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Increased risk of the abdominal aortic aneurysm in carriers of the *MTHFR 677T* allele

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Abstract. Abdominal aortic aneurysm (AAA) presents itself as a progressive dilation of the abdominal aorta, leading – if untreated – to rupture. It is a common disease of the elderly, with a complex etiology. Several genetic, biochemical and environmental factors are recognized as relevant for the pathogenesis of AAA. We determined the polymorphism of the *MTHFR* (methylenetetrahydrofolate reductase) gene within the fourth exon (*C677T*) in 63 patients with AAA and compared it to that in 75 subjects of the population sample. The frequencies of the *C/C*, *C/T* and *T/T* genotypes were 65%, 27%, and 8% in the population sample and 33%, 60%, and 6% in the patients. This corresponds to a 4.4-fold greater risk of AAA in subjects who have the *677C/T* variant of *MTHFR*, as compared with those who are *677C/C* ($p<0.0001$; 95% CI=2.11-9.34). The frequency of allele *MTHFR 677T* in patients (0.37) was higher than in the population sample (0.21; $p < 0.007$). This association between the common allele of the *MTHFR* gene – *MTHFR 677T* – and the development of AAA suggests that elevated homocysteine (Hcy) may disturb the function of the aortic wall. The disturbance may involve enhancement of elastin degradation, the process enhanced by mild hyperhomocysteinemia in minipigs. The magnitude of this effect, which refers to the AAA patients unselected for familial occurrence, indicates that the disturbance of aortic wall physiology caused by the presence of the *MTHFR 677T* allele is greater than the effect of the earlier described allele disequilibrium at the polymorphic alleles of the *PAI1* (plasminogen activator inhibitor 1) gene seen only in familial cases of AAA.

Key words: abdominal aortic aneurysm, elastin, homocysteinemia, *MTHFR* gene, *MTHFR 677T* allele, polymorphism.

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Introduction

Abdominal aortic aneurysm (AAA) is characterized by a progressive dilation of the abdominal aorta and thinning of the vessel wall secondary to the degradation of the media layer and the fragmentation of elastic and collagenous fibers decreasing their density in this tissue (RAMSBOTTOM et al. 1994). Ultrasonography is currently the most precise and commonly available method of early detection of AAA. Its prevalence in adult autopsy series varies between 1% and 6% (JOHNSON et al. 1985). In a recent international study of ASHTON and CASS group (2001) – the highest age-standardized AAA prevalence was recorded in the UK (7.7%) and the lowest in Denmark (4.5%). About 20% of AAA cases occur in families in which two or more persons are affected. The increased frequency of the *PAI1* gene allele *5D* was noted in familial cases of AAA, but not in the remaining patients. This was considered as an indication that the pathogenesis may be distinct in the familial versus the sporadic cases of AAA (ROSSAAK et al. 2000). The female patients of AAA occur more frequently in the familial type of the disease (VERLOES et al. 1995). The patients with AAA have plasma Hcy levels significantly higher than the normal values (BRUNELLI at al. 2000).

The current study was established to determine the frequency of the *MTHFR* (methylenetetrahydrofolate reductase) *677T* allele in 63 patients (5 women and 58 men) with AAA, as compared to that in 75 subjects of the population sample (26 women and 49 men).

Material and methods

Subjects studied

The group with AAA consisted of 63 patients (5 women and 58 men; mean age 64.9 ±7.9 years; age range 46-81). The diagnosis was confirmed by ultrasonography. The mean diameter of abdominal aorta was 6.0 ±1.6 cm (size range 3-10 cm). In all patients the abdominal aortic aneurysmectomy was performed. The population sample consisted of 75 subjects (26 women and 49 men) randomly selected from a local population. Patients and the population group were selected from the inhabitants of downtown Poznań. In the control group males and females did not differ when the *MTHFR* allele frequencies were compared (data not shown). Classic markers of the risk factors of vascular disease were determined in 51 of 63 AAA patients. The occurrence of other diseases, smoking habits and occurrence of overweight in the group of AAA patients were recorded. Overweight was defined as BMI (Body Mass Index, BMI [kg/m^2] = body weight [kg]/(height [m])^2) above 30.

Genotyping of *MTHFR*. Genomic DNA was isolated from peripheral blood leukocytes by the phenol extraction method. *MTHFR* polymorphism within

the fourth exon (*C677T*) was ascertained by the PCR-RFLP (polymerase chain reaction, restriction fragment length polymorphism) method (FROSST at al. 1995). This single nucleotide substitution results in a conversion of alanine to valine in the MTHFR (protein product). The amplified PCR fragment was digested with the *HinfI* restriction enzyme, which can recognize *C* to *T* substitution. If the *MTHFR 677T* allele is present, *HinfI* digests the 198-bp fragment into a 175-bp and 23-bp fragment. The fragments were resolved by electrophoresis in 10% polyacrylamide gels and stained with ethidium bromide. Genotypes were expressed as *C/C* for homozygous normal, *C/T* for heterozygous, and *T/T* for homozygous variant.

Other laboratory studies

Fasting serum triglyceride (TG), total serum cholesterol (TC), LDL-cholesterol (low density lipoprotein; LDL-C) and HDL-cholesterol (high density lipoprotein, HDL-C) were determined by routine laboratory assays. When direct measurement was not available, LDL-C levels were calculated from TC, TG and HDL-C by using Friedewald's formula (LDL-C [mmol/l] = TC [mmol/l] – HDL-C [mmol/l] – 0.45 × TG [mmol/l]; TG<4.6 mmol/l) (CHOTKOWSKa et al. 2001, KRAUSE et al. 1996).

Statistical analysis

Results of continuous data (e.g. cholesterol) are presented as mean values ±SD (standard deviations). Frequencies of *MTHFR* alleles and genotypes were compared between the study and the population groups by using a two-tailed Fisher's exact test. Odds ratio (OR) and 95% confidence interval (CI) were calculated on the basis of logistic regression analysis.

Results

Characteristics of the study population

Table 1 present the serum lipid parameters in AAA patients, compared to the range of normal values for the Polish population (CYBULSKA et al. 1994). The studied group displays increased median values of total cholesterol. Most (34) of the patients smoke (67%). In 9 (18%) of AAA patients, increased values of Body Mass Index (BMI>30) were noted. Heart diseases were diagnosed in 24 (47%) and diabetes in 4 (7%) of the AAA patients.

Frequency of the *MTHFR 677T* allele

Genotyping results are given in Table 2. In the group of 63 patients diagnosed with AAA, the *MTHFR 677T* allele frequency was 0.37 (Table 2A), whereas in 75 sub-

Table 1. Serum lipid parameters (mean values and SD) in AAA patients

Serum lipid parameter	AAA patients (51)	Range of normal values
Total cholesterol (TC) (mmol/l)	5.8 ±1.1	<5.2
HDL cholesterol (mmol/l)	0.96 ±0.23	>1.0(F); >0.8(M)
LDL cholesterol (mmol/l)	3.74 ±0.88	<4.0
Triglycerides (mmol/l)	1.8 ±0.8	<2.0

Table 2. A) The numbers and frequencies of *MTHFR* alleles in AAA patients and the population sample. B) *MTHFR* genotypes in AAA patients and the population sample. C) *MTHFR* genotypes in male AAA patients and males of the population sample. Adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for genotypes *677 C/T* and *677 C/T + 677 T/T*, using *677 C/C* as a reference.

A)

MTHFR allele	Number of *MTHFR* alleles	
	population sample N = 75 (%)	AAA patients N = 63 (%)
677 C	118 (79)	80 (63)
677 T	32 (21)[a]	46 (37)[a]

[a] $p<0.007$ (by Fisher's exact test)

B)

MTHFR genotype	Number of persons		OR	95% CI
	population sample N = 75 (%)	AAA patients N = 63 (%)		
677 C/C	49 (65)	21 (33)	1	–
677 C/T	20 (27)[a]	38 (60)[a]	4.43	2.11-9.34
677 T/T	6 (8)	4 (6)	ns	ns
677 C/T+T/T	26 (35)[b]	42 (67)[b]	3.77	1.86-7.65

[a] $p<0.0001$, [b] $p<0.0003$ (by Fisher's exact test)

C)

MTHFR genotype	Number of males		OR	95% CI
	population sample N = 49 (%)	AAA patients N = 58 (%)		
677 C/C	32 (65)	20 (34)	1	-–
677 C/T	13 (27)[a]	35 (60)[a]	4.31	1.85-10.05
677 T/T	4 (8)	3 (5)	ns	ns
677 C/T+T/T	17 (35)[b]	38 (66)[b]	3.58	1.61-7.96

[a] $p<0.001$, [b] $p<0.002$ (by Fisher's exact test)

jects of the population sample this value was significantly lower, amounting to 0.21 ($p<0.007$). The frequencies of the *C/C*, *C/T* and *T/T* genotypes among the subjects of the population sample were: 65%, 27%, and 8%. The corresponding frequencies among the patients with AAA were: 33%, 60%, and 6%. We observed a significant 4.4-fold increase in the risk of AAA among subjects who have the *677 C/T* variant of *MTHFR*, as compared with those who are *677 C/C* ($p < 0.0001$; 95% CI = 2.11-9.34). The presence of the *C677T* substitution in one or both alleles was associated with a 3.8-fold increase in the risk of AAA ($p < 0.0003$; 95% CI = 1.86-7.65; Table 2B). Following the analysis of *MTHFR* allele frequencies in the male patients compared to the male members of the population sample, the respective OR values were 4.31 (for *677 C/T* vs *C/C*) and 3.58 (for *677 C/T+T/T* vs *C/C*) (Table 2C).

Discussion

In this study we report the association between the susceptibility to AAA and the allele *677T* of the *MTHFR* gene. The data presented in Table 2A indicate that the risk of AAA is strongly associated with the *MTHFR 677T* allele. In the group of AAA patients (unselected for familial occurrence) an increase in the frequency of *MTHFR 677T* allele was noted, as compared to the persons of the population sample ($p < 0.007$, Table 2A). By contrast, the increase in the frequency of the *5G* allele of the plasminogen activator inhibitor 1 gene (*PAI1 5G*), reported by ROSSAAK et al. (2000), was noted only in familiar cases of AAA and at a lower magnitude ($p < 0.03$). Since the persons homozygous for the *5G* allele of the *PAI1* gene display the higher aneurysm growth rate, whereas the postrepair mortality in these cases is lower, the relations between the *PAI1 5G* allele and the pathogenesis of AAA may be considered rather complex (JONES et al. 2002). The effect of the *MTHFR 677T* allele on the risk of AAA is probably more straightforward, as compared to that of *PAI1 5G*.

The frequency of the heterozygous genotype was greater among the patients with AAA than in subjects of the population sample, and the difference was highly significant ($p < 0.0001$; OR = 4.4; 95% CI = 2.11-9.34; Table 2B). The *MTHFR 677T* allele and the *677 C/T* genotype frequencies in the studied population group were highly concordant with the corresponding values obtained in the larger (310 persons) population group from the Cracow district of Poland (SZCZEKLIK et al. 2001). An unexpected finding was the absence of the increase in the frequency of *MTHFR 677 T/T* persons in the AAA group. This may be related to allele disequilibrium between this allele and the *MTHFR 1298C* allele, described by VAN DER PUT et al. (1998). The observations presented indicate that this common *MTHFR* variant, which produces a mild elevation of plasma homocysteine, predisposes to AAA.

The etiology of aortic aneurysm formation is considered to be distinct from that of atherosclerosis (ROSSAAK et al. 2000). In patients with AAA, significantly elevated Hcy plasma levels were found (BRUNELLI et al. 2000). Hcy is a non-protein amino acid, which is involved in the one-carbon metabolism and methylation reactions. The high plasma levels of Hcy are toxic to the vascular cells and tissues, and then are an important risk factor in vascular disease. The two major ways of Hcy detoxification are: the trans-sulphuration pathway and the re-methylation pathway (ROSENBLATT 1989). CHEN et al. (1999) reported that the trans-sulphuration pathway is absent from the human cardiovascular system, which may sensitize these tissues to Hcy toxicity. Then in this tissue the metabolism of Hcy largely depends on remethylation reaction, which is catalysed by methionine synthase (MS). In this reaction the 5-methyltetrahydrofolate (5-MetTHF) serves as a methyl donor. This compound is produced in the irreversible reaction catalysed by MTHFR (EC 1.5.1.0). Consequently, the genetic deficiency of this enzyme results in excessive accumulation of Hcy, which particularly affects the vascular system. The *MTHFR 677T* allele produces the enzyme with reduced activity and increased thermolability (KANG et al. 1991) and is implicitly associated with mild hyperhomocysteinemia.

The *MTHFR C677T* polymorphism is common in all studied ethnic groups; however its frequency varies in a wide range. The highest frequencies of the low-activity thermolabile allele *MTHFR 677T* up to 0.38 was reported in French Canadians (FROSST et al. 1995) whereas the lowest (0.1) in African Americans (MCANDREW et al. 1996). The above-mentioned ethnic high frequency of the thermolabile variant of the *MTHFR* gene for Caucasians (0.25-0.38) coincides with the high frequency of AAA in Caucasian males (4.5%-7.7%). This compares to the low frequency of *MTHFR 677T* (0.1) in African American males and the parallel low frequency of AAA in this ethnic group (1.5%) (JOHNSON et al. 1985, LAMORTE et al. 1995).

To investigate *in vivo* the effects of hyperhomocysteinemia on the aortic wall, CHEN et al. (2001) generated *mthfr* knockout mice. Plasma total homocysteine levels in *mthfr* +/– and *mthfr* –/– mice were respectively 1.6- and 10-fold higher than those in wild-type littermates. At older age, abnormal lipid deposition in the proximal portion of the aorta was observed both in heterozygotes and in homozygotes, pointing to the involvement of hyperhomocysteinemia in pathologic changes in large vessels.

In vitro tissue culture studies show that Hcy thiolactone induces the caspase-independent vascular endothelial cell death with features of apoptosis (MERCIE et al. 2000). In *Saccharomyces* Hcy is converted by methionyl-, isoleucyl-, and leucyl-tRNA synthetases to the thioester Hcy thiolactone. The high energy costs of this process are detrimental to cell growth (JAKUBOWSKI 1991). Under physiological conditions Hcy thiolactone easily reacts with side-chain amino groups of lysine residues in proteins (JAKUBOWSKI 1999).

The pathogenicity of Hcy through formation of Hcy thiolactone or stimulation of production of oxygen radicals can be prevented by its S-nitrosylation with nitric oxide to S-nitroso-Hcy. Methionyl-tRNA synthetase binds S-nitroso-Hcy to $tRNA^{Met}$, which leads to translational incorporation of S-nitroso-Hcy into protein (JAKUBOWSKI 2001). However, the NO-producing endothelial cells are themselves susceptible to the toxicity of Hcy and the range of this compensation is limited. Translationally incorporated Hcy was also detected in the cultured human vascular endothelial cells (STAMLER et al. 1993). Both translationally and post-translationally incorporated Hcy was found in human blood proteins (JAKUBOWSKI 2002).

The degradation of elastin, which is the major structural component of the aortic wall, may be considered as the initial event in the formation of aneurysm (WHITE et al. 1993). Elastin is a very stable protein with a biological half–life of 70 years. The basic regions of this protein are rich in lysine, which forms covalent cross-links by extracellular oxidation (PATEL et al. 1995). Elastin is synthesized and deposited in early childhood and no significant synthesis occurs in adult life. The main factor that contributes to the thinning of the aortic wall with age, appears to be the intensity of the gradual loss of elastin from the aortic wall, which is not compensated by the synthesis of this protein. Then, the age-related decrease in elastin concentration is not due to the reduced transcription of the elastin gene. Presumably, the degradation of elastin may be enhanced by translational incorporation of S-nitroso-Hcy and/or its N-homocysteinylation in the presence of Hcy tiolactone.

The deterioration of the elastin structures of the media layer of the abdominal aorta was found in minipigs following the dietary-induced mild hyperhomocysteinemia (CHARPIOT et al. 1998). The presented observation of the increased risk of AAA in carriers of the *MTHFR 677T* allele may then be due to the enhancement of the processes of elastin degradation in these persons.

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