


# Blood pressure, arterial stiffness and endogenous lithium clearance in relation to AGTR1 A1166C and AGTR2 G1675A gene polymorphisms

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Marcin Cwynar<sup>1</sup>, Jerzy Gąsowski<sup>1</sup>, Anna Głuszewska<sup>1</sup>,  
 Jarosław Królczyk<sup>1</sup>, Henryk Bartoń<sup>2</sup>, Agnieszka Słowik<sup>3</sup>  
 and Tomasz Grodzicki<sup>1</sup>

## Abstract

**Introduction:** Although recently a matter of epidemiologic controversy, sodium overload and its interaction with genetic factors predispose to hypertension and related target organ complications.

**Methods:** In 131 (66 male) treated hypertensives, we measured peripheral and central arterial pressures and pulse wave augmentation indexes ( $Alx_p$ ,  $Alx_{C1}$ ,  $Alx_{C2}$ ), pulse wave velocity (PWV), daily urinary sodium excretion and did genetic studies of AGTR1 A1166C and AGTR2 G1675A polymorphisms. Proximal ( $FE_{Li}$ ) and distal ( $FDR_{Na}$ ) sodium reabsorption measurements were performed using endogenous lithium clearance.

**Results:** In men, we found interaction between  $FDR_{Na}$  and AGTR2 G1675A polymorphism with respect to  $Alx_{C1}$  ( $p_{INT}=0.01$ ),  $Alx_{C2}$  ( $p_{INT}=0.05$ ) and  $Alx_p$  ( $p_{INT}=0.006$ ). Arterial stiffness increased with higher sodium reabsorption in the distal tubule, in the presence of AGTR2 G allele with the opposite tendency in A allele carriers. In the subgroup with  $FDR_{Na}$  below median, as compared to those with  $FDR_{Na}$  above median, the  $Alx_{C1}$  ( $139.6\pm 3.8$  vs  $159.1\pm 5.7\%$ ;  $p=0.009$ ),  $Alx_{C2}$  ( $26.3\pm 1.8$  vs  $33.3\pm 1.7\%$ ;  $p=0.016$ ) and  $Alx_p$  ( $83.4\pm 2.5$  vs  $96.5\pm 2.6\%$ ;  $p<0.0001$ ) were lower, in the G allele carrying men and GG homozygous women.

**Conclusions:** The relation between sodium reabsorption in the distal tubule and the development of arterial stiffness depends on the AGTR2 G1675A polymorphism in blood pressure independent fashion.

## Keywords

Hypertension, arterial stiffness, distal sodium reabsorption, AGTR2 G1675A

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## Introduction

Results from several recent post-hoc analyses of prospective data showed the apparently divergent results concerning the relation between the level of sodium consumption and cardiovascular outcome. First, based on a large cross-European cohort of nuclear families, higher consumption of sodium in healthy subjects did not translate into a greater risk of cardiovascular events.<sup>1</sup> Another analysis of data revealed a J-shaped relationship between sodium intake and cardiovascular complications.<sup>2</sup> Thus far, neither the concordant nor the discrepant aspects of these analyses have found a possible unifying mechanistic explanation. Sodium load influences blood pressure values and facilitates damage to target organs.<sup>3,4</sup> The mechanisms involved

include an interaction between environmental factors such as sodium intake, and changes in renal sodium handling

<sup>1</sup>Department of Internal Medicine and Gerontology, Jagiellonian University, Poland

<sup>2</sup>Department of Food Chemistry and Nutrition, Jagiellonian University, Poland

<sup>3</sup>Department of Neurology, Jagiellonian University, Poland

### Corresponding author:

Marcin Cwynar, Department of Internal Medicine and Gerontology, Medical College, Jagiellonian University, 31-351 Krakow, Sniadeckich 10, Poland.

Email: [marcincw@poczta.onet.pl](mailto:marcincw@poczta.onet.pl)



caused, apart from kidney injuries, by mutations of a number of genes encoding cytoskeleton proteins, ion channels or hormones taking part in sodium metabolism.<sup>5,6</sup>

Angiotensin II (AT II), the effector hormone of the renin-angiotensin-aldosterone axis (RAA) axis, which is the main hormonal regulatory system in sodium-water homeostasis, exerts its biological effect via specific AT1 and AT2 receptors. Accumulating data established the role of the AT1 receptor in the classic actions of AT II including vasoconstriction and cardiovascular hypertrophy, whereas the AT2 receptor is suggested to exert direct functions in vasodilation and antigrowth effects.<sup>7</sup> The above observation raises the possibility that genetic variability in the AT1 and AT2 receptor genes (AGTR1 and AGTR2) has an impact on blood pressure, arterial stiffness and left ventricular hypertrophy. Such impact may indeed be mediated by sodium-dependent mechanisms and involve influence on renal sodium handling.

In the past all studies which investigated the relationship between target organ damage and sodium renal handling, and which involved endogenous lithium clearance methodology, were performed either in the general population of healthy adults<sup>8</sup> or untreated hypertensives,<sup>9</sup> and many experts advised against application of this methodology, or all similar studies in treated hypertensive patients, altogether.<sup>10,11</sup> On the other hand, as demonstrated by Hollenberg et al.<sup>12</sup> and Burnier et al.,<sup>13</sup> many of the pivotal antihypertensive medications such as Angiotensin converting enzyme (ACE)-inhibitors and angiotensin receptor blockers, do not materially change the inferences from the assessment of renal sodium handling in patients with high sodium intake as compared to results from medication-naïve individuals, leaving diuretics as the only classes of medications which are deemed to distort the results based on endothelium lithium clearance methodology. In daily practice we usually deal with treated hypertensive, rather than treatment-naïve, patients, and still less with the untreated healthy adults. And it is the treated patients who carry the bulk of the burden of cardiovascular risk. Thus, prepared to face criticism, we decided to study the relationship between renal sodium handling, angiotensin receptor gene polymorphisms and arterial stiffening in a group of treated hypertensive patients with high sodium intake, free from diuretics.

## Methods

### Study population

The study was approved by the Bioethical Committee of Jagiellonian University (KBET/141/B/2009). From April 2010–May 2013 in the framework of the National Research Centre grant (Nr. N N402 533239), we enrolled 140 hypertensive patients followed up at the reference hypertension clinic of the University Hospital in Krakow. The study design has been described in detail elsewhere.<sup>14</sup> Briefly, all

patients were requested to complete a standard epidemiologic questionnaire including detailed medical history, antihypertensive medications, and socioeconomic and demographic characteristics. The patients provided written informed consent to participate in the study. The patients with a history of malignancy, decompensated chronic diseases, cardiomyopathy of unknown aetiology, haemodynamically significant valvular heart disease or secondary hypertension were excluded from the study. To avoid interference between sodium excretion/reabsorption and the use of diuretic agents, only the patients not receiving long-term diuretic treatment or patients in whom hypertension was sufficiently well-controlled to allow discontinuation of diuretic treatment at least five days before enrolment were included in the study. The five-day washout period was deemed sufficient to eliminate an influence of diuretics on the analysed parameters.<sup>15</sup>

Nine patients were excluded from the statistical analysis: four because of diuretic treatment within five days before the 24-hour urine collection, three because of failure to complete the urine collection and two because of high serum lithium levels ( $>2.0 \mu\text{mol/l}$ ) and urinary lithium levels ( $>25.0 \mu\text{mol/l}$ ) that may have indicated external contamination or high dietary lithium intake. Thus, 131 subjects were included in the statistical analysis.

### Blood pressure and vascular stiffness measurements

Blood pressures were measured using the Omron HEM-705 device (Omron Healthcare, Kyoto, Japan). In every patient the measurements were performed on a non-dominant arm in a sitting position, after a minimum of 10-minute rest; blood pressure values were calculated as a mean of three measurements. Pulse pressure (PP) was calculated as a difference of the mean systolic blood pressure (SBP) and the mean diastolic blood pressure (DBP), and the mean blood pressure (MBP) was calculated as a sum of DBP and one-third of PP.

The measurements of arterial properties were performed by a one observer with the patient after a 15-minute rest and included central pulse wave analysis (PWA) (PWA module of the SphygmoCor 6.31 device; AtCor Medical Pty Ltd, West Ryde, New South Wales, Australia) and pulse wave velocity (PWV) (PWV module of the Complior device; Colson, France). PWA recordings were performed in eight-second periods using peripheral arterial tonometry. The measurements were performed on the radial artery of the non-dominant upper extremity using a SPC-301 micromanometer (Millar Instruments, Inc., Houston, Texas, USA). The following of the obtained parameters were considered in the statistical analyses: blood pressure parameters, including: central systolic blood pressure (SBP<sub>c</sub>), central diastolic blood pressure (DBP<sub>c</sub>), central mean blood pressure (MBP<sub>c</sub>) and central pulse pressure (PP<sub>c</sub>), and arterial

stiffness parameters, including: aortic pulse wave augmentation (AG) and peripheral (AIx<sub>p</sub>) and central (AIx<sub>c</sub>) aortic pulse wave augmentation indexes. AG (mm Hg) was calculated as a difference of the first and second systolic peak on the ascending arm of the aortic pulse wave. AIx<sub>p</sub> (%) and AIx<sub>c1</sub> (%) were calculated as a quotient of the first and second systolic peak on the ascending arm of the radial and aortic pulse wave, and AIx<sub>c2</sub> (%) was calculated as a product of AG and PP<sub>c</sub>. The averaged pulse waves showing <5% difference of the height and width of systolic and diastolic portions or >80 mV amplitude of the wave signal, were classified as normal. PWV (m/s) was measured between carotid and femoral arteries. Mean values of 10 consecutive measurements were included in the statistical analysis.

### Echocardiography

Structural and functional measurements of left ventricular (LV) myocardium were performed using transthoracic, 2D echocardiography with Doppler measurements (Vivid 4, General Electric, Wauwatosa, Wisconsin, USA), with a 2.5–3.5 MHz transducer. Pulsed-wave Doppler with a gate positioned on the mitral valve was used to assess the early (E) and late (A) mitral inflow, their proportion (E/A) and the E wave deceleration time (DT). The isovolumetric relaxation time (IVRT) was measured as the time from the end of aortic inflow to the beginning of the mitral inflow. Tissue Doppler imaging was used for the measurements of the movements of the mitral annulus with a gate positioned on the basal segment of the ventricular septum. These measurements included early diastolic velocity (e'), maximum velocity after atrial contraction (a') and their proportion (e'/a'); the E/e' index was also calculated. LV ejection fraction was assessed using the Simpson method. Stroke volume (SV) was calculated using planimetry in the four chamber apical view. Diastolic LV dysfunction was diagnosed on the basis of echocardiographic parameters for persons >50 years according to the European Society of Cardiology (ESC) guidelines.<sup>16</sup>

### Clinical measurements

Anthropometric measurements, including: height, body weight, waist circumference and hip circumference were obtained in every patient. Body mass index (BMI) was calculated as a ratio of the body weight (kg) and a square of the height (cm). Waist-to-hip ratio (WHR) was calculated as the ratio of the waist and hip circumferences. In the morning of cardiovascular measurements, a fasting blood sample was obtained from each patient for serum measurements of glucose, glycosylated haemoglobin A1c (HbA<sub>1c</sub>), serum lipid levels, N-terminal natriuretic propeptide B (NT-proBNP), sodium, potassium, creatinine and lithium. One day before, the patients recruited to the study

completed a 24-hour urine collection to measure 24-hour excretion of sodium (Na<sub>24</sub>), potassium, creatinine, lithium, and albumin.

### Renal sodium handling and genetic studies

We measured serum and urinary lithium concentrations necessary for the calculations of lithium clearance with atomic absorption spectrometry (AAS) using the 5100ZL AAS equipment (Perkin-Elmer Inc., Waltham, Massachusetts, USA). Urinary lithium levels were detected using flame injection AAS (FI-AAS) and graphite furnace AAS (GF-AAS) for urine measurements in the case of lithium concentrations below the level of 10 µg/l. Serum lithium concentrations were detected with GF-AAS. The analytic parameters of FI-AAS in urine were: limit of detection (LOD) at 1.4 µg/l, precision at Li concentration of 25.0 µg/l at 5%, recovery at 95–100%. The analytic parameters of GF-AAS for urine samples were as follows: LOD at 0.9 µg/l, precision at Li concentration of 10.0 g/l at 5%, recovery at 94%, and for serum, LOD 0.4–0.8 µg/l, precision at Li concentration of 5.0 g/l at 9%, recovery at 96%. Creatinine, sodium and lithium clearance (C) values were calculated using the formula:

$$C_x = V \times U_x / P_x$$

where V (m/min) was a 24-hour urine volume calculated per minute and U<sub>x</sub> and P<sub>x</sub> were molar creatinine, sodium or lithium concentrations in urine and serum, respectively. High lithium clearance or a small difference between the lithium and sodium clearances indicates increased capacity for renal excretion of excessive sodium loads. Fractional sodium excretion (FE<sub>Na</sub>) and fractional lithium excretion (FE<sub>Li</sub>) were expressed as percentages derived from the product of sodium or lithium clearance, respectively, and creatinine clearance:

$$FE_x (\%) = 100 \times C_x / C_{CR}$$

where x represents lithium or sodium. Since lithium is transported across the membranes of proximal renal tubular cells using the same pathways as sodium ions, FE<sub>Li</sub> is a very accurate marker of glomerular filtration rate (GFR)-dependent sodium reabsorption in the proximal tubules. Low FE<sub>Li</sub> indicates higher sodium and water load reabsorbed in the proximal tubule, which results in a lower sodium and water load filtered to the distal tubule. Measurements of both FE<sub>Li</sub> and FE<sub>Na</sub> allow for estimating the fractional distal sodium reabsorption (FDR<sub>Na</sub>) using the formula:

$$FDR_{Na} = ((FE_{Li} - FE_{Na}) / FE_{Li}) \times 100$$

FDR<sub>Na</sub> is an estimate of sodium that is not reabsorbed in the proximal tubule, but undergoes reabsorption in the

post-proximal segments of the renal tubules. High  $FDR_{Na}$  indicates higher sodium and water load reabsorbed in the distal tubule, which results in a lower sodium and water load that is filtered and subsequently excreted with urine.<sup>17</sup>

Genetic studies were performed at the Department of Neurogenetics, the Chair of Neurology of the Jagiellonian University using peripheral blood collected on ethylenediaminetetraacetic acid (EDTA). Genomic DNA was isolated using the QIAamp DNA Blood Mini Kit (QIAGEN, Germantown, Maryland, USA). We assayed the *A1166C* polymorphism of the *AGTR1* gene and *G1675A* of the *AGTR2* gene with the real-time polymerase chain reaction (RT-PCR) performed with ViiA 7 Real Time PCR System (Life Technologies, Carlsbad, California, USA). We used the TaqMan Pre-Designed SNP Genotyping Assay. The methodology has been described in detail elsewhere.<sup>18</sup>

### Statistical analysis

Database management and statistical analyses were performed using the SAS System 9.3 software (SAS Institute Inc., Cary, North Carolina, USA). The distributions of the analysed quantitative variables were compared with the normal distribution using the Shapiro-Wilk and Kolmogorov-Smirnov tests. In the descriptive statistics the quantitative data were expressed as mean values and standard deviations (for the data with normal distribution) or as median values and interquartile ranges (for the data that did not fulfil the criteria of normal distribution). The qualitative data were expressed as proportions. For the comparisons of the mean values in the groups of patients the Student's *t*-test was used. Alternatively, Wilcoxon and median tests were used in case of the skewed distribution of quantitative variables in the subgroups. The  $\chi^2$  Pearson's test or Fisher's test was used for qualitative variables. The correlations of quantitative sodium parameters were analysed using the standardised Spearman's correlation analysis. Simple linear regression analysis was performed to identify the factors associated with the analysed sodium parameters ( $FE_{Li}$ ,  $FDR_{Na}$ ,  $Na_{24}$ ).

The conformance of the distributions of the analysed genotypes with the distributions estimated using the Hardy-Weinberg principle was confirmed using  $\chi^2$  Pearson's test. Selected parameters of blood pressure and arterial stiffness were compared for the *AGTR1 A1166C* and *AGTR2 G1675A* gene polymorphisms using variance analysis in the whole study group and separately for male and female patients. The *G1675A* alleles are located on chromosome X, thus, to achieve a homogenous genetic background across genders the homozygotic women were analysed together with respective male groups. Then a multifactorial regression model (PROC GENMOD module of the SAS 9.2 software)<sup>19</sup> was applied to include the dependent variables and to calculate the standardised mean values and standard measurement error for the

respective genotypes and allele carriers. In the subsequent analyses a multifactorial regression model was applied to analyse the interactions between genetic and environmental factors with respect to blood pressure and parameters of arterial stiffness.

Our study was an association study with allelic variants treated as classification variables. The primary outcome measure was e.g. central augmentation index expressed as a percentage increase of central SBP ( $AIX_{C1}$ ). For calculations we employed the PROC GLMPOWER procedure as implemented in SAS 9.2. Assuming the standard deviation of  $AIX_{C1}$  to be 10%, about 50 persons would be needed per allelic group to achieve 80% power at alpha of 0.05 to detect 5% between  $FDR_{Na}$  group difference in  $AIX_{C1}$  in each allelic group, i.e. *G* vs *A* for *G1675A* polymorphism of *AGTR2* gene, separately. In our study the groups comprised 46 and 52 individuals, for *G* and *A* alleles, respectively.

## Results

### Characteristics of the study population

The study population consisted of 131 patients, including 66 men (50.3%) and 65 women (49.7%). The mean time from establishing the diagnosis of hypertension to enrolment to the study was 11.7 years. The daily sodium intake assessed based on 24-hour urine collection was  $193.9 \pm 95.3$  mmol. Male patients had higher BMI ( $30.4 \pm 4.3$  vs  $28.9 \pm 4.2$  kg/m<sup>2</sup>,  $p=0.04$ ), WHR ( $0.96 \pm 0.06$  vs  $0.85 \pm 0.06$ ,  $p<0.001$ ) and  $Na_{24}$  ( $236.6 \pm 99.5$  vs  $153.9 \pm 70.1$  mmol/l,  $p<0.001$ ) than female patients. However, no differences between the male and female patients were found for  $FE_{Na}$  ( $0.94 \pm 0.31$  vs  $0.81 \pm 0.19\%$ ,  $p=0.21$ ) and the parameters calculated using the endogenous lithium clearance:  $FE_{Li}$  ( $20.8$  ( $15.0$ – $34.7$ ) vs  $23.3$  ( $14.6$ – $44.8$ )%,  $p=0.49$ ) and  $FDR_{Na}$  ( $96.0$  ( $94.0$ – $97.6$ ) vs  $97.3$  ( $95.3$ – $98.1$ )%,  $p=0.07$ ). Clinical parameters of the study group, as well as the parameters of blood pressure, arterial stiffness, biochemical measurements in blood and 24-hour urine collections, calculated sodium, creatinine and lithium clearances and  $FE_{Na}$ ,  $FE_{Li}$  and  $FDR_{Na}$  in men and women analysed separately are summarised in Tables 1 and 2. There were no gender differences with respect to the treatment with ACE-inhibitors, angiotensin-receptor blockers, calcium-channel blockers and beta-blockers in the groups analysed in the study (all  $p \geq 0.12$ ).

Factors determining the peripheral and central blood pressure values and arterial stiffness parameters including PWV,  $AIX_{C1}$ ,  $AIX_{C2}$  and  $AIX_P$  were established using simple linear regression. In the study group, PWV showed a positive relationship with age ( $\beta=0.09$ , standard error (SE)=0.03;  $p=0.01$ ), WHR ( $\beta=0.06$ , SE=0.03;  $p=0.03$ ), SBP ( $\beta=0.07$ , SE=0.01;  $p=0.001$ ) and LV dysfunction ( $\beta=1.19$ , SE=0.56;  $p=0.03$ ).  $AIX_{C1}$  showed



**Table 1.** Clinical characteristics and arterial stiffness parameters in the study population.

	Male n=66	Female n=65	p
<i>Clinical characteristics</i>			
Age (years)	60.2 ± 7.4	64.2 ± 6.9	0.002
Height (cm)	174.9 ± 5.9	160.9 ± 6.5	0.001
Weight (kg)	93.0 ± 14.0	74.7 ± 11.5	0.001
BMI (kg/m <sup>2</sup> )	30.4 ± 4.3	28.9 ± 4.2	0.042
Waist (cm)	105.0 ± 10.8	93.3 ± 12.3	0.001
Hip (cm)	109.0 ± 8.3	109.7 ± 9.8	0.663
Waist-to-hip ratio (WHR)	0.96 ± 0.06	0.85 ± 0.06	0.001
Heart rate (beats/min)	69.8 ± 11.2	69.8 ± 8.7	0.995
SBP (mm Hg) <sup>a</sup>	144.4 ± 21.7	143.4 ± 20.0	0.775
DBP (mm Hg) <sup>a</sup>	83.1 ± 11.7	81.2 ± 10.3	0.344
MBP (mm Hg) <sup>a</sup>	103.5 ± 14.0	101.9 ± 12.3	0.498
PP (mm Hg) <sup>a</sup>	61.3 ± 15.2	62.1 ± 15.3	0.768
<i>Arterial stiffness parameters</i>			
PWV (m/s)	14.1 ± 3.0	13.2 ± 2.9	0.116
SBP <sub>C</sub> (mm Hg)	132.6 ± 21.3	132.8 ± 19.6	0.994
DBP <sub>C</sub> (mm Hg)	82.7 ± 11.4	80.3 ± 11.5	0.258
PP <sub>C</sub> (mm Hg)	50.3 ± 14.3	52.3 ± 14.1	0.424
AG (mm Hg)	15.1 ± 7.2	18.4 ± 8.1	0.019
Alx <sub>C1</sub> (%)	142.6 ± 22.0	154.7 ± 21.7	0.003
Alx <sub>C2</sub> (%)	28.8 ± 9.7	34.0 ± 9.4	0.003
Alx <sub>P</sub> (%)	87.1 ± 11.7	95.1 ± 13.1	0.001

AG: central pulse wave augmentation; Alx<sub>C1</sub> and Alx<sub>C2</sub>: central pulse wave augmentation indexes; BMI: body mass index; DBP: diastolic blood pressure; DBP<sub>C</sub>: central diastolic blood pressure; MBP: mean blood pressure; PP: pulse pressure; PP<sub>C</sub>: central pulse pressure; PWV: pulse wave velocity; SBP: systolic blood pressure; SBP<sub>C</sub>: central systolic blood pressure.

The data are presented as arithmetical means, <sup>a</sup>mean of three blood pressure measurements obtained at one visit.

**Table 2.** Biochemical studies in serum and 24-hour urine and the parameters obtained using endogenous lithium clearance in the study population. The data are presented as arithmetical mean ± SD and as median values with interquartile ranges.

	Male n=66	Female n=65	p
<i>Serum</i>			
Creatinine (μmol/l)	80.8 ± 12.2	65.9 ± 13.1	0.001
Sodium (mmol/l)	140.6 ± 1.9	140.6 ± 2.2	0.962
Potassium (mmol/l)	4.3 ± 0.4	4.3 ± 0.3	0.836
Lithium (μmol/l)	0.14 (0.08–0.23)	0.15 (0.06–0.22)	0.692
<i>24-hour urine</i>			
Volume (l)	1.87 ± 0.66	1.67 ± 0.62	0.081
Creatinine excretion (mmol)	15.3 ± 3.7	9.4 ± 3.0	0.001
Sodium excretion (mmol)	236.6 ± 99.5	153.6 ± 70.5	0.001
Potassium excretion (mmol)	69.1 ± 24.1	51.1 ± 18.0	0.001
Albumin excretion (mg)	19.0 ± 24.4	20.1 ± 32.5	0.829
Albumin/creatinine ratio (mg/mmol)	1.2 ± 1.4	2.3 ± 4.7	0.080
Creatinine clearance (ml/min/1.73 m <sup>2</sup> )	135.3 ± 36.0	103.8 ± 36.2	0.963
Sodium clearance (ml/min/1.73 m <sup>2</sup> )	1.17 ± 0.50	0.77 ± 0.35	0.001
<i>Endogenous lithium clearance</i>			
Lithium excretion (μmol/24 h)	5.42 (3.80–7.47)	4.06 (2.72–6.01)	0.010
Lithium clearance (ml/min/1.73 m <sup>2</sup> )	26.0 (20.0–41.1)	23.6 (14.3–39.4)	0.066
FE <sub>Na</sub> (%)	0.94 ± 0.61	0.81 ± 0.49	0.201
FE <sub>Li</sub> (%)	20.8 (15.0–34.7)	23.3 (14.6–44.8)	0.495
FDR <sub>Na</sub> (%)	96.0 (94.0–97.6)	97.3 (95.3–98.1)	0.065

FE<sub>Na</sub>: fractional urinary sodium excretion; FE<sub>Li</sub>: fractional urinary lithium excretion; FDR<sub>Na</sub>: fractional sodium reabsorption in distal tubules.

positive relation with age ( $\beta=0.70$ ,  $SE=0.27$ ;  $p=0.01$ ), female sex ( $\beta=12.25$ ,  $SE=3.87$ ;  $p=0.002$ ) and SBP ( $\beta=0.18$ ,  $SE=0.09$ ;  $p=0.05$ ), and negative relation with WHR ( $\beta=-0.58$ ,  $SE=0.23$ ;  $p=0.01$ ).  $AIx_{C2}$  showed positive relation with age ( $\beta=0.35$ ,  $SE=0.12$ ;  $p=0.004$ ), female sex ( $\beta=5.37$ ,  $SE=1.69$ ;  $p=0.002$ ) and SBP ( $\beta=0.09$ ,  $SE=0.04$ ;  $p=0.04$ ), and negative relation with WHR ( $\beta=-0.29$ ,  $SE=0.10$ ;  $p=0.04$ ).  $AIx_p$  showed positive relation with age ( $\beta=0.42$ ,  $SE=0.16$ ;  $p=0.008$ ), female sex ( $\beta=8.23$ ,  $SE=2.23$ ;  $p=0.0003$ ) and SBP ( $\beta=0.12$ ,  $SE=0.05$ ;  $p=0.02$ ), and negative relation with WHR ( $\beta=-0.42$ ,  $SE=0.13$ ;  $p=0.002$ ). On the basis of the obtained data, arterial stiffness parameters were standardised for age, sex, SBP, WHR, antihypertensive treatment and presence or absence of diastolic LV dysfunction.

The distributions of the analysed polymorphisms of the AGTR1 and AGTR2 genes were consistent with the Hardy-Weinberg principle: AGTR1 *A1166C* gene polymorphism ( $p=0.42$ ) and AGTR2 *G1675A* gene polymorphism ( $p=0.79$ ). The respective frequencies of the genotypes of the AGTR1 *A1166C* polymorphism were as follows: AA genotype (52.6%), AC genotype (39.1%) and CC genotype (8.3%), while the respective frequencies of the A and C allele were 72.6% and 27.4%, respectively. As the AGTR2 *G1675A* polymorphism is located on the X chromosome, further analyses were performed in men and women separately. In women, the respective frequencies of the genotypes were as follows: GG genotype (24.6%), GA genotype (50.8%) and AA genotype (24.6%). The frequencies of the G and A allele in women were 50.0% and 50.0% and in men were 45.6% and 54.6%, respectively.

### **Relationships of the AGTR1 *A1166C* polymorphism and sodium parameters with respect to peripheral and central blood pressures and arterial stiffness parameters**

For AGTR1 *A1166C* polymorphism, in the entire population and in male and female patients analysed separately, we found no statistically significant differences between the patients with various genotypes and the peripheral (all  $p \geq 0.15$ ), central (all  $p \geq 0.27$ ) blood pressures and arterial stiffness parameters: PWV ( $p \geq 0.85$ ),  $AIx_{C1}$  ( $p \geq 0.58$ ),  $AIx_{C2}$  ( $p \geq 0.63$ ) and  $AIx_p$  ( $p \geq 0.62$ ).

To demonstrate the relationships between sodium parameters and the genotypes of the AGTR1 *A1166C* polymorphism and their combined influence on blood pressure and arterial stiffness parameters we analysed the interactions between such polymorphism and  $FE_{Li}$  (all  $p_{INT} \geq 0.10$ ),  $FDR_{Na}$  (all  $p_{NT} \geq 0.14$ ) and  $Na_{24}$  (all  $p_{NT} \geq 0.22$ ). Moreover, in the analyses of the relationships with AGTR1 *A1166C* genotypes and sex, no statistically significant interactions were observed with respect to blood pressure and arterial stiffness parameters (all  $p_{INT} \geq 0.71$ ).

### **Relationships of the AGTR2 *G1675A* polymorphism and sodium parameters with respect to peripheral and central blood pressures and arterial stiffness parameters**

The analyses of the relationships of the AGTR2 *G1675A* polymorphism, conducted both in the whole study group and in the subgroups of male and female patients, revealed no significant differences between the allele carriers and patients with various genotypes of the polymorphism with respect to blood pressures (Table 3), as well as arterial stiffness parameters (Table 4).

To demonstrate the relationships between sodium parameters and the genotypes of the AGTR2 *G1675A* polymorphism and their combined influence on blood pressure and arterial stiffness parameters, in male and female patients separately, we analysed the interactions between such polymorphism and  $FE_{Li}$ ,  $FDR_{Na}$  and  $Na_{24}$ . The analyses of the polymorphism interactions with  $FE_{Li}$  (male:  $0.08 \leq p_{INT} \leq 0.93$ ; female:  $0.17 \leq p_{INT} \leq 0.91$ ) and  $Na_{24}$  (male:  $0.06 \leq p_{INT} \leq 0.33$ ; female:  $0.09 \leq p_{INT} \leq 0.73$ ) revealed no statistically significant interactions with respect to blood pressure and arterial stiffness parameters.

However, in men we revealed statistically significant relationships between the AGTR2 *G1675A* polymorphism and  $FDR_{Na}$  with respect to  $AIx_{C1}$ :  $p_{INT}=0.01$ ,  $AIx_{C2}$ :  $p_{INT}=0.05$  and  $AIx_p$ :  $p_{INT}=0.006$ . In the analyses of the relationships with  $FDR_{Na}$  in the women group, no statistically significant interactions were observed with respect to peripheral ( $0.21 \leq p \leq 0.34$ ), central ( $0.08 \leq p_{INT} \leq 0.33$ ) blood pressures and arterial stiffness parameters ( $0.09 \leq p_{INT} \leq 0.98$ ). In both sexes combined (with exclusion of heterozygous women), we observed statistically significant relationships between the AGTR2 *G1675A* polymorphism and  $FDR_{Na}$  with respect to  $AIx_{C1}$ :  $p_{INT}=0.01$ ,  $AIx_{C2}$ :  $p_{INT}=0.04$  and  $AIx_p$ :  $p_{INT}=0.01$ .

Thus, the subsequent analyses of the relationships between arterial stiffness parameters and the AGTR2 *G1675A* polymorphism were conducted using multifactorial regression analysis for the subgroups with sex-specific distribution of  $FDR_{Na}$  below and above the median value. In 30 *G* allele carrying men,  $AIx_{C1}$  (17.3% increase per 5% higher  $FDR_{Na}$ ;  $p=0.05$ ) and  $AIx_p$  (11.1% increase per 5% higher  $FDR_{Na}$ ;  $p=0.01$ ) with the similar trend for  $AIx_{C2}$ , arterial stiffness increased with higher sodium reabsorption in distal tubule, with the opposite tendency in *A* allele carriers. On the other hand, in 36 *A* allele carrier men,  $AIx_{C1}$  (8.7% borderline insignificantly decreasing per 5% higher  $FDR_{Na}$ ;  $p=0.06$ ) with the similar trend for  $AIx_{C2}$  and  $AIx_p$ , arterial stiffness decreased with higher sodium reabsorption in distal tubule. Additionally, in the subgroup with  $FDR_{Na}$  below the sex-specific median (96%) value,  $AIx_{C1}$  was 15.8% higher ( $p=0.004$ ),  $AIx_{C2}$  was 7.1% higher ( $p=0.01$ ) and  $AIx_p$  was 10.0% higher ( $p=0.007$ ) in the *A* allele compared with the *G* allele carriers.

**Table 3.** Analysis of the relationships between peripheral and central blood pressures and the AGTR2 *G1675A* polymorphism in the male and female patients.

Phenotype	Standardised mean $\pm$ SE			<i>p</i>
	<i>G</i>	.....	<i>A</i>	
<b>Male</b>				
<i>Number</i>	60	.....	72	
SBP, mm Hg	144.6 $\pm$ 3.9	.....	144.3 $\pm$ 3.5	0.954
DBP, mm Hg	82.3 $\pm$ 2.1	.....	83.7 $\pm$ 2.1	0.603
MBP, mm Hg	103.0 $\pm$ 2.5	.....	103.9 $\pm$ 2.5	0.798
PP, mm Hg	62.3 $\pm$ 2.8	.....	60.5 $\pm$ 2.5	0.639
SBP <sub>C</sub> , mm Hg	132.1 $\pm$ 4.0	.....	132.9 $\pm$ 3.5	0.889
DBP <sub>C</sub> , mm Hg	83.0 $\pm$ 2.1	.....	82.4 $\pm$ 1.8	0.847
PP <sub>C</sub> , mm Hg	49.0 $\pm$ 2.7	.....	51.2 $\pm$ 2.3	0.532
AG, mm Hg	14.2 $\pm$ 1.4	.....	15.8 $\pm$ 1.2	0.400
<b>Female</b>	<b>GG</b>	<b>GA</b>	<b>AA</b>	
<i>Number</i>	16	33	16	
SBP, mm Hg	143.3 $\pm$ 4.7	142.7 $\pm$ 3.3	144.9 $\pm$ 4.9	0.943
DBP, mm Hg	80.0 $\pm$ 2.4	82.3 $\pm$ 1.7	80.2 $\pm$ 2.6	0.668
MBP, mm Hg	101.1 $\pm$ 2.9	102.5 $\pm$ 2.1	101.7 $\pm$ 3.0	0.926
PP, mm Hg	63.3 $\pm$ 3.6	60.4 $\pm$ 2.6	64.7 $\pm$ 3.8	0.613
SBP <sub>C</sub> , mm Hg	129.9 $\pm$ 4.8	133.9 $\pm$ 3.3	133.5 $\pm$ 4.7	0.786
DBP <sub>C</sub> , mm Hg	78.5 $\pm$ 3.0	81.5 $\pm$ 2.1	79.8 $\pm$ 2.9	0.708
PP <sub>C</sub> , mm Hg	50.2 $\pm$ 3.4	53.3 $\pm$ 2.3	52.5 $\pm$ 3.3	0.765
AG, mm Hg	17.1 $\pm$ 1.9	19.2 $\pm$ 1.3	17.9 $\pm$ 1.8	0.649

AG: central pulse wave augmentation; DBP: diastolic blood pressure; DBP<sub>C</sub>: central diastolic blood pressure; MBP: mean blood pressure; PP: pulse pressure; PP<sub>C</sub>: central pulse pressure; SBP: systolic blood pressure; SBP<sub>C</sub>: central systolic blood pressure.

The data were standardised for age, waist-to-hip ratio (WHR), antihypertensive treatment and presence or absence of left ventricular (LV) diastolic dysfunction. Blood pressure parameters are presented as standardised arithmetic mean  $\pm$  standard error (SE). Values of *p* refer to the differences between the *G* and *A* alleles and between the *GG*, *GA* and *AA* genotypes.

**Table 4.** Analysis of the relationships between arterial stiffness parameters and the AGTR2 *G1675A* polymorphism in the male and female patients.

Phenotype	Standardised mean $\pm$ SE			<i>p</i>
	<i>G</i>	.....	<i>A</i>	
<b>Male</b>				
<i>Number</i>	60	.....	72	
PWV, m/s	13.8 $\pm$ 0.5	.....	14.3 $\pm$ 0.5	0.547
Alx <sub>C1</sub> , %	141.2 $\pm$ 4.3	.....	143.7 $\pm$ 3.7	0.677
Alx <sub>C2</sub> , %	26.9 $\pm$ 1.9	.....	30.3 $\pm$ 1.6	0.193
Alx <sub>p</sub> , %	84.9 $\pm$ 2.3	.....	88.7 $\pm$ 2.0	0.234
<b>Female</b>	<b>GG</b>	<b>GA</b>	<b>AA</b>	
<i>Number</i>	16	33	16	
PWV, m/sek	13.4 $\pm$ 0.7	13.3 $\pm$ 0.5	13.0 $\pm$ 0.7	0.903
Alx <sub>C1</sub> , %	153.8 $\pm$ 5.4	156.3 $\pm$ 3.8	152.4 $\pm$ 5.3	0.829
Alx <sub>C2</sub> , %	32.4 $\pm$ 2.3	35.7 $\pm$ 1.6	32.3 $\pm$ 2.2	0.353
Alx <sub>p</sub> , %	93.6 $\pm$ 3.1	96.8 $\pm$ 2.1	93.2 $\pm$ 3.2	0.561

Alx<sub>C1</sub> and Alx<sub>C2</sub>: central pulse wave augmentation indexes; Alx<sub>p</sub>: peripheral pulse wave augmentation index; PWV: pulse wave velocity.

The data were standardised for age, waist-to-hip ratio (WHR), systolic blood pressure (SBP), antihypertensive treatment and presence or absence of left ventricular (LV) diastolic dysfunction. Arterial stiffness parameters are presented as standardised arithmetic mean  $\pm$  standard error (SE). Values of *p* refer to the differences between the *G* and *A* alleles and between the *GG*, *GA* and *AA* genotypes.

Comparative analysis of the relationships between arterial stiffness parameters and the AGTR2 *G1675A* polymorphism were also conducted in women in the subgroups with FDR<sub>Na</sub> below and above the sex-specific

median (97.4%) value and no significant differences were observed. However, in both sexes combined (with the exclusion of heterozygous women), we received convergent results. In 46 *G* allele carriers,  $AIx_{C1}$  (15.3% increasing per 5% higher  $FDR_{Na}$ ;  $p=0.05$ ) and  $AIx_p$  (9.2% increasing per 5% higher  $FDR_{Na}$ ;  $p=0.02$ ) with the similar trend for  $AIx_{C2}$ , arterial stiffness increased with higher sodium reabsorption in the distal tubule, with the opposite tendency in *A* allele carriers. On the other hand, in 52 *A* allele carriers,  $AIx_{C1}$  did not significantly decrease (6.5% decrease per 5% higher  $FDR_{Na}$ ;  $p=0.07$ ) with the similar trend for  $AIx_{C2}$  and  $AIx_p$ , arterial stiffness decreased with higher sodium reabsorption in the distal tubule. Additionally, in the subgroup with  $FDR_{Na}$  below the all-population median (96.5%) value,  $AIx_{C1}$  was 10.9% higher ( $p=0.02$ ),  $AIx_{C2}$  was 5.5% higher ( $p=0.02$ ) and  $AIx_p$  was 7.3% higher ( $p=0.02$ ) in the *A* allele compared with the *G* allele carriers. The analysis of the relationships of the AGTR2 *G1675A* polymorphism with arterial stiffness parameters in the subgroups with  $FDR_{Na}$  below and above the sex-specific for men and all-group median value are presented on Figure 1.

## Discussion

Our study, conducted in a population characterised by high dietary sodium intake and long-standing history of hypertension, revealed that increased sodium reabsorption in distal tubules is related to the development of arterial stiffness, which, in turn, is related to the expression of the AGTR2 *G1675A* polymorphism. Further, we found this relation to be independent of blood pressure values. In men and in both sexes combined (with the exclusion of heterozygous women),  $AIx_{C1}$  and  $AIx_p$  increased with higher sodium reabsorption in distal tubule, in the presence of AGTR2 *G* allele with the opposite tendency in *A* allele carriers. In *G* allele carrying men and *GG* homozygous women, the  $AIx_{C1}$  (139.6±3.8 vs 159.1±5.7%;  $p=0.009$ ),  $AIx_{C2}$  (26.3±1.8 vs 33.3±1.7%;  $p=0.016$ ) and  $AIx_p$  (83.4±2.5 vs 96.5±2.6%;  $p<0.0001$ ) were lower in the subgroup with  $FDR_{Na}$  below the median value, compared to the subgroup with  $FDR_{Na}$  above the median value. Our results indicating the relationship of the increased distal sodium reabsorption, as measured using endogenous lithium clearance, with the AGTR2 *G1675A* polymorphism with respect to arterial stiffness parameters, are to the best of our knowledge the first such data in the literature. The AGTR1 *A1166C* polymorphism was unrelated to arterial stiffness.

The progressive interstitial and perivascular fibrosis, induced in part by AT II, contribute to an increase in arterial and cardiac muscle remodelling/hypertrophy and development of hypertension and diastolic dysfunction. Of the two major AT II receptor isoforms, AT1 and AT2, it is generally accepted that most of the traditional AT II

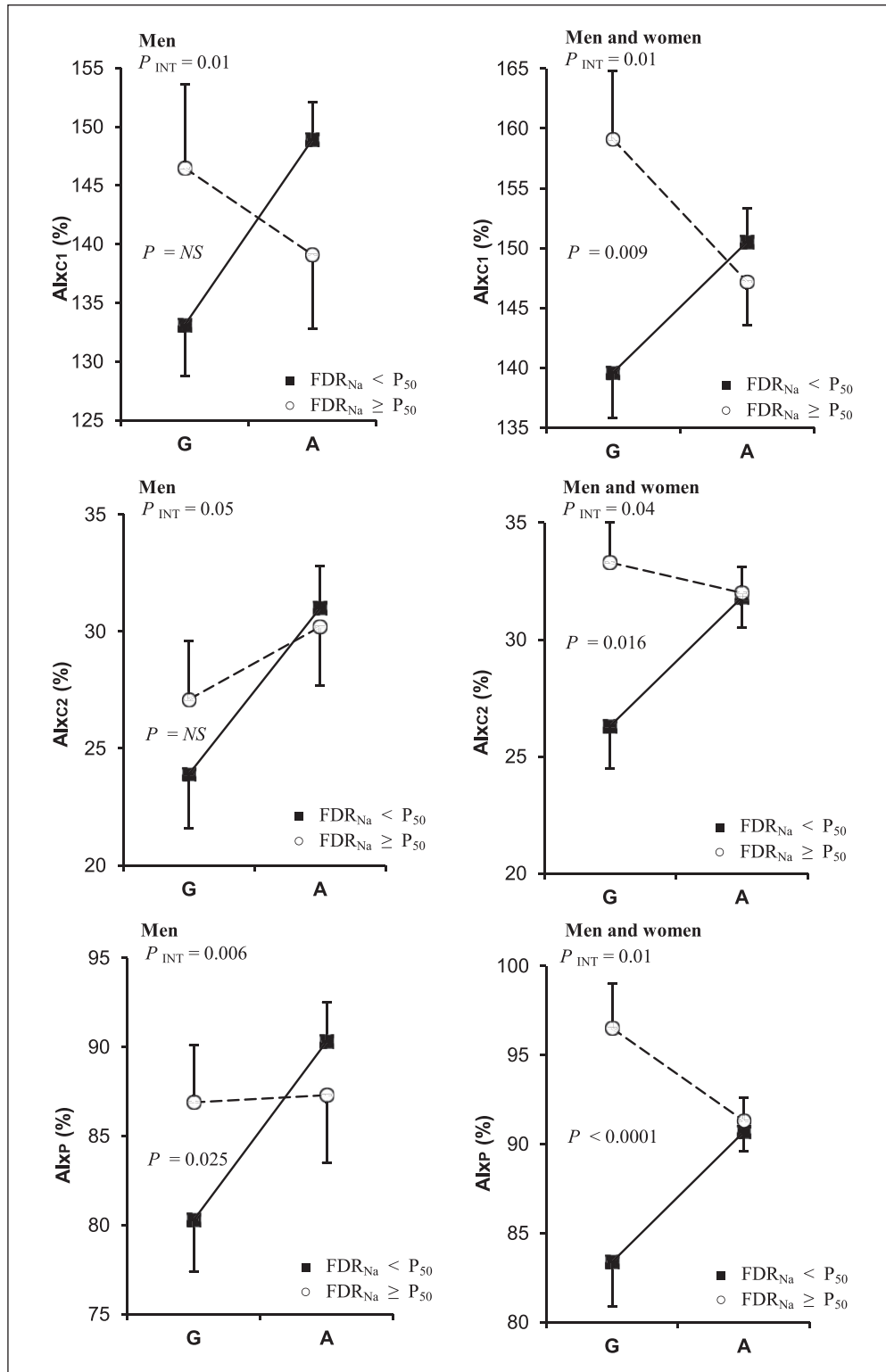
functions in the cardiovascular system are attributable to AT1. The anti-proliferative actions of the AT2 receptor offset the growth promoting effects mediated by the AT1.<sup>7</sup> However, several reports of in vivo as well as in vitro studies elucidated roles of AT2 in medial hypertrophy and fibrosis in aorta,<sup>20</sup> heart<sup>21</sup> and cultured cells.<sup>22</sup> Levy et al.<sup>20</sup> demonstrated that chronic AT2 blockade in AT II-induced hypertensive rats markedly suppressed arterial hypertrophy and fibrosis and proposed that the vasotrophic effects of AT II may be at least partially mediated via the AT2 receptor. This effect may be due to the fact that the AT2 receptor is expressed at low levels in normal tissues but upregulated in pathological states, including hypertension, vascular injury, myocardial infarction, stroke and heart failure.<sup>23</sup>

The AGTR2 *G1675A* polymorphism is located on the X chromosome and consists of three exons interspaced by two introns. Regulatory elements are located in the promoter area and in the first intron, whereas the third exon contains the complete protein-coding sequence, which encode the 363-amino acid G-protein-coupled receptor.<sup>24</sup> AGTR2 gene contains several polymorphic sites. A common *G/A* polymorphism occurs at position (rs1403543) relative to the transcription start. It is also known as *G1332A* relative to the start codon in the first intron. The *G1675A* polymorphism is probably functional and leads to differences in AT2 receptor expression,<sup>25</sup> however, studies on cell cultures yield conflicting results regarding the roles of particular alleles.<sup>26</sup> The studies did confirm that AGTR2 *G1675A* polymorphism may lead to differences in AT2 receptor expression. Warnecke et al.<sup>25</sup> indicated that individuals carrying the *G* allele may express higher levels of AT2 receptor protein, however another study suggested that the *A* allele might be associated with increased AT2 receptor transcription.<sup>26</sup>

The current literature which abounds in studies on the relation between the *G1675A* (equivalent to *G1332A*) AGTR2 gene polymorphism and the cardiovascular risk factors, predominantly the various indices of LV hypertrophy, shows conflicting results. Schmieder et al.<sup>27</sup> found in 120 young men with normal or mildly elevated blood pressure, that hypertensive but not normotensive *A* allele carriers had a higher LV mass (LVM) and LV mass index (LVMI) than *G* allele carriers, because of an increased wall thickness. Likewise, in a study by Herrmann et al.,<sup>28</sup> AGTR2 *A* allele carriers were more common in males with ECG LV hypertrophy, than in those without hypertrophy. However, in older hypertensive patients, LVM as measured by magnetic resonance imaging was higher in AGTR2 *G* allele carriers.<sup>29</sup>

In a study based on the European Project On Genes in Hypertension (EPOGH) database published by Kuznetsowa et al.,<sup>18</sup> the effect of the AGTR2 *G1675A* polymorphism on LVM differed according to sodium intake. Without taking sodium excretion into account, there was no difference in LVMI between carriers of the *G*





**Figure 1.** Parameters of arterial stiffness in the G and A allele carriers of the AGTR2 G1675A gene polymorphism in the subgroups with fractional sodium reabsorption in distal tubules (FDR<sub>Na</sub>) below and above the sex-specific for men and all-group median value. The data in the median FDR<sub>Na</sub> subgroups were standardized for age, sex, SBPWHR, antihypertensive treatment and presence or absence of diastolic LV dysfunction. The data were presented as arithmetic means ± standard error (SE). Values of  $p$  indicate the difference of stiffness parameters in the G-allele carriers, according to FDR<sub>Na</sub> level (above or below median). Alx<sub>C1</sub> and Alx<sub>C2</sub>: central pulse wave augmentation indexes; Alx<sub>P</sub>: peripheral pulse wave augmentation index.

or *A* allele. However, in man and in both sexes combined with exclusion of heterozygous women, LVMI and LV internal diameter increased with higher sodium excretion in the presence of *G* allele with the opposite tendency in *A* allele carriers. Further analyses dichotomised according to median sodium excretion showed that when sodium excretion was below the median, LVMI was lower in *G* than *A* allele carriers. This association has been validated in a study involving of young Caucasian males with and without mild hypertension with results in line with those of an earlier study.<sup>27</sup> After accounting for sodium, LVM was higher in the *G* allele carriers with high rather than low salt intake, in blood pressure independent fashion.<sup>30</sup> Both studies conform that AGTR2 *G* allele appears to be susceptible to a modifying effect of increased salt intake on LVM. Some other studies also reported the association of the *G* allele with hypertension and salt sensitivity.<sup>31</sup> The aforementioned results are stand in line with our own data on vascular stiffness parameters, where likewise independent of blood pressure, the renal sodium handling (assessed by  $FDR_{Na}$ ) turned out to modify gene effects. The studies in animal models strengthen the evidence by indicating that dietary sodium depletion increases the expression and density of the AT2 receptor in mature adult rat kidneys.<sup>32</sup> Renal AT2 receptors, through their influence on sodium balance and the circulating plasma volume, might at least partly explain these findings.

Studies on spontaneously hypertensive rats (SHRs) revealed that increased dietary sodium intake is associated with vascular<sup>33</sup> and myocardial<sup>34</sup> hypertrophy and remodelling which involved not only an increased tunica media thickness but also an elevated extracellular elastin and collagen. Of the diagnostic methods available for many years in studies on sodium sensitivity in hypertension the assessment of sodium reabsorption in the proximal and distal renal tubules using endogenous lithium clearance again comes to light. Only a limited number of publications on cardiovascular remodelling and target organ damage of hypertension included endogenous lithium clearance measurements. Seidlerova et al.<sup>10</sup> studied 1069 untreated subjects from the general population. They observed the relationships between the increased diameter of the brachial artery and increased sodium reabsorption in proximal tubule ( $FPR_{Na}$ ) and between the impaired compliance and distensibility of femoral artery and increased  $FDR_{Na}$ . Jin et al.<sup>11</sup> analysed the parameters of LV hypertrophy in the context of sodium parameters. They showed an independent increase of LVMI associated with an increased 24-hour urinary sodium excretion. The authors also analysed the effects of increased  $FPR_{Na}$  and  $FDR_{Na}$  in the context of LVMI increase and found no relationship; however, the increase of  $FDR_{Na}$  affected indirect parameters of LV hypertrophy. In our study we found statistically significant relationships between the AGTR2 G1675A polymorphism and  $FDR_{Na}$  with respect to arterial stiffness parameters of

$AIx_{C1}$ ,  $AIx_{C2}$  and  $AIx_p$ . However, we did not find such a relationship for  $FE_{Li}$  and  $Na_{24}$ , and the analysed indices of sodium load are each other closely related to each other. Before and after the standardisation  $FDR_{Na}$  showed significant negative correlation with  $Na_{24}$  (partial  $r=-0.36$ ;  $p<0.0001$ ), suggesting association between increased sodium intake and decreased sodium reabsorption in the distal tubule, and significant positive correlation with  $FE_{Li}$  (partial  $r=0.52$ ;  $p<0.0001$ ). These results are concordant with the ones derived from a general population.<sup>10</sup> In this context,  $FRD_{Na}$  seems to emerge stronger than the  $Na_{24}$  modulator of sodium-dependent genetic polymorphism-cardiovascular phenotype response to sodium overload.

Our study should be interpreted in the context of its limitations and strengths. The main limitation was that the study group of only 131 subjects did not allow for measurements in the respective tertiles of  $FDR_{Na}$  because of limited numbers of patients in the groups. The second limitation related to the enrolment of patients with long-standing hypertension to the studies on renal sodium regulation lies in the fact that they are treated with antihypertensive drugs. To avoid the interference of diuretics with renal sodium excretion and reabsorption the patients enrolled in the study were required to receive no long-term diuretic therapy or to have their hypertension controlled effectively enough to allow the discontinuation of diuretics at least five days before the enrolment. Nevertheless, other drugs, including ACE-inhibitors, angiotensin-receptor blockers, calcium channel blockers and beta-blockers were not discontinued for both ethical and medical reasons. This is certainly the most important limitation of our study. This issue is particularly important in the case of ACE-inhibitors and angiotensin-receptor blockers, as they increase renal blood flow and decrease both total and fractional sodium reabsorption. Nevertheless, in the case of ACE-inhibitors it has been observed that this effect was more pronounced in persons with high activity of the RAA system, which is observed in normal subjects with low dietary sodium intake.<sup>12</sup> Finally the enrolment was a non-randomised procedure. However, the key inclusion criterion was homogenous age, which allowed for the selection of two sex-specific groups with virtually no age difference. On the other hand, our results need separate confirmation in larger groups of patients, in a study powered enough to analyse men and women separately.

## Conclusions

Target organ damage, as an intermediate phenotype, has been linked in many studies with cardiovascular complications. Thus our inferences based on the assessment of such entities as vascular structure and function can to some extent shed mechanistic light upon the disparate results of recent epidemiologic studies which assessed relation between sodium consumption and cardiovascular

outcome. In the study by Stolarz-Skrzypek et al.<sup>1</sup> estimated sodium consumption was not related to hard cardiovascular outcome. However, the authors based their estimates on a pool of data obtained from healthy populations based on the nuclear-family principle. Another large study showed that there was a J-shaped relationship between sodium intake and risk of cardiovascular complications.<sup>2</sup> Neither study included genetic factors. It is possible, that the mutated polymorphism of the AT II gene stays phenotypically dormant in normotensive individuals, but its potentiating action reveals itself in the context of both hypertension and high sodium load, in individuals with impeded renal sodium handling. Such a hypothesis needs further testing, but can be a part of a unifying mechanistic explanation for the puzzling epidemiologic results.

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