

Effects of the renin-angiotensin system genes and salt sensitivity genes on blood pressure and atherosclerosis in the total population and patients with type 2 diabetes

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Abstract

Most studies on the genetic determinants of blood pressure and vascular complications of type 2 diabetes have studied the effects of single genes. These studies have often yielded conflicting results. Therefore, we examined the combined effects of three renin-angiotensin system (RAS) genes and three salt sensitivity genes in relation to blood pressure and atherosclerosis in the total population and type 2 diabetic patients.

The study was a part of Rotterdam Study, a population-based cohort study. We have genotyped three RAS gene polymorphisms, and three salt sensitivity gene polymorphisms. Diabetic patients with three risk genotypes of the RAS genes had a 6.9 mmHg higher systolic blood pressure (p for trend = 0.04) and a 6.0 mmHg higher pulse pressure (p for trend = 0.03) than those who did not carry any risk genotypes. Diabetic patients with three risk genotypes of the salt sensitivity genes had a 9.0 mmHg higher systolic blood pressure ($p = 0.19$) and a 13.1 mmHg higher pulse pressure ($p = 0.02$). Diabetic patients who carried three risk genotypes for the RAS genes had a higher mean intima media thickness than those with two risk genotypes (mean difference = 0.04 mm, $p = 0.02$).

We found that among type 2 diabetic patients mean systolic blood pressure, pulse pressure and risk of hypertension increased with the number of risk genotypes for the RAS genes and the salt sensitivity genes.

Introduction

The renin-angiotensin system (RAS) is an endocrine system predominantly responsible for the regulation of systemic blood pressure, as well as salt and water homeostasis and the maintenance of vascular tone.(1) Moreover, RAS is involved in the blood pressure response to salt intake.(2) Patients with diabetes tend to have a salt-sensitive type of hypertension.(3-5) Therefore, genes involved in the RAS and salt sensitivity system are considered candidate genes for blood pressure regulation and hypertension in type 2 diabetes in particular.

In subjects with hypertension or complications of type 2 diabetes consistent associations between three gene variants of the RAS (angiotensin converting enzyme (*ACE*; insertion/deletion (I/D),(6) angiotensinogen (*AGT*) M235T,(7) and angiotensin II type 1 receptor (*AT1R*) C573T(8)) have been reported. Similarly, three genes involved in the salt sensitivity pathway have been associated with hypertension (alpha adducin 1 (*ADD1*) G460T,(9) beta 3 subunit of heterotrimeric G proteins (*GNβ3*) C825T,(10) and cytochrome P-450 3A5 (*CYP3A5*) A6986G.(11)

Most studies on the genetic determinants of blood pressure and vascular complications of type 2 diabetes have focused on a single gene. These studies have often yielded conflicting results. This may be explained in part by the fact that the effects of a single gene are often very small. Combining the effects of a modest number of genes, whose products are known to act in a pathophysiological pathway, could be an alternative method. The RAS and the salt sensitivity pathways are well characterized and allow to evaluate the joint effects of multiple genes in these pathways. This may be explained in part by the fact that genes are part of a large pathway while the effects of a single gene are often very small.

We examined in the total population and in patients with type 2 diabetes the

combined effects of three gene variants in the RAS pathway and three genes involved in the salt sensitivity pathway on blood pressure as well as atherosclerosis.

Materials and methods

This study was conducted within the framework of the Rotterdam Study, an ongoing prospective population-based cohort study among subjects aged 55 years or older, living in Ommoord, a suburb of Rotterdam, the Netherlands. The design of the study has been described elsewhere.(12) Baseline data were collected between 1990 and 1993. The medical ethics committee of Erasmus University has approved the study and written informed consent was obtained from all participants.

At the baseline examination, information concerning medical history, medication use, and smoking status was obtained. Height and weight were assessed, and body mass index (BMI) (in kg/m²) was calculated. At the research center, blood pressure was measured. Hypertension was defined as a systolic blood pressure level > 160 mmHg and/or a diastolic blood pressure > 100 mmHg and/or the use of anti-hypertensive medication. We included as anti-hypertensive medication: β-blockers, diuretics, and other anti-hypertensive (such as calcium antagonists and ACE-inhibitors). Diabetes mellitus was defined as the use of glucose-lowering medication and/or random or post-load serum glucose level 11.1 mmol/L or over.(13) Total serum cholesterol and high-density lipoprotein (HDL) cholesterol were determined. Pulse pressure was calculated as following: systolic blood pressure – diastolic blood pressure. Mean arterial pressure was calculated as following: diastolic blood pressure + 1/3 (systolic blood pressure – diastolic blood pressure).

Carotid ultrasonography

Carotid atherosclerosis was assessed by duplex scan ultrasonography of the carotid arteries,

using a 7.5 MHz linear array transducer (ATL, Ultramark IV). Measurements of intima media thickness (IMT) were performed offline from the still images recorded on videotape. Details about these measurements have been published previously.(14) Results from a reproducibility study of IMT measurements have been published elsewhere.(15)

Genotyping

Genomic DNA was isolated from blood samples using standard methods.(16) The II, ID, and DD genotypes of the *ACE* gene were detected using the polymerase chain reaction (PCR) technique according to the method of Lindpaintner et al.(17) with some modifications. We utilized TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA, USA) for genotyping the *AGT* gene M235T, the *AT1R* gene C573T, the *ADD1* gene G460T, the *GNβ3* gene rs2301339 G/A, and *CYP3A5* gene A6986G polymorphisms.

Forward and reverse primer sequences were 5'-AGGTTTGCCTTACCTTGAAGTG-3' and 5'-GCTGTGACAGGATGGAAGACT-3' for *AGT*. The minor groove binding probes were 5'-CTGGCTCCCATCAGG-3' (VIC) and 5'-CTGGCTCCCGTCAGG-3' (FAM). We used the reverse strand design for the *AT1R* gene polymorphism. Forward and reverse primer sequences were 5'-TGTGCTTTCCATTATGAGTCCCAA-3' and 5'-CAGAAAAGGAAACAGGAAACCCAGTATA-3'. The minor groove binding probes were 5'-CTATCGGGAGGGTTG-3' (VIC) and 5'-CTATCGGAAGGGTTG-3' (FAM).

For *ADD1*, the forward and reverse primer sequences were 5'-GAGAAGACAAGATGGCTGAACTCT-3' and 5'-GTCTTCGACTTGGGACTGCTT-3'. The minor groove binding probes were 5'-CATTCTGCCCTTCCTC-3' (VIC) and 5'-ATTCTGCCATTTCCTC-3' (FAM). For *GNβ3*, the forward and reverse primer sequences were 5'-GGCAGGGCTGCTTCTCA-3' and 5'-

GCAAGCCGCTGCTCTCA-3'. The minor groove binding probes were 5'-AAACCAAGGAAGGGACA-3' (VIC) and 5'-ACCAAGGGAGGGACA-3' (FAM). For *CYP3A5*, the forward and reverse primer sequences were 5'-CGAATGCTCTACTGTCATTTCTAACC A-3' and 5'-TGAAGGGTAATGTGGTCCAAACAG-3'. The minor groove binding probes were 5'-TTTTGTCTTTCAATATCTC-3' (VIC) and 5'-TTTTGTCTTTCAGTATCTC -3' (FAM).

The assays utilized 5 nanogram of genomic DNA and 2 microliter reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 minutes at 95 degrees preceded 40 cycles of denaturation at 95 degrees for 15 seconds and annealing and extension at 50 degrees for 60 seconds. Allele-specific fluorescence was then analyzed on an ABI prism 7900HT Sequence Detection System v2.1 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Hardy-Weinberg equilibrium was tested with the chi-square test. Based on biological pathway the polymorphisms were defined in two groups: RAS genes and salt sensitivity genes. The genotypes considered at risk for an increased blood pressure or atherosclerosis for the RAS genes or the salt sensitivity genes, respectively, were coded 1 in the analysis, while the genotypes not at risk were coded 0.

Based on previous studies, we chose as risk alleles the D allele for *ACE* I/D,(18) the T allele for *AGT* M235T,(19) and the T allele for *AT1R*(20) C573T polymorphism in the RAS pathway. For the salt sensitivity pathway, we chose as risk alleles the T allele for G460T *ADD1*,(21) the G for the *GNβ3* rs2301339 G/A, and the A allele for the *CYP3A5* A6986G polymorphism.(22) The risk allele for the *GNβ3* rs2301339 G/A polymorphism was based on the results of a single gene in our study sample, which showed a higher systolic blood pressure in diabetic subjects with the GG genotype than

in subjects with the AA genotype (147.1 versus 142.1 mmHg).

The dominant genetic model was chosen in the RAS pathway based on previous studies for the *ACE* I/D,(18) and the *AGT* M235T(19) polymorphisms, and in the salt-sensitive hypertension genes on previous findings for the *ADD1*(G460T)(21) and the *CYP3A5* A6986G(22) polymorphisms. The rs2301339 G/A polymorphism of *GNB3* is in linkage disequilibrium with the C825T polymorphism which has been reported as being dominant.(10) The C573T polymorphism of the *AT1R* is in linkage disequilibrium with a well-known polymorphism (A1166C) which was reported as dominant.(23)

This approach resulted for the RAS genes in the following groups: *ACE* genotype: ID/DD=1, II=0, *AGT* genotype: MT/TT=1, MM=0, and *AT1R* genotype: CT/TT=1, CC=0. For the salt-sensitive combined group, genotypes were coded for *ADD1* as GT/TT=1, GG=0, for *GNB3*: AG/GG=1, AA=0, and for *CYP3A5* genotype: GA/AA=1, GG=0. Then, to assess the combined effects of the three genotypes of the RAS genes, a variable was created which included the three polymorphisms, which resulted in a score ranging from 0-3. The latter number represents the total number of risk genotypes.

A comparable procedure was used to assess the combined effects of risk genotypes of the three salt sensitivity genes. A variable was created using three genotypes of the salt sensitivity genes, which resulted in a score ranging from 0-3. The latter number represents the total number of risk genotypes.

Furthermore same procedure was used to assess the combined effects of risk genotypes of the both the three RAS genes and the three salt sensitivity genes. A variable was created using six genotypes of the both the RAS and the salt sensitivity genes, which resulted in a score ranging from 0-6. The latter number represents the

total number of risk genotypes.

When adding the genes together in a multiple gene scale there are only very few individuals who carry non risk genotypes. The population carries 2 or 3 risk genotypes for RAS genes and 1 or 2 risk genotypes for the salt sensitivity genes, and 3 or 4 risk genotypes for the both RAS and salt sensitivity genes. Therefore, we have considered the reference group according to the most frequent risk genotype: for RAS genes, the group with two risk genotypes, for the salt sensitivity genes the group with one risk genotype, and for both the RAS and the salt sensitivity genes the group with four risk genotypes. For the both RAS and salt sensitivity genes, the number of individuals with zero risk genotype was very low, therefore in our analysis we combined zero and one risk genotype together.

To investigate the associations between systolic and diastolic blood pressure, mean arterial pressure, pulse pressure, and common carotid IMT with each combined genotype group, an analysis of covariance (ANCOVA) was performed. All analyses were adjusted for age, gender, and blood pressure-lowering medication and additionally for BMI, total cholesterol, and smoking. The analysis on common carotid IMT was also adjusted for the systolic and diastolic blood pressures. Interaction between the RAS genes and the salt sensitivity genes was modeled with an interaction term obtained from the product of main effects in relation to blood pressure values, hypertension, and common carotid IMT. Post-hoc pairwise tests employed a Bonferroni correction for multiple comparisons. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated by logistic regression analyses, adjusted for gender and age. All analyses were performed using the SPSS for Windows software package, version 11.0 (SPSS Inc., USA).

Results

Table 1 presents the baseline characteristics of the study population. In the total

population, 7,983 participants (38.9% men) included 36.1% hypertensive patients, of whom 33.1% were on blood pressure-lowering drugs, and 9.6% had diabetes. In diabetic patients (39.0% men) 54.3% were hypertensive, of whom 50.2% were on blood pressure-lowering drugs.

Genotype frequencies were in Hardy-Weinberg equilibrium. In the total population with an increasing number of risk genotypes for the RAS genes there was a significant increase in systolic blood pressure (p for trend = 0.01) and mean arterial pressure (p for trend = 0.02) (Table 2). With an increasing number of risk genotypes (0 →3) systolic blood pressure increased with 2.5 mmHg and pulse pressure increased with 1 mmHg (Table 2). There was a significantly lower systolic blood pressure ($p = 0.02$), mean arterial pressure ($p = 0.04$), and pulse pressure ($p = 0.03$) in carriers of one risk genotype compare to carriers of two risk genotypes (Table 2). In diabetic patients a significant increase was found with an increasing number of the RAS risk genotypes in systolic blood pressure (p for trend = 0.04) and pulse pressure (p for trend = 0.03). Diabetic patients with three risk genotypes had a 6.9 mmHg higher systolic blood pressure and a 6.0 mmHg higher pulse pressure than those who did not have any risk genotypes (Table 2). In diabetic patients carriers of one risk genotype had a significantly lower systolic blood pressure ($p = 0.03$) and pulse pressure ($p = 0.03$) than those with two risk genotypes for the RAS genes (Table 2).

To examine whether anti-hypertensive medication is modifying the effect on blood pressure values across RAS, and salt sensitivity genes, we adjusted all the analysis for anti-hypertensive medication. No significant differences were observed in frequency of anti-hypertensive medication among the group of RAS genes ($p = 0.52$), among the group of salt sensitivity genes ($p = 0.75$), and among the group in which RAS and salt sensitivity genes were combined ($p = 0.20$). Although

many diabetic patients were treated with anti-hypertensive medication (50.2%), the frequencies of the RAS, salt sensitivity, and both the RAS and salt sensitivity genotypes were not statistically different among those with and without a treatment ($p = 0.24$, $p = 0.84$, $p = 0.89$, respectively). After stratification by using different anti-hypertensive medication the results remained unchanged (data not shown). In the total population these frequencies were only different in combined RAS and salt sensitivity in those who treated with β -blocker ($p = 0.04$), and in salt sensitivity in those who treated with diuretics ($p = 0.05$).

For the salt sensitivity genes no associations were observed between an increasing number of risk genotypes and blood pressure values in the total population (Table 3). In diabetic patients, however, those who had three risk genotypes had a 9.0 mmHg higher systolic blood pressure than those who did not have any risk genotypes. This difference became statistically significant after further adjustment for putative risk factors ($p = 0.05$). Furthermore, diabetic patients who had three risk genotypes had a 13.1 mmHg significantly higher pulse pressure ($p = 0.02$, Table 2). Diabetic patients who carried three risk genotypes for the salt sensitivity genes had a significant higher pulse pressure than those with one risk genotype ($p = 0.01$, Table 2).

For both RAS and salt sensitivity genes no associations were observed between an increasing number of risk genotypes and blood pressure values in the total population and in the diabetic patients (Table 4). In diabetic patients, those who carried two risk genotypes had a 7 mmHg lower systolic blood pressure than those who carried four risk genotypes ($p = 0.04$, Table 4). Diabetic patients who had two risk genotypes had a 6.8 mmHg lower pulse pressure ($p = 0.04$), and those who had five risk genotypes had a 5.7 higher pulse pressure than those who had four risk genotypes ($p = 0.01$, Table 4).

In the total population, no significant associations were found between the risk of hypertension and the number of risk genotypes (Figure 1). In diabetic patients, a threshold effect was observed in that the risk of hypertension in those without any risk genotypes was 0.2 (95% CI: 0.1 – 0.7), with one risk genotype was 1.0 (95% CI: 0.6 – 1.6), and with three risk genotypes this was 0.9 (95% CI: 0.6 – 1.3) lower than those with two risk genotypes of the RAS genes (p for trend = 0.46, Figure 1A). Diabetic patients who carried every risk genotype had a 4.9 (95% CI = 1.4 – 17.6, p = 0.02) times higher risk of hypertension than those who did not carry any risk genotypes of the RAS genes (data not shown).

For the salt sensitivity genes the risk of hypertension in diabetic patients, in those without any risk genotype was 0.5 times (95% CI: 0.2 – 1.3) lower and in those who had two risk genotypes was 1.1 times (95% CI: 0.7 – 1.5) higher than in those with one risk genotype (Figure 1B). Diabetic patients who carried every risk genotype had a 1.9 (95% CI = 0.8 – 4.3, p = 0.14) times higher risk of hypertension than those who did not carry any risk genotypes of the salt sensitivity genes (data not shown). No such a result was observed in the total population.

For both the RAS and the salt sensitivity genes in diabetic patients, the risk of hypertension in those with zero and one risk genotype was 0.3 (95% CI: 0.1 – 1.4), and with two risk genotypes was 0.8 (95% CI: 0.4 – 1.4) lower than those with four risk genotypes. Furthermore, the risk of hypertension in those with three risk genotypes was 1.2 (95% CI: 0.8 – 1.7), and with five risk genotypes was 1.2 (95% CI: 0.7 – 2.1) higher than those with four risk genotypes (Figure 1C).

In the total population, no significant associations were observed between mean common carotid IMT and the number of risk genotypes (Figure 2). In contrast, in diabetic patients there was a significant trend by increasing the number of risk genotypes for the RAS genes (p for trend =

0.01, Figures 2A). Diabetic patients with three risk genotypes had a higher mean common carotid IMT compared to those with two risk genotypes for the RAS genes (mean difference = 0.04 mm, p = 0.02, Figure 2A). Diabetic patients who carried every risk genotype had a higher mean common carotid IMT than those who did not carry any risk genotype for the RAS genes (mean difference = 0.05 mm, p = 0.24) (data not shown).

Figure 2B shows that diabetic patients who carried two risk genotypes for the salt sensitivity genes had a higher mean common carotid IMT compared to one risk genotype (mean difference = 0.03 mm, p = 0.04). These results remained after further adjustments. Diabetic patients who carried every risk genotype had a higher mean common carotid IMT than those who did not carry any risk genotype for the salt sensitivity genes (mean difference = 0.07 mm, p = 0.05) (data not shown). No significant association was found by including the interaction term of the RAS genes and salt sensitivity genes in relation to blood pressure values, hypertension, and common carotid IMT in both the total population and type 2 diabetic patients (data not shown). Figure 2C shows that diabetic patients who carried three risk genotypes for the both RAS and salt sensitivity genes had a lower mean common carotid IMT compared to four risk genotypes (mean difference = 0.06 mm, p = 0.02).

Discussion

In this population-based cohort study, we found that when studying three RAS and three salt sensitivity genes, most of the individuals in the population carried at least one or two risk genotypes and very few carried none at all. We found that among type 2 diabetes mean systolic blood pressure, pulse pressure and risk of hypertension increased with the number of risk genotypes for the RAS genes and the salt sensitivity genes. Furthermore, we observed that mean common carotid IMT was increased in diabetic patients who had

an increasing number of risk genotypes of the RAS genes.

To our knowledge, the present study is the first to study the combined effects of the RAS genes, and the salt sensitivity genes, in relation to blood pressure, pulse pressure, and common carotid IMT. Previous studies on the effects of single genetic markers of the RAS or salt sensitivity on blood pressure have reported on conflicting results.(24-27) However, a single susceptibility gene is expected to exert only a small effect on the development of vascular complications. Studying multiple genes from a single pathway simultaneously will allow to find more pronounced effects, in particular high risk subgroups such as patients with type 2 diabetes.

Several lines of evidence support the possibility that the RAS contributes to the etiology of vascular complications of diabetes.(21) The ACE (I/D) polymorphism is one of the RAS gene polymorphisms that is associated with alterations in circulating ACE levels, the DD genotype being 50% higher than that in the II genotype.(28) The association between ACE I/D alleles and cardiovascular disease (CVD) in diabetic patients has been examined in several studies.(6, 29) In AGT gene the T allele of M235T polymorphism is associated with increased plasma AGT levels, hypertension, and carotid atherosclerosis.(7) Furthermore, the AT1R C573T polymorphism has a protective effect against blood pressure-induced microalbuminuria in type 2 diabetes.(8) These findings prompted us to investigate the role of RAS genes as a single pathway involved in complications of diabetes.

Moreover, salt sensitivity genes play probably a role in the development of cardiovascular complications, insulin resistance, and hypertension.(30, 31) It has been found that the ADD1 polymorphism leads to higher activity of the sodium-potassium pump, and hence increases renal tubular sodium reabsorption, which increases the risk of salt-sensitive

hypertension, and cardiovascular disease.(9, 32) The *GNβ3* gene, which enhances G protein activation and increases the activity of the sodium-proton exchanger.(33, 34) Furthermore, insulin action and effect on glucose transport partly depends on a G protein-sensitive mechanism.(35) The *CYP3A5* gene can mediate the metabolism of sodium transport in renal epithelia.(36) Polymorphic renal expression of the *CYP3A5*1*(37) may contribute to metabolized cortisol, which reduces insulin sensitivity, and salt sensitive hypertension.(38) Therefore, it is reasonable to expect that in salt sensitivity pathway multiple genes be involved in complications of diabetes and act as a single pathway.

A large number of methodological problems still need to overcome in studies on multiple genes. Even in a large study such as ours problems arise with the number of individuals in the subcategories; for instance in our study only 15 diabetic patients had no risk genotype for the RAS genes. To overcome this problem we chose the reference group based on the largest number of individuals with either the RAS genes or the salt sensitivity genes or these two genes combined.

Complete evaluation of genetic interactions, for instance between three polymorphisms of the RAS genes presented in this study, would require comparisons of the 27 possible combinations of genotypes. Even a large study such as the Rotterdam Study does not allow to study so many comparisons. To overcome this latter problem, the variation in the three polymorphisms was reduced to one variable. A key issue for this analysis is to identify which variants are the potential risk alleles. We have chosen the risk alleles based on previous studies for four polymorphisms,(18-22) and for two polymorphisms we defined the risk allele based on the frequency observed in our study. The strategy of choosing the “risk” alleles and verification of risk profile using the same data is potentially dangerous and may lead to false-positive results. We tried

to follow this approach for only two genes. Nevertheless, our results on risk profile should be considered with caution and need to be verified in other study populations.

The basis of our genetic model was a joint effect of different genetic sites, as recently described in studies on, for example, insulin resistance,(39) and the interaction between genetic variants at different loci.(40-42) Statistically, there is multiple ways to define the joint effects of genes. The risk profile was composed of the sum of the risk genotypes, which basically suggests an additive model underlying the joint effect of genes. It has been suggested that additive models may be of biological relevance.(41) Another issue ignored in this type of analysis is that different alleles may have different effects, for example the observed associations may differ across different alleles.

High systolic blood pressure is associated with vascular complications in patients with diabetes. Each 10 mmHg reduction in systolic blood pressure is associated with a 13% reduction in the risk of vascular complications.(43) Furthermore, diabetic patients require two to three anti-hypertensive agents to achieve their targets.(44, 45) Our findings that the systolic blood pressure and risk of hypertension and atherosclerosis were increased with an increasing number of risk genotypes of the RAS risk and the salt sensitivity risk genotypes, may have important clinical implications for therapeutic approaches or in the assessment

of vascular complications of patients with type 2 diabetes who are likely to receive several medications for diabetes. Although many diabetic patients were treated with anti-hypertensive medication (50.2%), but the frequencies of the RAS genes, and salt sensitivity genes were not statistically different among those who had a treatment.

Our findings are in line with the decreased incidence of complications that has been reported in several large clinical studies, when subjects with type 2 diabetes were treated with either ACE-inhibitors(46) or angiotensin II receptor antagonists.(47) Given the pronounced role of RAS blockade in diabetes, hypertension,(48-50) and salt-sensitive type of hypertension,(3-5, 22) our findings are not unexpected from a medical perspective.

The results of the present study show the value of combining information of multiple genes in a single analysis of complex traits. Many of the negative results for single locus studies of blood pressure or atherosclerosis in type 2 diabetes may have been due to the effect of multiple genes in a complex pathway. More powerful statistical methods are needed to explore the combined effects of more genetic variants on these traits. We suggest a combined effect of the RAS genes and a combined effect of the salt sensitivity genes on blood pressure and atherosclerosis in type 2 diabetes. Furthermore, our data shows the importance of analysing multiple genes in a biological pathway. An independent confirmation in another cohort can substantiate our conclusion.

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Table 1. General characteristics of the total population at baseline

	Total population	Diabetic patients
Total number	7983	748
Age (years)	70.6 ± 9.8	74.0 ± 9.2
Male gender (%)	38.9	39.0
Body mass index (kg/m ²)	26.3 ± 3.1	26.8 ± 4.2
Total cholesterol (mmol/L)	6.6 ± 1.2	6.5 ± 1.3
HDL cholesterol (mmol/L)	1.3 ± 0.3	1.3 ± 0.4
Systolic blood pressure (mmHg)	139.5 ± 22.4	147.9 ± 24.0
Diastolic blood pressure (mmHg)	73.7 ± 11.7	72.9 ± 12.7
Hypertension (%)	36.1	54.3
Using anti-hypertensive medication (%)	33.1	50.2
<u>β-blocker</u>	<u>14.2</u>	<u>19.9</u>
<u>Diuretics</u>	<u>16.5</u>	<u>27.7</u>
<u>Others</u>	<u>13.5</u>	<u>22.6</u>
Type 2 diabetes mellitus (%)	9.4	
Current smoking (%)	22.6	22.3
<i>ACE</i> (II/ID/DD) (%)	22.1/49.9/28.1	19.4/46.9/21.4
<i>AGT</i> (MM/MT/TT) (%)	36.3/47.9/15.5	36.4/49.1/14.5
<i>AT1R</i> (CC/CT/TT) (%)	27.6/49.1/23.3	29.3/49.3/21.4
<i>ADD1</i> (GG/GT/TT) (%)	62.1/33.1/4.8	64.4/31.4/4.3
<i>GNβ3</i> (AA/AG/GG) (%)	9.3/41.9/48.8	9.4/40.5/50.1
<i>CYP3A5</i> (GG/GA/AA) (%)	85.8/13.5/0.7	84.8/14.7/0.5

HDL cholesterol: High-density lipoprotein cholesterol. *ACE*: angiotensin converting enzyme gene, *AGT*: angiotensinogen gene, *AT1R*: angiotensin II type 1 receptor gene, *ADD1*: alpha-adducin 1 gene, *GNβ3*: G-protein β3 subunit gene, *CYP3A5*: Cytochrome P-450 3A5 gene.

Table 2. Association of systolic and diastolic blood pressure and mean arterial pressure in the total population and diabetic patients by combined genotypes of three RAS genes (*ACE*, *AGT*, *AT1R*)

	Number of risk genotypes*				<i>P</i> for trend
	0	1	2	3	
<i>Total population</i>	134	982	2659	2123	
Systolic blood pressure (mmHg)	137.3 ± 1.9	137.7 ± 0.7*	139.6 ± 0.4	139.8 ± 0.5	0.01
Diastolic blood pressure (mmHg)	72.4 ± 1.0	73.3 ± 0.4	73.9 ± 0.2	74.0 ± 0.3	0.07
Mean arterial pressure (mmHg)	94.1 ± 1.2	94.8 ± 0.4*	95.8 ± 0.3	95.9 ± 0.3	0.02
Pulse pressure (mmHg)	64.8 ± 1.4	64.4 ± 0.5*	65.8 ± 0.3	65.9 ± 0.4	0.10
<i>Diabetes</i>	15	89	251	189	
Systolic blood pressure (mmHg)	142.30 ± 6.1	142.7 ± 2.5†	149.0 ± 1.5	149.2 ± 1.7	0.04
Diastolic blood pressure (mmHg)	72.4 ± 3.1	72.3 ± 1.3	73.3 ± 0.8	73.2 ± 0.9	0.59
Mean arterial pressure (mmHg)	95.7 ± 3.7	95.8 ± 1.5	98.5 ± 0.9	98.6 ± 1.0	0.14
Pulse pressure (mmHg)	70.0 ± 5.0	70.4 ± 2.0†	75.7 ± 1.2	76.0 ± 1.4	0.03

Adjusted for age, gender and using blood pressure-lowering medication. Values are presented as mean ± standard error. RAS: renin-angiotensin system, *ACE*: angiotensin converting enzyme gene, *AGT*: angiotensinogen gene, *AT1R*: angiotensin II type 1 receptor gene. *: Numbers indicate the number of the risk genotype for RAS genes (*ACE*: ID/DD=1, *AGT*: MT/TT=1, *AT1R*: T/TT=1). † *p* < 0.05 compared with reference group (2 risk genotypes).

Table 3. Association of systolic and diastolic blood pressure and mean arterial pressure in the total population and diabetic patients by combined genotypes of three salt sensitivity genes (*ADD1*, *GNβ3*, *CYP3A5*)

	Number of risk genotypes*				<i>P</i> for trend
	0	1	2	3	
Total population	260	3162	2263	288	
Systolic blood pressure (mmHg)	140.4 ± 1.3	139.4 ± 0.4	138.8 ± 0.5	140.5 ± 1.3	0.53
Diastolic blood pressure (mmHg)	74.3 ± 0.7	73.9 ± 0.2	73.5 ± 0.2	73.3 ± 0.7	0.14
Mean arterial pressure (mmHg)	96.3 ± 0.8	95.7 ± 0.2	95.3 ± 0.3	95.7 ± 0.8	0.25
Pulse pressure (mmHg)	66.1 ± 1.0	65.5 ± 0.3	65.3 ± 0.4	67.1 ± 1.0	0.84
<i>Diabetes</i>	25	302	198	27	
Systolic blood pressure (mmHg)	141.3 ± 4.7	147.2 ± 1.4	148.5 ± 1.7	149.9 ± 4.5	0.19
Diastolic blood pressure (mmHg)	70.9 ± 2.4	73.5 ± 0.7	72.5 ± 0.9	66.5 ± 2.3†	0.08
Mean arterial pressure (mmHg)	94.4 ± 2.8	98.1 ± 0.8	97.9 ± 1.0	94.3 ± 2.7	0.79
Pulse pressure (mmHg)	70.3 ± 3.8	73.6 ± 1.1	76.0 ± 1.4	83.4 ± 3.7†	0.01

Values are presented as mean ± standard error.

Adjusted for age, gender and using blood pressure-lowering medication.

ADD1: alpha-adducin 1 gene, *GNβ3*: G-protein β3 subunit gene. *: Numbers indicate the number of the risk genotype for salt sensitivity genes (*ADD1*: GT/TT=1, *GNB3*: AG/GG=1, *CYP3A5*: GA/AA=1). † *p* < 0.05 compare to reference group (1 risk genotype).

Table 4. Association of systolic and diastolic blood pressure and mean arterial pressure in the total population and diabetic patients by combined genotypes of six RAS and salt sensitivity genes (*ACE*, *AGT*, *AT1R*, *ADD1*, *GN β 3*, *CYP3A5*)

	Number of risk genotypes*						<i>P</i> for trend
	0/1	2	3	4	5	6	
Total population	110	667	1812	2104	899	94	
Systolic blood pressure (mmHg)	138.6 \pm 2.0	139.3 \pm 0.8	138.6 \pm 0.5	139.5 \pm 0.5	140.0 \pm 0.7	140.2 \pm 2.2	0.19
Diastolic blood pressure (mmHg)	72.8 \pm 1.1	73.8 \pm 0.4	74.0 \pm 0.3	74.0 \pm 0.2	74.0 \pm 0.4	73.0 \pm 1.2	0.55
Mean arterial pressure (mmHg)	94.7 \pm 1.3	95.6 \pm 0.5	95.2 \pm 0.3	96.0 \pm 0.3	96.0 \pm 0.4	95.4 \pm 1.4	0.29
Pulse pressure (mmHg)	65.8 \pm 1.6	65.5 \pm 0.6	65.0 \pm 0.4	66.0 \pm 0.4	66.1 \pm 0.6	67.3 \pm 1.7	0.20
<i>Diabetes</i>	9	64	186	179	74	11	
Systolic blood pressure (mmHg)	147.4 \pm 4.8	140.9 \pm 2.9 [†]	147.9 \pm 1.7	148.0 \pm 1.7	153.5 \pm 2.7	143.3 \pm 7.1	0.07
Diastolic blood pressure (mmHg)	70.4 \pm 4.0	71.5 \pm 1.5	74.6 \pm 0.9	72.8 \pm 0.9	71.6 \pm 1.4	68.7 \pm 3.6	0.23
Mean arterial pressure (mmHg)	96.1 \pm 4.7	94.6 \pm 1.8	99.0 \pm 1.0	97.9 \pm 1.1	98.9 \pm 1.6	93.6 \pm 4.3	0.45
Pulse pressure (mmHg)	77.1 \pm 6.4	69.4 \pm 2.4 [†]	73.4 \pm 1.4	75.1 \pm 1.4	81.9 \pm 2.2 [†]	74.6 \pm 5.8	< 0.01

Values are presented as mean \pm standard error. Adjusted for age, gender and using blood pressure-lowering medication.

ADD1: alpha-adducin 1 gene, *GN β 3*: G-protein β 3 subunit gene. *ACE*: angiotensin converting enzyme gene, *AGT*: angiotensinogen gene, *AT1R*: angiotensin II type 1 receptor gene. RAS: renin-angiotensin system. *: Numbers indicate the number of the risk genotype (*ADD1*: GT/TT=1, *GNB3*: AG/GG=1, *CYP3A5*: GA/AA=1, *ACE*: ID/DD=1, *AGT*: MT/TT=1, *AT1R*: CT/TT=1). [†] *p* < 0.05 compare to reference group (4 risk genotypes).

Figures legends

Figure 1. The odds ratios of hypertension by combined RAS (A), or salt sensitivity (B), or both the RAS and the salt sensitivity genes (C) in the total population and diabetic patients

RAS: Renin-angiotensin system.

Numbers indicate the number of the risk genotype for salt sensitivity genes (*ADD1*: GT/TT=1, *GNB3*: AG/GG=1, *CYP3A5*: GA/AA=1) and for RAS genes (*ACE*: ID/DD=1, *AGT*: MT/TT=1, *AT1R*: CT/TT=1).

* Significantly different from reference group, $p = 0.02$.

Data are adjusted for age and gender.

Figure 2. Mean common carotid intima media thickness by combined RAS (A), or salt sensitivity (B) genes, or both the RAS and the salt sensitivity genes (C) in total population and diabetic patients

RAS: Renin-angiotensin system.

Numbers indicate the number of the risk genotype for salt sensitivity genes (*ADD1*: GT/TT=1, *GNB3*: AG/GG=1, *CYP3A5*: GA/AA=1) and for RAS genes (*ACE*: ID/DD=1, *AGT*: MT/TT=1, *AT1R*: CT/TT=1).

* $p = 0.02$ compared with reference group (for the RAS genes: two risk genotypes, and for both the RAS and salt sensitivity genes: four risk genotypes).

** $p = 0.04$ compared with reference group (one risk genotype).

Data are adjusted for age, gender, and blood pressure-lowering medication.



