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PhD THESIS RESUME

CORRELATIONS BETWEEN GENETIC AND EPIGENETIC FACTORS IN THE ACUTE CORONARY ARTERY DISEASE

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THE STUDY MOTIVATION

Coronary artery disease is a widely spread cardiac pathology, both in eastern and western countries. Despite the decrease of its frequency in the developped countries, this pathology is still responsible for one third of the death causes for patients over 35 year-old. In the United States of America, 15.5 million persons over 20 suffer from ischemic heart disease, according to the 2016 analysis of the American Heart Society. Moreover, its prevalence growths with age and it is estimated that every 42 seconds, an american citizen has a heart attack. The situation os equally concerning in Romania, as 60% of the cardiovascular death are caused by different forms of coronary heart disease. Almost 40 romanian adults die of acute myocardial infarction every day.

The Framingham study is the most complex study ever performed on cardiovascular risk factors, its results being the fundament for primary and secondary prevention strategies around the world.

Apart from the sympthomatic coronary events, some patients experience silent myocardial ischemia. Those patients display a high risk for sudden cardiac death. However, it seems that those situations are not completely unannounced, as those patients often have high risk coronary profiles and presymphomatic signs, which they usually neglect. Almost 2-4 % of the general populations suffers from silent myocardial ischemia, which although mainly asymptomatic, could be detected by stress tests and 24 hours or 7 days loop recorders. Those events mainly occur in males, with more than two cardiovascular risk factors.

Acute myocardial infarction remains the most severe form of acute coronary disease, because of its possible complications, like dilatative cardiomiopathy and heart failure, cosequences of the severe wall motion abnormalities and thus severely diminished ejection fraction.

RO-STEMI (Romanian Acute Myocardial Infarction with ST Elevation) is the most important romanian analysis on acute myocardial infarction. It included 15.076 patients from 19 interventional cardiology centers and 45 non-interventional centers and was initiated in 2011. For each patient, the authors recorded demographical, clinical and therapeutical (medical or interventional treatment) informations. Results were similar with the ones from other european studies. The most important predictors for in-hospital mortality were interventionally treated patients were advanced Killip class, reduced contractility, old age and anterior myocardial infarction. On the other side, percutaneous revascularization, beta-blockers, angiotensin converting enzyme inhibitors or angiotensin receptor blockers have been associated with a lower in-hospital mortality.

The experimental part of this thesis was performed under the European Frame Programme CHRONEX 2007-2013, contract number POSDRU/159/1.5/S/133377. This project phocused on scientifical investigations of chronical diseases, in the attempt to elaborate better prevention strategies and treatment algorithms.

There has been a complex trend of medical studied dedicated to the genetic background of patients, which generates different treatment responses and disease evolutions. The center idea of many studies from various regions of the world, that the D allele of the angiotenson converting enzyme polymorphism offers a predisposition to coronary artery disease with severe lesions of more than one coronary artery, was the starting point of our thesis. Because dyslipidemia is a major cardiovascular risk factor and most of our patients had lipid metabolism alterations, we extended our genetical exploration to mutations of the B100 apoproteine gene, a component of LDL-cholesterol. Our study is the first in out country dedicated to those mutations and the coronary disease. As active members of the scientifical community, it is very important to remain up-to-date with the hypothesis studied in other regions and to test them in our country as well.

STUDY PURPOSE

This thesis has the main purpose of a better understanding of how the I/D angiotensin converting enzyme gene polymorphism influences the development and severity of the acute coronary artery disease alone and by interacting with epigenetic risk factors.

The main objectives were:

1. Evaluation of classic epigenetic factors for the included patients
2. Determination of apoprotein B100 gene mutations on the 3500 codone (A/G – the replacement of adenine by guanine)
3. Determination of angiotensin converting enzyme (ACE) gene mutations (I/D, insertions and deletions)
4. Analysis of the interactions between risk factors and echocardiographical parameters (left ventricular hypertrophy, wall motion abnormalities) with the identified genotypes (II, ID and DD).

MATERIAL AND METHOD

Our study was designed on patients with acute coronary disease (unstable angina and acute myocardial infarction) admitted to the Institute for Cardiovascular Disease Prof. Dr. George I.M. Georgescu from Iași. All the patients underwent coronary angiography in order to evaluate the coronary status and intervational revascularisation was performed on all patients who had criteria according to the Guidelines of the European Society of Cardiology.

We only included patients with minimum two cardiovascular risk factors, defined according to the guidelines (Graham et al., 2007):

1. Dyslipidemia: total Cholesterol> 200mg/dl, LDL> 100mg/dl and/or HDL< 40 mg/dl for men and <50 mg/dl for women and triglycerides>150 mg/dl or patients with normal lipid profile values, under statin treatment

2. High Blood Pressure- over 140/90 mmHg identified in three different occasions or the necessity for treatment for high blood pressure
3. Diabetes mellitus- fasting glycemia over 126 mg/dl in three different occasions or the necessity for hypoglicemic drugs or insulin
4. Family history of cardiovascular disease in first degree relatives (under 50 or after climax)
5. Smoking habbit
6. Body mass index (BMI) weight (kg)/height $(m^2)^2$, with mean normal values between 18 and 24.9, overweight 25-29.9 and obesity over 30.

The patient interviews and clinical examinations provided the following informations: admission symptoms, family medical history, personal medical history, work environment, tobacco and alcohol ingestion.

We recorded the blood pressure, heart rate, weight, height, heart auscultation (presence/absence of heart murmurs).

We investigated the following laboratory analysis: red blood cells and white blood cells parameters, platelet number, inflammatory syndrome (sedimentation speed, C reactive protein, fibrinogen), renal function (urea, creatinine), liver function (TGP, TGO), glycemia, lipid profile (cholesterol, LDL, HDL, triglycerides). We used 2 ml of blood for the genetical determinations of apoprotein B100 mutations on the 3500 codone (A/G – the replacement of adenine by guanine) and the determination of angiotensin converting enzyme mutations (I/D, insertions and deletions.

We recorded the electrocardiogram (for the rythm analysis, ST segment and T wave evaluation) and echocardiography measurements (diameters, motility, ejection fraction, evaluation of heart valves).

The severity of the coronary disease was evaluated according to the evidence from coronary angiography (no/one/two/three vessel disease). Stenosis over 70% were considered as significant.

The study protocole was approved by the Ethical Committee of our university. All patients read, understood and signed an informed consent designed according to the Declaration of Helsinki II.

Our study had two parts. The first one consisted of 58 patients classified in two groups, according to the clinical form: unstable angina (UA) and acute myocardial infarction (AMI). We evaluated the mutations of the apoprotein B100 gene and the ACE gene in all the patients and analised tthe possible correlations with the epigenetic factors.

For the second part of the study we added 96 patients and because the results from the first part suggested that a distribution of the patients according to the severity of the coronary disease instead of the clinical form would be better for the interpretations, we distributed the patients from this part consequently. Therefore, the new grouos were 0C (no vessel disease), 1C (one vessel disease), 2C (two vessel-disease)|, 3C (three vessel disease). We analysed the same epigenetic factors as for the first part, but for the genetic evaluation we only determined ACE gene mutations, because those were found to be significant in the first part. As for the apoB100 mutations, due to the fact the we identified no mutation present for the first patients, we did not proceed with the tests in the second part.

We used the **MutaGel ACE** kit for the evaluation of ACE gene mutations, which determins insertions and deletions from the ALU sequence of intron 16. One kit allows 24 determinations. The kit contains specific primers for the I allele (fragment length 490 bp) and the D allele (fragment length 190 bp), the Master Mix for the polimerase chain reaction (PCR) (Taq enzyme, $MgCl_2$, dNTP, buffer solution), positive and negative controls and high purity solution for PCR. We also used DNA extraction kit KBR3005 and gel electrophoresis reatives. The first step was verifying the DNA purity and concentration. The PCR steps were: denaturing (heating at 94 degrees for 5 minutes), followed by annealing (30 cycles at different temperatures) and the extending stage (final hold 72 degrees for 5 minutes, then 4 degrees). In order to separate the DNA fragments by their length, we used gel electrophoresis (agarose gel 1.5%), we coloured the fragments with fluorocrome SybrGreen and analysed them by UV light (312 nm). In the end, we used the DNA ladder to identify the fragments.

For the apoB100 mutations, we used the **MutaGel ApoB100 kit,** especially designed for the determination of 3500 codon mutations. One kit allows 24 determinations and contains the PCR mix (Taq enzyme, MgCl2, dNTP, buffer solution and specific nucleotides for the 3500 codon region), the positive and the negative controls, buffer solution for the restriction enzyme, restriction enzyme (specific for the 3500-A mutation). The PCR steps were: denaturing (heating at 94 degrees for 5 minutes), followed by annealing (30 cycles at different temperatures) and the extending stage (final hold 72 degrees for 5 minutes, then 4 degrees). Afterwards, the fragments were separated by digestion, for 3 hours, followed by 3.5% agarosis gel electrophoresis. In the end, we coloured the fragments with fluorocrome SybrGreen and analysed them by UV light (312 nm).

The statistical analysis was performed with SPSS 18.0.

EVALUATION OF THE EPIGENETIC RISK FACTORS CONTRIBUTION – RESULTS FROM THE FIRST PART OF THE STUDY

The first part of the study consisted of 58 patients classified in two groups, according to the clinical form: unstable angina (UA) and acute myocardial infarction (AMI).

PERSONAL PATHOLOGICAL HISTORY – THE PRESENCE OF CORONARY RISK FACTORS

Diabetus mellitus 60% of the patients with acute myocardial infarction and 60.7% of the patients with unstable angina had **diabetus mellitus,** but the differences were not statistically significant (p=0,956).

Dyslipidemia had a high incidence in both groups, with almost equal percentages (90% *vs* 89,3%; p=0,929).

Obezity In the AMI group, 30% had stage I obesity, 23.3% stage II obesity, with no significant differences fro tm the group UA patients, were we recorded 46.4% stage I obesity and 21.4% stage II obesity (p=0,753).

Smoking Almost half our patients were smokers (46,7% of the AMI group and 50% of the UA group) (p=0,800).

In conclusion, personal history parameters did not represent a supplementary risk for AMI versus UA (90% vs 89,3%; p=0,929).

The total cholesterol ranged between 90 to 298 mg/dl, 39.7% exceeded the maximum limit of 200 mg/dl. The highest values were in the UA with severe coronary lesions (169,83 *vs* 209,54 mg/dl; p=0,002).

LDL-cholesterol ranged between 38 and 219 mg/dl, 36,2% of the patients exceeded the maximum limit (140 mg/dl). The highest mean values were in the UA group (101,03 *vs* 138,50 mg/dl; p=0,001).

HDL-cholesterol ranged between 22 to 81 mg/dl, 41,4% of the patients had values bellow the lower reference value (40 mg/dl). There were no significant differences between the study groups (43,77 *vs* 44,04 mg/dl; p=0,928).

Triglyceridele ranged between 54 and 440 mg/dl, 37,9% of the mean values eceeding the maximum range. The highest mean values were in the UA group, but the differences between groups were not significant (150,67 *vs* 155,29 mg/dl; p=0,814).

INFLAMMATORY STATUS

The Sedimentation Speed (SS) ranged between 2 and 130 mm/h, 62,1% of the mean values exceeded the maximum limit (20 mm/h). The highest values were in the UA with severe coronary lesions (31,63 *vs* 35,07 mm/h; p=0,660).

C Reactive Protein (CRP) ranged between 0,30-77 mg/dl, 53,4% exceeded the maximu reference value (4 mg/dl). The mean values in the AMI group were slightly elevated compared to the UA group, but we recorded no statistical significance between AMI and UA (8,61 *vs* 7,57 mg/dl; p=0,715).

Fibrinogen Mean individual values ranged between 276 and 1012 mg/dl, 51,7% exceeding the upper normal limit (500 mg/dl). There were no statistically significant differences between groups (554 *vs* 545 mg/dl; p=0,845).

CORONARY ARTERIES STATUS – CORONAROGRAPHY RESULTS

We noticed a clear predominance of the one-vessel disease patients (41,4%), but the two-vessel and three-vessel diasease patients together represented 57% of the total number included (fig.1).

We recorded the following results in our study groups (p=0,584):

- Most AMI patients were 1C (43,3%), with only one patient with non-significant coronary lesions (0C) (3,3%);
- In the UA group, there was a discrete predominance of the 2C (35,7% *vs* 30%) and 3C cases (25% *vs* 23,3%) compared to the AMI group.

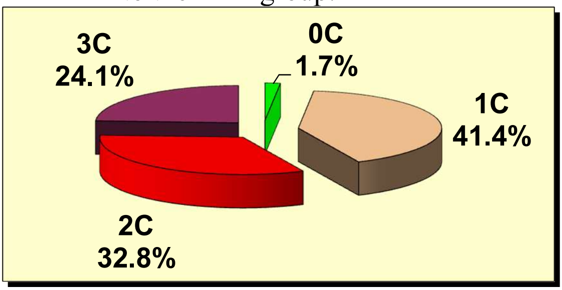

Fig.1. *The cases distribution according to the coronary status (OC =non significant lesions; 1C= one vessel disease, 2C= two vessel disease and 3C=three vessel disease)*

The coronary arteries status had the following particularities depending on the epidemiological characteristics and the personal history of the patient:

➢ AMI group

  - One vessel disease has a high incidence among patients over 65 year-old (52,6%; p=0,046) and obese patients (55,5%; p=0,05);
  - Two vessel disease was mainly recorded in heavy smokers (42,9%; p=0,026);
  - Three vessel disease was mainly recorded in diabetic patients (38,9%; p=0,037)

- UA group
  - Male patients had mainly one vessel disease (47,6%; p=0,013); we encountered the same status in the dyslipidemic patients (40%; p=0,001);
  - Two vessel disease patients were mainly over 65 year-old (46,2%; p=0,851) and obese (42,1%; p=0,087);
  - While three vessel disease patients were mainly diabetic (35,1%; p=0,345) and obese (31,6%; p=0,087).

2C patients from the AMI group had high CRP (p=0,001) and TGP (p=0,05) values. 3C patients from the AMI group had high triglycerides (p=0,044).

2C patients from the UA group had high fibrinogen (p=0,05) and SS (p=0,028) and 3C patients had high triglycerides (p=0,021).

EVALUATION OF THE EPIGENETIC RISK FACTOR'S CONTRIBUTION TO THE DEVELOPMENT OF THE CORONARY ARTERY DISEASE – RESULTS FROM THE SECOND PART OF THE STUDY

For this part of the study we enrolled **154 pacients** with acute coronary artery disease, divided in four groups according to the severity of the lesions: 0C (24 patients with non significant coronary lesions), 1C (48 pacients with one vessel disease), 2C (48 pacients with two vessel disease) and 3C (34 pacients with three vessel disease). We added two echocardiographical parameters for the analysis: left ventricle hypertrophy and wall motion abnormalities (hypokinesia and akinesia). The statistical analysis was also performed with SPSS 18.0 programme.

PERSONAL PATHOLOGICAL HISTORY – PRESENCE OF THE CARDIOVASCULAR RISK FACTORS

Diabetes mellitus 94,1% of the 3C patients and 62,5% of the 1C patients were diabetic, statistically significant when compared to the 0C patients (16,7%) (p=0,001).

Dyslipidemia was present in 83,3% of the OC and 1C patients and 100% of the 3C patients (p=0,016).

As for **obesity,** we noticed the following aspects (p=0,05):

- 1C group: 29,2% had stage II obesity and 8,3% had stage III obesity;
- 2C group: 45,8% had stage I obesity and 16,7% stage II obesity;
- 3C group: 52,9% had stage I obesity and 11,8% had stage II obesity;

Smoking Almost half of the patients with severe coronary lesions were heavy smokers (p=0,062).

ECHOCARDIOGRAPHICAL PARAMETERS

Akinesia 3C patients were mainly akinetic (82,4%; p=0,001).

Hypokinesia 3C patients had the lowest number of hypokinetic patients (17,6%; p=0,002).

Left Ventricle Hypertrophy (LVH) The biggest incidence of LVH was recorded in the 3C group (88,2%; p=0,001).

In conclusion, dyslipidemia, diabetes, obesity, smoking, family history, akinesia and LVH were mainly associated with 3C patients.

LIPID PROFILE

Total Cholesterol ranged between 101 and 320 mg/dl, 44,8% of the mean values exceeded the maximum range limit (200 mg/dl), but there were no significant differences between groups (p=0,922).

LDL-cholesterol ranged between 48 and 252 mg/dl, 40,9% of the mean values exceeded the upper range limit (140 mg/dl); there were no significant differences between groups (p=0,793).

HDL-cholesterol ranged between 22 and 81 mg/dl, 40,3% of the mean values were bellow the range limit (40 mg/dl). 2C and 3C patients had statistically significant low values, compared to 0C and 1C patients (p=0,011).

Triglyceridele ranged between 44 and 455 mg/dl, 40,3% of the patients exceeded the upper range limit (140 mg/dl). 3C patients had higher mean values then the other groups (p=0,001).

INFLAMMATORY STATUS

SS ranged between 2 and 130 mm/h, 58,4% of the mean values exceeded the upper range limit (20 mm/h). 2C patients had the highest SS values (p=0,001).

Individual **CRP** values ranged between 0,30 and 25 mg/dl, 52,6% of the mean values exceeded the upper range limit (4 mg/dl). Similar to the SS values, CRP values were also high mainly in the 2C group (p=0,001).

Individual **fibrinogen** values ranged between 130 and 1029 mg/dl, 48,7% of the mean values exceeded the upper range values (500 mg/dl). 2C patients had the highest fibrinogen values (p=0,001). The ROC curves show that inflammatory status markers were good predictors for two vessel disease (AUC>0,600).

EVALUATION OF THE GENETIC RISK FACTOR'S CONTRIBUTION – RESULTS FROM THE FIRST PART OF THE STUDY

APOB100 GENE MUTATIONS

Contrary to our expectations, we did not identify any mutation of this gene in our patients.

The amplified gene fragments, non digested, have the length of 170 bp. After the digestion process, we only obtained normal, wild fragments, of 140 bp. There were no 110 bp fragments (mutant fragments) (fig.2).

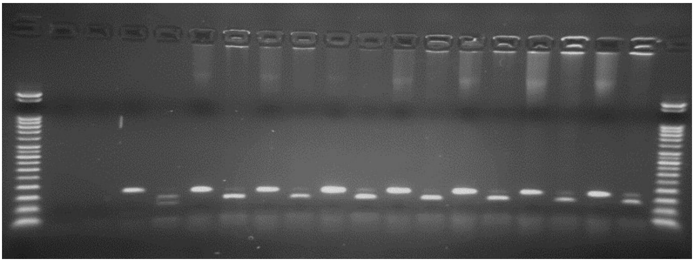

Fig.2. *Results from the apoB100 gene determinations: undigested fragments (170 bp), positive control (110 bp) and the fragments from our patients (140 bp)*

ANGIOTENSIN CONVERTING ENZYME GENE MUTATION

We identified the ACE polymorphisms by the length of the migrated fragments, using agarose gel electrophoresis for separation and UV light for recognition. The 190 bp amplicon bears the D allele, while the 480 bp amplicon bears the I allele. The positive controle is an example of ID genotype. We identified all three possible genotypes in our patients: II, ID, DD (fig.3). There were 6 II genotypes, 19 ID genotypes and 5 DD genotypoes in the UA group. In the AMI group, we found 2 II genotypes, 16 ID genotypes and 10 DD genotypes.

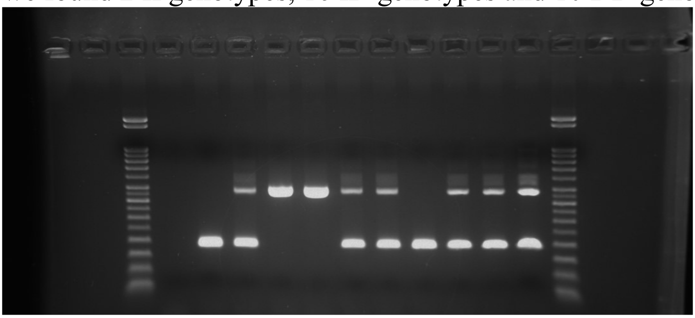

Fig.3. *Migrarea ampliconilor ECA în gel: II (480 bp), ID (480/190 bp) şi DD (190 bp)*

The correlations between ACE gene mutations and the clinical parameters revealed the following aspects:

- AMI group
  - The prevalence of II genotype was between 16,7% and 22,2%%;
  - There was a significant association between ID genotype and age over 65, diabetes mellitus, dyslipidemia, obesity and smoking ($p<0,05$);
  - Most of the diabetic patients had three vessel disease (27,8%);
- UA group
  - The prevalence of II genotype was <10%;
  - There was a significant association between ID genotype and males (52,4%; p=0,05), dyslipidemia (52%; p=0,050), smoking (74,4%; p=0,001);
  - DD genotype was mainly found in diabetic patients (53,8%; p=0,045); its frequency was similar to that of ID for patients over 65 year-old (47,4%) and for obese patients (47,1%).

AMI patients with ID genotype had high CRP levels (p=0,05), while patients with DD genotype had higher triglycerides (p=0,011).

UA patients with ID genotype had high HDL (p=0,05) and SS (p=0,016) levels, while for the DD carriers, we recorded high potasium values (p=0,046).

ASSESSMENT OF THE CONTRIBUTION OF GENETIC RISK FACTORS FROM THE SECOND STAGE OF THE STUDY

The **angiotensin conversion enzyme gene mutations**, correlated with the clinical parameters, highlighted the following aspects:

• **lot 0C**

o 21/24 patients (87.5%) had genotype II, of which 76.2% men, 57.1% over 65 years, with dyslipidemia (81%) and obesity (71.4%);

o ID genotype was detected in 3 patients, over 65 years of age, obese **and with dyslipidemia;**

• **lot 1C**

o ID genotype (83.3%) is predominant in males (80%), over 65 years of age (65%), functionaries (55%), in patients with dyslipidemia (80%) and diabetes (55%);

o Genotype II was identified in 16.7% of patients with unicoronary changes: 6 men with diabetes, dyslipidemia and obesity, only 3 smokers;

• **lot 2C**

o In 75% of patients, the ID genotype was identified, of which 61.1% were male, 58.3% were under 65 years of age, 88.9% were patients with dyslipidemia and 61.1% were obese.

o Genotype II was identified in 8.3% of patients with bicoronary changes: 4 men, over 65 years, smokers, with dyslipidemia;

o DD genotype was identified in 16.7% of patients with bicoronary changes: 8 men, over 65 years, with diabetes, dyslipidemia and obesity, only 2 smokers;

• **lot 3C**

o in 82.4% of patients, the DD genotype was identified, of which 82.1% male, 57.1% over 65 years, all with dyslipidemia, 92.9% diabetics and 57.1% with obesity, half of them smokers;

o ID genotype was identified in 14.3% of patients with tricoronary changes: 3 women and 3 men with diabetes, dyslipidemia and obesity.

LIPID PROFILE AND THE D ALLELE

In patients with 2C changes (206.63 mg / dl; p = 0.028) and 3C (200.71 mg / dl; p = 0.005) - DD genotype was significantly higher in **total cholesterol**.

The mean **LDL-cholesterol** level was significantly higher in patients with 2C changes (151.88 mg / dl; p = 0.016) and 3C (133.25 mg / dl; p = 0.001) - DD genotype.

The mean **HDL-cholesterol** level was significantly lower in patients with 3C changes - DD genotype (39.79 mg / dl; p = 0.027).

The mean **triglyceride** level was significantly higher in patients with 3C changes - DD genotype (230.21 mg / dl; p = 0.017).

INFLAMMATION AND THE D ALLELE

In patients with 2C (12.60 mg / dl; p = 0.018) and 3C (6 mg / dl; p = 0.033) changes - DD genotype was significantly higher in **CRP**. The mean **fibrinogen** and **VSH** levels did not show statistically significant differences according to RCT and genotype.

EJECTION FRACTION AND THE D ALLELE

The ejection fraction varied between 30-60%, individual values below the reference limit (60-70%) in all patients. Study groups showed a significantly lower mean value in the group with tricoronary changes (37.65%; p = 0.001).

The mean value of the ejection fraction was significantly lower in patients with DD genotype (36.39%; p = 0.001).

ARTERIAL HYPERTENSION (HTA) AND THE D ALLELE

Among patients with HTA std. I, 85.7% had genotype II; 64% of patients with HTA std. II, had genotype ID, and 35.6% of patients with HTA grade III, showed the DD genotype (p = 0.001) (fig. 4).

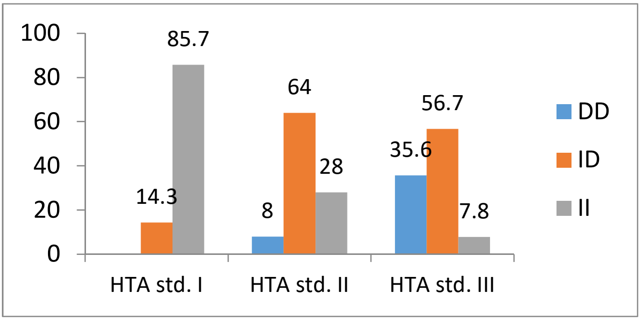

***Fig. 4**. Genotype correlation with HTA*

ECOCARDIOGRAPHICAL CHANGES AND THE D ALLELE

Of the patients with akinesia, 54% had DD genotype, 39.7% genotype ID and only 6.3% genotype II (p = 0.001).

Of the patients with hypokinesis, 2.9% had DD genotype, 75.7% genotype ID and 21.4% genotype II (p = 0.001).

Of the patients with left ventricular hypertrophy (HVS), 41.4% had DD genotype, 52.9% genotype ID and only 5.7% genotype II (p = 0.001).

DISCUSSIONS

The current stage of the research focused on the study of the risk factors for coronary heart disease, depending on its severity (uni / bi / tricoronary character versus the presence of insignificant stenoses, as a control group). We performed the statistical analysis of the results in two stages, the first one regarding the connection of the classic risk factors, the environment with the coronary disease and its severity (epigenetic analysis), and the second stage we dedicated it to the study between the genetic factors and the severity of the coronary disease, as well as the interaction between genetics and epigenetics in the determinism of this complex pathology.

Tobacco consumption is a major epigenetic risk factor. In the first part of the study, we observed that half of the patients were smokers, without statistically significant differences between myocardial infarction and angina (46.7% smokers with infarction and 50% smokers with unstable angina). Moreover, the negative balance tends to tilt in favor of unstable angina. The association between smoking and alcohol was strongly associated with myocardial infarction, although this difference was statistically significant only for smoking (Merry et al., 2011), different from the results of our study.

The close link between diabetes mellitus and ischemic disease has been long studied, with both pathologies increasing worldwide. The prevalence of diabetes among our patients has been very high. In the first part of the study, myocardial infarction versus unstable angina, the share of diabetes was approximately equal between groups (60% of those with infarction and 60.7% of those with unstable angina). These results are in contradiction with other studies, which support the higher prevalence of diabetes in those with heart attack. Esteghamati et al. included in their research 514 patients with unstable angina or acute myocardial infarction, of whom 30% were diabetics, with an average age of the disease between 13.4 +/- 8.7 years. The results showed a significantly higher proportion of myocardial infarction among diabetic patients. However, the location and extent of infarction, as well as the extent of enzyme increases, did not differ between diabetics and non-diabetics, contrary to findings in our study (Esteghamati et al., 2006). In the second part of the study, with the increase of the group size and the redistribution of the patients according to the severity of the vascular disease, we highlighted new associations. Of the total 154 patients, 90 were diabetic (58%). Moreover, we observed an association between the severity of coronary heart disease and diabetes, as the latter was present in 94.1% of tricoronarians (32 patients out of 34) and 62.5% of bicoronarians (30 patients out of 48). of 16% in the control group (4 of 24) ($p = 0.001$). This discovery is in consistent with other previous studies. The FREEDOM trial has shown that diabetic patients have more extensive and more severe lesions, often requiring aorto-coronary bypass (Dangas et al., 2014). To evaluate the extent of coronary artery disease, they used the Coronary Score, consisting of the segmental distribution of lesions and the morphological analysis of the atherosclerotic plaque. Coronary score was significantly higher in diabetic patients versus control group ($P <0.001$). Also, diabetics have had a greater number of proximal lesions. Morphological analysis revealed a high proportion of vulnerable plaques (24.37% vs. 15.33%; $P <0.01$) and more severe stenoses (70-90%) in the diabetic group (70.53% vs. 57.33%; $P <0.05$). Therefore, the fact that the lesions were more severe and widespread in the control group suggests the role of diabetes as an independent predictor of atherosclerosis (Uddin et al., 2005).

Lipid profile disorders are another major epigenetic factor. Evidence regarding the link between coronary heart disease progression and LDL and triglyceride levels is multiple and conclusive. Oxidized LDL particles damage the endothelium and induce plaque rupture. Also regarding lipoproteins, we can talk about an inverse relationship between HDL and coronary heart disease progression. The low level of HDL is an independent and potent risk factor for myocardial infarction and stroke, although there are also patients with infarction who have a normal level of HDL (Perez-Mendez et al., 2014). A study by Xenophontos et al. Looked at the link between low HDL (<40 mg / dl), smoking and myocardial infarction in male patients in Cyprus, and found that smoking reduces HDL and apoprotein concentrations and increases LDL and triglycerides. , thus contributing to the risk of myocardial infarction (Xenophontos et al., 2008). Moreover, the study by Virmani et al., Showed atheromas that have a thin cap, with high risk of rupture, are most commonly found in patients with low HDL and high total cholesterol / HDL ratio (Virmani et al., 2006) . In the first part of our study, 41.4% of the patients had HDL below 40 mg / dl, but we did not find statistical differences between the two groups (unstable angina and acute myocardial infarction). However, in the second part of the study, we observed a statistically significant preponderance of the reduced level of HDL in patients with bi and tricoronary lesions. Regarding total cholesterol and LDL level, we did not observe significant differences between groups. At first glance, the results appear to be unexpected, but given that 90% of patients have a history of dyslipidemia and were already on statin treatment at the time of inclusion in the study, we can infer that lipid profile was influenced by hypolipidemic treatment. The important role of the proaterogenous particles of LDL is well known, as shown by the results of many studies, including those of White et al. and Holmes et al. (Holmes et al., 2015; White et al., 2016), as well as the results of randomized clinical trials focused on hypolipidemic treatment (Baigent et al., 2010; Mihaylova et al., 2012).

Regarding triglycerides, 40.3% of the patients analyzed by us had values above the reference limit, with significantly higher values in tricoronary patients. Dyslipidemia, inflammation and the immune response to abnormal lipid metabolism are the basis for the production and development of cardiovascular disease. Our results are consistent with those of other studies in the field. Soeiro et al., showed that triglyceride levels above 1.13 mmol / L significantly increase cardiovascular risk (Soeiro et al., 2015). It seems that the role of triglycerides is greater than previously thought (Kolovou et al., 2011; Boren et al., 2014; Matsumoto et al., 2014; Tenenbaum et al., 2014). The higher the triglyceride level, the higher the risk of heart attack (Jiao et al., 2018).

Obesity is another traditional risk factor for cardiovascular disease. In the first part of the study, we observed that 56.7% of patients with myocardial infarction were obese and 43.3% had a normal-weight. The obesity stage was higher in the group with unstable angina, although the differences between groups had no statistical significance ($p = 0.860$). These results are arguments for the association between overweight and coronary heart disease. Zhu Jun et al., conducted a meta-analysis to demonstrate the involvement of overweight and obesity in the determinism of coronary heart disease. Both overweight and obesity increased the risk of heart attack. Metabolic syndrome, including abdominal obesity, has doubled cardiovascular risk and increased mortality 1.5 times (Zhu et al., 2014). Moreover, once diagnosed with heart disease, the presence of obesity accelerates the progression of the lesions. Overweight and obese patients are prone to insulin resistance and diabetes, which aggravates their prognosis (Savage, 2017).

Classically, the assessment of the severity of coronary heart disease is performed by coronarography, which divides patients into uni, bi, tricoronary or common trunk lesions and stenoses of over 50% or over 70% (Ringqvist et al., 1983). Combining these parameters results in coronary risk scores (Dash et al., 1977; Gensini et al., 1983). Rubinstein noted that obese patients sent for coronary artery disease were younger and had a smaller percentage of common trunk (Rubinshtein et al., 2006).

Inflammation, the central process in the development of coronary heart disease, appears as a response of coronary arteries to lipid oxidation, injury and sometimes infections. The classic risk factors, such as high blood pressure, diabetes, smoking are aggravated by oxidized LDL molecules and initiate a chronic inflammatory process, with the appearance of the vulnerable plaque, prone to rupture and thrombosis. Epidemiological studies have demonstrated the important link between inflammation markers and the risk of cardiovascular events (Ross, 1999). The inflammatory markers evaluated in our research were sedimentation speed (SS), fibrinogen and C-reactive protein (CRP).

In the first part of the study, 62.1% of patients had SS above the baseline, 53.4% high CRP and 51.7% increased fibrinogen, but without significant differences between acute myocardial infarction and unstable angina. These findings advocate the association between acute coronary syndrome and inflammatory syndrome, of which both pathologies are included. Regarding the role of inflammation in the severity of the lesions, we observed high values of fibrinogen in the group with bicoronary changes ($p = 0.001$). Fibrinogen plays an important role in the final stage of the coagulation cascade, namely fibrin formation, thus being both a marker of inflammation and a coagulation factor (Danesh et al., 2000). Inflammation is dependent on the interaction between fibrinogen and leukocytes, mediated by integrins (Stefanadi et al., 2010). The role of fibrinogen, alone or associated with other markers, in the determinism of coronary events has long been investigated. Xu et al. hypothesized that fibrinogen may be a link between stress and coronary syndromes (Xu et al., 2012). Fibrinogen is also associated with other risk factors, such as high blood pressure and hypercholesterolemia.

CRP is synthesized by the liver in response to interleukin 6 and significantly contributes to atherogenesis, atheroma plaque alteration and thrombosis. Thrombin and platelet-derived growth factor (PDF) induce increased production of interleukin 6, which increases the release of PCR in the liver, thus exacerbating inflammation and thrombosis. Additionally, CRP activates the complement system and amplifies tissue factor release from monocytes (Libby, 2001; Ridker, 2003). These mechanisms explain the involvement of CRP in the pathophysiological constellation that leads to the appearance of acute coronary syndromes, being not only a predictive factor for the occurrence of acute events in apparently healthy middle-aged adults, but also for the post-installation evolution of an acute coronary syndrome (Ridker, 2002).

The study of Brunetti et al. included 192 patients with coronary syndromes, of whom 138 with acute myocardial infarction (28 with non-Q infarction) and 54 with angina unstable. The authors measured CRP, creatinine kinase MB, LDH and troponin I, evaluated the ejection fraction by echocardiography and myocardial destruction using the plasma level of myocardial cytolysis enzymes. Patients were monitored for 6 months after the acute event. Different from our results, which did not record differences between the infarction group versus the unstable angina group, the authors found significantly higher CRP values in patients with Q wave infarction compared to non-Q infarction and unstable angina, as well as between non-Q infarction and unstable angina ($p < 0.01$). Regarding the risk of major cardiac adverse events at 6 months, this was higher for patients with Q wave infarction who had peak CRP values. However, by contrast to our research, which underlines the high CRP association with bicoronary lesions, the study of Brunetti et al. did not identify CRP-coronary angiography correlations.

The next step was the analysis of the genetic mutations. The first mutation investigated was the apoB100 gene polymorphism. The close relationship between apoprotein B100 and the circulating level of atherogenic LDL particles is unanimously recognized. Binding of LDL to its receptor is essential for the clearance of atherogenic molecules. Therefore, mutations in the apoB100 gene or in the LDL receptor affect this process. Replacement of arginine with glutamine at codon 3500 results in hypercholesterolemia (Frostegard et al., 1990). Given the important role of hyperlipidemia in atherosclerosis, the main process underlying coronary heart disease, we decided to include this parameter in our research. Contrary to our expectations, the genetic dosages performed in the included patients did not detect the presence of this mutation.

Since about 2/3 of the circulating cholesterol is incorporated into the LDL particles, the LDL-LDL Receptor complex is essential in maintaining proper lipid metabolism (Brown, Goldstein, 1986). Mutations may occur at the LDL or receptor level. Familial hypercholesterolemia increases the risk of myocardial infarction. However, as receptor mutations are responsible for only 5% of coronary heart disease in patients under the age of 60 (Brown, Goldstein, 1986; Goldstein, Brown, 1989), attention has shifted to apoB100 mutations, the most studied being the mutation from codon level 3500. Based on our research, we deduce that in addition to the criterion of hypercholesterolemia in the coronary patients we included, in further studies, it would be useful to initially dose the serum level of apoB100 and to perform the genetic study only in those patients with values above the upper reference limit.

The association between the mutation of this gene and coronary heart disease is still under research worldwide, the results being controversial. The studies presented in the specialized literature demonstrate the presence of the large number of mutations in the area of central Europe, with the decrease of the incidence as we move towards the south and the east of the continent (Horvath et al., 2002). Our study is the first in Romania on this topic. Therefore, this mutation may be rare in the territory of our country, but it is certainly a good research topic in the future, on a larger number of patients from several areas of the country, with the application of this additional criterion of inclusion in the country. only study of those with high serum level of apoB100.

Coronary artery disease (CAD) is a polygenic pathology. Its triggering and severity depend on the interaction between genetic and environmental factors. DD, ID and II genotypes are associated with high, intermediate and low levels of ACE (Cambien et al., 1994). Our study shows that all three genotypes influence the severity of coronary heart disease by interacting with conventional risk factors. The relationship between BAC and genetic polymorphism (DD genotype) was first discussed by Cambien et al. Since then, the topic has long been debated, the results being still unclear, with some positive studies (Gardemann, 1998), and others negative (Ferries et al., 1999; Canavy et al., 2000). I / D polymorphism is responsible for 20-50% of plasma ACE differences, which means that 50-80% come from the influence of environmental factors or from the interaction between these polymorphisms and environmental factors (Cambien et al., 1994).

The present research identified a strong correlation between ACE mutations and male sex, age over 65, smoking, diabetes and obesity, especially in groups with bicoronary lesions. Regarding smoking, the correlation of this parameter with the ACE in our patients was registered especially in the groups with bicoronary and tricoronary lesions, suggesting an important contribution in the development of coronary heart disease. Nicotine enhances ACE synthesis, leading to increased plasma and tissue activity of angiotensin (Zhang et al., 2001). Furthermore, allele D has been associated with endothelial dysfunction, especially in smokers (Buttler et al., 1999). Both smoking and the D allele were correlated with an increased level of angiotensin II, resulting in superoxide anion formation and nitric oxide degradation, and ultimately endothelial dysfunction (Drexler, Hornig, 1999). Smoking alters antioxidant protective mechanisms by altering ECA gene expression (Zhang et al., 2001).

It seems that the DD genotype, as opposed to the II genotype, is strongly associated with the risk of diabetes and the development of its complications (Marre et al., 1994) and with high blood pressure (Zee, 1992). However, there are studies that do not support this association (Chiang, 1997; Daimon, 2003). Furthermore, a study in Australia has revealed a strong link between allele I and familial hypertension in the Australian population (Zee et al., 1992). An important study in this direction conducted in Saudi Arabia has shown a high weight of the D allele in hypertensive and diabetic patients with or without hypertension ($p < 0.0001$), results similar to those of an important study in Taiwan (Hsieh et al. , 2000). These findings are supported by other research from other regions, which confirms the increased prevalence of DD genotype and D allele in diabetic and hypertensive patients (Jeng et al., 1997; Daimon et al., 2003). The current research is among the positive studies, the correlation of RCT polymorphisms with diabetes being statistically significant in the single and bicoronary patients ($p = 0.004$, respectively $p = 0.001$). Stephens J.W. analyzed the prevalence ACE mutations in diabetic and non-diabetic Caucasian patients and demonstrated the prevalence of DD, ID genotypes among diabetic patients ($p < 0.001$), as well as a positive correlation with family history of diabetes ($p = 0.03$) (Stephens et al., 2005). The link between diabetes and the D allele has created many controversies over time. Surprisingly, Zhou D. et al's meta-analysis of the Chinese population, published in 2012, which included 41 studies (4708 cases and 5368 control subjects), negated the association between ECA polymorphism and type 2 diabetes mellitus (Zhou et al. al., 2012).

By contrast, in the Egyptian population, Zarouk et. al., have pointed out the association of the D allele with both diabetes and high blood pressure, suggesting that in this category of patients, early initiation of therapy with ACE inhibitors and ACE receptor blockers may be useful in preventing complications further (Zarouk et al., 2009).

Of the inflammatory markers analyzed, we obtained a statistically significant correlation only between C-reactive protein (CRP) and ACE polymorphism, fibrinogen and SS not registering significant differences according to ACE genotype. The highest CRP values were identified in patients with DD genotype with bicoronary and tricoronary lesions. Angiotensin II has been shown to stimulate the inflammatory process by inducing cytokine synthesis, such as interleukin 6 and tumor necrosis factor alpha, and inflammation plays a key role in atherosclerosis, the pathophysiological basis of coronary heart disease (Kranzhofer et al., 1999). ACE activity and its plasma concentrations are determined by genetic polymorphisms (Rigat et al., 1992). DD genotype has a plasma activity 85% higher than genotype II (Rigat et al., 1990). The high level of CRP in patients with coronary heart disease appears to be an even more potent prognostic predictor than LDL cholesterol (Ridker et al., 2002). High-risk patients generally have vulnerable atheroma plaques and may consequently develop acute myocardial infarction following vessel closure (Ridker, 2003). The high level of CRP is thought to be a witness to plaque vulnerability, rather than to the extent of atherosclerosis, whereas angiotensin II has a greater role on the extent of atheromatosis than on vulnerability (Libby, Ridker, 2004).

Essential hypertension is another known cardiovascular risk factor. The RAAS contributes to maintaining the hydro-electrolyte balance and thus to regulating blood pressure. Through its two major functions, one activating angiotensin II, one potent vasoconstrictor and the other inactivating bradykinin, ACE plays a central role in tension homeostasis. Thus, it is expected that the polymorphisms of this gene, associated with lower or higher plasma activity ACE mutations in diabetic and non-diabetic Caucasian patients and demonstrated the prevalence of DD, ID genotypes among diabetic patients ($p < 0.001$), as well as a positive correlation with family history of diabetes ($p = 0.03$) (Stephens et al., 2005). The link between diabetes and the D allele has created many controversies over time. Surprisingly, Zhou D. et al's meta-analysis of the Chinese population, published in 2012, which included 41 studies (4708 cases and 5368 control subjects), negated the association between ECA polymorphism and type 2 diabetes mellitus (Zhou et al. al., 2012). By contrast, in the Egyptian population, Zarouk et. al., have pointed out the association of the D allele with both diabetes and high blood pressure, suggesting that in this category of patients, early initiation of therapy with ACE inhibitors and ACE receptor blockers may be useful in preventing complications. further (Zarouk et al., 2009).

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Essential hypertension is another known cardiovascular risk factor. The SRAA contributes to maintaining the hydro-electrolyte balance and thus to regulating blood pressure. Through its two major functions, one activating angiotensin II, one potent vasoconstrictor and the other inactivating bradykinin, ACE plays a central role in tension homeostasis. Thus, it is expected that the polymorphisms of this gene, associated with lower or higher plasma activity high, to cause tension imbalances (Badaruddoza et al., 2009). Our study highlights the statistically significant association between a lower degree of hypertension and allele I and a high degree of hypertension and allele D.

Left ventricular hypertrophy (LVH), one of the pathological markers of high blood pressure, is, in reality, the consequence of adapting the left ventricle to the growth of postsarcinoma. At the heart of this mechanism is the heart's attempt to maintain a normal heart rate, under conditions of pressure overload. Thus, LVH functions as an optimal compensatory mechanism up to a certain level, beyond which there is decompensation, with the installation of heart failure. A patient with LVF has a 4-fold increased risk of developing acute myocardial infarction than a patient with the same pressure level but without hypertrophy (Cosenso-Martin et al., 2015).

In recent years there has been a growing interest in the association between ACE polymorphism / ACE, angiotensin II serum level and high blood pressure, LVH. However, the results of these studies are controversial. Our study is part of this line of positive research. Of the patients with HVS, 41.4% had DD genotype, 52.9%

genotype ID and only a small percentage of 5.7% had genotype II. Cardoso et al., Found a higher frequency of hypertension in women with genotype II, whereas males had predominantly ID or DD (Cardoso et al., 2008. In our study, no differences were observed statistically significant between the two sexes regarding LVH.

CONCLUSIONS

1. Most of the patients with acute coronary syndrome were males and the prevalence of men was observed both in acute myocardial infarction and in unstable angina, but there were no significant differences between the sexes between the one/two or three-vessel disease patients.

2. More than half of the patients were smokers and, although we did not identify significant differences between infarction and unstable angina, considering that both are acute, severe pathologies, we can say that tobacco remains an important epigenetic risk factor for cardiac events.

3. Positive family history has been found predominantly in patients with severe lesions (three-vessel disease), which brings into question both the role of genetic predisposition in this pathology and the role of healthy versus unhealthy life style habits in the family.

4. Almost half of the patients had low HDL values, and half of them were smokers, which highlights the harmful influence of tobacco on this protective, anti-atherogenic metabolic parameter.

5. Atherogenic lipoproteins were increased in our group, but not at the level we expected, considering the severity of the lesions. But as the anamnesis showed that 90% had previously diagnosed dyslipidemia and were already undergoing statin therapy at the time of the acute coronary event, we can explain the small variations of cholesterol and LDL through this treatment.

6. More than half of the patients with acute myocardial infarction were obese, with an even higher stage of obesity in the two and three-vessel disease patients, compared to those without significant and unicoronary lesions. These aspects reinforce the well-known role of obesity in the determinism of ischemic heart disease.

7. Our results confirm the involvement of diabetes in the acceleration of coronary atherosclerosis, with the onset of acute cardiac events.

8. Fibrinogen and C-reactive protein were increased in bicoronary patients, without significant differences between infarction and unstable angina. These changes were especially observed in those patients in the study who were diabetic, hypertensive, smokers and dyslipidemic, which is an argument for all these epigenetic factors inducing inflammation and imbalance of the atheroma plaque, with the appearance of the unstable, vulnerable plaque.

9. Contrary to our expectations, in none of the included patients we obtained mutations of the B100 apoprotein gene, possible explanations could be the geographical area (these mutations are rare and predominant in central Europe) and the small number of patients.

10. The most important novelty elements of this research were obtained from the study of the genetic polymorphism of the ECA. Thus, in the control group (insignificant lesions) almost all the patients were carriers II (87.5%), the weight of the D allele increasing with the aggravation of the coronary lesions, to a 82% of DD carriers in the tricoronary patients group.

In conclusion, the clinical importance of this study lies in the fact that all these findings could explain the different response to ACE-inhibitor drugs, a response determined by the genotype of each patient. In this regard, further studies are needed to evaluate the individual pharmacogenetic effects of ACE polymorphism to improve therapeutic and prognostic strategies in acute coronary syndromes.

LIST OF “IN EXTENSO” ARTICLES PUBLISHED BY THE PHD STUDENT FROM THE THESIS

1. **Apăvăloaie (Vladeanu) M.C**., Bararu Bojan I., Bădulescu O.V., Pleşoianu C.E., Jitaru D., Bojan A., Iliescu D., Sîrbu P.D., Ciocoiu M., Bădescu M., Arsenescu Georgescu C. Determination of platelet parameters with impedance automated analyzers in diabetic patients with coronary artery disease. *Rev Chim-Bucharest* 2019; 70(4): 1242-1244.
2. **Apăvăloaie M.C.**, Bararu I., Pleșoianu C.E., Jitaru D., Dragoș L., Ciocoiu M., Arsenescu Georgescu C., Bădescu M.. Inflammatory and genetic markers (Apo B100 and angiotensin-converting enzyme gene) in the coronary artery disease. *Rev.Med.Chir*. 2016; 120 (3):530-536 ISSN: 0048-7848.
3. **Apăvăloaie M.,** Bararu I., Jitaru D., Ciocoiu M., Bădescu M., Arsenescu Georgescu C. Genetic determinism in the acute coronary syndrome. *RJAC* 2016; 4(10): 173-181, ISSN 2327-5707, e ISSN 2473-6562.
4. **Apăvăloaie M.,** Bararu I., Ciocoiu M., Bădescu M., Arsenescu Georgescu C. Angiotensin converting enzyme gene polymorphisms in coronary artery disease-a controversial role. *Rev Med Chir.* 2015; 119(3):784-790, ISSN 0048-7848.

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