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Authors Milica Ćučuz Jokić*, Vesna Ilić*, Bojana Cikota -Aleksić*, Slobodan Obradović†‡, Zvonko Magić*‡, Vojnosanitetski pregled (2018); Online First October, 2018.

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Milica Ćućuz Jokić*, Vesna Ilić*, Bojana Cikota -Aleksić*, Slobodan Obradović†‡, Zvonko Magić*‡

*Institut of Medical Research, Military Medical Academy, Belgrade, Serbia
†Clinic for Emergency and Internal Medicine, Military Medical Academy, Belgrade, Serbia
‡University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia

Korespondirajući autor: Milica Ćućuz Jokić, Institut of Medical Research, Military Medical Academy, Crnotravska 17, Belgrade. Telefon: 0113608482, mobilni: 062449558, mail adresa: milicacujo@gmail.com.

Kratak naslov rada: Povezanost polimorfizama gena za faktore koagulacije II, V i metilentetrahydrofolat reduktazu sa različitim formama infarkta miokarda

Doprinos koautora izradi studije i objavljivanju rada:

Milica Ćućuz Jokić- doprinela ideji, planu, prikupljanju podataka i analizi i tumačenju podataka i izrađivanju rukopisa.

Vesna Ilić- doprinela ideji, planu, prikupljanju podataka i analizi i tumačenju podataka i izrađivanju rukopisa.

Bojana Cikota–Aleksić-doprinela ideji, planu, prikupljanju podataka i analizi i tumačenju podataka i izrađivanju rukopisa.

Slobodan Obradović- doprino ideji, planu, prikupljanju podataka i analizi i tumačenju podataka i ispravlja kritički značajni intelektualni sadržaj rukopisa.
Zvonko Magić - doprino ideji, planu, prikupljanju podataka i analizi i tumačenju podataka i ispravlja kritički značajni intelektualni sadržaj rukopisa.

Abstract

**Background /Aim,** Coagulation Factor II G20210A and Factor V G1691A variants are moderately associated with a coronary artery disease (CAD). Polymorphism of the methylenetetrahydrofolate reductase (MTHFR) gene C677T is associated with a myocardial infarction (MI) in some ethnical groups. At the present time there are rare studies which try to differentiate the two forms of MI, ST-elevation MI (STEMI) and non ST-elevated MI (NSTEMI) according to the genetic background. We tried to examine genetic factors that would be different in those two entities, included in response to plaque rupture, and occlusion of a coronary artery. To investigate the association of polymorphisms of Factor II G20210A, Factor V G1691A and MTHFR C677T, to different forms of myocardial infarction: STEMI and NSTEMI.

**Methods.** The groups comprised 82 patients, divided into two cohorts: patients with STEMI (49 patients) and NSTEMI (33 patients). The peripheral blood lymphocytes were used as a DNA source. Genotypes were determined on a polymerase chain reaction (PCR) based methodology. **Results.** The frequency of MTHFR C677T CT genotype was higher in the patients with NSTEMI, in comparison with the patients with STEMI, with odds ratio (OR) of 3.33 (95% confidence interval (CI) 1.22-9.15) p=0.02. Logistic regression analysis shows MTHFR CT genotype as an independent prognostic factor for development of NSTEMI, OR is 3.15 (95% CI 1.20-8.29), with p=0.02. There were no differences between two patients groups in frequency of Factor II G20210A and Factor V G1691A gene polymorphism. **Conclusion.** MTHFRC677T CT genotype was significantly associated with the development of NSTEMI, in examined patients.

**Key words:** coagulation factor II, coagulation factor V, methylenetetrahydrofolate reductase, genetic polymorphism, myocardial infarction, STEMI, NSTEMI.

Apstrakt

**Uvod / Cilj.** Varijante faktorakoagulacije II G20210A I faktora V G1691A su umereno udružene sa koronarnom arterijskom bolešću. Polimorfizam gena za metilentetrahidrofolat reduktazu (eng. methylenetetrahydrofolatereductase MTHFR) C677T je udružena sa infarktom miokarda u nekim etničkim grupama. Retke su aktualne studije koje pokušavaju da diferentijuju dve forme infarkta miokarda, sa elevacijom ST segmenta (eng. ST-elevation myocardial infarction -STEMI) i infarkta miokarda bez elevacije ST segmenta (eng. non ST-elevated myocardial infarction NSTEMI), u odnosu na genetičku osnovu. Pokušali smo da ispitamo genetičke faktoare koji bi bili različiti u ova dva entiteta, uključene u rupturu plaka i okluziju koronarne arterije. Utvrdili povezanost polimorfizama faktora II G20210A, faktoraV G1691A I MTHFRC677T sa različitim formama infarkta miokarda: STEMI I NSTEMI. **Metode.** Grupa se sastojala od 82 pacijenta, podeljena u dve kohorte: pacijente sa STEMI (49 bolesnika) i pacijente sa NSTEMI (33 bolesnika). Korišćeni su limfociti periferne krvi kao izvor DNK. Genotipovi su određivani po metodologiji zasnovanoj na lančanoj rekciji polimeraze. **Rezultati.** Učestalost MTHFRC677TCT genotipa je bila veća
u pacijenata sa NSTEMI u poredjenju sa pacijentima sa STEMI, sa odnosom šansi (eng. oddsratio OR) od 3.33 i 95% intervalom poverenja (eng. confidence interval CI) od 1.22-9.15 sa p=0.02. Logistička regresionalna analiza pokazuje da je MTHFR CT genotip nezavisan prognostički factor za razvoj NSTEMI, OR je 3.15 (95% CI 1.20-8.29), sa p= 0.02. Nije bilo razlike izmedju dve grupe pacijenata u učestalosti faktora II G20210A I faktora V G1691A genskih polimorfizama. Zaključak. MTHFR C677T CT genotip je značajnopovezansarazvojem NSTEMI formemiokardnoginfarkta u ispitivanimbolesnikama.

Ključne reči:
faktor koagulacije II, faktor koagulacije V, metilentetrahidrofolat reduktaza, genetički polimorfizam, infarct miokarda, STEMI, NSTEMI.

Introduction

Myocardial infarction (MI), and an ischemic heart disease are the leading mortality factor world-wide (1). According to the third universal definition of the myocardial infarction, this disease is diagnosed upon specific electrocardiogram (ECG) patterns, as STEMI and NSTEMI (2). The clinical characteristics are different in these two forms of MI, as the the histology of plaque. Patients with STEMI show more severe plaque rupture of the coronary culprit lesions than patients with NSTEMI (3). Also, the fibroatheromas in NSTEMI were more calcified than in the STEMI (4). If the patients have recurrent infarctions, the type of infarction is often of the same type, which suggest that the individual patient is prone for developing of either STEMI or NSTEMI (5). There are established 50 genetic risk variants in the genome-wide association studies (GWAS) for the coronary artery disease (CAD), and MI (6). Among this genetic risk variants for the CAD there are some genes that are more related to the plaque rupture and thrombosis than atherosclerosis. Unfortunately, in the many genetic studies there are no clear distinguish of the different forms of CAD and MI, leading to complicated conclusions of the impact of genetics in development of the particular disease. The literature is poor of the genetic risk factors which differentiate STEMI and NSTEMI (7). Most of the reported single nucleotide polymorphisms (SNPs) were demonstrated to be associated with the CAD, and not specifically with the MI, so we presumed that genetic risk factors involved in the atherosclerosis, common condition for CAD and MI, are same in the STEMI and NSTEMI. We tried to examine the genetic factors that would be different in those two entities, included in a response to the plaque rupture, and an occlusion of coronary artery. We supposed that the SNP of the coagulation factors II, V and polymorphism in the gene involved in metabolism, MTHFR, also known as risk factor for CAD (8) and a vein thrombosis (9), would have different distribution in STEMI and NSTEMI. Factor V single base polymorphism, G1691A (factor V Leiden) leads to change in a functional protein which reduce a protein C cleavage sites from three to only one site, and leads to an increased thrombin production. Prothrombin G20210A polymorphisms affects a single base, but in a promoter region of gene. This polymorphisincreases the prothrombin production to levels of 30%, and 70% higher in the heterozygous and homozygous individuals, respectively, than in those who does not have it (10). It is shown in meta-analysis that polymorphisin factor V Leiden and factor II is associated with the CAD, and that per-allele relative risk is 1.17 for factor V and 1.31 for factor II mutation (11). MTHFR C677T gene polymorphism, (alanine to valin substitution)
which results in a thermo labile form of the enzyme, is established as a risk factor for developing CAD, with clear evidence for the TT, and a trend towards an increased risk, for the CT genotype (12). There are some studies which demonstrated association of MTHFR polymorphism with early onset CAD, (13) and others do not (14). The homozygous form of the MTHFR C677T gene polymorphism is associated with elevated homocysteine in plasma. Experimental evidence concluded the homocysteine is involved in an endothelial dysfunction and injury, followed by activation of the platelets and thrombus formation (15,16). Some studies showed mean homocysteine concentrations modestly increased in CT heterozygotes in comparison with CC homozygotes (17,18). In our study we analyzed the factor II G20210A, factor V G1691A and the MTHFR C677T variants in the patients with STEMI and NSTEMI, who underwent a percutaneous transluminal coronary angioplasty (PTCA) with a bare metal stent implantation. The aim of the study was to determine impact of the genetic polymorphism of the factor V, factor II and MTHFR on different forms of MI among patients with STEMI and NSTEMI.

Methods

Study Population

In this observational, retrospective study, eighty two patients with myocardial infarction were included, 62 men (76%) and 20 women (24%), who went to PTCA, and the bare metal stents implantation. The patients were admitted to the Clinic of Emergency Internal Medicine between periods of 2008 to 2010. Patients with acute and chronic autoimmune conditions, and the malignant diseases were excluded from the study. Among all patients, 49 (60%) were presented with STEMI and 33 (40%) were presented with NSTEMI. Patients with STEMI and NSTEMI were diagnosed and treated according to the criteria of European Society of Cardiology/ACCF/AHA/World Heart Federation Task Force for the Universal Definition of Myocardial Infarction (19,20). The main risk factors (elevated cholesterol, hypertension, obesity, smoking, diabetes mellitus and family history of CAD) were documented for each patient. Diabetes mellitus was diagnosed as elevated fasting plasma glucose level of more than 11 mol/L, or self-reported by patients, who used insulin or oral hypoglycemic agents. Hypertension was documented when a blood pressure was more than 140/90mmHg, or when patients use antihypertensive therapy. Total cholesterol values used in the study were obtained from the fasting plasma samples and included values of more than 4.64 mmol/L for man and 4.76 mmol/L for woman. Positive family history was defined if there is a history of CAD in at least one first or second relative degree. Obesity was categorized as body mass index (BMI) of more than 25kg/m². Levels of a C-reactive protein (CRP) were determined (normal range up to 4mg/L), at the hospital admission, and every day, up to end of a hospital stay. Creatine kinase-MB fraction (CK-MB), (normal ranges from 0.00 to 25 U/L), was measured at the hospital admission, and determined CKMB-maximal value in the follow up to 48 hours of a hospital admission, every 6 hours. Triglyceride and a total cholesterol were measured at the time of hospitalization. Information about a current smokers, were documented, too. Thrombolysis in myocardial infarction (TIMI) flow grade at baseline was determined by an angiography. All the patients gave a written informed
consents, and the study was approved by the Ethical Committee of Military Medical Academy.

**Polymorphism analysis**

Sodium citrate anticoagulant blood was obtained from each patient by a peripheral venipuncture. DNA was extracted by the salting-out method (21). The factor II G20210A (rs 1799963), factor V Leiden G1691A (rs 6025) and the MTHFR C677T (rs 1801133), was genotyped using the AttomolQuicktype PCR kit (Germany), according to a manufacturer’s instructions. Both alleles of gene of interest were specifically amplified. Examinations of the amplified products were performed by an agarose gel electrophoresis.

**Statistical analysis**

Results were presented as means ± standard deviation, for the numerical data with a normal distribution, or median, with 25% to 75% percentile for numerical, nonparametric data, and frequency distributions for the categorical variables. For the numerical variables the statistical significance was determined by Student t test, or Mann-Whitney test for the nonparametric numerical data. Statistical significance within the categorical variables, genotype frequencies, between patients with STEMI and NSTEMI was tested by Chi-square test. Association between the factor II G20210A, factor V Leiden G1691A and the MTHFR C677T genotypes with different forms of a MI, was determined by Odds ratio (OR), with two-tailed p values and 95% confidence interval (CI). P values less than 0.05 were considered significant. Logistic regression analysis was used to determine the independent predictors in patients with STEMI and NSTEMI. We use the Statistical Package for the Social Science (SPSS), version 13.0, Chicago, for windows.

**Results**

**Clinical characteristics of patients**

Characteristics of patients with STEMI and NSTEMI are presented in table 1.
Table 1

| Characteristics of patients | STEMI (n = 49) | NSTEMI (n = 33) | p Value
|-----------------------------|----------------|----------------|---------|
| Age (years)                 | 57.84 ± 9.99   | 57.97 ± 9.74   | 0.48    |
| Male/female                 | 40/9           | 22/11          | 0.12    |
| **CAD risk factors:**       |                |                |         |
| Hypertension†               | 31 (63.26%)    | 22 (66.67%)    | 0.75    |
| Diabetes mellitus†          | 16 (32.65%)    | 14 (42.42%)    | 0.37    |
| Positive family history†    | 22 (44.89%)    | 18 (54.55%)    | 0.39    |
| Current smoker‡             | 22 (44.89%)    | 12 (36.37%)    | 0.44    |
| Obesity (BMI>25kg/m²)†      | 32 (65.31%)    | 28 (84.85%)    | 0.05    |
| Hypercholesterolemia†       | 34 (69.38%)    | 16 (48.49%)    | 0.06    |
| **Laboratory data:**        |                |                |         |
| Triglyceride (mmol/L)§      | 1.95 ± 0.98    | 1.36 ± 0.53    | <0.01   |
| CRP (mg/L)§                 | 39.20 (21.00-52.90) | 20.10 (11.90-35.10) | <0.01   |
| Fibrinogen (g/L)§           | 3.95 ± 1.37    | 3.61 ± 0.90    | 0.100   |
| Total cholesterol (mmol/L)§ | 5.59 ± 1.10    | 4.91 ± 0.94    | <0.01   |
| CKMB at hospital admission (UL)§ | 23.00 (14.00-34.00) | 27.00 (14.00-33.00) | 0.71   |
| CKMB Max (UL)§              | 262.00 (150.00-384.00) | 59.00 (46.00-123.00) | <0.01   |
| **Coronary angiographic data:** |          |                |         |
| Multivessel coronary disease† | 22 (44.90%)    | 11 (33.33%)    | 0.29    |
| TIMI flow grade 3 at baseline§ | 13 (26.53%)    | 17 (51.51%)    | **0.02** |
| **Medications at the time of PTCA** |    |                |         |
| Aspirin†                    | 49 (100.00%)   | 33 (100.00%)   | 1.00    |
| Clopidogrel†                 | 49 (100.00%)   | 33 (100.00%)   | 1.00    |
| Heparin/Enoxaparin†         | 49 (100.00%)   | 33 (100.00%)   | 1.00    |
| Beta blockers§               | 24 (48.97%)    | 15 (45.45%)    | 0.75    |
| ACE inhibitors§              | 10 (20.41%)    | 9 (27.27%)     | 0.44    |
| Calcium blockers§            | 12 (24.49%)    | 13 (39.39%)    | 0.15    |
| Statins§                     | 39 (79.59%)    | 25 (75.76%)    | 0.68    |

CAD = coronary artery disease, ACE = angiotensin-converting enzyme, BMI = body mass index, CKMB = creatine kinase-MB form, determined at hospital admission, CKMB Max = creatine kinase-MB form-maximal value up to 48h of hospital admission, CRP = C-reactive protein maximal values measured up to 72h of hospital admission, STEMI = ST elevation myocardial infarction, NSTEMI = non ST elevation myocardial infarction, TIMI = thrombolysis in myocardial infarction, * = statistical significance determined by Student t-test, † = statistical significance determined by Chi-square test, ‡ = statistical significance determined by Mann-Whitney test.

The analyzed groups of the patients had significantly different triglyceride levels (1.95 ± 0.98 mmol/L in STEMI vs 1.36 ± 0.53 mmol/L in NSTEMI, p<0.01) and total cholesterol levels (5.59 ± 1.10 mmol/L in STEMI vs 4.91 ± 0.94 mmol/L in NSTEMI, p<0.01). The median value of maximal CRP measured up to 72h of a hospital admission was 39.20 (21.00-52.90) mg/L in the STEMI group and 20.10 (11.90-35.10) mg/L in the NSTEMI group (p<0.01). Median CKMB max, measured up to 48h of a hospital admission was
262.00 (150.00-384.00) U/L in the patients with STEMI and 59.00 (46.00-123.00) U/L in patients with NSTEMI (p<0.01). Thrombolysis in myocardial infarction (TIMI) flow grade 3 at baseline was present in 13 (26.53%) patients with STEMI and in 17 (51.51%) patients with NSTEMI (p=0.02).

The usage of all medications (beta blockers, aspirin, clopidogrel, heparin, angiotensin converting enzyme - ACE inhibitors, calcium blockers and statins) was not significantly different between STEMI and NSTEMI group.

The frequency of the risk factors for MI (smoking, hypertension, obesity, hypercholesterolemia, and positive family history) was not significantly different between patients with STEMI and NSTEMI.

Association between genotypes and disease characteristics

Distribution of FII G20210A, FV G1691A and MTHFR C677T genotypes in the patients with STEMI and NSTEMI is presented in the table 2.

Table 2

| Genotype  | STEMI group | NSTEMI group | Odds ratio | P value |
|-----------|-------------|--------------|------------|---------|
|           | n (%)       | n (%)        |            |         |
| Factor II G20210A |             |              |            |         |
| GG        | 47 (95.92)  | 32 (96.97)   | 1.36 (0.12-15.66) | 1.00    |
| GA        | 2 (4.09)    | 1 (3.03)     |            |         |
| Factor V G1691A |             |              |            | 0.39    |
| GG        | 44 (89.80)  | 32 (96.96)   | 3.64 (0.41-32.65) |         |
| GA        | 5 (10.20)   | 1 (3.03)     |            |         |
| MTHFR C677T |   |   |   |
|------------|---|---|---|
| CC         | 24 | 9 (27.27) | 3.33 (1.22-9.15) |
| CT         | 16 | 20 (60.61) | 0.84 (0.21-3.44) |
| TT         | 9  | 4 (12.12)  | 2.56 (0.99-6.63) |
| CT+TT      | 25 | 24 (72.73) | 1.00 |

GG genotypes were used as reference for Factor II G20210A and Factor V G1691A gene polymorphism, CC genotype for MTHFR C677T gene polymorphism, for testing null hypothesis by calculating Chi square test. There were no patients with AA genotype, for both Factor II and Factor V gene polymorphisms.

The frequency of MTHFR CT genotype (p=0.02) and combined CT and TT genotype of MTHFR C677T polymorphism was higher in patients with NSTEMI (p=0.05). In the analyzed group of 82 patients with MI, GG genotype of FII G20210A was found in 79 (96%) patients while three (4%) patients had GA genotype. FV G1691A GG genotype was present in 76 (93%) and GA genotype in six (7%) patients. MTHFR C677T CC genotype was found in 33 (40%) patients while 36 (44%) and 13 (16%) patients had CT and TT genotypes, respectively.

Association between FII G20210A, FV G1691A and MTHFR C677T genotypes and the clinical characteristics (male vs female, age ≤ 45 years vs > 45 years, univessel vs multivessel disease, STEMI vs NSTEMI) was not statistically significant.

We used the traditional risk factors, which were different among our patients with STEMI and NSTEMI, along with the genetic factors, to analyze prediction for NSTEMI development. Logistic regression analysis revealed that MTHFR C677CT genotype alone was predictor for NSTEMI development, OR is 3.15 with p=0.02 (Table 3).

### Table 3

| Predictive factors for outcome | Odds Ratio (95% CI) | p value |
|-------------------------------|---------------------|---------|
| Hypercholesterolemia          | 0.52 (0.11-1.42)    | 0.20    |
| Factor II G20210A             | 1.01                | 1.00    |
Factor V G1691A        0.61   0.672
      (0.06-6.14)          0.02
MTHFR C677T CT genotype 3.15
      (1.20-8.29)          0.15
Triglyceride
      (0.17-1.32)

NSTEMI = non ST - elevated myocardial infarction, 95% CI = 95% confidence interval, MTHFR C677T CC+TT genotype and Factor II G20210A GG, factor V G1691A GG genotype were used as a reference for OR calculation in logistic regression model

We also tried to use combined MTHFR C677T CT+TT genotype, as predictor for the NSTEMI development. MTHFR C677T CC genotype was used as a reference for OR calculation in the logistic regression model. According to the results of logistic regression analysis, combined genotypes CT and TT of MTHFR C677T polymorphism showed statistical trend of increased risk for NSTEMI, OR is 2.47, with 95% CI (0.92 - 6.62), p=0.07, but did not reach a statistical significance.

Discussion

Patients from our study showed no difference according to the traditional risk factors (hypertension, diabetes mellitus, hypercholesterolemia, BMI, history of smoking and positive family history, for developing CAD and MI, in groups of STEMI and NSTEMI. Similar results were published in study of Ţaliaduonytė-Pekšienė D et al. (22). No difference according to the coronary risk factors among patients with STEMI and NSTEMI were obtained in the study of Miyachi H et al. (23).

The patients with STEMI, in our study, had mean total cholesterol and triglyceride levels higher than the patients with NSTEMI. In the previously mentioned study of Ţaliaduonytė-Pekšienė D et al., there were no difference in a mean total cholesterol and triglyceride levels in the two patients groups. In the study of Belle L. et al., in France, patients with NSTEMI showed higher triglyceride levels than patients with NSTEMI. These differences can be explained by larger number of the patients in last study, which can increase a precision in statistics (24).

STEMI patients in our study had a higher absolute level of CRP, and maximal CK-MB levels, than the patients with NSTEMI. It may be due to a different inflammatory response to myocardial injury, in those two MI groups, or a difference in some inflammation mediators, which are included in a pathogenesis of MI. Similar results were observed in the
study of Di Stefano (25), where patients with STEMI had higher values of the inflammatory markers at a hospital admission. Significant difference among STEMI and NSTEMI patients, according to the higher peak CRP levels, were demonstrated in the study of Habib SS et al. (26).

There are no many documented genetic data, associated with MI, to differentiate STEMI and NSTEMI. (7.) There are no genetic data which differentiate polymorphisms of the coagulation factor II and V with STEMI and NSTEMI.

In our study, there were no differences in the frequency of polymorphism of the factor II G20210A, and factor V G1691A, between patients with STEMI and NSTEMI. In the study of Sode BF et al, there was no association of the factor V Leiden and prothrombin G20210A polymorphisms with the myocardial infarction. In their study there were no differentiation of two categories of MI, STEMI and NSTEMI (27). On the contrary, in the study of Ezzat H. et al, in the Egyptian population, the prevalence of heterozygous FV Leiden, and also recessive homozygous AA was higher in the patients with MI than in a control group. In this study there was no differentiation between STEMI and NSTEMI (28).

In our study, we found that there is a difference between patients with two forms of MI, STEMI and NSTEMI, according to the genotypes of MTHFR C677T. The frequency of CT genotype of MTHFR C677T is higher among the patients with NSTEMI in surveyed population, and lower for STEMI patients group.

When we use dominant model, namely CT+TT, in comparison with CC, we also found that this genotypes have increased risk for NSTEMI. There are no data about the different genotypes of MTHFR in the two categories of MI, STEMI and NSTEMI. In the study of Xuan C et al., in meta-analysis, they found that there were significant risk of MI in the model TT versus CT, for the MTHFR gene, in Caucasian population (30). The results of our study are in accordance with a previously published study by Isordia-Salas I. et al. in young Mexican patients (younger than 45 years). Polymorphism of the MTHFR C677T was not associated with development of STEMI in the young Mexican patients (14). It is known that the MTHFR C677T genotypes have different ethnical and geographical distribution (31). The TT genotype was common in Mexico (32%), southern Italy (26%), and northern China (20%). There was sort of a geographic increase in the Europe (north to south) and China (north to south decrease). In the big meta-analysis of Alizadeh S and coworkers (32) in the population of patients with MI there were differences in the polymorphism of MTHFR according to the ethnical groups. Their results showed that the T allele of C677T polymorphism is not associated with an increased risk for MI in European, Asian and North American population, but is associated in African population. In this analysis the CT genotype was associated with decreased risk of MI in North American population and in elderly people. Again, this meta-analysis did not separate the clinical forms of MI. Hyperhomocysteinemia is one of possible underlying mechanism for development of the MI and CAD. In the study of Chao-Hung Ho (33), is suggested that plasma homocysteine is important risk factor for the CAD, and some other diseases, but it
is important to include factors, as the MTHFR polymorphism, vitamin B12, triglycerides, total cholesterol, that can affect homocysteine metabolism.

In the meta analysis of Kluijtmans (34) is shown that all three MTHFR C677T genotypes confer different levels of the atherothrombotic risks. The CT heterozygotes had elevated risk according to the CC homozygotes. The first explanation is that the CT genotype actively confers atherothrombotic risk. Second explanation proposed by these authors is that the CC is a protective genotype, for development of atherothrombotic disease. We did not find studies which differentiated STEMI and NSTEMI according the polymorphism distribution of the MTHFR C677T. Possible explanation for finding no statistical difference between the TT and the CC MTHFR C677T genotypes is a small group of our patients, which implicated small number of the TT homozygous individuals. It is not clear, what underlines our finding of higher frequency of the CT genotypes of MTHFR C677T, in cohort of NSTEMI patients.

Discrepancies observed in all these aforementioned studies demands us for better grouping of MI patients, according to the age, gender, ethnic background, food intake, folic acid supplementation and most important the different forms of disease.

**Conclusion**

The limitation of study was a small number of patients. The MTHFR C677T, CT genotype was significantly associated with development of NSTEMI among MI patients. As MI is a multifactorial disease in which combination of the environmental factors and the genetic background, both play role in its development, more studies are needed to determine clear association of the MTHFR C677T gene polymorphism for development of NSTEMI.

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