Original Article

The A1166C Polymorphism of the Angiotensin II Type-1 Receptor in Acute Myocardial Infarction

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Objective

To assess the association of the A1166C polymorphism of the angiotensin II type-1 receptor (AT1R) gene with acute myocardial infarction and also with the severity of coronary artery disease.

Methods

A prospective, cross-sectional study was carried out with 110 patients with acute myocardial infarction, who, on coronary angiography, had significant lesions (> 50%) assessed according to 3 criteria of severity: number of vessels affected, morphology of the atherosclerotic plaque, and coronary risk score. The control group comprised 104 individuals with no coronary lesions. The A1166C polymorphism of AT1R gene was determined by polymerase chain reaction in the DNA of leukocytes in peripheral blood. The classic coronary risk factors were analyzed in all individuals.

Results

When stratifying the genotypes in regard to risk factors, only smoking predominated in the AC heterozygous patients (P = 0.02). The genotypic frequency in the infarcted patients was as follows: AA = 54.5%; AC = 35.5%; and CC = 10%, which was similar and nonsignificant in regard to that in the control group (P = 0.83). No risk increase occurred for acute myocardial infarction when comparing the genotypes as follows: CC vs AA (OR = 1.35; 95% CI = 0.50 – 3.59); AC vs AA (OR = 1.03; 95% CI = 0.58 – 1.84); and AA+AC vs AA (OR = 1.33; 95% CI = 0.51 – 3.45). None of the severity criteria showed a significant correlation with the genotypes.

Conclusion

According to our results, no correlation exists between the A1166C polymorphism of the angiotensin II type-1 receptor (AT1R) gene and acute myocardial infarction or the severity of coronary artery disease.

Key words

A1166C polymorphism, angiotensin II, acute myocardial infarction, coronary artery disease

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The renin-angiotensin system comprises a cascade of enzymatic reactions, which results in the production of angiotensin II from the angiotensinogen substrate. The physiological effects of angiotensin II are mediated by a final common pathway, through angiotensin II binding to specific receptors located on the cell membrane [1,2]. Two isoforms of endothelial receptors for angiotensin II are known so far: AT1 and AT2. Most of their physiological effects are mediated by the activation of AT1-subtype receptors. The receptors belong to the superfamily of the G-protein-coupled receptors, and, in the case of AT1 receptors (AT1R), coupling occurs via Gq proteins. Consequently, stimulation of AT1 receptors activates phospholipase C, increases the levels of diacylglycerol (DAG) and inosotol triphosphate (IP3), elevates the intracellular Ca^{+2} concentration, and activates several kinases, modulating cell functions [3,4]. Angiotensin II acts as a mitogen in vascular smooth muscle cells by activating several signaling pathways, such as that of phospholipase C, phospholipase A_2, and phospholipase D, as well as by activating a large number of kinases, such as tyrosine kinases, mitogen-activated protein kinases (MAPKs), c-src kinase, Janus-associated tyrosine kinase, and receptors with tyrosine-kinase activity. Angiotensin II also stimulates transcription factors, such as the activating protein 1, signal transduction and transcription activators (STATs), and the nuclear factor kappa B (NFkB) [5,6]. Several studies have reported that the proliferative effects of angiotensin II are mediated by the activation of AT1 receptors [7]. More recently, a study including patients with myocardial infarction and high circulating levels of angiotensin II reported that the administration of AT1R antagonists had a significant pharmacotherapeutic implication [8].

Cloning of cDNA of the AT1 receptor provided the identification of a polymorphism in the nontranslated region 3' (A1166C), corresponding to an A→C transversion (adenine replaced by cytosine) in the position of the nucleotide 1166 of the mRNA sequence, resulting in 1 heterozygous (AC) and 2 homozygous (CC and AA) genotypes [9]. The homozygous CC genotype seems to be associated with a greater incidence of myocardial infarction [10].

Increasing evidence has shown the importance of the participation of the renin-angiotensin system in the process of endothelial proliferation and coronary atherosclerosis. The action of the AT1 receptor suggests its participation in the genesis of coronary atherosclerosis and myocardial infarction. This study aimed at assessing the correlation of the A1166C polymorphism of the AT1R gene with acute myocardial infarction and with the severity of coronary artery disease.

Methods

A prospective cross-sectional study was carried out with 110 consecutive patients (66.6% of the male sex, and 36.4% of the female sex, mean age = 61.82 ± 10.81 years) diagnosed with acute myocardial infarction based on clinical, electrocardiographic, and enzymatic data [11], and confirmed through coronary angiography. The control group comprised 104 patients (57.7% of the female sex, and 42.3% of the male sex, mean age = 56.69 ± 11.52 years), who were renal transplant donors or had valvular heart disease or atypical chest pain, but had lesions neither in the coronary arteries nor in the cardiac muscle on coronary angiography. All patients underwent assessment of the following classical coronary risk factors: first-degree familial history of coronary artery disease; serum levels of cholesterol (HDL < 35 mg/dL, LDL > 159 mg/dL) and triglycerides (> 200 mg/dL); smoking (active smoker or someone who had quit smoking within the previous 12 months); systemic arterial hypertension (systolic blood pressure > 140 mmHg and diastolic blood pressure > 90 mmHg); diabetes mellitus (patients treated with insulin or oral hypoglycemic agents, or fasting glucose level = 140 mg/dL); sedentary lifestyle (practice of aerobic physical activity at a frequency lower than 3 times a week); overweight; and obesity [body mass index (BMI) > 25 kg/m^2]. Coronary angiography was used to confirm the presence or assess the extension of coronary artery disease, and patients with obstructive lesions greater than 50% were selected for the study.

Severity of the coronary artery disease was assessed according to the following criteria: 1) number of vessels affected (single-vessel or multivessel disease) [12]; 2) morphological characteristics of the atherosclerotic plaque according to Ellis et al [13] (A: proximal concentric lesion < 10 mm; B1: eccentric lesion between 10 and 20 mm, with moderate proximal tortuosity; B2: eccentric lesion between 10 and 20 mm, with moderate distal, ostial tortuosity, or tortuosity in the bifurcations; C: lesion > 20 mm, or total occlusion); and 3) coronary risk score, which is a simple method to estimate the amount of cardiac muscle at risk based on the location (proximal, middle, or distal) and degree of stenosis of the obstructive lesion. The greatest scores are associated with low left ventricular ejection fraction, providing greater prognostic information, according to Califf et al [14].

Patients with the following conditions were excluded: systemic malignant disease, terminal chronic illness, previous myocardial infarction, coronary artery bypass grafting, coronary angioplasty.

The research protocol was assessed and approved by the committee on ethics. All patients were instructed about the potential risks and benefits of the study and signed a written informed consent.

Analysis of the polymorphism of the AT1R gene was performed by use of the polymerase chain reaction technique (PCR). The DNA extractions were performed according to the DNA isolation method of Sambrook et al [15]. Peripheral blood was collected in a 5-mL vacutainer tube with EDTA, stored at 4ºC, and centrifuged for separation of the leukocyte layer. A 500-μL aliquot of the leukocyte layer was transferred to a 1.5-mL Eppendorf tube, and 1.0 mL of the buffer solution of lytic red blood cells [155 mM of NH_4Cl, 10 mM of $KHCO_3$, and 1mM of EDTA] was added to each sample, which was then mixed by inversion for 30 seconds. This mixture was centrifuged at 2,500 rpm for 5 minutes for nuclear pelletization. The floating material was ruled out, and the process was repeated 2 to 3 times, until the pellet acquired a light beige color. Then, 150 μL of the buffer solution of nuclear lysis [10 mM of Tris - HCl (pH 8.0), 2 mM of EDTA, 400 mM of NaCl] was added, and the pellet was resuspended. Then, 5 μL of cold 10% sodium dodecyl sulfate and 2.5 μL of proteinase K (20 mg/mL) were added to the solution, and the samples were incubated for 6 hours at 60 ºC. After that, 100 μL of saturated NaCl was added to the samples, which were placed on ice for 10 minutes.

The samples were centrifuged at 2,500 rpm for 15 minutes, the floating material was removed, and 800 μL of cold absolute ethanol were added, mixing by inversion until precipitation occurred. The tube was horizontally rotated on its axis, so that DNA attached to the wall of the tube, and the floating material was ruled out. The pellet was washed in 1.0 mL of 70% ethanol. The alcohol was ruled out and the pellets were resuspended in a variable volume of TE [10 mM of TRIS - HCl, 1 mM of EDTA (pH 7.4)] according to their size. Then, the DNA concentration was calculated with a spectrophotometer at 260 nm. Each reaction consisted of 100 ng of genomic DNA, 25 pmol of primer, 1.5 mmol/L of $MgCl_2$, 0.5 mmol/L of each dNTP, 1 U of Taq DNA polymerase, and Taq DNA polymerase buffer 1X in 30 mL of the reaction. The PCR used the following program: 30 cycles at 93ºC for 1.5 minute, 58ºC for 2 minutes, and 72ºC for 2 minutes. A fragment of the AT1R gene containing 856 pb, located in the nontranslated 3' region was amplified by the pair of primers: 5'-AAT GCT TGT AGC CAA AGT CAC CT-3'; 5'-GGC TTT GCT TTG TCT TGT TG-3'. This region houses the 1166 variant, characterized by the Adenine→Cytosine (A→C) transversion in the nucleotide occupying that position.

Each PCR was performed with 100 ng of genomic DNA, Taq-DNA polymerase buffer, 10 pmoles of each primer, 200 mM of each dNTP, 2.5 mM of $MgCl_2$, and 1.5 U of the Taq-DNA polymerase enzyme. The program used for amplification of the fragment consisted of initial denaturation for 5 minutes at 95ºC, followed by 34 cycles comprising denaturation at 94ºC for 30 seconds, annealing at 58ºC for 1 minute, and extension at 72ºC for 35 seconds. The amplified product was visualized in 1.5% agarose gel stained with ethidium bromide. Ten microliters of the amplified product were digested with 3 units of the Dde I restriction enzyme for 5 hours at 37ºC, according to the instructions of the manufacturer (Life Technologies). This enzyme recognizes the following restriction site: 5'- C ↓ TNAG –3'; 3'- GANT ↑ C –5'.

In the AT1R gene fragment studied, a restriction site occurs in the mutant allele, because the replacement of an A by a C creates another site of recognition of the Dde I enzyme. Therefore, the A allele of the AT1R gene has a restriction site for that enzyme generating 2 fragments of 600 and 256 pb, and the mutant C allele has 2 restriction sites producing 3 fragments of 600, 146, and 110 pb each. The restriction product was visualized in 2% agarose gel stained with ethidium bromide after electrophoresis as follows: homozygous individuals for the A allele have 2 bands of 600 and 256 pb; homozygous individuals for the C allele have 3 bands of 600, 146, and 110 pb; and AC heterozygous individuals have 4 bands of 600, 256, 146, and 110 pb [10].

The Statistica 5.5 Statsoft, Inc. program [16] was used to perform the analyses. An error $\alpha < 5\%$ ($P < 0.05$) was considered to indicate the statistical significance level. Pearson chi-square test was used to analyze the dichotomous variables. The variables were transformed into numerals by attributing a zero value for the absen-

ce and 1 for the presence. The continuous variables were expressed as mean ± standard deviation, and the differences between the groups were compared using the Student *t* test. The relative risk was assessed by calculating the odds ratios (OR) and their confidence intervals (CI). The differences between the frequencies of patients in regard to the conventional risk factors and the genotypical classes were assessed by use of logistic regression [17].

Results

The prevalence of smoking, familial history of coronary artery disease, systemic arterial hypertension, diabetes mellitus, and high levels of total cholesterol, LDL-cholesterol and triglycerides was significantly high in patients with acute myocardial infarction as compared with that of the control group. Only obesity (assessed through BMI), sedentary lifestyle, and HDL-cholesterol showed no statistical significance in the groups (tab. I).

Genotyping of the patients and controls for A1166C polymorphism of the AT1R gene by use of Dde I enzyme restriction resulted in a differentiated band pattern for the AA, AC, and CC genotypes. This was due to the modification in the amplified fragment of 856 pb, when replacement of an A (adenine) by a C (cytosine) occurred, creating a new restriction site for that enzyme. Therefore, the following band pattern was observed in the agarose gel stained with ethidium bromide: AA homozygous, 600 and 256 pb bands; AC heterozygous: 600, 256, 146, and 110 pb bands; and mutant CC homozygous: 600, 146, and 110 pb bands (fig. 1).

Table I - Risk factors: patients with acute myocardial infarction and controls

Variables	Infarction n = 110 (%)	Controls n = 104 (%)	P
Familial history of CAD	51 (46.4%)	33 (31.7%)	0.028
Arterial hypertension	92 (83.6%)	70 (67.3%)	0.005
Smokers	52 (47.3%)	26 (25.0%)	0.001
Diabetes mellitus	27 (24.5%)	13 (12.5%)	0.024
BMI > 25 kg/m²	53 (48.2%)	57 (54.8%)	0.332
Sedentary lifestyle	71 (64.5%)	60 (57.7%)	0.304
Cholesterol (mg/dL)			
Total > 200	78 (70.9%)	41 (39.4%)	0.0001
HDL < 35	17 (15.5%)	15 (14.4%)	0.833
LDL > 159	93 (84.5%)	62 (59.6%)	0.0001
Triglycerides > 200 mg/dL	28 (25.5%)	15 (14.4%)	0.044

CAD - coronary artery disease.

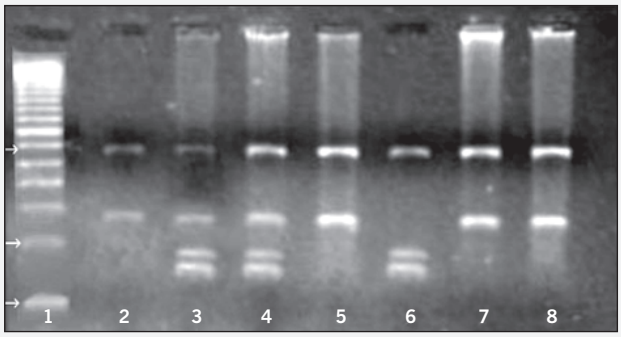

Fig. 1 - Products of enzymatic restriction of a fragment of the AT1R gene with the Dde I enzyme; column 1: marker of molecular weight of 100 pb; columns 2, 5, 7, and 8: 600 and 256-pb fragments representing the AA genotype; columns 3 and 4: 600, 256, 146, and 110-pb fragments representing the AC genotype; column 6: 600, 146, and 110-pb fragments representing the CC genotype.

Stratifying the population of the infarcted and control patients by the AT1R genotypes, no sex predominance was observed between the genotypes in the control group (P = 0.212) and in the infarcted patients (P = 0.739), the mean age being similar in both groups (56.3 ± 12.9 years). Analyzing the classical coronary risk factors in the controls stratified by the AT1R genotypes, no significant statistical difference was observed (tab. II). This same analysis in the patients with acute myocardial infarction showed that smoking was the only variable whose prevalence differed among them, being greater in the AC and AA genotypes (tab. III).

Table IV shows the distribution of the genotypes of the A1166C polymorphism of the AT1R gene in the patients with acute myocardial infarction and in the controls. No significant difference was observed between the groups ($\chi^2 = 0.367$; P = 0.832), and, even when the presence of the A allele (AA+AC) was assessed, its frequency did not statistically differ between the groups. The relative risk of acute myocardial infarction was analyzed by comparing the genotypes, which provided the following results: CC vs AA (OR = 1.35; 95% CI = 0.5079 – 3.5992); AC vs AA (OR = 1.03; 95% CI = 0.5828 – 1.8433); and AC+AA vs CC (OR = 0.90; 95% CI = 0.3640 – 2.2255) (fig. 2).

The severity of coronary artery disease was assessed by using the following criteria: number of vessels affected (single-vessel or multivessel disease); morphological characteristic of the atherosclerotic plaque (A, B1, B2, C); and the coronary risk score. No statistical difference was observed between the AT1R genotypes. The comparisons of the genotypes (CC vs AC vs AA) showed no difference between the groups (P < 0.05), a fact also observed in the comparisons CC vs AC and CC vs AA (table V).

Table II - Coronary risk factors in controls stratified according to the genotypes of angiotensin II AT1 receptors

Variables	CC n = 08 (%)	AC n = 37 (%)	AA n = 59 (%)	P
Familial history of CAD	03 (37.5)	12 (32.4)	18 (30.5)	0.918
Arterial hypertension	06 (75.0)	24 (64.9)	40 (67.8)	0.851
Smokers	02 (25.0)	07 (18.9)	17 (28.8)	0.552
Diabetes mellitus	01 (12.5)	04 (10.8)	08 (13.6)	0.924
BMI > 25 kg/m²	04 (50.0)	21 (56.8)	32 (54.2)	0.933
Sedentary lifestyle	08 (100.0)	20 (54.1)	32 (54.2)	0.052
Cholesterol (mg/dL)				
Total > 200	05 (62.5)	16 (43.2)	20 (33.9)	0.251
HDL < 35	02 (25.0)	05 (13.5)	08 (13.6)	0.675
LDL > 159	06 (75.0)	24 (64.9)	32 (54.2)	0.383
Triglycerides > 200 mg/dL	01 (12.5)	06 (16.2)	08 (13.6)	0.925

Table III - Coronary risk factors in patients with acute myocardial infarction stratified according to the genotypes of angiotensin II AT1 receptors

Variables	CC n = 11 (%)	AC n = 39 (%)	AA n = 60 (%)	p
Familial history of CAD	6 (54.5%)	16 (41%)	29 (48.3%)	0.658
Arterial hypertension	7 (63.6%)	34 (87.2%)	51 (85.0%)	0.161
Smokers	3 (27.3%)	25 (64.1%)	24 (40.0%)	0.024
Diabetes mellitus	1 (9.1%)	13 (33.3%)	13 (21.7%)	0.191
BMI > 25 kg/m²	7 (63.6%)	16 (41.0%)	30 (50.0%)	0.381
Sedentary lifestyle	8 (72.7%)	24 (61.5%)	39 (65.0%)	0.786
Cholesterol (mg/dL)				
Total > 200	7 (63.6%)	29 (74.4%)	42 (70.0%)	0.767
HDL < 35	3 (27.3%)	9 (23.1%)	5 (8.3%)	0.073
LDL > 159	8 (72.7%)	32 (82.1%)	53 (88.3%)	0.364
Triglycerides > 200mg /dL	5 (45.5%)	7 (17.9%)	16 (26.7%)	0.172

Table IV - Distribution and statistical analysis of the correlation of the genotypes of angiotensin II AT1 receptors in patients with acute myocardial infarction and controls

Genotype	Infarction n = 110 (%)	Controls n = 104 (%)	χ^2	P	Odds ratio	95% CI
CC	11 (10.0)	08 (07.7)	-	-	1.3521	0.5079 - 3.5992
AC	39 (35.5)	37 (35.6)	-	-	1.0365	0.5828 - 1.8433
AA	60 (54.5)	59 (56.7)	0.367	0.832	-	-
AA+AC	99 (90.0)	96 (92.3)	-	-	1.3333	0.5141 - 3.4581

Odds ratio: measure of the relative risk of coronary artery disease in individuals with the CC genotype in regard to the AA, AC and AA, and of the A allele (genotypes AA+AC) in regard to individuals with the AA genotypes.

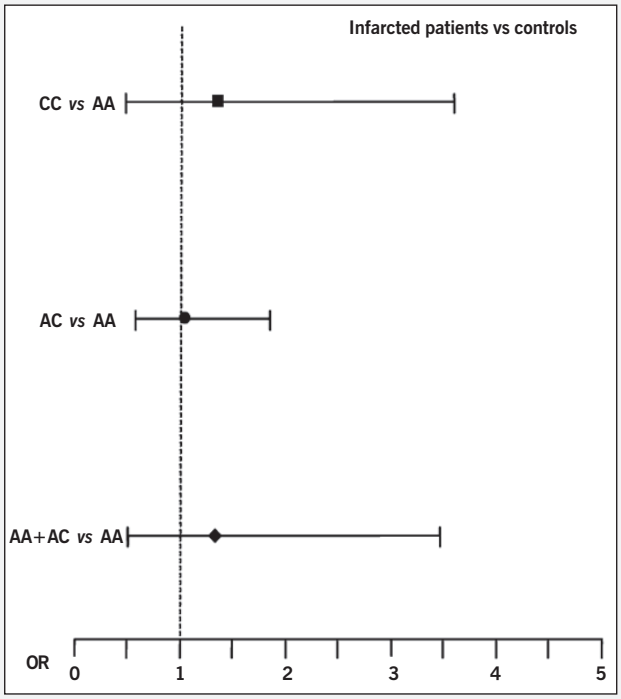

Fig. 2 - Estimate of the risk of having acute myocardial infarction in association with the A1166C polymorphism of the AT1R gene. Odds ratio according to the comparisons of the following genotypes: CC *vs* AA, AC *vs* AA, and AA+AC *vs* AA.

Table V - Statistical analysis of the severity of coronary artery disease according to the genotypes of the angiotensin II AT1 receptors

Variables*	CC n = 11 (%)	AC n = 39 (%)	AA n = 60 (%)
Number of vessels affected			
Usingle-vessel disease	2 (18.2)	11 (28.2)	13 (21.7)
Multivessel disease	9 (81.8)	28 (71.8)	47 (78.3)
Morphology of the plaque			
A	0	1 (2.7)	1 (1.7)
B1	1 (10.0)	7 (18.9)	19 (32.8)
B2	2 (20.0)	7 (18.9)	11 (19.0)
C	7 (70.0)	22 (59.5)	27 (46.6)
Risk score			
2	2 (18.2)	9 (23.1)	12 (20.0)
4	2 (18.2)	10 (25.6)	14 (23.3)
6	4 (36.4)	14 (35.9)	26 (43.3)
8	3 (27.3)	6 (15.4)	5 (8.3)
10	0	0	3 (5.0)

* Criteria of severity with no statistical difference between the genotypes of the A1166C polymorphism of AT1R.

Discussion

Atherosclerosis, the basic process of coronary artery disease, and its major manifestation, myocardial infarction, has become the major cause of death in the occident world. It is usually triggered by destabilization of the atherosclerotic plaque and sudden obstruction of the coronary artery by formation of a local thrombus [18]. The participation of an inherited genetic component, represented by the familial history of coronary artery disease, already described in the literature [19], was confirmed in this study by its greater prevalence in the group of infarcted patients (P = 0.028). Smoking, systemic arterial hypertension, and hypercholesterolemia (total and LDL) are the major risk factors, contributing in approximately equal degrees to ischemic heart disease [20,21], facts confirmed in this study with elevated statistical significance (P = 0.001; P = 0.005; and P = 0.0001, respectively). Hypertriglyceridemia is the lipid alteration most commonly associated with diabetes mellitus [22,23], a correlation observed in this study, in which the proportion of diabetic individuals among the infarcted patients corresponded to almost twice that among controls. Obesity and sedentary lifestyle, contrary to that which has been reported in the literature [24,25], were not important risk factors in that population.

The studies about the correlation between myocardial infarction and polymorphic variations in candidate genes or markers (noncodifying DNA sequence) are used to demonstrate the role played by genetic factors in the etiology of this multifactorial disease. As angiotensin II is the fundamental agent in the sequence of reactions that culminate with its physiological and pathological actions, its major receptor, AT1R, is an important element for the studies assessing the role of the renin-angiotensin system (RAS) in acute myocardial infarction.

This study showed that the classical coronary risk factors did not predominate among the AT1R genotypes in controls, except for sedentary lifestyle (P = 0.05). In the group of infarcted patients, only smoking had a significant statistical difference, greater for the AC genotype, indicating that an interaction of a highly prevalent environmental factor with genotype may exist [26]. As the other coronary risk factors had a similar distribution among the genotypes of controls and infarcted individuals, the AT1R genotypes are likely not to be associated with these factors, in regard to the development of myocardial infarction.

In this study, homozygosis for the A allele of the A1166C polymorphism in the locus of the AT1 receptor was more frequent both in controls and infarcted individuals. In the control group, the following distribution was observed: AA = 56.7%; AC = 35.6%; CC = 7.7%; and the genotypic combination AA/AC = 92.3%. In the infarcted individuals, the distribution was as follows: AA = 54.5%; AC = 35.5%, CC = 10.0%; and the genotypic combination AA/AC = 90.0%. The results showed no statistical significance

between the groups (P = 0.83) and that the chance of developing acute myocardial infarction did not increase, which was observed in the comparisons of the genotypes CC vs AA (OR = 1.35; 95% CI = 0.50 – 3.59), AC vs AA (OR = 1.03; 95% CI = 0.58 – 1.84), and AA+AC vs AA (OR = 1.33; 95% CI = 0.51 – 3.45). In a study about the correlation between myocardial infarction and AT1R, Berge et al [27] reported the following frequencies of the genotypes in controls: AA = 52.1%; AC = 42.7%; CC = 5.2%; and the combination of genotypes AA+AC = 94.8%. In our study, the frequencies of the genotypes in infarcted patients were as follows: AA = 49.3%; AC = 41.3%; CC = 9.4%, and the combination of genotypes AA+AC = 90%. Therefore, this study corroborated those results and also showed no correlation between that polymorphism and acute myocardial infarction. However, Canavy et al [28] reported a positive correlation between AA1166C polymorphism and the high prevalence of the C allele in patients with myocardial infarction [10,29]. Duncan et al [30], in a recent literature review of the studies on the associations of the AT1 gene focused on clinical goals, reported the controversies of the association between that gene and myocardial infarction.

The present study showed no association between the A1166C polymorphism of the AT1R gene and the severity of the coronary lesions, when comparing the genotypes CC vs AC vs AA, CC vs AC, and AC vs AA. Similar results allowed other authors to reach the same conclusion [31].

Analyzing A1166C polymorphism of the AT1R gene considering the results of this study, one can conclude that no correlation exists between that gene polymorphism and acute myocardial infarction, and the severity of coronary artery disease. The outcomes of the major publications about these facts have not been completely determined, probably due to the way the analyses are performed, the limitations of the data obtained, and the bias of the predominance of studies reporting positive results. Several other studies on gene polymorphisms, particularly those related to the renin-angiotensin system, are being carried out and may definitely establish the causal role played by these genes.

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