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STRAUSS E.[1], GŁUSZEK J.[2], PAWLAK A.L.[1]

AGE AND HYPERTENSION RELATED CHANGES IN GENOTYPES OF *MTHFR 677C>T, 1298A>C* AND *PON1 -108C>T* SNPs IN MEN WITH CORONARY ARTERY DISEASE (CAD)

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The phenotypes of CAD related to arterial hypertension co-occurrence were analysed in 174 male patients and 117 control men for the associations with the polymorphisms of the *MTHFR* gene (*677C>T* and *1298A>C*) and the *PON1* gene (*-108C>T*) in relation to age at diagnosis (less or equal and more than 50 years). We noted the increased frequency of the three *MTHFR* genotypes: *CC/AC*, *CT/AA* and *CC/CC* in the CAD group (65.5%) in comparison to the control group (45.3%), corresponding to the 2.3-fold increased risk of CAD for men with these genotypes (95%CI (1.4-3.7); p=0.0005). The higher increase in risk of CAD was noted for the younger men (OR=3.6; 95%CI(1.6-8.3); p=0.002) and lower for the older (OR=1.8; 95%CI(1.0-3.4); p=0.03). In the normotensive men the greater impact on CAD risk had the homozygous genotypes; the 2.3-fold higher risk was associated with *MTHFR CC/AC*, *CC/CC* and *TT/AA* genotypes (95%CI(1.2-4.4); p=0.01). After adjustment for age, the association between CAD and *MTHFR* was significant only for the younger normotensive men (OR=2.8; 95%CI (1.0-8.0); p=0.04). Additionally, we found that the younger part of the control group was characterized by higher frequency of the low expression *PON1 -108T* allele and *PON1 -108TT* genotype (0.54 and 31.9% respectively) in comparison to the older men (0.41 and 17.1% respectively; p=0.03).

Keywords: *coronary artery disease, CAD, age, hypertension, MTHFR, PON1, polymorphism, homocysteine, homocysteine thiolactone, case-control study, retrospective study*

INTRODUCTION

The prevalence of vascular diseases increases with age. The earlier occurrence of vascular diseases was noted also in patients with arterial

hypertension (AH) (1). We assumed that the individual differences in age of onset of coronary artery disease (CAD) and co-occurrence of AH may be associated with the different genetic profiles related to homocysteine (Hcy) metabolism. To add some knowledge to the role of these factors in pathogenesis of CAD we undertook a study of the associations between the distribution of genetic polymorphisms of the *MTHFR* and *PON1* genes recognized as the factors influencing homocysteine metabolism (2, 3) and AH as related to the phenotype of CAD in the group of 174 male patients differentiated for the age of CAD diagnosis (less or equal and more than 50 years) and 117 age-matched control males.

MATERIAL AND METHODS

Cases and controls

The study group consisted of 174 male patients with CAD diagnosed at age between 35 and 79 years, who were consecutively admitted to the Department of Arterial Hypertension, at Institute of Cardiology of the University School of Medical Sciences in Poznan between March 2002 and October 2003. The control group consisted of 117 males (43% of blood donors and 57% of patients treated for the posttraumatic disabilities). The study was approved by the Ethical Comittee of the University School of Medical Sciences in Poznan.

Demographic and clinical characteristics

In all patients the diagnosis was confirmed by coronarography. CAD was definied as the stenosis of at least one epicardial coronary artery exceeding 50%. Men from the control group had no history of CAD and no sings of angina pectoris. Blood pressure was measured under standarized conditions. Hypertension was diagnosed when the blood pressure was equal or exceeded 140/90 mmHg. The age at CAD diagnosis was determined retrospectively by reviewing the medical records.

Determination of MTHFR and PON1 polymorphisms

Genomic DNA was isolated from 5 ml of whole blood by the phenol extraction method. The *MTHFR 677C>T*, *1298A>C* and *PON1 -108C>T* polymorphisms were ascertained by PCR-RFLP (polymerase chain reaction-restriction fragments length polymorphism) method according to previously described methods (4-6). The polymorphisms were identified by digestion of the PCR-amplified products with *HinfI* restriction enzyme for *MTHFR 677C>T*, *MboII* for *1298A>C* and *FnuDII* for the *PON1 -108C>T*. DNA fragments were separated by electrophoresis in polyacrylamide or agarose gels and visualized by ethidium bromide staining.

Statistical analyses

Differences in baseline characteristics between the patients and the control groups were assessed by Chi-square test for categorical variables and t-test for continous parameters. Concordance of genotype frequencies with Hardy-Weinberg equilibrium was tested by a Chi-square goodness-of-fit test. Allele frequencies and prevalence of genotypes were compared between the

groups using Fisher's exact test. Odds ratio (OR) and 95% confidence intervals (CI) were calculated based on logistic regression analysis.

RESULTS

Characteristics of the study population

The basal characteristics of studied groups and prevalence of AH are shown in *Table 1.* The patients group consisted of 174 men. The mean age at CAD diagnosis was 55.2±10.3 (age range between 30 and 79). The control group consisted of 117 men and mean age was 53.8±9.4 (age range between 30 and 80). The groups were differentiated by the age into two subgroups: less or equal and more than 50 years old (patients by the age at diagnosis). There were no significant differences in age between the whole group of CAD patients and controls as well as betwen the older and younger subgroups. The prevalence of AH was significantly higher in the CAD group (n=115, 66%) in comparison to the control group (n=12, 10%, p<0.0001).

Prevalence of MTHFR and PON1 alleles and genotypes in relation to age

The distribution of *MTHFR* and *PON1* polymorphisms in CAD patients and controls are shown in *Table 2a* and *2b*.

The observed frequencies of *MTHFR* and *PON1* genotypes were comparable to those previously reported in the Caucasian populations and were in Hardy-Weinberg equilibrium in both groups. We noted the complete linkage

Table 1. Basal characteristics of 174 men with CAD and 117 control subjects (the age of patients refers to the age at CAD diagnosis).

	Controls N=11)	**CAD N=174**	**p=**
Age (yr)			
Mean ± SD	53.8±9.4	55.2±10.3	0.2
Range	30-80	30-79	-
Age ≤ 50 years (yr)			
n (%)	47 (40.2)	59 (33.9)	-
Mean ± SD	45.8±4.6	44.2±5.8	0.2
Age > 50 years (yr)			
n (%)	70 (59.8)	115 (66.1)	-
Mean ± SD	59.1±8.0	60.9±7.0	0.1
Arterial hypertension			
Yes; n (%)	12 (10.2)	115 (66.1)	<0.0001
No; n (%)	98 (83.8)	59 (33.9)	
unknown	7 (6.0)	0	

Table 2. Distribution of *MTHFR* and *PON1* alleles (a) and genotypes (b) in 174 men with CAD and 117 control subjects in relation to age.

a)

	Number and frequency of alleles					
	Controls			CAD		
	all N=117	**age ≤ 50* N=47**	**age > 50* N=70**	**all N=174**	**age ≤ 50 N=59**	**age > 50 N=115**
***MTHFR 677 C>T* alleles**						
677C	161	63	98	238	79	159
677T	73	31	42	110	39	71
677T frequency	0.31	0.33	0.30	0.32	0.33	0.31
***MTHFR 1298 A>C* alleles**						
1298A	168	67	101	238	79	159
1298C	66	27	39	110	39	71
1298C frequency	0.28	0.29	0.28	0.32	0.33	0.31
***PON1 -108 C>T* alleles**						
-108C	126	43	83	177	55	122
-108T	108	51	57	171	63	108
-108T frequency	0.46	0.54	0.41	0.49	0.53	0.47

**PON1 -108C vs T*: p=0.03 (Fisher's exact test)

b)

	Number and frequency of genotypes					
	Controls			CAD		
	all N=117 (%)	**age ≤ 50* N=47 (%)**	**age > 50* N=70 (%)**	**all N=174 (%)**	**age ≤ 50 N=59 (%)**	**age > 50 N=115 (%)**
***MTHFR 677 C>T* genotypes**						
677 CC	58 (49.6)	22 (46.8)	36 (51.4)	82 (47.1)	27 (45.8)	55 (47.8)
677CT	45 (38.5)	19 (40.4)	26 (37.1)	74 (42.5)	25 (42.4)	49 (42.6)
677 TT	14 (12.0)	6 (12.8)	8 (11.4)	18 (10.3)	7 (11.9)	11 (9.6)
***MTHFR 1298 A>C* genotypes**						
1298 AA	59 (50.4)	23 (48.9)	36 (51.4)	86 (49.4)	28 (47.4)	58 (50.4)
1298 AC	50 (42.7)	21 (44.7)	29 (41.4)	66 (37.9)	23 (39.0)	43 (37.4)
1298 CC	8 (6.8)	3 (6.4)	5 (7.1)	22 (12.6)	8 (13.6)	14 (12.2)
***PON1 -108 C>T* genotypes**						
-108 CC	36 (30.8)	11 (23.4)	25 (35.7)	48 (27.6)	13 (22.0)	35 (30.4)
-108 CT	54 (46.1)	21 (44.7)	33 (47.1)	81 (46.6)	29 (49.2)	52 (45.2)
-108 TT	27 (23.1)	15 (31.9)	12 (17.1)	45 (25.9)	17 (28.8)	28 (24.3)

**PON1 -108CC+CT vs T T*: p=0.03 (Fisher's exact test)

disequilibrium between the two *MTHFR* polymorphisms in our sample; no genotype indicating the occurrence of *677T* and *1298C* alleles in *cis* configuration was found.

The younger groups of patients and controls were characterized by the higher frequency of the *MTHFR 677T*, *1298C* and *PON1 -108T* alleles as well as of the carriers of the rarer alleles in comparison to the older groups. In the younger part of control group (age <=50, n=47), the low expression *PON1* allele (*PON1 -108 T*) occurred at the higher frequency (0.54) as compared to that in the older age

control group (0.41, p=0.03; n=70). Also the low expression *PON1* genotype (*PON1 -108 TT*) occurred at the higher frequency (31.9%) in the younger part of control group, as compared to that in the older age control group (17.1%; p=0.03).

Prevalence of combined MTHFR genotypes in relation to age and AH

The distribution of *MTHFR 677C>T/1298A>C* combined genotypes is shown in *Table 3*. We noted the increased frequency of the three *MTHFR* genotypes: *CC/AC*, *CT/AA* and *CC/CC* in the CAD group in comparison to the control group. The genotypes were present in 65.5% in the CAD group and in 45.3% in the control group (p=0.0005). On basis of these observations the 2.3-fold increased risk of CAD was assessed for subjects with these combinations (95%CI (1.4-3.7)). In the younger group the respective OR value for these genotypes was higher (3.6; 95%CI(1.6-8.3); p=0.002) whereas it was lower in the older group of patients (1.8; 95%CI (1.0-3.4); p=0.03) (*Table 4*).

In the normotensive patients the increased risk of CAD was associated with the slightly different *MTHFR 677/1298* genotype combinations, namely *CC/AC*, *CC/CC* and *TT/AA*. These genotypes were found in 59.0% in the CAD group and in 38.8% among the controls, which corresponds with 2.3-fold higher risk of CAD for normotensive men (95%CI (1.2-4.4); p=0.01). In the normotensive groups differentiated by the age of 50, the significant increase in risk of CAD for these genotypes was noted only in the younger group (OR=2.8; 95%CI (1.0-8.0); p=0.04). The impact of the CAD risk combinations of *MTHFR* genotypes in the older group without hypertension was absent.

Table 3. Distribution of the combined *MTHFR 677C>T/1298 A>C* genotypes in 174 men with CAD and 117 control subjects in relation to the age and hypertension.

	Number and frequency of combined genotypes					
	Controls			**CAD**		
	all N (%)	**age ≤ 50 N (%)**	**age > 50 N (%)**	**all N (%)**	**age ≤ 50 N (%)**	**age > 50 N (%)**
***MTHFR 677C>T / 1298 A>C* genotypes**						
all subjects	**117 (100.0)**	**47 (100.0)**	**70 (100.0)**	**174 (100.0)**	**59 (100.0)**	**115 (100.0)**
CC/AA	25 (21.4)	9 (19.1)	16 (22.8)	18 (10.3)	2 (3.4)	16 (13.9)
CC /AC	25 (21.4)	10 (21.3)	15 (21.4)	42 (24.1)	17 (28.8)	25 (21.7)
CT /AA	20 (17.1)	8 (17.0)	12 (17.1)	50 (28.7)	19 (32.2)	31 (27.0)
CT /AC	25 (21.4)	11 (23.4)	14 (20.0)	24 (13.8)	6 (10.2)	18 (15.6)
CC /CC	8 (6.8)	3 (6.4)	5 (7.1)	22 (12.6)	8 (13.5)	14 (12.2)
TT /AA	14 (12.0)	6 (12.8)	8 (11.4)	18 (10.3)	7 (11.9)	11 (9.6)
normotensive subjects	**98 (100.0)**	**43 (100.0)**	**55 (100.0)**	**61 (100.0)**	**24 (100.0)**	**37 (100.0)**
CC/AA	21 (21.4)	8 (18.6)	13 (23.6)	5 (8.2)	1 (4.2)	4 (10.8)
CC /AC	22 (22.4)	10 (23.2)	12 (21.8)	18 (29.5)	7 (29.2)	11 (29.7)
CT /AA	18 (18.4)	8 (18.6)	10 (18.2)	10 (16.4)	4 (16.6)	6 (16.2)
CT /AC	21 (21.4)	9 (20.9)	12 (21.8)	10 (16.4)	3 (12.5)	7 (18.9)
CC /CC	7 (7.1)	3 (7.0)	4 (7.3)	8 (13.1)	4 (16.6)	4 (10.8)
TT /AA	9 (9.2)	5 (11.6)	4 (7.3)	10 (16.4)	5 (20.8)	5 (13.5)

Table 4. Odds ratios (ORs) for risk of CAD associated with the combined *MTHFR 677C>T/1298A>C* genotypes, age and hypertension.

	Number and frequency of risk genotypes		OR	95%CI	p=*
	Controls N (%)	**CAD N (%)**			
CC/AC, CT/AA, CC/CC					
all	53 (45.3)	114 (65.5)	2.3	1.4-3.7	0.0005
age ≤ 50	21 (47.7)	44 (74.6)	3.6	1.6-8.3	0.002
age > 50	32 (4.7)	70 (60.9)	1.8	1.0-3.4	0.03
CC/AC, CC/CC, TT/AA					
all normotensive	38 (38.8)	36 (59.0)	2.3	1.2-4.4	0.01
age ≤ 50 *normotensive*	18 (41.9)	16 (66.7)	2.8	1.0-8.0	0.04
age > 50 *normotensive*	20 (45.5)	20 (54.0)	1.5	0.6-3.5	0.2NS

*p-Fisher's exact test

DISCUSSION

The study presented here deals with the associations between the combined genotypes of *MTHFR 677C>T* and *MTHFR 1298A>C* polymorphisms as well as the promotor polymorphism *PON1 -108C>T* and the susceptibility to the coronary artery disease (CAD). The assumed functional link between the studied polymorphisms and the phenotypes is that the low activity alleles in three studied SNPs were shown to increase the concentrations of either homocysteine (*MTHFR*) or its metabolite - homocysteine thiolactone (*PON1*) in tissues. The age and arterial hypertension are also influencing the tissue levels of Hcy, and all mentioned factors are common confounders of susceptibility to CAD.

The reduced enzyme activity accompanied by the proneness to hyperhomocysteinemia is common in persons with the *MTHFR* gene variants: *677T* alone and *1298C* in combination with *677T* (mixed heterozygotes *677CT/1298AC*). Homocysteine is a sulfur-containing intermediate product of normal metabolism of methionine. There are two main pathways in which Hcy is metabolised. This aminoacid is either methylated to metionine in a remethylation pathway or condensated with serine in a transsulfurylation pathway. The methyl donor in the former reaction is the 5-methyltetrahydrofolate, produced in a irreversible reaction catalysed by methylenetetrahydrofolate reductase (*MTHFR*). In the vascular smooth muscle cells (SMCs), but not in the endothelium, the cystathionine beta-synthase enzyme (CBS) is active in the transsulfuration pathway, which significantly influences the homocysteine metabolism in liver and muscle cells (7). As recently discovered, hyperhomocysteinemia has adverse effect on the left ventricle, it is inversely related with the left ventricular ejection fraction (LVEF) and predicts the cardiovascular mortality in high-risk coronary artery disease hypertensives. This important observation disclosed also the confounding interaction between the LVEF and the *MTHFR 677C>T* genotypes as the main factors influencing the Hcy levels. Following the stratification of the

Hcy concentrations and genotype data for the LVEF quartiles, the authors demonstrated the clear cut and significant differences in the Hcy concentrations as related to the patients *MTHFR 677* genotypes (8).

Homocysteine thiolactone - the highly reactive derivative of Hcy was identified as the endogenous substrate of the *PON1* protein (9). In serum this protein is associated with high density lipoprotein fraction (HDL) and active in detoxication of xenobiotic organic phosphates, such as the widely used herbicide - paraoxon from which the name paraoxonase has been formed. The proper name for this enzyme should be rather Hcy thiolactonase (9). Homocysteine thiolactone is produced from homocysteine when it is mis-incorporated in tRNA in place of methionine, leucine or isoleucine, and the aminoacylo t-RNA syntetases prevent the faulty synthesis of protein by the editing reaction in which this highly reactive Hcy derivative is formed (10). The polymorphism in the promoter region of *PON1* gene (*-108C>T*) studied in this project is the main genetic determinant of the paraoxonase (or Hcy thiolactonase) activity in plasma. It accounts for about 25% of the variation paraoxonase activity in serum and leads to the decreased expresion of the *PON1* protein in *PON1 -108 TT* subjects (11).

The genotype combinations *CC/AC*, *CT/AA* and *CC/CC* of the *MTHFR 677C>T/1298A>C* polymorphisms occurred with the higher frequency in the whole group of CAD patients as well as in the younger and the older age groups of CAD patients as compared to the respective control groups. Most of this effect occurred however in the younger CAD group. In the normotensive CAD patients a slightly different combination of *MTHFR* polymorphisms was associated with the increased risk of CAD. In place of the genotype *CT/AA* the homozygous genotype *TT/AA* occurred here with the higher frequency in the CAD group, what may be considered as increased impact of *MTHFR 677T* allele on CAD risk in the normotensives. In the normotensive persons the risk associated with carrying of the *MTHFR 677T* allele is also greater as refers to the development of abdominal aortic aneurysm (AAA), whereas no such association was noted (for AAA risk) in persons with hypertension (12).

The age effect was noted in the normotensive CAD patients, since in the older patients the risk associated with the genotypes specific for the younger group was negligible (*Table 4*). Since 2 of the 3 *MTHFR* combination genotypes singled out by us as the genotypes of risk for the CAD occurence in the younger age (<=50 y) contain the low activity *MTHFR 1298C* allele, our observation fits with the finding of Szczeklik (13), that the *MTHFR 1298CC* genotype occurs at increased frequency among the CAD patients in that age group. The involvement of *MTHFR 677C>T* polymorphism in longevity was confirmed by the difference in frequency of *TT* genotype 19% versus 7% between the healthy Japanese individuals aged <55 years versus >80 years (14). The lower frequency of *MTHFR 677T* allele was found also in the elderly control group as compared to the younger controls and the CAD group (15).

The age related differences in *PON1 -108* genotypes between the younger and the older groups of the control males noted in our study, in which the low expression *PON1 -108TT* genotype occurs at the higher frequency in the younger group (*Table 2*), confirm that this genotype may increase the risk of diseases developing from the age of 50. Our finding, that in the control group of males older than 50y (mean age 59.1) the frequency of *PON1-108 CC* genotype is higher than in the younger (<=50y; mean age 45.8) (Table 2) fits with the reports that the *PON1 -108CT* and *TT* genotypes increase the risk of early onset diseases. In the pooled random-effect analysis the risk of the coronary heart disease *PON1-108 CT* and *TT* subjects was found to be increased by 1.44 as compared to *PON1-108 CC* subjects (16). *PON1 -108CC* genotype which determines the high expression of the enzyme was found to be associated with the reduced risk of myocardial infarction in the younger CAD patients (17).

The associations between the *PON1* gene polymorphisms and the longevity were first reported with reference to the coding region *PON1 192Q>R* genotypes. Increased frequency of the carriers of *PON1 192R* allele has been found in the centenarians from Italy as compared to the young controls (18, 19). Increased frequency of *PON1 -108CC*, and higher HDL, as well as the paraoxonase activity has been found in the healthy Sicilian octagenarians when compared to the control group (age 60 y) (20). The authors consider this genotype as favoring the healthy aging and long living.

Elevated plasma Hcy concentration is recognized as a risk factor for atherosclerosis and coronary artery disease (CAD) (21). Hyperhomocysteinemia (HHcy) acts on the multiple endpoins. In his work on the homocysteine respondent genes Kokame, apart from the changes in expression of the specific genes has singled out two other molecular mechanisms: the slowing down of protein transfer from the endoplasmic reticulum either to the cell surface or to the extracellular space as well as the modification of structure and activity of proteins such as thrombomodulin or lipoprotein (a) (22). The elevated concentrations of Hcy prevent protein exit from the endoplasmic reticulum; this results in endoplasmic reticulum stress which dysregulates the cholesterol and triglyceride biosynthetic pathways (23). Among the genes induced in the vascular wall by Hcy highly relevant for the vascular aging is the T-cell death-associated gene 51 (*TDAG51*), specific for activation of the detachment mediated programed cell death (24, 25). Of great importance to the vascular pathology is also the induction of other genes which directly affect endothelial functions. Homocysteine can enhance the endothelial cell apoptosis by inducing the p53-dependent *Noxa* gene expression (27). The other Hcy-induced gene - *NDRG1* (N-Myc down-regulated gene 1), which also is co-regulated by p53, is required for the p53-mediated caspase activation and apoptosis (28). This gene is transcriptionally activated by DNA damage and thus may participate in vascular pathology associated with the genotoxic exposures.

The adverse effects of chronically elevated plasma homocysteine levels are thought to be mediated both by the increased oxidative stress and the reduced NO bioavailability (29). The elevation of an endogenous inhibitor of the endothelial NO synthase (eNOS) the asymmetric dimethylarginine (ADMA) in hyperhomocysteinemia would be an example of the second mechanism (30). The bioavailability of the endothelial nitric oxide (NO) may be decreased also in consequence of its reaction with the SH group of homocysteine (Hcy) forming the S-nitroso Hcy. In the recent assessment, the reduced NO bioavailability in hyperhomocysteinemia seems to be due to the decreased NO synthesis and release rather than to the NO destruction by oxidative stress (31).

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