hypoperfused canine hearts. *Hypertension*. 2005;46(1):113-7.
9. Mihailidou AS, Loan Le TY, Mardini M, Funder JW. Glucocorticoids activate cardiac mineralocorticoid receptors during experimental myocardial infarction. *Hypertension*. 2009;54(6):1306-12.
10. Schmidt K, Tissier R, Ghaleb B, Drogies T, Felix SB, Krieg T. Cardioprotective effects of mineralocorticoid receptor antagonists at reperfusion. *European heart journal*. 2010;31(13):1655-62.

11. Chai W, Garrelds IM, Arulmani U, Schoemaker RG, Lamers JM, Danser AH. Genomic and nongenomic effects of aldosterone in the rat heart: why is spironolactone cardioprotective? *British journal of pharmacology*. 2005;145(5):664-71.
12. van den Berg TN, Rongen GA, Frohlich GM, Deinum J, Hausenloy DJ, Riksen NP. The cardioprotective effects of mineralocorticoid receptor antagonists. *Pharmacology & therapeutics*. 2014;142(1):72-87.
13. Riksen NP, Rongen GA. Targeting adenosine receptors in the development of cardiovascular therapeutics. *Expert Rev Clin Pharmacol*. 2012;5(2):199-218.
14. Kharbanda RK, Peters M, Walton B, Kattenhorn M, Mullen M, Klein N, et al. Ischemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischemia-reperfusion in humans in vivo. *Circulation*. 2001;103(12):1624-30.
15. Liuni A, Luca MC, Gori T, Parker JD. Loss of the preconditioning effect of rosuvastatin during sustained therapy: a human in vivo study. *American journal of physiology Heart and circulatory physiology*. 2012;302(1):H153-8.
16. Loukogeorgakis SP, van den Berg MJ, Sofat R, Nitsch D, Charakida M, Haiyee B, et al. Role of NADPH oxidase in endothelial ischemia/reperfusion injury in humans. *Circulation*. 2010;121(21):2310-6.
17. Funder JW, Carey RM, Fardella C, Gomez-Sanchez CE, Mantero F, Stowasser M, et al. Case detection, diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. *The Journal of clinical endocrinology and metabolism*. 2008;93(9):3266-81.
18. De Man AJ, Hofman JA, Hendriks T, Rosmalen FM, Ross HA, Benraad TJ. A direct radio-immunoassay for aldosterone: significance of endogenous cortisol. *The Netherlands journal of medicine*. 1980;23(2):79-83.
19. Miwa Y, Masai H, Shimizu M. Differential effects of calcium-channel blockers on vascular endothelial function in patients with coronary spastic angina. *Circulation journal : official journal of the Japanese Circulation Society*. 2009;73(4):713-7.
20. Meijer P, Wouters CW, van den Broek PH, de Rooij M, Scheffer GJ, Smits P, et al. Upregulation of ecto-5'-nucleotidase by rosuvastatin increases the vasodilator response to ischemia. *Hypertension*. 2010;56(4):722-7.
21. Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, et al. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *American journal of physiology Heart and circulatory physiology*. 2011;300(1):H2-12.

22. Ramakers BP, Pickkers P, Deussen A, Rongen GA, van den Broek P, van der Hoeven JG, et al. Measurement of the endogenous adenosine concentration in humans in vivo: methodological considerations. *Current drug metabolism*. 2008;9(8):679-85.
23. Schreiber-Deturmeny E, Bruguerolle B. Simultaneous high-performance liquid chromatographic determination of caffeine and theophylline for routine drug monitoring in human plasma. *Journal of chromatography B, Biomedical applications*. 1996;677(2):305-12.
24. Smits P, Boekema P, De Abreu R, Thien T, van 't Laar A. Evidence for an antagonism between caffeine and adenosine in the human cardiovascular system. *Journal of cardiovascular pharmacology*. 1987;10(2):136-43.
25. Woodman RJ, Playford DA, Watts GF, Cheetham C, Reed C, Taylor RR, et al. Improved analysis of brachial artery ultrasound using a novel edge-detection software system. *Journal of applied physiology* (Bethesda, Md : 1985). 2001;91(2):929-37.
26. van den Munckhof I, Riksen N, Seeger JP, Schreuder TH, Borm GF, Eijsvogels TM, et al. Aging attenuates the protective effect of ischemic preconditioning against endothelial ischemia-reperfusion injury in humans. *American journal of physiology Heart and circulatory physiology*. 2013;304(12):H1727-32.
27. Headrick JP, Ashton KJ, Rose'meyer RB, Peart JN. Cardiovascular adenosine receptors: expression, actions and interactions. *Pharmacology & therapeutics*. 2013;140(1):92-111.
28. Anderson JL, Habashi J, Carlquist JF, Muhlestein JB, Horne BD, Bair TL, et al. A common variant of the AMPD1 gene predicts improved cardiovascular survival in patients with coronary artery disease. *Journal of the American College of Cardiology*. 2000;36(4):1248-52.
29. Riksen NP, Rongen GA, Boers GH, Blom HJ, van den Broek PH, Smits P. Enhanced cellular adenosine uptake limits adenosine receptor stimulation in patients with hyperhomocysteinemia. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25(1):109-14.
30. van den Berg TN, Deinum J, Bilos A, Donders AR, Rongen GA, Riksen NP. The effect of eplerenone on adenosine formation in humans in vivo: a double-blinded randomised controlled study. *PloS one*. 2014;9(10):e111248.
31. Meijer P, Wouters CW, van den Broek PH, Scheffer GJ, Riksen NP, Smits P, et al. Dipyridamole enhances ischaemia-induced reactive hyperaemia by increased adenosine receptor stimulation. *British journal of pharmacology*. 2008;153(6):1169-76.
32. Meijer P, Oyen WJ, Dekker D, van den Broek PH, Wouters CW, Boerman OC, et al. Rosuvastatin increases extracellular adenosine formation in humans in vivo: a new perspective on cardiovascular protection. *Arteriosclerosis, thrombosis, and vascular biology*. 2009;29(6):963-8.

33. Mahaffey KW, Puma JA, Barbagelata NA, DiCarli MF, Leesar MA, Browne KF, et al. Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction: results of a multicenter, randomized, placebo-controlled trial: the Acute Myocardial Infarction STudy of ADenosine (AMISTAD) trial. *Journal of the American College of Cardiology*. 1999;34(6):1711-20.
34. Ross AM, Gibbons RJ, Stone GW, Kloner RA, Alexander RW. A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). *Journal of the American College of Cardiology*. 2005;45(11):1775-80.
35. Goto M, Miura T, Iliodoromitis EK, O'Leary EL, Ishimoto R, Yellon DM, et al. Adenosine infusion during early reperfusion failed to limit myocardial infarct size in a collateral deficient species. *Cardiovascular research*. 1991;25(11):943-9.
36. Vander Heide RS, Reimer KA. Effect of adenosine therapy at reperfusion on myocardial infarct size in dogs. *Cardiovascular research*. 1996;31(5):711-8.
37. Xu Z, Downey JM, Cohen MV. Amp 579 reduces contracture and limits infarction in rabbit heart by activating adenosine A2 receptors. *Journal of cardiovascular pharmacology*. 2001;38(3):474-81.
38. Desmet W, Bogaert J, Dubois C, Sinnaeve P, Adriaenssens T, Pappas C, et al. High-dose intracoronary adenosine for myocardial salvage in patients with acute ST-segment elevation myocardial infarction. *European heart journal*. 2011;32(7):867-77.
39. Nazir SA, McCann GP, Greenwood JP, Kunadian V, Khan JN, Mahmoud IZ, et al. Strategies to attenuate micro-vascular obstruction during P-PCI: the randomized reperfusion facilitated by local adjunctive therapy in ST-elevation myocardial infarction trial. *European heart journal*. 2016;37(24):1910-9.
40. Methner C, Schmidt K, Cohen MV, Downey JM, Krieg T. Both A2a and A2b adenosine receptors at reperfusion are necessary to reduce infarct size in mouse hearts. American journal of physiology *Heart and circulatory physiology*. 2010;299(4):H1262-4.
41. Riksen NP, Zhou Z, Oyen WJ, Jaspers R, Ramakers BP, Brouwer RM, et al. Caffeine prevents protection in two human models of ischemic preconditioning. *Journal of the American College of Cardiology*. 2006;48(4):700-7.
42. Buchheiser A, Ebner A, Burghoff S, Ding Z, Romio M, Viethen C, et al. Inactivation of CD73 promotes atherogenesis in apolipoprotein E-deficient mice. *Cardiovascular research*. 2011;92(2):338-47.

43. Anderson TJ, Uehata A, Gerhard MD, Meredith IT, Knab S, Delagrange D, et al. Close relation of endothelial function in the human coronary and peripheral circulations. *Journal of the American College of Cardiology*. 1995;26(5):1235-41.

44. Matsuzawa Y, Kwon TG, Lennon RJ, Lerman LO, Lerman A. Prognostic Value of Flow-Mediated Vasodilation in Brachial Artery and Fingertip Artery for Cardiovascular Events: A Systematic Review and Meta-Analysis. *Journal of the American Heart Association*. 2015;4(11).

45. Piot CA, Padmanaban D, Ursell PC, Sievers RE, Wolfe CL. Ischemic preconditioning decreases apoptosis in rat hearts in vivo. *Circulation*. 1997;96(5):1598-604.

46. Nishizaka MK, Zaman MA, Green SA, Renfroe KY, Calhoun DA. Impaired endothelium-dependent flow-mediated vasodilation in hypertensive subjects with hyperaldosteronism. *Circulation*. 2004;109(23):2857-61.

47. Matsumoto T, Oki K, Kajikawa M, Nakashima A, Maruhashi T, Iwamoto Y, et al. Effect of aldosterone-producing adenoma on endothelial function and Rho-associated kinase activity in patients with primary aldosteronism. *Hypertension*. 2015;65(4):841-8.

48. Chou CH, Chen YH, Hung CS, Chang YY, Tzeng YL, Wu XM, et al. Aldosterone Impairs Vascular Smooth Muscle Function: From Clinical to Bench Research. *The Journal of clinical endocrinology and metabolism*. 2015;100(11):4339-47.

49. Vlachopoulos C, Xaplanteris P, Aboyans V, Brodmann M, Cifkova R, Cosentino F, et al. The role of vascular biomarkers for primary and secondary prevention. A position paper from the European Society of Cardiology Working Group on peripheral circulation: Endorsed by the Association for Research into Arterial Structure and Physiology (ARTERY) Society. *Atherosclerosis*. 2015;241(2):507-32.


59
67
78
f
p
sf
Adagio
88
1
3/4
p cresc.
f
95
4
sfp
104
1
p cresc. f
Coda
112
2
p
124

CHAPTER 2.3

PLASMA GALECTIN-3 CONCENTRATIONS IN PATIENTS WITH PRIMARY ALDOSTERONISM

T.N.A. (Daniëlle) van den Berg [a,b] | Wouter C. Meijers [c]
A. Rogier T. Donders [d] | Antonius E. van Herwaarden [e]
Gerard A. Rongen [a,b] | Rudolf A. de Boer [c]
Jaap Deinum [b] | Niels P. Riksen [b]

[a] Department of Pharmacoloy-Toxicology and [b] Department of Internal Medicine, Radboud university medical center, Nijmegen, the Netherlands, [c] University of Groningen, University Medical Center Groningen, Department of Cardiology, Groningen, The Netherlands, [d] Department for Health Evidence and [e] Department of Laboratory Medicine, Radboud university medical center, Nijmegen, The Netherlands

J Hypertens. 2017 Sep;35(9):1849-1856

ABSTRACT

Background The incidence of cardiovascular events is higher in patients with primary aldosteronism (PA) than in patients with essential hypertension (EHT), despite similar blood pressure levels. This suggests detrimental cardiovascular effects of aldosterone. Amongst others, it has been suggested that galectin-3 (Gal-3) is a key mediator in aldosterone-induced myocardial fibrosis.

Objective We studied whether patients with PA have higher plasma Gal-3 concentrations than patients with EHT, and evaluated its reversibility after adrenalectomy.

Methods In a retrospective cohort from our tertiary referral centre, we measured plasma Gal-3 concentrations in 78 patients with PA, 39 cured PA patients after adrenalectomy, and 56 patients with EHT. Paired samples were available in 11 patients (pre- and post-adrenalectomy). We compared plasma Gal-3 levels by univariate analysis of covariance with correction for cardiovascular risk factors, plasma creatinine concentration, plasma potassium levels and alcohol intake.

Results Adjusted plasma Gal-3 concentrations in patients with PA, patients after adrenalectomy, and patients with EHT were 11.39 ± 0.60, 11.64 ± 0.81, and 11.41 ± 0.73 ng/mL respectively (mean ± SD; p=0.95). In 11 patients of whom paired samples were available, mean Gal-3 concentrations increased from 10.03 ± 1.67 ng/mL pre-adrenalectomy to 14.36 ± 2.07 ng/mL post-adrenalectomy (p<0.01).

Conclusions In patients with PA, plasma Gal-3 concentrations are not elevated when compared to patients with EHT, and levels do not decrease after adrenalectomy. These results are in contrast to previous studies and do not support a pathophysiological role of plasma Gal-3 in the increased cardiovascular risk in patients with PA.

INTRODUCTION

Primary aldosteronism (PA) is the most common cause of secondary hypertension. PA is characterized by high circulating aldosterone and low renin levels, indicating the autonomous overproduction of the mineralocorticoid hormone aldosterone, which increases blood pressure by stimulating renal sodium reabsorption. Importantly, patients with PA have an increased risk for cardiovascular events, including atrial fibrillation, stroke, heart failure and myocardial infarction, compared to patients with essential hypertension (EHT) with comparable blood pressure levels. [1, 2] Also in patients without PA, plasma aldosterone and the aldosterone-renin-ratio (ARR) is positively associated with an increased risk of cardiovascular events. [3] These findings suggest that aldosterone has direct adverse effect on the cardiovascular system, independent from its blood pressure-increasing effect.

Indeed, preclinical studies have provided data that aldosterone has direct adverse cardiovascular effects, such as promoting apoptosis, vasoconstriction and increasing the infarct size in animal models of myocardial infarction. [4-7] Administration of mineralocorticoid receptor (MR) antagonists consistently reduces myocardial infarct size in these animal models. [7-11] Furthermore, aldosterone stimulates vascular and myocardial fibrosis and hence plays an important pathophysiological role in remodeling of the heart and vessel wall. [12-15]

Galectin-3 (Gal-3) has recently been proposed as a mediator of aldosterone-induced myocardial fibrosis. This β-galactoside-binding lectin is expressed in epithelial cells, cells of the immune system (predominantly macrophages [16]), fibroblasts, vascular smooth muscle cells (VSMC), cardiomyocytes and endothelial cells. [17, 18] Consistent with the expression in various cell types, Gal-3 is involved in a variety of processes, such as cell growth, apoptosis, cell-cell adhesion, and cell-matrix interactions. [19] Various factors are involved in the regulation of Gal-3 expression, including activation of the transcription factor NF-κB, activation of HIF-1α, integrins and various pro-inflammatory cytokines. [20] In addition, in vitro studies and experimental murine studies have reported

that aldosterone can stimulate Gal-3 secretion and expression in VSMC, fibroblasts, macrophages, and cardiac homogenates via MR activation, and that Gal-3 mediates the pro-fibrotic effects of aldosterone. [15, 21-23]

In patients with heart failure, circulating Gal-3 is associated with extracellular matrix markers and with adverse long-term cardiovascular outcomes. [24, 25] Aldosterone levels are increased and treatment with MR antagonists improves outcome in these patients. However, several studies in HF patients could not detect an interaction between the use of MR antagonists and Gal-3 levels, which contradicts a linear relationship between the suggested MR mediated effects of aldosterone and Gal-3. [26-28]

In summary, aldosterone promotes vascular and myocardial fibrosis and preclinical studies have demonstrated that this is mediated by Gal-3 production and secretion. Patients with overproduction of aldosterone suffer from more cardiovascular damage, including left ventricular hypertrophy (LVH) and heart failure, than patients with EHT. It is currently unknown whether this organ damage is mediated by Gal-3.
Based on the pre-clinical and mechanistic studies, we propose that aldosterone excess stimulates Gal-3 secretion in various cells of the cardiovascular and immune system, and that subsequently Gal-3 induces pro-fibrotic and pro-atherogenic effects in the vascular wall and myocardium. This mechanism could contribute to the increased prevalence of cardiovascular adverse events in patients with PA compared to patients with EHT. To explore this hypothesis, we measured circulating Gal-3 concentrations in well-characterized patient groups with PA, EHT, and patients who were cured from PA (post-adrenalectomy).

METHODS

The Radboud university medical center serves as a tertiary referral centre for PA in the Netherlands. Patients with refractory hypertension are screened for PA according to current guidelines, by serum aldosterone and plasma renin measurement. Residual plasma samples are routinely stored for 2 years at -20 °C. We carefully selected patients from the outpatient clinic of our hospital with PA, EHT or after cure from PA to measure Gal-3 in residual plasma. The study protocol was approved by the Institutional Review Board of our centre.

SAMPLE SELECTION

We selected all serum aldosterone and/or plasma renin measurements that had been performed in the period of July 2014 to July 2015 (n=2432) and then excluded samples derived from patients from other hospitals, repeated samples per patient, children below 18 years of age, samples lacking aldosterone measurements and patients with adrenal venous sampling (AVS) only (i.e. patients referred for AVS in whom no baseline aldosterone and renin were measured in our centre). Furthermore, we excluded patients with high aldosterone concentrations (>0.42 nmol/L), but low ARR values (<0.09 nmol/mU), because these values are suggestive for secondary hyperaldosteronism. Of the 195 remaining evaluable patients, we selected patients who underwent a salt loading test (SLT) for confirmation of PA (n=79), and also patients with normal baseline aldosterone and ARR values (<0.42 nmol/L and 0.09 nmol/mU respectively) (n=116). This latter group of patients with normal aldosterone and ARR has been used as the control group of patients with EHT.

Patients using antihypertensive medication that affects the aldosterone concentration, or patients who did not suffer from PA or EHT were excluded. In addition to these samples, we collected samples from patients with proven PA and EHT from the Department of Vascular Medicine from our hospital that were obtained before or after July 2014 (n=88). In 11 PA patients, a paired sample after adrenalectomy was available. We included these 11 patients in both the PA group (sample pre-adrenalectomy) and post-adrenalectomy group (sample after adrenalectomy). See Figure 1 for an overview of the selection process.

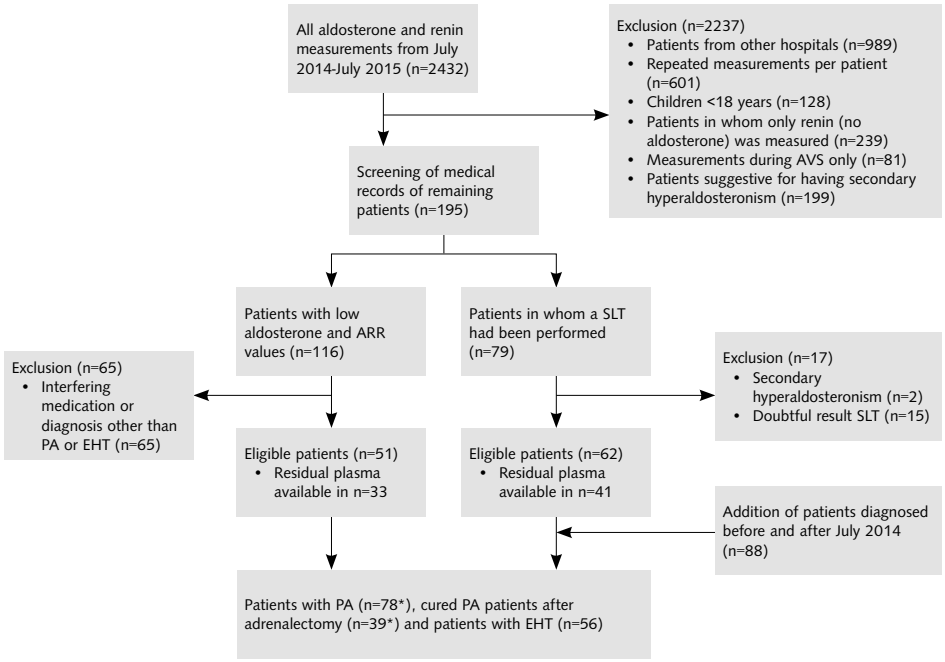


FIGURE 1. Flow of the selection process of the samples for Gal-3 measurements

** 11 patients are included in the PA- and post-adrenalectomy group*

Abbrevations: APA, aldosterone producing adenoma; ARR, aldosterone-renin-ratio; AVS, adrenal venous sampling; EHT, essential hypertension; PA, primary aldosteronism; SLT, salt loading test

DIAGNOSIS OF PA AND EHT

Concordant with the recently updated guideline of the Endocrine society and our local protocol, no antihypertensive drugs other than calcium channel blockers, doxazosin and/or hydralazin were taken in the four (for spironolactone, eplerenone, amiloride, triamterene or aliskiren) or two (for all other antihypertensive drugs) weeks prior to aldosterone and renin measurements. [29] When baseline aldosterone values exceeded 0.42 nmol/L and the ARR exceeded 0.09 nmol/mU, a SLT was performed by infusion of 2 liters of saline in 4 hours. PA was confirmed when the serum aldosterone level was >0.28 nmol/L after this test. Patients with EHT had a baseline serum aldosterone of <0.42 nmol/L and an ARR value of <0.09 nmol/mU, or a negative SLT (aldosterone < 0.14 nmol/L after saline infusion).

Patients with PA underwent sequential AVS of the right and left adrenal vein during cosyntropin infusion to assess uni- or bilateral aldosterone overproduction. A technically successful AVS was determined by an adrenal vein to peripheral vein cortisol ratio of >3. Criteria for a unilateral aldosterone overproduction were met when the left versus right (or vice versa) aldosterone-cortisol-ratio was ≥4.0 and the ratio of the contralateral site was ≤1.0, as an indication for contralateral suppression. Of the patients with a suspected unilateral aldosterone producing adenoma (APA), we screened the pathology reports to see whether the histology findings were in line with the diagnosis.

ANALYTICAL METHODS

Plasma renin and serum aldosterone concentrations were measured by the Department of Laboratory Medicine of our centre. Plasma active renin concentration was measured by an immunoradiometric assay (RENIN III generation, CIS Bio International). Serum Aldosterone concentration was measured after extraction and paper chromatography with recovery correction, as described earlier. [30]

Plasma Gal-3 levels were quantified in a blinded fashion using an enzyme-linked immunosorbent assay (BG medicine, Inc, Waltham, MA, USA). Intra-assay and interassay coefficients of variation of this assay are 3.2% and 5.6% respectively, with a lower detection limit of 1.13 ng/mL and an upper detection limit of 96.6 ng/mL, as published. [31]

CLINICAL PATIENT DATA

We screened the medical records of all included patients and collected the following data: the number of years of known hypertension, medication use, cardiovascular risk factors (body mass index (BMI), smoking, diabetes mellitus (DM), (family) history of cardiovascular diseases (CVD), and dyslipidemia), alcohol use and if available, the findings on echocardiography. The antihypertensive medication was expressed in a daily defined dose (DDD), as defined by the World Health Organisation (www.whocc.no/atc_ddd_index/). Furthermore, we recorded plasma creatinine levels, urinary albumin, systolic blood pressure (SBP), diastolic blood pressure (DBP), serum aldosterone, plasma renin, and plasma potassium levels. Apart from urinary albumin, these values were obtained just before or at the time at which the plasma samples, used for Gal-3 measurement, were collected.

STATISTICAL ANALYSIS

We used IBM SPSS Statistics 22 for the analysis of the data. We depicted normally distributed variables as mean ± SD and non-normally distributed variables as median (interquartile range (IQR)). The baseline characteristics were compared using a one-way independent ANOVA for normally distributed values and a Kruskal-Wallis test for non-normally distributed variables. We assessed differences between proportions with the Pearson chi-square test or Fisher's exact for smaller proportions. We compared the paired samples with a paired Student's t test or Wilcoxon Rank test, according to the distribution of the data. We assumed a significance level of ≤ 0.05.

Mean Gal-3 values between the patients with PA, the patients post-adrenalectomy and the patients with EHT were compared by univariate analysis of covariance with the following confounding factors: age, sex, BMI, smoking, DM, units (10 g) of alcohol/week, years of hypertension, SBP and DBP, plasma creatinine and potassium concentrations. We based adjustments for most of these covariates on previous studies in which strong correlations were found between these variables and plasma Gal-3 levels. [32, 33] In our cohort, we found the units of alcohol per week to be correlated to Gal-3 (β=-0.08, R^2=0.05, p<0.01). Since hypokalemia can suppress aldosterone production, plasma potassium might influence the relationship between aldosterone and Gal-3. [29] For these reasons, we added the units of alcohol per week and the plasma potassium concentration as covariates.

All variables were available, except for 27 missing plasma creatinine values (3 BAH, 3 APA, 3 post-adrenalectomy and 18 EHT), 2 missing values of alcohol intake (1 BAH and 1 APA) and 4 missing plasma potassium values (1 BAH, 2 post-adrenalectomy and 1 EHT).

RESULTS

PATIENTS

Residual plasma was available from 78 patients with PA, 39 patients cured from PA (post-adrenalectomy), and 56 patients with EHT. AVS (performed in n=75) revealed a bilateral aldosterone overproduction (bilateral adrenal hyperplasia; BAH) in 32 patients and an APA in 43 patients. In 3 patients no AVS was performed, but computed tomography showed a unilateral adenoma in 2 of these patients and bilateral adenoma's in 1. The baseline characteristics of the patients are presented in Table 1.

Of the 43 patients with lateralisation of aldosterone during adrenal venous sampling and 28 post-adrenalectomy patients, pathology reports were available in 46 patients. In 61% of the cases, an adenoma was confirmed by the pathologist and 13% had nodular hyperplasia with a dominant adenoma. In one patient the pathologist could not distinguish between nodular hyperplasia or a cortical adenoma and in another patient a pseudocyst was diagnosed. In the remaining 22%, histology revealed nodular hyperplasia.

Patients with PA had significantly higher aldosterone and ARR values, and a lower renin concentration, compared to post-adrenalectomy and EHT patients. As depicted in Table 1, all post-adrenalectomy patients were cured according to biochemical parameters. In addition, a SLT was performed in 33 of 39 adrenalectomized patients. The SLT was negative in all of these patients. Post-adrenalectomy, the ARR values were significantly lower in comparison to EHT patients (Table 1).

TABLE 1. Baseline characteristics of patients with PA (BAH, bilateral adrenal hyperplasia and APA, aldosterone producing adenoma), patients post-adrenalectomy and patients with EHT

	PA (n=78)		Post-adrenalectomy	EHT
	BAH (n=33)	**APA (n=45)**	**(n=39)**	**(n=56)**
Demographics				
Male (%)*	23 (69.7)	28 (62.2)	26 (66.7)	24 (42.9)
Median age (IQR)	53 (47-60)	51 (45-62)	50 (46-56)	55 (45-60)
Characteristics				
Median duration of known hypertension in years (IQR)	8.0 (1.3-13.5)†	7.0 (2.0-15.0)†	8.0 (4.0-14.0)†	2 (0.0-6.8)
Median DDD antihypertensives (IQR)	1.3 (0.6-2.3)†,‡	1.5 (0.8-2.9)†,‡	0 (0-1.0)	0 (0-0.8)
Blood pressure (mmHg)				
Median SBP (IQR)	173 (148-180)‡	167 (149-185)‡	130 (123-140)†	154 (137-173)
Median DBP (IQR)	97 (90-106)†,‡	96 (87-103)‡	84 (80-89)†	90 (84-100)
Median aldosterone level in nmol/L (IQR)	0.55 (0.38-0.94)†,‡	0.86 (0.55-1.40)†,‡	0.18 (0.14-0.23)	0.25 (0.20-0.30)
Median renin in mU/L (IQR)	5.9 (4.3-9.4)‡	5.5 (3.9-7.3)†,‡	14.0 (9.5-19.0)	9.0 (6.5-14.0)
Median ARR in nmol/mU (IQR)	0.10 (0.05-0.16)†,‡	0.18 (0.11-0.25)†,‡	0.01 (0.01-0.02)†	0.03 (0.02-0.04)
Mean potassium level in mmol/L ± SD	3.6 ± 0.4‡	3.6 ± 0.3†,‡	4.2 ± 0.4†	3.8 ± 0.3
Median units (10 g) alcohol/week (IQR)	2 (0-7)	1 (0-7)	1 (0-3)	2 (0-7)
Median creatinine in µmol/L (IQR)	78.5 (71.0-90.5) n=30	77.5 (64.0-87.3) n=42	83.0 (75.3-100.3)† n=36	73.5 (65.5-81.8) n=38
Median albumin-to-creatinine ratio in mg/mmol (IQR)	2.7 (1.7-9.4)‡ n=19	4.9 (1.1-22.8)†,‡ n=22	0.8 (0.0-1.3) n=10	1.4 (0.7-3.3) n=48
(Other) cardiovascular risk factors				
Median BMI in kg/m^2 (IQR)	28.9 (25.4-32.5)	29.1 (25.6-32.4)	28.4 (26.2-32.3)	28.4 (25.4-31.2)
Smoking (%)	8 (24.2)	6 (13.3)	6 (15.4)	6 (10.7)
Ex-smoker (%)	10 (30.3)	15 (33.3)	14 (35.9)	20 (35.7)
DM type 2 (%)*	5 (15.2)	3 (6.7)	2 (5.1)	0 (0)
Family history of CVD (%)	22 (66.7)	29 (64.4)	22 (56.4)	39 (69.6)

History of CVD (%)	2 (6.1)	5 (11.1)	3 (7.7)	2 (3.6)
Dyslipidemia (%)	10 (30.3)	6 (13.3)	6 (15.4)	8 (14.3)
Echocardiography				
Report available (%)*	17 (51.5)	19 (42.2)	8 (20.5)	39 (69.6)
Left ventricular hypertrophy (%)*	9 (52.9)	10 (52.6)	2 (25.0)	4 (10.3)
Diastolic dysfunction (%)*	10 (58.8)	10 (58.8)	4 (57.1)	15 (38.5)

** p≤0.05, † p≤0.05 compared to EHT, ‡ p≤0.05 compared to post-adrenalectomy*
Abbreviations: APA, aldosterone producing adenoma; ARR, aldosterone-renin-ratio; BMI, body mass index; CVD, cardiovascular diseases; DDD, daily defined dose; EHT, essential hypertension; PA, primary aldosteronism

In 11 patients of the APA-group, a paired residual plasma sample was available that had been obtained 9.0 + 4.6 months after adrenalectomy, without any interfering medication. The 11 patients both clinically and biochemically recovered after adrenalectomy, with a significant decrease in median SBP from 164 (140-178) to 133 (122-141) mmHg (p=0.01), a decrease in median serum aldosterone from 0.88 (0.61-1.11) to 0.14 (0.13-0.21) nmol/L (p<0.01), an increase in median plasma renin from 4.3 (3.6-8.1) to 19.0 (14.0-23.0) mU/L (p=0.01), a decrease in mean ARR from 0.20 ± 0.10 to 0.01 ± 0.01 nmol/mU (p<0.01) and an increase in mean plasma potassium concentration from 3.5 ± 0.3 to 4.5 ± 0.4 mmol/L (p<0.01). The median DBP tended to decrease from 98 (85-115) to 82 (74-90) mmHg and the median DDD of antihypertensives tended to decrease from 1 (0-2.25) to 0 (0-1) pre- and post-adrenalectomy respectively, but this decrease did not achieve statistical significance (p=0.06 and p=0.06).

PLASMA GAL-3 VALUES

Without correction for confounders, the mean Gal-3 concentration was not different between patients with PA, post-adrenalectomy and EHT (12.12 ± 0.36, 12.88 ± 0.50, and 12.42 ± 0.42 ng/mL respectively).

Mean plasma Gal-3 concentrations did not differ significantly between the groups after adjustments for covariates (11.39 ± 0.60, 11.64 ± 0.81 and 11.41 ± 0.73 ng/mL for PHA, post-adrenalectomy and EHT respectively). The Gal-3 concentrations in the different subgroups of patients with stepwise correction for covariates are described in Table 2.

TABLE 2. Plasma Gal-3 concentrations in ng/mL in the patients with PA, post-adrenalectomy, and the patients with EHT, without and with correction for confounders. Values are expressed as mean ± SD

Correction for	PA	Post-adrenalectomy	EHT	*P*-value
-	12.12 ± 0.36	12.88 ± 0.50	12.42 ± 0.42	0.46
Age, sex, BMI	12.17 ± 0.37	12.96 ± 0.51	12.39 ± 0.42	0.45
Age, sex, BMI, smoking, DM	11.04 ± 0.58	11.71 ± 0.72	11.05 ± 0.69	0.51
Age, sex, BMI, smoking, DM, years of hypertension, SBP and DBP	10.86 ± 0.61	11.98 ± 0.77	10.97 ± 0.70	0.29
Age, sex, BMI, smoking, DM, years of hypertension, SBP and DBP, creatinine	11.16 ± 0.60	12.20 ± 0.78	11.43 ± 0.75	0.42
Age, sex, BMI, smoking, DM, years of hypertension, SBP and DBP, creatinine, units alcohol/wk, potassium	11.39 ± 0.60	11.64 ± 0.81	11.41 ± 0.73	0.95

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; DM, diabetes mellitus; EHT, essential hypertension; PA, primary aldosteronism; SBP, systolic blood pressure

We did not find a correlation between serum aldosterone and plasma Gal-3 levels in the total cohort of 78 PA patients, 39 cured PA patients after adrenalectomy and 56 EHT patients (Spearman's correlation coefficient -0.11; p=0.17).

Within the group of patients with PA, we selected those who had undergone echocardiography (n=36) and compared the baseline characteristics between the patients with (n=19) and without LVH (n=17).

The blood pressure was similar in both groups, but patients with LVH used more antihypertensive drugs (DDD of antihypertensive drugs 2.0 (1.7-3.3) in patients with PA and LVH versus 0.9 (0-2.1) in patients with PA but without LVH; p=0.02). Otherwise, there were no differences in baseline characteristics between the patients with PA and LVH and patients with PA but without LVH (data not shown).

Mean Gal-3 levels did not differ between the patients with and without LVH (12.21 ± 0.68 and 13.03 ± 0.72 ng/mL respectively, p=0.42). Also after stepwise correction for confounders (age, sex, BMI, alcohol intake, diabetes, smoking, years of hypertension, blood pressure, plasma creatinine and potassium levels), Gal-3 levels were not different

between the PA patients with and without LVH (11.63 ± 1.00 versus 11.13 ± 1.02 ng/mL respectively, p=0.64) .

In the 11 paired samples of APA-patients, the Gal-3 concentration was 10.03 ± 1.67 ng/mL before adrenalectomy and increased to 14.36 ± 2.07 ng/mL post-adrenalectomy (p<0.01), as depicted in Figure 2. After correction for plasma creatinine levels, plasma potassium concentration and blood pressure, the mean Gal-3 concentration was 10.06 ± 0.81 and 14.54 ± 0.81 ng/mL pre- and post-adrenalectomy respectively (p<0.01).

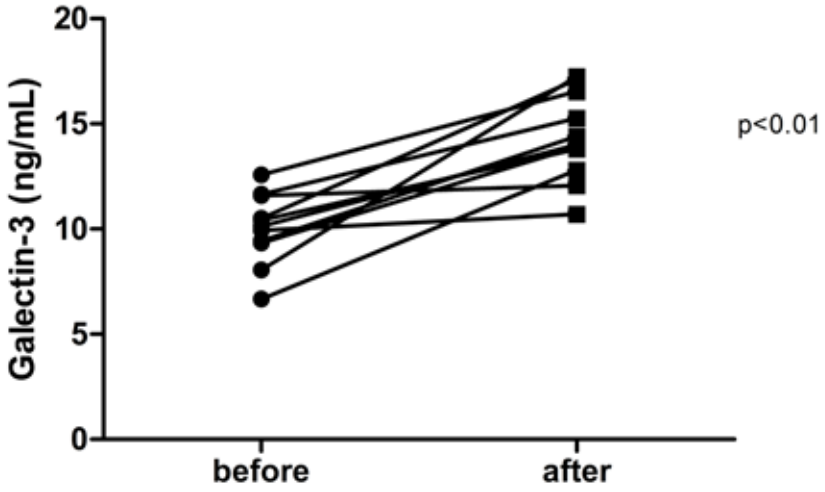


FIGURE 2. Mean plasma Gal-3 levels in the 11 patients of whom paired samples were available before and after adrenalectomy

DISCUSSION

Based on recent preclinical observations, we hypothesized that plasma Gal-3 levels are elevated in patients with PA, compared to patients with EHT, and that this may contribute to the increased cardiovascular risk of patients with PA. In our retrospective cohort study however, we did not find differences in the plasma Gal-3 concentrations between patients with PA, cured PA patients (post-adrenalectomy), and patients with EHT. Furthermore, in a subset of 11 patients with a unilateral producing adenoma in which blood samples were available before and after adrenalectomy, Gal-3 levels increased after adrenalectomy.

Our findings are in contrast to previous preclinical studies in which aldosterone, both *in vitro* and *in vivo*, consistently increased Gal-3 protein levels and expression. [15, 21-23] It should be appreciated though that the experimental animal models of hyperaldosteronism substantially differ from the patients with an autonomous overproduction of aldosterone. Another important issue that might explain the discrepancy between the preclinical findings and our *in vivo* observations, is the possibility of publication bias, which has shown to play a major role in other animal disease models. [34] In addition, in the preclinical studies, Gal-3 secretion and expression was measured in culture media, cell extracts and tissue homogenates, but not in the circulation *in vivo*. One of the potential explanations therefore, is that there is a differential regulation of Gal-3 in plasma and in the various tissue compartments, and that an increased Gal-3 expression in myocardial, renal or vascular tissue in patients with PA is not reflected in higher circulating Gal-3 levels.

Several studies demonstrated that the plasma Gal-3 concentrations are elevated in patients with heart failure. [35, 36] In these patients plasma Gal-3 concentrations predict morbidity and mortality. [37] Furthermore, persistently elevated levels of Gal-3 predict the onset of heart failure in the general population. [32, 38] The regulation of the plasma Gal-3 concentration in these patients with heart failure might be different however than in patients with PA. In this latter group of patients, only one study group has

investigated the circulating Gal-3 levels. [23] Higher plasma Gal-3 concentrations in patients with PA compared to EHT were observed by the TAIPAI study group. Mean Gal-3 levels returned to normal after adrenalectomy. [23, 39]

There are several differences between the studies of the TAIPAI study group and our present study that could explain the different results. First, the TAIPAI study group measured Gal-3 concentrations in relatively small cohorts of patients with PA (n=7 and n=11) and patients with EHT (n=11 and n=17). [23, 39] The selection process of these patients was not described and it is unclear whether the 2 studies share a majority of patients or not. We selected a large group of patients that had been diagnosed with PA and EHT according to current guidelines, and carefully corrected for potential confounders. In our large cohort of 78 patients with untreated PA, 39 patients post-adrenalectomy and 56 patients with EHT, no differences in Gal-3 concentrations were observed.

Secondly, we measured Gal-3 concentrations in plasma samples obtained for serum aldosterone and plasma renin measurements. At the time of sampling, patients did not take any medication that interfered with the renin-angiotensin-aldosterone system. Although Lin *et al* describe that patients who did not take the MR antagonist spironolactone within the previous 6 months were enrolled, it is unclear whether other medications interfering with the renin-angiotensin-aldosterone system were taken when venous blood was drawn for Gal-3 measurements. [23]

Next, there are differences in the analytical methods. For quantification of plasma Gal-3 concentrations we used the BG Gal-3 ELISA-kit. This kit has a reported within run and total precision of 2.1%–5.7% and 4.2%–12.0% respectively, and has received FDA clearance. [33] A different assay was used by the TAIPAI study group, and importantly, plasma Gal-3 concentrations were 10-fold lower in comparison to the observed mean Gal-3 values in our study. The Gal-3 concentrations in our study are in the same range of the Gal-3 concentration of 11.4 ± 4.0 ng/mL observed in historical controls from the PREVEND study, with comparable age (between 50 and 55 years). [33] Under other pathophysiological circumstances, such as heart failure, quantified Gal-3 levels are even higher (20-30 ng/mL), with values up to 60-100 ng/mL in patients with end stage renal disease. [25, 40]

Finally, differences in ethnic background of the included patients may explain the discrepant findings of our studies. In the studies of the TAIPAI study group Asians were enrolled, while our cohort consists of Caucasians.

In Table 1 we depicted the findings on echocardiography that were available in almost half of the PA and more than half of the EHT patients. Importantly, the prevalence of LVH and diastolic dysfunction was higher in PA patients than in EHT patients. We showed that Gal-3 levels did not differ between the patients with PA and LVH and the patients with PA but without LVH. The observation that the difference in cardiac damage is not reflected by differences in plasma Gal-3 concentrations, exclude a role of plasma Gal-3 in aldosterone-mediated target organ damage in humans *in vivo*.

Interestingly, although we did not observe differences in circulating Gal-3 between the patients with PA, patients with EHT, and patients treated for PA, we did observe a significant and consistent increase in Gal-3 concentrations in the subgroup of 11 patients from whom paired blood samples were available before and after adrenalectomy. We do not have a clear explanation for this interesting finding. There is no relevant difference between the subgroup and the total group with regard to serum aldosterone and ARR values, medication use, comorbidities or other baseline characteristics. In addition, there was no relevant difference between the timing of plasma samples that were taken 11.9 ± 4.8 months after adrenalectomy in the 39 post-adrenalectomy patients, compared to 9.0 ± 4.6 months in the 11 patients. Antihypertensive medication use might have influenced our results. As mentioned before, none of the patients used medication that affects serum aldosterone or plasma renin concentrations. It is unclear however, if the tolerated antihypertensive medication or the change in DDD have interfered with plasma Gal-3 concentrations.

The strength of our study is clearly the use of stringent diagnostic criteria for PA, and cure from PA after surgical treatment. Moreover, when investigating the Gal-3 concentrations in the 3 patient groups, the extensive phenotyping of the patients allowed us to rigorously correct for all potential confounders.
Nevertheless, our study has some limitations. First, our study is a retrospective analysis, which is prone to bias related to missing variables. As described, the number of missing plasma creatinine concentrations in our cohort was substantial (n=27). When we analyzed only those patients in whom there were no missing values for the confounders depicted in Table 2, unadjusted mean plasma Gal-3 levels were 12.25 ± 0.37, 13.00 ± 0.51 and 12.75 ± 0.50 ng/mL in PA patients, patients post-adrenalectomy and EHT patients

respectively; p=0.45. These levels do not differ from the unadjusted mean plasma Gal-3 concentrations in the total cohort (12.12 ± 0.36, 12.88 ± 0.50, and 12.42 ± 0.42 ng/mL). Also, adjusted Gal-3 variables in the patients without any missing variables did not differ from the results in Table 2 (data not shown). Therefore, we do not expect that the presence of missing variables in our retrospective analysis was a confounder itself.
In addition, echocardiography was performed in only half of the PA patients and more than half of the EHT patients, which might have introduced potential bias. Indeed, SBP levels were higher in the patients in whom echocardiography was performed (154 (138-176) versus 147 (133-168) mmHg in patients with and without echocardiography respectively; p=0.01). Adjusted plasma Gal-3 concentrations in patients in whom echocardiography was performed however did not differ between the subgroups (11.55 ± 0.88, 11.60 ± 1.69 and 11.56 ± 1.11 ng/mL for patients with PA, cured PA patients after adrenalectomy and patients with EHT respectively; p=1.00).
Secondly, our samples had been stored for a period of maximally 2 years, at -20 °C. The product insert of the BG medicine galectin-3 assay reports that galectin-3 is stable for at least 2 years when stored at -70 °C. In the study by Christenson *et al* on the assay characteristics of galectin-3, there were no major differences in galectin-3 levels between samples stored at -20 °C and samples stored at -70 °C, during at least 6 months of storage and 6 freeze/thaw cycles. (41) In our study, there was a maximum of 2 freeze/thaw cycles. Therefore, it is unlikely that our results are affected by analytical problems. Moreover, by adding the storage time as a covariate in the analysis, plasma Gal-3 levels remained comparable between the subgroups (11.76 ± 0.58, 11.98 ± 0.78 and 11.62 ± 0.70 ng/mL for PA, post-adrenalectomy and EHT patients respectively, p=0.91) and results did not change in the 11 paired samples (mean Gal-3 concentrations 9.77 ± 1.03 and 14.82 ± 1.03 ng/mL pre- and post-adrenalectomy respectively, p=0.02).

In conclusion, in our retrospective cohort study we did not observe differences in plasma Gal-3 concentrations between patients with PA, patients that had undergone adrenalectomy for PA, and patients with EHT. Our results are in contrast to preclinical studies and two small clinical studies in which Gal-3 has been proposed as a key mediator in aldosterone-mediated fibrosis.

PERSPECTIVES

We demonstrated, for the first time, that there is no association between aldosterone excess and plasma Gal-3 concentrations in humans *in vivo*. It is therefore highly unlikely that increases in plasma Gal-3 production contribute to the increased risk of cardiovascular events in patients with PA, compared to patients with EHT. This does not exclude however, a predictive role of Gal-3 on cardiovascular morbidity and mortality.

ACKNOWLEDGEMENTS

We would like to thank Sandra Oude Alink of the Department of Laboratory Medicine, who collected the residual plasma samples for this study.

REFERENCES

1. Milliez P, Girerd X, Plouin PF, Blacher J, Safar ME, Mourad JJ. Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. *Journal of the American College of Cardiology.* 2005;45(8):1243-8.
2. Savard S, Amar L, Plouin PF, Steichen O. Cardiovascular complications associated with primary aldosteronism: a controlled cross-sectional study. *Hypertension.* 2013;62(2):331-6.
3. Kisaka T, Ozono R, Ishida T, Higashi Y, Oshima T, Kihara Y. Association of elevated plasma aldosterone-to-renin ratio with future cardiovascular events in patients with essential hypertension. *Journal of hypertension.* 2012;30(12):2322-30.
4. De Angelis N, Fiordaliso F, Latini R, Calvillo L, Funicello M, Gobbi M, et al. Appraisal of the role of angiotensin II and aldosterone in ventricular myocyte apoptosis in adult normotensive rat. *Journal of molecular and cellular cardiology.* 2002;34(12):1655-65.
5. Burniston JG, Saini A, Tan LB, Goldspink DF. Aldosterone induces myocyte apoptosis in the heart and skeletal muscles of rats in vivo. *Journal of molecular and cellular cardiology.* 2005;39(2):395-9.
6. Fujita M, Minamino T, Asanuma H, Sanada S, Hirata A, Wakeno M, et al. Aldosterone nongenomically worsens ischemia via protein kinase C-dependent pathways in hypoperfused canine hearts. *Hypertension.* 2005;46(1):113-7.
7. Mihailidou AS, Loan Le TY, Mardini M, Funder JW. Glucocorticoids activate cardiac mineralocorticoid receptors during experimental myocardial infarction. *Hypertension.* 2009;54(6):1306-12.
8. Chai W, Garrelds IM, Arulmani U, Schoemaker RG, Lamers JM, Danser AH. Genomic and nongenomic effects of aldosterone in the rat heart: why is spironolactone cardioprotective? *British journal of pharmacology.* 2005;145(5):664-71.
9. Chai W, Garrelds IM, de Vries R, Danser AH. Cardioprotective effects of eplerenone in the rat heart: interaction with locally synthesized or blood-derived aldosterone? *Hypertension.* 2006;47(4):665-70.
10. Schmidt K, Tissier R, Ghaleb B, Drogies T, Felix SB, Krieg T. Cardioprotective effects of mineralocorticoid receptor antagonists at reperfusion. *European heart journal.* 2010;31(13):1655-62.

11. Loan Le TY, Mardini M, Howell VM, Funder JW, Ashton AW, Mihailidou AS. Low-dose spironolactone prevents apoptosis repressor with caspase recruitment domain degradation during myocardial infarction. *Hypertension.* 2012;59(6):1164-9.
12. Brilla CG, Matsubara LS, Weber KT. Antifibrotic effects of spironolactone in preventing myocardial fibrosis in systemic arterial hypertension. *The American journal of cardiology.* 1993;71(3):12A-6A.
13. Azibani F, Benard L, Schlossarek S, Merval R, Tournoux F, Fazal L, et al. Aldosterone inhibits antifibrotic factors in mouse hypertensive heart. *Hypertension.* 2012;59(6):1179-87.
14. Azibani F, Fazal L, Chatziantoniou C, Samuel JL, Delcayre C. Aldosterone mediates cardiac fibrosis in the setting of hypertension. *Current hypertension reports.* 2013;15(4):395-400.
15. Calvier L, Miana M, Reboul P, Cachofeiro V, Martinez-Martinez E, de Boer RA, et al. Galectin-3 mediates aldosterone-induced vascular fibrosis. *Arteriosclerosis, thrombosis, and vascular biology.* 2013;33(1):67-75.
16. Liu FT, Hsu DK, Zuberi RI, Kuwabara I, Chi EY, Henderson WR, Jr. Expression and function of galectin-3, a beta-galactoside-binding lectin, in human monocytes and macrophages. *The American journal of pathology.* 1995;147(4):1016-28.
17. de Boer RA, Voors AA, Muntendam P, van Gilst WH, van Veldhuisen DJ. Galectin-3: a novel mediator of heart failure development and progression. *European journal of heart failure.* 2009;11(9):811-7.
18. de Boer RA, Yu L, van Veldhuisen DJ. Galectin-3 in cardiac remodeling and heart failure. *Current heart failure reports.* 2010;7(1):1-8.
19. Dumic J, Dabelic S, Flogel M. Galectin-3: an open-ended story. *Biochimica et biophysica acta.* 2006;1760(4):616-35.
20. Wang L, Guo XL. Molecular regulation of galectin-3 expression and therapeutic implication in cancer progression. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie.* 2016;78:165-71.
21. Calvier L, Martinez-Martinez E, Miana M, Cachofeiro V, Rousseau E, Sadaba JR, et al. The impact of galectin-3 inhibition on aldosterone-induced cardiac and renal injuries. *JACC Heart failure.* 2015;3(1):59-67.
22. Martinez-Martinez E, Calvier L, Fernandez-Celis A, Rousseau E, Jurado-Lopez R, Rossoni LV, et al. Galectin-3 Blockade Inhibits Cardiac Inflammation and Fibrosis in Experimental Hyperaldosteronism and Hypertension. *Hypertension.* 2015.

23. Lin YH, Chou CH, Wu XM, Chang YY, Hung CS, Chen YH, et al. Aldosterone induced galectin-3 secretion in vitro and in vivo: from cells to humans. *PloS one.* 2014;9(9):e95254.
24. de Boer RA, Daniels LB, Maisel AS, Januzzi JL, Jr. State of the Art: Newer biomarkers in heart failure. *European journal of heart failure.* 2015;17(6):559-69.
25. Lopez-Andres N, Rossignol P, Iraqi W, Fay R, Nuee J, Ghio S, et al. Association of galectin-3 and fibrosis markers with long-term cardiovascular outcomes in patients with heart failure, left ventricular dysfunction, and dyssynchrony: insights from the CARE-HF (Cardiac Resynchronization in Heart Failure) trial. *European journal of heart failure.* 2012;14(1):74-81.
26. Koukoui F, Desmoulin F, Galinier M, Barutaut M, Caubere C, Evaristi MF, et al. The prognostic value of plasma galectin-3 in chronic heart failure patients is maintained when treated with mineralocorticoid receptor antagonists. *PloS one.* 2015;10(3):e0119160.
27. Gandhi PU, Motiwala SR, Belcher AM, Gaggin HK, Weiner RB, Baggish AL, et al. Galectin-3 and mineralocorticoid receptor antagonist use in patients with chronic heart failure due to left ventricular systolic dysfunction. *American heart journal.* 2015;169(3):404-11.e3.
28. Fiuzat M, Schulte PJ, Felker M, Ahmad T, Neely M, Adams KF, et al. Relationship between galectin-3 levels and mineralocorticoid receptor antagonist use in heart failure: analysis from HF-ACTION. *Journal of cardiac failure.* 2014;20(1):38-44.
29. Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, et al. The Management of Primary Aldosteronism: Case Detection, Diagnosis, and Treatment: An Endocrine Society Clinical Practice Guideline. *The Journal of clinical endocrinology and metabolism.* 2016;101(5):1889-916.
30. De Man AJ, Hofman JA, Hendriks T, Rosmalen FM, Ross HA, Benraad TJ. A direct radio-immunoassay for aldosterone: significance of endogenous cortisol. *The Netherlands journal of medicine.* 1980;23(2):79-83.
31. Meijers WC, van der Velde AR, de Boer RA. The ARCHITECT galectin-3 assay: comparison with other automated and manual assays for the measurement of circulating galectin-3 levels in heart failure. *Expert review of molecular diagnostics.* 2014;14(3):257-66.
32. Ho JE, Liu C, Lyass A, Courchesne P, Pencina MJ, Vasan RS, et al. Galectin-3, a marker of cardiac fibrosis, predicts incident heart failure in the community. *Journal of the American College of Cardiology.* 2012;60(14):1249-56.
33. de Boer RA, van Veldhuisen DJ, Gansevoort RT, Muller Kobold AC, van Gilst WH, Hillege HL, et al. The fibrosis marker galectin-3 and outcome in the general population. *Journal of internal medicine.* 2012;272(1):55-64.

34. Sena ES, van der Worp HB, Bath PM, Howells DW, Macleod MR. Publication bias in reports of animal stroke studies leads to major overstatement of efficacy. *PLoS biology*. 2010;8(3):e1000344.
35. Yu X, Sun Y, Zhao Y, Zhang W, Yang Z, Gao Y, et al. Prognostic value of plasma galectin-3 levels in patients with coronary heart disease and chronic heart failure. *International heart journal*. 2015;56(3):314-8.
36. Edelmann F, Holzendorf V, Wachter R, Nolte K, Schmidt AG, Kraigher-Krainer E, et al. Galectin-3 in patients with heart failure with preserved ejection fraction: results from the Aldo-DHF trial. *European journal of heart failure*. 2015;17(2):214-23.
37. van der Velde AR, Gullestad L, Ueland T, Aukrust P, Guo Y, Adourian A, et al. Prognostic value of changes in galectin-3 levels over time in patients with heart failure: data from CORONA and COACH. *Circulation Heart failure*. 2013;6(2):219-26.
38. van der Velde AR, Meijers WC, Ho JE, Brouwers FP, Rienstra M, Bakker SJ, et al. Serial galectin-3 and future cardiovascular disease in the general population. *Heart (British Cardiac Society)*. 2016;102(14):1134-41.
39. Liao CW, Lin YT, Wu XM, Chang YY, Hung CS, Wu VC, et al. The relation among aldosterone, galectin-3, and myocardial fibrosis: a prospective clinical pilot follow-up study. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research*. 2016;64(6):1109-13.
40. Drechsler C, Delgado G, Wanner C, Blouin K, Pilz S, Tomaschitz A, et al. Galectin-3, Renal Function, and Clinical Outcomes: Results from the LURIC and 4D Studies. *Journal of the American Society of Nephrology : JASN*. 2015;26(9):2213-21.
41. Christenson RH, Duh SH, Wu AH, Smith A, Abel G, deFilippi CR, et al. Multi-center determination of galectin-3 assay performance characteristics: Anatomy of a novel assay for use in heart failure. *Clinical biochemistry*. 2010;43(7-8):683-90.


f
p
fp
p
crescendo
f
Var. III
f
p
Var. IV
fp
fp
fp

CHAPTER 3

THE EFFECTS OF MINERALOCORTICOID RECEPTOR ANTAGONISTS ON CARDIOVASCULAR DAMAGE


9
17
24
tr
tr
tr
FINALE
Molto allegro
f
5
11
17
25
6
p

CHAPTER 3.1

THE CARDIOPROTECTIVE EFFECTS OF MINERALOCORTICOID RECEPTOR ANTAGONISTS

T.N.A. (Daniëlle) van den Berg [a,b] | Gerard A. Rongen [a,b]
Georg Frohlich [c] | Jaap Deinum [b]
Derek Hausenloy [c] | Niels P. Riksen [b]

[a] Department of Pharmacoloy and Toxicology and [b] Department of Internal Medicine, Radboud university medical center, Nijmegen, the Netherlands, [c] The Hatter Cardiovascular Institute, University College London, United Kingdom

Pharmacol Ther. 2014 Apr;142(1):72-87

ABSTRACT

Despite state-of-the-art reperfusion therapy, morbidity and mortality remains significant in patients with an acute myocardial infarction. Therefore, novel strategies to limit myocardial ischemia-reperfusion injury are urgently needed. Mineralocorticoid receptor (MR) antagonists are attractive candidates for this purpose, since several clinical trials in patients with heart failure have reported a survival benefit with MR antagonist treatment. MRs are expressed by several cells of the cardiovascular system, including cardiomyocytes, cardiac fibroblasts, vascular smooth muscle cells, and endothelial cells. Experiments in animal models of myocardial infarction have demonstrated that acute administration of MR antagonists, either before ischemia or immediately at the moment of coronary reperfusion, limit infarct size. This action appears to be independent of the presence of aldosterone, which is the endogenous ligand for the MR. The cardioprotective effect is mediated by a nongenomic intracellular signaling pathway, including adenosine receptor stimulation, and activation of several components of the Reperfusion Injury Salvage Kinase (RISK) pathway. In addition to limiting infarct size, MR antagonists can improve scar healing when administered shortly after reperfusion and can reduce cardiac remodeling post myocardial infarction when initiated after initial infarct healing. Clinical trials are currently being performed studying whether early administration of MR antagonists can indeed improve prognosis in patients with an acute myocardial infarction, independent of the presence of heart failure.

INTRODUCTION

In 2008, more than 17 million people died of cardiovascular diseases worldwide, according to summary tables of the World Health Organisation. In low-, middle- and high-income countries, ischemic heart disease and cerebrovascular disease are the leading cause of death. Percutaneous coronary intervention and thrombolysis are the main strategies to restore perfusion in patients with an acute myocardial infarction. Despite state-of-the-art reperfusion strategies, mortality and morbidity in patients with an acute myocardial infarction remain significant. This is caused, at least in part, by the fact that reperfusion itself also contributes to tissue injury, a phenomenon which has been termed "lethal reperfusion injury". [1] Therefore, novel therapeutic options to limit ischemia-reperfusion (IR) injury are urgently needed to improve outcome in these patients.

It has been suggested that the mineralocorticoid receptor (MR) antagonists spironolactone and eplerenone could potentially serve this goal, because these drugs reduce mortality in patients with heart failure. [2-4] These reported effects of MR antagonists are consistent with the observations that the endogenous ligand for the MR, aldosterone, can have detrimental cardiovascular effects. Patients with autonomous adrenal overproduction of aldosterone (primary aldosteronism; PA) have an increased risk of cardiovascular and cerebrovascular events, and cardiac remodeling, independent of blood pressure levels. [5-7] Also, in patients without PA, a high aldosterone level or high aldosterone-to-renin ratio is associated with an increased risk of cardiovascular events. [8] In the setting of an acute myocardial infarction, aldosterone levels, even within the normal range, are associated with cardiovascular death and heart failure. [9]

Preclinical studies have now provided convincing evidence that MR antagonists have beneficial effects on various pathophysiological processes that contribute to cardiovascular morbidity and mortality (Figure 1). MR antagonists attenuate the process of atherosclerosis [10-15], modulate endothelial function and blood flow in animal studies [16-19] and in various patient groups [20-27], limit myocardial IR-injury, prevent IR-induced arrhythmias [28-35], and beneficially modulate cardiac remodeling. Protective effects against IR-injury have not only been reported in the heart, but also in the kidney [36, 37], the brain [38, 39], and the retina [40].

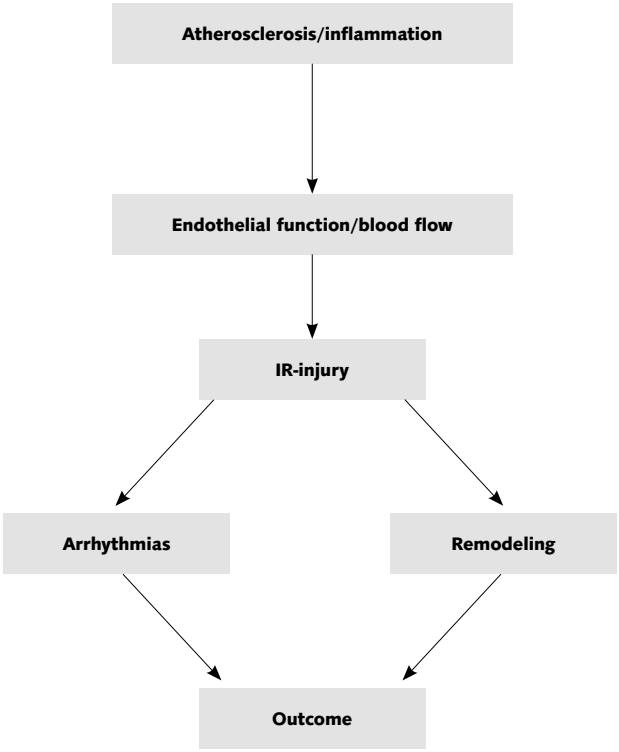


FIGURE 1. Overview of the various pathophysiological processes that ultimately lead to cardiovascular morbidity and mortality which can be affected by MR antagonists

In this review paper, we will summarize current knowledge on the effects of MR antagonists on myocardial IR-injury and post-infarction remodeling. To appreciate the molecular mechanisms underlying these beneficial effects, detailed knowledge about the distribution and function of the MR throughout the body is essential. Therefore, we will discuss the expression of the MR receptor in cardiovascular tissues, the effects of MR antagonists on IR-injury and the underlying mechanisms, and the effects of MR antagonists on post-infarction remodeling.

EXPRESSION AND FUNCTION OF THE MR IN THE CARDIOVASCULAR SYSTEM

CHARACTERISTICS OF THE MR

From the classical physiological perspective the MR is expressed in the distal tubular cells of the kidney. The endogenous ligand is aldosterone, which is synthesized in the zona glomerulosa of the adrenal cortex. In this paradigm the role of this major mineralocorticoid is to regulate blood pressure and water balance by promoting the reabsorption of sodium and the excretion of potassium. The MR displays similar affinity for aldosterone and glucocorticoid hormones. Despite a 100- to 1000-fold higher concentration of these latter hormones than aldosterone, the MR in aldosterone target cells is not stimulated by glucocorticoids under normal physiological conditions. The enzyme 11-β-hydroxysteroid dehydrogenase type 2 (11βHSD2), co-expressed with the MR, converts cortisol into the inactive metabolite cortisone (Figure 2). In rodents, where corticosterone instead of cortisol is the major glucocorticoid, corticosterone is metabolized to 11-deoxycorticosterone by 11βHSD2. Cortisone and 11-deoxycorticosterone have a much lower affinity for the MR. [41, 42] In this way 11βHSD2 protects the MR from stimulation by cortisol and confers specificity to aldosterone binding.

The isozyme 11βHSD type 1 (11βHSD1), *in vitro* catalyzes interconversion of cortisol and cortisone [43], but *in vivo* the enzyme converts cortisone into cortisol only. Taken together, this suggests that MR stimulation by aldosterone only takes place in cells that express 11βHSD2, but not in cells expressing 11βHSD1, both types, or none of these enzymes.

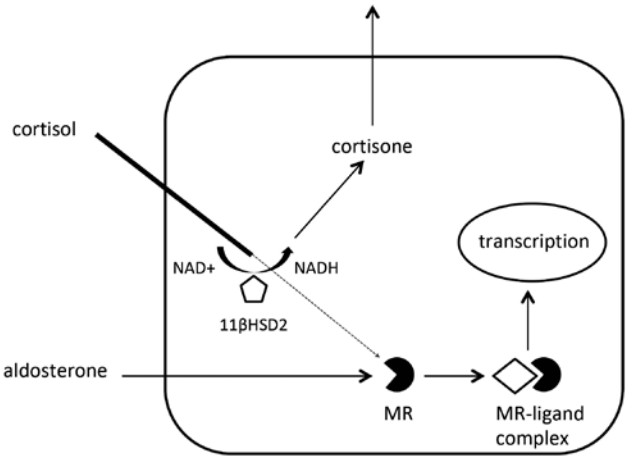


FIGURE 2. Mineralocorticoid target cell and the function of the enzyme 11-β-hydroxysteroid dehydrogenase type 2

Abbreviations: 11ßHSD2, 11-ß-hydroxysteroid dehydrogenase type 2; NAD+/NADH, nicotinamide adenine dinucleotide

Alternative mechanisms of mineralocorticoid selectivity for aldosterone, including intrinsic discriminating properties of the MR itself, and distinct molecular ligand-MR interactions, have been described. Kinetic studies have shown that aldosterone has a 5-times lower off-rate from the human MR than cortisol, pointing to an intrinsic discriminating property of the receptor, which leads to an increase in transcriptional activity by aldosterone [44]. In addition, there are indications that the expression of co-activators of the MR can increase aldosterone-mediated transactivation [45]. These mechanisms have been reviewed elsewhere [42, 46-49] and we will not further discuss them here since they do not seem to bear relevance to cardioprotection by MR antagonists.

EXPRESSION OF THE MR AND 11βHSD IN CELLS OF THE CARDIOVASCULAR SYSTEM

From the above it follows that the role of MR stimulation by aldosterone or by glucocorticoids can only be understood if it is known whether 11βHSD2 and perhaps 11βHSD1 are co-expressed with the MR. This is important, because the MR is expressed not only in the distal tubular cells of the kidney, but also in other cell types that are relevant to the cardiovascular system, including cardiomyocytes, cardiac

fibroblasts, vascular smooth muscle cells, endothelial cells, and mononuclear cells of the immune system. [42, 47, 48, 50, 51] Also in other tissues, including brain [47, 52, 53] and retina [54], expression of the MR has been reported. Not all of these cell types co-express 11βHSD. Table 1 provides an overview of the studies which examined the presence of 11βHSD type 1 and 2 within cells of the cardiovascular system. Before the 1990s researchers were not aware of the existence of two 11βHSD enzymes and, hence, no distinction was made between 11βHSD1 and 11βHSD2. Most studies at that time assessed the presence of 11βHSD in rat organ tissues by measurement of the conversion of ^{3}H-corticosterone to ^{3}H-11-deoxycorticosterone, assuming that this indicated the presence of 11βHSD type 2. However, *in vitro* 11βHSD1 converts the substrates bidirectionally [43], and therefore, conversion of corticosterone or cortisol into 11-deoxycorticosterone or cortisone, respectively, in *in vitro* experiments does not necessarily indicate the presence of 11βHSD2. From the 1990s onwards, Northern blot analysis permitted the differentiation between the two types of 11βHSD. [55, 56] Moreover, when it was discovered that both enzymes needed different co-factors, the addition of $NADP^{+}$ (11βHSD1) and NAD+ (11βHSD2) provided a pharmacological method of discrimination. Table 1 is not exhaustive for studies examining the presence of 11βHSD within cells in the cardiovascular system, but is limited to papers using appropriate assays to distinguish between the two types. In animal models, no 11βHSD2 was demonstrated in whole heart [57-64] and cardiac fibroblasts [64, 65], suggesting that in these cell types effects of MR stimulation are more likely to be mediated by cortisol binding. Studies in rat vascular endothelial cells, human cardiomyocytes and human vascular smooth muscle cells show expression of 11βHSD2. [66-71] In contrast to the studies which detected 11βHSD2 in human cardiac myocytes [66, 67], two other studies [58, 72] and all animal studies in Table 1 showed that cardiomyocytes lack 11βHSD2.

Under pathological conditions, however, modulation of the expression of 11βHSD1 and -2 in these cell types has been reported. In salt-sensitive rats and spontaneously hypertensive rats, activity and mRNA expression of 11βHSD1 and 11βHSD2 in the heart and mesenteric arteries was reduced, when compared to salt-resistant and normotensive rats respectively, but no association between these changes in 11βHSD expression and cardiac hypertrophy were found. Importantly, plasma aldosterone levels were not measured in the rats. [73] Takeda *et al* reported increased aldosterone levels and

an up-regulation of 11βHSD2 in young stroke-prone spontaneously hypertensive rats, which was further increased by high sodium intake. [74] Chronic intermittent hypobaric hypoxia leads to an up-regulation of 11βHSD2 in the free ventricular walls and septum tissues of rats *in vivo*. [75] In patients with rheumatic valve disease, the presence of atrial fibrillation was associated with increased mRNA expression of 11βHSD2 in atrial tissue. [76] Finally, mRNA expression of 11βHSD1 is enhanced in epicardial adipose and ascending aorta tissues of patients with the metabolic syndrome and coronary artery disease, compared to patients with the metabolic syndrome only. [77]

TABLE 1. Schematic overview of the co-expression of 11βHSD1 and -2 in different cell types

Reference	Species	11βHSD type 1 – and or 2	Detection	Finding
HEART				
Walker 1991 [155]	Rat Heart tissue	NADPH+ dependent 11βHSD present	Pharmacologically	Conversion of B into A
			Immunohistochemistry	Localized enzyme in cardiomyocytes and
			mRNA expression by in situ hybridization	Expression found in cardiac muscle
Walker 1992 [156]	Rat Heart tissue	NADPH+ dependent 11βHSD present	Pharmacological	Conversion of A to B
Albiston 1994 [72]	Human Heart tissue	11βHSD2 absent	mRNA detection by Northern blotting	No mRNA detection in heart tissue
Slight 1994 [157]	Ox, pig, horse, rat, rabbit, dog and human Heart tissue	NADPH+ dependent 11βHSD present	Pharmacologically	Conversion of B into A (highest in pig, rat and rabbit) No conversion of F to E in any of the species
	Rat Heart tissue	NADPH+ dependent 11βHSD present	Pharmacologically	Bidirectional conversion of A and B
Lombes 1995 [66]	Human Cardiomyocytes	NAD+ dependent 11βHSD present	Pharmacologically	Conversion of B to A
Agarwal 1995 [158]	Sheep Heart tissue	11βHSD2 present at low levels	mRNA detection	RT-PCR products, but no cDNA, detectable
	Human Heart tissue	11βHSD2 absent	RNA blot	No transcripts detectable
Brown 1996 [57]	Mouse from embryonic day 9-after birth Heart muscle	11βHSD2 absent	mRNA expression by 11βHSD2 sense in situ hybridizations	No detection of 11βHSD2 mRNA in embryo and fetus
Slight 1996 [67]	Human Tissue from failing explanted hearts and donor hearts	11βHSD1 absent and 11βHSD2 present	Pharmacologically	Conversion of B to A with NAD+ twice that formed with NADP+. No reduction of A into B in presence of NADP+
			mRNA detection	11βHSD2 expression
Smith 1996 [68]	Human Myocytes from diseased heart	11βHSD2 absent	Immunohistochemistry	No 11βHSD2 detected
Li 1996 [159]	Rat Heart tissue	11βHSD2 absent	Total RNA by Northern blotting	No 11βHSD2 detected

Smith and Krozowski 1996 [58]	Rat Heart tissue	11βHSD1 present and 11βHSD2 absent	Pharmacologically	Increased conversion of F, or B into A ,and E resp. after addition of NADP+. Inability of NAD+ to augment metabolism suggests absence of 11βHSD2
			mRNA detection	11βHSD1 gene expression
Roland 1996 [59]	Rat Heart tissue	11βHSD2 absent	RNA expression by in situ hybridization	Levels of 11βHSD2 RNA in heart below limits of detection
Condon 1997 [60]	Mouse Heart tissue	11βHSD2 absent	mRNA expression by northern blot analysis	No 11βHSD2 detectable
Náray-Fejes-Tóth 1998 [61]	Rabbit Heart tissue	11βHSD2 absent	Western blot with a 11βHSD2 antibody	No 11βHSD2 in heart present
			Immunohistochemistry	No11βHSD2 detected
Romero 2000 [62]	Ox Heart tissue	11βHSD2 absent	mRNA detection	No detectable levels of 11βHSD2 after RT-PCR
Moore 2000 [63]	Mouse Heart tissue	11βHSD1 present and 11βHSD2 absent	mRNA expression by labeled antisense probes	Detection of 11βHSD1 in heart
Brereton 2001 [65]	Rat Cardiomyocytes	11βHSD1 absent	Immunohistochemistry	No 11βHSD1 immunoreactivity
			Western blot	Low signal detected (presence 11βHSD1 in fibroblasts detected)
Sheppard 2002 [64]	Rat Cardiomyocytes	11βHSD1 present and 11βHSD2 absent	Pharmacologically	11βHSD1 acts as a reductase only, converting A to B
			mRNA expression	11βHSD1 detected
Thompson 2004 [160]	Mouse from embryonic day 12.5 until postnatal day 0.5 Heart tissue	11βHSD 1 present at low levels 11βHSD2 present at day 12.5	mRNA detection by in situ hybridization	After embryonic day 12.5 no 11βHSD2 expression
Klusonová 2008 [161]	Chicken Heart tissue	11βHSD 1 present at low levels	Pharmacologically	Little conversion of A into B
			mRNA expression	RT-PCR of 11βHSD1 products detectable
VASCULAR SMOOTH MUSCLE CELL				
Funder 1989 [162]	Rat in vivo Adrenalectomized at 9 d of age	11βHSD present	Pharmacologically	Conversion of B into A by pooled mesenteric vascular arcade and aortic minces
			Binding study	15 min after s.c. administration of aldosterone and corticosterone, mesenteric vascular arcade highly aldosterone-specific
Walker 1991 [155]	Rat Aorta, coronary artery, mesenteric artery and caudal artery	NADPH+ dependent 11βHSD present	Pharmacologically	Conversion of B into A
			Immunohistochemistry	Localized enzyme in smooth muscle cells throughout the vessel walls
			mRNA expression by in situ hybridization	Expression found in VSMCs of aorta and mesenteric artery
Slight 1994 [157]	Ox, pig, horse, rat, rabbit, dog and human Aortic tissue	NADPH+ dependent 11βHSD present	Pharmacologically	Conversion of B into A with high levels in pig No conversion of F to E in any of the species

>>

Kornel 1994 [163]	Rabbit VSMCs from aorta	NADP+ preferable 11βHSD present	Pharmacologically	Conversion of F to E
			Binding study	High affinity binding of ^{3}H-aldosterone, not interfered by GR blocker
Brem 1995 [164]	Rat Aorta	11βHSD present	Pharmacologically	Conversion of B into A and vice versa. 4 times greater oxo-reductase reaction. Greater activity in both directions in quiescent VSMCs
			mRNA expression by northern blot analysis	Decrease in 11βHSD-specific mRNA in growing fase
Brem 1998 [70]	Rat Aortic VSMC	11βHSD1 present and 11βHSD 2 nearly absent	mRNA detection	Little or no detectable RT-PCR products
Smith 1996 [68]	Human VSMCs of arterioles in skin, heart and saphenous vein	11βHSD2 present	Immunohistochemistry	Lower amounts in venous smooth muscle cells
Hatakeyama 1999 (69)	Human CASMC	Both types present	Pharmacologicallv	Bidirectional conversion of E and F, favoring oxoreduction of E to F
			mRNA detection	RT-PCR products of both enzymes detectable
VASCULAR ENDOTHELIAL CELL				
Brem 1998 [70]	Rat Aortic ECs and endothelium intact aorta	Both types present	Pharmacologically	Conversion of A to B greater than B to A. Oxoreductase activity was reduced half when a 11βHSD1 antisense oligomer was added Equal conversion with NAD+ or NADP+.
			mRNA detection	Readily detectable RT-PCR products
Souness 2002 [71]	Rat Aortic ECs	Both types present	Pharmacologically	Conversion of A to B, and contractile respons to PE decreased after 11βHSD2 antisense incubation. Vice versa after 11βHSD1 antisense incubation.
CARDIAC FIBROBLAST				
Slight 1993 [165]	Rat Cardiac fibroblasts	NADP+ independent 11βHSD present	Pharmacologically	Conversion of B to A. No conversion of F to E
Brereton 2001 [65]	Rat Interstitial fibroblasts of endocardium and adventitial fibroblasts of blood vessels	11βHSD1 present and 11βHSD2 absent	Immunohistochemistry	11βHSD1 detected
			Western blot	11βHSD1detected in fibroblasts of endocardium and blood vessels
Sheppard 2002 [64]	Rat Cardiac fibroblasts	11βHSD1 present and 11βHSD2 absent	mRNA expression	mRNA expression of 11βHSD1 only
			Pharmacologically	11βHSD1 acts as a reductase only, converting A to B

Abbreviations: A=^{3}H-11-dehydrocorticosterone, B = ^{3}H-corticosterone, E = cortisone, F = cortisol
CASMC, coronary artery smooth muscle cells; EC, endothelial cell; GR, glucocorticoid receptor; NAD+, Nicotinamide Adenine Dinucleotide; NADP+, Nicotinamide Adenine Dinucleotide Phospate; resp., respectively; s.c., subcutaneously; VSMC, vascular smooth muscle cell

If detrimental cardiovascular effects of aldosterone are mediated by MR stimulation, it may be expected that in the absence of 11βHSD2, cortisol may also elicit unfavorable effects. Indeed experimental evidence exists that confirms this hypothesis. [42] In isolated rat hearts, Mihailidou *et al* have shown that both aldosterone and cortisol increased infarct size, an effect which was blocked by spironolactone. [78] In contrast, evidence suggests that glucocorticoids can act as a MR antagonist in cells lacking 11βHSD2. The underlying mechanism is unknown, but it has been assumed that glucocorticoids prevent aldosterone-mediated transactivation, by occupying the MR but without inducing transcription. [79, 80]

GENOMIC AND NONGENOMIC EFFECTS OF MR STIMULATION

Despite the fact that glucocorticoids may also stimulate the MR, the great majority of studies have focused on aldosterone as the ligand of interest to cause cardiovascular damage. The higher risk of cardiovascular events and cardiac remodeling in patients with primary hyperaldosteronism [5-7], and the favorable effects of MR antagonists on cardiovascular mortality and morbidity [2-4], suggest a direct role of aldosterone on the different cell types within the cardiovascular system. These actions can be mediated via the MR, and in that case can be specific for aldosterone if 11βHSD2 is present, as outlined above, but it has also been suggested that there are MR-independent effects of aldosterone. Binding of the ligand to the cytosolic MR induces receptor dimerization and migration to the nucleus, followed by gene transcription, which is not a fast process and is called the 'genomic' pathway. [81] The rise in blood pressure in response to activation of the renin-angiotensin-aldosterone-system system is an example of a genomic effect of aldosterone.

Next to this relatively slow process, which may take hours, aldosterone has rapid, 'non-genomic' effects, which do not depend on transcription. Figure 3 provides a schematic overview of the genomic and non-genomic effects of aldosterone. The extra-nuclear 'non-genomic' signaling effects occur within minutes and can be provoked by binding of aldosterone to the MR localized in the cell membrane, or to other membrane receptor(s). Arguments that support non-genomic effects of aldosterone via a membrane receptor or membrane MR are, next to rapid response, that effects are insensitive to transcription inhibitors and that albumin-complexed aldosterone, that cannot enter

cells, induces similar responses. [49] A membrane-associated aldosterone receptor distinct from the MR has not yet been identified, although promising candidates have been proposed. [82] It is estimated that about 50% of the rapid effects of aldosterone occur without involvement of the MR [82], because various non-genomic effects of aldosterone have been described that cannot be blocked by MR antagonists. [42, 83] This does not, however, hold true for all studies, as shown in Table 2. Therefore, non-genomic effects of aldosterone can also be exerted via the classical MR. Glucocorticoids have been shown to mimic some of the non-genomic effects of aldosterone, although less potent. [83]

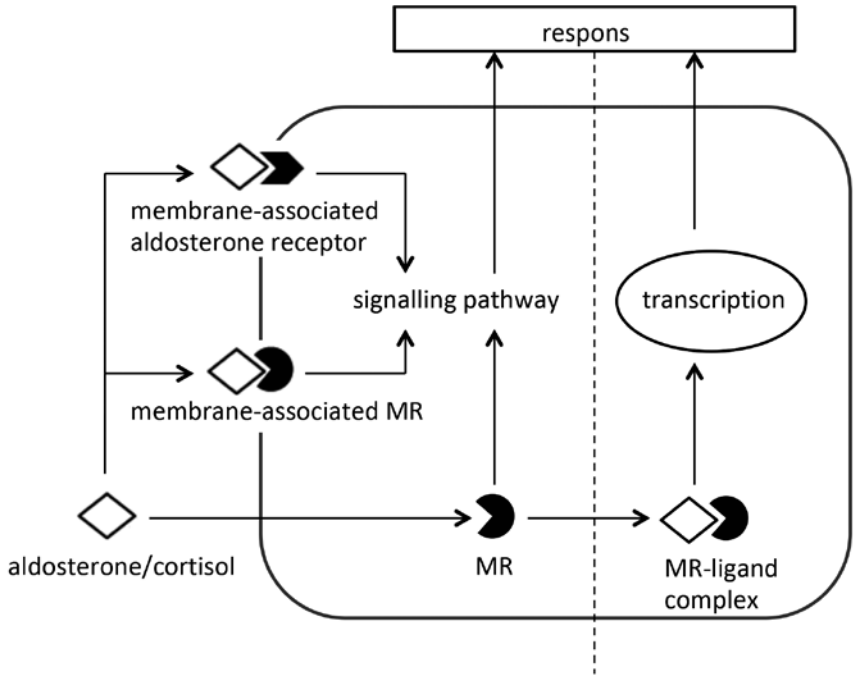


FIGURE 3. Schematic overview of the genomic (right) and non-genomic (left) effects of aldosterone

Very recently, Toda *et al* reviewed the effects of short-term as opposed to chronic exposure to aldosterone on endothelial function and vascular tone in humans, animals and isolated cells. [84] In healthy volunteers, there is a great inconsistency in the acute, non-genomic, effects of aldosterone infusion on endothelial function. Schmidt *et al* proposed that the effect (vasoconstriction, vasodilation or no effect) depends on the dosage of aldosterone infusion, with a vasodilator effect at high levels of aldosterone and a vasoconstrictive effect at low dosages. [85] In animal studies, short-term exposure to aldosterone most often led to vasodilation, mediated by endothelium-derived nitric oxide. After persistent administration of aldosterone to healthy subjects, a decrease in nitric oxide and an impairment of endothelial function was seen. When aldosterone was given into the coronary artery of hypoperfused dogs *in vivo* over 60 minutes, coronary blood flow was reduced and protein kinase C (PKC) was activated. The effect on CBF was blunted by a PKC inhibitor, and not by spironolactone. [86]

Other non-genomic effects of aldosterone were demonstrated in ventricular myocytes of rabbits and rats, in which aldosterone increased intracellular sodium concentrations via the $Na^+/K^+/2Cl$ co-transporter and the Na^+/H^+ exchanger [87] and caused cell swelling. [88] Also in endothelial cells, aldosterone led to an increase in absolute cell volume via the Na^+/H^+ exchanger. [89] In an *in vitro* study in cardiomyocytes of rats, aldosterone increased the percentage of apoptosis through calcineurin-dependent pathways.[90] In porcine coronary vascular smooth muscle cells (VSMCs), incubation with aldosterone led to increased levels of intracellular cyclic adenosine monophosphate. Since this effect was not blocked by the MR antagonist spironolactone, nor by inhibitors of transcription factors and protein synthesis, this effect of aldosterone on cyclic adenosine monophosphate appears to be non-genomic and independent of the MR. Aldosterone, together with isoproterenol, had synergistic effects on this phosphorylation.[91] In subsequent research, the influence of the adrenergic state on the effects of aldosterone was tested in healthy subjects. After pretreatment with the beta-blocking agent esmolol, aldosterone increased mean arterial pressure within minutes. After pretreatment with the beta-agonist, dobutamine, the mean arterial pressure was decreased by aldosterone. [92] Blunting of the human baroreceptor sensitivity by aldosterone supports the idea of an interaction between the adrenergic system and aldosterone. This effect of aldosterone occurred within a short time frame and pretreatment with canrenoate

could not prevent the effects of aldosterone on the baroreceptor sensitivity. Therefore, non-genomic effects, independent of the MR, were presumed to be responsible for this mechanism. [93] In VSMCs of rats, aldosterone has been shown to have a profibrotic role via c-Src (tyrosine kinase) pathways [94], to cause hypertrophy by upregulation of NADPH oxidase 1 via activation of Src and activating transcription factor 1 [95], to stimulate proliferation via big mitogen-activated protein kinase 1 [96], and aldosterone has been shown to increase intracellular sodium concentrations MR-independently [97]. Furthermore, aldosterone increased intracellular calcium concentrations in VSMCs, with involvement of phospholipase C and PKC. [83, 98] An increase in calcium concentration was also seen in porcine endothelial cells. [98, 99]

In conclusion, the 'classic' intracellular MR is present in many cardiovascular tissues, and for this reason it may play a role in cardiovascular (patho)physiological situations. Stimulation of the MR in these tissues may not always be due to aldosterone, as it may also be due to cortisol, depending on whether or not 11βHSD2 is co-expressed with the MR. To complicate matters further, membrane receptor(s) for aldosterone and membrane-associated MR may also exist in these tissues, which may explain the various rapid intracellular responses to aldosterone. These 'non-genomic' rapid actions are often, but not always blocked by MR antagonists.

We now turn to the effects of MR antagonists on IR injury and post-infarction cardiac remodeling. It appears that most of these actions are non-genomic, demonstrating that extrarenal MR physiology substantially differs from renal tubular MR physiology.

EFFECTS OF MR ANTAGONISTS ON IR INJURY

The effects of MR antagonists on myocardial IR injury have been studied in several animal models (rats, mice, and rabbits) of myocardial infarction, both *ex vivo* and *in vivo*. In animal *in vivo* studies of permanent coronary occlusion, the administration of eplerenone and spironolactone, either started before occlusion or immediately after occlusion, respectively, did not affect myocardial infarct size, suggesting that reperfusion is essential for infarct size-limitation by MR antagonists. [100-112] In Langendorff isolated perfused rat hearts, the administration of MR antagonists, commencing before coronary occlusion, consistently reduced myocardial infarct size (Table 2). Recently, it appeared that eplerenone not only confers cardioprotection when administrated before the induction of ischemia, but also when administration at the moment of reperfusion. [113]

The dosages of the MR antagonist used in the isolated perfused heart studies varied among the studies (Table 2). Loan Le *et al* studied the dose-effect relationship of MR antagonists on infarct size and found spirinolactone 10 nM and eplerenone 100 nM to be the lowest concentrations to significantly reduce infarct size. [114] In other studies, a 10-fold higher dose of spironolactone [115] and 10-fold and 100-fold higher dose of eplerenone [80] also reduced infarct size [113]. It has to be realized, however, that in this *ex vivo* infarction model, the endogenous ligands of the MR aldosterone and glucocorticoids are not present. Therefore, the results of these experiments do not necessarily predict the effects of MR antagonists *in vivo*.

Schmidt *et al* have studied the effects of the MR antagonist potassium canrenoate, which can be administered intravenously, on myocardial infarct size in rats, mice and rabbits *in vivo*. In mice, potassium canrenoate was given in incremental dosages as a bolus five minutes before coronary reperfusion to study dose-dependent effects on infarct size. Canrenoate in a dosage of 0.03 mg/kg significantly reduced infarct size, with a maximum effect at 1 mg/kg. Also, in a rabbit model of myocardial infarction *in vivo*, an intravenous bolus of 1 mg/kg canrenoate reduced infarct size, an effect that persisted after 72 hours of reperfusion. [113]

TABLE 2. Schematic overview of the effects of aldosterone and MR antagonists on IR injury, derived from animal studies

Reference	Animal Species and experimental design	Intervention	Effect on IR injury	Mechanistic insight
MYOCARDIAL ISCHEMIA				
Rochetaing 2003 [166]	Rat Langendorff, 25' of global low flow I, 30' R	Sp (50 mg/kg orally) for 1 month before surgery	Sp improved contractile recovery and reduced ventricular tachycardia during reperfusion	Sp increased reactive hyperemia
Fujita 2005 [86]	Dog Low flow (CPP 33% of control CBF)	Aldo (0.1 nM)	Aldo: ↓ CBF in ischemic heart (37.4±3.8 to 19.3±5.2 mL/100 g/min), further reduced FS and increased lactate extraction	Aldo might worsen IR-injury by ↓ CBF ↓ CBF and FS abolished by PKC inhibitor GF109203x, but not by spironolactone
Chai 2005 [115]	Rat Langendorff LAD occlusion 45' I, 3h R	Perfusion with Sp (100 nM), or Aldo (100 nM) starting 15' before I	Sp: IS ↓ 68 ± 2 to 45±3% and ↓ fibrillation Aldo: IS = 68 ± 2 to 71±5%	In hearts without IR Aldo increased LVP and decreased CBF; Sp alone did not affect these parameters, but blocked LVP increase by Aldo
Chai 2006 [80]	Rat Langendorff LAD occlusion 45'I, 3h R	Perfusion with Aldo (100 nM), Epl (1 μM) or combined Aldo+Epl starting 15'before I	Epl: IS ↓ 68±2 to 53±4% Aldo: IS = Epl + Aldo: IS ↓ (comparable to Epl alone)	In heart without IR, Aldo and Epl increased LVP, Aldo reduced CF. Epl did not block Aldo actions. Epl enhanced LVP recovery after IR, Aldo did not affect LVP and CF after IR.
Takeda [112]	Rat Permanent LAD occlusion	Sp (100 mg/kg/d orally) starting immediately after I, for 14 d	IS = (Massons's trichrome staining) Sp ↓ LVEDP, ↑LV dP/dT, ↓ apoptosis (3.5% vs 5.8% in controls)	Sp suppressed expression of MR and 11βHSD2 mRNA, which were upregulated in untreated MI group
Barbato 2002 [167]	Rat Langendorff	Perfusion with Aldo (10 nM), Sp (10 nM), or Aldo plus Sp (10 and 1000 nM resp)	Aldo: ↑ contractility (by 45%) in 2-4 min Sp: ↑ contractility (by 41%) in 2-4 min	Addition of Sp to Aldo: ↑ contractility (106%) instead of blocking Aldo effect
Mulder 2008 [168]	Rat Coronary artery occlusion during 3 weeks	Sp (80 mg/kd/d) or FAD286 (4 mg/kg/d) starting 8 d I for 7 or 90 d	Sp and FAD286 improved CO and reduced total peripheral resistance after 7 d	Sp and FAD286 reduced myocardial ROS concentration after 7 days FAD286 prevented decrease in myocardial AT_1 and AT_2 receptor, and ACE-2 expression
Mihailidou 2009 [78]	Rat Langendorff LAD occlusion 30' I, 2.5h R	Sp (10 nM, 1 μM), Aldo (1, 10 , 100 nM) starting before I	Aldo dose-dependently ↑ IS, prevented by Sp Sp ↓ IS	Cortisol also ↑ IS, blocked by Sp ↑ IS by Aldo and cortisol abolished by radical scavenger Tempol Aldo and cortisol ↑ apoptosis Sp ↓ apoptosis Sp also ↓ IS in adrenalectomized rats
Schmidt 2011 [113]	In situ mouse hearts LAD occlusion 30'I, 2h R	Can (0.003, 0.03, 0.3, 1, 10 mg/kg) iv bolus 5'before R	↓ IS, dose-dependent (37.8±7.5% to max 7.3±4.7 (1 mg/kg)) and ↓ troponin I release	Protection abolished in CD73 -/- mice and Ado_{2B} receptor -/- mice
	In situ rabbit hearts LAD occlusion 30'I 4h/72h R	Can (1 mg/kg) iv bolus 5'before R	↓ IS up to 72h	
	Rat Langendorff Coronary artery occlusion 30' I, 2h R	Epl (10 μM) in perfusate starting 5'before R, and Epl+inhibitors	Epl: ↓ IS (40.8±5.3 to 10.9±7.2%) Can: ↓ IS (40.8±5.3 to 10.2±3.8%)	Protection abolished by co-administration of 8-SPT, wortmannin, chelerythrine, and U0126 Protection abolished by co-administration of Aldo (500 nM) ↑ phosphorylation of Akt and ERK1/2 by MR blockade at reperfusion
Loan Le 2012 [114]	Rat Langendorff LAD occlusion 30' I, 2.5h R	Sp (1, 3, 10, 1000 nM), Epl (100, 1000 nM) starting 15' before I	Sp (10, 100 nM) en Epl (100, 1000 nM) ↓ IS	↓ IS by Sp not blocked by androgen receptor antagonist flutamide Sp and Epl attenuated apoptotic index Sp and Epl prevented degradation of antiapoptotic protein ARC In vitro: MR activation ↓ ARC expression, prevented by Sp

RENAL ISCHEMIA				
Mejia-Vilet 2007 [36]	Rat, bilateral renal I 20', 24h R	Sp (20 mg/kg orally) for 1, 2, or 3 days	Sp prevented fall in renal function, RBF and tubular injury (histologically, and urinary markers). Sp 2 and 3 d before I/R prevented apoptosis	Sp: ↑ eNOS phosphorylation, ↓ ROS formation, ↓ kidney lipoperoxidation,
Ramirez 2009 [124]	Rat, bilateral renal I 20', 24h R	Adx 3 days before I/R	Adx prevented renal dysfunction, tubular damage (histologically and by urinary markers), and decrease in renal plasma flow	Adx prevented IR-induced increasein oxidative stress and ↑ eNOS phosphorylation. Adx prevented increased expression of Rho-kinase and ET_A
Sanchez-Pozos 2012 [37]	Rat, bilateral renal I 20', 24h R	Sp (20 mg/kg orally) 0, 3, 6 and 9 h after I	Sp at 0 and 3h: prevented renal dysfunction, tubular damage, and decrease in RPF At 6h and 9h: partial and no effect resp.	Sp prevented increase in oxidative stress, and prevented upregulation of Rho kinase, endothelin, ET_A receptor, and AT1 receptor upregulation
CEREBRAL ISCHEMIA				
Dorrance 2001 [39]	Stroke-prone spontaneously hypertensive rats Permanent occlusion MCA for 6 h	Sp (3.3 mg/d orally) for 6 weeks	↓ IS (51.69±3.60 to 22.00±6.69%) after 6 h of occlusion	Sp: ↓ EGFR mRNA in aorta (no effect on EGF mRNA levels)
Iwanami 2009 [38]	Permanent occlusion left MCA	Epl (± 150 mg/kg/d orally) for 2 weeks (pretreatment)	↓ infarct volume (72.7 to 46.9 mm^3) after 24 h of occlusion	Epl: ↓ ROS and improvement in cerebral blood flow in penumbra
Frieler 2011 [125]	Myeloid specific MR -/- mice MCA occlusion 90' I, 24h R	-	↓ IS (32 to 11%) No difference in MCA blood flow during ischemia.	↓ activated microglia/macrophages ↓ TNF-α, IL-1β, MCP-1, IL-6, MIP-1α
HIND LIMB				
Kobayashi 2010 [126]	Rat Permanent unilateral hindlimb ischemia	Epl (30 mg/kg/d) for 3 weeks after I	↑ blood perfusion ratio (ischemic/normal)	↑ neovascularization (capillary density) ↑ number, colony formation, and migration of EPGs ↑ eNOS, VEGF, Ang 1, Ang 2, and eNOS protein expression ↓ NAD9P)H oxidases and MR expression
RETINA				
Liu 2012 [40]	Rat Raising intra-ocular pressure (unilateral) 45' I, 7d R	Sp (1, 10, 100 mg/kg/d) for 7 d, starting 1 d before I	Sp 10 and 100: prevented IR-induced retinal ganglion cell death, and prevented the reduction in IPL and INL thickness (10 and 100 mg/kg dose)	-

Abbreviations: ACINUS, Apoptotic chromatin condensation inducer in the nucleus; Adx, adrenalectomy; Ado, adenosine; Aldo, aldosterone; Ang, angiotensin; AT1, angiotensin 1; Can, canrenoate; CBF, coronary blood flow; CHEL, chelerythrine (PKC inhibitor); Cort, cortisol; CPP, coronary perfusion pressure; Dexa, dexamethasone; EGF, epithelial growth factor; EGFR, epithelial growth factor recetor; eNOS, endothelial nitric oxide synthase; EPG, endothelial progenitor cell; Epl, eplerenone; ERKi, ERK inhibitor; ET_A, endothelin A; Flut, flutamide; I, ischemia; ICAD, inhibitor of caspase-activated DNAse; IL, interleukin; INL, inner nuclear layer; IPL, inner plexiform layer; iv, intravenously; LVP, left ventricular pressure; MCA, middle cerebral artery; MCP-1, monocyte chemoattractant protein-1; Mife, mifepristone; MIP-1α, macrophage inflammatory protein-1α; R, reperfusion; RBF, renal blood flow; ROS, reactive oxygen species; sc, subcutaneously; Sp, spironolactone; SPT, 8-p-sulphophenyltheophylline (adenosine receptor blocker); TNF-α, tumor necrosis factor-α; WORT, wortmannin (PI3-kinase inhibitor)

MECHANISMS OF PROTECTION

There are several important questions which relate to the underlying mechanism of MR antagonist-induced cardioprotection. First, are the protective effects due to antagonism of endogenous ligands of the MR, including aldosterone and cortisol, or due to inverse agonism of the MR. Secondly, does the cardioprotective effect involve a classical 'genomic', or a 'nongenomic' effect? Thirdly, what is the intracellular pathway that is activated upon binding of the MR antagonist to the MR? These questions will be discussed in this paragraph.

The first question to be answered is whether endogenous aldosterone can aggravate myocardial IR-injury. Several Langendorff studies investigated the effect of administration of exogenous aldosterone on infarct size, but these studies reported inconsistent results. Chai *et al* showed that perfusion with 100 nM of aldosterone, commenced before coronary occlusion, did not affect infarct size. [80, 115] In the study by Schmidt *et al*, perfusion with 500 nM of aldosterone, starting immediately before reperfusion, did not affect infarct size either. [113] In contrast, Mihailidou *et al* reported a dose-dependent increase in infarct size with 1, 10, and 100 nM of aldosterone, administered before coronary occlusion. [78] These results, however, are not relevant to the potential effects of endogenous aldosterone. It has to be realized that *ex vivo* Langendorff perfused hearts are not exposed to circulating endogenous aldosterone. Therefore, the observation that MR antagonists confer cardioprotection in this model, suggests that the effect of MR antagonists is independent from the binding of endogenous aldosterone. It has been suggested previously that aldosterone is produced locally in the heart during ischemia [116], but more recent studies have provided convincing evidence against such a local cardiac aldosterone synthesis. [117, 118] Indeed, Chai *et al* showed in a pharmacokinetic study in Langendorff perfused rat hearts that cardiac aldosterone was derived from uptake of circulating aldosterone. During wash-out, aldosterone disappeared within minutes and during buffer perfusion there was no aldosterone detectable anymore in cardiac tissue. [80] A more definitive answer to the question whether endogenous aldosterone is required for the cardioprotective effects of MR antagonists was provided by the elegant study by Mihailidou *et al*, who studied the effect of spironolactone in rats after adrenalectomy. [78] Also in isolated perfused hearts from these rats, administration of spironolactone significantly

reduced myocardial infarct size. Surprisingly, adrenalectomy did not protect against IR injury, which might be expected since administration of exogenous aldosterone and glucocorticoids increased infarct size in this study. A possible explanation is that the administered dosages of aldosterone and cortisol exceed physiological levels, although the concentration of aldosterone reflects normal levels. [87] These observations suggest that spironolactone acts as an inverse agonist at the MR. Indeed, previous in vitro studies support this concept of inverse agonism by spironolactone. [119]
Finally, the role of glucocorticoids has been explored in the study by Mihailidou *et al*. [78] This is highly relevant, since, as discussed in paragraph 2, MRs have equal affinity for aldosterone, cortisol, and corticosterone, circulating levels of glucocorticoids are >100-fold higher than circulation aldosterone, and cardiomyocytes lack expression of 11βHSD2 (Table 1). It has been suggested, however, that in cardiomyocytes, endogenous glucocorticoids do not act as MR agonists, but rather MR antagonists. In the study by Mihailidou *et al* perfusion with cortisol also increased infarct size, which was prevented by spironolactone [78]. It was suggested that cortisol acted as MR agonist in the situation of ischemia and reperfusion because increased oxidative stress drives MR activation by glucocorticoids. The fact, however, that adrenalectomy did not abolish the infarct size-limiting effect of spironolactone excludes that endogenous glucocorticoids are involved in MR antagonist-induced cardioprotection.

Does the cardioprotective effect of MR antagonists involve a genomic or non-genomic mechanism? The observation that the administration of eplerenone or canrenoate immediately before reperfusion limits infarct size, measured two hours after reperfusion, suggests a rapid non-genomic effect. [113] Additional evidence in favor of a nongenomic effect is that the cardioprotective effect of canrenoate was abolished when its administration was delayed and given more than one minute after the onset of reperfusion. [113]

Several studies have tried to elucidate the underlying mechanism of MR antagonist-induced cardioprotection (Table 2). Limitation of IR-induced apoptosis plays a pivotal role in the cardioprotective effect of these drugs. In rat Langendorff experiments, the administration of spironolactone or eplerenone significantly reduced the number of apoptotic cells after IR. [78, 114] Also after permanent coronary ligation, spironolactone

reduced the occurrence of apoptosis. [112] These effects might represent antagonism of a pro-apoptotic effect of endogenous aldosterone. Indeed, the administration of aldosterone or cortisol increased the occurrence of apoptosis in isolated rat hearts [78], and in rat neonatal cardiac myocytes, aldosterone dose-dependently induced apoptosis and enhanced mitochondrial permeability transition pore opening [90]. The direct pro-apoptotic effects of aldosterone in isolated cardiomyocytes were attenuated by concomitant administration of MR antagonists. [120, 121] It has recently been reported that MR antagonists limit IR-induced apoptosis by preventing degradation of the anti-apoptotic protein apoptosis repressor with caspase recruitment domain (ARC) during IR. [114] Additional *in vitro* studies, have shown that MR activation by aldosterone promotes ARC degradation, which is blocked by spironolactone, demonstrating that this mechanism is mediated via the MR. [114]

In an elegant series of experiments in rat Langendorff-perfused hearts, Schmidt *et al* have studied whether MR antagonist-induced cardioprotection is mediated by similar pathways that are activated by ischemia pre- and postconditioning, which have been described in detail in previous reviews [122, 123]. [113] Indeed, adenosine receptor stimulation, and activation of the reperfusion injury salvage (RISK)-pathway appeared to be critical for MR-antagonist-induced cardioprotection. In a mouse model of myocardial infarction, the protective effect of the canrenoate was completely abolished in CD73 (the enzyme catalyzing extracellular conversion of AMP into adenosine) as well as adenosine A_{2b} receptor knock-out mice. In addition, the nonspecific adenosine receptor antagonist 8-p-sulphophenyltheophylline completely abolished the protective effect of eplerenone in isolated perfused rat hearts. In the same series of experiments, the cardioprotective effect of eplerenone was abolished by co-administration of the PKC inhibitor chelerythrine, the extracellular signal-regulated kinase inhibitor U0126, and the phosphoinositide 3-kinase inhibitor wortmannin. In addition, canrenoate and eplerenone increased phosphorylation of the extracellular signal-regulated kinase 1/2, and Akt, which was prevented by wortmannin. [113]

The effect of MR antagonists on IR-injury has not only been investigated in the heart, but also in the kidney, the brain, and the retina. These studies have revealed alternative pathways that can also contribute to the protective effects of these drugs. In a series of

experiments, Bobadilla's group has explored the effects of spironolactone on renal IR-injury (Table 2). Transient bilateral renal ischemia induced a rapid increase in circulating aldosterone, a reduction in renal plasma flow, severe tubular damage, and a decreased creatinine clearance. Pretreatment with spironolactone for one day completely prevented these changes. [36] In addition, adrenalectomy, performed three days before renal ischemia, similarly prevented IR-injury [124], suggesting that the beneficial effects of spironolactone are due to prevention of MR stimulation. Interestingly, the administration of spironolactone immediately or three hours after renal reperfusion also prevented tubular damage. [37] This is in contrast to previous myocardial models of IR-injury, where the protective effect of canrenoate was lost when its administration was delayed for more than one minute after reperfusion [113], illustrating that the infarct-size limiting effect of the heart probably differs from the renoprotective effect. The underlying mechanism of renoprotection appears to be prevention of the reduction of renal plasma flow by increased eNOS expression [36, 124], and prevention of up-regulation of the vasoconstrictor substances Rho-kinase and the endothelin ET_A receptor [37, 124], and further prevention of tubular injury by limiting oxidative stress [37].

Also in mouse models of stroke, pretreatment with MR antagonists have proven to be beneficial. [38, 39, 125] In two mouse models of permanent occlusion of the middle cerebral artery, pretreatment with spironolactone (6 weeks) or eplerenone (2 weeks) reduced cerebral infarct size. [38, 39] It was suggested that this is mediated by a beneficial effect of these drugs on the cerebral vessels, which would increase collateral blood flow to the penumbra. Indeed, administration of eplerenone increased blood flow in the peripheral region of the middle cerebral artery territory. [38] Therefore, the smaller infarct size in these studies could be mediated by a reduced ischemic burden, rather than increased cerebral intrinsic tolerance against ischemia and reperfusion. This increased blood flow was not observed in the study by Frieler *et al*, which measured cerebral infarct size after transient (90 min) middle cerebral artery occlusion in myeloid-specific MR knock-out mice. Infarct size appeared to be significantly reduced compared to wild type mice, suggesting that the MR receptor on monocytes and macrophages importantly modulates IR injury. In myeloid specific MR knock-out mice, there was a reduction in activated microglia and macrophages in the ischemic core, and a reduction in mRNA expression of pro-inflammatory cytokines including, TNF-α, IL-1β, MCP1, and

IL-6. [125] In retinal IR injury, spironolactone prevented a decrease in inner layer thickness and in the number of retinal ganglion cells. Aldosterone decreased the number of retinal ganglion cells in rat eyes exposed to retinal IR, but did not affect retinal thickness. [40] Finally, after unilateral hind limb ischemia in rats, eplerenone improved proliferation and function of endothelial progenitor cells. [126]

In conclusion, in animal studies in which the heart is subjected to IR, MR antagonists limit infarct size, when administered either before ischemia, or immediately after the onset of reperfusion. The infarct-size limiting effect of MR antagonists in *ex vivo* heart models in which endogenous aldosterone is absent, and in adrenalectomized animals suggests that this cardioprotective effect is independent from the endogenous ligand aldosterone. The cardioprotective effect of the MR antagonist canrenoate was completely abolished in CD73 and adenosine receptor knock-out mice, and after concomitant administration of pharmacological blockers of the signaling pathways that are also active in ischemic pre- and postconditioning, including the RISK pathway. These results suggest that the underlying mechanism of protection of MR antagonists depends on activation of adenosine and the RISK pathway. The observation that in mice, genetic deletion of the myeloid-specific MR reduces cerebral infarct size, suggests an important role for the MR on monocytes.

EFFECTS OF MR ANTAGONISTS ON POST-INFARCTION REMODELING

Cardiac remodeling can be defined as the process of cellular and interstitial changes in the myocardium with subsequent changes in morphology (hypertrophy), structure (chamber dilatation) and function (impairment) of the heart resulting from altered loading conditions and/or cardiac injury. Cardiac remodeling plays a critical role in the development of heart failure. Therefore, interventions that prevent excessive remodeling will prevent heart failure and improve survival. In this section, we focus on the effect of MR antagonists on cardiac remodeling after myocardial infarction. Infarct size is an important predictor of cardiac remodeling. [127, 128] MR antagonists can reduce infarct size in experimental settings as described in the previous paragraph.

In the current section, we aim to describe the effect of MR antagonists on the remodeling process itself and subsequent development of heart failure. In this context, the effect of MR antagonists on infarct size can be regarded as a confounder that should be taken into account when interpreting the studies that describe the effect of these drugs on cardiac remodeling. MR antagonists may not only affect remodeling, which typically occurs in the non-infarcted part of the heart, but may also affect repair and scar formation in the infarcted area. This potential action of MR antagonists could indirectly interfere with remodeling by affecting the loading conditions of the non-infarcted areas of the heart. Therefore, we will start with a brief description of the effect of MR antagonists on repair and scar formation in the infarcted area.

EFFECT OF MR ANTAGONISTS ON REPAIR AND SCAR FORMATION IN THE INFARCTED AREA IN ANIMAL STUDIES

Myocardial infarction results in a substantial and acute loss of myocardial cells as well as disruption of the microcirculation. Cell necrosis triggers an inflammatory response and invasion of inflammatory cells. Early invasion of inflammatory cells may contribute to reperfusion injury. However, approximately 7 days after coronary occlusion, infiltration of bone marrow derived dendritic cells in the infarct zone occurs and mediate differentiation of macrophages to the M2 phenotype which play an essential role in the orchestration of the repair phase and scar formation. [129] Aldosterone may modulate dendritic cell function. [130] Cardiac myofibroblasts (cells with characteristics of fibroblasts and smooth muscle cells) play an important role in scar formation and contraction. [131] Aldosterone may stimulate proliferation of these cells. [132]

In cardiomyocyte-specific MR knock-out mice, infarct healing was improved with increased infiltration of macrophages, enhanced infarct neovessel formation and collagen structural organization associated with reduced infarct expansion. [133] The authors claimed that MR signaling in cardiomyocytes hinders normal scar formation by reducing early macrophage-infiltration and subsequent scar formation. [133] Oral treatment of Wistar rats with high doses of eplerenone (100 mg/kg/day), starting immediately after coronary occlusion increased macrophage infiltration in the infarct zone and reduced subsequent thinning and dilation of the infarct zone, mimicking the observations in cardiomyocyte MR knock-out mice. This benefit of eplerenone

on infarct healing was not observed when treatment was initiated 3 days after start of the coronary occlusion. [101] However, in a study with Sprague Dawley rats, oral high dose eplerenone (150 mg/kg/day) was started 24 hours after coronary artery occlusion and did not affect infarct healing. [100] These limited preclinical data suggest that MR antagonists improves scar healing only when introduced immediately after reperfusion. However, the role of the MR in this effect of eplerenone has not yet been established nor is known whether clinically relevant doses have a similar effect. It is currently not known how MR antagonists affect angiogenesis, cardiac myofibroblasts, collagen formation or infiltration by bone marrow-derived progenitor cells in the infarct zone.

3.1 EFFECT OF MR ANTAGONISTS ON CARDIAC REMODELING IN THE NON-INFARCTED AREA IN ANIMAL STUDIES

Loss of contractile myocardial tissue immediately alters the loading conditions of the remaining functional myocardium, with increased wall stress and myocardial cell stretch. These alterations result in release of cytokines (including TNF-α, IL-1 and IL-6) and neurohormones (including the renin-angiotensin-aldosterone system), catecholamines and other transmitters of the sympathetic nervous system, endothelin-1, vasopressin). Acting in concert, these hemodynamic and neurohumoral factors trigger a remodeling process in the non-infarcted myocardium. This remodeling process involves an altered gene program, hypertrophy, apoptosis of myocardial cells, increased reactive oxygen species signaling and oxidative stress, mitochondrial dysfunction, altered intracellular calcium handling and changes in the extracellular matrix. Starting as an adaptive hypertrophic response, ongoing myocardial cell loss, fibrosis and metabolic derangements result in progressive myocardial dysfunction, ventricular dilatation and reduced ejection fraction, well known as the clinical syndrome of heart failure (reviewed elsewhere: [134, 135]).

Several lines of evidence point to a role for aldosterone and its receptor in cardiac remodeling in response to altered cardiac loading conditions:
First, plasma aldosterone levels are increased in patients who present with a myocardial infarction and the plasma aldosterone concentration at presentation of acute myocardial infarction is associated with prognosis including the occurrence of heart failure. [136] Likewise, in animal models of acute myocardial infarction, plasma aldosterone levels are increased. [101, 103]

Secondly, patients with primary hyperaldosteronism may develop cardiac hypertrophy in excess to what is expected from their increase in blood pressure [7, 137] and this hypertrophy is reversed by adrenalectomy. [138] However, the independence from blood pressure has been challenged by others. [139, 140] Furthermore, in studies in transgenic mice with genetically determined hyperaldosteronism, cardiac remodeling is only observed when hyperaldosteronism is combined with salt-induced hypertension [141], although the role of hypertension in the effect of salt intake in this model has recently been challenged by the same authors [142].

Another important point is that against a background of hypertension induced by increased salt intake, nephrectomy and nitric oxide-deficiency, which induced hypertension with normal aldosterone levels, wild type but not macrophage MR knock-out mice developed cardiac fibrosis, suggesting an important role for the MR in cardiac remodeling. [143] In contrast and to complicate matters, in mice with selective cardiomyocyte knock-down of the MR (using anti-sense mRNA) cardiac fibrosis and heart failure was induced in the absence of hypertension or chronic hyperaldosteronism.[144] As for aldosterone, these results suggest an essential modulating role of altered cardiac loading conditions (such as systemic blood pressure) in MR-related development of cardiac remodeling, although differences in target cells may play a role as well.

And lastly, after myocardial infarction, cardiomyocyte-specific MR knock-out mice show less hypertrophy, fibrosis, apoptosis and mitochondrial dysfunction in non-infarcted myocardium after coronary occlusion [133] as compared with non-engineered controls. However, in these experiments, the MR knock-out mice also showed improved infarct healing. Therefore, it is not clear whether the observations in the non-infarcted area occurred independent from reduced changes in loading conditions and neurohumoral activation after myocardial infarction due to improved infarct healing.

Based on these observational data in humans and experimental evidence in animals on the role of aldosterone and MR in cardiac remodeling, one might hypothesize that aldosterone receptor antagonists should reduce maladaptive cardiac remodeling of the non-infarcted myocardium independent from its potential action on infarct healing. Indeed, an increasing body of evidence in both animals and humans indicate that MR antagonists reduce cardiac remodeling post MI when initiated after initial infarct healing.

TABLE 3. Schematic overview of the effects of MR antagonists on post-infarction cardiac remodeling in animal studies

Reference	Animal species and experimental design	Intervention	Effect on cardiac remodeling	Mechanistic insight
Delyani 2001 [100]	Rat LCA ligation	Epl (150 mg/kg bid orally) for 3, 7, or 28 d, starting 24 h after MI	No effect on infarct healing ↓ fibrosis in viable myocardium	-
Suzuki 2002 [146]	Dog Intracoronary microembolizations	Epl (10 mg/kg bid orally) for 3 months, starting 2 weeks after last embolization	↓ LVEDV, LVESV, LVEF ↓ MCSA ↓ reactive fibrosis ↓ replacement fibrosis	-
Mill 2003 [169]	Rat LCA ligation	Sp (20 mg/kg orally) for 1 month, starting immediately after MI	Infarct size unaffected (by study design) ↓ collagen content No effect on ventricular hypertrophy.	Effect unrelated to hemodynamic adjustments
Fraccarollo 2003 [145]	Rat LCA ligation	Epl (100 mg/kg/d orally) for 9 weeks, starting from the 10th day post-MI	↓ LVEDP, and LVEDV collagen content in non-infarcted myocardium almost prevented	↓ β-MHC and ANP expression ↓ collagen I gene expression ↓ Ca-ATPase down regulation in non-infarcted area. Additive to ACEi.
Wang 2004 [147]	Mouse LCA ligation	Epl (200 mg/kg/d orally) for 12 weeks, starting 2 weeks after MI	↓ LV weight ↑LVEF, CO, and ↓ LVA ↓ interstitial collagen ↓ MCSA	Further improvement of EF, LVA, LV weight, and RV weight when combined with ACEi.
Matsumoto 2004 [105]	Rat LCA ligation	Sp (100 mg/kg/d orally), Can (10 mg/kg/d orally), or combined for 4 weeks, starting after MI	↓ LVEDP, LVEDV, LVDd, and E/A ratio Can: ↓ LV weight, ↑ LVEF	↓ transcriptional activity of AP-1 and NF-κB ↓ mRNA expression of ANP, BNP, collagen I and III in non-infarcted myocard Effects additive to ACEi.
Perrier 2004 [31]	Rat ventricular myocytes isolated 1 and 3 weeks after LCA ligation	RU28318 (50 µg/h s.c., starting after MI	Prevents electric remodeling: ↓ C_m and APD; ↑ slope conductance	RU28318 blunted $Ca_v1.2$ and $K_v4.2$ mRNA alterations
Masson 2004 [106]	Forty rat LAD ligation	Epl (average 120 mg/kg/d orally) for 3 months, starting 18 d post-surgery	Restoration of diastolic function (peak E velocity, E wave deceleration time, and IVRT)	↓ myocardial interstitial collagen and ↓ aortic fibrosis
Lal 2004 [107]	Rat LCA ligation	Sp (80 mg/kg/d orally) or Sp (100 ng/hr icv), for 6 weeks starting 1 and 3 (icv) d after MI	↑ LV dP/dt_{max} and ↓ LVEDP ↓ RV weight, and prevention increase in LV and RV internal circumference ↓ interstitial fibrosis Sp icv: ↑ LVPSP	↓ cardiomyocyte hypertrophy and Attenuation of laminin and fibronectin accumulation in septal and peri-infarct zones of LV Authors suggest contribution of CNS pathways (however, no plasma drug levels were reported)
Huang 2005 [108]	Rat LCA ligation	Sp (400 ng/kg/hr icv) for 4 weeks, starting 3 d after MI	↑ LVPSP, LV dP/dt_{max} and ↓ LVEDP	Prevention of sympathetic hyperactivity and impairment of baroreflex function
Enomoto 2005 [109]	Rat LCA ligation	Epl (100 mg/kg/d orally) for 4 weeks, starting after MI	↓ LVEDP, LVEDd, LVEDV and LV weight ↑ LVEF and ↓ E:A ratio Attenuation cardiomyocyte hypertrophy and interstitial fibrosis	↓ transcription activity of AP-1 and NF-κB; ↓ mRNA expression of ANP, BNP, collagen I and III, PAI-1, and MCP-1 in non-infarcted myocard

Fraccarollo 2005 [102]	Rat LCA ligation	Epl (100 mg/kg/d orally) for 9 weeks, starting from the 10th day post-MI	↓ LVEDP, LVESV, and LVEDV	↓ AT_1-receptor and ACE up-regulation, ↓ endothelin-1 expression in non-infarcted myocardium Attenuation of collagen I and –III expression
Nehme 2005 [170]	Rat LCA ligation	Sp (10 mg/kg/d orally) for 30 d, starting 3 months after MI	No significant effect on LVEDP and heart weight ↑ Einc of carotid artery	↓ LVEDP after treatment with Sp and lisinopril (1 mg/kd/d) ↓ collagen density in carotid media
Milliez 2005 [110]	Rat LCA ligation	Sp (10 mg/kg/d) for 30 d, starting 3 months after MI	↓ atrial and LV fibrosis ↓ P-wave duration No significant effect on LVEDP after sp or lisinopril alone	↓ LVEDP after treatment with Sp and lisinopril (1 mg/kd/d)
Kang 2006 [103]	Rat LAD ligation	Epl (30 mg/kg/d orally) for 6 weeks, starting 1 d after MI	↓ LVEDP, RV weight ↑ survival No effect on LVEF, LVEDV, and LVEDV/M	↓ plasma norepinephrine, TNA-α, IL-1β, IL-6 ↓ COX-2 staining in small blood vessels in PVN ↓ Fra-like activity, TNF-α, and IL-1β in PVN neurons
Takeda 2007 [112]	Rat LCA ligation	Sp (100 mg/kg/d orally) for 2 weeks, starting after MI	↓ LVEDP, ↑ dP/dt_{max} and dP/dt_{min} ↓ collagen volume fraction	↓ MR and 11βHSD2 RNA
Fraccarollo 2008 [101]	Rat LCA ligation vs sham-operated	Epl (100 mg/kg od orally) for 2, 3, or 7 d, starting after MI	↓ thinning and dilatation of infarcted wall ↓ LV dysfunction ↑ neovascularization	↑ macrophage infiltration ↑ MCP-1, TNF-α, IL-1β, IL-6, IL-10, IL-4, and factor XIIIa protein expression
Kanashiro-Takeuchi 2009	Rat male vs female LCA ligation	Epl (100 mg/kg/d orally) for 4 weeks, starting from the 3rd d post-MI	↓LVDd and LVSd, and ↑ EF in females ↓ cardiac fibrosis in females (not in males)	Altered gene expression rather in females than in males Igf1 signalling pathway putative candidate to explain sex-specific effects of Epl
Noda 2012 [111]	Rat LCA ligation	Sp (100 mg/kg/d orally) for 4 weeks, starting 3 weeks after MI	↓ LVEDP, ↑ dP/dt_{max} and dP/dt_{min}, ↓LVDd, ↑ FS ↓ reactive fibrosis	↓ mRNA expression of ANP, BNP, collagen I and III, and ↓ lipid peroxidation, ↓NADPH oxidase-dependent and ↓ mitochondrial superoxide production in non-infarcted myocardium Addition of olmesartan (10 mg/kg/d) synergistically improved oxidative stress and cardiac function

Abbreviations: ß-MHC, ß-myosin heavy chain; ACE, angiotensin converting enzyme; ACEi, angiotensin converting enzyme inhibitor; ANP, atrial natriuretic peptide; AP-1, activator protein-1; APD, action potential duration; AT_1-receptor, angiotensin-1-receptor; BNP, brain natriuretic peptide; dP/dt_{max} and dP/dt_{min}, maximal rates of pressure rise and decline; C_m, membrane capacitance; $C_v1.2$, Ca^{2+} channel alpha1C subunit; Can, canrenoate; CO, cardiac output; Einc, incremental elastic modulus; Epl, eplerenone; FS, fractional shortening; icv, intracerebroventricular; Igf, insulin-like growth factor; IL, interleukin; $K_v4.2$, K^+ channel 4.2 subunit; IVRT, isovolumic relaxation time; LCA, left coronary artery; LVA, left ventricular systolic area; LVDd, left ventricular end-diastolic dimension; LVEDP, left ventricular end diastolic pressure; LVEDV, left ventricular end diastolic volume; LVEDV/M, left ventricular end diastolic volume-to-mass ratio; LVEF, left ventricular ejection fraction; LVESV, left ventricular end systolic volume; LVPSP, left ventricular peak systolic pressure; LVSd, left ventricular end-systolic dimension; MCP-1, monocyte chemoattractant protein-1; MCSA, myocyte cross-sectional area; MI, myocardial infarction; NF-κB, nuclear factor κB; PAI-1, plasminogen activator protein-1; PVN, paraventricular nucleus; RV, right ventricle; Sp, spironolactone; TNF-α, tumor necrosis factor-α

In animals, this hypothesis has best been tested in models of permanent coronary artery occlusion to prevent any confounding by reduced infarct size [100-111] (see previous chapter). Most studies used dosages of spironolactone or eplerenone in the range of 100 – 150 mg/kg/day (see Table 3 for an overview). When treatment with eplerenone was started from the 10th day post-infarction (to prevent effects on infarct healing), beneficial effects on LV dilation, cardiac function, fibrosis, or collagen content are observed in most studies [102, 106, 111, 145-147]. However, at dosages in the range of 10-20 mg/kg/day, benefits on cardiac function are less consistent [103, 110] suggesting that the benefit of MR-antagonists may involve non-specific actions of these drugs, thus challenging the specificity of the high doses of MR antagonists for blocking the MR. To the best of our knowledge, the effects of MR-antagonists on post MI remodeling have not been studied in MR-knock out animals to further address the role of the MR in these observations.

CURRENT CLINICAL EVIDENCE FOR THE CARDIOPROTECTIVE EFFECTS OF MR ANTAGONISTS

Only a few studies have explored the effect of early post-MI treatment with MR antagonists in humans. In a randomized open-label clinical trial, 65 patients presenting with acute myocardial infarction were treated with blockade of MRs at reperfusion. This intervention improved LVEF and LVEDV at 1 month as compared with 69 patients who did not receive an MR antagonist. [148] In the EPHESUS trial, published in 2003, the vast majority of patients were on concomitant ACE inhibitor (86%) and beta-blocker (75%) treatment, reflecting current clinical practice. EPHESUS investigated 3313 patients after acute myocardial infarction complicated by heart failure (LVEF ≤40% on echo plus pulmonary congestion on X-ray or pulmonary rales or presence of a third heart sound or diabetes). Eplerenone was initiated 3-14 days after the event at 25 mg/d and up-titrated to 50 mg/d, as tolerated. Patients on eplerenone had a 15% relative risk (RR) reduction for overall mortality (95% CI 0.75-0.96, p=0.008), a 17% RR reduction to die from a cardiovascular cause (95% CI 0.72-0.94, p=0.005) and an 11% RR reduction (95% CI 0.64-0.97, p=0.03) to die from sudden cardiac death. The mean duration of follow-up was 16 months. [3]

Although these trials support the use of MR antagonists early after presentation with myocardial infarction, the role of improved scar formation versus reduced maladaptive remodeling in the non-infarct area in this clinical benefit is not known. Hopefully, recent advances in imaging may provide more insight on the mechanism of this observed benefit in humans. [149] More trials are needed to prove additional clinical benefit of early (immediately upon reperfusion) versus delayed (after initial infarct healing) initiation of therapy with MR antagonists. Moreover, the need for prolonged treatment with MR antagonists after an acute MI with preserved cardiac function has not been explored yet.

The effects of MR-antagonists on cardiac remodeling have been studied in relatively small trials (<350 patients per study arm) in patients who already have some degree of systolic myocardial dysfunction as expressed by a reduced LVEF (on average ranging from 20 to 51%), mainly as a result of a previous myocardial infarction (see [150] for a recent meta-analysis and references). In these patients, MR antagonists improved LVEF, LVEDV and LVESV indexes, and reduced a plasma marker of collagen turnover (amino-terminal peptide of procollagen type-III). These studies used relatively low doses of 12.5-100 mg spironolactone, 25-50 mg canrenoate or 25-50 mg eplerenone per day. MR antagonists do not only have an impact on cardiac function, but also reduce lethal arrhythmia's in patients with heart failure. In a recent meta-analysis including eight randomized trials which specifically investigated the impact of MR antagonism on sudden cardiac death in patients with an LVEF ≤45%, MR antagonists reduced odds for sudden cardiac death by 23% (95% CI 0.66-0.89, p=0.001). [151]

These beneficial effects of MR antagonists on cardiac remodeling have been substantiated by large clinical trials with clinically relevant endpoints (hospitalization and mortality) in patients with mild to severe systolic heart failure. [2, 4]
First, the RALES trial, published in 1999, enrolled 1663 patients with a history of at least one episode of New York Heart Association (NYHA) class IV heart failure within 6 months prior to randomization, a NYHA class ≥III at baseline and an ejection fraction ≤35%. Ischemic heart disease was the predominant underlying condition (54% of patients). Indeed, the relative risk for both, overall mortality (HR 0.70, 95% CI 0.60-0.82, p<0.001) and cardiovascular death (HR 0.69, 95% CI 0.58-0.82, p<0.001) was reduced by approximately one third in the spironolactone group, when compared

to placebo. [2] The main limitation of this study was the low number of patients treated with beta-blockers (≈10%) in these early days of therapy with MR antagonists.
The EMPHASIS trial, published in 2011, investigated the effect of eplerenone in 2737 patients with chronic systolic heart failure (LVEF ≤35%) and mild symptoms (NYHA class II). Ischemic heart disease was the underlying condition in 69% of patients. The RR for overall mortality was reduced by 22% (95% CI 0.64-0.95, p=0.01), while the RR for cardiovascular death was diminished by 23% (95% CI 0.62-0.96, p=0.02). (4) Reverse cardiac remodeling and an improved left ventricular function are directly attributed to treatment with MR antagonists and may explain, how this therapy improves long-term survival in a heart failure population. [150] This benefit occurs with low doses of the MR antagonist and even with optimal background pharmacotherapy with ACE inhibition or angiotensin receptor antagonism and beta blockade. [152]

In patients with diastolic dysfunction and preserved systolic left ventricular function (LVEF >50%) and NYHA class II-III, a recent small randomized double-blind placebo controlled trial observed a significant improvement of diastolic function in response to 1 year treatment with spironolactone (25 mg/day) but without any effect on exercise capacity. Larger clinical trials are needed to explore the clinical relevance of this observation for the treatment of diastolic heart failure. [153]

Based on the findings of the above mentioned randomized clinical trials, the European Society of Cardiology has updated their guidelines for the treatment of acute and chronic heart failure in 2012. [154] In particular, spironolactone or eplerenone are now recommended on top of ACE inhibitor and beta-blocker treatment for all heart failure patients with persisting symptoms (NYHA class II-IV) and a LVEF ≤35% (class I, level of evidence A). Eplerenone is recommended in patients with an acute coronary syndrome and acute heart failure in the presence of an LVEF ≤40% (class I, level of evidence B).

Preliminary results from the REMINDER trial (NCT 01176968) indicate that MR antagonist treatment might also be beneficial in patients after an acute coronary syndrome (ACS), even in the absence of heart failure. Early analyses were presented at the Conference of the American College of Cardiology in March 2013. In this study, 1012 patients with STEMI and a LVEF >40% were initiated on eplerenone or placebo

within 24 hours after the event. The hazard to hit the primary combined endpoint (comprising of time to first event of cardiovascular mortality, re-hospitalization or extended initial hospital stay due to diagnosis of heart failure, sustained ventricular tachycardia or fibrillation, LVEF ≤40% or BNP above age adjusted cut-off) fell by 43% (95% CI 0.44-0.74, $p<0.0001$) in the eplerenone group. Another study, the ALBATROSS trial (NCT 01059136), is currently recruiting patients (target: n=1600) presenting with ACS, with and without heart failure. MR antagonism will be initiated as early as possible after the event (<72 hours) and the first dose will be applied intravenously (potassium canrenoate), followed by spironolactone treatment. The primary combined endpoints consist of the 6 months rate of death, resuscitated cardiac arrest, potentially lethal ventricular arrhythmia, indication for implantable cardioversion devices and occurrence of heart failure. First results may be expected within the upcoming 12 months.

In summary, there is striking evidence in favor of MR antagonism in patients with heart failure for ischaemic and non-ischaemic causes. Further, some early promising data indicate that therapy with MR antagonists might also be extended to all patients after ACS in future.

CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, the expression of the MR on various cell types in the heart allows for direct effects of MR antagonists on ischemia-reperfusion injury and postinfarction remodeling. Administration of MR antagonists before ischemia or immediately upon reperfusion potently limit infarct size in animal models. This rapid nongenomic effect appears to be independent from the presence of the endogenous ligand aldosterone, since it also occurs in isolated heart preparations and in adrenalectomized rats. In addition to limiting infarct size, MR antagonist also appear to facilitate scar healing and reduce postinfarction remodeling. In patients with heart failure, there is strong evidence that MR antagonist improve prognosis. With interest we await further studies on the immediate administration of MR antagonists in patients with an acute myocardial infarction aiming to reduce infarct size.

REFERENCES

1. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *The New England journal of medicine*. 2007;357(11):1121-35.
2. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *The New England journal of medicine*. 1999;341(10):709-17.
3. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *The New England journal of medicine*. 2003;348(14):1309-21.
4. Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, et al. Eplerenone in patients with systolic heart failure and mild symptoms. *The New England journal of medicine*. 2011;364(1):11-21.
5. Milliez P, Girerd X, Plouin PF, Blacher J, Safar ME, Mourad JJ. Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. *Journal of the American College of Cardiology*. 2005;45(8):1243-8.
6. Stowasser M, Sharman J, Leano R, Gordon RD, Ward G, Cowley D, et al. Evidence for abnormal left ventricular structure and function in normotensive individuals with familial hyperaldosteronism type I. *The Journal of clinical endocrinology and metabolism*. 2005;90(9):5070-6.
7. Rossi GP, Sacchetto A, Pavan E, Palatini P, Graniero GR, Canali C, et al. Remodeling of the left ventricle in primary aldosteronism due to Conn's adenoma. *Circulation*. 1997;95(6):1471-8.
8. Kisaka T, Ozono R, Ishida T, Higashi Y, Oshima T, Kihara Y. Association of elevated plasma aldosterone-to-renin ratio with future cardiovascular events in patients with essential hypertension. *Journal of hypertension*. 2012;30(12):2322-30.
9. Beygui F, Collet JP, Benoliel JJ, Vignolles N, Dumaine R, Barthelemy O, et al. High plasma aldosterone levels on admission are associated with death in patients presenting with acute ST-elevation myocardial infarction. *Circulation*. 2006;114(24):2604-10.
10. Rajagopalan S, Duquaine D, King S, Pitt B, Patel P. Mineralocorticoid receptor antagonism in experimental atherosclerosis. *Circulation*. 2002;105(18):2212-6.

11. Keidar S, Hayek T, Kaplan M, Pavlotzky E, Hamoud S, Coleman R, et al. Effect of eplerenone, a selective aldosterone blocker, on blood pressure, serum and macrophage oxidative stress, and atherosclerosis in apolipoprotein E-deficient mice. *Journal of cardiovascular pharmacology.* 2003;41(6):955-63.
12. Takai S, Jin D, Muramatsu M, Kirimura K, Sakonjo H, Miyazaki M. Eplerenone inhibits atherosclerosis in nonhuman primates. *Hypertension.* 2005;46(5):1135-9.
13. Raz-Pasteur A, Gamliel-Lazarovich A, Gantman A, Coleman R, Keidar S. Mineralocorticoid receptor blockade inhibits accelerated atherosclerosis induced by a low sodium diet in apolipoprotein E-deficient mice. *Journal of the renin-angiotensin-aldosterone system : JRAAS*. 2012.
14. Deuchar GA, McLean D, Hadoke PW, Brownstein DG, Webb DJ, Mullins JJ, et al. 11beta-hydroxysteroid dehydrogenase type 2 deficiency accelerates atherogenesis and causes proinflammatory changes in the endothelium in apoe-/- mice. *Endocrinology.* 2011;152(1):236-46.
15. Suzuki J, Iwai M, Mogi M, Oshita A, Yoshii T, Higaki J, et al. Eplerenone with valsartan effectively reduces atherosclerotic lesion by attenuation of oxidative stress and inflammation. *Arteriosclerosis, thrombosis, and vascular biology.* 2006;26(4):917-21.
16. Sartorio CL, Fraccarollo D, Galuppo P, Leutke M, Ertl G, Stefanon I, et al. Mineralocorticoid receptor blockade improves vasomotor dysfunction and vascular oxidative stress early after myocardial infarction. *Hypertension.* 2007;50(5):919-25.
17. Schafer A, Vogt C, Fraccarollo D, Widder J, Flierl U, Hildemann SK, et al. Eplerenone improves vascular function and reduces platelet activation in diabetic rats. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society.* 2010;61(1):45-52.
18. Sanz-Rosa D, Oubina MP, Cediel E, De las Heras N, Aragoncillo P, Balfagon G, et al. Eplerenone reduces oxidative stress and enhances eNOS in SHR: vascular functional and structural consequences. *Antioxidants & redox signaling.* 2005;7(9-10):1294-301.
19. Kobayashi N, Hara K, Tojo A, Onozato ML, Honda T, Yoshida K, et al. Eplerenone shows renoprotective effect by reducing LOX-1-mediated adhesion molecule, PKCepsilon-MAPK-p90RSK, and Rho-kinase pathway. *Hypertension.* 2005;45(4):538-44.
20. Abiose AK, Mansoor GA, Barry M, Soucier R, Nair CK, Hager D. Effect of spironolactone on endothelial function in patients with congestive heart failure on conventional medical therapy. *The American journal of cardiology.* 2004;93(12):1564-6.
21. Joffe HV, Kwong RY, Gerhard-Herman MD, Rice C, Feldman K, Adler GK. Beneficial effects of eplerenone versus hydrochlorothiazide on coronary circulatory function in patients with diabetes mellitus. The Journal of clinical endocrinology and metabolism. 2007;92(7):2552-8.

22. Studen KB, Sebestjen M, Pfeifer M, Prezelj J. Influence of spironolactone treatment on endothelial function in non-obese women with polycystic ovary syndrome. *European journal of endocrinology / European Federation of Endocrine Societies.* 2011;164(3):389-95.
23. Syngle A, Vohra K, Kaur L, Sharma S. Effect of spironolactone on endothelial dysfunction in rheumatoid arthritis. *Scandinavian journal of rheumatology.* 2009;38(1):15-22.
24. Macdonald JE, Kennedy N, Struthers AD. Effects of spironolactone on endothelial function, vascular angiotensin converting enzyme activity, and other prognostic markers in patients with mild heart failure already taking optimal treatment. *Heart (British Cardiac Society).* 2004;90(7):765-70.
25. Farquharson CA, Struthers AD. Spironolactone increases nitric oxide bioactivity, improves endothelial vasodilator dysfunction, and suppresses vascular angiotensin I/angiotensin II conversion in patients with chronic heart failure. *Circulation.* 2000;101(6):594-7.
26. Fujimura N, Noma K, Hata T, Soga J, Hidaka T, Idei N, et al. Mineralocorticoid receptor blocker eplerenone improves endothelial function and inhibits Rho-associated kinase activity in patients with hypertension. *Clinical pharmacology and therapeutics.* 2012;91(2):289-97.
27. Rossi R, Nuzzo A, Iaccarino D, Lattanzi A, Origliani G, Monopoli DE, et al. Effects of antihypertensive treatment on endothelial function in postmenopausal hypertensive women. A significant role for aldosterone inhibition. *Journal of the renin-angiotensin-aldosterone system : JRAAS.* 2011;12(4):446-55.
28. Gao X, Peng L, Adhikari CM, Lin J, Zuo Z. Spironolactone reduced arrhythmia and maintained magnesium homeostasis in patients with congestive heart failure. *Journal of cardiac failure.* 2007;13(3):170-7.
29. Ramires FJ, Mansur A, Coelho O, Maranhao M, Gruppi CJ, Mady C, et al. Effect of spironolactone on ventricular arrhythmias in congestive heart failure secondary to idiopathic dilated or to ischemic cardiomyopathy. *The American journal of cardiology.* 2000;85(10):1207-11.
30. Barr CS, Lang CC, Hanson J, Arnott M, Kennedy N, Struthers AD. Effects of adding spironolactone to an angiotensin-converting enzyme inhibitor in chronic congestive heart failure secondary to coronary artery disease. *The American journal of cardiology.* 1995;76(17):1259-65.
31. Perrier E, Kerfant BG, Lalevee N, Bideaux P, Rossier MF, Richard S, et al. Mineralocorticoid receptor antagonism prevents the electrical remodeling that precedes cellular hypertrophy after myocardial infarction. *Circulation.* 2004;110(7):776-83.
32. Dabrowski R, Szwed H. Antiarrhythmic potential of aldosterone antagonists in atrial fibrillation. *Cardiology journal.* 2012;19(3):223-9.

33. Lammers C, Dartsch T, Brandt MC, Rottlander D, Halbach M, Peinkofer G, et al. Spironolactone prevents aldosterone induced increased duration of atrial fibrillation in rat. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology.* 2012;29(5-6):833-40.
34. Swedberg K, Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Shi H, et al. Eplerenone and atrial fibrillation in mild systolic heart failure: results from the EMPHASIS-HF (Eplerenone in Mild Patients Hospitalization And SurvIval Study in Heart Failure) study. *Journal of the American College of Cardiology.* 2012;59(18):1598-603.
35. Laszlo R, Bentz K, Konior A, Eick C, Schreiner B, Kettering K, et al. Effects of selective mineralocorticoid receptor antagonism on atrial ion currents and early ionic tachycardia-induced electrical remodelling in rabbits. *Naunyn-Schmiedeberg's archives of pharmacology.* 2010;382(4):347-56.
36. Mejia-Vilet JM, Ramirez V, Cruz C, Uribe N, Gamba G, Bobadilla NA. Renal ischemia-reperfusion injury is prevented by the mineralocorticoid receptor blocker spironolactone. *American journal of physiology Renal physiology.* 2007;293(1):F78-86.
37. Sanchez-Pozos K, Barrera-Chimal J, Garzon-Muvdi J, Perez-Villalva R, Rodriguez-Romo R, Cruz C, et al. Recovery from ischemic acute kidney injury by spironolactone administration. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association.* 2012;27(8):3160-9.
38. Iwanami J, Mogi M, Okamoto S, Gao XY, Li JM, Min LJ, et al. Pretreatment with eplerenone reduces stroke volume in mouse middle cerebral artery occlusion model. *European journal of pharmacology.* 2007;566(1-3):153-9.
39. Dorrance AM, Osborn HL, Grekin R, Webb RC. Spironolactone reduces cerebral infarct size and EGF-receptor mRNA in stroke-prone rats. *American journal of physiology Regulatory, integrative and comparative physiology.* 2001;281(3):R944-50.
40. Liu Y, Hirooka K, Nishiyama A, Lei B, Nakamura T, Itano T, et al. Activation of the aldosterone/mineralocorticoid receptor system and protective effects of mineralocorticoid receptor antagonism in retinal ischemia-reperfusion injury. Experimental eye research. 2012;96(1):116-23.
41. Odermatt A, Atanasov AG. Mineralocorticoid receptors: emerging complexity and functional diversity. *Steroids.* 2009;74(2):163-71.
42. Funder JW. Mineralocorticoid receptors: distribution and activation. *Heart failure reviews.* 2005;10(1):15-22.
43. Tomlinson JW, Walker EA, Bujalska IJ, Draper N, Lavery GG, Cooper MS, et al. 11beta-hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. *Endocrine reviews.* 2004;25(5):831-66.

44. Lombes M, Kenouch S, Souque A, Farman N, Rafestin-Oblin ME. The mineralocorticoid receptor discriminates aldosterone from glucocorticoids independently of the 11 beta-hydroxysteroid dehydrogenase. *Endocrinology.* 1994;135(3):834-40.
45. Pascual-Le Tallec L, Simone F, Viengchareun S, Meduri G, Thirman MJ, Lombes M. The elongation factor ELL (eleven-nineteen lysine-rich leukemia) is a selective coregulator for steroid receptor functions. *Molecular endocrinology (Baltimore, Md).* 2005;19(5):1158-69.
46. Farman N, Bocchi B. Mineralocorticoid selectivity: molecular and cellular aspects. *Kidney international.* 2000;57(4):1364-9.
47. Fuller PJ, Yao Y, Yang J, Young MJ. Mechanisms of ligand specificity of the mineralocorticoid receptor. *The Journal of endocrinology.* 2012;213(1):15-24.
48. Odermatt A, Kratschmar DV. Tissue-specific modulation of mineralocorticoid receptor function by 11beta-hydroxysteroid dehydrogenases: an overview. *Molecular and cellular endocrinology.* 2012;350(2):168-86.
49. Skott O, Uhrenholt TR, Schjerning J, Hansen PB, Rasmussen LE, Jensen BL. Rapid actions of aldosterone in vascular health and disease--friend or foe? *Pharmacology & therapeutics.* 2006;111(2):495-507.
50. Fuller PJ, Young MJ. Mechanisms of mineralocorticoid action. *Hypertension.* 2005;46(6):1227-35.
51. Lombes M, Oblin ME, Gasc JM, Baulieu EE, Farman N, Bonvalet JP. Immunohistochemical and biochemical evidence for a cardiovascular mineralocorticoid receptor. *Circulation research.* 1992;71(3):503-10.
52. Herman JP, Patel PD, Akil H, Watson SJ. Localization and regulation of glucocorticoid and mineralocorticoid receptor messenger RNAs in the hippocampal formation of the rat. *Molecular endocrinology (Baltimore, Md).* 1989;3(11):1886-94.
53. Seckl JR, Dickson KL, Yates C, Fink G. Distribution of glucocorticoid and mineralocorticoid receptor messenger RNA expression in human postmortem hippocampus. *Brain research.* 1991;561(2):332-7.
54. Golestaneh N, Picaud S, Mirshahi M. The mineralocorticoid receptor in rodent retina: ontogeny and molecular identity. *Molecular vision.* 2002;8:221-5.
55. Krozowski Z, Stuchbery S, White P, Monder C, Funder JW. Characterization of 11 beta-hydroxysteroid dehydrogenase gene expression: identification of multiple unique forms of messenger ribonucleic acid in the rat kidney. *Endocrinology.* 1990;127(6):3009-13.
56. Whorwood CB, Franklyn JA, Sheppard MC, Stewart PM. Tissue localization of 11 beta-hydroxysteroid dehydrogenase and its relationship to the glucocorticoid receptor. *The Journal of steroid biochemistry and molecular biology.* 1992;41(1):21-8.

57. Brown RW, Diaz R, Robson AC, Kotelevtsev YV, Mullins JJ, Kaufman MH, et al. The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. *Endocrinology*. 1996;137(2):794-7.
58. Smith RE, Krozowski ZS. 11 beta-Hydroxysteroid dehydrogenase type I enzyme in the hearts of normotensive and spontaneously hypertensive rats. *Clinical and experimental pharmacology & physiology*. 1996;23(8):642-7.
59. Roland BL, Funder JW. Localization of 11beta-hydroxysteroid dehydrogenase type 2 in rat tissues: in situ studies. *Endocrinology*. 1996;137(3):1123-8.
60. Condon J, Ricketts ML, Whorwood CB, Stewart PM. Ontogeny and sexual dimorphic expression of mouse type 2 11beta-hydroxysteroid dehydrogenase. *Molecular and cellular endocrinology*. 1997;127(2):121-8.
61. Naray-Fejes-Toth A, Fejes-Toth G. Extranuclear localization of endogenous 11beta-hydroxysteroid dehydrogenase-2 in aldosterone target cells. *Endocrinology*. 1998;139(6):2955-9.
62. Romero DG, Zhou M, Gomez-Sanchez CE. Cloning and expression of the bovine 11beta-hydroxysteroid dehydrogenase type-2. *The Journal of steroid biochemistry and molecular biology*. 2000;72(5):231-7.
63. Moore XL, Hoong I, Cole TJ. Expression of the 11beta-hydroxysteroid dehydrogenase 2 gene in the mouse. *Kidney international*. 2000;57(4):1307-12.
64. Sheppard KE, Autelitano DJ. 11Beta-hydroxysteroid dehydrogenase 1 transforms 11-dehydrocorticosterone into transcriptionally active glucocorticoid in neonatal rat heart. *Endocrinology*. 2002;143(1):198-204.
65. Brereton PS, van Driel RR, Suhaimi F, Koyama K, Dilley R, Krozowski Z. Light and electron microscopy localization of the 11beta-hydroxysteroid dehydrogenase type I enzyme in the rat. *Endocrinology*. 2001;142(4):1644-51.
66. Lombes M, Alfaidy N, Eugene E, Lessana A, Farman N, Bonvalet JP. Prerequisite for cardiac aldosterone action. Mineralocorticoid receptor and 11 beta-hydroxysteroid dehydrogenase in the human heart. *Circulation*. 1995;92(2):175-82.
67. Slight SH, Ganjam VK, Gomez-Sanchez CE, Zhou MY, Weber KT. High affinity NAD(+)-dependent 11 beta-hydroxysteroid dehydrogenase in the human heart. *Journal of molecular and cellular cardiology*. 1996;28(4):781-7.
68. Smith RE, Little PJ, Maguire JA, Stein-Oakley AN, Krozowski ZS. Vascular localization of the 11 beta-hydroxysteroid dehydrogenase type II enzyme. *Clinical and experimental pharmacology & physiology*. 1996;23(6-7):549-51.

69. Hatakeyama H, Inaba S, Miyamori I. 11beta-hydroxysteroid dehydrogenase in cultured human vascular cells. Possible role in the development of hypertension. *Hypertension.* 1999;33(5):1179-84.
70. Brem AS, Bina RB, King TC, Morris DJ. Localization of 2 11beta-OH steroid dehydrogenase isoforms in aortic endothelial cells. *Hypertension.* 1998;31(1 Pt 2):459-62.
71. Souness GW, Brem AS, Morris DJ. 11 beta-Hydroxysteroid dehydrogenase antisense affects vascular contractile response and glucocorticoid metabolism. *Steroids.* 2002;67(3-4):195-201.
72. Albiston AL, Obeyesekere VR, Smith RE, Krozowski ZS. Cloning and tissue distribution of the human 11 beta-hydroxysteroid dehydrogenase type 2 enzyme. *Molecular and cellular endocrinology.* 1994;105(2):R11-7.
73. Mazancova K, Kopecky M, Miksik I, Pacha J. 11beta-Hydroxysteroid dehydrogenase in the heart of normotensive and hypertensive rats. *The Journal of steroid biochemistry and molecular biology.* 2005;94(1-3):273-7.
74. Takeda Y. Pathophysiological roles of vascular 11beta-hydroxysteroid dehydrogenase and aldosterone. *The Journal of steroid biochemistry and molecular biology.* 2003;85(2-5):443-7.
75. Klusonova P, Rehakova L, Borchert G, Vagnerova K, Neckar J, Ergang P, et al. Chronic intermittent hypoxia induces 11beta-hydroxysteroid dehydrogenase in rat heart. *Endocrinology.* 2009;150(9):4270-7.
76. De-An P, Li L, Zhi-Yun X, Jin-Yu H, Zheng-Ming X, Min W, et al. Increased expression of mineralocorticoid receptor and 11beta-hydroxysteroid dehydrogenase type 2 in human atria during atrial fibrillation. *Clinical cardiology.* 2010;33(1):23-9.
77. Atalar F, Vural B, Ciftci C, Demirkan A, Akan G, Susleyici-Duman B, et al. 11beta-hydroxysteroid dehydrogenase type 1 gene expression is increased in ascending aorta tissue of metabolic syndrome patients with coronary artery disease. *Genetics and molecular research : GMR.* 2012;11(3):3122-32.
78. Mihailidou AS, Loan Le TY, Mardini M, Funder JW. Glucocorticoids activate cardiac mineralocorticoid receptors during experimental myocardial infarction. *Hypertension.* 2009;54(6):1306-12.
79. Gomez-Sanchez EP, Venkataraman MT, Thwaites D, Fort C. ICV infusion of corticosterone antagonizes ICV-aldosterone hypertension. *The American journal of physiology.* 1990;258(4 Pt 1):E649-53.
80. Chai W, Garrelds IM, de Vries R, Danser AH. Cardioprotective effects of eplerenone in the rat heart: interaction with locally synthesized or blood-derived aldosterone? *Hypertension.* 2006;47(4):665-70.

81. Losel R, Feuring M, Wehling M. Non-genomic aldosterone action: from the cell membrane to human physiology. *The Journal of steroid biochemistry and molecular biology.* 2002;83(1-5):167-71.
82. Wendler A, Albrecht C, Wehling M. Nongenomic actions of aldosterone and progesterone revisited. *Steroids.* 2012;77(10):1002-6.
83. Wehling M, Neylon CB, Fullerton M, Bobik A, Funder JW. Nongenomic effects of aldosterone on intracellular Ca2+ in vascular smooth muscle cells. *Circulation research.* 1995;76(6):973-9.
84. Toda N, Nakanishi S, Tanabe S. Aldosterone affects blood flow and vascular tone regulated by endothelium-derived NO: therapeutic implications. *British journal of pharmacology.* 2013;168(3):519-33.
85. Schmitt M, Gunaruwan P, Frenneaux MP. Rapid nongenomic aldosterone effects in the human forearm? *Hypertension.* 2004;43(1):e1; author reply e2.
86. Fujita M, Minamino T, Asanuma H, Sanada S, Hirata A, Wakeno M, et al. Aldosterone nongenomically worsens ischemia via protein kinase C-dependent pathways in hypoperfused canine hearts. *Hypertension.* 2005;46(1):113-7.
87. Mihailidou AS, Buhagiar KA, Rasmussen HH. Na+ influx and Na(+)-K+ pump activation during short-term exposure of cardiac myocytes to aldosterone. *The American journal of physiology.* 1998;274(1 Pt 1):C175-81.
88. Matsui S, Satoh H, Kawashima H, Nagasaka S, Niu CF, Urushida T, et al. Non-genomic effects of aldosterone on intracellular ion regulation and cell volume in rat ventricular myocytes. *Canadian journal of physiology and pharmacology.* 2007;85(2):264-73.
89. Schneider SW, Yano Y, Sumpio BE, Jena BP, Geibel JP, Gekle M, et al. Rapid aldosterone-induced cell volume increase of endothelial cells measured by the atomic force microscope. *Cell biology international.* 1997;21(11):759-68.
90. Mano A, Tatsumi T, Shiraishi J, Keira N, Nomura T, Takeda M, et al. Aldosterone directly induces myocyte apoptosis through calcineurin-dependent pathways. *Circulation.* 2004;110(3):317-23.
91. Christ M, Gunther A, Heck M, Schmidt BM, Falkenstein E, Wehling M. Aldosterone, not estradiol, is the physiological agonist for rapid increases in cAMP in vascular smooth muscle cells. *Circulation.* 1999;99(11):1485-91.
92. Schmidt BM, Georgens AC, Martin N, Tillmann HC, Feuring M, Christ M, et al. Interaction of rapid nongenomic cardiovascular aldosterone effects with the adrenergic system. *The Journal of clinical endocrinology and metabolism.* 2001;86(2):761-7.

93. Schmidt BM, Horisberger K, Feuring M, Schultz A, Wehling M. Aldosterone blunts human baroreflex sensitivity by a nongenomic mechanism. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association.* 2005;113(5):252-6.
94. Callera GE, Touyz RM, Tostes RC, Yogi A, He Y, Malkinson S, et al. Aldosterone activates vascular p38MAP kinase and NADPH oxidase via c-Src. *Hypertension.* 2005;45(4):773-9.
95. Shi G, Fu Y, Jiang W, Yin A, Feng M, Wu Y, et al. Activation of Src-ATF1 pathway is involved in upregulation of Nox1, a catalytic subunit of NADPH oxidase, by aldosterone. *Endocrine journal.* 2011;58(6):491-9.
96. Ishizawa K, Izawa Y, Ito H, Miki C, Miyata K, Fujita Y, et al. Aldosterone stimulates vascular smooth muscle cell proliferation via big mitogen-activated protein kinase 1 activation. *Hypertension.* 2005;46(4):1046-52.
97. Christ M, Douwes K, Eisen C, Bechtner G, Theisen K, Wehling M. Rapid effects of aldosterone on sodium transport in vascular smooth muscle cells. *Hypertension.* 1995;25(1):117-23.
98. Wehling M, Ulsenheimer A, Schneider M, Neylon C, Christ M. Rapid effects of aldosterone on free intracellular calcium in vascular smooth muscle and endothelial cells: subcellular localization of calcium elevations by single cell imaging. *Biochemical and biophysical research communications.* 1994;204(2):475-81.
99. Schneider M, Ulsenheimer A, Christ M, Wehling M. Nongenomic effects of aldosterone on intracellular calcium in porcine endothelial cells. *The American journal of physiology.* 1997;272(4 Pt 1):E616-20.
100. Delyani JA, Robinson EL, Rudolph AE. Effect of a selective aldosterone receptor antagonist in myocardial infarction. *American journal of physiology Heart and circulatory physiology.* 2001;281(2):H647-54.
101. Fraccarollo D, Galuppo P, Schraut S, Kneitz S, van Rooijen N, Ertl G, et al. Immediate mineralocorticoid receptor blockade improves myocardial infarct healing by modulation of the inflammatory response. *Hypertension.* 2008;51(4):905-14.
102. Fraccarollo D, Galuppo P, Schmidt I, Ertl G, Bauersachs J. Additive amelioration of left ventricular remodeling and molecular alterations by combined aldosterone and angiotensin receptor blockade after myocardial infarction. *Cardiovascular research.* 2005;67(1):97-105.
103. Kang YM, Zhang ZH, Johnson RF, Yu Y, Beltz T, Johnson AK, et al. Novel effect of mineralocorticoid receptor antagonism to reduce proinflammatory cytokines and hypothalamic activation in rats with ischemia-induced heart failure. *Circulation research.* 2006;99(7):758-66.

104. Cittadini A, Casaburi C, Monti MG, Di Gianni A, Serpico R, Scherillo G, et al. Effects of canrenone on myocardial reactive fibrosis in a rat model of postinfarction heart failure. *Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy.* 2002;16(3):195-201.

105. Matsumoto R, Yoshiyama M, Omura T, Kim S, Nakamura Y, Izumi Y, et al. Effects of aldosterone receptor antagonist and angiotensin II type I receptor blocker on cardiac transcriptional factors and mRNA expression in rats with myocardial infarction. *Circulation journal : official journal of the Japanese Circulation Society.* 2004;68(4):376-82.

106. Masson S, Staszewsky L, Annoni G, Carlo E, Arosio B, Bai A, et al. Eplerenone, a selective aldosterone blocker, improves diastolic function in aged rats with small-to-moderate myocardial infarction. *Journal of cardiac failure.* 2004;10(5):433-41.

107. Lal A, Veinot JP, Leenen FH. Critical role of CNS effects of aldosterone in cardiac remodeling post-myocardial infarction in rats. *Cardiovascular research.* 2004;64(3):437-47.

108. Huang BS, Leenen FH. Blockade of brain mineralocorticoid receptors or Na+ channels prevents sympathetic hyperactivity and improves cardiac function in rats post-MI. *American journal of physiology Heart and circulatory physiology.* 2005;288(5):H2491-7.

109. Enomoto S, Yoshiyama M, Omura T, Matsumoto R, Kusuyama T, Kim S, et al. Effects of eplerenone on transcriptional factors and mRNA expression related to cardiac remodelling after myocardial infarction. *Heart (British Cardiac Society).* 2005;91(12):1595-600.

110. Milliez P, Deangelis N, Rucker-Martin C, Leenhardt A, Vicaut E, Robidel E, et al. Spironolactone reduces fibrosis of dilated atria during heart failure in rats with myocardial infarction. *European heart journal.* 2005;26(20):2193-9.

111. Noda K, Kobara M, Hamada J, Yoshifuji Y, Shiraishi T, Tanaka T, et al. Additive amelioration of oxidative stress and cardiac function by combined mineralocorticoid and angiotensin receptor blockers in postinfarct failing hearts. *Journal of cardiovascular pharmacology.* 2012;60(2):140-9.

112. Takeda M, Tatsumi T, Matsunaga S, Hayashi H, Kimata M, Honsho S, et al. Spironolactone modulates expressions of cardiac mineralocorticoid receptor and 11beta-hydroxysteroid dehydrogenase 2 and prevents ventricular remodeling in post-infarct rat hearts. *Hypertension research : official journal of the Japanese Society of Hypertension.* 2007;30(5):427-37.

113. Schmidt K, Tissier R, Ghaleb B, Drogies T, Felix SB, Krieg T. Cardioprotective effects of mineralocorticoid receptor antagonists at reperfusion. *European heart journal.* 2010;31(13):1655-62.

114. Loan Le TY, Mardini M, Howell VM, Funder JW, Ashton AW, Mihailidou AS. Low-dose spironolactone prevents apoptosis repressor with caspase recruitment domain degradation during myocardial infarction. *Hypertension.* 2012;59(6):1164-9.

115. Chai W, Garrelds IM, Arulmani U, Schoemaker RG, Lamers JM, Danser AH. Genomic and nongenomic effects of aldosterone in the rat heart: why is spironolactone cardioprotective? *British journal of pharmacology.* 2005;145(5):664-71.

116. Silvestre JS, Heymes C, Oubenaissa A, Robert V, Aupetit-Faisant B, Carayon A, et al. Activation of cardiac aldosterone production in rat myocardial infarction: effect of angiotensin II receptor blockade and role in cardiac fibrosis. *Circulation.* 1999;99(20):2694-701.

117. Gomez-Sanchez EP, Ahmad N, Romero DG, Gomez-Sanchez CE. Origin of aldosterone in the rat heart. *Endocrinology.* 2004;145(11):4796-802.

118. Ye P, Kenyon CJ, MacKenzie SM, Jong AS, Miller C, Gray GA, et al. The aldosterone synthase (CYP11B2) and 11beta-hydroxylase (CYP11B1) genes are not expressed in the rat heart. *Endocrinology.* 2005;146(12):5287-93.

119. Chun TY, Bloem L, Pratt JH. Spironolactone increases integrin beta3 gene expression in kidney and heart muscle cells. *Molecular and cellular endocrinology.* 2002;194(1-2):175-82.

120. De Angelis N, Fiordaliso F, Latini R, Calvillo L, Funicello M, Gobbi M, et al. Appraisal of the role of angiotensin II and aldosterone in ventricular myocyte apoptosis in adult normotensive rat. *Journal of molecular and cellular cardiology.* 2002;34(12):1655-65.

121. Burniston JG, Saini A, Tan LB, Goldspink DF. Aldosterone induces myocyte apoptosis in the heart and skeletal muscles of rats in vivo. *Journal of molecular and cellular cardiology.* 2005;39(2):395-9.

122. Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: united at reperfusion. *Pharmacology & therapeutics.* 2007;116(2):173-91.

123. Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: underlying mechanisms and clinical application. *Atherosclerosis.* 2009;204(2):334-41.

124. Ramirez V, Trujillo J, Valdes R, Uribe N, Cruz C, Gamba G, et al. Adrenalectomy prevents renal ischemia-reperfusion injury. *American journal of physiology Renal physiology.* 2009;297(4):F932-42.

125. Frieler RA, Meng H, Duan SZ, Berger S, Schutz G, He Y, et al. Myeloid-specific deletion of the mineralocorticoid receptor reduces infarct volume and alters inflammation during cerebral ischemia. *Stroke; a journal of cerebral circulation.* 2011;42(1):179-85.

126. Kobayashi N, Fukushima H, Takeshima H, Koguchi W, Mamada Y, Hirata H, et al. Effect of eplerenone on endothelial progenitor cells and oxidative stress in ischemic hindlimb. *American journal of hypertension.* 2010;23(9):1007-13.

127. Masci PG, Ganame J, Francone M, Desmet W, Lorenzoni V, Iacucci I, et al. Relationship between location and size of myocardial infarction and their reciprocal influences on post-infarction left ventricular remodelling. *European heart journal.* 2011;32(13):1640-8.
128. Konermann M, Sanner BM, Grotz J, Laschewski F, Josephs W, Odenthal HJ, et al. [Effect of infarct size and site, patency of the infarct vessel and perfusion of vital myocardium on remodeling of the left ventricle--studies with cine-magnetic resonance tomography in the first 6 months following myocardial infarct]. *Zeitschrift fur Kardiologie.* 1996;85(12):906-16.
129. Anzai A, Anzai T, Nagai S, Maekawa Y, Naito K, Kaneko H, et al. Regulatory role of dendritic cells in postinfarction healing and left ventricular remodeling. *Circulation.* 2012;125(10):1234-45.
130. Herrada AA, Contreras FJ, Marini NP, Amador CA, Gonzalez PA, Cortes CM, et al. Aldosterone promotes autoimmune damage by enhancing Th17-mediated immunity. *Journal of immunology.* 2010;184(1):191-202.
131. van den Borne SW, Diez J, Blankesteijn WM, Verjans J, Hofstra L, Narula J. Myocardial remodeling after infarction: the role of myofibroblasts. *Nature reviews Cardiology.* 2010;7(1):30-7.
132. Neumann S, Huse K, Semrau R, Diegeler A, Gebhardt R, Buniatian GH, et al. Aldosterone and D-glucose stimulate the proliferation of human cardiac myofibroblasts in vitro. *Hypertension.* 2002;39(3):756-60.
133. Fraccarollo D, Berger S, Galuppo P, Kneitz S, Hein L, Schutz G, et al. Deletion of cardiomyocyte mineralocorticoid receptor ameliorates adverse remodeling after myocardial infarction. *Circulation.* 2011;123(4):400-8.
134. van Berlo JH, Maillet M, Molkentin JD. Signaling effectors underlying pathologic growth and remodeling of the heart. *The Journal of clinical investigation.* 2013;123(1):37-45.
135. Gullestad L, Ueland T, Vinge LE, Finsen A, Yndestad A, Aukrust P. Inflammatory cytokines in heart failure: mediators and markers. *Cardiology.* 2012;122(1):23-35.
136. Beygui F, Montalescot G, Vicaut E, Rouanet S, Van Belle E, Baulac C, et al. Aldosterone and long-term outcome after myocardial infarction: A substudy of the french nationwide Observatoire sur la Prise en charge hospitaliere, l'Evolution a un an et les caRacteristiques de patients presentant un infArctus du myocarde avec ou sans onde Q (OPERA) study. *American heart journal.* 2009;157(4):680-7.
137. Galetta F, Bernini G, Franzoni F, Bacca A, Fivizzani I, Tocchini L, et al. Cardiac remodeling in patients with primary aldosteronism. *Journal of endocrinological investigation.* 2009;32(9):739-45.
138. Lin YH, Lee HH, Liu KL, Lee JK, Shih SR, Chueh SC, et al. Reversal of myocardial fibrosis in patients with unilateral hyperaldosteronism receiving adrenalectomy. *Surgery.* 2011;150(3):526-33.

139. Yoshihara F, Nishikimi T, Yoshitomi Y, Nakasone I, Abe H, Matsuoka H, et al. Left ventricular structural and functional characteristics in patients with renovascular hypertension, primary aldosteronism and essential hypertension. *American journal of hypertension.* 1996;9(6):523-8.

140. Goldkorn R, Yurenev A, Blumenfeld J, Fishman D, Devereux RB. Echocardiographic comparison of left ventricular structure and function in hypertensive patients with primary aldosteronism and essential hypertension. *American journal of hypertension.* 2002;15(4 Pt 1):340-5.

141. Wang Q, Clement S, Gabbiani G, Horisberger JD, Burnier M, Rossier BC, et al. Chronic hyperaldosteronism in a transgenic mouse model fails to induce cardiac remodeling and fibrosis under a normal-salt diet. *American journal of physiology Renal physiology.* 2004;286(6):F1178-84.

142. Wang Q, Domenighetti AA, Schafer SC, Weber J, Simon A, Maillard MP, et al. Impact of salt on cardiac differential gene expression and coronary lesion in normotensive mineralocorticoid-treated mice. *American journal of physiology Regulatory, integrative and comparative physiology.* 2012;302(9):R1025-33.

143. Bienvenu LA, Morgan J, Rickard AJ, Tesch GH, Cranston GA, Fletcher EK, et al. Macrophage mineralocorticoid receptor signaling plays a key role in aldosterone-independent cardiac fibrosis. *Endocrinology.* 2012;153(7):3416-25.

144. Beggah AT, Escoubet B, Puttini S, Cailmail S, Delage V, Ouvrard-Pascaud A, et al. Reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes. *Proceedings of the National Academy of Sciences of the United States of America.* 2002;99(10):7160-5.

145. Fraccarollo D, Galuppo P, Hildemann S, Christ M, Ertl G, Bauersachs J. Additive improvement of left ventricular remodeling and neurohormonal activation by aldosterone receptor blockade with eplerenone and ACE inhibition in rats with myocardial infarction. *Journal of the American College of Cardiology.* 2003;42(9):1666-73.

146. Suzuki G, Morita H, Mishima T, Sharov VG, Todor A, Tanhehco EJ, et al. Effects of long-term monotherapy with eplerenone, a novel aldosterone blocker, on progression of left ventricular dysfunction and remodeling in dogs with heart failure. *Circulation.* 2002;106(23):2967-72.

147. Wang D, Liu YH, Yang XP, Rhaleb NE, Xu J, Peterson E, et al. Role of a selective aldosterone blocker in mice with chronic heart failure. *Journal of cardiac failure.* 2004;10(1):67-73.

148. Hayashi M, Tsutamoto T, Wada A, Tsutsui T, Ishii C, Ohno K, et al. Immediate administration of mineralocorticoid receptor antagonist spironolactone prevents post-infarct left ventricular remodeling associated with suppression of a marker of myocardial collagen synthesis in patients with first anterior acute myocardial infarction. *Circulation.* 2003;107(20):2559-65.

149. Yin G, Zhao S, Lu M, Ma N, Zuehlsdorff S, Cheng H, et al. Assessment of left ventricular myocardial scar in coronary artery disease by a three-dimensional MR imaging technique. *Journal of magnetic resonance imaging : JMRI*. 2012.

150. Li X, Qi Y, Li Y, Zhang S, Guo S, Chu S, et al. Impact of mineralocorticoid receptor antagonists on changes in cardiac structure and function of left ventricular dysfunction: a meta-analysis of randomized controlled trials. *Circulation Heart failure*. 2013;6(2):156-65.

151. Bapoje SR, Bahia A, Hokanson JE, Peterson PN, Heidenreich PA, Lindenfeld J, et al. Effects of mineralocorticoid receptor antagonists on the risk of sudden cardiac death in patients with left ventricular systolic dysfunction: a meta-analysis of randomized controlled trials. *Circulation Heart failure*. 2013;6(2):166-73.

152. Krum H, Shi H, Pitt B, McMurray J, Swedberg K, van Veldhuisen DJ, et al. Clinical Benefit of Eplerenone in Patients with Mild Symptoms of Systolic Heart Failure Already Receiving Optimal Best Practice Background Drug Therapy: Analysis of the EMPHASIS-HF Study. *Circulation Heart failure*. 2013.

153. Edelmann F, Wachter R, Schmidt AG, Kraigher-Krainer E, Colantonio C, Kamke W, et al. Effect of spironolactone on diastolic function and exercise capacity in patients with heart failure with preserved ejection fraction: the Aldo-DHF randomized controlled trial. *JAMA : the journal of the American Medical Association*. 2013;309(8):781-91.

154. McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. *European heart journal*. 2012;33(14):1787-847.

155. Walker BR, Yau JL, Brett LP, Seckl JR, Monder C, Williams BC, et al. 11 beta-hydroxysteroid dehydrogenase in vascular smooth muscle and heart: implications for cardiovascular responses to glucocorticoids. *Endocrinology*. 1991;129(6):3305-12.

156. Walker BR, Campbell JC, Williams BC, Edwards CR. Tissue-specific distribution of the NAD(+)-dependent isoform of 11 beta-hydroxysteroid dehydrogenase. *Endocrinology*. 1992;131(2):970-2.

157. Slight S, Ganjam VK, Weber KT. Species diversity of 11 beta-hydroxysteroid dehydrogenase in the cardiovascular system. *The Journal of laboratory and clinical medicine*. 1994;124(6):821-6.

158. Agarwal AK, Rogerson FM, Mune T, White PC. Analysis of the human gene encoding the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase. *The Journal of steroid biochemistry and molecular biology*. 1995;55(5-6):473-9.

159. Li KX, Smith RE, Ferrari P, Funder JW, Krozowski ZS. Rat 11 beta-hydroxysteroid dehydrogenase type 2 enzyme is expressed at low levels in the placenta and is modulated by adrenal steroids in the kidney. *Molecular and cellular endocrinology.* 1996;120(1):67-75.

160. Thompson A, Han VK, Yang K. Differential expression of 11beta-hydroxysteroid dehydrogenase types 1 and 2 mRNA and glucocorticoid receptor protein during mouse embryonic development. *The Journal of steroid biochemistry and molecular biology.* 2004;88(4-5):367-75.

161. Klusonova P, Kucka M, Ergang P, Miksik I, Bryndova J, Pacha J. Cloning of chicken 11beta-hydroxysteroid dehydrogenase type 1 and its tissue distribution. *The Journal of steroid biochemistry and molecular biology.* 2008;111(3-5):217-24.

162. Funder JW, Pearce PT, Smith R, Campbell J. Vascular type I aldosterone binding sites are physiological mineralocorticoid receptors. *Endocrinology.* 1989;125(4):2224-6.

163. Kornel L. Colocalization of 11 beta-hydroxysteroid dehydrogenase and mineralocorticoid receptors in cultured vascular smooth muscle cells. *American journal of hypertension.* 1994;7(1):100-3.

164. Brem AS, Bina RB, King T, Morris DJ. Bidirectional activity of 11 beta-hydroxysteroid dehydrogenase in vascular smooth muscle cells. *Steroids.* 1995;60(5):406-10.

165. Slight S, Ganjam VK, Nonneman DJ, Weber KT. Glucocorticoid metabolism in the cardiac interstitium: 11 beta-hydroxysteroid dehydrogenase activity in cardiac fibroblasts. *The Journal of laboratory and clinical medicine.* 1993;122(2):180-7.

166. Rochetaing A, Chapon C, Marescaux L, Le Bouil A, Furber A, Kreher P. Potential beneficial as well as detrimental effects of chronic treatment with lisinopril and (or) spironolactone on isolated hearts following low-flow ischemia in normal and infarcted rats. *Canadian journal of physiology and pharmacology.* 2003;81(9):864-72.

167. Barbato JC, Mulrow PJ, Shapiro JI, Franco-Saenz R. Rapid effects of aldosterone and spironolactone in the isolated working rat heart. *Hypertension.* 2002;40(2):130-5.

168. Mulder P, Mellin V, Favre J, Vercauteren M, Remy-Jouet I, Monteil C, et al. Aldosterone synthase inhibition improves cardiovascular function and structure in rats with heart failure: a comparison with spironolactone. *European heart journal.* 2008;29(17):2171-9.

169. Mill JG, Milanez Mda C, de Resende MM, Gomes Mda G, Leite CM. Spironolactone prevents cardiac collagen proliferation after myocardial infarction in rats. *Clinical and experimental pharmacology & physiology.* 2003;30(10):739-44.

170. Nehme JA, Lacolley P, Labat C, Challande P, Robidel E, Perret C, et al. Spironolactone improves carotid artery fibrosis and distensibility in rat post-ischaemic heart failure. *Journal of molecular and cellular cardiology.* 2005;39(3):511-9.


76
90
96
f
102
tr
111
f
120
3
p
129
scen
135
3

CHAPTER 3.2

THE EFFECT OF EPLERENONE ON ADENOSINE FORMATION IN HUMANS *IN VIVO*: A DOUBLE-BLINDED RANDOMISED CONTROLLED STUDY

T .N. A. (Daniëlle) van den Berg [a,b] | Jaap Deinum [b]
Albert Bilos [a] | A. Rogier T. Donders [c]
Gerard A. Rongen [a,b] | Niels P. Riksen [a,b]

[a] Department of Pharmacology and Toxicology,
[b] Department of Internal Medicine and [c] Department for Health Evidence,
Radboud university medical center, Nijmegen, the Netherlands

PLoS One. 2014 Oct 30;9(10):e111248

ABSTRACT

Background It has been suggested that mineralocorticoid receptor antagonists have direct cardioprotective properties, because these drugs reduce mortality in patients with heart failure. In murine models of myocardial infarction, mineralocorticoid receptor antagonists reduce infarct size. Using gene deletion and pharmacological approaches, it has been shown that extracellular formation of the endogenous nucleoside adenosine is crucial for this protective effect. We now aim to translate this finding to humans, by investigating the effects of the selective mineralocorticoid receptor antagonist eplerenone on the vasodilator effect of the adenosine uptake inhibitor dipyridamole, which is a well-validated surrogate marker for extracellular adenosine formation.

Methods In a randomised, double-blinded, placebo-controlled, cross-over study we measured the forearm blood flow response to the intrabrachial administration of dipyridamole in 14 healthy male subjects before and after treatment with placebo or eplerenone (50 mg bid for 8 days).

Results The forearm blood flow during administration of dipyridamole (10, 30 and 100 μg $\cdot min^{-1} \cdot dl^{-1}$) was 1.63 (0.60), 2.13 (1.51) and 2.71 (1.32) ml $\cdot dl^{-1} \cdot min^{-1}$ during placebo use, versus 2.00 (1.45), 2.68 (1.87) and 3.22 (1.94) ml $\cdot dl^{-1} \cdot min^{-1}$ during eplerenone treatment (median (interquartile range); p=0.51). Concomitant administration of the adenosine receptor antagonist caffeine attenuated dipyridamole-induced vasodilation to a similar extent in both groups. The forearm blood flow response to forearm ischemia, as a stimulus for increased formation of adenosine, was similar during both conditions.

Conclusion In a dosage of 50 mg bid, eplerenone does not augment extracellular adenosine formation in healthy human subjects. Therefore, it is unlikely that an increased extracellular adenosine formation contributes to the cardioprotective effect of mineralocorticoid receptor antagonists.

INTRODUCTION

Despite state-of-the-art reperfusion strategies, mortality and morbidity in patients with an acute myocardial infarction remain significant. This is caused, at least in part, by 'lethal reperfusion injury'. [1] Therefore, novel therapeutic options to further limit ischemia-reperfusion (IR) injury are urgently needed to improve outcome in these patients.

It has been suggested that the mineralocorticoid receptor (MR) antagonists spironolactone and eplerenone could potentially serve this goal, because these drugs reduce mortality in patients with heart failure. [2-4] Indeed, recent studies in murine models of myocardial infarction have shown that MR antagonists can directly limit infarct size [5-8], and have beneficial effects on left ventricular remodeling [9, 10].

The underlying mechanisms of the infarct size-limiting effect are not yet fully understood, but it has been suggested that the endogenous purine nucleoside adenosine is crucially involved. Adenosine is an endogenous purine nucleoside, which is formed by intra-, and extracellular degradation of adenosine monophosphate by the enzyme ecto-5'-nucleotidase (CD73). Degradation of adenosine only occurs in the intracellular compartment. As a consequence, facilitated diffusion of adenosine over the cellular membrane by the equilibrative nucleoside transporter (ENT) is normally directed inwards. Stimulation of membrane-bound adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) induces various effects, including vasodilation, inhibition of inflammation, and protection against IR-injury. Indeed, endogenous adenosine acts as a key mediator of the infarct size-limiting effect of several drugs, including statins and metformin. [11, 12] A recent series of experiments, using genetic and pharmacological approaches in mouse and rat models of myocardial infarction, convincingly demonstrated that the cardioprotective effects of the MR antagonists eplerenone and canrenoate were crucially dependent on extracellular adenosine formation by CD73 and adenosine receptor stimulation. [6]

Based on these previous animal studies, we now hypothesize that MR antagonists increase the extracellular adenosine concentration by activating CD73, which has previously also been reported for statins [13], and we aim to test this hypothesis in humans *in vivo*. Measurement of circulating endogenous adenosine is extremely difficult

[14], because the half life of adenosine in blood is very short, due to rapid uptake and degradation by circulating erythrocytes. [15] Therefore, we used the vasodilator effect of the ENT inhibitor dipyridamole as a read-out for endogenous adenosine formation by CD73, as previously described by our group.[16, 17] The results of this study will give novel insight into the pharmacology of MR antagonists, which can be used to optimize pharmacological cardioprotective strategies, for example avoid the use of adenosine receptor antagonists, such as caffeine, in patients treated with MR antagonists.

METHODS

3.2

STUDY POPULATION

After approval by the Institutional Review Board of our centre, we included 14 healthy male volunteers. They had no history of cardiovascular disease or asthma, did not smoke and did not use any medication. In all participants we performed a physical examination, electrocardiography, and laboratory investigation to exclude cardiovascular and pulmonary disease, hypertension (SBP >140 mmHg or DBP >90 mmHg), hypotension (SBP <100 mmHg or DBP <60 mmHg), diabetes mellitus (fasting venous glucose >6.9 mmol/l or random glucose of >11.0 mmol/l), renal dysfunction (MDRD <60 mL/min), liver enzyme abnormalities (alanine aminotransferase (ALAT) twice upper limit), and hyperkalemia (plasma potassium ≥4.8 mmol/l).

All volunteers provided written informed consent before enrollment. The study was conducted in accordance with Good Clinical Practices and the Declaration of Helsinki and was prospectively registered at ClinicalTrials.gov by number NCT01837108.

STUDY DESIGN

We performed a single center, double-blinded, randomised, placebo-controlled cross-over study. Tablets of 50 mg of eplerenone were over-encapsulated and fully mimicking placebos were created by the Department of Clinical Pharmacy of the Radboud University Medical Centre. Study medication was taken bid during 8 days. We advised the participants to have a diet low in potassium. On the 7th and 8th day of

treatment, we measured forearm blood flow (FBF) with the use of venous occlusion plethysmography. After a wash-out period of at least 4 weeks, the participants crossed-over to the alternative treatment arm. Blood pressure was measured at baseline, on day 3-5, on day 7 and 8.

VENOUS OCCLUSION PLETHYSMOGRAPHY

All experiments were performed in a temperature-controlled room (24 ± 0.5 °C), in the morning after an overnight fast and at least 24 hours of caffeine and alcohol abstinence. On the days of the experiments, 75 minutes after supervised intake of the study drug, a 27-gauge needle (B. Braun Medical B.V.) was inserted into the brachial artery of the non-dominant arm for intra-arterial drug administration. Fifteen minutes later, baseline FBF was measured during the infusion of normal saline. During the experiment, the total volume infused into the brachial artery was kept constant at 100 μl $\cdot min^{-1} \cdot dl^{-1}$ of forearm volume. We measured FBF in both arms with venous occlusion plethysmography, using mercury-in-silastic-strain gauges. The hand circulation was occluded during the measurements, as described previously. [18] Drugs were administered for 5 minutes per dose. On day 7, we performed 3 experiments, which were all separated by a wash-out period of 30 minutes to prevent any cross-over effects:

1. We measured FBF during the administration of incremental dosages of dipyridamole (10, 30 and 100 μg $\cdot min^{-1} \cdot dl^{-1}$ of forearm volume) into the brachial artery. [10]
2. Subsequently, we measured the vasodilator response to 2 minutes and 5 minutes of arterial occlusion ('post-occlusive reactive hyperemia' (PORH)). Forearm ischemia was induced by inflation of an upper arm cuff to 200 mmHg, as described previously. [13] PORH was used as a stimulus for increased endogenous extracellular adenosine formation.
3. Finally, on day 7, we measured FBF during simultaneous administration of dipyridamole (10, 30 and 100 μg $\cdot min^{-1} \cdot dl^{-1}$) and the adenosine receptor antagonist caffeine (90 μg $\cdot min^{-1} \cdot dl^{-1}$) into the brachial artery.

On day 8, we recorded the forearm vasodilator response to the administration of sodium nitroprusside (SNP) (0.6 and 0.06 μg $\cdot min^{-1} \cdot dl^{-1}$) and adenosine (1.5 and 5.0 μg $\cdot min^{-1} \cdot dl^{-1}$) to exclude non-specific effects of eplerenone on vasomotor function and adenosine sensitivity. Figure 1 illustrates the design of the study.

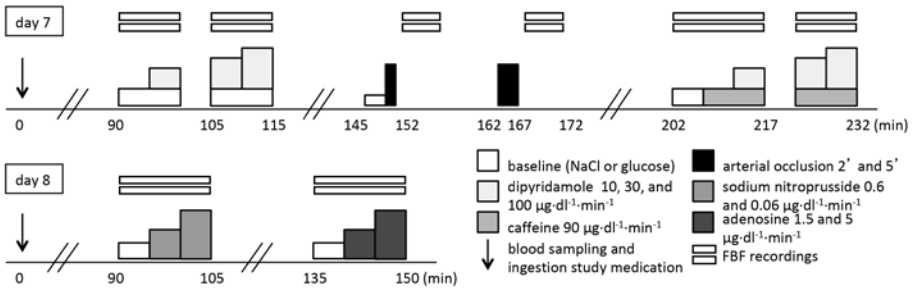


FIGURE 1. Schematic overview of the experimental protocol

3.2

BLOOD AND URINE SAMPLING

Three to 5 days after the start of the study medication, the plasma potassium concentration was measured in venous blood. Volunteers were excluded and study medication was discontinued if the plasma potassium was ≥5.1 mmol/l.

At day 6, the participants collected a 24-hours urine sample. Urinary sodium and creatinine were determined to ensure that salt intake was approximately the same during both treatment periods.

Before the experiment on day 7, blood was drawn for the determination of potassium, sodium, creatinine, plasma caffeine concentration (to check compliance with caffeine abstinence), aldosterone, renin, and plasma eplerenone concentration. On day 8 we measured the plasma caffeine concentration only. Subjects with a circulating caffeine concentration >1.0 mg/l were excluded from analyses.

ANALYTIC PROCEDURES

Plasma caffeine concentrations were determined using reversed-phase HPLC with ultraviolet detection set at 273 nm, according to Schreiber-Deturmeny and Bruguerolle. [19] After data monitoring and data lock, eplerenone concentrations were determined by LC-MSMS. Liquid chromatographic separation was performed at a temperature of 30 °C with a mobile phase consisting of solvent A (0.1% (v/v) formic acid (HCOOH) in water) and solvent B (0.1 % (v/v) HCOOH in acetonitrile). For the mass spectrometric analysis, heated electrospray ionization was operated at a spray

voltage of + 4.5 kV, a capillary temperature of 225 °C and a vaporizer temperature of 382 °C. Positive ion mode was used with selected reaction monitoring for the quantitative analysis of eplerenone, using the most abundant product ion for quantification.

OUTCOMES

The primary outcome was the FBF response to the intrabrachial administration of incremental dosages of dipyridamole, after treatment with eplerenone, compared to placebo.

Secondary outcomes were the FBF response to the intrabrachial administration of incremental dosages of dipyridamole with concomitant administration of caffeine, and the FBF response to incremental periods of arterial occlusion.

STATISTICAL ANALYSIS

For sample size calculation, we assumed a 25% increase in the primary endpoint, a standard deviation of the logarithm of the FBF of 0.35, and a correlation between both experiments of 0.7. This assumption was based on the previous findings that MR antagonists reduced infarct size in preclinical studies with approximately 30% [6], and that rosuvastatin, which increases CD73 activity with approximately 50% approximately doubles dipyridamole-induced forearm vasodilation [13,17]. The power of the study was set at 80% with a two-sided alpha of 0.05. As results, 12 evaluable subjects were needed. In order to have 12 evaluable subjects, we included 14 eligible participants.

FBF analyses were done offline before unblinding of the study. We averaged all individual FBF responses during the last 4 minutes of the baseline FBF (normal saline), the last 2 minutes of the FBF response to dipyridamole, sodium nitroprusside, and adenosine. For the PORH, we selected the highest FBF after reperfusion and also averaged FBF values for each consecutive minute. Results are expressed as the median absolute FBF (interquartile range) in $ml \cdot dl^{-1} \cdot min^{-1}$. In an additional analysis, to correct for any possible systemic effects on FBF during the experiments, we divided the FBF in the experimental arm by the FBF in the non-experimental arm. This analysis was performed with untransformed data.

A linear mixed model was used to compare differences between the treatments, with the log FBF during placebo and eplerenone treatment as the dependent variable with the following fixed factors: treatment (eplerenone versus placebo), dose, period, and

the interaction between treatment and dose. To correct for repeated measurements, we used a heterogeneous compound symmetry structure for the residuals.
For the comparison of the plasma potassium values, blood pressure, and heart rate on a single moment as well as the measurements in the 24 hours urine samples and plasma sodium, serum aldosterone, and plasma renin values, we used a paired sample t-test, after testing for normality.

RESULTS

3.2

SUBJECTS

We screened 20 subjects for eligibility. Two participants withdrew from participation and 4 participants were excluded, because of a DBP <60 mmHg (n=3), and a SBP >140 Hg (n=1). The baseline characteristics are depicted in Table 1.

TABLE 1. Baseline characteristics of the male participants

Clinical characteristics	**Value (mean ± SD)**
Age - years	21.9 ± 3.0
BMI - kg/m^2	23.6 ± 1.9
SBP: Syslic blood pressure (SBP) in mmHg	127 ± 8
DBP: Diastolic blood pressure (DBP) in mmHg	70 ± 8
HR – beats/min	59 ± 9
Blood plasma	
Potassium - mmol/L	3.9 ± 0.3
Creatinine - µmol/L	82.2 ± 9.7
Non-fasting glucose - mmol/L	5.3 ± 0.6
ALAT - U/L	30.0 ± 5.3
Non-fasting cholesterol - mmol/L	3.8 ± 0.6

Abbreviations: ALAT, alanine aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; HR, heart rate; SBP, systolic blood pressure; SD, standard deviation

FBF responses to the different stimuli were obtained in all 14 subjects, with the exception of the FBF response to SNP and adenosine in 1 subject, because insertion of the arterial needle failed. All baseline plasma caffeine concentrations were below 0.6 mg/l, and therefore none of the subjects had to be excluded from analysis. Please see Figure 2 for the flow diagram of the various different phases of this cross-over trial.

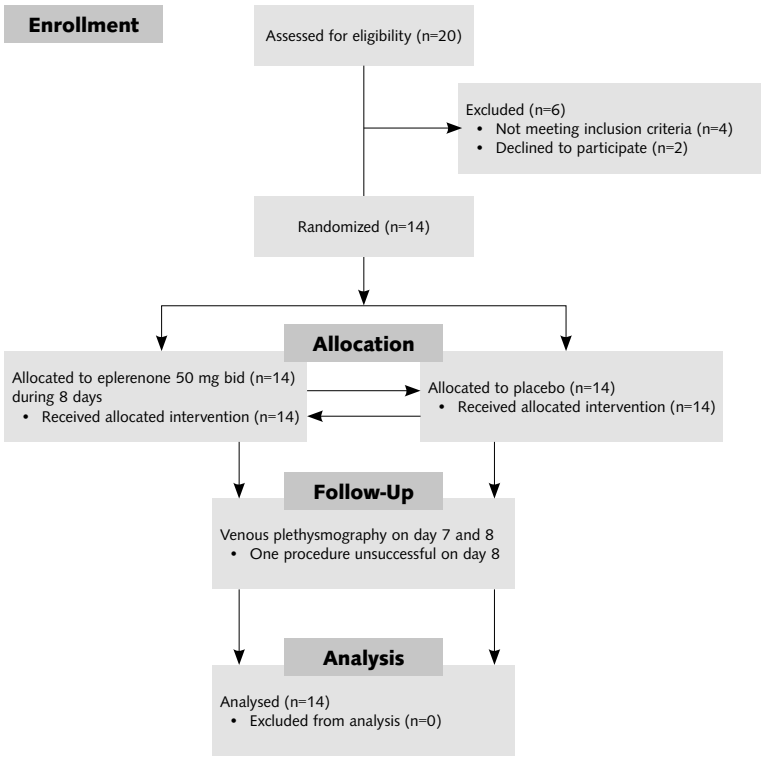


FIGURE 2. Consort 2010 flow diagram

Eplerenone treatment did not significantly affect blood pressure and plasma potassium, but there was a significant decrease in the plasma sodium concentration (Table 2). Urinary sodium concentration did not significantly differ between placebo and eplerenone treatment. Furthermore, eplerenone treatment almost doubled the serum aldosterone and plasma renin concentrations ($p<0.05$), with an unchanged aldosterone-to-renin-ratio (Table 2; $p=0.30$).

TABLE 2. Hemodynamic and laboratory values during the study (means ± SD)

	Placebo	Eplerenone	*p*-value
Blood plasma			
Potassium day 3-5 - mmol/L	3.9 ± 0.26	3.9 ± 0.28	0.78
Potassium day 7 - mmol/L	3.6 ± 0.23	3.7 ± 0.27	0.12
Sodium day 7 - mmol/L	140.0 ± 1.4	139.1 ± 1.4	<0.05
Creatinine day 7 - µmol/L	82.1 ± 6.8	84.6 ± 6.4	<0.05
Aldosterone day 7 - nmol/L	0.65 ± 0.41	1.20 ± 0.50	<0.05
Renin day 7- mE/L	27.7 ± 18.1	44.8 ± 26.7	<0.05
ARR	34.2 ± 21.0	38.0 ± 16.9	0.30
24hr urine (day 6)			
Total amount - mL	1475.6 ± 520.0	1635.4 ± 677.4	0.34
Sodium - mmol/L	85.7 ± 50.3	104.7 ± 44.4	0.15
Creatinin - mmol/L	13.3 ± 6.2	12.0 ± 5.2	0.52
Blood pressure - mmHg			
SBP day 3-5	126 ± 9	129 ± 7	0.57
DBP day 3-5	65 ± 7	67 ± 11	0.54
SBP day 7	123 ± 10	120 ± 8	0.19
DBP day 7	62 ± 10	62.6 ± 8	0.58
SBP day 8	124 ± 11	126 ± 10	0.52
DBP day 8	65 ± 8	63 ± 6	0.34
Heart rate - beats per minute			
HR day 3-5	63 ± 10	62 ± 9	0.42
HR day 7	59 ± 11	58 ± 10	0.51
HR day 8	61 ± 11	60 ± 7	0.74

Abbreviations: ARR, aldosterone-to-renin-ratio; DBP, diastolic blood pressure; HR, heart rate; SBP, systolic blood

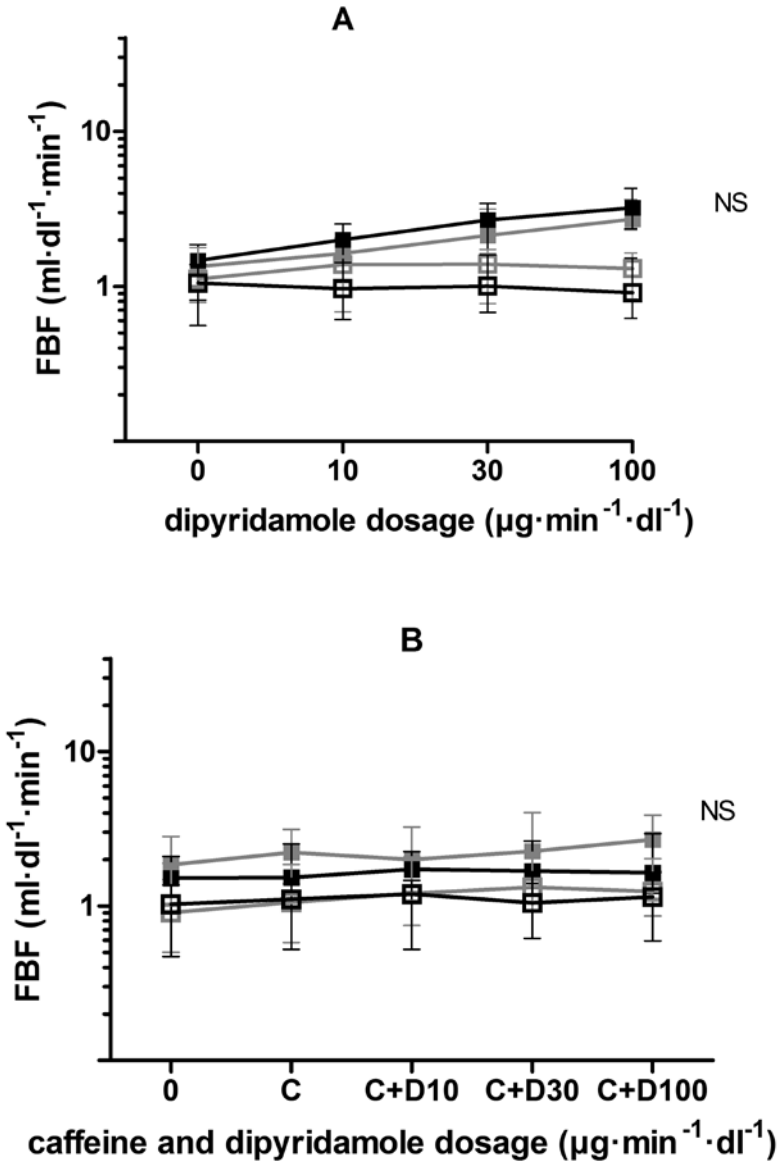


FIGURE 3. FBF response to **A** dipyridamole and **B** dipyridamole (D) in incremental dosages during concomitant administration of caffeine (C) in a constant dosage of 90 µg·dL^{-1}·min^{-1}, during placebo (grey) and eplerenone (black) treatment in the experimental (filled squares) and non-experimental (open squares) arm

OUTCOMES

Baseline FBF before intra-arterial infusion of dipyridamole was 1.34 (0.82) ml·dl^{-1}·min^{-1} during placebo and 1.47 (1.05) ml·dl^{-1}·min^{-1} during eplerenone treatment. The incremental dosages of dipyridamole increased FBF in the experimental arm to 1.63 (0.60), 2.13 (1.51) and 2.71 (1.32) ml·dl^{-1}·min^{-1}, and 2.00 (1.45), 2.68 (1.87) and 3.22 (1.94) ml·dl^{-1}·min^{-1} during placebo and eplerenone treatment, respectively. There was no significant increase in FBF response to dipyridamole during eplerenone treatment compared to the placebo experiment (Figure 3A; p=0.51). Similarly, the FBF ratio did not differ between placebo and eplerenone treatment (p=0.79). In none of the experiments changes in FBF in the non-experimental arm were observed.

Caffeine significantly blunted the dipyridamole-induced vasodilator response during placebo and eplerenone treatment (p<0.001), but there was no difference between both treatment periods (Figure 3B; p=0.98).

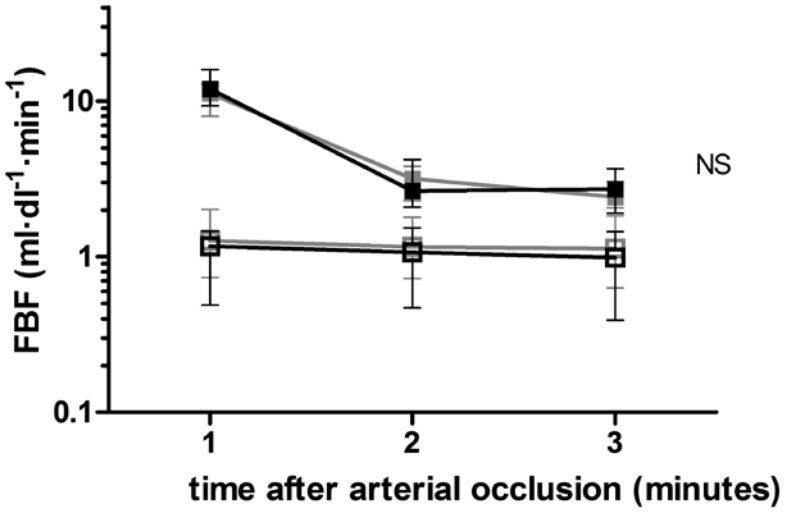


FIGURE 4. PORH after 2 minutes of arterial occlusion PORH in the first 3 minutes after 2 minutes of arterial occlusion during placebo (grey) and eplerenone (black) treatment in the experimental (filled squares) and non-experimental (open squares) arm

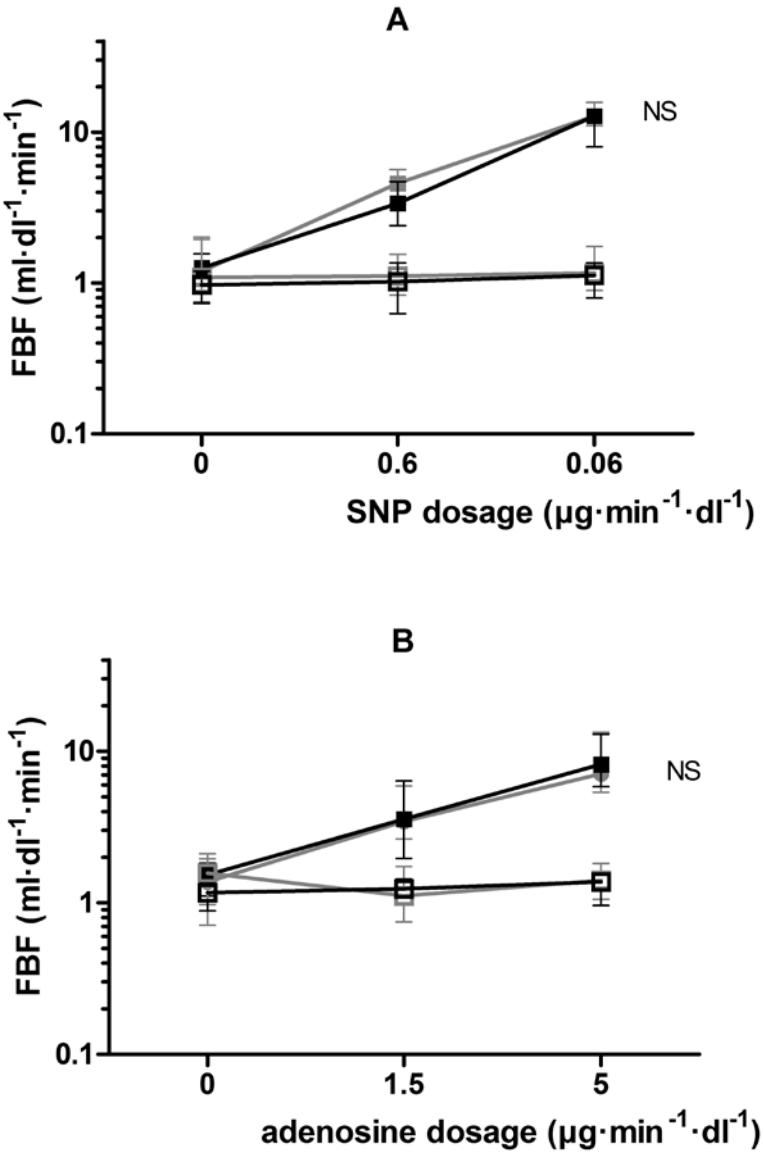


FIGURE 5. FBF response to **A** sodium nitroprusside (SNP) and **B** adenosine, during placebo (grey) and eplerenone (black) treatment in the experimental arm (filled squares) and non-experimental arm (open squares)

The peak (absolute) FBF's after 2 and 5 minutes of arterial occlusion were 20.00 (9.73) and 27.60 (7.45) ml·dl^{-1}·min^{-1} respectively during placebo, and 23.05 (12.35) and 27.75 (16.05) ml·dl^{-1}·min^{-1} respectively during eplerenone use (p=0.91). Figure 4 shows that the PORH after 2 minutes of arterial occlusion was not potentiated by eplerenone (p=0.73). The averaged FBF after 5 minutes of arterial occlusion was 11.24 (4.94) in the 1st minute, 3.18 (1.52) in the 2nd minute, and 2.42 (0.99) ml·dl^{-1}·min^{-1} in the 3rd minute after arterial occlusion during placebo use. During eplerenone treatment, FBF was 11.97 (6.60), 2.65 (2.14), and 2.72 (1.78) ml·dl^{-1}·min^{-1} in the first 3 minutes after 5 minutes of arterial occlusion. Eplerenone did not potentiate the PORH after 5 minutes of arterial occlusion (p=0.58).

The vasodilator response to SNP and adenosine did not differ between placebo and eplerenone treatment (Figure 5).

The plasma eplerenone concentration on t=0 was 0.17 ± 0.13 μg/ml (mean ± SD). During placebo, the eplerenone concentration was 0.0 μg/ml in all cases.

SAFETY

There were no serious adverse events. In none of the subjects, plasma potassium exceeded 4.6 mmol/l. Thirteen adverse events occurred in 9 different participants. During eplerenone treatment 1 subject experienced a short period of abdominal pain on day 4 of the treatment and another subject had a mild headache on day 7. During placebo use 2 subjects had a short period of headache, 1 subject experienced light headedness during sports activities, 1 subject had symptoms of hay fever and another subject developed a skin rash. Due to dislocation of the arterial needle, a very small amount of the infused volume was administered extra-arterially in 3 subjects. Another subject developed 3 small, pulsating, yellowish hives just medial from the arterial needle during dipyridamole infusion, which resolved spontaneously. One subject experienced pain in his left shoulder during the experiment, which resolved after we repositioned the participant. Finally, 1 subject developed a forearm hematoma after several unsuccessful attempts to cannulate the brachial artery on day 8.

DISCUSSION

This study indicates that the selective MR antagonist eplerenone does not have an effect on the extracellular adenosine formation in humans *in vivo*, excluding this mechanism as an explanation for the beneficial cardiovascular effects of MR antagonism observed in patients with heart failure.

The Randomized Aldactone Evaluation Study (RALES), Eplerenone Post–acute myocardial infarction Heart failure Efficacy and Survival Study (EPHESUS), and Eplerenone in Mild Patients Hospitalization And Survival Study in Heart Failure (EMPHASIS-HF) showed that treatment with MR antagonists reduces the mortality and the number of hospitalizations in patients with mild to severe systolic heart failure [2-4]. In animal models of myocardial infarction, MR antagonists limit infarct size when administered either prior to ischemia or just before the onset of reperfusion, and protect against cardiac remodeling, as reviewed by van den Berg *et al.* [20]

The underlying mechanism of the beneficial effects of MR antagonists in patients is not yet understood. However, results from animal studies suggest that extracellular adenosine formation is crucial for the protective effect of MR antagonists against myocardial IR injury. In an elegant series of experiments in mice and rabbits, Schmidt *et al* showed that the MR antagonists canrenoate and eplerenone reduce infarct size in a dose-dependent manner. Moreover, by using pharmacological approaches and models of targeted gene deletion, the investigators convincingly demonstrated an important role for endogenous adenosine in the cardioprotective effect. The infarct size-limiting effect of canrenoate was completely abolished in CD73 knock-out mice and in adenosine A_{2b} receptor knock-out mice. Similar results were obtained in isolated rat hearts. In rats, administration of eplerenone at 10 μM at the moment of reperfusion resulted in a reduction of the infarct size from 40 to 10%, which was completely blocked by co-administration of the adenosine receptor blocker 8p-sulfophenyladenosine. [6]

Based on this series of experiments, we hypothesized that the selective MR antagonist eplerenone increases extracellular formation of adenosine, by activation of the enzyme CD73. Interestingly, increased endogenous adenosine receptor stimulation has also been

implicated in the cardioprotective effect of other drugs, including statins, methotrexate and metformin. [12, 21, 22]

It is difficult to investigate the potential effects of drugs on endogenous adenosine because the half life of adenosine in blood is extremely short due to rapid uptake and degradation of adenosine by erythrocytes and endothelial cells. [16] In a normal physiological situation, the transmembranous adenosine concentration gradient drives extracellular adenosine into the cytosol. [23] The ENT-inhibitor dipyridamole inhibits this facilitated diffusion and thereby increases the extracellular adenosine concentration, which can subsequently activate membrane-bound adenosine receptors at the site of adenosine formation. [16, 24] These observations justify the use of dipyridamole-induced vasodilation as a read-out of extracellular adenosine formation. We have previously shown, using a similar experimental design as the present study, that rosuvastatin augments dipyridamole-induced forearm vasodilation and post-occlusive reactive hyperemia and limits forearm ischemia-reperfusion injury via adenosine receptor stimulation. [13, 17] Moreover, rosuvastatin increased the activity of CD73 on circulating human mononuclear cells. [13]

In the present study, we could not confirm a relation between treatment with eplerenone and the extracellular adenosine system in healthy humans *in vivo*. We showed that a one-week treatment with eplerenone 50 mg bid does not potentiate dipyridamole-induced vasodilation and does not affect PORH after 2 and 5 minutes of arterial occlusion. These findings are in sharp contrast to the previous observations in animal models of myocardial infarction. [6]

How can this discrepancy be explained? First, it has been recognized that many promising findings in animal studies cannot be confirmed in human studies. [25] In general, this translational failure can be explained by methodological shortcomings in animal studies, e.g. the lack of a formal sample size calculation, selection bias due to the lack of randomization, unblinded researchers, and inadequate statistical analyses. [25] The scientific basis for our hypothesis is based on only one preclinical study by Schmidt *et al.* [6] This study, however, described a series of experiments in which a pivotal role for endogenous adenosine in the infarct size-limiting effect of the MR antagonists

canrenoate and eplerenone was consistently observed in different animal species and by using various methodological approaches, including pharmacological inhibitor studies and gene deletion models.

Translational failure could also result from fundamental differences between the animal models and the human situation. With regard to adenosine metabolism, it is known for fundamental differences exists between rats and humans (e.g. the rat ENT1 transporter is less sensitive to inhibition with dipyridamole than the human ENT1). [26] However, other drugs that modulate adenosine levels seem equally effective in humans and in animal models. [17, 21, 22, 27]

Thirdly, a major difference in drug concentration between the animal studies and the human situation is often present. In our study, the circulating eplerenone concentration immediately before the intake of the last medication dose (trough concentration) averaged 0.17 μg/ml (0.41 μM). Given a bioavailability of 70%, and an apparent volume of distribution of 43-90 liters, the eplerenone concentration at the moment of the experiment is approximately 1.7 μM. In a rat Langendorff model, in which the heart is perfused with a buffer solution [6], a cardioprotective effect of eplerenone was observed with 10 μM, but not with 1 μM. Given a plasma protein binding of 50%, the effective concentration in the rat study is approximately 10-times higher than the concentration in our study. We used 50 mg of eplerenone bid, which is higher than the dosages administered in the clinical trials in patients with heart failure in which eplerenone showed beneficial effects. [3, 4] Therefore, we cannot exclude that eplerenone does affect adenosine formation at higher concentrations, which are not relevant however for the clinical situation. In addition, in our study, eplerenone was administered during one week, in contrast to the single dose administration in the animal studies. As expected, the serum aldosterone and plasma renine levels had almost doubled after 6 days of treatment with eplerenone. Although this increase in aldosterone does probably not affect our results, because eplerenone blocks the MR, MR-independent effects of aldosterone have been described previously [20]. In theory, any non-MR-mediated effects of aldosterone on adenosine metabolism might have influenced our result. Finally, it is important to realize that our experiments were performed in healthy subjects, and not in patients with cardiovascular disease or heart failure. This was done because we aimed to demonstrate a general pharmacological mechanism of eplerenone and also because the preclinical studies that were fundamental to our hypothesis were performed

in healthy animals. We cannot exclude, however, that the effects of MR antagonists are different in patients with cardiovascular disease, such as heart failure.

ACKNOWLEDGEMENTS

We want to thank Ms. E.P. Verhofstad for the measurement of the plasma caffeine concentrations.

REFERENCES

1. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *The New England journal of medicine.* 2007;357(11):1121-35.
2. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *The New England journal of medicine.* 1999;341(10):709-17.
3. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *The New England journal of medicine.* 2003;348(14):1309-21.
4. Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, et al. Eplerenone in patients with systolic heart failure and mild symptoms. *The New England journal of medicine.* 2011;364(1):11-21.
5. Loan Le TY, Mardini M, Howell VM, Funder JW, Ashton AW, Mihailidou AS. Low-dose spironolactone prevents apoptosis repressor with caspase recruitment domain degradation during myocardial infarction. *Hypertension.* 2012;59(6):1164-9.
6. Schmidt K, Tissier R, Ghaleb B, Drogies T, Felix SB, Krieg T. Cardioprotective effects of mineralocorticoid receptor antagonists at reperfusion. *European heart journal.* 2010;31(13):1655-62.
7. Mihailidou AS, Loan Le TY, Mardini M, Funder JW. Glucocorticoids activate cardiac mineralocorticoid receptors during experimental myocardial infarction. *Hypertension.* 2009;54(6):1306-12.
8. Chai W, Garrelds IM, de Vries R, Danser AH. Cardioprotective effects of eplerenone in the rat heart: interaction with locally synthesized or blood-derived aldosterone? *Hypertension.* 2006;47(4):665-70.
9. van den Borne SW, Isobe S, Zandbergen HR, Li P, Petrov A, Wong ND, et al. Molecular imaging for efficacy of pharmacologic intervention in myocardial remodeling. *JACC Cardiovascular imaging.* 2009;2(2):187-98.
10. Kessler-Icekson G, Schlesinger H, Freimann S, Kessler E. Expression of procollagen C-proteinase enhancer-1 in the remodeling rat heart is stimulated by aldosterone. *The international journal of biochemistry & cell biology.* 2006;38(3):358-65.

11. Riksen NP, Rongen GA. Targeting adenosine receptors in the development of cardiovascular therapeutics. *Expert review of clinical pharmacology.* 2012;5(2):199-218.
12. Paiva M, Riksen NP, Davidson SM, Hausenloy DJ, Monteiro P, Goncalves L, et al. Metformin prevents myocardial reperfusion injury by activating the adenosine receptor. *Journal of cardiovascular pharmacology.* 2009;53(5):373-8.
13. Meijer P, Wouters CW, van den Broek PH, de Rooij M, Scheffer GJ, Smits P, et al. Upregulation of ecto-5'-nucleotidase by rosuvastatin increases the vasodilator response to ischemia. *Hypertension.* 2010;56(4):722-7.
14. Ramakers BP, Pickkers P, Deussen A, Rongen GA, van den Broek P, van der Hoeven JG, et al. Measurement of the endogenous adenosine concentration in humans in vivo: methodological considerations. *Current drug metabolism.* 2008;9(8):679-85.
15. Moser GH, Schrader J, Deussen A. Turnover of adenosine in plasma of human and dog blood. *The American journal of physiology.* 1989;256(4 Pt 1):C799-806.
16. Riksen NP, Rongen GA, Yellon D, Smits P. Human in vivo research on the vascular effects of adenosine. *European journal of pharmacology.* 2008;585(2-3):220-7.
17. Meijer P, Oyen WJ, Dekker D, van den Broek PH, Wouters CW, Boerman OC, et al. Rosuvastatin increases extracellular adenosine formation in humans in vivo: a new perspective on cardiovascular protection. *Arteriosclerosis, thrombosis, and vascular biology.* 2009;29(6):963-8.
18. Meijer P, Wouters CW, van den Broek PH, Scheffer GJ, Riksen NP, Smits P, et al. Dipyridamole enhances ischaemia-induced reactive hyperaemia by increased adenosine receptor stimulation. *British journal of pharmacology.* 2008;153(6):1169-76.
19. Schreiber-Deturmeny E, Bruguerolle B. Simultaneous high-performance liquid chromatographic determination of caffeine and theophylline for routine drug monitoring in human plasma. *Journal of chromatography B, Biomedical applications.* 1996;677(2):305-12.
20. van den Berg TN, Rongen GA, Frohlich GM, Deinum J, Hausenloy DJ, Riksen NP. The cardioprotective effects of mineralocorticoid receptor antagonists. *Pharmacology & therapeutics.* 2013.
21. Sanada S, Asanuma H, Minamino T, Node K, Takashima S, Okuda H, et al. Optimal windows of statin use for immediate infarct limitation: 5'-nucleotidase as another downstream molecule of phosphatidylinositol 3-kinase. *Circulation.* 2004;110(15):2143-9.
22. Asanuma H, Sanada S, Ogai A, Minamino T, Takashima S, Asakura M, et al. Methotrexate and MX-68, a new derivative of methotrexate, limit infarct size via adenosine-dependent mechanisms in canine hearts. *Journal of cardiovascular pharmacology.* 2004;43(4):574-9.

23. Deussen A. Metabolic flux rates of adenosine in the heart. *Naunyn-Schmiedeberg's archives of pharmacology.* 2000;362(4-5):351-63.
24. Riksen NP, van Ginneken EE, van den Broek PH, Smits P, Rongen GA. In vivo evidence against a role for adenosine in the exercise pressor reflex in humans. *Journal of applied physiology* (Bethesda, Md : 1985). 2005;99(2):522-7.
25. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, et al. Can animal models of disease reliably inform human studies? *PLoS medicine.* 2010;7(3):e1000245.
26. Yao SY, Ng AM, Muzyka WR, Griffiths M, Cass CE, Baldwin SA, et al. Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues. *The Journal of biological chemistry.* 1997;272(45):28423-30.
27. Riksen NP, Barrera P, van den Broek PH, van Riel PL, Smits P, Rongen GA. Methotrexate modulates the kinetics of adenosine in humans in vivo. *Annals of the rheumatic diseases.* 2006;65(4):465-70.


p
tr
cresc.
f
cresc.
ff
tr
dimin.
pp
ritard.
p
crescendo
f
dim.
cresc.
p
cresc.
f
p cresc.
f
ff
E a tempo
tr
f
tr
tr
tr
inf
dimin.

CHAPTER 3.3

EPLERENONE DOES NOT LIMIT ISCHEMIA-REPERFUSION INJURY IN HUMAN MYOCARDIAL TISSUE

T.N.A. (Daniëlle) van den Berg [a,b] | Henry A. van Swieten [c]
J.C. (Annemieke) Vos [a] | Viviënne Verweij [a]
Alfons C. Wouterse [a] | Jaap Deinum [b] | Wim J. Morshuis [c]
Gerard A. Rongen [a,b] | Niels P. Riksen [b]

[a] Department of Pharmacology and Toxicology, [b] Department of Internal medicine and [c] Department of Cardiothoracic Surgery, Radboud university medical center, Nijmegen, The Netherlands

Int J Cardiol. 2016 Aug 1;216:110-3

ABSTRACT

Background Despite rapid reperfusion, mortality and morbidity in patients with an acute myocardial infarction remain significant. Therefore, novel pharmacological strategies to further limit ischemia-reperfusion (IR) injury are warranted. In animal models of myocardial infarction, mineralocorticoid receptor antagonists potently limit infarct size. In the current study we aimed to translate these findings to the human situation and investigated for the first time in human myocardial tissue whether eplerenone limits IR-injury.

Methods In 24 patients undergoing elective cardiac surgery, the right atrial appendage was harvested, and two trabeculae were dissected from each appendage and suspended in an organ bath. We induced contraction by electrical field stimulation. Recovery of contractile force after a period of simulated ischemia and reperfusion was used as well-validated endpoint of IR-injury. From each patients, the trabeculae were randomized to either ischemic preconditioning (IP) or no IP (n=12; positive control experiment) or to superfusion with eplerenone (10 μM) or vehicle (n=12) in a paired approach.

Results IP improved recovery from 19.9 (SEM 3.3) % to 26.3 (SEM 4.3)% ($p<0.05$). During vehicle and eplerenone superfusion, mean recovery of contractile function after simulated ischemia and reperfusion was 45.2 (SEM 5.6)% and 36.5 (SEM 4.1)% ($p=0.14$).

Conclusion Eplerenone does not limit IR-injury in human atrial tissue ex vivo. Our results are in sharp contrast to preclinical studies demonstrating cardioprotective effects of mineralocorticoid receptor antagonist. With great interest we await the results of the MINIMISE-STEMI study, in which the effect of MR antagonism on myocardial infarct size in humans is currently under investigation.

INTRODUCTION

Rapid myocardial reperfusion is essential to limit infarct size in patients with a myocardial infarction. Paradoxically, reperfusion itself can also aggravate injury ("reperfusion injury"). [1] Therefore, mortality and morbidity of these patients remain high, and novel strategies to reduce ischemia-reperfusion (IR) injury are needed. It has been suggested that mineralocorticoid receptor (MR) antagonists might serve this goal, since these drugs reduce morbidity and mortality in patients with heart failure. [2-4]
Indeed, direct cardioprotective effects of these drugs are consistently demonstrated in several murine models of myocardial infarction. [5-10] The acute administration of MR antagonists, either before the onset of ischemia or at the moment of reperfusion, profoundly reduced infarct size. (Reviewed in [11]) In an elegant series of experiments it has been shown that the cardioprotective effects of the MR antagonists eplerenone and canrenoate depend on extracellular adenosine formation. [9] Adenosine is an endogenous purine nucleoside, and stimulation of membrane-bound adenosine receptors induces various effects, including attenuation of inflammation, vasodilation and protection against IR. [12]

Whether these cardioprotective effects of MR antagonists also hold true in humans is yet unknown. In the current study, we aimed to translate the preclinical findings to the human situation for the first time and test the hypothesis that the MR antagonist eplerenone limits IR-injury in human myocardial tissue. We used the recovery of contractile function after a period of simulated IR in human atrial trabeculae as a well-established model of myocardial IR-injury. [13-15]

METHODS

PATIENTS

Adult patients undergoing elective coronary artery bypass surgery (CABG), valve surgery or aortic surgery, with extracorporeal circulation were asked to participate. Exclusion criteria were atrial arrhythmias, right ventricular failure, known atrial enlargement, the use of mineralocorticoid receptor antagonists, and the use of oral antiarrhythmics (except beta-blockers), sulfonylurea derivates, dipyridamole, or theophylline. Patients were asked to abstain from caffeine consumption 24 hours before surgery, since caffeine is an effective adenosine receptor antagonist which prevents the protective effects of ischemic preconditioning and might interfere with any protective effects of eplerenone. [14] All volunteers provided written informed consent before enrollment. The study protocol was approved by the Institutional Review Board of our centre. The study was conducted in accordance with Good Clinical Practices and the Declaration of Helsinki and was prospectively registered at ClinicalTrials.gov (NCT02118753).

EXPERIMENTAL DESIGN

We used the experimental set up as described previously. [13, 14] Briefly, the right atrial appendage was harvested by the cardiothoracic surgeon before the introduction of the extracorporal circulation and immediately placed in cold (4°C) modified Tyrode's solution (NaCl 118.5 mmol/l, KCl 4.8 mmol/l, $NaHCO_3$ 24.8 mmol/l, KH_2PO_4 1.2 mmol/l, $MgSO_4$ 1.4 mmol/l, $CaCl_2$ 1.8 mmol/l, glucose 10.0 mmol/l, and pyruvate 10.0 mmol/l), which was gassed with 95% oxygen and 5% CO_2. Two atrial trabeculae were dissected, vertically suspended in an organ bath, and linked to a force transducer. Each trabecula was superfused with pre-oxygenated Tyrode's buffer. Electrical field stimulation was performed in unstretched condition at 1 Hz using platinum ring electrodes placed on both sides of the trabeculae (pulse duration 60 ms; pulse current 40 mA). After 30 minutes of stimulation at unstretched conditions to allow recovery from transportation and preparation, trabeculae were gradually stretched over 15 minutes until maximal contractile force was achieved. After 20 minutes of equilibration, a baseline recording was performed during 10 min. Those trabeculae that failed to produce at least 0.2 g of

developed force at the end of baseline were excluded. After 30 minutes of equilibration, the trabeculae were subjected to 90 minutes of simulated ischemia, followed by 105 minutes of reperfusion. Simulated ischemia was accomplished by superfusing the trabeculae with substrate-free modified Tyrode's solution (7.0 mM choline chloride substituted for glucose and pyruvate) and rapid pacing at 3 Hz. The superfusate was pumped into an artificial lung filled with 95% N_2/5% CO_2, which resulted in a low pO_2 of 10 to 20 mm Hg. For each patient, the trabeculae were randomized to either an intervention or control to allow paired analysis of the effects of the interventions.

First, as a positive control experiment, we studied the effect of ischemic preconditioning (IP) in 12 pairs of trabeculae, according to the experimental design we published previously. [14] Briefly, immediately after baseline recordings, the trabeculae of each patient (n = 12) were randomly assigned to either a stimulus for IP or continued superfusion with Tyrode's solution. IP was induced by 5 min of simulated ischemia and 5 min of simulated reperfusion.

Subsequently, the trabeculae of 12 patients were randomly assigned to superfusion with eplerenone (10 μM, kindly provided by Pfizer) or vehicle (dimethyl sulfoxide, DMSO <0.1 %). Administration of eplerenone started 10 minutes before simulated ischemia and continued throughout the experiment.

DATA RECORDING AND STATISTICAL ANALYSIS

The differences between the maximal tension during contraction and the minimal tension during relaxation were averaged for every 5 minutes ('contractile force'). Recovery of contractile force in the last 10 minutes of reperfusion as a percentage of the last 10 minutes of equilibration was used as the primary endpoint.

For sample size calculation, we assumed a mean averaged percentage recovery of 18.7 ± 3.20 %, and a test-to-test correlation coefficient of 0.5. A total of 12 subjects was required to detect a 37.5 % change in averaged percentage recovery, which is a relevant reduction of ischemia-reperfusion injury, with a power of 80% at a significance level of 0.05.

Data are presented as mean (SEM) and were analysed with SPSS 22 for Windows. We assumed a one-sided (IP) and two-sided (eplerenone versus vehicle) significance level of 0.05. Data were checked for normality and analyzed accordingly (Wilcoxon Rank test or Student's t-test).

RESULTS

Of the 83 patients who initially signed their informed consent, successful paired experiments in two two trabeculae were performed in 24 patients. The reasons for exclusion of the patients, and the reasons for drop-out are depicted in Figure 1. The baseline characteristics of the patients are presented in Table 1.

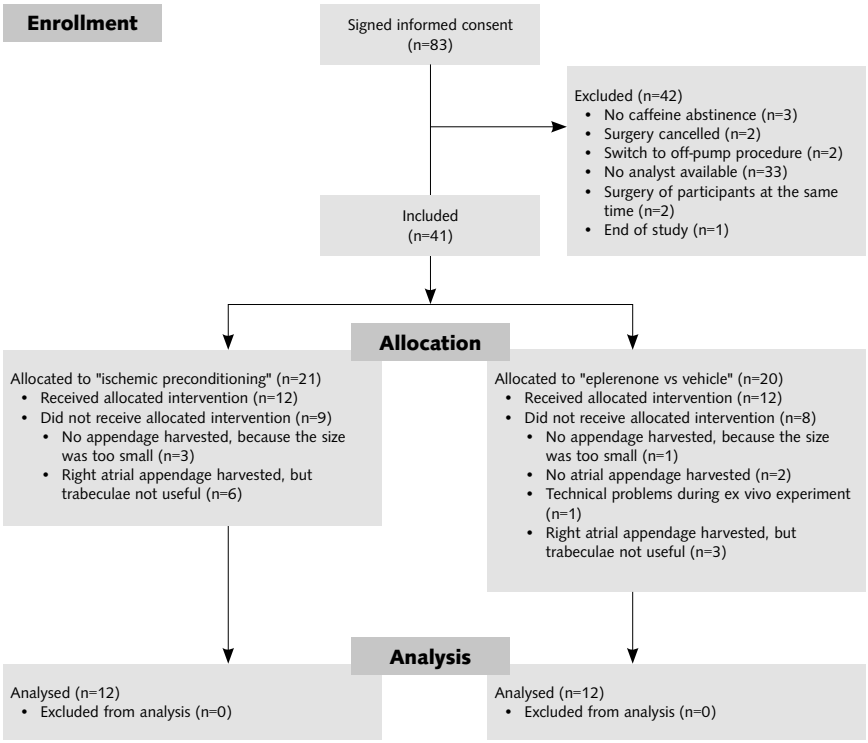


FIGURE 1. Progress of participants during the experiment

TABLE 1. Baseline characteristics

Variable (n)	Setting 1 preconditioning (n=12)	Setting 2 eplerenone vs control (n=12)
Men	11	12
Age (mean ± SD)	55.4 ± 11.9	66.4 ± 8.1
Type of surgery		
CABG	6	8
AVR	4	2
CABG + AVR	0	1
Aortic surgery	2	1
Cardiovascular risk factors		
Hypertension	4	10
Diabetes mellitus	1	1
Current of former smoker	4	5
Positive family history	3	4
Dyslipidemia	7	4
BMI > 30 kg/m^2	2	1
Medication		
Aspirin	8	10
Beta blocker	10	9
ACE-inhibitor	4	5
ATII receptor antagonist	2	4
Ca-channel antagonist	1	5
Diuretic	1	5
Nitrate	1	1
Lipid lowering drug	9	4
Glucose lowering therapy	1	1

Abbreviations: ACE, angiotensin converting enzyme; ATII, angiotensin II; AVR, aorthic valve surgery; BMI, body mass index; Ca, calcium; CABG, coronary artery bypass grafting

In the positive control experiment, IP improved recovery from 19.9 (SEM 3.3)% to 26.3 (SEM 4.3)% (p=0.048), corroborating our previous finding. [14] In the second set of experiments mean recovery of contractile function after reperfusion was 45.2 (SEM 5.6)% and 36.5 (SEM 4.1)%, during vehicle and eplerenone superfusion, respectively (p=0.14; Wilcoxon Rank test). After exclusion of one outlier with a recovery of 98.6% in the control trabecula, the results remained unchanged (40.4 (SEM 3.1)% and 36.0 (SEM 4.4)% for vehicle and eplerenone respectively; Figure 2; p=0.17 (Student's t-test)).

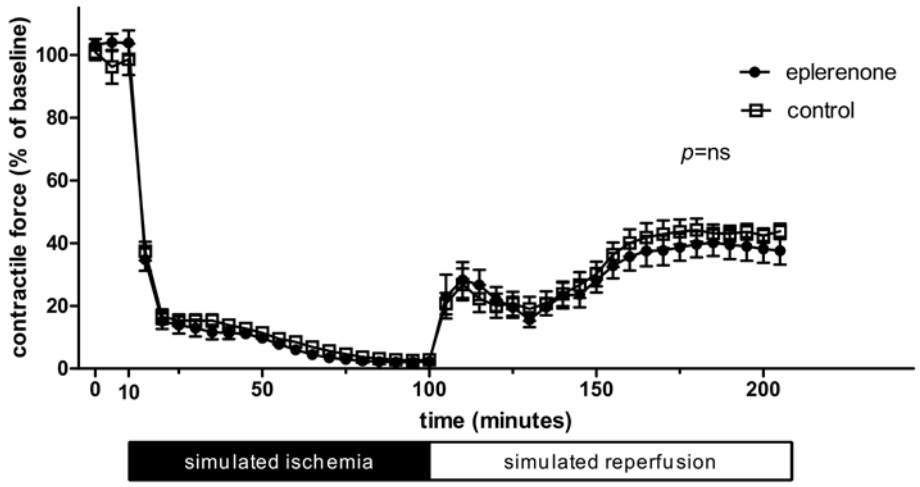


FIGURE 2. The contractile force (difference between maximal tension during contraction and minimal tension during relaxation) in paired trabeculae (n=11) during eplerenone exposure (10 µM) (filled circles) or control (open squares); exclusion of 1 outlier

DISCUSSION

Our study is the first to investigate the direct effects of mineralocorticoid receptor antagonism on ischemia-reperfusion injury in human cardiac tissue. In contrast to various recent preclinical studies, in which administration of MR antagonists consistently reduced myocardial infarct size in animal models of myocardial infarction [11], we did not observe any protective effect of eplerenone against IR-injury in human myocardial tissue.

Post-ischemic recovery of contractile force of human atrial trabeculae has been validated previously as a reliable and reproducible surrogate model of human myocardial IR-injury. Ischemic preconditioning consistently reduced IR-injury in this model in previous studies [13, 14], which was reproduced by the first series of experiments in our current study. Moreover, the protective effect of IP in this model is critically dependent on adenosine receptor stimulation [14], opening of adenosine triphosphate-dependent potassium (K_{ATP}) channels, and activation of protein kinase C [13]. These mechanisms are of similar importance in IP-mediated cardioprotection in animal models of myocardial infarction using histological infarct size as endpoint of IR-injury [16]. These findings highlight that the mechanism of cardioprotection by IP is largely similar in animal models of myocardial infarction and the model we used in the current study.

In models of acute myocardial infarction in mice, rats, and rabbits, acute administration of spironolactone, eplerenone, or canrenoate profoundly limits infarct size. [6-10] Pathways analysis revealed that increased extracellular adenosine formation and subsequent stimulation of adenosine A_{2B} receptors is critical for eplerenone-induced cardioprotection. [9] Why do these consistent findings not translate to a cardioprotective effect of eplerenone in our current study?

First, it is known that in general the external validity of animal studies is limited due to biological differences between animals en humans. This is illustrated by the fact that about 500 neuroprotective treatment strategies improve outcome in animal models of ischemic stroke, while only two have proven effective in patients. [17] In addition, we used the recovery of contractile function as a marker for IR-injury. This differs from

the studies in animals, in which histological infarct size is used as the primary endpoint. However, the protective effect of IP and pivotal involvement of adenosine, PKC, and K_{ATP}-channels that has been demonstrated in many animal models of myocardial infarction, could previously be confirmed in the atrial trabeculae model. [13, 14]

A second potential explanation relates to the presumed mechanism of eplerenone-induced cardioprotection. In animals, increased adenosine formation and receptor stimulation is critical for this effect. We have recently demonstrated, however, that eplerenone does not increase extracellular adenosine formation at relevant dosages in humans in vivo. [18] This finding is consistent with the lack of a cardioprotective effect in humans.

Thirdly, one could argue that the presence of the endogenous ligand aldosterone is required for the protective effect of MR antagonists. However, cardioprotective effects were also observed in hearts from adrenalectomized rats [8] and in Langendorff perfusion models [11], showing that the cardioprotective effect of MR antagonists in animals is independent from the presence of aldosterone.

Finally, it is important to consider the dose and timing of administration of eplerenone. The concentration of eplerenone that we used (10 μM), has been shown to limit IR-injury in a previous study [9] and is approximately 6 times higher than the calculated (peak) plasma levels in healthy adults after a one week treatment of eplerenone 50 mg bid. [18] Administration of eplerenone was started only 10 minutes before simulated ischemia. However, the limited duration of exposure to eplerenone does not explain its lack of benefit in the current study, since also in the animal studies, acute administration of MR antagonists effectively reduced infarct size. [11]

In summary, eplerenone does not limit IR-injury in human myocardial tissue. These results are in sharp contrast to previous observations in animal models of IR. Based on these promising preclinical data, a large clinical trial was recently initiated to investigate whether MR antagonist limit infarct size in patients with an acute myocardial infarction. [19] Therefore, patients with a STEMI will be randomized to an intravenous bolus of canrenoate before coronary reperfusion, followed by three months of oral spironolactone. With great interest we await the results of this randomized controlled trial. [19]

ACKNOWLEDGEMENTS

We would like to thank all cardiothoracic surgeons that have been involved in harvesting the atrial tissue and the secretarial staff of the Department of Cardiothoracic surgery for their help during the inclusion of patients. Eplerenone was kindly provided by Pfizer.

REFERENCES

1. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *The New England journal of medicine.* 2007;357(11):1121-35.
2. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *The New England journal of medicine.* 1999;341(10):709-17.
3. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *The New England journal of medicine.* 2003;348(14):1309-21.
4. Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, et al. Eplerenone in patients with systolic heart failure and mild symptoms. *The New England journal of medicine.* 2011;364(1):11-21.
5. Rochetaing A, Chapon C, Marescaux L, Le Bouil A, Furber A, Kreher P. Potential beneficial as well as detrimental effects of chronic treatment with lisinopril and (or) spironolactone on isolated hearts following low-flow ischemia in normal and infarcted rats. *Canadian journal of physiology and pharmacology.* 2003;81(9):864-72.
6. Chai W, Garrelds IM, Arulmani U, Schoemaker RG, Lamers JM, Danser AH. Genomic and nongenomic effects of aldosterone in the rat heart: why is spironolactone cardioprotective? *British journal of pharmacology.* 2005;145(5):664-71.
7. Chai W, Garrelds IM, de Vries R, Danser AH. Cardioprotective effects of eplerenone in the rat heart: interaction with locally synthesized or blood-derived aldosterone? *Hypertension.* 2006;47(4):665-70.
8. Mihailidou AS, Loan Le TY, Mardini M, Funder JW. Glucocorticoids activate cardiac mineralocorticoid receptors during experimental myocardial infarction. *Hypertension.* 2009;54(6):1306-12.
9. Schmidt K, Tissier R, Ghaleb B, Drogies T, Felix SB, Krieg T. Cardioprotective effects of mineralocorticoid receptor antagonists at reperfusion. *European heart journal.* 2010;31(13):1655-62.

10. Loan Le TY, Mardini M, Howell VM, Funder JW, Ashton AW, Mihailidou AS. Low-dose spironolactone prevents apoptosis repressor with caspase recruitment domain degradation during myocardial infarction. *Hypertension.* 2012;59(6):1164-9.
11. van den Berg TN, Rongen GA, Frohlich GM, Deinum J, Hausenloy DJ, Riksen NP. The cardioprotective effects of mineralocorticoid receptor antagonists. *Pharmacology & therapeutics.* 2014;142(1):72-87.
12. Riksen NP, Rongen GA. Targeting adenosine receptors in the development of cardiovascular therapeutics. *Expert review of clinical pharmacology.* 2012;5(2):199-218.
13. Speechly-Dick ME, Grover GJ, Yellon DM. Does ischemic preconditioning in the human involve protein kinase C and the ATP-dependent K+ channel? Studies of contractile function after simulated ischemia in an atrial in vitro model. *Circulation research.* 1995;77(5):1030-5.
14. Riksen NP, Zhou Z, Oyen WJ, Jaspers R, Ramakers BP, Brouwer RM, et al. Caffeine prevents protection in two human models of ischemic preconditioning. *Journal of the American College of Cardiology.* 2006;48(4):700-7.
15. El Messaoudi S, Nederlof R, Zuurbier CJ, van Swieten HA, Pickkers P, Noyez L, et al. Effect of metformin pretreatment on myocardial injury during coronary artery bypass surgery in patients without diabetes (MetCAB): a double-blind, randomised controlled trial. *The lancet Diabetes & endocrinology.* 2015;3(8):615-23.
16. Riksen NP, Smits P, Rongen GA. Ischaemic preconditioning: from molecular characterisation to clinical application--part I. *The Netherlands journal of medicine.* 2004;62(10):353-63.
17. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, et al. Can animal models of disease reliably inform human studies? *PLoS medicine.* 2010;7(3):e1000245.
18. van den Berg TN, Deinum J, Bilos A, Donders AR, Rongen GA, Riksen NP. The effect of eplerenone on adenosine formation in humans in vivo: a double-blinded randomised controlled study. *PloS one.* 2014;9(10):e111248.
19. Bulluck H, Frohlich GM, Mohdnazri S, Gamma RA, Davies JR, Clesham GJ, et al. Mineralocorticoid receptor antagonist pretreatment to MINIMISE reperfusion injury after ST-elevation myocardial infarction (the MINIMISE STEMI Trial): rationale and study design. *Clinical cardiology.* 2015;38(5):259-66.


MENUETTO.
Tempo di Menuetto.
TRIO.
Presto.

CHAPTER 4

SUMMARY AND GENERAL DISCUSSION

SUMMARY

The adrenocortical hormone aldosterone plays a key role in blood pressure regulation. Autonomous overproduction of aldosterone, which occurs in patients with primary aldosteronism (PA), results in hypertension and/or hypokalemia. Next to the well known risks of hypertension on cardiovascular events [1, 2], it has been suggested that aldosterone itself has direct toxic effects on the cardiovascular system: preclinical data demonstrate that aldosterone promotes oxidative stress, fibrosis and apoptosis, and increases infarct size (IS) in animal models of coronary occlusion [3-8], and treatment with mineralocorticoid receptor (MR) antagonists consistently reduces IS in these models. [8] Similarly, in the human *in vivo* situation, patients with PA display a higher risk of atrial fibrillation, heart failure, myocardial infarction (MI), stroke and left ventricular hypertrophy (LVH) than patients with essential hypertension (EHT) and similar blood pressure levels [9], and treatment with MR antagonists reduces morbidity and mortality in patients with heart failure. [10-12]

To be able to improve treatment of patients with PA to prevent cardiovascular disease, it is important to better understand how aldosterone impacts on the cardiovascular system and how MR antagonists can prevent these effects. This thesis is dedicated to unravelling the adverse cardiovascular effects of aldosterone and beneficial effects of MR antagonists with the ultimate aim to improve diagnosis and treatment of patients with PA.

In chapter 2.1, we investigated whether patients with PA already have cardiovascular damage early in the disease, using an active screening strategy in patients with hypertension. Patients with a newly diagnosed hypertension from more than 50 primary care centres were systematically screened for PA. Of 361 hypertensive patients in whom an aldosterone-to-renine ratio (ARR) was calculated, the diagnosis of PA (with a positive salt loading test (SLT)) was made in 9 individuals. [13] We assessed cardiovascular risk markers in 6 of these patients with PA and 24 matched control patients with EHT. Interestingly, 2 of the patients with PA (33.3 %) compared to none of the patients

with EHT had LVH (p=0.04). We did not observe differences in ankle-brachial index, carotid intima media thickness, flow-mediated dilation (FMD), pulse wave velocity, central aortic blood pressure and urinary albumin-creatinine-ratio between patients with PA and patients with EHT. The absence of differences in these latter outcome measures can be explained by the lack of power. Nevertheless, given the difference in LVH, we conclude that in patients with PA target organ damage is present already in the early phase of their disease, suggesting that early active diagnosis and treatment might be of benefit.

The exact underlying mechanisms of the deleterious effects of aldosterone as well as the beneficial effects of MR antagonists on the cardiovascular system are not yet fully elucidated. Most of the suggested mechanisms are retrieved from preclinical studies. [8] In an elegant series of animal experiments, it has been suggested that the protective effects of MR antagonists on ischemia-reperfusion (IR) injury depend on adenosine receptor signalling. [14] Indeed, the endogenous nucleoside adenosine has powerful cardioprotective properties. [15]

In chapter 2.2, we aimed to translate these findings to the human *in vivo* situation. We hypothesized that patients with PA have reduced circulating adenosine levels and increased susceptibility to IR compared to patients with EHT. This could, at least in part, explain the higher risk of future cardiovascular events in these patients.
Although measurement of circulating adenosine levels is challenging due to its rapid half-life of less than a second, we previously validated a method to overcome the continuation of adenosine formation and breakdown in the vacutainer after blood drawing. Using a purpose-built syringe, the blood mixes immediately at the end of the needle with a solution containing pharmacological blockers of the proteins involved in adenosine metabolism. We measured forearm FMD before and after 20 minutes of ischemia and 20 minutes of reperfusion of the upper arm as a safe and well-validated model to study IR injury in the in human *in vivo* situation.
For the inclusion of our patients we used stringent diagnostic criteria to diagnose and exclude PA. Although we did not match for baseline characteristics, we ended up with 2 comparable groups of 20 patients with PA and 20 patients with EHT. There were no differences in sex distribution, age, duration of hypertension and blood pressure

between the study groups. We found that patients with PA have significantly lower levels of circulating adenosine, but unaffected susceptibility to IR.
The reduced circulating adenosine level in patients with PA provides a novel and exciting explanation for the increased risk of cardiovascular events in these patients compared to patients with EHT. Drugs beneficially affecting the adenosine metabolism could therefore potentially reduce the risk of future cardiovascular events in patients with PA.

We explored yet another underlying mechanism of the increased risk for cardiovascular complications in patients with PA in chapter 2.3. In preclinical studies, aldosterone promotes vascular and myocardial fibrosis and experimental studies demonstrate that this is mediated by galectin-3 (Gal-3) production and secretion. [16-19] We proposed that aldosterone excess stimulates Gal-3 secretion in various cell types of the cardiovascular and immune system, and that subsequently Gal-3 induces pro-fibrotic and pro-atherogenic effects in the vascular wall and myocardium in humans *in vivo*. We measured circulating Gal-3 concentrations in a retrospective cohort compromising 78 patients with PA, 56 patients with EHT, and 39 patients who had been cured from PA (post-adrenalectomy). In these well-characterized patient groups, we did not observe a difference in plasma Gal-3 values. Also after correction for potential confounders, circulating Gal-3 values were not higher in patients with PA compared to patients with EHT or cured patients with PA. Therefore, we concluded that it is highly unlikely that increases in plasma Gal-3 production contribute to the increased risk of cardiovascular events in patients with PA, compared to patients with EHT.

Finally, in chapter 3, we focused on the cardioprotective effects of MR antagonists. In chapter 3.1, we reviewed the literature on the beneficial effects of MR antagonists on IR injury and post MI remodeling. In preclinical studies, MR antagonists limit IR injury when administered either before the onset of ischemia, or just before reperfusion. [8] These cardioprotective effects of MR antagonists therefore appear to be mediated by nongenomic (rapid) intracellular signaling pathways. As described above, one of the suggested underlying mechanisms of cardioprotection by MR antagonism is adenosine receptor stimulation. [14]
In chapter 3.2, we aimed to confirm this proposed action of MR antagonists in the human *in vivo* situation. We investigated whether treatment with the MR antagonist eplerenone

increases extracellular adenosine concentrations in humans *in vivo*. To circumvent the methodological difficulties of directly measuring circulating adenosine concentrations, we measured the forearm blood flow response to the intrabrachial administration of dipyridamole as a read out parameter for extracellular adenosine formation.

To understand this model of extracellular adenosine assessment, it is important to bear in mind the human adenosine metabolism: adenosine is an endogenous purine nucleoside, which is formed by intra-, and extracellular degradation of adenosine monophosphate by the enzyme ecto-5'-nucleotidase, which is also named CD73. Degradation of adenosine occurs in the intracellular compartment. As a consequence, facilitated diffusion of adenosine over the cellular membrane by the equilibrative nucleoside transporter (ENT) is normally directed inwards. Stimulation of membrane-bound adenosine receptors induces various effects, including vasodilation, inhibition of inflammation, and protection against IR-injury. [15, 20] Dipyridamole increases the extracellular endogenous adenosine concentration by inhibition of the ENT transporter and induces local vasodilation. [20] Therefore, the vasodilator effect of dipyridamole accurately reflects extracellular adenosine formation by the CD73 enzyme. [21]

From our placebo-controlled cross-over study described in chapter 3.2 we concluded that a one-week treatment with eplerenone in a dosage of 50 mg bid does not affect extracellular adenosine formation in healthy volunteers. It is therefore unlikely that an increased extracellular adenosine formation contributes to the cardioprotective effect of mineralocorticoid receptor antagonists in the human *in vivo* situation.

However, healthy volunteers substantially differ from older patients with comorbidities. Therefore, we readdressed our hypothesis in a human *ex vivo* experiment including patients scheduled for cardiac surgery. Of these patients, we used the right atrial appendage that was harvested by the cardiothoracic surgeon before the introduction of the extracorporal circulation. We dissected two trabeculae from each appendage and suspended these in an organ bath. We induced contraction by electrical field stimulation and used the recovery of contractile force after a period of simulated IR as a well-validated endpoint of IR-injury. [22-24] From each patient, the trabeculae were randomized to either superfusion with eplerenone (10 μM) or vehicle. As described in chapter 3.3, eplerenone did not limit IR-injury in human atrial tissue *ex vivo*. Our results are conflicting with the findings in preclinical studies summarized in chapter 3.1, in which MR antagonists limit IR injury when administered just before the onset of IR.

GENERAL DISCUSSION, CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

Despite the serious risk of cardiovascular events in patients with PA, which necessitates early diagnosis and treatment, it is recognized that there is a mean delay in diagnosis of PA of approximately 8 years. [25] Most likely this is caused by the absence of the typical clinical characteristic of hypokalemia in 37-56 % of the patients with PA [25], as well as unawareness of PA amongst physicians or the presumption that PA is a rare disease not to be looked for routinely. In contrast, PA appears to be the most common cause of secondary hypertension. [26] In the primary care hypertensive population prevalence rates of PA of 3-12 % have been reported, versus 1-30 % in referral centres. [27, 28]

By systematic screening for PA in the primary care setting, in combination with extensive measurements of cardiovascular adverse effects, and by exploring novel mechanisms that could mediate the increased cardiovascular risk of patients with PA, this thesis might be helpful for improving the diagnostic and therapeutic strategy in these patients in the future.

In chapter 2.1, we reported that patients with PA display LVH already in the early phase of their disease, when early diagnosis is actively pursued by systematic screening of all patients with a novel diagnosis of hypertension in the primary care setting. Our finding, although with cautions given the small sample size, should encourage physicians to screen for PA, at least in high risk patient groups, to prevent (further) cardiovascular damage. [13] When adhering to the current international guideline however [13], none of the patients that participated in our study described in chapter 2.1 would be eligible for ARR measurement, because these patients did not have clinical clues for PA, i.e. therapy resistant hypertension or hypokalemia.

Therefore, one might suggest to screen all patients with a newly diagnosed hypertension for PA. It has been reported that ARR testing in the hypertensive population leads to a 10-fold increase in detection rate of PA and 10-fold increase in adrenalectomies. [29] Screening for PA in primary care however has proven to be

challenging: in a previous study from our centre examining the prevalence of PA in patients with a newly diagnosed, never treated hypertension, less than 10 % of the 3748 patients with a newly diagnosed hypertension were actually screened. [30] More importantly, the low prevalence rate of PA of 2.6 % (CI 1.4-4.9 %) in this primary care population questions health benefits and cost-effectiveness of screening all patients with a newly diagnosed hypertension for PA.

We included the 9 patients with PA who were diagnosed in this previous study from our centre. [30] Due to the lower than expected prevalence rate of PA, our study in chapter 2.1 did not have sufficient power to detect differences in ankle-brachial index, carotid intima media thickness, FMD, pulse wave velocity, central aortic blood pressure and urinary albumin-creatinine-ratio between patients with PA and patients with EHT. Another important limitation of our study is the use of antihypertensive drugs during the vascular experiments that may have altered the outcome measures.

A possible explanation for the low screening rate as well as low prevalence rate is that PA might be a continuous pathologic disorder, with mild symptoms and biochemical disturbances in the early phase of the disease and the development of typical, more severe clinical characteristics when PA progresses. Mildly elevated blood pressure levels, as well as the absence of hypokalemia might have withheld the general practitioners to measure aldosterone and renin concentrations in the patients with a newly diagnosed hypertension. [30] Furthermore, the sensitivity of the screening test for PA might be lower in patients with a recently diagnosed hypertension. Indeed, in a primary care hypertensive population, 43 % of patients with PA, diagnosed by a positive confirmation test, have aldosterone levels below 0.44 nmol/l. [31] Despite lowering of the cut-off value of aldosterone from 0.42 nmol/l to 0.40 nmol/l in our primary care population, it is likely that we have falsely excluded patients with hypertension from having PA. [30]

Next to limitations in the screening test for PA, no confirmation test is currently considered to be the golden standard. [13] In the studies described in this thesis, we used the SLT as confirmation test for PA. Next to safety issues because of volume overloading (2 L in 4 hours) in specific patient groups, (e.g. heart failure or severe therapy resistant hypertension), sensitivity and specificity of the SLT are restricted. When compared to the fludrocortison suppression test, which is generally accepted as a reliable confirmation test for PA, sensitivity and specificity are 85-90 % and 92-84 % respectively. [32, 33] Importantly, these numbers have to be interpreted with caution, since

the fludrocortison suppression test itself is not the golden standard. When compared to the finding of significant lateralisation of aldosterone production on AVS, in patients with a unilateral aldosterone producing adenoma (APA), sensitivity and specificity of the SLT are 83 and 75 % respectively. [34] In a recent study 29 % of patients with PA had lateralisation on AVS despite a post-SLT aldosterone <0.139 nmol/l, which is similar to the cut-off value we use to exclude PA in our centre. [35] Interestingly, these 12 patients all had potassium values between 2.7 and 3.5 mmol/l. Therefore, the authors suggest to avoid confirmation testing and perform AVS in all patients with a positive screening test for PA and hypokalemia. [35] This suggestion was adopted in the recently updated international guideline on the diagnosis of PA [13], but has not yet been implemented in our local protocol. Major drawbacks of avoiding the SLT and proceed to AVS quickly might be the costs and patients' safety.

It follows that diagnosis of PA is still challenging. Therefore, it would be highly relevant to study novel biomarkers for the diagnosis of PA. An attractive candidate is urinary prostasin, which is involved in epithelial sodium channel activation. [36, 37] In our centre, specific omics profiles for patients with PA are currently being investigated. Future studies are needed to validate their role in the identification of PA.

Using the current strategy of diagnosis and treatment of PA, patients with PA are at increased risk of cardiovascular events compared to patients with EHT. [9] In this thesis, we reported for the first time that patients with PA have lower levels of circulating adenosine than control patients with EHT and similar blood pressure levels. This finding might, at least in part, explain their higher risk of cardiovascular events compared to patients with EHT.

Indeed, endogenous adenosine induces powerful cardioprotective effects, including vasodilation and inhibition of atherosclerosis, inflammation and fibrosis, and limitation of IR injury. [15] The potential importance for this substance to impact on cardiovascular outcome is illustrated by studies reporting that genetic variants in the adenosine metabolism leading to increased endogenous adenosine formation are associated with improved cardiovascular survival in patients with coronary artery disease. [38] In addition, in patients with an acute MI, administration of exogenous adenosine limits IR injury [39, 40], although not consistently, and might protect against the development of heart failure [41]. Although increasing adenosine concentrations can limit IR injury,

most studies report that prevention of adenosine signalling does not further increase IR injury. [23, 42]

From these observations we can appreciate that a reduction in circulating adenosine levels might impair cardiovascular function, as our research group previously hypothesized in a study in patients with hyperhomocysteinemia in whom the risk of cardiovascular events is increased. In these patients, adenosine-induced vasodilation was impaired due to an increased uptake of adenosine into the intracellular compartment, limiting adenosine receptor stimulation. [43]

From our results it follows that modulation of the adenosine metabolism might reduce the excess risk of cardiovascular events in patients with PA. In chapter 3.2, we showed that treatment with MR antagonists does not increase extracellular adenosine formation in healthy humans *in vivo*. [44] It is therefore interesting to explore the effects of drugs known to increase extracellular adenosine levels. Statins are known to upregulate the enzyme CD73 [21], which converts adenosine monophosphate to adenosine. We propose that these drugs are attractive candidates to lower the risk of cardiovascular events in patients with PA. Also, dipyridamole, which is an ENT-blocker resulting in increased extracellular adenosine concentrations [45], could potentially serve this goal.

Follow up studies would definitely benefit from measurements of circulating adenosine levels before and after adrenalectomy. Also, future studies should focus on unravelling the metabolic changes driving lower adenosine levels, to pave the way towards novel pharmacological treatment of PA. The circulating concentration of adenosine is the sum of adenosine production, cellular uptake, and intracellular degradation. Activity of the enzyme CD73 did not differ between the patients with PA and EHT. Therefore, increased cellular uptake and degradation of adenosine most probably explains the lower adenosine concentration, comparable to patients with hyperhomocysteinemia. [43]

As mentioned before, endogenous adenosine has multiple effects that potentially protect against cardiovascular disease, including limitation of atherosclerosis, inhibition of thrombocyte aggregation, inhibition of inflammation and fibrosis, vasodilatation, and limiting ischemia-reperfusion injury. [15, 20] In our study, we investigated the susceptibility to forearm IR. The significant reduction in circulating adenosine levels but unaffected susceptibility to IR injury in patients with PA compared to patients with EHT can have several potential explanations. First, the beneficial effect of adenosine on IR injury

is controversial, at least in humans *in vivo*. Whilst administration of adenosine before reperfusion diminished IS in patients with an anterior wall MI [39, 40], several preclinical studies [46-48] and clinical studies [49, 50] failed to show an effect of exogenous adenosine on IR injury. In addition, even if enhanced adenosine receptor stimulation might limit IR injury, this does not necessarily mean that a reduction in adenosine receptor stimulation would augment IR injury, particularly considering the fact that many endogenous substances regulate IR susceptibility. For example, adenosine receptor antagonists did not increase IS itself in preclinical models of IR-injury [14], although these antagonists did significantly prevent the beneficial effects of ischemic pre- and post-conditioning. [23, 42]

Future studies in patients with PA should therefore not focus on IR injury, but on alternative determinants of cardiovascular damage. An attractive topic to study is atherosclerosis for several reasons. First, patients with PA have an increased risk of atherosclerotic complications, including myocardial infarction and stroke, compared to patients with EHT. [9] Second, in animal models aldosterone increases atherosclerosis and promotes plaque formation via the MR. [51, 52] Third, adenosine has anti-atherosclerotic properties [20] and in genetic deletion models, inactivation of the adenosine metabolism leads to progression of atherosclerosis [53]. We therefore propose that the reduced circulating adenosine levels in patients with PA may, at least in part, contribute to progression of atherosclerosis.

Another appealing mechanism of the excess risk of cardiovascular events in PA appeared to be overproduction of the β-galactoside-binding lectin Gal-3. Aldosterone increases Gal-3 protein levels and expression in *in vitro* and *in vivo* studies [16-19] and in patients with heart failure, circulating Gal-3 is associated with extracellular matrix markers and with adverse long-term cardiovascular outcomes. [54, 55] Also in these patients, aldosterone levels are increased and treatment with MR antagonists improves mortality and morbidity. [10-12] Indeed, higher plasma Gal-3 concentrations in patients with PA compared to EHT were observed by an Asian group (the TAIPAI study group), and levels return to normal after adrenalectomy. [19, 56] In our study described in chapter 2.3 however, we did not find differences in plasma Gal-3 concentrations between patients with PA, cured PA patients (post-adrenalectomy), and patients with EHT. [57]

Despite limitations due to the retrospective character, our study benefits from major strengths. In comparison to the small cohort of the TAIPAI study group [19, 56], we selected a large group of patients. The patients in our cohort did not use treatment interfering in the renin-angiotensin-aldosterone system and we carefully corrected our results for potential confounders. Levels of circulating Gal-3 were 10-fold lower in the cohort of the Asian study group, which may be explained by a difference in analytical method or ethnic background of the patients. [57]
In a subanalysis of our study in chapter 2.3, we compared Gal-3 levels in patients with PA with and without LVH. The observation that the difference in cardiac damage is not reflected by differences in plasma Gal-3 concentrations, excludes a role of plasma Gal-3 in aldosterone-mediated target organ damage in humans *in vivo*. [57]

In contrast to the direct cardioprotective effects of MR antagonists on IR injury and cardiac remodelling in preclinical models as described in our review in chapter 3.1 [8], we could not translate these findings to the human *in vivo* situation. [44, 58] Eplerenone did not protect against IR injury in human cardiac tissue [58], and we could not confirm a key role of adenosine receptor signalling in the beneficial effect of eplerenone. [44]
It has been recognized that many promising findings of preclinical (cardiovascular) research cannot be confirmed in human studies. [59, 60] This so-called 'translational failure' in general can be explained by methodological shortcomings in animal studies, such as the lack of a formal sample size calculation, selection bias due to the lack of randomization, unblinding, and inadequate statistical analyses. [59, 60] Also, animals substantially differ from humans with regard to physiology and pathophysiology. Alarmingly, only 2 of the 500 protective strategies regarding cerebral IR injury in animal models have been proven to be effective in humans. [59]
Another important difference between preclinical and clinical studies is the use of different end points. In chapter 2.2, we measured brachial FMD before and after upper arm ischemia, as a model for endothelial IR injury. In chapter 3.3, we used the recovery of contractile function as a marker for IR-injury. [58] These models significantly differ from the studies in animals, in which histological infarct size is used as the primary endpoint. However, the protective effect of, amongst others, IP has been confirmed in the FMD and atrial trabeculae models. [22, 23, 61]

The effect of early administration of MR antagonists on IR injury and post-infarction LV remodeling in patients with an acute MI, independent of the presence of heart failure, is currently under investigation in the MINIMISE STEMI trial. [62] From our study described in chapter 3.3, we concluded that administration of eplerenone just before the onset of ischemia does not protect against IR injury in human atrial tissue *ex vivo*. One might argue that the presence of the endogenous ligand aldosterone is required for the protective effect of MR antagonists. However, MR antagonists also confer cardioprotection in adrenalectomized rats [63] and in *ex vivo* Langendorff perfusion models [8], showing that the cardioprotective effect of MR antagonists is independent from the presence of aldosterone. The calculated concentration of eplerenone in our human atrial tissue *ex vivo* model was approximately 6 times higher than the calculated (peak) plasma levels in healthy adults after a one week treatment of eplerenone 50 mg bid. [44] In the MINIMISE STEMI trial, patients receive the parenterally available MR antagonist potassium canrenoate in a dosage of 200 mg, followed by spironolactone 50 mg od during 3 months. [62] The dosage of canrenoate is comparable and the maintenance dose of spironolactone is slighty higher than the dosages used in the ALBATROSS trial, which failed to show a beneficial effect of MR antagonism on major cardiovascular outcomes in patients admitted for MI. [64] Given these results and our own observations [58], we await the results of the MINIMISE STEMI trial with interest and scepticism.

Taken together, we showed that an active screening strategy for PA might not prevent the development of cardiac damage in these patients, as reflected by our finding that LVH is already present at the time of diagnosis of hypertension. This does not exclude however, an important role for future studies aiming at improving the diagnostic strategy for PA. Currently, there is a delay in diagnosis of PA of approximately 8 years [25], which leads to (progression of) cardiovascular damage in these patients.
Therefore, we aimed to unravel underlying mechanisms of aldosterone-mediated cardiovascular damage, with the ultimate goal to develop novel treatment strategies in patients with PA. Our exciting finding of reduced circulating adenosine levels in comparison to patients with EHT could potentially serve this goal. Future studies are needed to confirm our finding and to unravel the underlying pathway of these reduced adenosine levels. Pharmacological modulation of the adenosine metabolism could

then be a way to improve mortality and morbidity in patients with PA and bilateral aldosterone overproduction, who cannot be surgically treated. These patients are continuously exposed to high aldosterone levels and its toxic effects on the cardiovascular system, that may not be prevented completely by treatment with MR antagonists. MR antagonists have been shown to protect against IR injury and post-infarction cardiac remodelling in preclinical models, but we could not translate an effect of eplerenone on IR injury in human cardiac tissue. We are therefore reluctant to further study the effect of MR antagonism on IR injury in humans *in vivo*. Furthermore, MR antagonists did not increase extracellular adenosine formation in healthy humans *in vivo*. Future studies are needed to unravel mechanisms -other than upregulation of extracellular adenosine levels- of the cardioprotective effect of MR antagonists.

REFERENCES

1. Lawes CM, Vander Hoorn S, Rodgers A. Global burden of blood-pressure-related disease, 2001. *Lancet.* 2008;371(9623):1513-8.
2. 2013 Practice guidelines for the management of arterial hypertension of the European Society of Hypertension (ESH) and the European Society of Cardiology (ESC): ESH/ESC Task Force for the Management of Arterial Hypertension. *Journal of hypertension.* 2013;31(10):1925-38.
3. Park YM, Park MY, Suh YL, Park JB. NAD(P)H oxidase inhibitor prevents blood pressure elevation and cardiovascular hypertrophy in aldosterone-infused rats. *Biochemical and biophysical research communications.* 2004;313(3):812-7.
4. Pu Q, Neves MF, Virdis A, Touyz RM, Schiffrin EL. Endothelin antagonism on aldosterone-induced oxidative stress and vascular remodeling. *Hypertension.* 2003;42(1):49-55.
5. Azibani F, Fazal L, Chatziantoniou C, Samuel JL, Delcayre C. Aldosterone mediates cardiac fibrosis in the setting of hypertension. *Current hypertension reports.* 2013;15(4):395-400.
6. De Angelis N, Fiordaliso F, Latini R, Calvillo L, Funicello M, Gobbi M, et al. Appraisal of the role of angiotensin II and aldosterone in ventricular myocyte apoptosis in adult normotensive rat. *Journal of molecular and cellular cardiology.* 2002;34(12):1655-65.
7. Fujita M, Minamino T, Asanuma H, Sanada S, Hirata A, Wakeno M, et al. Aldosterone nongenomically worsens ischemia via protein kinase C-dependent pathways in hypoperfused canine hearts. *Hypertension.* 2005;46(1):113-7.
8. van den Berg TN, Rongen GA, Frohlich GM, Deinum J, Hausenloy DJ, Riksen NP. The cardioprotective effects of mineralocorticoid receptor antagonists. *Pharmacology & therapeutics.* 2014;142(1):72-87.
9. Monticone S, D'Ascenzo F, Moretti C, Williams TA, Veglio F, Gaita F, et al. Cardiovascular events and target organ damage in primary aldosteronism compared with essential hypertension: a systematic review and meta-analysis. *The lancet Diabetes & endocrinology.* 2018;6(1):41-50.
10. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *The New England journal of medicine.* 1999;341(10):709-17.
11. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *The New England journal of medicine.* 2003;348(14):1309-21.

12. Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, et al. Eplerenone in patients with systolic heart failure and mild symptoms. *The New England journal of medicine.* 2011;364(1):11-21.
13. Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, et al. The Management of Primary Aldosteronism: Case Detection, Diagnosis, and Treatment: An Endocrine Society Clinical Practice Guideline. *The Journal of clinical endocrinology and metabolism.* 2016;101(5):1889-916.
14. Schmidt K, Tissier R, Ghaleb B, Drogies T, Felix SB, Krieg T. Cardioprotective effects of mineralocorticoid receptor antagonists at reperfusion. *European heart journal.* 2010;31(13):1655-62.
15. Headrick JP, Ashton KJ, Rose'meyer RB, Peart JN. Cardiovascular adenosine receptors: expression, actions and interactions. *Pharmacology & therapeutics.* 2013;140(1):92-111.
16. Calvier L, Miana M, Reboul P, Cachofeiro V, Martinez-Martinez E, de Boer RA, et al. Galectin-3 mediates aldosterone-induced vascular fibrosis. *Arteriosclerosis, thrombosis, and vascular biology.* 2013;33(1):67-75.
17. Calvier L, Martinez-Martinez E, Miana M, Cachofeiro V, Rousseau E, Sadaba JR, et al. The impact of galectin-3 inhibition on aldosterone-induced cardiac and renal injuries. *JACC Heart failure.* 2015;3(1):59-67.
18. Martinez-Martinez E, Calvier L, Fernandez-Celis A, Rousseau E, Jurado-Lopez R, Rossoni LV, et al. Galectin-3 Blockade Inhibits Cardiac Inflammation and Fibrosis in Experimental Hyperaldosteronism and Hypertension. *Hypertension.* 2015.
19. Lin YH, Chou CH, Wu XM, Chang YY, Hung CS, Chen YH, et al. Aldosterone induced galectin-3 secretion in vitro and in vivo: from cells to humans. *PloS one.* 2014;9(9):e95254.
20. Riksen NP, Rongen GA. Targeting adenosine receptors in the development of cardiovascular therapeutics. *Expert review of clinical pharmacology.* 2012;5(2):199-218.
21. Meijer P, Wouters CW, van den Broek PH, de Rooij M, Scheffer GJ, Smits P, et al. Upregulation of ecto-5'-nucleotidase by rosuvastatin increases the vasodilator response to ischemia. *Hypertension.* 2010;56(4):722-7.
22. Speechly-Dick ME, Grover GJ, Yellon DM. Does ischemic preconditioning in the human involve protein kinase C and the ATP-dependent K+ channel? Studies of contractile function after simulated ischemia in an atrial in vitro model. *Circulation research.* 1995;77(5):1030-5.
23. Riksen NP, Zhou Z, Oyen WJ, Jaspers R, Ramakers BP, Brouwer RM, et al. Caffeine prevents protection in two human models of ischemic preconditioning. *Journal of the American College of Cardiology.* 2006;48(4):700-7.

24. El Messaoudi S, Nederlof R, Zuurbier CJ, van Swieten HA, Pickkers P, Noyez L, et al. Effect of metformin pretreatment on myocardial injury during coronary artery bypass surgery in patients without diabetes (MetCAB): a double-blind, randomised controlled trial. *The lancet Diabetes & endocrinology.* 2015;3(8):615-23.
25. Dhanjal TS, Beevers DG. Delay in the diagnosis of Conn's syndrome: a single-center experience over 30 years. *Hypertension.* 2008;52(3):e22; author reply e3.
26. Stowasser M, Gordon RD. Primary Aldosteronism: Changing Definitions and New Concepts of Physiology and Pathophysiology Both Inside and Outside the Kidney. *Physiological reviews.* 2016;96(4):1327-84.
27. Kayser SC, Dekkers T, Groenewoud HJ, van der Wilt GJ, Carel Bakx J, van der Wel MC, et al. Study Heterogeneity and Estimation of Prevalence of Primary Aldosteronism: a Systematic Review and Meta-regression Analysis. *The Journal of clinical endocrinology and metabolism.* 2016:jc20161472.
28. Hannemann A, Wallaschofski H. Prevalence of primary aldosteronism in patient's cohorts and in population-based studies--a review of the current literature. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme.* 2012;44(3):157-62.
29. Stowasser M, Gordon RD. Primary aldosteronism--careful investigation is essential and rewarding. *Molecular and cellular endocrinology.* 2004;217(1-2):33-9.
30. Kayser SC, Deinum J, de Grauw WJ, Schalk BW, Bor HJ, Lenders JW, et al. Prevalence of primary aldosteronism in primary care: a cross-sectional study. *The British journal of general practice : the journal of the Royal College of General Practitioners.* 2018;68(667):e114-e22.
31. Mosso L, Carvajal C, Gonzalez A, Barraza A, Avila F, Montero J, et al. Primary aldosteronism and hypertensive disease. *Hypertension.* 2003;42(2):161-5.
32. Song Y, Yang S, He W, Hu J, Cheng Q, Wang Y, et al. Confirmatory Tests for the Diagnosis of Primary Aldosteronism: A Prospective Diagnostic Accuracy Study. *Hypertension.* 2018;71(1):118-24.
33. Mulatero P, Milan A, Fallo F, Regolisti G, Pizzolo F, Fardella C, et al. Comparison of confirmatory tests for the diagnosis of primary aldosteronism. *The Journal of clinical endocrinology and metabolism.* 2006;91(7):2618-23.
34. Rossi GP, Belfiore A, Bernini G, Desideri G, Fabris B, Ferri C, et al. Prospective evaluation of the saline infusion test for excluding primary aldosteronism due to aldosterone-producing adenoma. *Journal of hypertension.* 2007;25(7):1433-42.

35. Cornu E, Steichen O, Nogueira-Silva L, Kupers E, Pagny JY, Grataloup C, et al. Suppression of Aldosterone Secretion After Recumbent Saline Infusion Does Not Exclude Lateralized Primary Aldosteronism. *Hypertension.* 2016;68(4):989-94.
36. van der Lubbe N, Jansen PM, Salih M, Fenton RA, van den Meiracker AH, Danser AH, et al. The phosphorylated sodium chloride cotransporter in urinary exosomes is superior to prostasin as a marker for aldosteronism. *Hypertension.* 2012;60(3):741-8.
37. Pizzolo F, Chiecchi L, Morandini F, Castagna A, Zorzi F, Zaltron C, et al. Increased urinary excretion of the epithelial Na channel activator prostasin in patients with primary aldosteronism. *Journal of hypertension.* 2017;35(2):355-61.
38. Anderson JL, Habashi J, Carlquist JF, Muhlestein JB, Horne BD, Bair TL, et al. A common variant of the AMPD1 gene predicts improved cardiovascular survival in patients with coronary artery disease. *Journal of the American College of Cardiology.* 2000;36(4):1248-52.
39. Mahaffey KW, Puma JA, Barbagelata NA, DiCarli MF, Leesar MA, Browne KF, et al. Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction: results of a multicenter, randomized, placebo-controlled trial: the Acute Myocardial Infarction STudy of ADenosine (AMISTAD) trial. *Journal of the American College of Cardiology.* 1999;34(6):1711-20.
40. Ross AM, Gibbons RJ, Stone GW, Kloner RA, Alexander RW. A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). *Journal of the American College of Cardiology.* 2005;45(11):1775-80.
41. Bulluck H, Sirker A, Loke YK, Garcia-Dorado D, Hausenloy DJ. Clinical benefit of adenosine as an adjunct to reperfusion in ST-elevation myocardial infarction patients: An updated meta-analysis of randomized controlled trials. *International journal of cardiology.* 2016;202:228-37.
42. Methner C, Schmidt K, Cohen MV, Downey JM, Krieg T. Both A2a and A2b adenosine receptors at reperfusion are necessary to reduce infarct size in mouse hearts. *American journal of physiology Heart and circulatory physiology.* 2010;299(4):H1262-4.
43. Riksen NP, Rongen GA, Boers GH, Blom HJ, van den Broek PH, Smits P. Enhanced cellular adenosine uptake limits adenosine receptor stimulation in patients with hyperhomocysteinemia. *Arteriosclerosis, thrombosis, and vascular biology.* 2005;25(1):109-14.
44. van den Berg TN, Deinum J, Bilos A, Donders AR, Rongen GA, Riksen NP. The effect of eplerenone on adenosine formation in humans in vivo: a double-blinded randomised controlled study. *PloS one.* 2014;9(10):e111248.

45. Meijer P, Wouters CW, van den Broek PH, Scheffer GJ, Riksen NP, Smits P, et al. Dipyridamole enhances ischaemia-induced reactive hyperaemia by increased adenosine receptor stimulation. *British journal of pharmacology.* 2008;153(6):1169-76.
46. Goto M, Miura T, Iliodoromitis EK, O'Leary EL, Ishimoto R, Yellon DM, et al. Adenosine infusion during early reperfusion failed to limit myocardial infarct size in a collateral deficient species. *Cardiovascular research.* 1991;25(11):943-9.
47. Vander Heide RS, Reimer KA. Effect of adenosine therapy at reperfusion on myocardial infarct size in dogs. *Cardiovascular research.* 1996;31(5):711-8.
48. Xu Z, Downey JM, Cohen MV. Amp 579 reduces contracture and limits infarction in rabbit heart by activating adenosine A2 receptors. *Journal of cardiovascular pharmacology.* 2001;38(3):474-81.
49. Desmet W, Bogaert J, Dubois C, Sinnaeve P, Adriaenssens T, Pappas C, et al. High-dose intracoronary adenosine for myocardial salvage in patients with acute ST-segment elevation myocardial infarction. *European heart journal.* 2011;32(7):867-77.
50. Nazir SA, McCann GP, Greenwood JP, Kunadian V, Khan JN, Mahmoud IZ, et al. Strategies to attenuate micro-vascular obstruction during P-PCI: the randomized reperfusion facilitated by local adjunctive therapy in ST-elevation myocardial infarction trial. *European heart journal.* 2016;37(24):1910-9.
51. Moss ME, Jaffe IZ. Mineralocorticoid Receptors in the Pathophysiology of Vascular Inflammation and Atherosclerosis. *Frontiers in endocrinology.* 2015;6:153.
52. van der Heijden C, Deinum J, Joosten LAB, Netea MG, Riksen NP. The mineralocorticoid receptor as a modulator of innate immunity and atherosclerosis. *Cardiovascular research.* 2018;114(7):944-53.
53. Buchheiser A, Ebner A, Burghoff S, Ding Z, Romio M, Viethen C, et al. Inactivation of CD73 promotes atherogenesis in apolipoprotein E-deficient mice. *Cardiovascular research.* 2011;92(2):338-47.
54. de Boer RA, Daniels LB, Maisel AS, Januzzi JL, Jr. State of the Art: Newer biomarkers in heart failure. *European journal of heart failure.* 2015;17(6):559-69.
55. Lopez-Andres N, Rossignol P, Iraqi W, Fay R, Nuee J, Ghio S, et al. Association of galectin-3 and fibrosis markers with long-term cardiovascular outcomes in patients with heart failure, left ventricular dysfunction, and dyssynchrony: insights from the CARE-HF (Cardiac Resynchronization in Heart Failure) trial. *European journal of heart failure.* 2012;14(1):74-81.
56. Liao CW, Lin YT, Wu XM, Chang YY, Hung CS, Wu VC, et al. The relation among aldosterone, galectin-3, and myocardial fibrosis: a prospective clinical pilot follow-up study. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research.* 2016;64(6):1109-13.

57. van den Berg T, Meijers WC, Donders ART, Van Herwaarden AE, Rongen GA, de Boer RA, et al. Plasma galectin-3 concentrations in patients with primary aldosteronism. *Journal of hypertension.* 2017;35(9):1849-56.

58. van den Berg TN, van Swieten HA, Vos JC, Verweij V, Wouterse AC, Deinum J, et al. Eplerenone does not limit ischemia-reperfusion injury in human myocardial tissue. *International journal of cardiology.* 2016;216:110-3.

59. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, et al. Can animal models of disease reliably inform human studies? *PLoS medicine.* 2010;7(3):e1000245.

60. Ramirez FD, Motazedian P, Jung RG, Di Santo P, MacDonald ZD, Moreland R, et al. Methodological Rigor in Preclinical Cardiovascular Studies: Targets to Enhance Reproducibility and Promote Research Translation. *Circulation research.* 2017;120(12):1916-26.

61. Kharbanda RK, Peters M, Walton B, Kattenhorn M, Mullen M, Klein N, et al. Ischemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischemia-reperfusion in humans in vivo. *Circulation.* 2001;103(12):1624-30.

62. Bulluck H, Frohlich GM, Mohdnazri S, Gamma RA, Davies JR, Clesham GJ, et al. Mineralocorticoid receptor antagonist pretreatment to MINIMISE reperfusion injury after ST-elevation myocardial infarction (the MINIMISE STEMI Trial): rationale and study design. *Clinical cardiology.* 2015;38(5):259-66.

63. Mihailidou AS, Loan Le TY, Mardini M, Funder JW. Glucocorticoids activate cardiac mineralocorticoid receptors during experimental myocardial infarction. *Hypertension.* 2009;54(6):1306-12.

64. Beygui F, Cayla G, Roule V, Roubille F, Delarche N, Silvain J, et al. Early Aldosterone Blockade in Acute Myocardial Infarction: The ALBATROSS Randomized Clinical Trial. *Journal of the American College of Cardiology.* 2016;67(16):1917-27.


F
pp
fp
do
ff
H
mf p
ff
I
pp
Tempo di Menuetto.
p
cre
sce
do
p
dim.
p
dimin.
p
cresc.
ff

CHAPTER 5

NEDERLANDSE SAMENVATTING EN DISCUSSIE

SAMENVATTING

Het hormoon aldosteron, dat in onze bijnierschors wordt geproduceerd, speelt een belangrijke rol in de bloeddrukregulatie. Overproductie van aldosteron, zoals bij patiënten met primair hyperaldosteronisme (PA), resulteert in hypertensie (hoge bloeddruk) en/of hypokaliëmie (een laag kaliumgehalte in het bloed). Bij de meeste patiënten met hypertensie is de oorzaak hiervan overigens niet bekend. We spreken in dat geval van essentiële hypertensie (EHT).

Naast de bekende negatieve gevolgen van hypertensie op hart- en vaatziekten [1, 2], wordt gesuggereerd dat aldosteron zelf directe schadelijke effecten heeft op ons hart- en vaatstelsel: laboratorium- en dierexperimentele studies hebben aangetoond dat aldosteron zorgt voor meer schade door reactieve zuurstofverbindingen, bindweefselvorming, celdood en een toename in de infarctgrootte bij dieren met een hartinfarct [3-8].

Behandeling met medicamenten die de werking van aldosteron blokkeren, zogenaamde mineralocorticoïd receptor (MR) antagonisten, verminderen op consequente wijze de infarctgrootte in deze diermodellen. [8]

Patiënten met PA blijken een hoger risico op bezoemfibrilleren (een hartritmestoornis), hartfalen, het krijgen van een hartinfarct en het ontwikkelen van linkerventrikelhypertrofie (verdikte hartspier) te hebben dan patiënten met EHT en vergelijkbare bloeddrukken. [9] Behandeling met MR antagonisten vermindert de totale sterfte, sterfte door hart- en vaatziekten en het aantal ziekenhuisopnames voor hart- en vaatziekten bij patiënten met hartfalen. [10-12]

Om de behandeling van patiënten met PA te kunnen optimaliseren met als doel om hart- en vaatziekten bij deze patiënten te voorkomen, is het belangrijk om te begrijpen welke effecten aldosteron heeft op ons hart- en vaatstelsel en hoe MR antagonisten dit kunnen voorkomen. In dit proefschrift is gepoogd om de onderliggende schadelijke effecten van aldosteron alsmede de gunstige effecten van MR antagonisten op ons hart- en vaatstelsel te ontrafelen, met het uiteindelijke doel om diagnostiek naar en behandeling van PA te verbeteren.

In hoofdstuk 2.1 onderzochten we door middel van een actieve screeningsmethode of patiënten met PA al hart- en vaatschade hebben in de vroege fase van hun ziekte. Patiënten met een nieuw gediagnosticeerde hypertensie uit meer dan 50 huisartspraktijken werden gescreend op het hebben van PA. Screening op PA geschiedt door middel van het meten van aldosteron en renine in een bloedmonster. Vervolgens dient de diagnose te worden bevestigd. In het Radboudumc wordt hiervoor de zoutbelastingstest gebruikt, waarbij patiënten in 4 uur tijd 2 L fysiologisch zout via een infuus krijgen toegediend. Bij gezonde personen zorgt het toedienen van vocht voor een onderdrukking van de aanmaak van renine en aldosteron. Bij patiënten met PA blijft de aldosteronproductie echter verhoogd direct na de test.
Van de 361 hypertensieve patiënten bij wie een aldosteron en renine in een bloedmonster werd bepaald, werd bij uiteindelijk 9 patiënten de diagnose PA gesteld door middel van een positieve zoutbelastingstest. [13] We vergeleken 6 verschillende markers voor hart- en vaatschade tussen 6 patiënten met PA en 24 patiënten met EHT. Wij vonden dat in 2 van de patiënten met PA (33.3 %), vergeleken met geen enkele patiënt met EHT, reeds linkerventrikelhypertrofie aanwezig was. We vonden geen verschillen in de andere 5 markers voor hart- en vaatschade tussen de patiënten met PA en patiënten met EHT, hetgeen verklaard kan worden door het kleine aantal proefpersonen.
Echter, vanwege het significant vaker voorkomen van linkerventrikelhypertrofie hebben wij geconcludeerd dat bij patiënten met PA orgaanschade al aanwezig is in de vroege fase van hun ziekte. Het is daarom belangrijk om deze patiënten in een vroeg stadium op te sporen en ze tijdig te behandelen.

De onderliggende mechanismen van de schadelijke effecten van aldosteron alsmede de gunstige effecten van MR antagonisten op ons hart- en vaatstelsel zijn nog niet volledig opgehelderd. De meeste voorgestelde mechanismen werden verkregen uit laboratorium- en dierexperimentele onderzoeken. Zo is van MR antagonisten aangetoond dat zij kunnen beschermen tegen ischemie-reperfusie (IR) schade. [8] IR schade is de schade die resteert na een periode van ischemie (zuurstofgebrek door een verminderde of afwezige bloeddoorstroming) ondanks reperfusie (het herstellen van de bloeddoorstroming). In een elegante serie van dierexperimenten werd aangetoond dat het beschermende effect van MR antagonisten op IR schade afhankelijk is van

stimulatie van de adenonisinereceptor. [14] Adenosine is een bio-organische verbinding met krachtige beschermende effecten op hart- en bloedvaten. [15]

In hoofdstuk 2.2 vertaalden we de bevindingen van deze dierexperimentele studie naar de mens. Onze hypothese was dat patiënten met PA lagere adenosineconcentraties in hun bloed hebben en (hierdoor) en verhoogde gevoeligheid voor IR dan patiënten met EHT. Dit zou, in ieder geval gedeeltelijk, het hoger risico op toekomstige hart- en vaatziekten bij deze patiënten kunnen verklaren.
Het meten van adenosineconcentraties in het bloed is zeer gecompliceerd, omdat binnen een seconde de helft alweer wordt afgebroken. In het verleden is op de afdeling Farmacologie-Toxicologie echter een betrouwbare methode ontwikkeld om de adenosinevorming en –afbraak na bloedafname te voorkomen. Hiervoor wordt een speciale spuit gebruikt, waarbij het bloed aan het eind van de naald meteen gemixt wordt met een oplossing van blokkers van eiwitten die betrokken zijn in het adenosinemetabolisme.
Voor het meten van IR schade bij de mens gebruikten we een alternatieve, maar veilige en betrouwbare methode, namelijk het meten van de mate van vaatverwijding in de armslagader, vóór en na 20 minuten durende ischemie en 20 minuten durende reperfusie. Ischemie van de bovenarm werd gecreëerd door met een volledig opgepompte bloeddrukband de bloedtoevoer naar de arm te onderbreken.
Voor de selectie van de patiënten in deze studie gebruikten we strenge diagnostische criteria om PA te bevestigen dan wel uit te sluiten. We eindigden met 2 vergelijkbare groepen van 20 patiënten met PA en 20 patiënten met EHT. Er waren geen verschillen in man-vrouw verdeling, leeftijd, duur van hypertensie en de hoogte van de bloeddruk tussen de studiegroepen. De belangrijkste bevinding in onze studie was dat patiënten met PA significant lagere adenosineconcentraties in het bloed hebben dan patiënten met EHT, maar dat er geen verschil is in de gevoeligheid voor IR tussen beide studiegroepen. De verlaagde adenosineconcentraties bij patiënten met PA verschaft een nieuwe en spannende verklaring voor het verhoogd risico op hart- en vaatziekten bij deze groep patiënten, in vergelijking met patiënten met EHT. Medicamenten die op een gunstige manier het adenosinemetabolisme stimuleren, zouden mogelijkerwijs het risico op toekomstige hart- en vaatziekten bij patiënten met PA kunnen verlagen.

We bestudeerden een ander onderliggend mechanisme van het verhoogd risico op hart- en vaatschade bij patiënten met PA in hoofdstuk 2.3. Aldosteron heeft in laboratorium- en dierexperimentele onderzoeken laten zien bindweefselvorming in bloedvaten en het hart te stimuleren en uit experimentele studies bleek dat dit effect werd bewerkstelligd via galectine-3 (Gal-3)-productie en -uitscheiding. [16-19] Naast bindweefselvorming is Gal-3 belangrijk in veel andere biologische processen in ons lichaam, zoals celgroei en celdood.
Onze hypothese in hoofdstuk 2.3 luidde dat overproductie van aldosteron Gal-3-uitscheiding stimuleert in verschillende celtypes van ons hart- en vaatstelsel en het immuunsysteem, leidend tot bindweefselvorming en slagaderverkalking in de vaatwand en het hart bij mensen. Bij 78 patiënten met PA, 56 patiënten met EHT en 39 patiënten die na bijnierverwijdering van PA genezen waren, hebben we Gal-3 concentraties in een bloedmonster gemeten. In deze goed gekarakteriseerde patiënten zagen wij geen verschillen in Gal-3 waarden tussen de 3 groepen. Ook na correctie voor mogelijke beïnvloedende variabelen bleken de Gal-3 concentraties niet hoger in patiënten met PA dan in de patiënten met EHT of de van PA genezen patiënten. Hieruit concludeerden we dat het onaannemelijk is dat Gal-3 in het bloed bijdraagt aan het verhoogd risico op hart- en vaatziekten bij patiënten met PA ten opzichte van patiënten met EHT.

Tenslotte hebben we onze aandacht in hoofdstuk 3 op de beschermende effecten van MR antagonisten op het hart- en bloedvaten gericht. In hoofdstuk 3.1 hebben wij een overzicht van de literatuur over de beschermende effecten van MR antagonisten op IR schade en remodellering van het hart na een hartinfarct gegeven. Laboratorium- en dierexperimentele studies hebben een gunstig effect van MR antagonisten op IR schade aangetoond wanneer zij werden toegediend net voor de fase van ischemie, of net voordat gestart werd met reperfusie. [8] Deze beschermende effecten van MR antagonisten lijken derhalve te worden veroorzaakt door non-genomische (snelle) signaalroutes in de cel. Zoals hierboven reeds beschreven, is een van de voorgestelde onderliggende mechanismen van deze beschermende effecten op het hart door MR antagonisten stimulering van de adenosinereceptor. [14]
In hoofdstuk 3.2 onderzochten we dit veronderstelde effect van MR antagonisten bij de mens. We onderzochten of behandeling met de MR antagonist eplerenon bij gezonde vrijwilligers zorgt voor een toename in de adenosineconcentratie in het bloed. Om de

moeilijkheden van metingen van adenosineconcentraties in bloed te omzeilen, gebruikten we een indirecte manier om de hoeveelheid adenosine te evalueren. We gebruikten een van de effecten van adenosine als uitleesmaat voor de hoeveelheid gevormde adenosine in het bloed, namelijk de mate van vaatverwijding in de arm als respons op toediening van het medicijn dipyridamol in de onderarmsslagader. Om dit model van adenosinevorming te kunnen begrijpen, is het belangrijk om het adenosinemetabolisme van de mens te kennen: adenosine wordt gevormd uit de afbraak van adenosine monofosfaat door het enzym ecto-5'-nucleotidase, ook wel CD73 genaamd. Verdere afbraak van adenosine gebeurt in de cel. Als gevolg daarvan zal adenosine over het celmembraan via een transporter (de equilibratieve nucleoside transporter (ENT)) de cel in gaan. Stimulatie van adenosinereceptoren op het celmembraan induceert verschillende effecten, zoals vaatverwijding, remming van ontsteking en bescherming tegen IR schade. [15, 20] Dipyridamol verhoogt de adenosinedeconcentratie buiten de cel door de ENT te remmen (zodat adenosine niet de cel in kan gaan) en zorgt dus voor lokale vaatverwijding. [20] Derhalve weerspiegelt het vaatverwijdende effect van dipyridamol de adenosinevorming door het enzym CD73. [21]

Uit onze in hoofdstuk 3.2 beschreven studie concludeerden we dat de MR antagonist eplerenon, in een dosering van tweemaal per dag 50 mg gedurende een week, geen invloed heeft op de adenosinevorming in gezonde mannelijke vrijwilligers. Het is daarom onwaarschijnlijk dat een verhoogde adenosinevorming bij mensen bijdraagt aan de beschermende effecten van MR antagonisten op het hart.

Echter, gezonde vrijwilligers verschillen substantieel van oudere patiënten met één of meerdere ziekten. Daarom onderzochten we onze hypothese opnieuw, maar dan in een experiment met menselijk hartweefsel, van patiënten die een open hartoperatie ondergingen. Van deze patiënten gebruikten we het rechter hartoor, dat verwijderd werd door de hartchirurg voor aansluiting van de hart-longmachine. Uit het hartoor van elke patiënt werden 2 afzonderlijke hartspiertjes vrijgemaakt en gefixeerd in een orgaanbad. We induceerden contractiliteit (samentrekking) van deze hartspiertjes door elektrische stimulatie en gebruikten het herstel van de contractiliteit na een periode van ischemie en reperfusie als betrouwbaar eindpunt voor IR schade. [22-24] Van elke patiënt werd één hartspiertje blootgesteld aan eplerenon en de andere aan het oplosmiddel. Zoals beschreven in hoofdstuk 3.3 zorgde eplerenon niet voor een vermindering van IR schade in menselijk hartspierweefsel. Onze resultaten conflicteren met de bevindingen

samengevat in hoofdstuk 3.1, waarin MR antagonisten IR schade beperken wanneer ze worden toegediend op het moment voor IR.

DISCUSSIE, KLINISCHE IMPLICATIES EN TOEKOMSTPERSPECTIEVEN

Ondanks het verhoogd risico op hart- en vaatziekten bij patiënten met PA, waardoor vroegtijdig diagnosticeren en behandeling noodzakelijk is, weten we dat er een vertraging van gemiddeld 8 jaar bestaat in het diagnosticeren van PA. [25] Waarschijnlijk komt dit door de afwezigheid van de typische hypokaliëmie in 37-56 % van de patiënten met PA [25], alsmede het gebrek aan aandacht voor PA onder artsen of de aanname dat PA zeldzaam is en derhalve niet standaard onderzocht hoeft te worden. Integendeel, PA blijkt de meest voorkomende secundaire vorm van hypertensie. [26] In de huisartsenpraktijk komt PA bij 3-12 % van de patiënten met hypertensie voor, vergeleken met 1-30 % in ziekenhuizen. [27, 28]

Door het systematisch screenen op PA in de huisartsenpraktijk in combinatie met uitgebreide evaluaties van markers van hart- en vaatschade en door ontrafeling van nieuwe onderliggende mechanismen die bijdragen aan het verhoogd risico op hart- en vaatziekten bij patiënten met PA, draagt dit proefschrift bij aan de verbetering van de diagnostische en therapeutische behandeling van deze patiënten in de toekomst.

In hoofdstuk 2.1 lieten we zien dat patiënten met PA al linkerventrikelhypertrofie hebben in de vroege fase van hun ziekte, namelijk op het moment van vaststellen van hypertensie door de huisarts. Onze bevinding, hoewel deze met voorzichtigheid moet worden geïnterpreteerd vanwege de kleine aantallen, zou artsen moeten aanmoedigen om te zoeken naar PA om (verdere) hart- en vaatschade te beperken. [13] Als we echter kijken naar de adviezen in de huidige internationale richtlijn [13], zou in geen van de patiënten in onze studie een aldosteron en renine bepaald zijn, omdat deze patiënten geen aanwijzingen voor PA hadden, zoals therapieresistente hypertensie of hypokaliëmie.

Daarom zou men kunnen voorstellen om alle patiënten met een nieuw gediagnosticeerde hypertensie te screenen op PA. Het is beschreven dat screening op PA bij patiënten met hypertensie leidt tot een tienvoudige toename in het detecteren van PA en tevens een tienvoudige toename in het aantal bijnierverwijderingen. [29] Het screenen op PA in de huisartsenpraktijk is echter uitdagend gebleken: in een voorgaande studie waarin de prevalentie (het vóórkomen) van PA bij patiënten met een nieuw gediagnosticeerde hypertensie werd onderzocht, bleek de screening in minder dan 10 % van de 3748 patiënten te zijn uitgevoerd. [30] Belangrijker nog: PA bleek nauwelijks voor te komen in de huisartsenpraktijk, namelijk maar bij 2.6 % (BI 1.4-4.9 %) van de patiënten met hypertensie, hetgeen gezondheidswinst en kosteneffectiviteit van een dergelijke screening op PA in twijfel trekt.

We includeerden 6 patiënten met PA, die in de voorgaande studie werden gediagnosticeerd. [30] Door de veel lager dan verwachte prevalentie van PA had onze studie in hoofdstuk 2.1 niet genoeg kracht om verschillen in 5 van de 6 markers voor hart- en vaatschade tussen de patiënten met PA en patiënten met EHT aan te tonen. Een andere belangrijke beperking van onze studie was het gebruik van bloeddrukverlagende medicijnen tijdens de vaatmetingen, waardoor onze resultaten vertroebeld kunnen zijn. Een mogelijke verklaring voor het lage screeningspercentage alsmede de lage prevalentie van PA is de mogelijkheid dat PA een langzaam voortschrijdende ziekte is, met milde symptomen en milde bloedafwijkingen in de beginfase van de ziekte en het ontwikkelen van meer typische en ernstigere karakteristieken wanneer PA zich verder ontwikkelt. Mild verhoogde bloeddrukken en de afwezigheid van hypokaliëmie zou huisartsen ervan weerhouden kunnen hebben om aldosteron -en renineconcentraties te meten bij de patiënten met een nieuw gediagnosticeerde hypertensie. [30] Ook zou het zo kunnen zijn dat de waarde van de screeningstest voor PA beperkter is bij patiënten met een nieuw gediagnosticeerde hypertensie. Inderdaad is gebleken dat bij een groep patiënten met hypertensie in de huisartsenpraktijk 43 % van de patiënten met PA een lage aldosteronwaarde in het bloed had. [31] Ondanks het verlagen van de ondergrens van de afkapwaarde van aldosteron van 0.42 nmol/l naar 0.40 nmol/l, is het zeer goed mogelijk dat we bij patiënten met hypertensie de diagnose PA ten onrechte hebben uitgesloten. [30]

Naast beperkingen van de screeningstest voor PA, is geen enkele bevestigingstest bestempeld als de gouden standaard. [13] In de studies in dit proefschrift hebben

we de zoutbelastingstest als bevestigingstest voor PA gebruikt. Het toedienen van het volume van 2 L kan uitdagend zijn, met name bij patiënten met hartfalen of therapieresistente hypertensie. Daarnaast heeft de zoutbelastingstest een beperkte sensitiviteit en specificiteit. Deze 2 termen beschrijven de waarde van een medische test. Sensitiviteit beschrijft het percentage terecht positieve uitslagen onder zieke patiënten en specificiteit beschrijft het percentage terecht negatieve uitslagen onder niet-zieke patiënten. Vergeleken met een fludrocortison suppressietest, hetgeen wordt beschouwd als een betrouwbare bevestigingstest voor PA, zijn de sensitiviteit en specificiteit van de zoutbelastingstest respectievelijk 85-90 % en 92-84 %. [32, 33] Uiteraard dienen deze getallen met voorzichtigheid te worden geïnterpreteerd, omdat de fludrocortison suppressietest zelf ook geen gouden standaard is. Als we de zoutbelastingstest vergelijken met bijnieradersampling bij patiënten met een eenzijdige overproductie van aldosteron (dus in één van de beide bijnieren), dan zijn de sensitiviteit en specificiteit respectievelijk 83 en 75 %. [34] In een recente studie bleek 29 % van de patiënten met PA eenzijdige overproductie bij bijnieradersampling te hebben ondanks een aldosteron van <0.139 nmol/l na de zoutbelastingstest, hetgeen ook de afkapwaarde is om PA uit te sluiten in ons ziekenhuis. [35] Interessant genoeg bleek deze 29 % een kaliumwaarde in het bloed tussen 2.7 en 3.5 mmol/l te hebben (normaalwaarde: 3.5-5.0 mmol/L). Daarom suggereren de auteurs van de studie om bevestigingstesten over te slaan bij patiënten met een positieve screeningstest voor PA én hypokaliëmie. [35] Deze suggestie is overgenomen in de nieuwe versie van de internationale richtlijn over de diagnose en behandeling van PA [13], maar nog niet in ons lokale protocol. Nadelen van het overslaan van de bevestigingstest en dus meteen naar bijnieradersampling door te pakken zouden de kosten en patiëntveiligheid kunnen zijn.

Het is duidelijk dat diagnostiek van PA nog altijd uitdagend is. Daarom zou het heel waardevol zijn om nieuwe biomarkers voor de diagnose van PA te vinden. Het eiwit prostasine in de urine, dat betrokken is in het openen van natriumzoutkanalen in onze cellen, is een aantrekkelijke kandidaat. [36, 37] In ons ziekenhuis worden momenteel specifieke profielen van markers voor patiënten met PA onderzocht. Uiteraard is hierin nog veel onderzoek nodig.

Met de huidige diagnostische strategie en behandeling hebben patiënten met PA een verhoogd risico op hart- en vaatziekten vergeleken met patiënten met EHT en

vergelijkbare bloeddrukken. [9] In dit proefschrift hebben wij voor het eerst aangetoond dat patiënten met PA lagere adenosineconcentraties in hun bloed hebben dan patiënten met EHT en vergelijkbare bloeddrukken. Deze bevinding zou, in ieder geval gedeeltelijk, een verklaring kunnen zijn voor dit verhoogd risico bij patiënten met PA.

Het is inderdaad zo dat het lichaamseigen adenosine krachtige beschermende effecten op het hart- en vaatstelsel heeft, zoals vaatverwijding en het beperken van IR schade, slagaderverkalking, ontsteking en bindweefselvorming. [15] De mogelijke gunstige invloed van adenosine op hart- en vaatziekten wordt geïllustreerd door onderzoeken die rapporteren dat genetische varianten in het adenosinemetabolisme die leiden tot een verhoogde adenosinevorming, geassocieerd zijn met een langere overleving bij patiënten met kransslagaderziekten. [38] Daarnaast beschermt het toedienen van adenosine bij patiënten met een acuut hartinfarct tegen IR schade [39, 40], hoewel dit niet consequent in de literatuur wordt beschreven. Toediening van adenosine zou mogelijk ook beschermen tegen het ontwikkelen van hartfalen. [41] Hoewel oplopende adenosineconcentraties IR schade kunnen beperken, beschrijven de meeste studies dat het voorkomen van adenosinesignallering IR schade niet verder vergroot. [23, 42]

Uit deze observaties kunnen we herleiden dat een afname in adenosinewaarden in het bloed een negatieve invloed op ons hart- en vaatstelsel heeft, zoals ook eerder door onze onderzoeksgroep werd voorgesteld bij patiënten met de stofwisselingsziekte hyperhomocysteïnemie, bij wie het risico op hart- en vaatziekten is vergroot. Bij deze patiënten bleek het vaatverwijdende effect van adenosine beperkt door een verhoogde opname van adenosine in de cel, waarbij stimulatie van de adenosinereceptor voorkomen werd. [43]

Uit onze resultaten zouden we kunnen opmaken dat beïnvloeding van het adenosinemetabolisme het verhoogd risico op hart- en vaatziekten bij patiënten met PA kan verlagen. In hoofdstuk 3.2 hebben we laten zien dat MR antagonisten de adenosinevorming buiten de cel niet verhogen bij gezonde vrijwilligers. [44] Het is daarom interessant om medicamenten te testen waarvan we weten dat ze adenosineconcentraties in het bloed wél verhogen. Van statines (cholesterolverlagers) is bekend zij dat het enzym CD73, dat adenosinemonofosfaat omzet in adenosine, stimuleren. [21] Een statine is dan ook een aantrekkelijke kandidaat om het risico op hart- en vaatziekten bij patiënten met PA te verlagen. Ook dipyridamol, dat een ENT-blokker is en leidt tot een toename van adenosineconcentraties buiten de cel [45], zou dit effect kunnen hebben.

Het zou absoluut interessant zijn om in toekomstig onderzoek bij patiënten met PA adenosineconcentraties in het bloed te meten voor en na bijnierverwijdering. Ook zou in toekomstig onderzoek de nadruk gelegd moeten worden op het ontrafelen van de oorzaak van de lagere adenosineconcentraties bij patiënten met PA, zodat nieuwe therapeutische opties beschikbaar kunnen komen. De adenosineconcentratie in het bloed is de som van adenosineproductie, opname van adenosine in de cel en afbraak van adenosine in de cel. In onze studie was geen verschil in activiteit van het enzym CD73 tussen patiënten met PA en patiënten met EHT, dus een verhoogde opname in de cel of verhoogde afbraak van adenosine in de cel is de meest waarschijnlijke verklaring voor de verlaagde adenosineconcentraties bij patiënten met PA, net zoals bij patiënten met hyperhomocysteïnemie. [43]

Zoals hierboven vermeld, heeft adenosine verschillende effecten die mogelijk bescherming bieden voor het ontwikkelen van hart- en vaatziekten, zoals beperken van slagaderverkalking, remming van bloedplaatjesklontering, remming van ontsteking en bindweefselvorming, vaatverwijding en beperking van IR schade. [15, 20] In ons onderzoek hebben we de gevoeligheid voor onderarms-IR onderzocht. Het feit dat deze niet verschilde tussen patiënten met PA en patiënten EHT, terwijl de adenosineconcentratie in het bloed wel significant lager was bij patiënten met PA, kan verklaard worden door verschillende zaken. Ten eerste is het gunstige effect van adenosine op IR schade controversieel, in ieder geval bij mensen. Terwijl toediening van adenosine vóór reperfusie (herstel van bloedtoevoer) de hartinfarctgrootte verminderde bij patiënten met een hartinfarct [39, 40], hebben verschillende dierexperimenten [46-48] en klinische onderzoeken [49, 50] geen effect van toediening van adenosine op IR schade kunnen constateren. En ook al zou stimulatie van de adenosinereceptor IR schade kunnen beperken, dan betekent dit nog niet meteen dat een afname in stimulatie van de adenosinereceptor IR schade vergroot, vooral vanwege het gegeven dat veel andere lichaamseigen stoffen de gevoeligheid voor IR reguleren. Als voorbeeld hebben adenosinereceptorantagonisten in diermodellen van IR schade laten zien de hartinfarctgrootte niet te vergroten [14], hoewel deze antagonisten wel de gunstige effecten van pre- en post-conditionering voorkomen. [23, 42] Pre- en postconditionering is het herhaaldelijk toebrengen van korte periodes van zuurstoftekort, respectievelijk voor of na IR, om IR schade te voorkomen.

Toekomstige onderzoeken bij patiënten met PA zouden zich daarom niet moeten richten op IR schade, maar op alternatieve determinanten van hart- en vaatschade. Om verschillende redenen is slagaderverkalking een aantrekkelijk onderwerp om te bestuderen. Ten eerste hebben patiënten met PA een hoger risico op complicaties van slagaderverkalking dan patiënten met EHT, zoals een hartinfarct en beroerte. [9] Ten tweede is in diermodellen aangetoond dat aldosteron slagaderverkalking verergert en dat verkalkingen ontstaan via stimulatie van de MR. [51, 52] Ten derde heeft adenosine de eigenschap om slagaderverkalking tegen te gaan [20] en inactivatie van het adenosinemetabolisme leidt tot progressie van slagaderverkalking [53]. Daarom suggereren we dat de verlaagde circulerende adenosinewaarden bij patiënten met PA zouden kunnen bijdragen aan de progressie van slagaderverkalking.

Een andere aantrekkelijke verklaring voor het verhoogd risico op hart- en vaatziekten bij patiënten met PA leek overproductie van galectine-3 (Gal-3). Aldosteron verhoogt Gal-3 eiwitconcentraties en expressie in laboratoriumonderzoek en diermodellen [16-19] en bij patiënten met hartfalen is Gal-3 geassocieerd met slechte uitkomsten. [54, 55] Ook in deze patiënten zijn aldosteronconcentraties verhoogd en behandeling met MR antagonisten heeft een gunstig effect op de overleving en het aantal ziekenhuisopnames. [10-12] Inderdaad is in Aziatisch onderzoek gevonden dat Gal-3 concentraties verhoogd zijn in het bloed van patiënten met PA ten opzichte van patiënten met EHT. De verhoogde Gal-3 waarden bij deze patiënten met PA herstelden weer naar normaal na bijnierverwijdering. [19, 56] In onze studie beschreven in hoofdstuk 2.3 echter, zagen wij geen verschillen in Gal-3 concentraties tussen patiënten met PA, genezen patiënten met PA (na bijnierverwijdering) en patiënten met EHT. [57]
Ondanks beperkingen van het achteraf samenstellen van de groep patiënten, heeft onze studie sterke punten. Vergeleken met de kleine Aziatische studies [19, 56], selecteerden wij een groot cohort (groep van) patiënten. De patiënten in ons cohort gebruiken geen medicatie die invloed kon hebben op aldosteron- en reninewaarden en we corrigeerden onze resultaten met zorg voor mogelijke beïnvloedende factoren. De waarden van Gal-3 in bloed waren tien maal zo laag als in het Aziatische cohort, hetgeen verklaard kan worden door een verschil in analysemethode of de etnische achtergrond van de patiënten. [57]

In een analyse van een deel van onze studie beschreven in hoofdstuk 2.3 vergeleken we Gal-3 waarden binnen de groep van patiënten met PA mét en zónder linkerventrikelhypertrofie. Onze observatie dat een verschil in hartschade niet werd weerspiegeld door een verschil in Gal-3 concentraties, sluit een rol van plasma Gal-3 in de door aldosteron veroorzaakte orgaanschade uit bij mensen. [57]

In tegenstelling tot de direct beschermende effecten van MR antagonisten op IR schade en remodellering van het hart in dierexperimenteel onderzoek, zoals beschreven in ons overzicht in hoofdstuk 3.1 [8], konden we deze bevindingen niet vertalen naar de menselijke situatie. [44, 58] De MR antagonist eplerenon beschermde niet tegen IR schade in menselijk hartweefsel [58] en ook konden we geen sleutelrol van signalering van de adenosinereceptor in het beschermende effect van eplerenon aantonen. [44]
Het is bekend dat veel veelbelovende bevindingen van dierexperimenteel onderzoek niet kunnen worden vertaald naar onderzoek bij mensen. [59, 60] Dit zogenaamd 'falen van vertaling' kan in zijn algemeenheid worden verklaard door methodologische tekortkomingen bij dierstudies, zoals het ontbreken van een goede berekening van de steekproefgrootte, het ontbreken van randomisatie, het niet blinderen van het onderzoek en inadequate statistische analyses. [59, 60] Ook verschillen dieren fysiologisch en pathofysiologisch gezien substantieel van mensen. Alarmerend genoeg zijn slechts 2 van de 500 beschermende strategieën bij IR schade van het brein bij dieren ook effectief gebleken bij mensen. [59]
Een ander belangrijk verschil tussen laboratorium- en dierstudies en onderzoek bij mensen is het verschil in eindpunten. In hoofdstuk 2.2 hebben we de mate van vaatverwijding van de armslagader gemeten vóór en na ischemie van de bovenarm, als model voor IR schade. In hoofdstuk 3.3 gebruikten we het herstel van de contractiliteit (het samentrekken) van hartspierweefsel als maat voor IR schade. [58] Deze modellen verschillen substantieel van de dierexperimentele studies, waarin infarctgrootte werd gebruikt als uitkomstmaat. Het beschermende effect van bijvoorbeeld pre-conditionering is echter ook bevestigd in het model van vaatverwijding van de armslagader en het model met hartspiertjes. [22, 23, 61]
Het effect van MR antagonisten op IR schade en remodellering van het hart bij patiënten met een acuut hartinfarct wordt momenteel onderzocht in de MINIMISE STEMI trial. [62] Uit onze studie in hoofdstuk 3.3 concludeerden we dat toediening van

eplerenon net voor een ischemische prikkel niet beschermt tegen IR schade in menselijk hartweefsel. Men zou kunnen beargumenteren dat de aanwezigheid van lichaamseigen aldosteron nodig is voor het beschermende effect van MR antagonisten. Echter bieden MR antagonisten ook bescherming bij ratten bij wie de bijnieren zijn verwijderd [63] en in hartmodellen waarin geen aldosteron aanwezig is [8], hetgeen betekent dat het beschermende effect van MR antagonisten onafhankelijk van de aanwezigheid van aldosteron is.

De berekende eplerenonconcentratie in ons hartweefselexperiment was 6 keer hoger dan de berekende eplerenonconcentratie in het bloed van gezonde vrijwilligers (na één week behandeling met eplerenon tweemaal daags 50 mg). [44] In de MINIMISE STEMI trial ontvangen patiënten de MR antagonist canrenoaat in een dosering van 200 mg via het infuus, gevolgd door de MR antagonist spironolacton in een dosering van eenmaal daags 50 mg gedurende 3 maanden. [62] De dosering canrenoaat is vergelijkbaar met de dosering die werd gebruikt in de ALBATROSS trial en de dosering spironolacton is iets hoger. In de ALBATROSS trial werd echter geen gunstig effect van MR antagonisten bij patiënten met een acuut hartinfarct op hart- en vaatschade gezien. [64] Vanwege deze bevindingen en onze eigen observaties [58] wachten wij met grote interesse en scepticisme op de resultaten van de MINIMISE STEMI trial.

Samenvattend hebben wij aangetoond dat het actief opsporen van PA het ontwikkelen van hart- en vaatschade niet voorkomt, omdat er al linkerventrikelhypertrofie aanwezig is op het moment dat deze patiënten zich voor het eerst met hypertensie bij de huisarts presenteren. Dit betekent echter niet dat studies die zich richten op de verbetering van het diagnostische proces van PA niet belangrijk zijn. Er is namelijk een gemiddelde vertraging in het diagnosticeren van PA van 8 jaar [25], hetgeen leidt tot (progressie van) hart- en vaatschade bij deze patiënten.

Wij hebben daarom gepoogd onderliggende mechanismen van de schadelijke effecten van aldosteron op ons hart- en vaatstelsel te ontrafelen, met als uiteindelijk doel om nieuwe behandelingsstrategieën voor patiënten met PA te ontwikkelen. Onze spannende bevinding van de verlaagde adenosinewaarden bij patiënten met PA ten opzichte van patiënten met EHT zou dit doel wel eens kunnen dienen. Toekomstig onderzoek is nodig om onze bevinding te bevestigen en om de onderliggende route van deze verlaagde adenosineconcentratie op te helderen. Beïnvloeding van het

adenosinemetabolisme met medicatie zou de overleving en ziektelast van patiënten met PA en tweezijdige aldosteronoverproductie kunnen verbeteren, omdat deze patiënten niet kunnen worden behandeld door een bijnierverwijdering. Deze patiënten zijn dus continu blootgesteld aan hoge aldosteronconcentraties met schadelijke effecten op ons hart- en vaatstelsel, die mogelijk niet geheel voorkomen kunnen worden door behandeling met MR antagonisten. MR antagonisten hebben in laboratorium –en dierexperimentele studies aangetoond te beschermen tegen IR schade en remodellering van het hart na een hartinfarct, maar wij zagen geen effect van eplerenon op IR schade in menselijk hartweefsel. We zijn daarom terughoudend in het bestuderen van de effecten van MR antagonisten op IR schade bij mensen. Daarnaast verhoogden MR antagonisten de adenosinevorming niet bij gezonde vrijwilligers. Meer onderzoek is nodig om de mechanismen van het beschermende effect van MR antagonisten op ons hart- en vaatstelsel te ontrafelen.

REFERENCES

1. Lawes CM, Vander Hoorn S, Rodgers A. Global burden of blood-pressure-related disease, 2001. *Lancet*. 2008;371(9623):1513-8.
2. 2013 Practice guidelines for the management of arterial hypertension of the European Society of Hypertension (ESH) and the European Society of Cardiology (ESC): ESH/ESC Task Force for the Management of Arterial Hypertension. *Journal of hypertension*. 2013;31(10):1925-38.
3. Park YM, Park MY, Suh YL, Park JB. NAD(P)H oxidase inhibitor prevents blood pressure elevation and cardiovascular hypertrophy in aldosterone-infused rats. *Biochemical and biophysical research communications*. 2004;313(3):812-7.
4. Pu Q, Neves MF, Virdis A, Touyz RM, Schiffrin EL. Endothelin antagonism on aldosterone-induced oxidative stress and vascular remodeling. *Hypertension*. 2003;42(1):49-55.
5. Azibani F, Fazal L, Chatziantoniou C, Samuel JL, Delcayre C. Aldosterone mediates cardiac fibrosis in the setting of hypertension. *Current hypertension reports*. 2013;15(4):395-400.
6. De Angelis N, Fiordaliso F, Latini R, Calvillo L, Funicello M, Gobbi M, et al. Appraisal of the role of angiotensin II and aldosterone in ventricular myocyte apoptosis in adult normotensive rat. *Journal of molecular and cellular cardiology*. 2002;34(12):1655-65.
7. Fujita M, Minamino T, Asanuma H, Sanada S, Hirata A, Wakeno M, et al. Aldosterone nongenomically worsens ischemia via protein kinase C-dependent pathways in hypoperfused canine hearts. *Hypertension*. 2005;46(1):113-7.
8. van den Berg TN, Rongen GA, Frohlich GM, Deinum J, Hausenloy DJ, Riksen NP. The cardioprotective effects of mineralocorticoid receptor antagonists. *Pharmacology & therapeutics*. 2014;142(1):72-87.
9. Monticone S, D'Ascenzo F, Moretti C, Williams TA, Veglio F, Gaita F, et al. Cardiovascular events and target organ damage in primary aldosteronism compared with essential hypertension: a systematic review and meta-analysis. *The lancet Diabetes & endocrinology*. 2018;6(1):41-50.
10. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *The New England journal of medicine*. 1999;341(10):709-17.
11. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *The New England journal of medicine*. 2003;348(14):1309-21.

12. Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, et al. Eplerenone in patients with systolic heart failure and mild symptoms. *The New England journal of medicine*. 2011;364(1):11-21.
13. Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, et al. The Management of Primary Aldosteronism: Case Detection, Diagnosis, and Treatment: An Endocrine Society Clinical Practice Guideline. *The Journal of clinical endocrinology and metabolism*. 2016;101(5):1889-916.
14. Schmidt K, Tissier R, Ghaleb B, Drogies T, Felix SB, Krieg T. Cardioprotective effects of mineralocorticoid receptor antagonists at reperfusion. *European heart journal*. 2010;31(13):1655-62.
15. Headrick JP, Ashton KJ, Rose'meyer RB, Peart JN. Cardiovascular adenosine receptors: expression, actions and interactions. *Pharmacology & therapeutics*. 2013;140(1):92-111.
16. Calvier L, Miana M, Reboul P, Cachofeiro V, Martinez-Martinez E, de Boer RA, et al. Galectin-3 mediates aldosterone-induced vascular fibrosis. *Arteriosclerosis, thrombosis, and vascular biology*. 2013;33(1):67-75.
17. Calvier L, Martinez-Martinez E, Miana M, Cachofeiro V, Rousseau E, Sadaba JR, et al. The impact of galectin-3 inhibition on aldosterone-induced cardiac and renal injuries. *JACC Heart failure*. 2015;3(1):59-67.
18. Martinez-Martinez E, Calvier L, Fernandez-Celis A, Rousseau E, Jurado-Lopez R, Rossoni LV, et al. Galectin-3 Blockade Inhibits Cardiac Inflammation and Fibrosis in Experimental Hyperaldosteronism and Hypertension. *Hypertension*. 2015.
19. Lin YH, Chou CH, Wu XM, Chang YY, Hung CS, Chen YH, et al. Aldosterone induced galectin-3 secretion in vitro and in vivo: from cells to humans. *PloS one*. 2014;9(9):e95254.
20. Riksen NP, Rongen GA. Targeting adenosine receptors in the development of cardiovascular therapeutics. *Expert review of clinical pharmacology*. 2012;5(2):199-218.
21. Meijer P, Wouters CW, van den Broek PH, de Rooij M, Scheffer GJ, Smits P, et al. Upregulation of ecto-5'-nucleotidase by rosuvastatin increases the vasodilator response to ischemia. *Hypertension*. 2010;56(4):722-7.
22. Speechly-Dick ME, Grover GJ, Yellon DM. Does ischemic preconditioning in the human involve protein kinase C and the ATP-dependent K+ channel? Studies of contractile function after simulated ischemia in an atrial in vitro model. *Circulation research*. 1995;77(5):1030-5.
23. Riksen NP, Zhou Z, Oyen WJ, Jaspers R, Ramakers BP, Brouwer RM, et al. Caffeine prevents protection in two human models of ischemic preconditioning. *Journal of the American College of Cardiology*. 2006;48(4):700-7.

24. El Messaoudi S, Nederlof R, Zuurbier CJ, van Swieten HA, Pickkers P, Noyez L, et al. Effect of metformin pretreatment on myocardial injury during coronary artery bypass surgery in patients without diabetes (MetCAB): a double-blind, randomised controlled trial. *The lancet Diabetes & endocrinology*. 2015;3(8):615-23.
25. Dhanjal TS, Beevers DG. Delay in the diagnosis of Conn's syndrome: a single-center experience over 30 years. *Hypertension*. 2008;52(3):e22; author reply e3.
26. Stowasser M, Gordon RD. Primary Aldosteronism: Changing Definitions and New Concepts of Physiology and Pathophysiology Both Inside and Outside the Kidney. *Physiological reviews*. 2016;96(4):1327-84.
27. Kayser SC, Dekkers T, Groenewoud HJ, van der Wilt GJ, Carel Bakx J, van der Wel MC, et al. Study Heterogeneity and Estimation of Prevalence of Primary Aldosteronism: a Systematic Review and Meta-regression Analysis. *The Journal of clinical endocrinology and metabolism*. 2016:jc20161472.
28. Hannemann A, Wallaschofski H. Prevalence of primary aldosteronism in patient's cohorts and in population-based studies--a review of the current literature. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme*. 2012;44(3):157-62.
29. Stowasser M, Gordon RD. Primary aldosteronism--careful investigation is essential and rewarding. *Molecular and cellular endocrinology*. 2004;217(1-2):33-9.
30. Kayser SC, Deinum J, de Grauw WJ, Schalk BW, Bor HJ, Lenders JW, et al. Prevalence of primary aldosteronism in primary care: a cross-sectional study. *The British journal of general practice : the journal of the Royal College of General Practitioners*. 2018;68(667):e114-e22.
31. Mosso L, Carvajal C, Gonzalez A, Barraza A, Avila F, Montero J, et al. Primary aldosteronism and hypertensive disease. *Hypertension*. 2003;42(2):161-5.
32. Song Y, Yang S, He W, Hu J, Cheng Q, Wang Y, et al. Confirmatory Tests for the Diagnosis of Primary Aldosteronism: A Prospective Diagnostic Accuracy Study. *Hypertension*. 2018;71(1):118-24.
33. Mulatero P, Milan A, Fallo F, Regolisti G, Pizzolo F, Fardella C, et al. Comparison of confirmatory tests for the diagnosis of primary aldosteronism. The Journal of clinical endocrinology and metabolism. 2006;91(7):2618-23.
34. Rossi GP, Belfiore A, Bernini G, Desideri G, Fabris B, Ferri C, et al. Prospective evaluation of the saline infusion test for excluding primary aldosteronism due to aldosterone-producing adenoma. Journal of hypertension. 2007;25(7):1433-42.
35. Cornu E, Steichen O, Nogueira-Silva L, Kupers E, Pagny JY, Grataloup C, et al. Suppression of Aldosterone Secretion After Recumbent Saline Infusion Does Not Exclude Lateralized Primary Aldosteronism. Hypertension. 2016;68(4):989-94.

36. van der Lubbe N, Jansen PM, Salih M, Fenton RA, van den Meiracker AH, Danser AH, et al. The phosphorylated sodium chloride cotransporter in urinary exosomes is superior to prostasin as a marker for aldosteronism. *Hypertension*. 2012;60(3):741-8.
37. Pizzolo F, Chiecchi L, Morandini F, Castagna A, Zorzi F, Zaltron C, et al. Increased urinary excretion of the epithelial Na channel activator prostasin in patients with primary aldosteronism. *Journal of hypertension*. 2017;35(2):355-61.
38. Anderson JL, Habashi J, Carlquist JF, Muhlestein JB, Horne BD, Bair TL, et al. A common variant of the AMPD1 gene predicts improved cardiovascular survival in patients with coronary artery disease. *Journal of the American College of Cardiology*. 2000;36(4):1248-52.
39. Mahaffey KW, Puma JA, Barbagelata NA, DiCarli MF, Leesar MA, Browne KF, et al. Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction: results of a multicenter, randomized, placebo-controlled trial: the Acute Myocardial Infarction STudy of ADenosine (AMISTAD) trial. *Journal of the American College of Cardiology*. 1999;34(6):1711-20.
40. Ross AM, Gibbons RJ, Stone GW, Kloner RA, Alexander RW. A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). *Journal of the American College of Cardiology*. 2005;45(11):1775-80.
41. Bulluck H, Sirker A, Loke YK, Garcia-Dorado D, Hausenloy DJ. Clinical benefit of adenosine as an adjunct to reperfusion in ST-elevation myocardial infarction patients: An updated meta-analysis of randomized controlled trials. *International journal of cardiology*. 2016;202:228-37.
42. Methner C, Schmidt K, Cohen MV, Downey JM, Krieg T. Both A2a and A2b adenosine receptors at reperfusion are necessary to reduce infarct size in mouse hearts. *American journal of physiology Heart and circulatory physiology*. 2010;299(4):H1262-4.
43. Riksen NP, Rongen GA, Boers GH, Blom HJ, van den Broek PH, Smits P. Enhanced cellular adenosine uptake limits adenosine receptor stimulation in patients with hyperhomocysteinemia. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25(1):109-14.
44. van den Berg TN, Deinum J, Bilos A, Donders AR, Rongen GA, Riksen NP. The effect of eplerenone on adenosine formation in humans in vivo: a double-blinded randomised controlled study. *PloS one*. 2014;9(10):e111248.
45. Meijer P, Wouters CW, van den Broek PH, Scheffer GJ, Riksen NP, Smits P, et al. Dipyridamole enhances ischaemia-induced reactive hyperaemia by increased adenosine receptor stimulation. *British journal of pharmacology*. 2008;153(6):1169-76.

46. Goto M, Miura T, Iliodoromitis EK, O'Leary EL, Ishimoto R, Yellon DM, et al. Adenosine infusion during early reperfusion failed to limit myocardial infarct size in a collateral deficient species. *Cardiovascular research*. 1991;25(11):943-9.
47. Vander Heide RS, Reimer KA. Effect of adenosine therapy at reperfusion on myocardial infarct size in dogs. *Cardiovascular research*. 1996;31(5):711-8.
48. Xu Z, Downey JM, Cohen MV. Amp 579 reduces contracture and limits infarction in rabbit heart by activating adenosine A2 receptors. *Journal of cardiovascular pharmacology*. 2001;38(3):474-81.
49. Desmet W, Bogaert J, Dubois C, Sinnaeve P, Adriaenssens T, Pappas C, et al. High-dose intracoronary adenosine for myocardial salvage in patients with acute ST-segment elevation myocardial infarction. *European heart journal*. 2011;32(7):867-77.
50. Nazir SA, McCann GP, Greenwood JP, Kunadian V, Khan JN, Mahmoud IZ, et al. Strategies to attenuate micro-vascular obstruction during P-PCI: the randomized reperfusion facilitated by local adjunctive therapy in ST-elevation myocardial infarction trial. *European heart journal*. 2016;37(24):1910-9.
51. Moss ME, Jaffe IZ. Mineralocorticoid Receptors in the Pathophysiology of Vascular Inflammation and Atherosclerosis. *Frontiers in endocrinology*. 2015;6:153.
52. van der Heijden C, Deinum J, Joosten LAB, Netea MG, Riksen NP. The mineralocorticoid receptor as a modulator of innate immunity and atherosclerosis. *Cardiovascular research*. 2018;114(7):944-53.
53. Buchheiser A, Ebner A, Burghoff S, Ding Z, Romio M, Viethen C, et al. Inactivation of CD73 promotes atherogenesis in apolipoprotein E-deficient mice. *Cardiovascular research*. 2011;92(2):338-47.
54. de Boer RA, Daniels LB, Maisel AS, Januzzi JL, Jr. State of the Art: Newer biomarkers in heart failure. *European journal of heart failure*. 2015;17(6):559-69.
55. Lopez-Andres N, Rossignol P, Iraqi W, Fay R, Nuee J, Ghio S, et al. Association of galectin-3 and fibrosis markers with long-term cardiovascular outcomes in patients with heart failure, left ventricular dysfunction, and dyssynchrony: insights from the CARE-HF (Cardiac Resynchronization in Heart Failure) trial. *European journal of heart failure*. 2012;14(1):74-81.
56. Liao CW, Lin YT, Wu XM, Chang YY, Hung CS, Wu VC, et al. The relation among aldosterone, galectin-3, and myocardial fibrosis: a prospective clinical pilot follow-up study. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research*. 2016;64(6):1109-13.
57. van den Berg T, Meijers WC, Donders ART, Van Herwaarden AE, Rongen GA, de Boer RA, et al. Plasma galectin-3 concentrations in patients with primary aldosteronism. *Journal of hypertension*. 2017;35(9):1849-56.

58. van den Berg TN, van Swieten HA, Vos JC, Verweij V, Wouterse AC, Deinum J, et al. Eplerenone does not limit ischemia-reperfusion injury in human myocardial tissue. *International journal of cardiology*. 2016;216:110-3.
59. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, et al. Can animal models of disease reliably inform human studies? *PLoS medicine*. 2010;7(3):e1000245.
60. Ramirez FD, Motazedian P, Jung RG, Di Santo P, MacDonald ZD, Moreland R, et al. Methodological Rigor in Preclinical Cardiovascular Studies: Targets to Enhance Reproducibility and Promote Research Translation. *Circulation research*. 2017;120(12):1916-26.
61. Kharbanda RK, Peters M, Walton B, Kattenhorn M, Mullen M, Klein N, et al. Ischemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischemia-reperfusion in humans in vivo. *Circulation*. 2001;103(12):1624-30.
62. Bulluck H, Frohlich GM, Mohdnazri S, Gamma RA, Davies JR, Clesham GJ, et al. Mineralocorticoid receptor antagonist pretreatment to MINIMISE reperfusion injury after ST-elevation myocardial infarction (the MINIMISE STEMI Trial): rationale and study design. *Clinical cardiology*. 2015;38(5):259-66.
63. Mihailidou AS, Loan Le TY, Mardini M, Funder JW. Glucocorticoids activate cardiac mineralocorticoid receptors during experimental myocardial infarction. *Hypertension*. 2009;54(6):1306-12.
64. Beygui F, Cayla G, Roule V, Roubille F, Delarche N, Silvain J, et al. Early Aldosterone Blockade in Acute Myocardial Infarction: The ALBATROSS Randomized Clinical Trial. *Journal of the American College of Cardiology*. 2016;67(16):1917-27.

DANKWOORD

PUBLICATION LIST

CURRICULUM VITAE

PHD PORTFOLIO

DANKWOORD

Mijn proefschrift zou nooit tot stand gekomen zijn zonder de inzet van proefpersonen. Mijn dank voor de inzet, tijd, moeite en interesse in het onderzoek van de gezonde mannelijke vrijwilligers en patiënten is dan ook erg groot.

Prof. dr. N.P. Riksen, beste Niels, ik had me geen betere promotor kunnen wensen. Ik bewonder je talenten en de efficiënte wijze waarop je deze benut, maar vooral wil ik je danken voor de fijne en goede begeleiding. De snelheid waarmee jij manuscripten leest, beoordeelt en verbetert, is ongekend. Aanvankelijk schrok ik van de grote lappen rode tekst in de verbeterde manuscripten, maar gelukkig werd dat met de tijd minder.
Je hebt een fijne persoonlijkheid met een goed gevoel voor humor. Hard werken zonder dat het je stress oplevert (of lijkt op te leveren), met welverdiend succes als gevolg en daarnaast tijd hebben voor veel andere dingen, maakt dat je een voorbeeld bent voor velen. Dank je wel voor alles!

Prof. dr. G.A.P.J.M. Rongen, beste Gerard, ook jou wil ik hartelijk danken voor het begeleiden van mijn promotietraject. Ik dank je voor je kritische blik en bewonder de integere wijze waarop je onderzoek verricht. Ook jouw gedrevenheid in zaken buiten het ziekenhuis vind ik bewonderenswaardig en dan met name jouw fascinatie voor elektronische gadgets, motor rijden, Snickers, zingen, het lopen van marathons en viool spelen. Eigenlijk zouden we wel eens vaker duetten voor hobo en viool kunnen spelen.

Dr. J. Deinum, beste Jaap, voor mij ben jij een voorbeelddokter. Ik dank je voor jouw superviserende rol en hetgeen wat ik van jou geleerd heb en nog mag leren. Je hebt daarnaast een hele brede interesse voor alles wat buiten het ziekenhuis gebeurt en hoe je hier tijd voor weet te vinden, is me een raadsel. Vooral jouw meest recent gestarte project ondersteun ik van harte. Ik hoop dat de mooie klankkleur hetgeen is dat je overhaalde om hobo te gaan spelen en niet de fysiologische effecten van het blazen, die wellicht helemaal niet zo goed voor ons hart- en vaatstelsel zijn.

Prof. dr. P. Smits, beste Paul, de start van mijn promotietraject lag in jouw handen toen ik als zesdejaars geneeskundestudent bij je aanklopte met de vraag of ik als arts-onderzoeker op –toen nog "jouw"- afdeling farmacologie-toxicologie aan de slag kon gaan. Je koppelde me aan Niels, van wie ik destijds nog nooit gehoord had en hiervoor ben ik je heel dankbaar. In de afgelopen jaren bleef je geïnteresseerd in de voortgang van mijn promotieonderzoek en ik vind het dan ook ontzettend leuk dat je plaatsvervangend rector kunt en wilt zijn op deze bijzondere dag.

Hoewel er een letterlijke en figuurlijke scheiding bestaat tussen de arts-onderzoekers in de prekliniek en de basale wetenschappers in de toren, voelde ik me prima op mijn gemak bij het "torentuig". Om mijn actie met droogijs konden jullie gelukkig wél lachen. Elke dag ging ik met plezier naar mijn werk en daar speelden mijn collega's (van de toren en prekliniek) een belangrijke rol in. Dank jullie wel!
Een aantal personen wil ik graag in het bijzonder noemen. Janny, dank voor het (opnieuw) leren van de celkweek. Tom S, ik vind het jammer dat ons gezamenlijke project (nog) niet is doorgegaan. Je bent een erg fijne collega! Gaby en Jolien, bij dezen stel ik mijn placenta voor jullie placenta-perfusiemodel beschikbaar. Saloua, helaas overlapte onze onderzoekstijd maar ten dele. Ik waardeer de lol die wij samen konden hebben. Annemieke en Fons, dankzij jullie hebben we de hartoortjesstudie kunnen uitvoeren. Ik waardeer het heel erg dat jullie soms ook in jullie eigen tijd zoveel moeite hebben gedaan voor dit project. Karl, samen met Michel hebben we ervoor gezorgd dat na het afdelingsuitje van "MiDaKa" Magic in de auto stappen of het oprapen van een stuk papier te moeilijk bleek voor velen. Heerlijk dat we samen zo hebben kunnen genieten van het achterlaten van deze onuitwisbare indruk.

Prof. dr. F.G.M. Russel, beste Frans, dank voor jouw scherpe opmerkingen en kritische blik tijdens het bespreken van mijn onderzoeken tijdens de labmeetings op de dinsdagochtend. Beste opleiders van de interne geneeskunde, Prof. dr. J. de Graaf, Prof. dr. J. Smit, Dr. G. Vervoort, Dr. P. Netten en Dr. W. Smit, beste Jacqueline, Jan, Gerald, Paetrick en Watske, graag wil ik jullie danken voor de flexibiliteit binnen de opleiding tot internist om mijn onderzoek mogelijk te maken. Beste Prof. C. Kramers, beste Kees, dank dat je het mogelijk maakte om de laatste metingen binnen een gedeelte van de opleiding tot klinisch farmacoloog te kunnen doen. Het delen van een kamer

was niet per se bevorderlijk voor mijn snelheid van schrijven, maar zorgde wel voor vertier en vermaak. Beste collega's van de interne geneeskunde, dank voor jullie hulp bij het includeren van patiënten voor de FMD-studie. Beste collega's van de vasculaire geneeskunde, heel veel dank voor het warme bad waarin ik me momenteel bevind.

Beste Dr. H. Monajemi, beste Houshang, dank voor de prettige samenwerking en je hulp met het includeren van patiënten met essentiële hypertensie voor onze FMD-studie. Erg leuk dat je zitting neemt in de corona. Graag wil ik de dames van het planbureau van de cardiothoracale chirurgie en het CRCN danken voor hun ondersteuning van twee van onze studies. Prof. dr. R. de Boer en Dr. W. Meijers, samen hebben we een fraai stuk over galectine-3 bij primair hyperaldosteronisme gepubliceerd. Dank voor de prettige samenwerking. Beste dr. van Mil, beste Anke, jou ben ik veel dank verschuldigd. Jij hebt vrijwel alle FMD's van hoofdstukken 2.1 en 2.2 verricht. Ik vond het heel prettig met je samen te werken en dank je voor je gezelligheid, flexibiliteit en kundigheid. Heel veel succes met je nieuwe baan! Ook Bregina, Martijn en Tim wil ik hartelijk danken voor hun hulp en fijne samenwerking. Beste Prof. dr. D. Thijssen, beste Dick, ik voelde me altijd welkom op de afdeling fysiologie. Dank voor jouw kritische blik en grote bijdrage aan de opzet van de FMD-studie. Beste collega's van de eerstelijnsgeneeskunde en in het bijzonder kersverse Dr. Sabine Käyser, ondanks de tegenslagen in het PAGODE-onderzoek hebben we waardevolle gegevens vergaard. Dank voor de leerzame samenwerking. Beste Inge van den Munckhof, dames van het vaatlaboratorium en met name Marie-José Beelen, dank voor het leren van de vaatmetingen! Beste Markus Bösch, het bleek best uitdagend een student medische biologie te begeleiden en me weer opnieuw bezig te houden met fundamenteel onderzoek. Ik dank je voor jouw inzet en wens je heel veel succes in je verdere carrière!

Lieve Petra, het lag al heel vroeg voor de hand dat jij mijn paranimf zou zijn. De afdeling farmacologie-toxicologie voelde voor mij als een warm bad en jij hebt daar een erg belangrijke bijdrage aan geleverd. Als analist ben je onmisbaar voor de afdeling. Je bent de creatieve geest achter de *in vitro* onderzoeken in dit proefschrift en niet gepubliceerde studies. We hebben niet alleen tijd samen op het lab doorgebracht, maar vooral ook daarbuiten. Je stimuleert werkbesprekingen onder het genot van een drankje

in de Aesculaaf en ik kan deze arbeidsethos alleen maar waarderen. Dank je voor jouw gezelligheid en onze vriendschap.

Lieve Lilibeth, we schelen enkele jaren in leeftijd. Biologisch is het verschil stukken minder groot dan het aantal kalenderjaren en dat wordt bepaald door jouw geweldige looks. Ik bewonder je smaak en stijl, je humor met sarcasme en jouw precisie. Tal van onderzoekers heb jij al ondersteund en ik voel me dan ook heel vereerd dat jij achter mij wilt staan op deze belangrijke dag. Uitgerekend jij zult het vast heel vervelend hebben gevonden dat je weer een nieuwe jurk met accessoires hebt moeten uitzoeken.

Met Roos en Karin is ons clubje compleet. Toen ik bij ons eerste etentje als enige "jongeling" zou aanschuiven, voelde ik me bezwaard. M aar wat was het gezellig! Gelukkig lukt het de etentjes voort te zetten, hoewel het misschien best vaker mag. Binnenkort ook weer eens met de mannen?

Ik kan me geen leven zonder het maken van muziek voorstellen. Ik heb in de loop der jaren ontzettend veel mensen in de muziek leren kennen, met veel mensen mogen musiceren en samen erg mooie muziek gemaakt. Beste muziekvrienden, dank jullie wel!

Lieve vrienden, stuk voor stuk tref ik het met jullie! Een aantal personen wil ik in het bijzonder noemen. Lieve Rick en Juul, Laurens en Margit, Nathalie en Freek, Lara en Wim, dank voor onze fijne vriendschap. We accepteren van elkaar dat we elkaar niet wekelijks kunnen zien en dat vind ik fijn. Als we samen zijn, is alles bespreekbaar. Vaak gaat het over de leuke dingen, maar ook in moeilijke tijden zijn jullie er voor mij. Dank jullie wel. Lieve Linda, Milou en Rosanne, jammer dat we onze jaarlijkse stedentripjes niet meer hebben voortgezet. Etentjes zijn tot die tijd een erg leuk alternatief. De volgende keer met de mannen? Lieve Laura en Marcel, onze band is de laatste jaren heel sterk geworden en dat is me/ons ontzettend kostbaar!

Lieve schoonfamilie, wat kom ik graag bij jullie over de vloer. Dank jullie voor de fijne tijd die we samen hebben en de interesse in mij en alles wat ik doe. Lieve familie Van den Berg en familie Van der Biezen, met de meesten van jullie heb ik een erg goede band en dat waardeer ik zeer. Het is jammer dat we elkaar niet vaker zien.

Lieve Yvonne, Stephanie en Ralph, als we bij elkaar zijn hebben we zoveel om over te kletsen, dat het eigenlijk niet vaak over werk gaat. Wellicht is jullie ook pas na vandaag duidelijk geworden waar ik me nu precies mee heb bezig gehouden in de afgelopen jaren. We weten elkaar altijd feilloos aan onze slechte eigenschappen te herinneren en spreken wellicht te weinig uit wat we zo aan elkaar waarderen. Ik houd heel veel van jullie en ben heel blij dat het nu zo goed gaat met ons viertjes.
Lieve Nathan, Jeroen & Benjamin, Brenda & Jesse en Fedde, dank jullie wel voor het zo gelukkig maken van mijn sibbes. We zijn een gezellige club met z'n allen!

Lieve papa en mama, jullie hebben altijd gestimuleerd de dingen te doen die ik leuk vind en daar ben ik jullie erg dankbaar voor. Hoewel het niet vaak uitgesproken wordt, spat de trots op jullie kinderen er vanaf. Andersom geldt dat ik te weinig uitspreek hoeveel ik van jullie houd, in goede en slechte tijden. Bij dezen! Ik denk dat ik heel veel goede dingen vanuit huis heb meegekregen en op veel van deze eigenschappen ben ik erg trots. Dat mijn voorletters afwijken van mijn roepnaam is ontzettend onhandig, zoals ook blijkt uit mijn publicatielijst. D.N.A. van den Berg was toch ook heel mooi geweest?

Lieve Sander, jij bent de liefde van mijn leven en mijn allerbeste maatje. Ik voel mij zo fijn bij jou. Wij genieten extreem van het leven en de life events vliegen ons om de oren. Ik ben ontzettend benieuwd naar onze mini-we! Ik houd zielsveel van jou.

Liefs, Daniëlle

PUBLICATION LIST

Hendriks GJ, **van den Berg TN**, Witteman C, Lucassen P. Tunnelvisie benadeelt patiënt (klinische les). *Submitted*

van den Berg TN and Deinum J. Epidemie van hypertensie na nieuwe richtlijn (in het kort). *Ned Tijdschr Geneeskd.* 2018;162:D3315 1

van den Berg TN, Käyser SC, van Mil AC, Riksen NP, Schermer TR, Schalk BW, Thijssen DH, Rongen GA, de Grauw W, Lenders JW, Biermans MC, Deinum J. Do patients with primary aldosteronism have cardiovascular damage at time of diagnosing hypertension in primary care? *Submitted*

van den Berg TN, Thijssen DH, van Mil AC, van den Broek PH, Rongen GA, H. Monajemi, Deinum J, Riksen NP. Plasma levels of the cardiovascular protective endogenous nucleoside adenosine are reduced in patients with primary aldosteronism. *Submitted*

van den Berg TN, Meijers WC, Donders AR, van Herwaarden AE, Rongen GA, de Boer RA, Deinum J, Riksen NP. Plasma galectin-3 concentration in patients with primary aldosteronism. *J Hypertens. 2017 Sep;35(9):1849-1856*

van den Berg TN, van Swieten HA, Vos JC, Verweij V, Wouterse AC, Deinum J, Morshuis WJ, Rongen GA, Riksen NP. Eplerenone does not limit ischemia-reperfusion injury in human myocardial tissue. *Int J Cardiol. 2016 Apr 22;216:110-113*

van den Berg TN, El Messaoudi S, Rongen GA, van den Broek PH, Bilos A, Donders AR, Gomes ME, Riksen NP. Ticagrelor Does Not Inhibit Adenosine Transport at Relevant Concentrations: A Randomized Cross-Over Study in Healthy Subjects In Vivo. *PLoS One. 2015 Oct 28;10(10):e0137560.*

Tunjungputri RN, van der Ven AJ, Riksen N, Rongen G, Tacke S, **van den Berg** TN, Fijnheer R, Gomes ME, Dinarello CA, van de Veerdonk FL, Gasem M, Netea MG, Joosten LA, De Groot PG, de Mast Q. Differential effects of platelets and platelet inhibition by ticagrelor on TLR2- and TLR4-mediated inflammatory responses. *Thromb Haemost. 2015 Apr 29;113(5):1035-45.*

van den Berg TN, Deinum J, Bilos A, Rongen GA, Riksen NP. The effect of eplerenone on adenosine formation in humans in vivo: a double-blinded randomised controlled study. *PLoS One. 2014 Oct 30;9(10):e111248.*

van den Berg TN, Rongen GA, Fröhlich GM, Deinum J, Hausenloy DJ, Riksen NP. The cardioprotective effects of mineralocorticoid receptor antagonists. *Pharmacol Ther. 2014 Apr;142(1):72-87*

van den Berg DT, Deinum J, Postma CT, van der Wilt GJ, Riksen NP. The efficacy of renal angioplasty in patients with renal artery stenosis and flash oedema or congestive heart failure: a systematic review. *Eur J Heart Fail. 2012 Jul; 14(7):773-81*

Tromp M, Hulscher M, Bleeker-Rovers CP, Peters L, **van den Berg DT**, Borm GF, Kullberg BJ, van Achterberg T, Pickkers P. The role of nurses in the recognition and treatment of patients with sepsis in the emergency department: A prospective before-and-after intervention study. *Int J Nurs Stud. 2010 Dec;47(12):1464-73*

Vroegop MP, Franssen EJ, van der Voort PH, **van den Berg TN**, Langeweg RJ, Kramers C. The emergency care of cocaine intoxications. *Neth J Med. 2009 Apr;67(4):122-6.*

Tromp M, **van den Berg TN**, Peters L, Hulscher M, van Achterberg T, Bleeker-Rovers CP, Kullberg BJ, Pickkers P. Patiënten met sepsis op de SEH van het UMC St Radboud. *Triage. 2008;2:7-10*

CURICULUM VITAE

Daniëlle van den Berg werd op 1 maart 1985 geboren te Berlicum. Na het behalen van haar eindexamen aan het Gymnasium Bernrode te Heeswijk-Dinther, startte zij in 2003 met de studie Biomedische Wetenschappen aan de Radboud Universiteit te Nijmegen. Na een tweede maal te zijn uitgeloot voor de studie Geneeskunde besloot zij haar bachelor in de Biomedische Wetenschappen te voltooien. Haar afsluitende stage volbracht zij op het Laboratorium voor Kindergeneeskunde en Neurologie onder leiding van Dr. D.J. Lefeber. In 2006 startte zij met een verkorte opleiding Geneeskunde, tevens aan de Radboud Universiteit. Tijdens deze studie werkte zij als preparateur op de afdeling Anatomie en was zij betrokken bij de uitvoering van onderzoek naar sepsis op de spoedeisende hulp van het Radboudumc, onder leiding van Dr. M. Tromp en Prof. dr. P. Pickkers. Dat zij tijdens haar bachelor Biomedische Wetenschappen plezier kreeg in het verrichten van onderzoek, bleek tevens uit het feit dat zij op eigen initiatief in 2008 een keuzevak verruilde voor onderzoek naar dengue in het Rumah Sakit Hasan Sadikin te Bandung op Java, Indonesië. Ze rondde haar studie Geneeskunde af met een tropencoschap in het Sengerema Designated District Hospital te Sengerema, Tanzania. Na het behalen van haar artsendiploma startte zij direct met de opleiding tot internist in het Jeroen Bosch Ziekenhuis met Dr. P.M. Netten als opleider. Zij ontving een AGIKO (assistent geneeskundige in opleiding tot klinisch onderzoeker) stipendium beurs van het Radboudumc om deze opleiding te kunnen combineren met promotieonderzoek op de afdelingen Farmacologie-Toxicologie en Interne Geneeskunde van het Radboudumc, onder leiding van Prof. dr. N.P. Riksen, Prof. dr. G.A.P.J.M. Rongen en Dr. J. Deinum. Het onderzoek naar de effecten van aldosteron en mineralocorticoïd receptorantagonisten op hart- en vaatschade heeft geleid tot dit proefschrift.

Tijdens haar onderzoeksperiode volgde zij de door de European Society of Hypertension georganiseerde Hypertension Summer School te Sirmione in Italië. Momenteel zet zij haar opleiding tot internist voort, met verdere differentiatie in de vasculaire geneeskunde. Tevens is zij in opleiding tot klinisch farmacoloog en in dit kader schreef zij samen met haar opleider Prof. dr. C. Kramers een nascholing voor huisartsen over antistolling.

In haar vrije tijd is Daniëlle een fanatiek hoboïste. Zij won de kampioenswedstrijd voor solisten en blazersensembles van de Federatie voor Katholieke Muziekgezelschappen, kreeg een eervolle vermelding op het Prinses Christina Concours en was hoboïste van het NJHO (Nederlands Jeugd HarmonieOrkest). Zij speelt momenteel in blazersensemble La Spiritata en remplaceert daarnaast in verschillende amateur- en semi-professionele orkesten.
Daniëlle trouwde met Sander den Besten op 19 augustus 2017 en in juni hopen zij hun eerste kind te krijgen.

Institute for Health Sciences
Radboudumc

PHD PORTFOLIO

Name PhD candidate: T.N.A. (Daniëlle) van den Berg Department: Pharmacology-Toxicology and Internal Medicine Graduate School: Radboud Institute for Health Sciences	PhD period: 01-12-2012 – 01-06-2014 and 01-06-2015 – 01-12-2016 Promotors: Prof. N.P. Riksen and Prof. G.A.P.J.M. Rongen Co-promotor: Dr J. Deinum

	Year(s)	ECTS
TRAINING ACTIVITIES		
a) Courses & Workshops		
NVVG AIOS course	2012	0.25
BROK course	2013	1.5
NCEBP Science day	2013	0.25
NCEBP Introduction course	2013	1.5
PhD Cardiovasculair training course Hartstichting (vasculair biology)	2013	2.0
PhD course How to convince the editor	2013	0.1
Presentation course (Spies & Spreken)	2014	0.2
PhD workshop Poster design and presentation	2014	0.2
Internal medicine science days	2014 and 2016	0.5
Workshop vascular ultrasound (EACPT focus meeting)	2014	0.2
PhD Cardiovasculair training course Hartstichting (atherosclerosis and thrombosis)	2015	2.0
ESH Hypertension Summer School (Sirmione, Italy) (oral)	2016	2.25
Masterclass A crucial role of the MR (oral)	2016	0.65
b) Seminars & lectures		
Radboud Research Rounds and Grand Rounds	2012-2018	0.5
Acute Kidney Failure by Prof. Lameire (Gent, Belgium)	2013	0.1

c) Symposia & congresses		
NHG (oral)	2012	0.75
NFU symposium Radboudumc	2013	0.1
CardioVasculaire Conferentie (oral)	2014	0.75
EACPT focus meeting (oral)	2014	0.75
NHG	2014	0.1
FIGON Dutch Medicine Days (poster and oral)	2015	0.75
CarVasz congress (oral)	2017	0.75
EAS (Lisbon, Portugal) (poster)	2018	1.25
d) Other		
Lab meetings	2012-2016	1.0
Vascular Damage theme meetings	2012-2018	1.0
Radboud Adrenal Centre (RAC) meetings	2012-2018	0.5
Reviewing scientific publications	2016-2018	0.4
Monitoring other studies	2013 and 2015	1.0
TEACHING ACTIVITIES		
d) Lecturing		
5O104 Beweging en sturing – bloeddrukregulatie als regelkring	2013	0.1
5OMZ1 Pathofysiologie en genetic - ontsteking	2013 and 2015	0.2
5O201 Circulatie 2 – patiënten met risicofactoren voor hart- en vaatziekten	2014	0.1
5O208 Regulatie en integratie 2 – farmacotherapie van pijn	2014	0.1
5O101 Circulatie en respiratie 1 – integratie van respiratie en circulatie	2014	0.1
3MFD Farmacodynamiek – toegepaste farmacodynamiek	2016	0.1
Ontsteking – sepsis (studenten medische biologie)	2016 and 2018	0.2
e) Supervision of internships / other		
BOSA praktijkoriëntatie	2013	0.5
OMB2 onderzoeksstage biomedische wetenschappen	2013	0.5
MED-MIN01 onderzoeksstage geneeskunde	2015	0.5
Markus Bösch (bachelor medical biology) – The role of the MR on CD73 activity	2016	3.0
TOTAL		**26.7**

Institute for Health Sciences
Radboudumc