Angiotensinogen 235T Allele “Dosage” Is Associated With Blood Pressure Phenotypes

Alexandre C. Pereira, Glória F.A. Mota, Roberto S. Cunha, Fernando L. Herbenhoff, José G. Mill, José E. Krieger

Abstract—The genetic mechanisms underlying interindividual blood pressure variation among humans may reflect, at least in part, clustering of functional gene variants belonging to complex blood pressure control systems. In this study, we investigated the association of specific functional gene variants of the renin-angiotensin system, ACE (I/D) and angiotensinogen (M/T) genes, with blood pressure phenotypes (systolic, mean, diastolic, and pulse pressure), in an ethnically mixed urban population in Brazil. Individuals (n=1421) were randomly selected from the general population of the Vitoria City Metropolitan area. Neither gender, age, smoking status, total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol, or diabetes was associated with ACE or AGT polymorphism in univariate analysis. No association was found between ACE variants and blood pressure phenotypes. However, a statistically significant association was revealed between the AGT 235T variant and all blood pressure phenotypes, consistent with an additive/codominant mode of action even after adjustment for age and gender (P<0.01). Genotypic analysis contemplating both ACE and AGT variants in the same model did not show any significant interaction between both genetic polymorphisms. In addition, the AGT 235T allele was significantly associated with hypertension in a recessive model, which remained as an independent risk factor for hypertension even after adjustment for age, gender, and ethnicity (OR, 1.33; 95% CI, 1.04 to 1.70). Taken together, these data indicate a linear relation between AGT 235T allele number (“dosage”) and blood pressure in an ethnically mixed urban population and confirmed its role as an independent risk factor for hypertension in men and women when in homozygosity. (Hypertension. 2003;41:25-30.)

Key Words: angiotensinogen ■ angiotensin-converting enzyme ■ hypertension, genetic ■ blood pressure ■ polymorphism

The interplay between environmental and genetic factors is a major determinant of the final phenotype in hypertension.1 The genetic pathways underlying this complex disease remain largely elusive because late age of onset, polygenic inheritance, genetic heterogeneity, incomplete penetrance, unknown mode of action of disease alleles, quantitative variability of blood pressure phenotypes, ethnicity, age, gender, and environmental factors, such as diet, physical activity, or smoking status, are confounding variables that may interfere in this process in an unknown fashion.

In this scenario, the study of components of important physiological control systems known to contribute to the regulation of blood pressure can offer a unique opportunity to evaluate potential genetic mechanisms or pathways underlying interindividual blood pressure variation among humans.

The renin-angiotensin system (RAS) plays a key role in the maintenance of cardiovascular homeostasis. Two allelic variants in RAS components have been undoubtedly associated with particular intermediate phenotypes that could help explain some of the pathophysiological derangement observed in cardiovascular homeostasis.

The insertion/deletion (I/D) ACE gene polymorphism2 has been shown to predict approximately half of the interindividual variability in the serum2-3 and tissue4 levels of ACE. In addition, association of the ACE I/D polymorphism with myocardial infarction,5 sudden death,6 left ventricular hypertrophy,7 venous thrombosis,8 and nephropathy progression9 in a number of studies have been published, with contradictory findings. There is no consistent correlation between plasma ACE levels and hypertension or between ACE DD genotype and hypertension in a number of studies.10-13

In 1992, Jeunemaitre et al14 showed that a specific variant in the angiotensinogen gene leading to the substitution of a methionine (M) for a threonine (T) at the codon 235 of the gene was significantly linked to hypertension and was also associated with a modestly elevated plasma angiotensinogen (AGT) concentration. Since this first publication, several studies have also been performed, with conflicting results.15

In the current study, the hypothesis that the ACE I/D polymorphism and the angiotensinogen M235T genetic variants were associated with blood pressure phenotypes in an
ethnically admixed urban population was tested. This is one of the largest studies conducted in the general population to test this hypothesis with both gene variants, and it is the largest conducted in an ethnically admixed urban population to date considering blood pressure both as a continuous and as a qualitative variable.

Methods

Study Population
A cross-sectional study of risk factors for cardiovascular diseases was performed in the urban population of Vitoria, Brazil, using the WHO-MONICA project guidelines. A sample of 2044 individuals (from an eligible population of 137,330) of either gender, 25 to 64 years of age, were chosen according to the nearest birthday after a random selection of domiciles.

Participants (n=1507) attended the clinic visit and were further evaluated for height, weight, smoking habits, blood pressure measurements, and use of medicines. Blood glucose, total cholesterol, lipoprotein fractions, and triglycerides were assayed by standard techniques in 12-hour fasting blood samples.

Subjects were classified as white or black, according to a set of phenotypic characteristics (skin color, hair texture, shape of the nose, aspect of the lip, and jaw position), or racially mixed.17,18

Blood Pressure Phenotype Determination
Blood pressure was measured in the sitting position with the use of a standard mercury sphygmomanometer on the left arm after 5 minutes’ rest. The first and fifth phases of Korotkoff sounds were used for systolic and diastolic pressure, respectively. Systolic and diastolic blood pressures were calculated from two readings taken by two different observers, with a minimal interval of 10 minutes. Hypertension was defined as mean systolic blood pressure of ≥140 mm Hg and/or diastolic blood pressure of ≥90 mm Hg.19 Pulse pressure was the difference between systolic and diastolic blood pressures.

Assessment of ACE and Angiotensinogen Gene Polymorphism Genotype
The ACE gene I/D polymorphism was determined by means of a 3-primer system6,20 and the M235T variant of the ATG gene by a standard polymerase chain reaction detection method.21,22

Quality control for these assays was assessed by randomly selecting 50 samples to be re-genotyped by 3 independent technicians.

Statistical Analysis
Allele and genotype frequencies among study participants were analyzed by means of the χ² test and multivariate logistic regression by use of the Statistical Package StatView for Windows version 5.0 (SAS Institute Inc), with no corrections for multiple comparisons. Continuous and categorical variables were compared by means of the Student t test and the χ² test, respectively.

Hardy-Weinberg equilibrium for the distribution of genotypes was estimated by the χ² test. The odd ratios (OR) for different association models were calculated with 95% CI and 2-tailed probability values. Genetic models of action of the studied variants were constructed by combining genotypes (ie, dominant=heterozygous+hozygous for the polymorphism associated with increased levels of the gene product; recessive=homozygous for the polymorphism associated with increased levels of the gene product).

The unpaired t test was used to determine association between genotype and blood pressure phenotypes. Correlation between a particularly chosen genetic model and blood pressure phenotype was examined by means of simple and multiple linear regression.

Logistic regression analysis that allowed for age, gender, smoking status, diabetes mellitus, plasma cholesterol, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol, and ethnicity explored the association between genotype and risk of hypertension.

Results
Demographic data are summarized in Table 1. In Table 2, we present allelic and genotypic data for the studied population. AGT M/T alleles were in Hardy-Weinberg equilibrium, for a significant level of 0.01. ACE D/I alleles were not in Hardy-Weinberg equilibrium, which may be explained by a shift toward a higher frequency of DI individuals instead of DD individuals in our population. Of note, both ACE D/I and

<table>
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<tr>
<th>TABLE 1. Demographic Data</th>
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<tr>
<td>No. 1425</td>
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<tr>
<td>Male (female) 45.4 (54.6)</td>
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<tr>
<td>Mean age, y (range) 44.9 (24–65)</td>
</tr>
<tr>
<td>Ethnicity, %</td>
</tr>
<tr>
<td>European descent 36.7</td>
</tr>
<tr>
<td>Mulatto 49.8</td>
</tr>
<tr>
<td>African descent 7.4</td>
</tr>
<tr>
<td>Other 6.1</td>
</tr>
<tr>
<td>Current smoking, % 20.6</td>
</tr>
<tr>
<td>Total Cholesterol 216</td>
</tr>
<tr>
<td>Triglyceride 133.7</td>
</tr>
<tr>
<td>HDL-Chol 45.8</td>
</tr>
<tr>
<td>LDL-Chol 140.0</td>
</tr>
<tr>
<td>VLDL-Chol 25.9</td>
</tr>
<tr>
<td>Glucose 104.8</td>
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<tr>
<td>Diabetes mellitus, % 7.6</td>
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</table>

Metabolic data are expressed in mg/dL. Diabetes mellitus was defined as fasting glucose >125 mg/dL.

In addition, a linear regression–based genetic model (ie, dominant, recessive, or additive/codominant) characterizing the effects of alleles in the studied loci was also determined by assigning simple indicator variables to the individuals, based on genotype information. The best-fitting genetic model of the three for each marker was determined as the model that produced the smallest least-squares error and was taken to characterize the allelic effects of each loci.23

Sample size and power calculations were conducted with the use of EpiInfo (version 6.0). A value of P<0.05 on a 2-sided test was considered significant.

<table>
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<th>TABLE 2. Polymorphism Allelic and Genotypic Frequencies</th>
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<tr>
<td>ACE D/I Population</td>
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<td>A. Allelic frequencies</td>
</tr>
<tr>
<td>D allele 56.60</td>
</tr>
<tr>
<td>I allele 43.40</td>
</tr>
<tr>
<td>B. Genotypic frequencies</td>
</tr>
<tr>
<td>DD 29.10</td>
</tr>
<tr>
<td>DI 54.90</td>
</tr>
<tr>
<td>III 15.99</td>
</tr>
<tr>
<td>C. Clustering frequencies</td>
</tr>
<tr>
<td>MM</td>
</tr>
<tr>
<td>II 2.75</td>
</tr>
<tr>
<td>DI 10.73</td>
</tr>
<tr>
<td>DD 6.38</td>
</tr>
</tbody>
</table>
We have also studied the relation of these polymorphisms with the development of hypertension in our population. Univariate analysis failed to show any association of the ACE I/D polymorphism with any of the proposed genetic models (data not shown). In contrast, the angiotensinogen M235T allele was significantly associated with hypertension in our population. The best-fitting model of this association was the recessive for the T variant \((P=0.02; \text{OR}, 1.33 [1.04 to 1.7])\). Multiple logistic regression models to better characterize the relation between the M235T allele and other risk factors for the development of hypertension failed to show an effect of smoking status, triglycerides, HDL-cholesterol, LDL-cholesterol, or diabetes status (defined as fasting blood glucose >125 mg/dL). In contrast, adding ethnicity to the model reduced the importance of the M235T allele in defining hypertension (from OR of 1.5 to OR of 1.33 after adjustment). It is important to note, however, that the TT genotype (the recessive genotype of the M235T allele) still remained as an independent risk factor even after adjustment for age \((P<0.0001; \text{OR}, 1.07 [1.06 to 1.08])\), sex \((\text{female}, P<0.0001; \text{OR}, 0.49 [0.38 to 0.62])\), and ethnicity \((\text{mixed race}, P<0.0001; \text{OR}, 1.69 [1.30 to 2.20]; \text{black}, P<0.0001; \text{OR}, 2.81 [1.76 to 4.49])\).

**Discussion**

Both circulating and tissue RAS components have been implicated in a series of pathophysiological derangements leading to cardiovascular disease phenotypes.\(^{24,25}\) However, results of studies trying to associate functional variants of the RAS with such cardiovascular phenotypes have been contradictory.\(^{26}\)

Possible explanations for these contradictory findings are inadequate sample size for the majority of studies, leading to reduced statistical power; differences both within and between studies in the criteria used to select patients and control subjects; differences in environmental and ethnic/genetic background; differences in age of the subjects studied; the relatively low informativeness of the marker system used; and perhaps publication bias toward positive association.\(^{26}\)
Important characteristics of the current study circumvents some of these issues. The sample size and the selection criteria adopted have been designed to select a large and unbiased sample from the general population of the studied city. The Vitoria city metropolitan area is characteristically a region with a small migration flux, reducing both genetic and environmental variability. Finally, by using a strategy not restricted to the operational definition of hypertension, we were able to study blood pressure as a quantitative trait (blood pressure levels throughout the whole studied population) and qualitative (hypertensive versus nonhypertensive subjects), reducing bias that could be operant in erroneously matched case-control studies (ie, differences in genetic population structure).

We found no association of the ACE I/D polymorphism with any of the blood pressure phenotypes studied in our population. Similarly, Agerholm-Larsen et al\(^\text{27}\) failed to find any significant association of the D allele with any phenotypic variation in recognized risk factors for ischemic heart disease, including hypertension, in a case-referent study with 10,150 women and men. In addition, results from a meta-analysis study with a total of 32,715 white subjects found no association of these alleles with blood pressure.\(^\text{28}\)

In contrast, several lines of evidence based on experimental and genetic data suggest that angiotensinogen may be involved in blood pressure elevation.\(^\text{29–31}\)

TABLE 4. Linear Regression and Gender Stratification

<table>
<thead>
<tr>
<th>Blood Pressure Phenotypes</th>
<th>Women (n=647)</th>
<th>Men (n=776)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>R²</td>
</tr>
<tr>
<td>Mean blood pressure</td>
<td>0.117</td>
<td>0.014</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>0.138</td>
<td>0.019</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>0.090</td>
<td>0.008</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>0.119</td>
<td>0.014</td>
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</table>
This possibility was elegantly demonstrated by the establishment of transgenic mice expressing high levels of rat AGT and having elevated blood pressure,\textsuperscript{32} by the development of a knock-out model of the angiotensinogen gene, showing a significantly lower blood pressure than wild-type mice\textsuperscript{33} and by the development of knock-out/knock-in mice strains harboring 0 to 4 copies of the AGT gene. In these animals, plasma angiotensinogen levels increase progressively from zero in the null animals to 145\% of normal in the animals, plasma angiotensinogen levels increase progressively from zero in the null animals to 145\% of normal in the 3-copy animals, and blood pressure increased significantly and almost linearly at $\approx 8$ mm Hg per gene copy.\textsuperscript{34} It should be noted that our findings, different from any previous epidemiologic study, are in agreement with the results obtained with animal models by showing blood pressure elevation in a T-allele, dose-dependent manner.

Although, the association between the M235T allele and AGT levels has been more constantly reproduced,\textsuperscript{35} the association of this variant and blood pressure levels or hypertension has been more difficult to be established.

Kunz et al,\textsuperscript{15} in a meta-analysis performed to examine the association between the AGT 235T allele and hypertension comprising 5493 white patients from studies published between 1992 and 1996 reached a common odds ratio associated with the T allele, compared with the M variant, of 1.20 (95\% CI, 1.11 to 1.29). The odds ratio increased to 1.42 (95\% CI, 1.25 to 1.61) in subjects with a positive family history of hypertension and to 1.39 (95\% CI, 1.20 to 1.62) in hypertensive patients recruited from referral centers.\textsuperscript{25} Our findings show an odds ratio of 1.33 (95\% CI, 1.04 to 1.70) of hypertension in individuals with the TT genotype compared with individuals with the MT and MM genotypes. It is important to emphasize that our sample is from the general population and that the reported results were obtained after model adjustment for age, gender, and ethnicity. In particular, adjustment for ethnicity still conferred a significant association of the genotype and risk of hypertension, suggesting that the TT genotype may act independent of ethnic origin. More recently, Sethi et al\textsuperscript{36} demonstrated an association of the TT genotype with elevated blood pressure, isolated elevated systolic blood pressure, mildly elevated blood pressure, and use of antihypertensive medication in women but not in men from the Copenhagen City Heart Study.

Our findings expand these observations by demonstrating the association of the TT genotype independent of gender and by showing a significant and linear relation between T allele “dosage” and all the studied blood pressure phenotypes. Stratification of our data by gender supports the findings of a gender-genotype interaction in this locus. For each of the studied blood pressure phenotypes in our data, the best-fitting regression model was obtained in female subjects, even though there is a clear association independent of gender, either in regression models adjusting for gender or in regression models conducted only in the male sample of our population.

We have not tested the dosage effect of the 235T allele in any formal statistical analysis. However, the best-fitting genetic model of the three proposed models for all blood pressure phenotypes was consistent with an additive/codominant mode of action for the 235T allele. In addition, the statistical approach used to reach this conclusion, based on the smallest least-squares error, has been used by several groups to choose for the best-fitting model in genetic association studies. Despite these facts, a dominant mode of inheritance is also a statistically possible explanation for the observed data. Nonetheless, one cannot underemphasize the linear tendency of our data.

Our findings have potential limitations. Analysis of the genotype on each ethnic group was not able to show a statistically significant association with hypertension, which is most likely related to the small sample size and reduced statistical power associated to the subgroup analysis. Indeed, statistical power was significantly reduced for ethnic subgroup analysis (0.28 for the white population; 0.43 for the mixed race population, and only 0.11 for the black population) compared with the whole population (0.70).

The adopted definition of hypertension may also limit the interpretations. First, blood pressure measurements were taken in only one visit instead of the more accepted procedure on three different medical visits. Second, data on antihypertensive drug use (16.7\% of individuals) were not included in the definition of hypertensive individuals, since only 30\% of these treated subjects had normal blood pressure. Finally, blood pressure measurements were analyzed both as quantitative and qualitative variables. The results were able to uncover for the first time an association of the M235T genotype, with blood pressure as a continuous variable in a dose-dependent manner, and confirmed its role when it is considered as a dichotomized one (normotension versus hypertension). It is highly unlikely that a systematic measurement error could be responsible for all these concordant findings.

**Perspectives**

Taken together, these results suggest that there is a linear relation between angiotensinogen 235T allele number (“dosage”) and blood pressure in an ethnically mixed urban population and confirmed its role as a risk factor for hypertension both in men and women when in homozigosity. The magnitude of blood pressure variation associated to T allele “dosage” (3 to 4 mm Hg per copy of T allele) may qualify it as a potential player in the determination of a complex phenotype. This is consistent with the elevation of $\approx 8$ mm Hg per angiotensinogen gene copy, established by Smithies et al\textsuperscript{34} in mice with uniform genetic background under controlled experimental conditions. These results, which may apply to some but not other populations, highlight the challenges involved in the identification of sets of genes and the determination of their relative roles within individualized genetic and environmental context. This type of knowledge is a prerequisite for the development of the so-awaited individualized and more objective medicine.

**Acknowledgments**

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