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Classic Genotypes of the ACE Gene do Not Interfere in Blood Pressure Responses to Reactivity Test in Male Adolescents

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JFVNM and CSGC designed the study and collected the data. Authors VCS and OTN wrote the protocol and performed the DNA genotyping. Authors OL, MMS, SRM and FOC participated in the writing of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: The Angiotensin Converting Enzyme (ACE) is responsible for converting Angiotensin I into Angiotensin II, which has vasoconstrictive properties. Polymorphisms in the *ACE* gene have been associated to higher levels of Angiotensin II and, therefore, higher blood pressure values.
Aims: To verify if classic genotypes of the *ACE* gene could interfere in blood pressure reactivity responses to a reactivity test.
Study Design: Cross-sectional study aiming to associate genotypes of the *ACE* gene with blood pressure reactivity responses.

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Place and Duration of Study: Male adolescents from the city of Petrolina, Pernambuco, Brazil participated in the study from February to October of the year 2013.
Methods: One hundred and sixty (160) male adolescents, aged 14 to 20 years, students from three high schools from the city of Petrolina, Pernambuco, Brazil, participated in the study. The participants underwent measurements of body mass, height, waist circumference and skinfold thickness. Body mass index and waist-to-height ratio were calculated. DNA was extracted from blood samples in order to determine the genotypes of the rs4646994 of the *ACE* gene. Blood pressure was measured at rest, during the application of a reactivity test (Cold Pressor Test), and 1 min after the test. One-Way ANOVA was used to verify the differences between the genotypes of the *ACE* gene and the anthropometric and blood pressure variables. Odds ratio was calculated in order to attest if the D allele carriers presented higher chances of having increased resting blood pressure values and of being hyper-reactive to the Cold Pressor Test.
Results: No statistically significant differences were found between genotypes when comparing anthropometric and blood pressure values at rest as well as responses to the reactivity test.
Conclusion: Classic genotypes of the *ACE* gene do not seem to interfere in blood pressure responses to a reactivity test in male adolescents.

Keywords: ACE gene polymorphism; blood pressure; adolescents; cold pressor test.

ABBREVIATIONS

ACE : Angiotensin Converting Enzyme
RAAS :Renin-Angiotensin-Aldosterone System
BP : Blood Pressure
DNA : Deoxyribonucleic Acid
EDTA : Ethylenediamine Tetraacetic Acid
BMI : Body Mass Index
SBP : Systolic Blood Pressure
DBP : Diastolic Blood Pressure
WC : Waist Circumference
RNA : Ribonucleic Acid
mRNA : Messenger RNA
AGT : Angiotensinogen
AGTR1 : Angiotensin II Receptor Type 1

1. INTRODUCTION

The Angiotensin Converting Enzyme (ACE) is an important regulatory enzyme of the Renin-Angiotensin-Aldosterone System (RAAS), which is a complex system that acts in the maintenance of the homeostasis of blood pressure (BP). In addition to converting Angiotensin I into Angiotensin II, the vasoconstrictive properties of ACE involve the inactivation of Bradikinin and Calidin [1,2].

The *ACE* gene is located in the chromosome 17 and contains a polymorphism that distinguishes itself by the presence (insertion, I) or absence (deletion, D) of a segment of 287 base pairs in the intron 16 [3,4]. The I/D polymorphism of the *ACE* gene is responsible for 47.0% of the variation of ACE in the blood stream, where individuals homozygous for the D allele present higher plasma levels of this enzyme [4].

Due to its vasoconstrictive characteristic, studies involving classic genotypes of the *ACE* gene have been performed with the aim of verifying to what extension the D allele can influence in the BP responses [2,3]. Regarding children and adolescents, specifically, some studies have reported that the presence of the D allele influences BP responses and other variables [5-7].

In addition to its behavior at rest, BP responses to stress agents have been considered an important risk factor for cardiovascular disease [8-10]. In this scenario, a simple way of verifying BP responses after being exposed to a stress agent is through the Cold Pressor Test [11-12].

Therefore, the aim of the present study was to verify if classic genotypes of the *ACE* gene could interfere in BP reactivity responses, induced by a stress agent, in male adolescents.

2. MATERIALS AND METHODS

2.1 Sample and Ethical Procedures

Three high schools from the city of Petrolina, state of Pernambuco, Brazil, were randomly selected and all male students enrolled in the schools were invited to participate in the study. Only the ones who returned the informed consent term signed by a parent (or himself if aged 18 or more) participated in the study. In total, 160 adolescents, aged 14 to 20 years, participated (20.78%). The present study has a cross-sectional design, therefore, follow-up was not necessary.

2.2 Anthropometric Evaluation

Anthropometric evaluation was performed through measurements of body mass, height, BMI, waist circumference, waist-to-height ratio and body fat percentage.

Body mass was measured with a digital scale (Wiso®, Brazil) and height was assessed with a portable stadiometer (Wiso®, Brazil). During both measurements the participants wore light clothes and were barefeet. BMI was calculated dividing body mass (kg) by the squared value of height (m^2).

Waist circumference was measured with a non-extendible tape (CESCORF®, Brazil). The measurement was made between the last rib and the iliac crest. Waist-to-height ratio was calculated dividing waist circumference (cm) by height (cm).

Triceps and calf skinfolds were measured according to Heyward & Stolarczyk [13] and body fat percentage was calculated according to Slaughter et al. [14].

2.3 DNA Extraction and Genotyping of the rs4646994

Blood samples were obtained from the antecubital vein by a trained professional. Three to five ml of blood was drawn in EDTA-containing tubes. DNA was obtained from whole blood using a DNA extraction kits (QIAGEN, Germany) [15] and stored at -80℃ for subsequent analysis.

The I/D polymorphism of the *ACE* gene (rs4646994) was determined by inspection of the electrophoretic profile of polymerase chain-reaction (PCR) products, as performed by Moraes et al. [16]. Briefly, the 490 bp (I allele) or the 190 bp (D allele) products were amplified using primers: 5′-CTGCAGACCACTCCCATCCTTTCT-3′ and 5′-GATGTGGCCATCACATTCGTCAGAT-3′. Reaction tubes contained 100 ng DNA, 10 mmol/l Tris–HCl pH 8.3, 75 mmol/l KCl, 3.5 mmol/l MgCl2, 0.2 mmol/l dNTP, 20 pmol of each primer, 0.5 µg of purified chicken albumin and 1 U of Taq DNA polymerase (Phoneutria®, Brazil) in a final volume of 25 µl. After 1 min of hot start at 80℃ and an initial denaturation for 2 min at 94℃, the amplifications were carried out for 30 cycles of 40 s at 94℃, 45 s at 64℃ and 50 s at 72℃ followed by a final 5 min extension at 72℃. Inspection of DD subjects was carried out using oligonucleotides (5′-TGGGACCACAGCGCCCGCCACTAC-3′ and 5′-TCGCCAGCCCTCCCATGCCCATAA-3′) specific to amplify a 335 bp fragment of the insertion sequence. All PCR products were separated by electrophoresis on 2% agarose gels containing ethidium bromide at 50 µg/ml, visualized by using CCD camera (Vilber Lourmat®, Deutschland), examined using the gel analysis software enclosed (Photo Capt 1D), and confirmed by visual inspection.

2.4 Blood Pressure Measurements

Blood pressure was measured using a validated automatic oscillometric device (BP A100, Microlife®, China) [17] in three distinct moments: (a) at rest, after 10 min in a seated position; (b) during the application of the Cold Pressor Test (at the 60-sec mark); and (c) 1 min after the Cold Pressor Test. All measurements were performed in a seated position in a quiet room with the subjects' left arm at the level of the heart, as recommended by the Brazilian Cardiology Society [18]. Resting blood pressure was considered high when SBP and / or DBP presented values equal or above the 95th percentile for the subjects' age, sex, and height percentile [19].

2.5 Cold Pressor Test

In this test, participants immersed their right hand up to the wrist in a container filled with cold water (4.0℃ to 5.0℃) for 1 min. The temperature of the water was controlled using an infrared thermometer (Cason® CA380, China). Blood pressure reactivity was assessed at the 60-sec mark of the Cold Pressor Test and 1-min after finishing the test. Subjects that presented an increase in blood pressure equal or above 25 mmHg or 20 mmHg for systolic and/or diastolic blood pressure, respectively, when compared to blood pressure at rest, were considered hyper-reactive. Lower values were considered as normoreactive [12].

2.6 Statistical Procedures

A descriptive analysis of the data was performed and values were expressed in frequency (%), mean ± standard deviation and range. Data normality was assessed through skewness and kurtosis values, respecting an interval between -1.00 and +1.00 [20]. The correction of the extreme values (outliers) was performed by adding one unit to the extreme value [21]. The

Chi-Squared test was used to verify the equilibrium between the genotypes of the rs4646994 of the *ACE* gene [22].

A One-Way Analysis of Variance (ANOVA), with Bonferroni's *post hoc*, was performed to verify the differences between the genotypes of the rs4646994 of the *ACE* gene and the other variables. Finally, Odds Ratio was calculated, using the Crosstabs option, with a confidence interval of 95%, in order to attest if the D allele carriers presented higher chances of having increased resting BP values and of being hyper-reactive to the Cold Pressor Test.

The level of significance adopted was P≤0.05 and the software used in the statistical analysis was the Statistical Package for the Social Sciences (SPSS), version 15.0.

3. RESULTS AND DISCUSSION

3.1 Results

The main characteristics of the sample are shown in Table 1. The distribution of the genotypes of the polymorphism of the *ACE* gene showed that 34 participants (21.3%) were homozygous for the I allele, 67 (41.9%) were heterozygous (ID), and 59 (36.9%) presented the DD genotype. The Chi-Squared test revealed equilibrium between the genotypes (X^2=3.21; P>0.05).

The One-Way ANOVA with Bonferroni's *post hoc* did not detect any significant differences between the main characteristics of the participants when divided by the *ACE* gene's genotypes, as shown in Table 2.

Sixteen percent (n=26) of the participants presented increased resting BP values, and 33.7% (n=55) were hyper-reactive to the Cold Pressor Test. The comparison between BP values at rest, during, and 1 min after the Cold Pressor Test also did not show statistical differences when comparing the polymorphisms of the *ACE* gene (Table 3).

Table 1. Main characteristics of the sample (n=160). Values expressed in mean ± standard deviation and range

Variable	Mean ± SD	Range
Age (years)	16.2 ± 1.3	14.0 – 20.0
Body mass (kg)	65.1 ± 12.7	38.1 – 107.0
Height (cm)	172.8 ± 6.5	154.0 – 189.0
Body mass index (kg/m²)	21.7 ± 3.6	14.7 – 31.8
Waist circumference (cm)	74.6 ± 7.7	56.5 – 101.0
Waist-height ratio	0.4 ± 0.0	0.3 – 0.5
Body fat (%)	15.1 ± 5.4	7.6 – 28.9

Lastly, the Odds Ratio analysis revealed that none of the allelic groups exhibited chances of having increased resting BP values when compared to the participants homozygous for the I allele. Despite the trend for a 50.0% higher and 5.0% lower chance for increased blood pressure values at rest and hyper-reactivity to the Cold Pressor Test, respectively, the present results did not reach statistical significance (X^2=0.49 and X^2=0.02, respectively; P>0.05), as shown in Table 4.

3.2 Discussion

In the present study no statistically significant differences were found between BP responses to a reactivity test and the classical genotypes of the *ACE* gene. However, the D allele carriers of the I/D polymorphism of the *ACE* gene presented 50.0% more chances of having increased BP values at rest, but without statistical significance. The lack of significant alterations in BP at rest and during the reactivity test considering the different genotypes of the *ACE* gene may be attributed to several factors. Among them, the phenotypes related to morphological variations, and also the physical fitness of the studied population, since no differences were found when comparing body composition parameters.

Table 2. Main characteristics of the sample according to the genotypes of the *ACE* gene (n=160). Values expressed in mean ± standard deviation

Variable	II (n=34)	ID (n=67)	DD (n=59)	P value
Age (years)	16.3 ± 1.4	16.1 ± 1.2	16.3 ± 1.3	0.553
Body mass (kg)	68.7 ± 13.6	63.8 ± 12.3	64.7 ± 12.4	0.174
Height (cm)	173.1 ± 6.3	172.1 ± 6.9	173.5 ± 6.0	0.474
BMI (kg/m²)	22.9 ± 4.0	21.4 ± 3.2	21.4 ± 3.7	0.120
WC (cm)	76.7 ± 8.5	73.7 ± 7.0	74.3 ± 8.0	0.167
Waist-Height ratio	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.180
Body fat (%)	16.6 ± 5.9	14.4 ± 4.7	15.1 ± 5.7	0.142

BMI=body mass index; WC=waist circumference

Table 3. Blood pressure responses at rest, during, and 1 min after performing the cold pressor test according to the different genotypes of the *ACE* gene

	Genotype	Rest	During	1 min After
SBP	II (n=34)	118.50 ± 13.65	133.29 ± 21.42	123.12 ± 17.70
(mmHg)	ID (n=67)	121.67 ± 13.07	135.03 ± 20.25	122.52 ± 16.90
	DD (n=59)	121.32 ± 10.78	133.80 ± 18.83	123.46 ± 14.85
	P value	0.452	0.901	0.949
DBP	II (n=34)	69.12 ± 9.51	83.32 ± 13.57	74.00 ± 11.91
(mmHg)	ID (n=67)	68.92 ± 8.34	83.39 ± 14.21	71.25 ± 9.86
	DD (n=59)	70.76 ± 8.48	84.51 ± 12.54	72.54 ± 10.36
	P value	0.457	0.134	0.454

SBP=systolic blood pressure; DBP=diastolic blood pressure

Table 4. Odds Ratio analysis between *ACE* gene's genotypes

***ACE* gene genotypes**	**Increased resting blood pressure**		
	Odds ratio	**CI (95%)**	**P value**
II (n=34)	1	-	
DD/ID (n=126)	1.50	0.478 – 4.707	0.485
***ACE* gene genotypes**	**Hyper-reactivity to cold pressor test**		
	Odds ratio	**CI (95%)**	**P value**
II (n=34)	1	-	
DD/ID (n=126)	0.95	0.429 – 2.101	0.899

CI=confidence interval

However, several studies have reported increased chances of the development of hypertension in individuals that carry the D allele of the *ACE* gene. Lemes et al. [5] reported a 2.44 higher chance of hypertension in obese children and adolescents that carried the D allele. Also, Wu et al. [6] stated that, in hypertensive children, the D allele is significantly more frequent than in normotensive children.

On the other hand, Barretti et al. [23] demonstrated that overweight, aerobically trained Zucker rats presented lower cardiac *ACE* mRNA, ACE activity and Angiotensin II in the left ventricle when compared to their overweight sedentary pairs. In addition, the authors demonstrated that, when comparing the aerobically trained and sedentary rats, increased RAAS activity directly impacted the pathological hypertrophy of the left ventricle. Therefore, these results suggest that the present study's sample can present variations in its functional aspect, where adolescents with the same genotype can show a within group variability in the expression of RAAS intermediaries, such as ACE and Angiotensin II.

Even though no statistically significant differences between the *ACE* genotypes and the responses of BP to the reactivity test were found, it was observed that the carriers of the D allele presented slightly higher BP values when compared to the participants homozygous for the I allele. Therefore, the results of the present study suggest that the BP responses to the Cold Pressor Test are not influenced by the polymorphisms of the *ACE* gene. However, when analyzing the values of the total sample, an increase in systolic and diastolic blood pressure during the test is observed. This indicates an increase in the activity of the sympathetic nervous system, and consequent release of norepinephrine [24], as well as an increase in the activity of RAAS intermediaries [23], resulting in a constriction of the blood vessels.

Alternatively, other studies that aimed to compare the BP responses to reactivity tests found different results when compared to the present study. Wang et al. [25], analyzing two other genes that are related to RAAS (*AGT* and *AGTR1*), reported significant differences between genotypes when performing the Cold Pressor Test. In addition, Mei et al. [26] and Roy-Gagnon et al. [27], when studying Chinese and American families, respectively, reported similar responses to stress induced by the Cold Pressor Test in subjects from the same family. Thus, the possibility of a genetic influence in the BP responses to a stress agent cannot be discarded.

In adults, the association between high values of BP at rest and the presence of the D allele seems to be positive. He et al. [28] found chances 94.0% higher of hypertension in D allele carriers. In Brazilian adults, Freitas et al. [29] reported a systolic blood pressure 7.8 mmHg higher in individuals that carried the D allele.

The present study showed that D allele carriers presented slightly higher non statistically significant blood pressure values at rest when compared to individuals homozygous for the I allele. It is possible that due to the participant's low mean age (~16 years old) the differences derived from the distinct genotypes are yet to appear. Nonetheless, in adults, 2.0 mmHg differences in systolic and diastolic blood pressure values have been associated with decreases in the prevalence of hypertension, coronary heart disease and stroke [30,31].

While the present study brings important contributions, it is not without limitations. First, there were no direct measurements of the intermediaries of RAAS (ACE and Angiotensin II, for instance). Therefore, it is not possible to establish a cause-effect relation, or to determine the mechanisms by which BP increases when submitted to a stress agent (Cold Pressor Test). Moreover, no information regarding the physical fitness of the participants was collected. Thus, there is no way of knowing how physical activity levels can interfere in BP responses to reactivity tests.

4. CONCLUSION

The comparisons between the classic genotypes of the *ACE* gene did not reveal statistically different values in the different moments of the BP reactivity test (rest, during and 1 min after). Although the comparisons between the genotypes were not different, the presence of the D allele of the *ACE* gene polymorphism was associated with an increase of 50.0% in the chances of presenting high resting BP values.

Clinically, the results found in the present study have an important application, since individuals that carry the D allele can present higher RAAS activity, and, consequently, higher risks of developing cardiovascular diseases in the future.

CONSENT

All authors declare that written informed consent was obtained from the participant's parents or guardians, or themselves (if aged 18 years or older).

ETHICAL APPROVAL

The present study was approved by the ethics committee of the Catholic University of Brasília (protocol number 195/2010) and all procedures were in accordance with the 1964 Declaration of Helsinki and the resolution 466/2012 of the Brazilian National Health Council.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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