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Association Between *M235T*-AGT and *I/D*-ACE Polymorphisms and Carotid Atheromatosis in Hypertensive Patients: A Cross-Sectional Study

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Abstract. *Background/Aim: The renin-angiotensin-aldosterone system (RAAS) may be implicated in carotid atheromatosis (CA) development. We aimed to assess the relationship of M235T-angiotensinogen (AGT) and insertion/deletion of angiotensin conversion enzyme (I/D-ACE) genotypes with CA in patients with essential hypertension (EHT). Patients and Methods: We determined the M235T-AGT and I/D-ACE genotypes, using PCR-RFLP methods, in 162 hypertensive subjects from three tertiary regional medical centers. The relationship between the studied RAAS gene polymorphisms and CA was assessed by multiple logistic regressions. Results: Hypertensive patients carrying the MT/TT235-AGT and MT235-AGT genotypes had a 2.17-fold (p=0.033) and 2.24-fold (p=0.036) increased risk to develop CA, respectively. These genotypes, MT/TT 235-AGT (OR=2.17, p=0.033) and MT235-AGT (OR=2.24, p=0.036), remain independent risk factors for CA in hypertensive patients according to the multivariate model. Conclusion: There is a statistically significant association between M235T-AGT and CA, when adjusting for several confounders and controlling for hypertension.*

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Essential hypertension (EHT) is associated with cardiovascular risk factors, such as carotid atheromatosis (CA), dyslipidemia, obesity, left ventricular hypertrophy (LVH), and presents a high risk for ischemic coronary and cerebrovascular events (1-4).

According to the World Health Organization (WHO), ischemic cardiovascular diseases are responsible for 31% of all deaths worldwide. Stroke and myocardial infarction (MI) represent 80% of all deaths of ischemic vascular cause (5).

CA and the increase in carotid intima-media thickness (cIMT) in the carotid-vertebral axis are manifestations of atherosclerotic disease and diagnostic markers for ischemic heart disease (IHD) and ischemic cerebrovascular disease (6, 7).

The genetic polymorphisms of the renin-angiotensin-aldosterone system (RAAS) are associated with uncontrolled EHT (8, 9). RAAS influences atherosclerosis (ATS) through oxidative stress, an increase in NADPH activity, reactive oxygen species production and low-density lipoprotein cholesterol (LDL-C) peroxidation (10, 11).

The components of RAAS are angiotensinogen (AGT), angiotensin-converting enzyme (ACE), angiotensin I (AngI), angiotensin II (AngII), angiotensin II type 1 receptor (AngII R1) and angiotensin II type 2 receptor (AngII R2) (12). AGT is secreted by the liver and is converted to AngI upon the action of renin (REN). ACE converts AngI to AngII,

stimulates aldosterone secretion from the adrenal cortex, and influences blood pressure (BP) (13-15). ACE is a risk factor for ischemic cerebrovascular ischemic events, high levels of plasma ACE determines atheromatosis and increased cIMT. High levels of plasma AngII induces proliferation of arteriolar smooth muscle, collagen deposition and apoptosis of smooth muscle cells (16-18). ACE activity in arterial endothelial and smooth muscle cells is increased, contributing to the development of atherosclerotic lesions, independently of BP values or lipid profile (19).

Met235Thr (*M235T-T704C*) is a point mutation in the gene encoding AGT, which is associated with EHT and ATS, through the induction of endothelial dysfunction and increase in vascular wall inflammation (20). Meta-analyses have reported that Caucasian patients diagnosed with EHT, carriers of the *MT235* heterozygous genotype or the *TT235* homozygous genotype, had higher AGT levels compared to *MM235* homozygotes. In some studies, patients carrying the *TT235* genotype were diagnosed with malignant hypertension (HT) (21, 22).

I/D represents the insertion/deletion of a fragment of the ACE gene, which results in an increase in plasma ACE levels and activity. This genetic variation closely correlates with EHT (23, 24).

There is a genetic predisposition of pateints with the *M235T*-AGT and *I/D*-ACE genotypes to IHD, ischemic stroke, EHT, and diabetes mellitus (DM) (5, 25, 26).

The aim of our study was to analyze the *M235T*-AGT and *I/D*-ACE gene polymorphisms in patients with EHT, and their relationship with CA, as predictors of ischemic cerebrovascular events.

Patients and Methods

Study design and setting. We designed a prospective cross-sectional study in EHT patients from the ambulatory, internal medicine, and cardiology wards of three tertiary regional medical centers (4th Medical Clinic, 5th Medical Clinic, and Polaris Hospital in Cluj-Napoca, Romania), between June 2015 - December 2017.

The study included 162 patients diagnosed with EHT (on treatment), as well as patients newly diagnosed with EHT. Systolic BP (SBP) and diastolic BP (DBP) were measured 5 minutes after the beginning of the examination. According to EHT management guidelines, three successive measurements were performed 2-5 min apart, in sitting or supine position, with a manual blood pressure monitor. EHT was defined according to guidelines at values higher than or equal to 140/90 mmHg, glycemia was considered increased at values over 100 mg/dl, high total cholesterol (TC) was considered at values >200 mg/dl, increased low-density lipoprotein cholesterol (LDL-C) was considered at values higher than 100 mg/dl, hypertriglyceridemia (HTG) was considered at triglyceride (TG) values over 150 mg/dl.

A detailed history was recorded for each patient including age, gender, and body mass index (BMI) (weight/height - kg/cm^2).

The following laboratory tests were performed in the patients included in the study: TC, TG, high-density lipoprotein cholesterol (HDL-C), LDL-C, renal function (urea, creatinine), liver function [alanine transaminase (ALT) and aspartate transaminase (AST)], using standard laboratory methods.

Associated risk factors, such as dyslipidemia, DM, and non-alcoholic hepatic steatosis (NAHS), were monitored for the presence of the metabolic syndrome. The general objective of the examination was aimed the detection of the presence of possible signs of EHT-mediated organ involvement (cardiovascular, neurological changes) and other causes of secondary HT, such as the presence of neurofibromatosis or hyperthyroidism.

Patients who refused participation in the study, patients with secondary HT, with acute or chronic renal disease, with a glomerular filtration rate (GFR) below 30 ml/min/1.72 m^2, with functional New York Heart Association (NYHA) class III and IV heart failure, cancer disease and liver failure, or alcohol-induced hepatic steatosis (HS) were excluded from the study.

Written consent was obtained from all participants before their inclusion in the study. The study was approved by the Ethics Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy (study no. 333/2.06.2015).

Carotid ultrasonography. At the time of recruitment, all patients underwent B-mode Doppler ultrasound of the carotid-vertebral axis, abdominal ultrasound, using Samsung H60 (Samsung Medison, Seoul, South Korea), and Aloka Alpha 7 (Aloka Medical Company Ltd., Tokyo, Japan) ultrasound systems, with 3-14 MHz linear probes and 1-7 MHz convex probes, respectively. The participants were examined in the supine position. The presence of ATS plaques was examined in transverse and longitudinal sections in the common carotid arteries (CCA), carotid bulb, internal carotid artery (ICA) and external carotid artery (ECA), bilaterally.

For cIMT and CA examination, measurements in 3 CCA locations, where no ATS plaques were present and at 1.5-1 cm distance from the CCA bifurcation were performed. Patients were diagnosed as having CA if they presented a cIMT greater than or equal to 1 mm or more than 50% focal narrowing of the vessel wall or ATS plaques. The severity of stenosis was classified: no stenosis, mild <50%, moderate 50-70%, severe 70-99%, and occlusion.

Also, the presence or absence of HS was examined by abdominal ultrasound, according to standard examination protocols: 1) attenuation of the image quickly within 4-5 cm of depth, 2) echogenic diffusely but particularly important to note brightness within the first 2-3 cm of depth, 3) liver uniformly heterogeneous, and 4) thick subcutaneous depth (>2 cm). No complimentary examinations for the diagnosis of HS were performed (liver puncture biopsy, abdominal CT, FibroScan), because the aim of this study was not to analyze the relationship between gene polymorphism and HS.

Genetic determinations. For genetic determinations, venous blood was collected in EDTA vacutainers. Genomic DNA was isolated from peripheral leukocytes using a DNA isolation kit (Quick-DNA-Miniprep Kit-Zymo Research Corporation, Freiburg, Germany).

Identification of M235T-AGT genotypes. The patients' *M235T*-AGT genotypes were identify using the following specific primers: forward primer, 5'CAGGGTGCTGTCCACACTGGACCCC-3' and reverse primer, 5'-CCGTTTGTGCAGGGCCTGGCTCTCT-3'. Amplification was performed in an iCycler (Bio-Rad Life Science, Hercules, CA, USA) in 25 μl reaction mixture and comprised 20 ng DNA, 0.2 μM primers, 2.0 mM $MgCl_2$, 200 μM dNTPs, 2U *Taq*

Table I. *Comparison of patient characteristics between essential hypertension subjects with and without carotid atheromatosis.*

Carotid atheromatosis	Yes (73)	No (89)	*p*-Value
Age (years), mean (SD)	64.4 (9.08)	52.67 (12.44)	<0.001
Age ≥60 years (yes), n (%)	55 (75.34)	33 (37.08)	<0.001
Gender (Female), n (%)	33 (45.21)	50 (56.18)	0.164
Gender (Male), n (%)	40 (54.79)	39 (43.82)	
BMI (kg/cm^2), mean (SD)	29.43 (4.25)	29.54 (4.21)	0.87
Weight status			0.704
Normal weight	9 (12.33)	13 (14.61)	
Obesity	34 (46.58)	45 (50.56)	
Overweight	30 (41.1)	31 (34.83)	
LDL cholesterol (mg/dl), mean (SD)	125.82 (33.71)	127.85 (36.72)	0.717
LDL cholesterol >100 mg/dl, n (%)	50 (68.49)	56 (62.92)	0.458
Total cholesterol (mg/dl), mean (SD)	199.62 (46.99)	204.97 (40.58)	0.438
Total cholesterol >200 mg/dl, n (%)	39 (53.42)	51 (57.3)	0.621
Triglycerides (mg/dl), mean (SD)	158.14 (77.75)	130.83 (71.25)	0.021
Triglycerides >150 mg/dl, n (%)	34 (46.58)	28 (31.46)	0.049
cIMT, n (%)	45 (61.64)	7 (7.87)	<0.001
Left ventricular hypertrophy, n (%)	55 (75.34)	46 (51.69)	0.002
Hepatic steatosis, n (%)	58 (79.45)	56 (62.92)	0.022
Glucose basal level, median (IQR)	102 (92-113)	96 (90-105)	0.033
Diabetes mellitus, n (%)	14 (19.18)	9 (10.11)	0.1

BMI: Body mass index- weight (kg)/height (cm)^2; LDL-cholesterol: low-density lipoprotein cholesterol; cIMT: increased intima-media thickness; SD: standard deviation; IQR: interquartile range.

polymerase in specific buffer, 10X *Taq* buffer with $(NH_4)_2SO_4$ [750 mM tris-HCl (pH 8.8 at 25˚C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20]. The amplification program included an initial denaturation stage of 10 min at 95˚C, followed by 34 amplification cycles with a denaturation stage of 10 s at 94˚C, a primer hybridization stage of 20 s at 64˚C, an elongation stage of 20 s at 72˚C, followed by a final elongation stage of 5 min at 72˚C. The amplified fragment of 165 bp was subjected to enzymatic digestion in 10 µl mixture with 5U of the specific restriction enzyme *Tth111I,* for 3 h at 65˚C. The normal *M235* allele is not digested while the mutated *T235* allele forms two fragments of 141 and 24 bp (1).

Identification of I/D-ACE genotypes. To identify patients' *I/D*-ACE genotypes, the forward 5'-CTGGAGACCACTCCCATCCTTTCT-3' and reverse 5'-GATGTGGCCATCACATTCGTCAGC-3' primers were used. Amplification was performed in an iCycler (Bio-Rad) in 25 µl reaction mixture, and comprised 20 ng DNA, 0.2 µM primers, 2.0 mM $MgCl_2$, 200 µM dNTPs, 2U *Taq polymerase* in specific buffer, 10X *Taq* buffer with $(NH_4)_2SO_4$ [750 mM tris-HCl (pH 8.8 at 25˚C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20]. The amplification program included an initial denaturation stage of 5 min at 95˚C, followed by 34 amplification cycles with a denaturation stage of 10 s at 94˚C, a primer hybridization stage of 20 s at 69˚C, an elongation stage of 20 s at 72˚C, followed by a final elongation stage of 5 min at 72˚C.The *I*-ACE allele generates a 390 bp fragment, while the *D*-ACE allele generates a 290 bp fragment (1).

The amplified and enzymatic digestion fragments were visualized by UV light through migration in agarose gels stained with ethidium bromide 10 mg/ml.

All PCR-RFLP reagents were from Fermentas (Thermo Fischer Scientific Inc., Waltham, MA, USA) and Jena Bioscience (Analytik Jena AG, Jena, Thuringia, Germany), except for primers, which were from Eurogentec (Kaneka Eurogentec S.A., Liege Science Park, Seraing, Liege, Belgium).

Bias. We took special care to use objective methods to assess the two genetic polymorphisms, outcome (CA), and confounders, as explained above. We excluded important confounding pathologies, and we used multiple logistic regressions to control for known confounders.

Statistical analysis. Categorical variables were described by counts and percentages. We used means and standard deviations or medians and interquartile ranges for continuous data (for normal and skewed data). The Hardy-Weinberg equilibrium was assessed using an exact test. The associations between single nucleotide polymorphisms (SNP) and CA were assessed using the codominant, dominant, recessive, overdominant, and log-additive models, showing the odds ratio, *p*-value and the Akaike information criterion of the model. Next, we built several logistic regression models to explore the relationship between polymorphisms and CA. We built four models, two for each polymorphism (one for codominant and one for dominant models). Each model included the polymorphisms that were adjusted for age, gender, HS, LDL-C. The adjusted variables were chosen based on literature search and on the results found in univariate analysis. For all the models, we checked the presence of multicollinearity, misspecifications (Stukel test for interaction and Osius-Rojek test), and their goodness of fit (using Hosmer-Lemeshow test). For continuous variables, we checked the linearity with the log-odds (using a general additive model with smoothing). The results of the regression are expressed as odds ratios with 95% confidence intervals. For all statistical tests, we showed the two-tailed *p*-value with a 0.05 level of significance.

All statistical analyses were performed in R environment for statistical computing and graphics (R Foundation for Statistical Computing, Vienna, Austria), version 3.6.2. For genetic associations, we used the package SNPassociation 1.9-2.

Results

Our study included 162 patients with EHT with a mean age of 58.01 (SD=12.43) years, range 23-86 years. Of the 162 patients diagnosed with EHT, 73 had CA. Of these, 40 (54.79%) were men and 33 (45.21%) were women. The age of hypertensive patients with CA was significantly older compared to that of hypertensive patients without CA ($p<0.001$). The results obtained showed a statistically significantly higher number of hypertensive patients with CA aged over 60 years compared to those without CA ($p<0.001$).

Table II. *M235T-AGT model associations with carotid atheromatosis in essential hypertensive subjects.*

Model	With CA	%	Without CA	%	OR	95% CI lower	95% CI upper	*p*-Value	AIC
Codominant									
MM235	15	20.5	32	36.0	1.00			0.09134	224.2
MT235	41	56.2	39	43.8	0.45	0.21	0.95		
TT235	17	23.3	18	20.2	0.50	0.20	1.22		
Dominant									
MM235	15	20.5	32	36.0	1.00			0.02988	222.3
MT235-TT235	58	79.5	57	64.0	0.46	0.23	0.94		
Recessive									
MM235-MT235	56	76.7	71	79.8	1.00			0.63787	226.8
TT	17	23.3	18	20.2	0.84	0.39	1.77		
Overdominant									
MM235-TT235	32	43.8	50	56.2	1.00			0.11747	224.5
MT235	41	56.2	39	43.8	0.61	0.33	1.14		
Log-additive									
0,1,2	73	45.1	89	54.9	0.69	0.44	1.07	0.09751	224.3

CA: Carotid atheromatosis; OR: odds ratio; CI: confidence interval; AIC: Akaike information criterion.

No significant differences were found regarding gender (p=0.164) and weight status (BMI) (p=0.87) between hypertensive patients with and without CA. Regarding the lipid profile, there were no statistically significant differences in plasma LDL-C (p=0.717) and TC (p=0.438) levels between the two groups of hypertensive patients, with and without CA. In contrast, a statistically significant association between the presence of CA and HTG was observed (p=0.021). Also, basal glycemia was evaluated, the difference being statistically significant between the group of hypertensive patients with CA compared to those without CA (p=0.033).

Hypertensive patients with CA had TG >130 mg/dl (p=0.049), an increase in cIMT >1 mm in the carotid axis (p<0.001), and LVH (p=0.002) in a significantly higher proportion than hypertensive patients without CA.

The characteristics of hypertensive patients with and without CA are shown in Table I.

Distribution of RAAS genotypes in the study groups. The distribution of *M235T*-AGT genotypes in the group of hypertensive patients with CA compared to the group of hypertensive patients without CA was as follows: *MT235* (56.2% *vs*. 43.8%), *TT235* (23.35 *vs*. 20.2%), *MM235* (20.5% *vs*. 36%). The *M235T*-AGT genetic variant was statistically significantly associated with CA in the dominant model (p<0.029), but not in the other models (codominant, recessive, log-additive).

Regarding the *I/D*-ACE gene variant, the distribution of genotypes in the group of hypertensive patients with CA compared to those without CA was as follows: I/D (20.5% *vs*. 29.2%), D/D (37% *vs*. 34.8%), I/I (42.5% *vs*. 36), but the differences were not statistically significant in the codominant model.

Genotype distribution and the relationship of *M235T*-AGT and *I/D*-ACE with CA in hypertensive patients are presented in Tables II and III.

We found deviations from Hardy-Weinberg equilibrium for the *I/D*-ACE polymorphism, but not for *M235T*-AGT (Table IV).

For the *M235T*-AGT polymorphism and for the I/D-ACE gene variant, the codominant and dominant models in relation to CA were examined.

With regard to *M235T*-AGT polymorphism, in the dominant model, the chance for a patient to have CA was 2.17 times (95%CI=1.08-4.53) higher for the *MT235-TT235* genotype compared to *MM*235, the association being statistically significant (p=0.0298). The association was maintained even after adjustment for confounders (age, sex, HS, and LDL-C), with 2.3 times (95%CI=1.05-5.26) higher chance of having CA (p=0.041) for patients carrying *MT235-TT235* genotypes. In the codominant model, the chance for a patient to have CA was 2.24 times (95%CI=1.07-4.86) higher in the case of patients carrying the *MT235* genotype compared to carriers of the *MM*235 genotype, the association being statistically significant (p=0.036), and 2.01 times higher for carriers of the *TT235* genotype compared to patients carrying the *MM235* genotype, but the association was not statistically significant. Following adjustment for confounders, the chance for a patient to have CA was 2.32 times higher in the case of carriers of the *MT235* genotype compared to carriers of the *MM235* genotype, but the association was not statistically significant (p=0.052).

Table III. *I/D-ACE model associations with carotid atheromatosis in essential hypertensive subjects.*

Model	With CA	%	Without CA	%	OR	95% CI lower	95% CI upper	*p*-Value	AIC
Codominant									
I/I	31	42.5	32	36.0	1.00			0.4281	227.3
I/D	15	20.5	26	29.2	1.68	0.75	3.76		
D/D	27	37.0	31	34.8	1.11	0.54	2.27		
Dominant									
I/I	31	42.5	32	36.0	1.00			0.3979	226.3
I/D-D/D	42	57.5	57	64.0	1.31	0.70	2.48		
Recessive									
I/I-I/D	46	63.0	58	65.2	1.00			0.7760	226.9
D/D	27	37.0	31	34.8	0.91	0.48	1.74		
Overdominant									
I/I-D/D	58	79.5	63	70.8	1.00			0.2043	225.4
I/D	15	20.5	26	29.2	1.60	0.77	3.31		
Log-additive									
0,1,2	73	45.1	89	54.9	1.06	0.74	1.52	0.7494	226.9

CA: Carotid atherosclerosis; OR: odds ratio; CI: confidence interval; AIC: Akaike information criterion.

For the *I/D*-ACE gene variant, no statistically significant association with CA was observed without adjustment for confounding variables nor after adjustment. The chance of having CA was 0.6 times higher in carriers of the *I/D* genotype compared to the *I/I* genotype without adjustment, and 0.48 times higher after adjustment for confounders. The chance to have CA was 0.9 times higher in carriers of the *D/D* genotype compared to the *I/I* genotype without adjustment, and 0.7 times higher after adjustment for confounders. The chance to have CA was 0.76 times higher in carriers of the *D/D* or *I/D* genotype compared to carriers of the *I/I* genotype without adjustment, and 0.61 times higher after adjustment for confounders.

The results of univariate and multivariate regression analysis are presented in Table V.

Table IV. *Hardy-Weinberg equilibrium exact test p-values for M235T-AGT and I/D-ACE polymorphisms in essential hypertension subjects.*

Single nucleotide polymorphism	With CA	Without CA
M235T-AGT	0.3541	0.3845
I/D-ACE	<0.0001	0.0001

CA: Carotid atheromatosis.

Discussion

Genetic and environmental factors (race, ethnicity, geographical region) influence the development and progression of ATS (27, 28). Some studies confirm the relationship of the *I/D*-ACE gene polymorphism with IHD and EHT, while others refute this association (29-35). Therefore, there are few and contradictory data regarding the relationship between CA and the gene variations involving RAAS.

Therefore, the aim of our study was to determine the relationship between the *M235T*-AGT, *I/D*-ACE gene variants, and EHT as predictors of CA.

It has been established that *M235T*-AGT and *I/D*-ACE gene variants play a role in the etiology of IHD and the development of MI in Caucasian and Japanese subjects (24, 36, 37). Ethnicity, geographical area, and environmental factors influence this association (21).

Hazzani *et al*. (2014) have demonstrated that the *TT235* genotype increases susceptibility to coronary disease in the Egyptian population, independently of other risk factors such as smoking, DM, lipid profile (38). Min *et al*. (2019) have mentioned that a Chinese population carrying the *M235T*-AGT genetic variant was susceptible to MI (39). Jeng *et al*. (1999), in a study on a population of Chinese origin, have shown that in patients with EHT, the *TT235* genotype might be considered a risk factor for the development of LVH, but not CA (40).

The mechanism of association between *M235T*-AGT and *I/D*-ACE genetic variants with EHT and CA is not clear. Bonfim-Silva *et al*. (2016) have shown that a Brazilian population of Caucasian origin had an increased risk for EHT in the case of carriers of the *TT235* genotype, possibly due to the high plasma AGT concentration in association with this genotype (21). However, Pontremoli *et al*. (2000) have shown that carriers of the *M235T*-AGT genetic variation had increased SBD and DBD values and presented

Table V. *Univariate and multivariate logistic regressions predicting carotid atheromatosis in association with M235T-AGT and I/D-ACE genetic variations.*

Parameters	OR unadjusted	(95%CI)	*p*-Value	OR adjusted*	(95%CI)	*p*-Value
M235T-AGT (*MT235*heterozygous *vs*. *MM235* negative)	2.24	(1.07-4.86)	0.036	2.32	(1.01-5.52)	0.052
M235T-AGT (*TT235*homozygous *vs*. *MM235* negative)	2.01	(0.82-5.04)	0.128	2.27	(0.81-6.54)	0.121
I/D-ACE (*I/D*heterozygous *vs*. *I/I* negative)	0.6	(0.26-1.32)	0.207	0.48	(0.18-1.22)	0.13
I/D-ACE (*D/D*homozygous *vs*. *I/I* negative)	0.9	(0.44-1.84)	0.77	0.7	(0.31-1.59)	0.402
M235T-AGT-dominant (*TT*, *MT vs*. *MM*)	2.17	(1.08-4.53)	0.033	2.3	(1.05-5.26)	0.041
I/D-ACE-dominant (*D/D*, *I/D vs*. *I/I*)	0.76	(0.4-1.44)	0.398	0.61	(0.29-1.25)	0.181

BMI: Body mass index; AGT: angiotensinogen; LDL-C: low-density lipoprotein cholesterol; OR: odds ratio; CI: confidence interval; *adjusted for age, gender, hepatic steatosis.

CA (41). Chapman *et al*. (2001), in a study conducted in a population of Chinese origin, determined the risk of developing CA and found an increase in cIMT in the case of patients carrying the *M235T*-AGT genetic variant (42). Furthermore, studies conducted by Min *et al*. (2019) and Van Rijn *et al*. (2007) have established that the *M235T*-AGT gene polymorphism plays an independent role in the pathophysiology and evolution of CA, through the increase in cIMT and progression of ATS, even if EHT is controlled by treatment (39, 43). The study conducted by Kretowski *et al*. (2007) has demonstrated that type 1 DM associated with *TT235* genotype could lead to CA progression (44).

With regard to the *I/D*-ACE genetic variant it has been reported that it is involved in the development and progression of IHD, LVH, CA and cerebrovascular disease (36, 41, 45). In addition, in a study carried out by Zhang *et al*. (2004), the *D/D*-ACE genotype was considered an independent risk factor and predisposes to cerebral microatheromatous disease, as well as to lacunar stroke (46). Forgo *et al*. (2018), in a meta-analysis including 9800 Asian and Caucasian hypertensive patients diagnosed with cerebrovascular ischemic disease, type 1 and 2 DM, have demonstrated that the *I/D*-ACE genotype was associated with an increase in cIMT >1 mm (5). A comparative study in a European and Asian population conducted by Xia *et al*. (2019) ihas shown that the *D* allele was associated with an increased risk of ATS in European compared to Asian subjects (28). In a study performed by Islam *et al*. in Finland in 2006, which included healthy subjects, no association of the presence of *I/D*-ACE and *M235T*-AGT polymorphisms with the predisposition to EHT or CA was demonstrated (47).

Our prospective cross-sectional study showed a statistically significant association between *M235T*-AGT and CA in the dominant model. The odds of having CA were 2.17 higher for the *MT-TT* genotype than for the *MM* genotype of the *M235T*-AGT polymorphism in hypertensive patients, even after adjustment - OR=2.3 (95%CI=1.05-5.26) - for confounders (age, gender, HS, LDL-C). Univariate analysis showed that the MT235-TT235 genotypes represent risk factors for CA in the case of hypertensive patients. Multivariate analysis implies that the *MT235-TT235* genotype of the *M235T*-AGT polymorphism is an independent risk factor for CA in hypertensive patients.

We found no statistically significant association between the *I/D*-ACE genetic variation and CA in hypertensive patients, either in univariate or in multivariate analyses.

A number of studies have evaluated other risk factors for CA independently of the patient's genetic profile. The study conducted by Arnett *et al*. (1998) in hypertensive middle-aged patients without a history of CA, non-carriers of *D*-ACE or *T235*-AGT alleles reported CA progression in these patients (48).

The study carried out by Min *et al*. (2019) has confirmed the association of CA with metabolic syndrome and HS (39). Also, the study performed by Targher *et al*. (2006) has confirmed that CA reflects generalized ATS and ischemic vascular disease and is influenced by age, sex, the geographical area, smoking, EHT, type 2 DM, high LDL-C (49). Moreover, it has been shown that pathophysiologically, the process of aging, hypercholesterolemia, EHT, DM are responsible for the development of CA and increase in cIMT (50-52). Forgo *et al*. (2018) have demonstrated that risk factors associated with EHT influence the location of the carotid ATS plaque, DM, and smoking cause ATS in the carotid bulb and high LDL-C induces ATS in the ICA (5). Our study observed an association between CA and age ($p<0.001$), HTG ($p=0.049$) and NAHS ($p=0.022$).

The limitations of our study refer to the fact that our study sample consisted of patients with EHT from regional tertiary centers. Thus the results are generalizable to this type of

population. We had a limited number of exclusion criteria, and this is an argument in favor of generalization.

The strength of our study is the fact that we had a good sample size, having enough power to show the association between *M235T*-AGT and ATS. The association was present in our sample that used exclusion criteria to limit the influence of important confounders. Furthermore, the observed relationship held even after adjustment for potential confounders.

In the future, we aim to include a greater number of patients in the study and to extend investigations by analyzing more genetic variants associated with RAAS as risk factors for CA in hypertensive patients.

Conclusion

Our results showed an association between *M235T*-AGT and CA, after adjustment for several confounders and controlling for HT, the study being conducted only in hypertensive subjects. The presence of similar results in other studies is a good argument in favor of this association. Nevertheless, ATS is a complex process, and the importance of this finding and its relationships with other characteristics associated with it represent an open field for different studies.

Conflicts of Interest

There are no known conflicts of interest regarding this study.

Authors' Contributions

OM: Conception of the study, performed collection of subjects, clinical work and ultrasound procedures, preparation of the database, drafted the manuscript; DR, EB: conception of the study, performed collection of subjects, clinical work and ultrasound procedures; AC: performed collection of subjects, clinical work and ultrasound procedures; DCL: performed the statistical analysis, contribution to manuscript preparation; LMP: conception and coordination of the study, performed genotyping of samples and laboratory assays, main supervisor, critical revision and final approval of the article. All Authors approved the final version of the manuscript.

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