Angiotensin-converting enzyme gene D-allele and the severity of coronary artery disease

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Abstract. Coronary artery disease (CAD) is the first cause of morbidity and mortality worldwide. An important goal is to diagnose patients in early stages, in order to reduce acute cardiovascular events. The angiotensin-converting enzyme (ACE) is an important element for the cardiovascular system, through its actions on hydro-salin balance and vascular tone. ACE polymorphism consists of insertions (I)/deletions (D) and there are 3 genotypes: II, ID, DD. It is speculated that the DD genotype may be a genetic basis for severe CAD, while the II genotype may have a protective effect on the coronary arteries. The present study included 154 patients with acute coronary syndroms admitted to the Institute for Cardiovascular Disease 'George I.M. Georgescu', Iasi. The patients underwent coronary angiography in order to assess the severity of the lesions and the ACE genotypes were determined for each patient. The genotypes were correlated with the severity of the vessel-disease and the exposure to classic risk factors. It was concluded that the D-allele is associated with a greater risk for acute coronary events and severe coronary stenosis, especially when risk genotype and risk phenotype interact.

Introduction

Coronary artery disease (CAD) is the first cause of morbidity and mortality all around the world. During the last 30 years, important progress was made in order to reduce the morbi-mortality of atherosclerotic disease. Correction of cardiovascular risk factors and improving treatments for acute coronary syndromes have represented the first line of study. An important goal is to diagnose patients in early stages, in order to reduce acute cardiovascular events. Latest discoveries revealed that mutations in the angiotensin-converting enzyme (ACE) gene may influence the onset and the severity of CAD (1). Therefore, studying the association between genotype and phenotype would be an important cornerstone in cardiology.

The angiotensin-converting enzyme is common to RAAS (renin-angiotensin-aldosterone system) and the kinin-kalicrein system. ACE is a zinc metalloproteinase that cleaves the terminal dipeptide (His-Leu) of Ang I, turning it into Ang II, a highly constricting substance. Due to interference on the two systems, ACE also inactivates bradykinin, a vasodilating substance. Through its functions, ACE maintains the hydro-salin balance and vascular tone (2,3).

The ACE gene is found on the long arm of chromosome 17, at heading 23 (17q23), measures 21 kb and includes 26 exons and 25 introns (4).

ACE polymorphism consists of insertions (I)/deletions (D) at the level of intron 16. Thus, from the polymorphism of intron 16, there are 3 genotypes: Insertional homozygote (II), heterozygote (ID) and teletional homozygote (DD). Serum levels of ACE are determined by genetic polymorphism in the following order: DD>ID>II.

The central goal of our study was to investigate the correlation between the presence of the D-allele and severe stenosis or occlusion of one or several coronary arteries.

Patients and methods

The present study included 154 patients with acute coronary syndroms (acute myocardial infarction and unstable angina) admitted to the Institute for Cardiovascular Disease 'George I.M. Georgescu', Iasi. The study was approved by the Ethics Committee of the 'Grigore T. Popa' University of Medicine and Pharmacy (Iasi, Romania) on 09.06.2015, according to the law of medical research, no. 206 from 27.05.2004. Informed consent was obtained from all patients included in

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the study. The inclusion criteria were the following, according to the European Guidelines (5): i) diagnosed coronary artery disease; ii) exposure to minimum two cardiovascular risk factors: dyslipidemia (total cholesterol >200 mg/dl, LDL-cholesterol >100 mg/dl, HDL-cholesterol <40 mg/dl for males and <50 mg/dl for females, triglycerides >150 mg/dl or normal lipid profile under cholesterol lowering medication), high blood pressure (>140/90 mmHg or normal blood pressure under medication), diabetes mellitus, family history of cardiovascular disease at young age (<50 year-old for male relatives or at climax for female relatives), smoking, overweight (BMI between 25-29.9) or obesity (BMI >30).

Anamnesis helped us extract the following information: symptoms, family history (CAD, stroke, lower extremity artery disease and other consequences of severe atherosclerosis), personal cardiovascular history, work exposure to toxic substances and smoking habit.

Through clinical examination (for the BMI calculation) weight and height, blood pressure, heart rate and cardiac ascultation were recorded.

Basic haematological and biochemical blood analysis included: Red blood cell count, haemoglobin, mean haemoglobin concentration, mean erythrocyte volume, white blood cell count and leucocitary formula, platelet count, mean platelet volume, inflamatory markers [erythrocyte sedimentation rate (ESR); C reactive protein (CRP); fibrinogen (Fg)], glycemia, glicated haemoglobin, lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides), renal function (urea, creatinine), liver function (AST, ALT), cardiac necrosis enzymes (CK-MB, LDH, TGO).

An electrocardiogram was recorded for each patient (for rythm, ST segment and T wave analysis) and echocardiography (for ejection fraction, left ventricle contractility, cardiac dimension and valve analysis) was performed.

All patients underwent coronary angiography in order to adequately define the coronary status and those who met the criteria from the revascularisation guidelines, were implanted percutaneous stents in order to restore myocardial perfusion.

Apart from the blood for standard analysis, 2 extra mililiters of blood for genetic analysis was also collected for the determination of angiotensin-converting enzyme gene polymorphisms, deletions or insertions of genes.

Polymerase chain reaction (PCR) was used for the amplification of genetic material. The method is based on the DNA-polymerase ability to synthesize new strands of complementary DNA, starting from a base chain. DNA-polymerase can only add nuleotide to a pre-existing 3'-OH group, so it takes a primer to add the first nucleotide. In the end, the amplified sequence will be found in billions of copies. The process has several stages: DNA distortion, bonding of primers, extension of primers and consists of several cycles.

To determine the genetic polymorphism of the ACE gene, the MutaGel ACE kit was used. It determines mutations such as insertions (I)/deletions (D) based on analysis of the ALU sequence at intron 16. A kit allows 24 determinations. The MutaGel ACE kit contains specific primers for identifying both mutations: I-allele (fragment 490 bp) and D-allele (190 bp fragment), master mixture (Master Mix) for chain polymerization (Taq enzyme, $MgCl_2$, dNTP, buffer solution), positive control for genotypes containing I-allele, positive control for genotypes containing D-allele, negative controls and high purity solution for PCR. In addition to this kit KBR3005 DNA extraction kit and gel electrophoresis reagents were also used. In the first stage, the purity and concentration of the DNA was checked. Then the following amplification program was used: initial hold step (initial distortion) at 95 degrees for 5 min, followed by 30 exponential amplification cycles, each cycle having 3 temperature variations (distortion at 94 degrees for 60 sec, hybridization at 57 degrees for 60 sec and elongation at 72 degrees for 90 sec) and final hold step 72 degrees 5 min and then 4 degrees. Next the amplified material was separated by electrophoresis in the 1.5% agarose gel, coloring SYBR Green fluorochrome fragments and analyzing them using UV spectrophotometry with 312 nm wavelengths. Finally, the identification of the fragments was made by comparing them with the DNA ladder.

Statistical analysis was performed using SPSS 18.0. Primary processing, i.e. data systematization through centralization and grouping, led to the obtaining of primary indicators, which are presented in the form of absolute sizes. On the basis of the primary indicators, different statistical processes of comparison, abstraction and generalization were obtained of the derivative indicators. Derivative indicators are intended to highlight the qualitative aspects of an ensemble, targeting the relationship between different parts of a group of patients or different characteristics, interdependence links between variables. The following derivative indicators were used, described by the ANOVA test: Indicators of the mean value (simple arithmetic mean, median, module, minimum and maximum values) and indicators of dispersion (standard deviation, coefficient of variation). The following statistical tests and correlation methods were also used: i) Chi-square test, qualitative non-parametric test, compares frequency distributions. ii) Student's t-test, parametric test that compares the average values recorded in 2 groups with normal distributions. iii) The F test (ANOVA) used when comparing 3 or more groups with normal distributions. iv) The Kruskall-Wallis correlation compares ordinal variables in 3 or more groups. v) Correlation coefficient 'Pearson' (r) is the correlation of 2 variables in the same group, the direct/indirect correlation given by the coefficient sign.

Results

The patients were divided into four groups, according to the severity of the CAD, as revealed by the coronary angiography: 0C (non-significant stenosis, considered control group); 1C (one-vessel disese); 2C (two-vessel disease); and 3C (three-vessel disease).

The sex distribution revealed that patients were predominantly males (74%), sex ratio M/F=2.85/1, regardless of the coronary status (Chi-square=1.688; df=3; P=0.640) (Fig. 1).

Age ranged between 35 and 85, the group mean was 64.5±10,85 years, slightly elevated for the one-vessel disease patients (65.44 years; P=0.931) (Table I).

Women with altered coronary status were older than those in the control group (P>0.05) (Fig. 2).

In conjunction with the average age of the study group (~65 years), the age group distribution shows a 62.5% share of patients in group 1C over 65 years of age, while in group 3C the

Table I. Age descriptive indicator (years) comparison between groups.

Group	N	Mean	Standard deviation	Standard error	Confidence interval 95% -95% CI	Confidence interval 95% +95% CI	Min	Max	Test F (ANOVA) P-value
0C	24	64.13	14.21	2.90	58.12	70.13	36	78	0.931
1C	48	65.44	9.53	1.38	62.67	68.20	35	85	
2C	48	64.21	12.29	1.77	60.64	67.78	34	80	
3C	34	64.15	7.70	1.32	61.46	66.83	44	76	
Total	154	64.56	10.85	0.87	62.84	66.29	34	85	

0C, non-significant stenosis, considered control group; 1C, one-vessel disese; 2C, two-vessel disease; and 3C, three-vessel disease.

Table II. Group structure according to age.

	0C		1C		2C		3C	
Age group	n	%	n	%	n	%	n	%
<65 years	9	37.5	18	37.5	21	43.8	16	47.1
≥65 years	15	62.5	30	62.5	27	56.2	18	52.9

0C, non-significant stenosis, considered control group; 1C, one-vessel disese; 2C, two-vessel disease; and 3C, three-vessel disease.

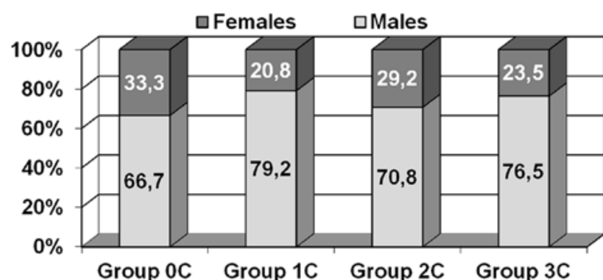

Figure 1. Sex distribution within groups (0C, non-significant stenosis, considered control group; 1C, one-vessel disese; 2C, two-vessel disease; and 3C, three-vessel disease).

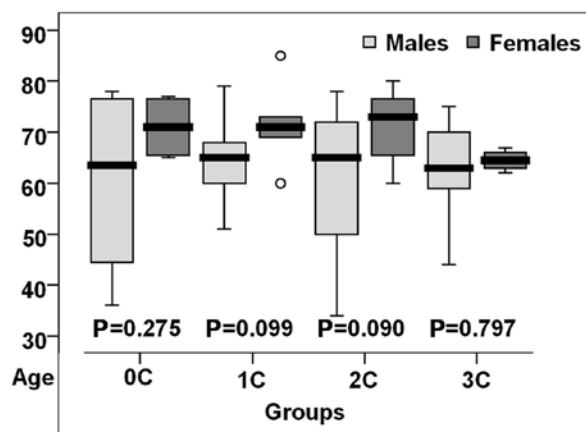

Figure 2. Comparison of mean age values between study groups (0C, non-significant stenosis, considered control group; 1C, one-vessel disese; 2C, two-vessel disease; and 3C, three-vessel disease).

frequency of patients under 65 years of age was 47.1%. However, these frequency distributions showed no statistically significant differences, suggesting the homogeneity of the study group by age group (Chi-square=1.007; df=3; P=0.800) (Table II).

The identification of ACE genotypes was carried out according to the length of the amplicons after migration into the gel. The 190 bp amplicon signals the presence of allele D and the 480 bp amplicon signals the presence of allele I. Positive control is heterozygous. The three possible genotype variants: II, ID and DD were identified (Fig. 3). In the OC group 21 patients with genotype II, 3 patients with genotype ID and no patients with genotype DD were identified. In the 1C group 8 patients with genotype II, 40 patients with genotype ID and no patients with genotype DD were identified. In the 2C group 4 patients with genotype II, 36 patients with genotype ID and 8 patients with genotype DD were identified. In the 3C group no patients with genotype II, 6 patients with genotype ID and 28 patients with genotype DD were identified.

The exposure to cardiovascular risk factors of DD patients with severe coronary lesions: in the 3C group 82.4% of patients had the genotype DD, 82.1% were male, 57.1% were >65 years of age, all with dyslipidemia, 92.9% diabetics and 57.1% were obese, half were smokers (Table III).

Discussion

Coronary artery disease is a polygenic pathology. Its onset and severity depend on the interaction between genetic and environmental factors. The DD, ID and II genotypes are associated with high, intermediate and low levels of ACE. Our study shows that the three genotypes influence the severity of CAD, interacting with conventional risk factors. The relationship

Table III. Genotype-phenotype correlations for each study group.

	Group 0C (n=24)				Group 1C (n=48)			
Risk factor	II (n=21)	ID (n=3)	DD (n=0)	P-value	II (n=8)	ID (n=40)	DD (n=0)	P-value
Male	76.2	-	-	0.006	75.0	80.0	-	0.755
>65 years	57.1	100.0	-	0.080	50.0	65.0	-	0.430
Diabetes	19.0	-	-	0.278	100.0	55.0	-	0.004
Dyslipidemia	81.0	100.0	-	0.278	100.0	80.0	-	0.073
Obesity	71.4	100.0	-	0.028	100.0	50.0	-	0.001
Smoking	23.8	-	-	0.219	37.5	55.0	-	0.364
	Group 2C (n=48)				Group 3C (n=34)			
Risk factor	II (n=4)	ID (n=36)	DD (n=8)	P-value	II (n=0)	ID (n=6)	DD (n=28)	P-value
Male	100.0	61.1	100.0	0.007	-	50.0	82.1	0.113
>65 years	100.0	41.7	100.0	0.001	-	33.3	57.1	0.287
Diabetes	-	44.4	100.0	0.001	-	100.0	92.9	0.370
Dyslipidemia	100.0	88.9	100.0	0.298	-	100.0	100.0	1.000
Obesity	-	61.1	100.0	0.001	-	100.0	57.1	0.012
Smoking	100.0	47.2	25.0	0.022	-	-	50.0	0.007

0C, non-significant stenosis. considered control group; 1C, one-vessel disease; 2C, two-vessel disease; and 3C, three-vessel disease.

Figure 3. ACE amplicon migration in gel electrophoresis: M (DNA ladder), NC (negative control), PC (positive control), II (480 bp), ID (480/190 bp) and DD (190 bp). ACE, angiotensin-converting enzyme.

between CAD and genetic polymorphism (genotype DD) was first discussed by Cambien *et al* (6). Since then, the topic has long been debated, the results are still unclear, with some positive studies (7), and others negative (8,9). Polymorphism I/D is responsible for 20-50% of the differences in plasma level of ACE, which assumes that 50-80% come from the influence of environmental factors or from the interaction between these polymorphisms and environmental factors (6). Our results in this direction are indisputable, since we demonstrated a growing precentage of the genotype DD proportional with the growing severity of the coronary disease, from absent genotype DD in the no-vessel disease, to 82.5% DD carriers among the three-vessel disease patients. Also, II genotype, which was predominant in the control group, was absent among the 3C patients, who were all D-allele carriers (17.64% genotype ID and 82.35% genotype DD). This was similar to the results of Nakai *et al* (10), Vargas-Alarcón *et al* (11) Jamil *et al* (12) and Acarturk *et al* (13), who demonstrated an association between CAD and ACE gene I/D polymorphism. By contrast, Ragia *et al* (14), Qiu *et al* (15), and Ramakrishnan *et al* (16) found no association between those two parameters.

Traditional risk factors such as smoking, high blood pressure, diabetes mellitus, family history of cardiovascular disease at a young age, obesity influence RAAS by stimulating ACE synthesis, leading to excessive vasoconstriction, enhancing ischemic risk. Freitas *et al* (17) concluded that genotype DD induces onset and severity of coronary lesions only when exposure to common risk factors is present, like hypertension and diabetes. Also, the case control study of Niemec *et al* (18) demonstrated correlations between ACE gene polymorphisms and CAD in the presence of hypercholesterolemia. The present study revealed that three-vessel disease genotype DD patients were diabetic and dyslipidemic and half of them were obese and heavy smokers.

The present study has some limitations. First, the sample size of included patients is small, that may reduce the statistical power. Second, this is a single-center clinical study, which might cause some selection bias.

In conclusion, by analysing all the study groups, significant associations were obtained between the D-allele and male sex, age over 65 years, smoking, diabetes mellitus, obesity, dyslipidemia, which illustrates the strong interaction between ACE polymorphism and environmental factors (genetic-epigenetic interaction) in initiation and worsening of coronary heart disease. Based on this study it is concluded that the D-allele is associated with a greater risk for acute coronary events and severe coronary stenosis, especially when risk genotype and risk phenotype interact.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MCV and IBB acquired the data. MCV, IBB, OVB and AB analyzed the data and drafted the manuscript. MB, MC and CAG designed the study and supervised data analysis. MCV, MCB and DI were involved in the design of the study and analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the 'Grigore T. Popa' University of Medicine and Pharmacy (Iasi, Romania) on 09.06.2015, according to the law of medical research, no. 206 from 27.05.2004. Informed consent was obtained from all patients included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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