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Plasma levels of the cardiovascular protective endogenous nucleoside adenosine are reduced in patients with primary aldosteronism without affecting ischaemia-reperfusion injury: A prospective case-control study

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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 ischaemia-reperfusion (IR) injury by increasing extracellular adenosine formation and adenosine receptor stimulation. Adenosine is an endogenous compound with profound cardiovascular protective effects. Firstly, 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.

Design: 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] vs 22.7 [19.4-36.8] nmol/L, respectively, $P < .01$). The reduction in FMD after IR, however, did not differ between patients with PA and patients with EHT ($-1.0 \pm 2.9\%$ vs $-1.6 \pm 1.6\%$, respectively, $P = .52$).

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.

KEYWORDS
adenosine, aldosterone, hypertension, ischaemia-reperfusion injury, primary aldosteronism

1 | 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 improves 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] it stimulates vascular and myocardial fibrosis[9-11] and vascular inflammation,[12,13] and aldosterone increases infarct size in animal models of myocardial infarction,[14] although this latter result has not been reported in other studies.[15,16] In addition, the administration of MR antagonists consistently reduces myocardial infarct size in these animal models.[17]Schmidt et al[15] 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 signalling. Adenosine is formed by intracellular 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.[18] 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 nonpharmacological strategies.[18]

Firstly, we 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.[19] This protocol of IR results in an immediate decrease in brachial artery FMD, which reflects IR-induced endothelial dysfunction.[20,21]

2 | DESIGN

We performed a prospective case-control study including patients with PA and patients with EHT from two centres in the Netherlands from October 2013 to March 2017. All patients with PA were recruited in the Radboud university medical center, which 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.[22] 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 as follows: 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: nonsteroidal 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. Reporting of the study conforms to STROBE statement along with references to STROBE statement and the broader EQUATOR guidelines.[23]

2.1 | 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

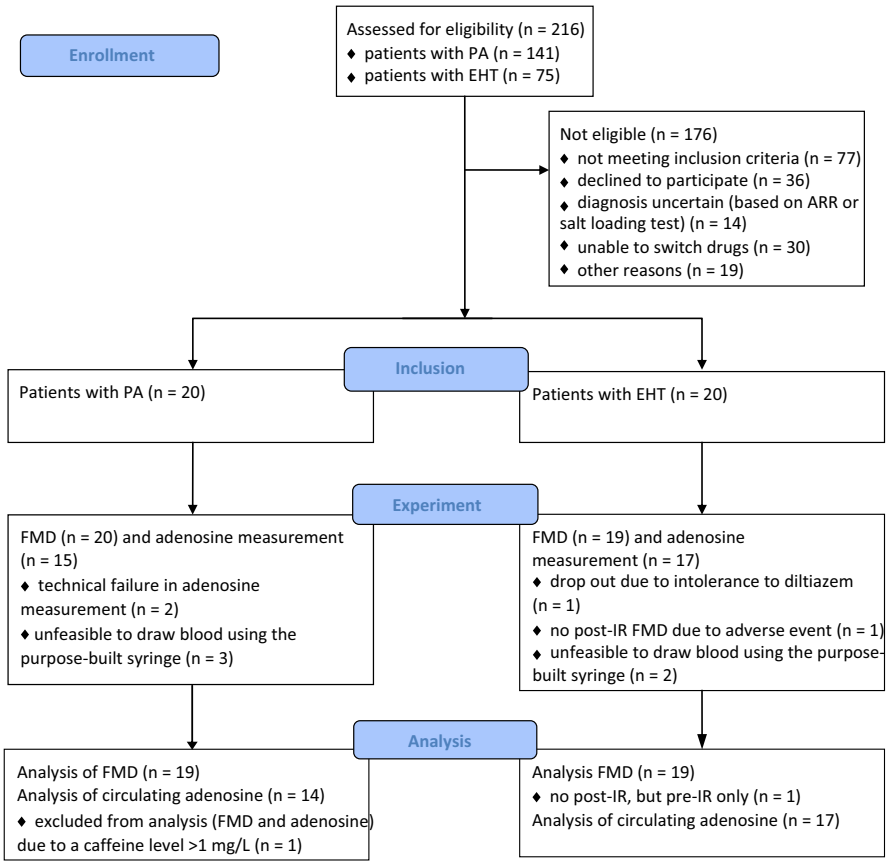

FIGURE 1 Overview of the selection process of patients. Abbreviations: EHT, essential hypertension; FMD, flow-mediated dilation; IR, ischaemia reperfusion; and PA, primary aldosteronism

spironolactone, eplerenone, amiloride, triamterene or aliskiren) or 2 (for all other antihypertensive drugs) weeks prior to aldosterone and renin measurements.[22]

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.[24] In patients from the Rijnstate Hospital, plasma aldosterone and plasma renin activity 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 vs 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/h

2.2 | 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.[25] At least 1 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 1 week before the experiments.[26] In addition, we aimed to avoid hypokalaemia 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.[27]

2.3 | 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.[28] 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 analysed using reversed-phase high-performance liquid chromatography and fluorescence detection with excitation and emission wavelength set at 280 and 420 nm. Separation took place on a Polaris column (Varian, Polaris 3 μm C18-A 150 × 4.6 mm) with a mobile phase containing 50 mmol/L $NH_4H_2PO_4$ 5 mmol/L of hexane sulphonic acid (pH 3.0). Acetonitrile was used as the organic modifier.

2.4 | 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 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4 and 1.8 mmol/L 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.[26]

2.5 | Additional blood drawing

Before start of the experiment we drew blood to determine plasma potassium levels and caffeine concentrations, as described previously.[29] Subjects with a circulating caffeine concentration >1.0 mg/L were excluded from analysis, since caffeine is a potent adenosine receptor antagonist.[30]

2.6 | 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 5 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), according to the guideline of Thijssen et al.[27]

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 ischaemic stimulus distal from the brachial artery, leading to reactive hyperaemia 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 mm Hg. 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 mm Hg (or 50 mm Hg 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.[31] 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 × 100%). Other outcome measures that were assessed include time to peak (sec) and shear rate (area under the curve).

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)	.21
Screening			
Mean SBP (SD)	155 (19)	155 (25)	1.00
Mean DBP (SD)	91 (14)	91 (12)	.88
Mean heart rate (SD)	71 (17)	70 (13)	.86
Median duration of known hypertension in years (IQR)	7.5 (2.6-12.5)	6.0 (4.0-11.5)	.84
Median baseline aldosterone in nmol/L (IQR)	0.81 (0.61-0.93)	0.22 (0.11-0.28)	<.01
Median baseline ARR in nmol/mU (IQR) (n = 15)	0.22 (0.17-0.25)	0.01 (0.01-0.02)	<.01
Median baseline ARR in nmol/L per ng/mL/h (IQR) (n = 5)	–	0.41 (0.15-0.49)	–
Mean plasma sodium in mmol/L (SD)	141.9 (2.7)	141.0 (1.7)	.14
Median plasma potassium (IQR)	3.8 (3.6-4.0)	4.0 (3.8-4.1)	.03
Median plasma creatinine in μmol/L (IQR)	75.0 (68.0-84.0)	84.5 (71.5-89.5)	.13
Median kidney function (MDRD) in mL/min (IQR)	84 (76-91)	79 (75-91)	.57
Mean total plasma cholesterol in mmol/L (nonfasting) (SD)	5.2 (1.0) n = 19*	4.9 (1.0) n = 19*	.33
Median plasma glucose in mmol/L (nonfasting) (IQR)	5.3 (4.9-5.9) n = 20	5.2 (4.8-5.8) n = 18†	.59
Risk factors			
History of smoking (%)	8 (40)	10 (50)	.75
Median units of alcohol per week (IQR)	3.5 (0-7.0)	2.0 (0-10.0)	.67
Mean BMI (SD)	27.5 (5.6)	27.4 (4.3)	.96
Dyslipidaemia (%)	4 (20)	6 (30)	.72
1st grade family history of hypertension or CVD (%)	12 (60)	16 (80)	.30

Abbreviations: ARR, aldosterone-to-renin ratio; BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; EHT, essential hypertension; IQR, interquartile range; MDRD, modification of diet in renal disease; PA, primary aldosteronism, PY, pack years; SBP, systolic blood pressure; and SD, standard deviation.

*in 1 patient a value of a fasting (vs nonfasting) cholesterol was available.

†in 2 patients a value of a fasting (vs nonfasting) glucose value was available.

2.7 | 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%.[32] The average reduction in FMD by IR was 2.0% (standard deviation [SD] 2.4%). To demonstrate a twofold 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

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)	<.01
Mean SBP in mm Hg (SD)	158 (19)	155 (21)	.66
Mean DBP in mm Hg (SD)	96 (9)	93 (10)	.37
Median heart rate in x/min (IQR)	64 (60-68)	60 (60-68)	.47
Median number of antihypertensive drugs (IQR)	1 (0-1)	1 (0-1)	.31
Median DDD antihypertensive drugs (IQR)	0.83 (0.00-1.00)	0.75 (0.00-0.83)	.20
Mean duration in min between reperfusion and post-FMD measurement (SD)†	21.7 (1.9)	22.7 (2.7)	.20

Abbreviations: DBP, diastolic blood pressure; DDD, daily defined dosage; EHT, essential hypertension; FMD, flow-mediated dilation; IQR, interquartile range; IR, ischaemia reperfusion; PA, primary aldosteronism; SBP, systolic blood pressure; and SD, standard deviation.

*n = 18 in both patient groups.

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

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.

3 | RESULTS

3.1 | 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 ischaemia, because of sudden appearance of petechiae of the ischaemic 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 patients 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).

3.2 | 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 ($P = .008$).

3.3 | CD73 activity

There was no significant difference in CD73 activity of intact mononuclear cells of patients with PA vs patients with EHT. Patients with PA had a CD73 activity of 0.43 (0.19-0.55) vs 0.54 (0.33-0.80) nmol/min per mg protein in patients with EHT; $P = .21$.

3.4 | 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%, $P = .001$). The decrease in % FMD was not significant within the group of patients with PA (4.4 ± 2.1 to 3.3 ± 2.7, $P = .14$).

Post-IR % FMD minus pre-IR % FMD did not differ between patients with PA and patients with EHT (−1.0 ± 2.9% vs −1.6 ± 1.6% respectively, $P = .52$).

4 | 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.

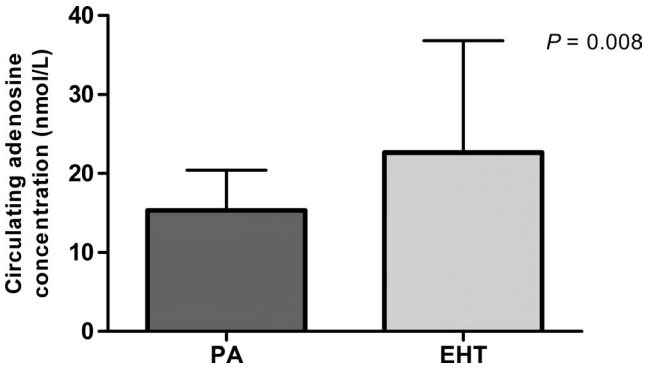

FIGURE 2 Circulating adenosine concentrations in nmol/L in patients with primary aldosteronism (PA; n = 14) and patients with essential hypertension (EHT; n = 17)

Adenosine is an endogenous purine nucleoside with several beneficial effects on the cardiovascular system, including vasodilation, anti-atherosclerotic effects, inhibition of inflammation and fibrosis, and limitation of IR injury.[33] 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.[34]

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 hyperhomocysteinaemia, 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.[35]

In our study, we have now shown that also in patients with PA the endogenous adenosine concentration is reduced. Although aldosterone might have multiple potentially adverse cardiovascular effects that are independent from lowering aldosterone, we 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.[36] 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.[37,38]

The circulating concentration of adenosine is the sum of adenosine production, cellular uptake and intracellular degradation. Activity of the enzyme CD73, which catalyses the extracellular formation of adenosine from adenosine

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)	.86	.18
Mean FMD in mm (SD)	0.441 (0.076)	0.437 (0.083)	0.483 (0.081)	0.445 (0.080)	.90	.16
Mean FMD in % (SD)	4.4 (2.1)	4.9 (1.9)	3.3 (2.3)	3.3 (2.0)	.47	1.00
Median time to peak in sec (IQR)	54 (38-89)	50 (40-74)	63 (32-75)	50 (31-77)	.58	.73
Mean shear rate AUC (SD)	21945 (10103)	19968 (8572)	17623 (11737)	19293 (11910)	.52	.67

Abbreviations: AUC, area under the curve; EHT, essential hypertension; FMD, flow-mediated dilation; IQR, interquartile range; IR, ischaemia reperfusion; PA, primary aldosteronism; and SD, standard deviation.

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

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 hyperhomocysteinaemia.[35] 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 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 discrepant finding. Firstly, 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[41-43] and clinical studies[44,45] failed to show an effect of exogenous adenosine on IR injury. Secondly, 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. For example, adenosine receptor antagonists did not increase infarct size itself in preclinical models of IR injury,[15] although these antagonists did significantly prevent the beneficial effects of ischaemic pre-conditioning and post-conditioning.[46,47] Thirdly, many endogenous substances and underlying mechanisms other than the adenosine metabolism regulate IR susceptibility, including an increase in reactive oxygen species, a decrease in intracellular pH and a reduction in bioavailability of nitric oxide.[48]

Future studies in patients with PA should therefore not focus on IR injury, but on alternative determinants of cardiovascular damage.

One of the processes that might be affected by adenosine in the context of PA is atherosclerosis. 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[18] and in genetic deletion models, inactivation of the adenosine metabolism leads to progression of atherosclerosis.[49]

Similarly, fibrosis and inflammation might be the link between the reduced concentration of circulating adenosine in patients with PA and their increased risk of cardiovascular events. In preclinical studies, aldosterone stimulates vascular and myocardial inflammation and fibrosis and hence plays an important pathophysiological role in remodelling of the heart and vessel wall.[9-13] Since adenosine has anti-fibrotic and anti-inflammatory properties,[18] the reduced levels of circulating adenosine in patients with PA compared to patients with EHT might contribute to the increased prevalence of cardiovascular morbidity and mortality in general in patients with PA compared to patients with EHT.

We therefore propose that the reduced circulating adenosine levels in patients with PA may contribute to progression of atherosclerosis or fibrosis and inflammation, rather than IR injury. Our study benefits from the use of stringent criteria for the diagnosis of PA and EHT, detailed clinical characterization 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.[27]

Nevertheless, some aspects of our methods and results merit critical discussion. First, although some patients dropped out of the study, the group size was according to the sample size calculation with regard to measuring IR injury. Circulating adenosine concentrations could be measured in 14 patients with PA and 17 patients with EHT, due to difficulties in the sample collection or processing of the samples. Nevertheless, the difference in the adenosine concentrations was statistically significant ($P = .008$).

Secondly, 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,[27,50] and brachial FMD is a good predictor of future cardiovascular events.[51] The reduction in FMD immediately after a period of forearm ischaemia 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.[19,52]

Thirdly, 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.[53-55] Several explanations can be found for the discrepancies between our study and these studies. In contrast to the study of Nishizaka et al,[53] we used stringent diagnostic criteria for PA and EHT, concordant to international guidelines. Importantly, we standardized antihypertensive treatment to diltiazem with or without hydralazin and/or doxazosin. In previous studies,[53-55] different antihypertensive drugs may have modulated endothelial function, and therefore, their results have to be interpreted with caution.[56] Furthermore, the above mentioned studies do not describe any dietary restrictions before FMD measurement.[53-55] In our study, patients were 24 hours free of alcohol and caffeine before FMD measurement, as recommended in the expert guideline.[27] Next, we excluded patients with an history of cardiac failure, atherosclerotic disease, severe renal dysfunction, diabetes mellitus and/or current smoking. Chou et al[55] do not describe any of these baseline characteristics. 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.[55] Finally, in the study by Matsumoto et al,[54] a reduction in % FMD was seen only in those patients with PA who suffered from aldosterone-producing adenomas, and not idiopathic aldosteronism. Likewise, of the 35 patients with PA in the study by Chou et al,[55] 91% had an aldosterone-producing adenoma. 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% vs 4.3 ± 2.5% respectively; $P = .67$). 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; $P = .13$. 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. However, the adverse cardiovascular effects of aldosterone probably involve multiple pathways, and the effects of increasing adenosine remains to be tested.

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CONFLICT OF INTEREST

GAR is a member of a data safety monitoring board of Mereo BioPharma. All other authors have nothing to declare.

AUTHOR CONTRIBUTIONS

TNAvdB, DHJT, GAR, PHvdB, JD and NPR conceived or designed the study. TNAvdB and HM contributed to inclusion of patients. TNAvdB and ACCMvM collected the data. TNAvdB, DHT, ACCMvM and PHvdB analysed and interpreted the data. TNAvdB and NPR drafted the article. All authors contributed to critical revision of the article. All authors contributed to final approval of the version to be published.

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