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Association of Methylenetetrahydrofolate (MTHFR) and Apolipoprotein E (Apo E) Genotypes with Homocysteine, Vitamin and Lipid Levels in Carotid Stenosis

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A B S T R A C T

The aim of the study was to investigate the association between methylenetetrahydrofolate (MTHFR) genotypes and levels of homocysteine (Hcy), folate, vitamin B12 and lipids as well as the association between apolipoprotein E (apo E) genotypes and levels of lipids in a Croatian healthy control group and a group of patients with >70% carotid stenosis (CS). The study included 98 Croats, 38 patients with >70% carotid stenosis and 60 age- and sex-matched controls. The MTHFR and apo E genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), Hcy by enzyme immunoassay, vitamins by immunochemiluminiscence, and lipids by spectrophotometric method. There was no difference between control subjects and CS patients in the distribution of C677T MTHFR genotypes ($p=0.786$) and alleles ($p=0.904$), however, differences in the frequencies of apo E genotypes ($p=0.012$) and alleles ($p=0.029$) were statistically significant. The odds ratio for apo E 3/4 genotype was 3.93 (95% CI 1.23–12.61). Hyperhomocysteinemia (≥15 μmol/L) was found in 11% of CS patients and 5% of control subjects. Total cholesterol, triglycerides, vitamin B12 and folate were statistically different in »all MTHFR genotypes« ($p<0.001$, $p<0.01$, $p=0.044$ and $p=0.036$, respectively), and in TC/TT ($p<0.001$, $p=0.003$, $p=0.030$ and $p=0.032$, respectively) groups. The levels of total cholesterol, LDL cholesterol and triglycerides in the apo E 3/3, and total cholesterol in the apo E 3/4 group yielded statistical difference. An association was found of apo E 3/4 genotype but not of MTHFR genotypes with the risk of CS. MTHFR and apo E affect blood lipid levels, which was statistically confirmed. An association was also recorded between hyperhomocysteinemia and patients with CS. Vitamin status in CS showed a statistically verified association with TC/TT MTHFR genotype. In the group of patients with TC/TT MTHFR genotype, lower vitamin B12 and higher folate values were recorded. The results of multiple logistic analysis showed that there was no statistical significance of Hcy levels (OR 2.403, $p=0.334$) or conventional vascular risk factors such as smoking habit (OR 0.505, $p=0.149$), age (OR 1.048, $p=0.087$) or sex (OR 2.037, $p=0.112$) in predicting CS.

***Key words**: MTHFR, apo E, genotype, lipids, vitamin status, homocysteine, carotid stenosis*

Introduction

Extracranial atherosclerotic carotid disease is the leading cause of stroke and transient ischemic attacks (TIA) in Western populations[1]. In addition to the established risk factors for atherosclerosis (diabetes, tobacco use, decreased high-density lipoprotein level, hyperlipidemia, hypertension, positive family history, etc.), epidemiologic data indicate that elevated homocysteine (Hcy) concentrations are associated with an increased risk of

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cardiovascular disease, including coronary artery disease, cerebrovascular disease and peripheral arterial occlusive disease[1,2].

Hcy is a putatively atherothrombotic sulfur amino acid produced during methionine metabolism. It is catabolized to cystathionine and cysteine by cystathionine β-synthase. Significant amounts of methionine may be regenerated by the remethylation pathway, a reaction catalyzed by methionine synthase. Vitamin B12 is a cofactor and methyltetrahydrofolate a substrate in this reaction. Methyltetrahydrofolate, the predominant circulating form of folate found in the blood, is formed in a reaction catalyzed by 5,10-methylene tetrahydrofolate reductase (MTHFR). This enzyme has a strong indirect influence on Hcy remethylation[3]. The main determinants of mild hyperhomocysteinemia are folate and B12 intake, impaired renal function, and genetic factors[3].

A common missense mutation, C677T, has been identified in the MTHFR gene where alanine is substituted by valine, which results in a thermolabile variant of the reductase[4,5]. The data have shown that the frequency of the mutation varies among different populations[6,7], and the association of MTHFR genotype and carotid artery atherosclerosis has been controversial[7–10].

High levels of total and low density lipoprotein (LDL) cholesterol and low levels of high density lipoprotein (HDL) cholesterol are known to predispose to the development of atherosclerosis[11,12]. Also, lipid metabolism in humans is strongly affected by polymorphisms of a number of genes[11]. The apolipoprotein E (apo E) gene, which also belongs to this group, is located on chromosome 19 and has three common allelic variants known as apo E*2 (ε2), apo E*3 (ε3) and apo E*4 (ε4)[12]. Studies of apo E allele distributions in different ethnic groups have shown similar patterns for most Caucasian populations. The ε3 allele is most common, with frequencies between 0.70 and 0.85, ε4 is less frequent, 0.10–0.20, and ε2 is the rarest one with a frequency of 0.05–0.10[13]. Also, it is already known that the prevalence of apo E allele differs among populations[14]. Additionally, studies on apo E and carotid atherosclerosis have yielded inconsistent results[10,15–20]. As this association has not yet been fully clarified, additional research is needed for definite conclusions[21].

The aim of our study was to investigate the association between MTHFR genotypes and levels of Hcy, folate, vitamin B12 and lipids as well as the association between apo E genotypes and levels of lipids in a Croatian healthy control group and group of patients with >70% carotid stenosis.

Materials and Methods

Subjects

In this study, C677T MTHFR and apo E genotypes as well as blood lipid, homocysteine and vitamin (vitamin B12 and folate) levels were determined in 98 subjects, residents of Zagreb. Patients (38 patients; 9 female and 29 male, mean age 62±9 years) with a severe carotid stenosis (>70%) according to the ECST criteria were included[22]. All patients had a clinical diagnosis of atherosclerosis based on ultrasonography color Doppler imaging findings. The control group included 60 sex- and age-matched healthy volunteers. EDTA-blood was collected by venipuncture after an overnight fast and important exclusion criteria was using of multivitamins. Patient group was under medication treatment (aspirin, antihypertensive or oral antidiabetics) which was administered after blood drawing. An informed consent was received from each subject prior to analysis. The study was approved by the Ethics Committee of the University Hospital »Sestre milosrdnice«, Zagreb.

C677T MTHFR and apo E genotyping

The C677T MTHFR gene mutation and apo E genotypes were detected by a DNA-based method consisting of DNA isolation and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[4,7,23]. Leukocyte DNA was isolated by the phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation method[24,25].

The C677T substitution in the MTHFR was identified as previously described[4,7]. The apo E genotypes were identified by a slightly modified method described by Dallinga-Thie et al.[24]. Briefly, target DNA (0.3 μg) was amplified by PCR using PCR Core Kit (Roche Diagnostics, Mannheim, Germany) and two specific primers (MWG Biotech, Ebersberg, Germany) in a DNA thermal cycler (ProGene thermal cycler, Techne, Duxford, Cambridge, UK). The sequence of primers used for MTHFR gene amplification was: 5′- tgaaggagaaggtgtctgcggga-3′ and 5′-aggacggtgcggtgagagtg-3’; and the sequence of primers for apo E gene amplification was: P1:5′- agaattcgccccggcctggtacac-3′ and P2: 5′-taagcttggcacggctgtccaagga-3′. The fragments were amplified separately, in reaction volume of 50 μL using 0.25 μM primers, 200 μM dNTP, 1X PCR buffer with 1.5 mM $MgCl_2$ and 1 U Taq polymerase. Formamide (1 μL/50 μL) was added in the reaction mixture of apo E gene amplification as a procedure modification resulting in a higher quantity of specific PCR product with a very sharp and bright band on the gel photography.

Each reaction mixture was subjected to 30 cycles of 30 s at 95 °C, 30 s at 60 °C and 60 s at 72 °C, initial denaturation for 5 min at 95 °C, final extension for 5 min at 72 °C. PCR products (198 bp of MTHFR gene, and 244 bp of apo E gene) were checked electrophoretically in a Clearose BG-ET gel (Elchrom Scientific AG, Basel, Switzerland) using SEA 2000 apparatus (Guest Elchrom Scientific AG, Basel, Switzerland) in 30 mM Tris-acetic-EDTA (TAE) buffer. Negative quality controls generated by inclusion of all reagents except for DNA and positive quality control containing a known genotype were included in every run. Positive quality control containing a known apo E genotype was obtained by the courtesy of Dallinga-Thie GM, Department of Medicine and Endocrinology, Utrecht, The Netherlands.

The specific MTHFR and apo E amplified products were digested using Hinf I (G↓AnTC) and Cfo I (GCG↓C) (Roche Diagnostics, Mannheim, Germany), respectively. Upon digestion, the MTHFR cleaved products were submitted to electrophoresis on 12% PolyNAT gel (Guest Elchrom Scientific AG, Basel, Switzerland), while the apo E cleaved products were analyzed on Spredex EL 400 gel (Guest Elchrom Scientific AG, Basel, Switzerland). Both gels were stained for 30 min by SYBER Green I day (Molecular Probes, Leiden, The Netherlands) and destained overnight by distilled water. Restriction fragments were visualized under UV transillumination and photographs were obtained. MTHFR and apo E genotypes were detected on the basis of restriction fragment length and unique genotype-dependent band combination.

Plasma Hcy determination

Total plasma Hcy was measured by the enzyme immunoassay method (Axis-Shield AS, Oslo, Norway) described by Frantzen *et al*.[26]. The reference range of Hcy level was 5–15 μmol/L.

Plasma vitamin B12 and folate determinations

Vitamin B12 and folate were determined by the chemiluminescence method using test packages (Bayer Corp., NY, USA) for the Bayer ACS:180 Plus autoanalyzer. The reference values of plasma vitamin B12 and folate were 148–664 pmol/L and 2.5–45.4 nmol/L, respectively.

Plasma lipid determinations

Total cholesterol, HDL cholesterol, and triglycerides were measured by standard methods using available tests (Olympus Diagnostica GmbH, Clare, Ireland) for the Olympus AU 600 autoanalyzer. Total cholesterol and triglycerides were detected spectrophotometrically, whereas HDL cholesterol was analyzed by a combination of spectrophotometric and immunoinhibition method. LDL cholesterol levels were calculated using Friedewald equation, LDL-cholesterol = (total cholesterol/2.18 – HDL cholesterol).

Statistical analysis

To analyze Hcy, vitamin B12, folate and lipid differences, comparisons between groups (classified according to genotype and two study groups) were carried out with Mann-Whitney test. The χ^2-test was used to compare C677T MTHFR and apo E allele and genotype frequencies as well as distribution in value-dependent (Hcy, vitamins and lipids) groups between patients and controls.

The association between homocystein levels (independent variable) and CS (dependent variable) was examined by means of a multiple logistic regression model, with adjustment for age, sex, and smoking as conventional vascular risk factors. Results were expressed as odds ratio (OR) together with their 95% confidence interval (CI). Statistical significance was taken as $p<0.05$.

The statistical programs used were SigmaStat (version 2.0, Jandel Corporation, Chicago, IL, USA) for Mann-Whitney, χ^2-test and multiple logistic regression analysis, and a program found on Internet for OR calculation[27].

Results

C677T MTHFR and apo E genotype analysis

The frequencies of C677T MTHFR and apo E genotypes in controls and patients with >70% carotid stenosis (CS) are presented in Figure 1.

The frequencies of MTHFR genotypes (CC/TC/TT) and alleles (C/T) were 45/50/5 and 70/30 in the control group, and 47/45/8 and 70/30 in the patient group, respectively. C677T MTHFR genotypes were in Hardy-Weinberg equilibrium (HWE) for CS patients (p=0.972) and controls (p=0.178). There was no statistically significant difference between the study groups in the distribution of C677T genotypes (p=0.786) and alleles (p=0.904).

All three common alleles of apo E were detected, whereas apo E 2/2 and 4/4 genotypes were not found among study subjects. The frequencies of apo E genotypes (3/3, 3/4, 2/3 and 2/4) and alleles (2/3/4) were 80/8/9/3 and 6/88/6 and in the control group, and 50/26/21/3 and 12/74/14 in the patient group, respectively. Apo

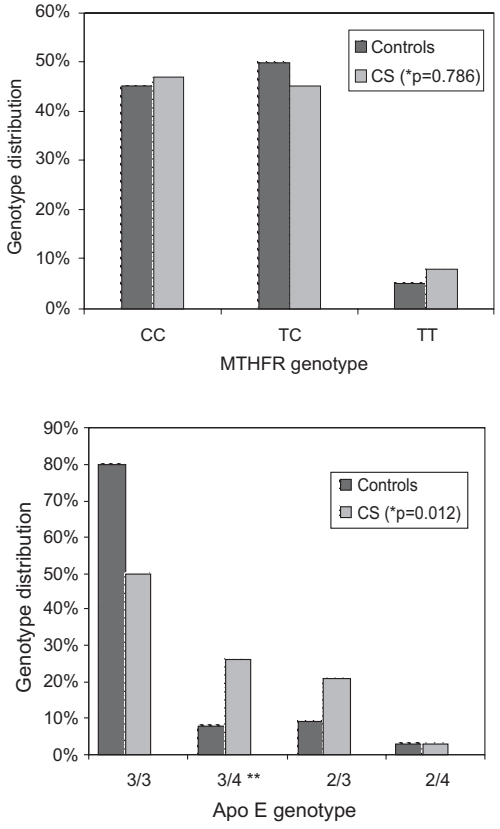

*Fig. 1. MTHFR (upper panel) and apo E (down panel) genotype distribution in control subjects (N=60) and carotid stenosis patients (CS, N=38). *p-value (χ^2-test) for differences among study group genotype frequencies, **OR (odds ratio) of apo E 3/4 genotype was 3.93 (95% CI 1.23–12.61), CI – confidence interval.*

TABLE 1
HOMOCYSTEINE, VITAMIN B12, FOLATE AND LIPIDS IN CONTROL SUBJECTS AND CAROTID STENOSIS PATIENTS WITH DIFFERENT MTHFR GENOTYPES, AND DISTRIBUTION OF STUDY SUBJECTS ACCORDING TO PARAMETER REFERENCE RANGES

	Controls (N=60), CC genotype (N=27), *TC/TT genotype (N=33) median (range)	**CS (N=38), CC genotype (N=18), *TC/TT genotype (N=20) median (range)	p-value
Homocysteine (μmol/L)			
CC MTHFR	8.75 (0.60–15.20)	8.00 (1.30–19.90)	ns
TC/TT MTHFR	8.60 (3.00–18.40)	7.35 (2.90–15.90)	ns
All genotypes	8.70 (0.60–18.40)	7.50 (1.30–19.90)	ns
≥15.00 μmol/L, N (%)	3 (5)	4 (11)	ns
<15.00 μmol/L, N (%)	57 (95)	34 (89)	
Vitamin B12 (pmol/L)			
CC MTHFR	406.53 (75.99–1011.52)	324.63 (57.55–799.04)	ns
TC/TT MTHFR	472.19 (127.64–1274.92)	241.63 (15.49–602.68)	0.030
All genotypes	418.33 (75.99–1274.92)	287.00 (15.49–799.04)	0.044
<148.00 pmol/L, N (%)	3 (5)	9 (23.7)	0.021
148.00–664.00 pmol/L, N (%)	51 (85)	25 (65.8)	
>664.00 pmol/L, N (%)	6 (10)	4 (10.5)	
Folate (nmol/L)			
CC MTHFR	7.37 (0.23–46.08)	11.46 (0.23–46.31)	ns
TC/TT MTHFR	2.50 (0.23–41.09)	19.07 (0.23–43.36)	0.032
All genotypes	3.18 (0.23–46.08)	11.46 (0.23–46.31)	0.036
***<2.5npmol/L, N (%)	26 (43.3)	8 (21)	0.041
≥2.5 nmol/L, N (%)	34 (56.7)	30 (79)	
Total cholesterol (mmol/L)			
CC MTHFR	3.78 (1.98–7.04)	5.92 (3.44–9.94)	<0.001
TC/TT MTHFR	3.36 (2.54–8.82)	5.62 (4.24–8.24)	<0.001
All genotypes	3.70 (1.98–8.82)	5.89 (2.06–12.00)	<0.001
LDL cholesterol (mmol/L)			
CC MTHFR	2.00 (1.00–4.60)	3.60 (0.85–7.20)	0.003
TC/TT MTHFR	2.30 (1.00–6.90)	2.92 (0.75–9.60)	ns
All genotypes	2.20 (1.00–6.90)	3.50 (0.75–9.60)	<0.001
HDL cholesterol (mmoll/L)			
CC MTHFR	1.04 (0.49–1.86)	0.99 (0.67–1.71)	ns
TC/TT MTHFR	0.93 (0.62–1.59)	0.92 (0.68–8.24)	ns
All genotypes	0.95 (0.49–1.86)	0.97 (0.67–1.71)	ns
Triglycerides (mmol/L)			
CC MTHFR	1.08 (0.38–6.01)	1.38 (0.65–8.67)	ns
TC/TT MTHFR	1.32 (0.39–3.43)	1.74 (0.85–3.90)	0.003
All genotypes	1.32 (0.38–6.01)	1.70 (0.65–8.67)	<0.01

Statistical analysis: χ^2-test for differences among value-dependent (homocysteine, vitamin B12 and folate) groups, and Mann-Whitney test for differences among genotype-dependent groups.
* For statistical analysis TC and TT MTHFR genotypes were pooled because of the small number of subjects with TT genotype.
** CS – carotid stenosis.
*** Subjects were divided into two groups because only two subjects in control group and one subject in patient group with folate higher than upper (45.4 nmol/L) level of reference range were found

E genotypes were also in HWE for CS patients (p=0.447) and controls (p=0.920). There was a statistically significant difference between CS patients and controls in the distribution of apo E genotypes (p=0.012) and alleles (p=0.029). The odds ratio (OR) was 3.93 (95% CI 1.23–12.61) for apo E 3/4 genotype, 2.93 (95% CI 0.88–9.77) for apo E 2/3 genotype and 0.78 (95% CI 0.07–8.95) for apo E 2/4 genotype.

TABLE 2
MULTIPLE LOGISTIC REGRESSION ANALYSIS OF RISK FACTORS AND HOMOCYSTEIN LEVELS IN ASSOCIATION WITH CAROTIDE STENOSIS

	β*	SE**	OR (95%CI)***	p-value
Homocystein	0.877	0.907	2.403 (0.406–14.217)	0.334
Smoking	–0.684	0.475	0.505 (0.199–1.279)	0.149
Sex	0.711	0.447	2.037 (0.848–4.896)	0.112
Age	0.046	0.027	1.048 (0.993–1.105)	0.087

*β – estimated coefficient, **SE – standard error; ***OR – adjusted odds ratio with 95% CI, confidence interval

C677T MTHFR genotype and levels of Hcy, vitamin B12, folate and lipids in control subjects and CS patients

Table 1 shows the distribution of Hcy, vitamin B12, folate and lipid values (median and range) among control subjects and CS patients grouped according to MTHFR genotype.

For statistical analysis, heterozygous, TC, and homozygous, TT genotypes were pooled because of the small number of subjects with TT genotype (Figure 1; only 5% of TT genotype in control and 8% in patient group were found, respectively).

There were no statistically significant differences Hcy and HDL cholesterol among study groups. Triglycerides, vitamin B12 and folate were statistically significantly different in the »all genotypes« group ($p<0.01$, $p=0.044$ and $p=0.036$, respectively), and combined (TC/TT) genotype group ($p=0.003$, $p=0.030$ and $p=0.032$, respectively), but not in the group of CC MTHFR genotype. Differences in total cholesterol concentrations reached statistical significance in all three groups ($p<0.001$), whereas LDL cholesterol did not in the TC/TT group.

Statistical analysis allowed for the results obtained to summarize. The CS patient group with TC/TT genotype showed statistically different vitamin B12, folate, total cholesterol and triglyceride levels in comparison with control subjects with the same genotype. On comparison, only total cholesterol and LDL cholesterol reached statistical difference between CS patients with CC MTHFR genotype and controls with the same genotype. Comparison of Hcy, vitamin B12, folate and lipids irrespective of MTHFR and apo E genotype, between patients and controls yielded statistically significant differences for vitamin B12, folate, total cholesterol, LDL cholesterol and triglycerides, but not for HDL cholesterol. All these values showed higher median in patients than in control subjects, with the exception of Hcy and vitamin B12.

Value-dependent Hcy groups were not statistically different (Table 1), however, hyperhomocysteinemia (≥15 μmol/L) was found in 11% of patients and 5% of controls. Distribution frequencies of vitamin B12 value-dependent groups showed statistical difference ($p=0.021$) with 24% of patients and 5% of controls under the lower normal value. Only two-folate value-dependent groups were considered because of the low frequency of patients and controls (two subjects in control and one subject in patient group) with folate higher than upper (45.5 nmol/L) level of reference range were found. Distributions of these groups showed statistical difference ($p=0.041$).

The findings from the univariate analysis were further investigated in a multiple logistic model with inclussion of age (in years), sex and smoking habit as a conventional vascular risk factors (Table 2).

The results of multiple logistic analysis showed that there was no statistical significance of homocystein levels (OR 2.403, $p=0.334$) or conventional vascular risk factors such as smoking habit (OR 0.505, $p=0.149$), age (OR 1.048, $p=0.087$) or sex (OR 2.037, $p=0.112$) in predicting CS.

Apo E genotype and levels of lipids in control subjects and CS patients

Table 3 shows the results of lipid analysis in relation to apo E genotype.

Comparison between the control and CS patient groups classified according to genotype yielded statistical differences for some measured lipids ($p<0.05$). The levels of total cholesterol, LDL cholesterol and triglycerides in the apo E 3/3 genotype group yielded statistical difference. In the group of the apo E 3/4 genotype statistically significant result was found only for total cholesterol. Levels of all measured lipids were not different in the group of apo E 2/3 genotype. Statistical analysis was not performed in the group of apo E 2/4 genotype group because only one CS patient had this genotype.

Discussion

In the present study, the association of MTHFR genotype with plasma Hcy, vitamin B12, folate and lipids as well as between apo E genotype and lipids was investigated in the groups of Croatian healthy subjects and patients with significant (>70%) carotid artery stenosis. The frequency of C677T MTHFR genotype in study subjects is presented in Figure 1, showing the genotype distribution to be the same/very similar as previously published[7,28], although the present study included a smaller number of subjects. We found no association between CS and MTHFR genotype. Literature data show controversial results concerning the association of MTHFR genotype and carotid artery atherosclerosis, ischemic cerebrovascular disease (ICVD) and stroke[7–10,29].

TABLE 3
LIPID LEVELS IN CONTROL SUBJECTS AND CAROTID STENOSIS PATIENTS WITH DIFFERENT APO E GENOTYPES

	Control (N=60); Genotypes: 3/3 (N=48), 3/4 (N=5), 2/3 (N=5) and 2/4 (N=2); (median (range)	*CS (N=38); Genotypes 3/3 (N=19), 3/4 (N=10), 2/3 (N=8) and 2/4 (N=1); (median (range)	**p-value
Total cholesterol (mmol/L)			
3/3	4.04 (1.98–8.82)	6.09 (3.44–9.94)	<0.001
3/4	3.29 (2.96–5.59)	6.50 (3.78–8.36)	0.003
2/3	3.42 (3.10–3.88)	4.08 (2.06–12.00)	ns
2/4	5.14 (5.07–5.20)	5.07	–
LDL cholesterol (mmol/L)			
3/3	2.30 (1.00–6.90)	3.70 (1.22–7.20)	0.007
3/4	1.80 (1.20–3.80)	3.50 (0.75–5.70)	ns
2/3	1.85 (1.00–2.50)	2.40 (0.90–9.60)	ns
2/4	2.53 (2.45–2.60)	2.60	–
HDL cholesterol (mmol/L)			
3/3	1.04 (0.62–1.86)	1.03 (0.67–1.71)	ns
3/4	0.92 (0.49–1.33)	0.94 (0.80–1.51)	ns
2/3	1.04 (0.95–1.11)	0.96 (0.71–1.41)	ns
2/4	1.07 (0.93–1.20)	0.93	–
Triglycerides (mmol/L)			
3/3	1.44 (0.38–3.43)	2.00 (0.77–5.00)	0.035
3/4	1.27 (0.45–6.01)	2.21 (0.82–8.67)	ns
2/3	1.10 (0.70–2.35)	1.44 (0.75–4.00)	ns
2/4	3.40 (3.35–3.45)	3.45	–

*CS – carotid stenosis, **Mann-Whitney test

Comparison of plasma Hcy level between the patients and control group matched for sex, age and place of residence did not show elevated Hcy levels in the CS group (Table 1). The Hcy median was within the normal range in both groups. However, hyperhomocysteinemia (≥15.00 μmol/L) was detected in 5% of controls and in 11% of patients (Table 1), but did not reach statistical difference. Epidemiologic data show that hyperhomocysteinemia is an independent risk factor for atherosclerosis in general[2,3] and carotid atherosclerosis in particular[29,30], however, there also is a study report that failed to demonstrate the association between Hcy and CS[9].

Vitamin B12 and folate among controls and CS patients (Table 1) were statistically different in general (group of »all genotypes«) and in the group of TC/TT MTHFR subjects, however, the median of all values was within the normal range. Surprisingly, in the group of patients with TC/TT MTHFR genotype, lower vitamin B12 and higher folate values were recorded in comparison to the controls. Additionally, vitamin B12 concentration below the lowest normal value was found in 24% of patients and only 5% of controls, while there were 79% of patients and 57% of controls with folate over the lowest normal value (Table 1).

The finding of lower vitamin B12 and higher folate concentrations in patients as compared with controls is apparently paradoxical, since neither group of subjects received folate supplementation. It seems that this finding could be explained by the linked metabolism of folic acid and vitamin B12, and by the reaction that transfers methyl group from N^5-methyltetrahydrofolate to cobalamin[30]. Namely, in case of cobalamin deficiency, folate is »trapped« as N^5-methyltetrahydrofolate. It is »metabolically dead« in the absence of vitamin B12 and cannot be recycled as tetrahydrofolate back into the folate pool.

The collected questionnaires of CS patients showed that 80% were regular smokers and alcohol consumers, 70% had elevated blood pressure, and 30% had insulin dependent diabetes mellitus (IDDM). Although a complex disease background of our patient group would predispose to elevated Hcy levels, they were not recorded in our study. In contrast to our results, other authors observed positive Hcy association with alcohol and caffeine intake, smoking, IDDM as well as with hormonal changes, renal failure, cancer, and some drugs[3,31]. Additionally, Bo *et al*. conclude that a high level of alcohol intake plays a role as an independent risk factor in carotid atherogenesis. Alcohol intake is independent from other risk factors such as arterial hypertension, dyslipidemia, diabetes mellitus, smoking, social status and family history of cardio-cerebrovascular disease[32].

Selhub *et al.* showed that Hcy exhibited strong inverse association with folate and weaker association with vitamin B12 in extracranial carotid artery stenosis in the elderly[33]. Streifler *et al*. conclude that hyperhomocys-

teinemia and human platelet antigen (HPA)-1 a/b polymorphism are independent risk factors for ischemic events in patients with significant CS (≥50%)[34].

In contrast, Kostulas *et al.* report that C677T gene polymorphism did not exert any major influence on the risk of developing ICVD or internal carotid artery (ICA) stenosis, and did not cause Hcy level increase as observed in ICA stenosis. Also, it was shown that MTHFR genotype did not correlate with the levels of either Hcy, folic acid or vitamin B12, and did not affect Hcy levels, even in the presence of low blood folate[9].

The negative results of multiple logistic regression analysis of homocystein levels, smoking habit, age and sex in predicting CS is almost expected because the Hcy median was within the normal range in both groups. There is substantial problem of choosing so called control or healthy group of patients in genetic study. The reason is because there is no warranty that any of age and sex match control subject will get illness for a few years, so in the moment of testing have hyperhomocysteinaemia showing false positive values in control group.

The statistically significant difference in the distribution of apo E genotypes ($p=0.012$) and alleles ($p=0.029$) between CS cases and controls indicated the association between apo E polymorphism and CS. Additionally, OR was 3.93 (95% CI 1.23–12.61) for apo E 3/4 genotype. The association of apo E with CS (>50%) has been previously proposed[10], and was now confirmed and additionally established in severe stenosis (>70%) by the present study. In contrast, Catto *et al.* find no relationship between apo E genotype and cerebrovascular disease[35]. Also, Frikke-Schmidt *et al.* showed that apo E polymorphism is a risk factor for Alzheimer's disease and other dementia independent of lipid and lipoprotein levels but does not affect the risk of ICVD[36]. Additionally, no association of apo E polymorphism and carotid atherosclerosis was reported by others[15,17,18], however, there also are reports that confirm the existence of such an association[16,19,20].

Concerning the results on the association of apo E and MTHFR genetic polymorphism with blood lipids in patients with severe CS, we observed some gene effects on the lipids in the study population. To our knowledge, there are little or no data on such associations in patients with severe SC. Our results demonstrated the association of triglycerides with TC/TT MTHFR ($p=0.003$) but not with wild-type MTHFR genotype. HDL cholesterol was not associated with either wild-type or mutant genotypes, while total cholesterol was associated with both groups of genotypes. LDL cholesterol was statistically different only for CC MTHFR genotype. It has been reported that fasting and postprandial triglyceride-rich lipoproteins (but not LDLs) are elevated in patients with ≥50% CS as compared with controls and particularly identify echolucent, rupture-prone carotid plaques (37). The study of Yatsu *et al.* showed that in Caucasians, carotid artery stenosis was associated with increased plasma total cholesterol and LDL levels and an atherogenic profile but not with Sac I polymorphism for apoprotein AI[38].

Comparison between the control and CS patient groups classified according to genotype yielded statistical differences for some measured lipids as it was shown in Table 3. In the group of the apo E 3/4 genotype statistically significant result was found only for total cholesterol, but in the group of the apo E 3/3 genotype total cholesterol, LDL cholesterol and triglycerides were significant. Levels of all measured lipids were not different in the group of apo E 2/3 genotype. Some studies on carotid atherosclerosis showed that total cholesterol and LDL cholesterol were lower in E2 than in E3 and E4 allele carriers[16,19], whereas lower HDL cholesterol and higher LDL cholesterol were found in the apo E4 group of subjects[39]. Similar results, the association of apo E genotypes with lipids, were obtained for the group of coronary heart disease in middle-aged women[40].

Our study indicated an association of apo E 3/4 genotype but not of MTHFR genotype with the risk of CS. Also, the MTHFR and apo E genotypes were shown to affect blood lipid levels, which was statistically confirmed. This effect was demonstrated through medians of all values (the HDL cholesterol values of controls and CS patients were very close) that were higher in the CS group compared to control subjects with the same genotype. Study results showed an association of hyperhomocisteinemia with heterozygous/mutant MTHFR genotype in the CS group, although not statistically confirmed. Also, the CS group vitamin status was found to be associated with heterozygous/mutant MTHFR genotype, which was confirmed statistically, in comparison to controls with the same genotype. Namely, in the group of patients with TC/TT MTHFR genotype, lower vitamin B12 and higher folate values were recorded in comparison to the controls. Our findings are consistent with the concept according to which neither genes nor the environment but their interactions are responsible for the etiopathogenesis of complex diseases such as vascular disease. This preliminary study of the association between MTHFR genotype and Hcy, vitamin and lipid levels, and between apo E genotype and lipids revealed that there were associations that could be clarified and confirmed in further studies.

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POVEZANOST GENOTIPOVA METILENTETRAHIDROFOLAT REDUKTAZE (MTHFR) I APOLIPOPROTEINA E (APO E) S RAZINOM HOMOCISTEINA, VITAMINA I LIPIDA U ISPITANIKA SA STENOZOM KAROTIDA

S A Ž E T A K

Cilj istraživanja bio je ispitivanje povezanosti genotipova metilentetrahidrofolat reduktaze (MTHFR) s razinom homocisteina (Hcy), folata, vitamina B12 i lipida kao i ispitivanje povezanosti genotipova apolipoproteina E (apo E) s razinom lipida u zdravoj hrvatskoj kontrolnoj skupini i skupini ispitanika s >70% stenoze karotida (CS). U studiju je bilo uključeno 98 Hrvata, 38 ispitanika s >70% stenozom karotida i 60 kontrolnih uzoraka usklađenih po dobi i spolu. Genotipovi MTHFR i apo E određeni su metodom lančane reakcije polimerazom-polimorfizmom duljine restrikcijskih fragmenata (PCR-RFLP), Hcy enzimskim imunotestom, vitamini imunokemiluminiscencijom, i lipidi spektrofotometrijski. Statistički značajna razlika između kontrola i ispitanika s CS nije nađena u distribuciji C677T MTHFR genotipova ($p=0.786$) i alela ($p=0.904$), dok je učestalost apo E genotipova ($p=0.012$) i alela ($p=0.029$) statistički značajna. Faktor rizika (eng. odds ratio, OR) za apo E 3/4 genotip iznosio je 3.93 (95% CI 1.23–12.61). Hiperhomocisteinemija (≥15 μmol/L) nađena je u 11% CS ispitanika i 5% kontrola. Ukupni kolesterol, trigliceridi, vitamin B12 i folati statistički su značajni u skupinama »svi MTHFR genotipovi» ($p<0.001$, $p<0.01$, $p=0.044$ i $p=0.036$), i TC/TT genotipovi ($p<0.001$, $p=0.003$, $p=0.030$ i $p=0.032$). Statistički značajna razlika dobivena je i za ukupni kolesterol, LDL kolesterol i triglyceride u skupini s apo E 3/3 genotipom, i za ukupni kolesterol u skupini s apo E 3/4 genotipom. Nađena je povezanost apo E 3/4 genotipa, ali ne i MTHFR genotipova s rizikom od CS. Statističkom značajnošću potvrđen je utjecaj MTHFR i apo E na razinu lipida u krvi. Također je zabilježena povezanost hiperhomocisteinemije i ispitanika s CS. Statistički je potvrđena povezanost vitaminskog statusa s TC/TT MTHFR genotipom. U skupini ispitanika s TC/TT genotipom nađena je niža razina vitamina B12 i viša razina folata. Rezultati multiple logističke analize pokazali su da u predskazivanju pojavnosti CS nema statističke značajnosti ni razina Hcy (OR 2.403, $p=0.334$) niti konvencionalni vaskularni faktori rizika, kao što su pušenje (OR 0.505, $p=0.149$), godine (OR 1.048, $p=0.087$) ili spol (OR 2.037, $p=0.112$).