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Humoral markers of endothelial dysfunction and systemic inflammatory response in patients with acute myocardial infarction depending on genes polymorphism of ACE (I/D) and eNOS (894G>T)

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The dynamics of endothelial dysfunction (ED) humoral factors: a soluble form of vascular cell adhesion molecule 1 (sVCAM-1), total NO metabolites and systemic inflammatory response - C-reactive protein (CRP) in patients with acute myocardial infarction (MI) under the influence of treatment and depending on genes polymorphism – angiotensin converting enzyme (ACE, I/D) and endothelial nitric oxide synthase (eNOS, T894G) were evaluated. The presence of DD-genotype of ACE gene is associated with a significantly greater decrease of sVCAM-1 and CRP levels under the influence of treatment (better with thrombolytic therapy (TLT), $p<0.05$); in T- allele carriers of eNOS gene the level sVCAM-1 under TLT decreased by 30.7-31.2%. Content of NO metabolites decreased more in D-allele carriers of ACE gene as well as after combined treatment with TLT (39.1% and 35.2%) and did not depend on the allele state of eNOS gene.

Keyword: ACE (I/D), eNOS (894G>T) genes, myocardial infarction, endothelium, treatment

1. Introduction

The imbalance between protective and disturbing humoral factors (nitric monoxide (NO), activity of endothelial nitric oxide synthase (eNOS), endothelial hyperpolarizing factor, PgI, endothelin-1, thromboxan-A2 etc.) as well as anti-inflammatory cytokines, chemoattractants, adhesion molecules (soluble form of vascular cell adhesive molecule (sVCAM), L-, E-selectins, intensification of oxidizing stress processes, hemodynamic changes (under conditions of intact endothelium the pressure of laminar blood flow on the vascular wall (shear stress)) result in the release of endogenic NO [1-3]. On the contrary, blood flow turbulence in case of vasospasm or in the places of arterial trunk bifurcations changes orientation of endothelial cells and decreases NO release, dyslipidemia, mutations of associated genes etc., creating preconditions for the

development and progress both endothelial dysfunction and atherosclerosis [3-6]. Increased NO synthesis with accumulation of its final metabolites under conditions of acute endothelial damage with acute coronary syndrome (ACS) may have a cytotoxic effect, because accumulated superoxide radical (O_2^-), as a result of activation of free radical lipid damage processes, interacting with NO create a highly active oxidant – peroxynitrite ($ONOO^-$) causing additional oxidation of the damaged tissues leads to endothelial dysfunction (ED), with clinical consequences and myocardial infarction (MI) among them [2, 5, 7, 8]. The genetic dependence concerning the development and severity of ED with the aim to differentiate high risk groups of cardiovascular diseases or ACS occurrence has been extensively studied over the past decade [7, 9-11]. But in Eastern Europe, such studies are extremely insufficient [6, 7, 9, 12].

Consequently, the aim of our study was to analyze the dynamics of ED humoral factors (sVCAM-1, total NO metabolites) and systemic inflammatory response – C-reactive protein (CRP) in patients with ACS under the influence of treatment depending on genes polymorphism of angiotensin converting enzyme (ACE, I/D, rs 4646994) and eNOS (894G>T, rs1799983).

2. Materials and Methods

2.1 Compliance with bioethics

The study was performed in compliance with the Council of Europe Convention on Human Rights and Biomedicine and the Recommendations of the Committee on Bioethics of the Ministry of Public Health of Ukraine. Patients' Examination Cards and Patients' Informed Consent Forms were approved by the Biomedical Ethics Commission of Bukovinian State Medical University, the Ministry of Public Health of Ukraine (Chernivtsi, Ukraine). All enrolled patients were treated in the Regional Clinical Cardiologic Hospital (Chernivtsi, Ukraine) during 2010-2013 y.y. Genetic bench studies performed in the laboratory of the Department of Medical Biology and Genetics of Bukovinian State Medical University. After screening (matching inclusion/exclusion criteria) 102 acute MI patients were selected for further examination. The control group included 30 practically healthy individuals.

2.2 Inclusion / Exclusion criteria

Inclusion criteria. The presence of typical anginal pain at rest lasting more than 20 minutes, which is not eliminated by nitroglycerin; ECG changes (persistent elevation of ST segment, in at least two contiguous leads ≥0,25 mV in men before 40 years, ≥0,20 mV in men over 40 years, ≥0,15 mV in women in leads V_2-V_3, and/or ≥0,10 mV in other leads (in the absence of left ventricular hypertrophy (LVH), or blockade of left bundle branch block), first onset of left bundle branch block and/or pathological Q-wave), in accordance with national and ESC guidelines (2012) [13-15]; age above 20 y.o.; voluntary consent to participate in the study.

Exclusion criteria. We excluded patients with chronic heart failure (CHF) higher than II functional class (NYHA III-IV), true cardiogenic shock, type I diabetes, sub- and decompensated type II diabetes, malignant uncontrolled arterial hypertension (AH), sub- and decompensated diseases of the liver (three times over the norm level of aspartate aminotransferase, alanine aminotransferase) and kidneys (blood serum creatinine 200 mkmol/l and higher), bronchial asthma, chronic obstructive pulmonary disease of III-IV stage (GOLD 2011), exacerbated oncologic and infectious diseases or during unstable remission, psychological disorders.

2.3 Diagnosis of Myocardial Infarction

Selection of patients and their distribution into groups was performed according to the Recommendation of Ukrainian and European Society of Cardiology (ESC, 2012) [13-15]. The clinical diagnosis of MI was made on the basis of clinical findings, ECG and biochemical examinations, the biomarkers troponin-T (cTnT) of myocardium damage according to the current national and international recommendations [13-15].

All patients were examined comprehensively: general clinical, ECG, laboratory and instrumental examination (EchoCG).

2.4 Endothelium Dysfunction humoral factors and systemic inflammatory response investigation

Total stable metabolites of nitric oxide (NO_2^- +NO_3^-) and sVCAM-1 were detected in the blood plasma of patients with MI stabilized with EDTA (1 mg/mL): NO metabolites – by means of calorimetric method (Assay Kit) with the set of reagents of the "Total NO/NO_2^-/NO_3^-" (RDS, Great Britain); sVCAM-1 – by immune-enzyme analysis (ILISA) with the set of reagents of "Diaclone" (France). The content of CRP in the blood serum was studied by ELISA with the sets of reagents of "Quantikine®" (R&D Systems, Inc., USA). NO metabolites levels, sVCAM-1 and CRP were detected in 88 patients before and after the treatment.

2.5 Principles of Myocardial Infarction Management

Basic pharmacotherapy of patients with MI included into the study during the whole period of examination was conducted according to the current legal national and international recommendations and protocols [13-15]. Among them, 50 patients received thrombolytic therapy (TLT) with Streptokinase /or Alteplase/ or Tenecteplase according to the algorithm of administration for this medicine. The period of observation was 12 months ± 2-3 weeks.

2.6 Genotyping of the ACE – I/D and eNOS – 894G>T polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes using the "DNA-sorb-B" test system, with primers specific to the genes' alleles [16]. Detection of I/D polymorphism of ACE gene and 894G>T polymorphism of eNOS gene was performed by the multiplex polymerase chain reaction (PCR) according to the manufacturer's protocol. Allele-specific primers were used in the PCR (Table 1). The PCR products for 894G>T polymorphism of eNOS gene detection were digested overnight by restriction endonuclease Ban II (Eco241) for G-allele+ (Fermentas, Lithuania) at 37 °C. The PCR products (for ACE gene – II genotype 553 bp, ID – 553 and 263 bp, DD – 263 bp; for eNOS gene – TT genotype - 250 bp, GG – 160 and 90 bp, TG – 250, 160 and 90 bp) were separated by horizontal electrophoresis on 3% agarose gels, stained with 4 µl of ethidium-bromide and visualized by in the presence of molecular mass ladder (100-1000 bp) using a UV transilluminatior (Nyxtechnic, USA) (Figures 1, 2).

Table 1: Primer sequences for ACE (I/D) and eNOS (T894G) gene SNP and size of fragments

SNP locus	Primers	Primer sequences (5'-3')	Size of fragments, bp
ACE I/D	Forward	5–GCCGGGGACTCTGTAAGCCACTGC–3'	Allele I: 553 bp Allele D: 263 bp
	Reverse	5'–CCTTGTCTCGCCAGCCCTCCCA–3'	
eNOS 894G>T	Forward	5–ATGAAGGCAGGAGACAGTGGATGG–3'	Allele G: 160, 90 bp Allele T: 250 bp
	Reverse	5'–CCAGTCAATCCCTTTGGTGCTCA–3'	

bp – base pair

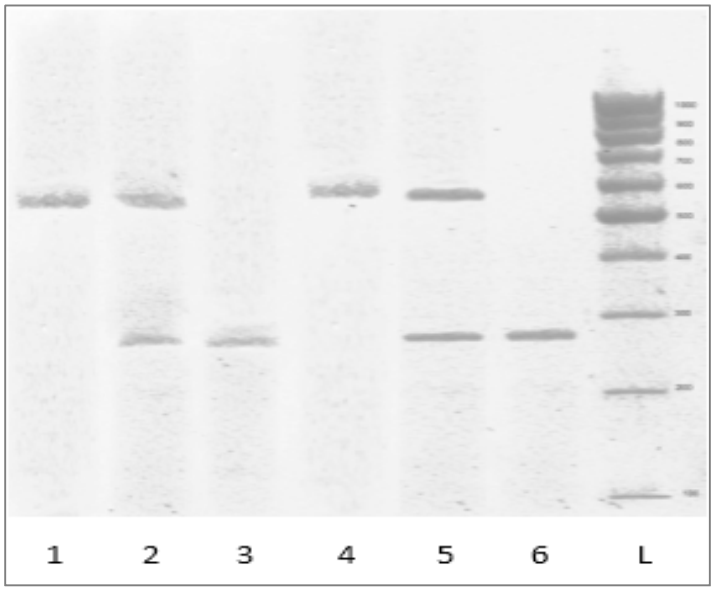

Fig 1: Electrophoregramm of human DNA PCR products amplification of ACE I/D gene polymorphism. Note: L – DNA Ladder "GeneRuler™ 100 bp" (1000-100 bp); lines 1, 4 – homozygous II genotype; lines 2, 5 – heterozygous ID variant; lines 3, 6 – homozygous DD variant.

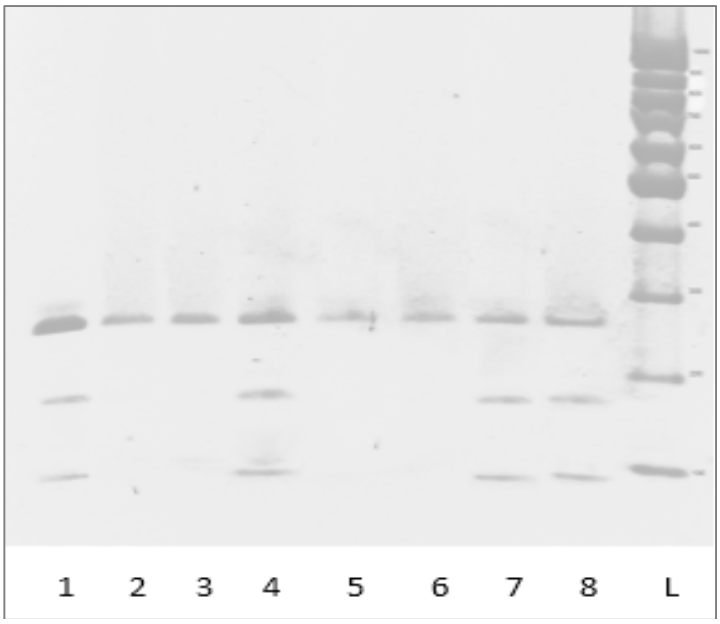

Fig 2: Electrophoregramm of human DNA PCR products amplification of eNOS 894T>G gene polymorphism. Note: L – DNA Ladder "GeneRuler™ 100 bp" (1000-100 bp); lines 1, 4, 7, 8 – heterozygous TG variant; lines 2, 3, 5, 6 – homozygous TT variant.

2.7 Statistical analysis: Statistical analysis was performed using Statistica 7.0 (StatSoft Inc, USA) software, by parametric and nonparametric methods of calculation. P value and odds ratio

(OR), with 95% confidence interval (CI) using a chi-square test were determined for the calculated frequencies of each allele and genotypes. Risk ratios (RR) were estimated by OR. Adjusted OR and 95%CI were estimated for an association between age, Q-MI, non-Q-MI and genetic polymorphism. Parametric data are reported as mean±S.D. Continuous variables were tested for normal distribution by the Kolmogorov-Smirnov test. When data were not normally distributed, they were logarithmically transformed before statistical analysis. P values <0.05 were considered statistically significant.

3. Results and Discussions

102 patients with acute MI were screened. Patients were selected and divided into groups by the type of myocardial infarction (MI) (Q-, not Q-MI), localization (anterior, inferior myocardial wall of the left ventricle (LV)), succession of development (first, repeated or recurrent) according to the Recommendation of the National Ukrainian (2013) and European Society of Cardiology (ESC, 2012) [13-15]: 92 (90.2%) patients with Q-MI and stable ST segment elevation, and 10 (9.8%) patients with non Q-MI without ST segment elevation. There were 15 women (14.7%) and 87 men (85.3%) with an average age 60.7±4.25 (from 22 to 83 y.o.). The reference group included 30 practically healthy individuals with corresponding sex distribution without relative relations with the study group patients.

The levels of sVCAM-1, NO metabolites and CRP in the blood of patients with Q-MI before treatment (Table 2) were 28.9%, 18.6% and 47.1% higher than in the non Q-MI patients respectively ($p<0.05$). A considerable dynamics of indices depending on the succession of MI occurrence was not found. Although, inferior localization of MI was accompanied by a 33.8% increase of sVCAM-1 content as compared with the anterior localization ($p=0.028$).

Table 2: Humoral Markers of Endothelial Dysfunction and Inflammation Depending on the Type of Myocardial Infarction, Sequence of Its Occurrence and Localization, M±S.D.

Observed Groups		sVCAM-1, ng/mL	$NO/NO_2^-/NO_3^-$, mkmol/L	CRP, mg/L
Control		953.0±147.3	21,05±2.71	2.15±0.84
Type of MI	Q-MI, n=78	1512.0±176.1 $p<0.01$	46.7±2.09 $p<0.001$	13.1±2.56 $p<0.001$
	non Q-MI, n=10	1075.0±201,9 $p_1=0.046$	38.0±3.97 $p<0.001$ $p_1<0.05$	6.93±2.12 $p<0.05$ $p_1=0.035$
Sequence of MI	Primary MI, n=63	1395.0±197.9	45.9±5.88 $p<0.001$	10.5±2.26 $p<0.001$
	Repeated MI, n=25	1364.0±85.1 $p<0.05$	42.5±3.17 $p<0.001$	7.65±2.12 $p<0.01$
Localization of MI	Anterior, n=46	1325.0±141.3	42.9±3.15 $p<0.001$	12.6±2.64 $p<0.001$
	Inferior, n=42	2002.0±391.4 $p<0.01$ $p_1=0.028$	47.2±4.94 $p<0.001$	9.90±3.61 $p<0.001$

Notes: MI – myocardial infarction; $NO/NO_2^-/NO_3^-$ – total NO/nitrite/nitrate; sVCAM-1 (CD 106) – soluble vascular cell adhesive molecule; CRP – C-reactive protein. p – reliability of differences considering the control group; p_1 – reliability of differences between Q-, non Q-MI; p_2 – reliability of differences of indices between patients with primary MI and a repeated / or recurrent MI; p_3 – reliability of differences between anterior and inferior MI.

Under the influence of treatment a reliable dynamics of humoral markers of ED and inflammation was observed (Table 3): decrease of sVCAM-1 plasma levels, total NO and serum CRP in 27-28 days and six months by 1.4-2.3 times ($p<0.01$-0.001) and 1.5-2.0 times ($p<0.01$-0.001) respectively. It should be noted that administered thrombolytic therapy (TLT) promoted a reliable additional 17.2% decrease of toxic levels of NO metabolites as compared with the treatment without TLT ($p<0.05$).

Table 3: Humoral Markers of Endothelial Dysfunction and Inflammation in Patients with Myocardial Infarction after 28 Days and 6 Months of Treatment and observation, M±S.D.

Parameters	**Before treatment, n=88**	**27-28 days of treatment, n =44**		**6 months of observation, n=44**
		After TLT, n=24	**Without TLT, n=20**	
sVCAM-1, ng/mL	1258.6±97.0	801.0±61.3 $p<0.001$	844.9±83.2 $p<0.001$	850.3±102.4 $p<0.01$
$NO/NO_2^-/NO_3^-$, mkmol/L	45.7±4.01	28.8±2.02 $p<0.001$	34.8±2.76 $p<0.01$ $p_1<0.05$	27.5±4.22 $p<0.001$ $p_2<0.05$
CRP, mg/L	10.3±1.02	4.15±0.97 $p<0.001$	4.74±1.01 $p<0.001$	5.08±1.42 $p<0.001$

Notes: TLT – thrombolytic therapy; $NO/NO_2^-/NO_3^-$ – total NO/nitrite/nitrate; sVCAM-1 (CD 106) – soluble form of vascular cell adhesive molecule; CRP – C-reactive protein. p – reliability of data differences concerning the condition before treatment; p_1 – reliability of differences with the results of patients 27-28 days after TLT treatment; p_2 – reliability of differences with the results of patients 27-28 days after treatment without TLT.

Table 4: Plasma Content of a Soluble Form of Vascular Cell Adhesive Molecule-1 (ng/mL) in Patients with Myocardial Infarction on the 28th Day of Treatment and 6 months of Observation Depending on ACE (I/D) and eNOS (894G>T) Genes Polymorphisms, M±S.D.

Genotypes of the analyzed genes		Before treatment, n=88	27-28 days of treatment, n=44		6 months of observation, n=44
			After TLT, n=24	Without TLT, n=20	
ACE	II	903.6±195.5	742.5±98.7	764.4±89.4	766.9±109.5
	I/D	875.4±143.9	779.2±80.1	800.1±113.6	803.9±125.3
	DD	1457±137.7 **II,I/D**	1029±99.0 $p<0.05$ **II. I/D**	1050±110.5 $p<0.05$ **II. I/D**	1103±92.8 $p<0.05$ **II, I/D**
eNOS	GG	1273±154.5	901.0±67.1 $p<0.05$	938.9±118.5	941.7±128.4
	TG, TT	1183±110.3	792.2±85.8 $p<0.05$	813.9±80.3 $p<0.05$	819.6±104.3 $p<0.05$

Notes: TLT – thrombolytic therapy. p – reliability of differences concerning the condition before treatment separately for every genotype; p_1 – reliability of differences after TLT separately for every genotype; p_2 – reliability of differences without TLT separately for every genotype. 3. Squared genotype – reliability of indices differences ($p<0.05$) concerning the genotype indicated within every gene (vertically) separately 28 days after treatment and 6 months of observation.

Depending on the analyzed genes' genotypes distribution (Table 4) statistically important decrease of sVCAM-1 after treatment both on the 28th day at the hospital and 6 months of the observation was found in the DD-genotype carriers of ACE gene, better with basic TLT – by 29.4% and 27.9% ($p<0.05$) and 24.3% ($p<0.05$) respectively. Herewith, sVCAM-1 in DD-genotype carriers was still higher than in the "wild" I-allele carriers ($p<0.05$). Depending on the genotypes of eNOS gene, TLT promoted a reliable decrease of sVCAM-1 in both groups by 29.2% and 33.0% respectively ($p<0.05$). Although, without TLT after 6 months treatment and observation considerable changes were found only in the "mutant" T-allele carriers by 31.2% and 30.7% respectively ($p<0.05$). Reliable differences between the genotypes of eNOS gene were not found

CRP reliably decreased after treatment in all the groups observed with statistically important benefit in DD-genotype carriers of ACE gene: with TLT – by 2.84 times ($p<0.001$), without TLT – by 2.15 times ($p<0.001$). In addition, the use of TLT promoted a more considerable decrease of CRP – on 24.3% ($p<0.05$) (Table 5). Despite a strong effect of the treatment the level of CRP in the DD-genotype carriers was higher than in I-allele carriers ($p<0.05$). Reliable differences of CRP content during treatment between the eNOS gene genotypes were not found.

Table 5: Serum Content of C-reactive Protein (mg/L) in Patients with Myocardial Infarction on the 28th Day of Treatment and after 6 Months of Observation Depending on ACE (I/D) and eNOS (894G>T) Genes Polymorphisms, M±S.D.

Genotypes of genes analyzed		Before treatment, n=88	27-28 days of treatment, n=44		6 months of observation, n=44
			After TLT, n=24	Without TLT, n=20	
ACE	II	11.7±4.08	4.18±0.52 $p<0.001$	5.03±0.69 $p<0.01$	5.38±1.44 $p<0.05$
	I/D	11.6±1.68	3.98±0.71 $p<0.001$	4.67±0.93 $p<0.001$	4.97±1.05 $p<0.001$
	DD	19.6±2.86 **II, I/D**	6.90±1.04 **II, I/D** $p<0.001$	9.12±1.10 **II, I/D** $p<0.001$ $p_1=0.049$	8.80±1.27 **II, I/D** $p<0.001$
eNOS	GG	12.7±1.90	4.95±1.09 $p<0.001$	5.43±0.97 $p<0.001$	5.51±0.82 $p<0.001$
	TG, TT	12.1±1.55	4.18±0.65 $p<0.001$	4.69±1.12 $p<0.001$	5.04±1.07 $p<0.001$

Note: similarly to Table 4.

The plasma content of total stable NO metabolites was reliably decreased after treatment (Table 6): in the D-allele carriers of ACE gene with TLT – by 39.1% and 35.2% ($p<0.001$) and without TLT – by 26.6% and 25.4% ($p<0.001$), with a reliable difference between them – by 17.0% and 13.2%, respectively ($p<0.05$). In addition, during the whole period of treatment NO level in the DD-genotype carriers were higher than in the II-genotype carriers by 16.6-27.1% ($p<0.05$). eNOS gene genotypes distribution did not influence reliably on the NO metabolites concentration during the course of treatment (Table 6). Although, TLT promoted 13.0% decrease of NO in the GG-genotype carriers ($p<0.05$) and 11.1% decrease in the T-allele carriers of eNOS gene ($p<0.05$).

Table 6: Total NO Metabolites Plasma Content (mkmol/L) in Patients with Myocardial Infarction on the 28th Day of Treatment and 6 Months of Observation Depending on ACE (I/D) and eNOS (894G>T) Genes Polymorphisms, M±S.D.

Genotypes of genes analyzed		Before treatment, n=88	27-28 days of treatment, n=44		6 months of observation, n=44
			After TLT, n=24	Without TLT, n=20	
ACE	II	43.0±3.22	26.5±2.17 p<0.001	28.0±3.90 p<0.001	25.6±2.15 p<0.001
	I/D	45.8±5.15	27.9±1.88 p<0.001	33.6±2.75 p<0.001 p_1<0.05	27.8±2.29 p<0.001 p_2<0.05
	DD	47.7±2.64	30.9±1.38 II p<0.001	35.6±1.20 II p<0.001 p_1<0.05	32.0±2.07 II p<0.001 p_2=0.051
eNOS	GG	40.1±2.10	26.8±2.41 p<0.001	30.8±1.13 p<0.001 p_1<0.05	27.1±2.46 p<0.001 p_2=0.053
	TG, TT	46.6±4.78	28.1±2.20 p<0.001	31.6±1.06 p<0.001 p_1<0.05	27.9±3.19 p<0.001

Note: similarly to Table 4.

Epidemiological analysis showed that sVCAM-1 is a potential risk factor of Q-MI (OR=4.51, 95%CI OR=1.57-13.0, p=0.003), irrespective of MI localization and sequence of occurrence (more pronounced in case of inferior localization and repeated occurrence OR=5.40, 95% CI OR=1.77-17.1, p=0.002 and OR=7.50, 95%CI OR=2.15-26.2, p=0.001, respectively). Increased content of NO metabolites (≥45 mkmol/L) is a risk marker of MI regardless of its type, localization and sequence. CRP increases the risk of Q-MI occurrence by 2.16 times (OR=3.75, 95%CI OR=1.49-9.46, p=0,004), on the anterior myocardial wall of the left ventricle – by 2.77 times (OR=7.79, 95% CI OR=2.75-22.1, p<0.0001), for the first time MI onset – by 2.08 times (OR=3.44, 95% CI OR=1.33-8.88, p=0.009).

In general, our results coincide with the findings of other researchers. Some of them proved that moderate NO hyperproduction improves tissue perfusion by means of vasodilatation, inhibits adhesion and aggregation of platelets, reveals anti-thrombotic action, and the reduction of leukocyte-endothelial adhesion can prevent occurrence of a critical stage of an inflammatory reaction [1, 4, 5, 8]. Other researchers describe absolutely opposite effects of an increased NO level [3, 11], considering that NO hyperproduction results in cytotoxic effects, apoptosis initiation, dilation of the peri-infarction area, aggravating further course and prognosis of MI. There is convincing evidence proving that endothelial dysfunction is interrelated with the systemic inflammation factors – CRP, pro-inflammatory cytokines, lipid and carbohydrate metabolism disorders, oxidant stress etc. in patients with MI [11, 17]. The combination of the above mentioned factors increases the risk of MI development [11, 17].

4. Conclusion

Q-MI is accompanied with 18.6-47.1% growth of humoral markers of endothelial damage (sVCAM-1, total stable NO metabolites) and inflammation (CRP), that intensified with inferior localization of MI. DD-genotype of ACE gene and TT-genotype of eNOS gene are associated with higher levels of sVCAM-1 and CRP by 19.1-40.8%, as well as NO metabolites in the TT-genotype carriers by 23.7%, which is indicative of more severe endothelial dysfunction due to

excessive synthesis of nitrite-anion and absence of an adequate coronary vessels compensatory response.

Increasing of sVCAM is a potential risk factor of Q-MI (OR=4.51), regardless of its localization and sequence of occurrence. Increase of NO metabolites increases the risk of MI by 4.43-4.80 times, irrespective of its type, localization and sequence. CRP increases the risk of Q-MI occurrence in 2.16 times (OR=3.75), on the anterior myocardial wall of the left ventricle – in 2.77 times (OR=7.79), primary occurrence – in 2.08 times (OR=3.44).

Complex therapy of MI promoted a reliable decrease of humoral markers of endothelial dysfunction and inflammation 27-28 days after treatment in 1.4-2.3 times, 6 months after observation – in 1.5-2.0 times. TLT administration promoted an additional reliable decrease of toxic levels of NO metabolites by 17.2%. DD-genotype of ACE gene is associated with a reliably greater decrease of sVCAM-1 and CRP levels after treatment, both 28 days in the hospital and 6 months of observation (better after TLT); in T-allele carriers of eNOS gene the level of sVCAM-1 after TLT was 30.7-31.2% lower. The NO metabolite content became less after the treatment with the use of TLT in the D-allele carriers of ACE gene (by 39.1% and 35.2%) and it did not depend on the allele content of eNOS gene.

Prospects of Research: molecular-genetic investigations of MI patients; prognosis of acute MI depending on genetic factors.

5. Conflict of Interest: None declared.

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