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Association between genetic and environmental factors and the risk of Alzheimer's disease

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Abstract

*The only well confirmed genetic risk factor for sporadic Alzheimer's disease (AD) is the possession of apolipoprotein E (*APOE*) ε4 allele. As it contributes to 40-70% of AD cases, a large proportion of genetic variance may be determined by additional loci.*

*Our aim was to estimate how reported genetic factors (*APOE, NOS3, MTHFR*) interact to increase the risk for AD and combine them with environmental factors (homocysteine, vitamin B_{12}, cholesterol). Genotyping was performed in 154 AD patients and 176 healthy controls. Levels of homocysteine, vitamin B_{12} and cholesterol were assessed in subgroups of 100 AD patients and 100 controls.*

We found a difference in APOE *ε4 and* NOS3 *G/G distribution between groups ($p<0.005$). Plasma total homocysteine was increased and vitamin B_{12} decreased in AD patients ($p<0.001$). The influence of* APOE *ε4 and* NOS3 *G alleles on the risk of AD was independent of homocysteine, vitamin B_{12} levels and* MTHFR *status.*

Key words: *Alzheimer's disease, APOE, NOS3, MTHFR, homocysteine.*

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with complex pathogenesis [2]. Among genetic factors that contribute to the development of the late-onset form of Alzheimer's disease, apolipoprotein E (APOE) polymorphism is the most important one. The association between the disease and the *APOE* ε4 allele was first reported in 1993 and strongly supported later [3,33,37]. The *APOE* ε4 allele increases the risk of AD in a dose -related fashion and is associated with about 40-70% of late-onset cases. Nevertheless, *APOE* genotyping does not provide sufficient sensitivity or specificity to be used alone as a diagnostic test for AD. This explains the attempts to identify other genetic and environmental factors associated with Alzheimer's disease [32]. Potential candidates may be: genes encoding endothelial nitric oxide synthase (*NOS3* gene) and methylenetetrahydrofolate reductase (*MTHFR* gene), increased homocysteine levels, decreased levels of vitamin B_{12} or increased cholesterol levels.

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Endothelial isoform of nitric oxide synthase is involved in regulation of vascular tone and blood pressure, but also in the processes of learning and memory by generation of nitric oxide (NO), as retrograde messenger, during long-term potentiation [29]. NO may affect many other physiological processes such as oxidative phosphorylation in mitochondria, oxidative stress, inflammatory reaction and apoptosis. Moreover, NOS3 is an important regulator of iNOS (and likely other genes) expression *in vivo* [7]. Many variants have been detected in the *NOS3* gene and its promoter; however, the only common variation leading to amino acid substitution is 894 G/T or Glu298Asp. It has been reported that the possession of *NOS3* gene G/G genotype of common 894 G/T polymorphism is associated with an increased risk for AD [9,15,41]. Other studies have shown negative results [8,16,26].

5,10-methylenetetrahydrofolate reductase (MTHFR) is a major enzyme involved in folate-dependent regulation of homocysteine concentrations in humans. There are several different *MTHFR* mutations that cause severe homocystinuria or moderate or mild hyperhomocysteinaemia. The *MTHFR* gene has been mapped to chromosome 1p36.3 [14]. The most frequent mutation of the *MTHFR* gene 677C/T, rendering the MTHFR enzyme thermolabile, is linked to moderate hyperhomocysteinaemia [12]. Homocysteine levels correlate inversely with cognitive performance [10]. It has been postulated that hyperhomocysteinaemia elevates the risk of mild cognitive impairment and dementia of both vascular and Alzheimer type [4,40]. The most recent study showed that an increase in plasma homocysteine is an independent risk factor for the development of AD [34].

An association between AD and low concentration of vitamin B_{12} in serum and CSF has been described [19]. Some data suggest that high intake of vitamin B_{12} is related to a low risk of AD. It has been recently demonstrated that B vitamins may efficiently decrease the plasma level of Aβ40 and thus have a role in the prevention of AD [11]. However, no association has been found between risk of developing Alzheimer's disease and vitamin B_{12} intake [27].

Cholesterol is a factor possessing direct implications for AD. It has been shown that animals fed a diet supplemented with 2% cholesterol contained an increased load of Aβ in the brain cortex and hippocampus [35]. It has been suggested that cholesterol-lowering drugs that inhibit ACAT1 (acyl-coenzyme a: cholesterol acyltransferase 1) could inhibit gamma-secretase [18]. Recently, it was proposed that impaired brain cholesterol dynamics is the cause of Alzheimer's disease [22].

The aim of this study was to estimate the association of previously reported genetic factors (*APOE*, *NOS3* 894 G/T, *MTHFR* 677C/T) with levels of cholesterol, vitamin B_{12} and homocysteine in Alzheimer's disease. The design of the study was case-control in all factors with the exception of cholesterol.

Material and Methods

All human studies have been approved by the appropriate ethics committee and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All persons gave their informed consent prior to their inclusion in the study. The AD group consisted of 154 patients (F-102, M-52, mean age of onset = 71.5±4.67) recruited from the Department of Neurodegenerative Disorders at the Mossakowski Medical Research Centre in Warsaw. In all AD patients the disease was diagnosed as probable according to the NINCDS-ADRDA criteria. All of them were examined by a neurologist, a neuropsychologist (evaluation in MMSE, Global Deterioration Scale, Alzheimer's Disease Assessment Scale – cognitive subscale (ADAS-cog) and Blessed Dementia Rating Scale) and a psychiatrist. In addition, a CT scan with assessment of hippocampal fissure was obtained for each patient. The control group consisted of 176 non-demented people (F-120, M-56, mean age = 72.7±6.23; MMSE score > 27). Blood samples were taken after obtaining written consent from the patient or his/her representative. DNA was isolated from leukocytes using standard protocols. *APOE*, *NOS3* 894 G/T and *MTHFR* 677C/T genotyping was done using a method of restriction genotyping described by [6,9,12].

Total fasting homocysteine concentrations in blood plasma were measured with a fluorescence polarization assay (Abbott IMx Homocysteine Assay), and plasma vitamin B_{12} by immunoassay. Reference ranges are from 4 to 12 µmol/L for homocysteine and 157-1059 pg/ml for vitamin B_{12}. Homocysteine and vitamin B_{12} were assessed in subgroups of subjects: 100 patients with AD and 100 controls.

Statistical analysis

Chi-square test and Kruskal-Wallis rank sum test were used in statistical analysis. Statistica 6.0 was used for the statistical analysis.

Results

The frequencies of *APOE* ε4, ε3 and ε2 alleles were 0.31, 0.65, 0.04 in the AD group and 0.11, 0.84, 0.05 in the control group, respectively (Table I). There was a highly significant overrepresentation of the *APOE* ε4 allele in AD patients compared with controls (χ^2 test, p<0.005). Our study revealed an increased frequency of ε4/4 genotype in AD patients (9% vs. 2%, p=0.017). Also *APOE* ε3/4 genotype frequency was increased in the AD group (p<0.005). Genotype *APOE* ε3/3 was found more often in the control group compared to the AD one.

Frequencies of *NOS3* genotypes G/G, G/T and T/T in the AD group were: 0.69: 0.25: 0.06 (Table II). In the control group those frequencies were: 0.56: 0.35: 0.09, respectively. Genotype G/G was significantly more frequent in the AD group than in the control group (χ^2 test, p=0.024), and the "G" allele was also significantly overrepresented in the AD group (χ^2 test, p=0.025).

There were no significant differences in the frequencies of the *MTHFR* alleles and genotypes among AD and cognitively intact elderly people. Frequencies of *MTHFR* genotypes C/C, C/T and T/T in the AD group were: 0.51: 0.38: 0.11. In the control group those frequencies were: 0.55: 0.38: 0.07, respectively.

The mean plasma total homocysteine levels were significantly higher in patients with AD (17.5±7.7 µmol/l) than in controls (14.4±4.5 µmol/l) (p<0.0001) and vitamin B_{12} levels were significantly lower in AD patients (312.1±129.2 pg/ml) in comparison to controls (413.8±242.5 pg/ml) (p=0.0006) (Table III). Lipids (total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides) were assessed only in a subgroup of 100 AD patients. The mean total cholesterol was slightly higher than the reference range in this group. There was no correlation between the severity of the disease and the level of cholesterol.

Table I. Frequencies of *APOE* alleles and genotypes

APOE	ε2	ε3	ε4	ε 2/2	ε 2/3	ε 2/4	ε 3/3	ε 3/4	ε 4/4
AD N=154	0.04	0.65	**0.31***	0 (0%)	6 (4%)	5 (3.2%)	61 (39.6%)	**73* (47.4%)**	**9*** ** (5.8%)
Controls N=176	0.05	0.84	0.11	0 (0%)	16 (9.1%)	3 (1.7%)	**124** (70.5%)**	31 (17.6%)	2 (1.1%)

** p<0.005; ** p<0.05, *** p=0.017.*

Table II. Frequencies of *NOS3* 894 G/T alleles and genotypes

NOS3	G allele	T allele	G/G	G/T	T/T
AD N=154	**0.81***	0.19	**106*** (68.8%)	38 (24.7%)	10 (6.5%)
Controls N=176	0.74	0.26	100 (56.8%)	60 (34.1%)	16 (9.1%)

** p=0.025.*

Table III. Plasma levels of homocysteine, vitamin B_{12} and lipids in AD and controls

	AD patients	Control Group	Reference range
N	100	100	
Homocysteine	17.5±7.7	14.4±4.5*	4-12 µmol/l
Vit. B_{12}	312.1±129.2	413.8±242.5**	157-1059 pg/ml
Total cholesterol	209.9±34.4	nd	150-200 mg/dl
LDL cholesterol	131±30. 9	nd	100-129 mg/dl
HDL cholesterol	55.7±14.4	nd	>45 mg/dl
Triglycerides	109±59.7	nd	60-150 mg/dl

*p<0.0001, ** p=0.0006, nd – not determined. Results are expressed in mean ± SD.*

Discussion

Our results demonstrate a highly significant difference in *APOE* ε4 and *NOS3* G/G distribution between healthy controls and AD patients. Moreover, plasma total homocysteine was increased and vitamin B_{12} decreased in AD patients. The effect of *APOE* 4 and *NOS3* G alleles on the risk of AD was independent of homocysteine, vitamin B_{12} levels and *MTHFR* polymorphism.

Many studies have confirmed that presence of the *APOE* ε4 allele is the most important genetic risk factor for late-onset AD [3,33,37]. Its frequency in AD patients ranges between 0.32 and 0.42; while in normal individuals this frequency is estimated as between 0.13 and 0.17. However, some ethnic variations in genotypic risk have been reported. The results of our study were consistent with those performed on Caucasian populations and revealed a statistically significant increase of *APOE* ε4 frequencies in the AD group compared to controls.

Our data suggest an association of *NOS3* 894 G/G genotype with AD. The significance of *NOS3* polymorphism in AD remains controversial. The polymorphism of codon 894 G/T is the only common polymorphism leading to amino acid substitution in mature protein, in which substitution of guanine by thymine results in the exchange of aspartate against glutamate at position 298 [17]. The impact of this mutation on gene expression or enzyme catalytic activity remains controversial. The Glu298Asp polymorphism is located on the surface of the protein structure, far from the active site or the dimerization interface [5]. These data suggest that the Glu/Glu genotype exerts its effect by a mechanism not related to synthase of nitric oxide. Analysis of recombinant protein eNOS Asp298 and Glu298 indicates that there is no difference in catalytic function [13]. However, in the cell NOS3 is regulated by a variety of mechanisms. The effect of Glu298Asp on the NOS3 activation process has never been studied, but it was observed that substitution of Glu at codon 298 by Asp increases susceptibility to cleavage of this protein [39]. Moreover, the impact of this polymorphism on blood flow was demonstrated, which suggests that NO availability may be altered [28].

The analysis of the G allele-related risk of late onset AD development in different populations has given both positive and negative results [9,8,15,16,26]. Our previous analysis also did not confirm that there is a genetic association between 894 G/T polymorphism of *NOS3* and the risk of AD [38]. However, the previous analysis was performed on a small group of patients and could be underpowered. In a recent study by Wang et al. [41] conducted on 338 AD patients and 378 controls in China, the association of the *NOS3* 894G/G genotype with AD was dependent on the *APOE* ε4 status, and the risk was increased only in *APOE* ε4 noncarriers. We did not find a similar interaction between the examined polymorphisms in Polish patients.

As demonstrated recently, significant diversity between association studies is frequent, and may be explained by bias or genuine population diversity [20]. Genetic association studies require cautious replication, and subsequent meta-analysis may detect previously unrevealed diversity, enabling the evaluation of interfering effects in further studies. Analysis of genetic associations including subgroup effects requires large numbers of subjects [20].

The association between total homocysteine level in plasma and AD found in our study was consistent with previous case-control and longitudinal prospective studies [1,24,30,34]. The data presented in this study are an extension of our previously described case-control study [31].

In some countries, but not in Poland, the fortification of food with folic acid has been started. It may have an effect on homocysteine levels and cognitive performance in the elderly population, but has to be evaluated in longer perspective. So far there is no evidence suggesting that food enrichment with folic acid and vitamins B_{12} and B_6 may repair the cognitive impairment seen in AD [23,25]. It has even been reported that increased vitamin B complex intake did not improve cognitive function in elderly patients with vascular lesions [36]. This may indicate that neuronal and synaptic damage is difficult to restore and maybe anti-hyperhomocysteinaemia interventions should be started early in the disease process. Anti-hyperhomocysteinaemia treatment seems to be a preventive rather than curative strategy in AD, and middle-age subjects (45-55 years old) should be included as a target population in clinical trials.

The data presented in our study indicate that a highly significant difference exists in *APOE* ε4 and *NOS3* G/G distribution in AD patients and healthy controls. The influence of the *APOE* 4 and *NOS3* G alleles on the risk of AD was independent of homocysteine, vitamin B_{12} levels and *MTHFR* status.

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