

ACE Gene I/D polymorphism and systemic arterial hypertension in different classes of hypertensive individuals

Abstract

Systemic arterial hypertension (SAH) is a multifactorial clinical condition characterized by high and sustained levels of blood pressure (BP). Some studies have reported that variants of the angiotensin-converting enzyme (*ace*) gene increase the risk of hypertension. The aim of this study was to verify the existence of the relationship between the insertion/deletion (I/D) polymorphism in the ACE gene and its genotypic variants with BP in four distinct groups of hypertensive individuals and also to genetically and epidemiologically characterize the investigated samples. The study was formed of 112 individuals arranged into the following groups: normotensive (control); hypertensive and non-obese; hypertensive and obese; and, hypertensive and with type 2 diabetes mellitus (T2DM). Epidemiological data and peripheral blood were collected from participants for DNA extraction and amplification by PCR (polymerase chain reaction). The allele (D=0.5446; I=0.4554) and genotype (DD =0.2411, ID =0.6071; II =0.1518) frequencies showed low genetic differentiation ($F_{st} < 0.05$) and were outside the Hardy-Weinberg equilibrium ($p < 0.05$). There was no significant difference between the groups ($\chi^2 = 4.4335$; $p = 0.6174$). There was no association of the D allele with SAH, reinforcing the hypothesis that environmental interferences are prevalent in the evolution of SAH.

Keywords: ACE, SAH, Obesity, type 2 Diabetes mellitus

Volume 4 Issue 1 - 2019

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Received: December 14, 2018 | **Published:** February 19, 2019

Introduction

Systemic arterial hypertension (SAH) is a multifactorial clinical condition characterized by high and sustained levels of blood pressure (BP).¹ Over the past 30 years there has been a large increase in the prevalence of illness and deaths due to chronic diseases such as cardiovascular diseases and diabetes mellitus^{1a} with SAH playing an important part in this trend.

The number of hypertensive patients in Brazil has increased considerably this is due to lifestyle, with high salt intake and a sedentary lifestyle, among other factors. Moreover, according to Young,² the epidemiological spread of SAH is due, in part, to the evolutionary history of humans in relation to adaptation to climate, first in Africa and later in the rest of the world. The same study suggests that populations originating from cold regions have more susceptibility to hypertension than those who live in hot and humid environments, due to the fact that salt and water retention is more common in cold climates.²

The renin-angiotensin-aldosterone system (RAAS) plays an important role in controlling BP.³ Many gene components of this system have been studied because of their role in the regulation of BP.⁴ The angiotensin-converting enzyme (ACE) is a key component of the RAAS in the regulation of BP.⁵ This enzyme plays an important role in the production of angiotensin II and the catabolism of bradykinin; the latter is involved in the modulation of vascular tone and the proliferation of smooth vascular muscle cells.⁶

It has been suggested that the *Ace* gene may have its activity genetically regulated by the polymorphism of insertion/deletion (I/D) of an ALU sequence of 278 bp, in the intron 16,⁷ close to the

^{1a}Ministério da Saúde. DATASUS. SISHiperdia. Presentation. National Coordination of HA and DM. Department of Basic Attention. Department of Health Care.

3' extremity of the intron.⁸ This I/D polymorphic variation produce three possible genotypes: II, ID and DD.⁸ Individuals with the DD genotype have increased plasma levels of ACE, as compared with the genotype II, suggesting that the I/D polymorphism regulates the circulating quantity of the enzyme.⁶ Currently, there are two schools of thought about the physiological role of this polymorphism concerning the control of BP. One hypothesis is that the physiological changes in the circulating quantity of the enzyme favours the emergence of hypertension, when the D allele and the DD and DI genotypes are unfavourable, constituting a specific clinical risk factor for the development of SAH.⁹ Alternatively, another hypothesis suggests that the polymorphism does not contribute to SAH and that it has no effect on the level of BP and homeostasis.¹⁰

Consequently, the present study aimed to characterize the sampled patients regarding epidemiological parameters for SAH and to demonstrate the relationship of the I/D polymorphism of the *Ace* gene and its genotypic variants with regard to BP, in relation to the following classes of patients: obese, non-obese, and those with type 2 diabetes mellitus (T2DM).

Methods

The study was conducted in the municipality of Pinhão, Paraná, Brazil in three Health Units, over two stages. In the first stage, the collection of anthropometric data (BMI–Body Mass Index and waist circumference) was performed, as well as medications in use, BP and data from recent routine laboratory tests (total cholesterol, triglycerides and fasting glucose). In the second stage, the collection of peripheral blood was performed for each patient. In total, 112 male and female individuals participated in the study, of which 83 were aged over fifty years and taking three or more medications to control BP. The sample were classified into three groups of hypertensive patients in different classes: non-obese (Group 2: SAH+NO), obese (Group

3: SAH+O), and obese with T2DM (Group 4: SAH+ O+T2DM). In addition, a group of 29 individuals was sampled and classified as negative control, normotensive (Group 1).

All the participants signed an informed consent according the Ethics Committee on Human Research (COEP, State University of Ponta Grossa; Resolution CNS 196/96 with the research protocol no. 13664/11 and file 47/2012).

The DNA samples were extracted from peripheral blood lymphocytes, using an Invitrogen PureLink® commercial kit, according to the manufacturer's instructions. The genotyping of the I/D polymorphism of the *Ace* gene for each individual was performed by PCR (polymerase chain reaction), followed by electrophoresis in 1% agarose gel.

The primers used in this analysis were obtained according to Lindpaintner et al.⁷ and had the following sequences: ACE 1F (forward): 5'-GCCCTGCAGGTGTCTGCAGCATGT3' and ACE 1R (reverse): 5'-GGATGGCTCTCCCCGCCTT GTCTC-3'. The PCR reactions were performed in a final volume of 15µL, under the following conditions: 1.5 µL of (1X) reaction buffer (containing 200 mM of Tris, pH 8.4 and 500 mM of KCL); 0.25µL of Taq DNA Polymerase (5U/µL) (Invitrogen®); 1µL of MgCl₂ (50mM); 1µL of dNTP (2mM) (Ludwig Biotec®); 0.6 µL of forward primer and 0.6 µL of reverse primer (10mM) (Ludwig Biotec®), and 20ng of genomic DNA obtained from study participants. The polymerization reactions were performed in a thermocycler (Biocycler®) under the following program: initial denaturation for 5minutes at 94°C; 5 cycles: 1 minute at 94°C, 30 seconds at 57.5°C, 45 seconds at 72°C; followed by 30 cycles of: 1 minute at 94°C, 30 seconds at 56°C, 45 seconds at 72°C, ending with a final extension of 7 minutes at 72°C. The samples were subjected to electrophoresis in 1.5% agarose gel stained with GelRed (Biotium®) to verify the amplification of DNA fragments.

For the analysis of the parameters of population genetics, the TFPGA (Tools for Population Genetic Analyses)¹¹ program was used

in order to verify the allelic and genotypic frequencies. The Hardy-Weinberg Equilibrium, the pattern of genetic differentiation between groups with Wright's F-statistics,¹² and Reynold's distances¹³ were also used. The genotype frequencies were compared between groups using Person's chi-square test, with 5% significance level, using the Statistica 7.0 (StatSoft, Inc. 2001) program. For the remaining BP data, sedentary or not, smoker or non-smoker, laboratory tests, family history of SAH, BMI and waist circumference, MANOVA multivariate analysis was performed using Wilks, Pillai's, Hotelling and Roy's tests, and *a posteriori* Tukey's test at 5% significance, as well as correlation analysis and multiple linear regression (p<0.05), all through the Statistica 7.0 (StatSoft, Inc. 2001) program.

Results

The epidemiological data from the sampled patients of each group are summarized in Table 1.

Multivariate analysis (MANOVA)

The MANOVA for the data of waist circumference, total cholesterol, triglycerides, glucose, systolic blood pressure (SBP) and diastolic blood pressure (DBP) did not result in significant differences for the variables, I/D polymorphism, smoking, and family history of SAH, when using Wilks, Pillai's Hotelling and Roy's tests (Table 2). Subsequently, Tukey's test, with 95% confidence (p<0.05), was performed in order to determine possible statistical differences for each categorical variable in relation to the dependent variables. The results are shown in Table 3.

The analysis between genders only revealed significant differences for total cholesterol, with the highest value assigned to the female sex (Table 3).

An analysis of the different age groups of the population sample indicated significance of data as to the value of triglycerides in the age groups 60-69 and 70-79, and regarding SBP, in the age groups 70-79 and 80-82 (Table 4).

Table 1 Epidemiological data

	Group 1 normotensive	Group 2 SAH+NO	Group 3 SAH+O	Group 4 SAH+DM2
	n: 29	n: 30	n: 24	n:29
Age	59.14 (±5.92)	63.43(±8.89)	64.17(±9.34)	65.17(±8.81)
SBP	118.62(±13.55)	135.3(±15.25)	134.17(±13.81)	139.66(±24.57)
DBP	78.45 (±8.77)	86.33(±9.99)	84.17(±9.29)	86.55(±12.89)
BMI	28.13(±4.75)	26.75(±2.51)	34.05(±4.75)	30.56(±5.33)
WC	93.14(±12.10)	92(±8.34)	109.50(±13.54)	102.66(±10.10)
TCT. cholest	190.45(±48.69)	188.26(±49.93)	186.88(±35.71)	148.38(±41.41)
Triglycerides	141.55(±74.10)	142.4(±90.90)	155.58(±53.34)	201.62(±132.73)
F. Glucose	90.14(±9.50)	89.23(±12.31)	90.58(±13.23)	141.03(±44.43)
Sedentary	58.60%	20.00%	83.30%	63.30%
Smoker	24.10%	23.30%	20.80%	23.30%
F. His. SAH	65.50%	80.00%	79.10%	80.00%

SAH, Systemic arterial hypertension; NO, non-obese; O, obese; T2DM, diabetes mellitus type 2; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; WC, waist circumference; TG, triglycerides; TCT cholest, total cholesterol; F Glucose, fasting glucose, F His. SAH, family history of SAH

Table 2 Wilks, Pillai's, Roy's and Hotelling's tests to verify the significance of the variables

	WC	Total cholesterol	Triglycerides	Fasting Glucose	SBP	DBP
Groups (4)	+	+	+	+	+	+
Gender	+	+	+	+	+	+
Age	+	+	+	+	+	+
Sedentary	+	+	+	+	+	+
BMI	+	+	+	+	+	+
I/D Polymorphism	n.s	n.s	n.s	n.s	n.s	n.s
Smoker	n.s	n.s	n.s	n.s	n.s	n.s
Family history of SAH	n.s	n.s	n.s	n.s	n.s	n.s

WC, waist circumference, SBP, systolic blood pressure, DBP, diastolic blood pressure, n.s, non-significant value, +, significant value (<0.05)

Table 3 Tukey's test for groups, gender and the variables WC, total cholesterol, triglycerides, fasting glucose, SBP and DBP

WC	Normotensive	SAH+NO	SAH+O	SAH+DM2	F	M
Normotensive		0.979006	0.000140	0.007635		
SAH and non-obese	0.979006		0.000138	0.001990		
SAH and obese	0.000140	0.000138		0.118112		
Diabetes and SAH	0.007635	0.001990	0.118112			
F						0.249780
M					0.249780	
Total cholesterol						
Normotensive		0.997714	0.991547	0.002901		
SAH and non-obese	0.997714		0.999541	0.004778		
SAH and obese	0.991547	0.999541		0.012191		
Diabetes and SAH	0.002901	0.004778	0.012191			
F						0.002493
M					0.002493	
Triglycerides						
Normotensive		0.999980	0.610200	0.124889		
SAH and non-obese	0.999980		0.580077	0.109875		
SAH and obese	0.610200	0.580077		0.814001		
Diabetes and SAH	0.124889	0.109875	0.814001			
F						0.584581
M					0.584581	
Fasting glucose						
Normotensive		0.993552	1.000000	0.000138		
SAH and non-obese	0.993552		0.994954	0.000138		
SAH and obese	1.000000	0.994954		0.000138		
Diabetes and SAH	0.000138	0.000138	0.000138			
F						0.732201
M					0.732201	
SBP						
Normotensive		0.000974	0.003893	0.000197		
SAH and non-obese	0.000974		0.996745	0.891478		
SAH and obese	0.003893	0.996745		0.813685		
Diabetes and SAH	0.000197	0.891478	0.813685			
F						0.564972
M					0.564972	
DBP						
Normotensive		0.019232	0.177632	0.016447		
SAH and non-obese	0.019232		0.872882	0.999838		
SAH and obese	0.177632	0.872882		0.840821		
Diabetes and SAH	0.016447	0.999838	0.840821			
F						0.653770
M					0.653770	

SAH, Systemic arterial hypertension; NO, non-obese; O, obese; DM2, diabetes mellitus type 2. The values in bold indicate significant differences

Table 4 Tukey's test for age, BMI and the variables, WC, total cholesterol, triglycerides, fasting glucose, SBP and DBP

WC	50-59	60-69	70-79	80-82		
50-59		0.990030	0.995848	0.576883		
60-69	0.990030		0.999997	0.658405		
70-79	0.995848	0.999997		0.681287		
80-82	0.576883	0.658405	0.681287			
Normal					0.000138	0.000114
Overweight					0.000138	0.000114
Obesity					0.000114	0.000114
Total cholesterol						
50-59		0.566577	0.050446	0.262158		
60-69	0.566577		0.419890	0.565605		
70-79	0.050446	0.419890		0.960444		
80-82	0.262158	0.565605	0.960444			
Normal					0.711409	0.760688
Overweight					0.711409	0.993417
Obesity					0.760688	0.993417
Triglycerides						
50-59		0.198305	0.421435	0.998004		
60-69	0.198305		0.012579	0.752900		
70-79	0.421435	0.012579		0.940501		
80-82	0.998004	0.752900	0.940501			
Normal					0.001333	0.000145
Overweight					0.001333	0.378305
Obesity					0.000145	0.378305
Fasting glucose						
50-59		0.748555	0.999991	0.978244		
60-69	0.748555		0.865792	0.999999		
70-79	0.999991	0.865792		0.982924		
80-82	0.978244	0.999999	0.982924			
Normal					0.412460	0.667112
Overweight					0.412460	0.837278
Obesity					0.667112	0.837278
SBP						
50-59		0.055033	0.986577	0.070455		
60-69	0.055033		0.313773	0.496648		
70-79	0.986577	0.313773		0.130265		
80-82	0.070455	0.496648	0.130265			
Normal					0.281706	0.014512
Overweight					0.281706	0.165675
Obesity					0.014512	0.165675
DBP						
50-59		0.658540	0.572367	0.140434		
60-69	0.658540		0.130284	0.337029		
70-79	0.572367	0.130284		0.040498		
80-82	0.140434	0.337029	0.040498			
Normal					0.641368	0.243000
Overweight					0.641368	0.583753
Obesity					0.243000	0.583753

Values in bold indicate significant differences.

Regarding BMI, a significant relationship was found between waist circumference and all classes of BMI. Triglyceride values were significant between the normal and overweight groups, and the normal and the obese groups, while in relation to SBP there was difference between the normal and obese individuals, with the highest values attributed to obesity (Table 4).

Regarding sedentarism, there was a significant increase in waist circumference for the individuals who did not practice any physical activity.

Linear regression showed a significant association between SBP and DBP. The R² value of the linear regression model between DBP

and SBP was 57.52%. The graph in Table 3 shows that the higher the DBP, the higher the SBP, with a 57.52% correlation coefficient (R²).

I/D polymorphism of the Ace gene

The I/D polymorphism for the *Ace* gene was efficiently amplified, as described by Lindpaintner et al.⁷ and its genotypes were identified. The genotypes were tabulated into groups, as shown in Table 5.

Taking into consideration the 112 individuals, the allele/gene and genotypic frequencies for the I/D polymorphism of the ACE gene can be seen in Table 5; all of the genotype frequencies were outside the Hardy-Weinberg equilibrium (HWE) (p<0.05).

Table 5 Genotypic, gender classification, gene frequencies (D, I) and genotypes (DD, ID, II) for the I/D polymorphism of the ACE gene and p value in all of the analyzed individuals and by groups. Group 1, normotensive; Group 2, hypertensive (SAH) and non-obese; Group 3, hypertensive and obese; and Group 4, hypertensive and diabetes mellitus type 2

Allele/Genotype	Total	Group 1 Normotensive	Group 2 SAH and non-obese	Group 3 SAH and obese	Group 4 SAH and DM2
n	112	29	30	24	29
DD		8	7	7	5
ID		17	21	12	18
II		4	2	2	6
F		15	16	15	15
M		14	14	9	14
Freq D	0.5446	0.5690	0.5833	0.5417	0.4828
Freq I	0.4554	0.4310	0.4167	0.4583	0.5172
Freq DD	0.2411	0.2759	0.2333	0.2917	0.1724
Freq ID	0.6071	0.5862	0.7000	0.5000	0.6207
Freq II	0.1518	0.1379	0.0667	0.2083	0.2069
P value	0.0245	0.4442	0.0276	1.0000	0.2771

Genotypes: deletion/deletion DD, insertion/deletion, ID, insertion/insertion II; M, male, F, female; n, number of individuals per group

The allele and genotype frequencies were obtained by taking into consideration the grouping of the population into four classes (groups), as shown in Table 5. The classes that were shown to be within the HWE were groups 1, 3 and 4, while group 2 was outside of HWE. The comparison of frequencies between the groups was assessed using Pearson's chi-square test, which determined the significance of the differences found between the two groups, and this showed that there was no significant difference between them (chi-square =4.4339, p = 0.6174).

The analysis of genetic differentiation (F statistic) was low (Fst <0.05). In order to measure the genetic distance between the population pairs, Reynold's et al.⁸ distance was used. The greatest distances were found between the SAH and non-obese group, and between the SAH and T2DM group (0.0032). The lowest distance was between the normotensive group, and the SAH and obese group (-0.0177).

Discussion

The I/D polymorphism of the ACE gene in the intron 16 region is attributed to a phenomenon known as linkage disequilibrium,¹⁴ which describes the degree of non-random association between two alleles at two different loci of the same chromosome.¹⁵ This polymorphism has been associated with SAH,⁹ atherosclerosis¹⁶ and

coronary heart disease, such as acute myocardial infarction.⁶ The D allele has been attributed with being responsible for an increase in vascular vasoconstriction,¹⁶ due to increased ACE levels in the plasma and tissues of individuals with the D allele.^{16,17} However, some studies have not found this association with the occurrence of coronary artery disease,⁷ brain injury,¹⁸ deep venous thrombosis and thromboembolism¹⁹ and SAH,⁵ leaving the biological significance of the I/D polymorphism of the ACE gene remaining an open question.

The present study did not reveal the relationship between the I/D polymorphism of the ACE gene and SAH because the genotypic differences between the four sampled groups was not significant. The family history of SAH was also not found to be associated, and these were the environmental factors that most contributed to the development of SAH in the analyzed sample.

Obesity and waist circumference were prevalent factors that contributed to the SAH profile in this study, with increased values in the SAH and obese group, and the SAH and T2DM group, which were related to increased values of BMI and sedentarism. Total cholesterol was prevalent in the SAH and T2DM group, and it was higher for females. Increased values of triglycerides were found for those aged 60-69 and in obese individuals. Increased SBP values were most evident in the three groups with SAH and in individuals with obesity, and those individuals with DBP aged over 80, as well as the SAH and

non-obese group, and the SAH and T2DM group. These data support the hypothesis that individuals with SAH are at increased risk of developing T2DM and dyslipidemia.²⁰

The close relationship between the increase in SBP and DBP reinforces the hypothesis of the elevated levels of hypertension in most of the individuals. Although increased DBP has been associated with the DD genotype,²¹ no correlation was observed in the present study between the ACE genotypes and SBP or DBP. Our data only indicated the associations between DBP and advanced age (over 80 years old), in the SAH and non-obese group, and the SAH and T2DM group, but not with respect to I/D polymorphism.

However, we do not rule out the possibility that genetic factors are associated with SAH. Hereditary factors, combined with environmental risk factors, are important in the onset of SAH. Montgomery et al.²² evaluated the relationship between I/D polymorphism of ACE and the growth of the left ventricle (LV) only in individuals with II and DD homozygous genotypes, undergoing 10 weeks of physical activity. Those individuals with the DD genotype had a greater hypertrophic response than those with the II genotype.

From an evolutionary standpoint, in the hypertensive and normotensive population that was studied, the genotypic frequencies were outside the HWE, but when they were considered in groups, such as subpopulation, only group 2 (SAH and non-obese) was outside the HWE. According to Beiguelman,²³ the HWE is only maintained in a population if evolutionary factors such as mutation, natural selection, genetic drift, gene flow of migrant populations, and endogamy are not present. As the heterozygous genotype frequency for the SAH and non-obese group was 0.700, this effect can be attributed to a selection in favor of the heterozygote, or random drift, due to the small population size. No genetic structure (Fst) was observed in the sample, thus there was no association of the genotype classes with the four groups.

In a study of 210 individuals, conducted in Brazil by Inácio et al.,²⁴ the allele frequency for the ACE gene was 0.39 and 0.61, for the I allele and the D allele, respectively, and the genotype frequencies were II: 0.20; ID: 0.43; and DD: 0.37. When conducted in southern Brazil, this was 0.35 and 0.65 for the I allele and D allele, respectively and the genotype frequencies were II: 0.22 ID: 0.24 and DD: 0.54, which differed from the Brazilian population as a whole, with increased DD homozygous genotype and decreased heterozygote. The findings were attributed to the different ethnic composition of the region and the small sample might have favoured random drift. The population investigated in the present study was also from southern Brazil, consisting of 112 individuals who obtained values of 0.4554 for the I allele and 0.5446 for the D allele. The values for the DD, ID and II genotypes were 0.2411, 0.6071 and 0.1518, respectively. These results differ from those obtained by Inácio et al.²⁴ for southern Brazil, with a greater frequency of the heterozygote genotype, and not for the DD homozygote, as had been expected. This result is even more surprising because the present study deliberately included patients screened for SAH and its comorbidities.

Conclusion

In conclusion, the analysis of the association of the I/D polymorphism of the ACE gene with SAH, taking into consideration different classes of hypertensive and normotensive individuals, did not result in statistically significant associations, leading to the conclusion

that environmental factors are more prevalent, even in relation to family history, in the determination of SAH in the population studied here.

Acknowledgments

None.

Conflicts of interest

Author declares no conflicts of interest.

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