GENETIC RISK FACTORS IN ALZHEIMER'S DISEASE

Ph.D. Thesis

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Szeged

2010

Articles the Thesis is based on

Fehér Á, Juhász A, Rimanóczy Á, Kálmán J, Janka Z. Association between BDNF Val66Met Polymorphism and Alzheimer's Disease, Dementia with Lewy Bodies and Pick's Disease. Azheimer's Disease and Associated Disorders. 2009. 23(3):224-228. **IF_{2008}: 3.220**

Fehér Á, Juhász A, Rimanóczy Á, Csibri É, Kálmán J, Janka Z. Association between a genetic variant of the alpha-7 nicotinic acetylcholine receptor subunit and four types of dementia. Dementia and Geriatric Cognitive Disorders. 2009. 28(1): 56-61. **IF_{2008}: 3.142**

Selected abstracts related to the thesis:

A Juhász, Á Rimanóczy, **Á Fehér**, J Kálmán, M Gálfi, Z Janka. Alpha-7 nicotinic acetylcholine receptor and apolipoprotein E polymorphisms in Alzheimer's dementia. The Journal of the European College of Neuropsychopharmacology. Vol. 17, Suppl. 4, p. S417; IF_{2007}: 3.794 (20^{th} Congress of European College of Neuropsychopharmacology, Vienna, Austria, 2007)

A Juhász, Á Rimanóczy, **Á Fehér**, J Kálmán, Z Janka. Apolipoprotein E and cyclooxygenase 2 -765 G>C polymorphisms in Alzheimer's dementia. The Journal of the European College of Neuropsychopharmacology. Vol. 18, Suppl. 4, p. S203; IF_{2008}: 3.661 (21^{st} Congress of European College of Neuropsychopharmacology, Barcelona, Spain, 2008)

A Juhász, Á Rimanóczy, **Á Fehér**, M Pákáski, J Kálmán, Z Janka. Association Analysis of BACE C786G and apolipoprotein E polymorphisms in Alzheimer's disease. European Psychiatry. 2008; 23: S195. (16^{th} AEP Congress, Nice, France, 2008)

Á Fehér, A Juhász, Á Rimanóczy, J Kálmán, Z Janka. BDNF Val66Met polimorfizmus vizsgálata demenciákban. *[BDNF Val66Met polymorphism in dementias]*. Neuropsychopharmacologia Hungarica, Volume X., Suppl 2, p. 18. (XI. Conference of Hungarian Neuropsychopharmacology, Tihany, Hungary, 2008)

A Juhasz, **Á Fehér**, Á Rimanóczy, J Kálmán, Z Janka. Interferon gamma +874 T/A gene polymorphism is not a risk factor in the pathogenesis of Alzheimer's disease. European Psychiatry. 2009; P01 95. (17^{th} European Congress of Psychiatry, Lisbon, Portugal, 2009)

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Á Fehér, A Juhász, Á Rimanóczy, J Kálmán, Z Janka. Genetic interaction of alpha-7 nicotinic acetylcholine receptor and brain-derived neurotrophic factor polymorphisms in Alzheimer's disease. Abstract book. p. 339. (10^{th} World Congress of Biological Psychiatry, Paris, France, 2009)

Á Fehér, A Juhász, Á Rimanóczy, J Kálmán, Z Janka. Gyulladással kapcsolatos génpolimorfizmusok vizsgálata Alzheimer-kórban. *[Neuronflammation-related gene polymorphisms in Alzheimer's disease]*. Volume XI., Suppl 3, p. 15-16. (XII. Conference of Hungarian Neuropsychopharmacology, Tihany, Hungary, 2009)

Á Fehér, A Juhász, Á Rimanóczy, J Kálmán, Z Janka. Serotonin system gene polymorphisms in Alzheimer's disease. European Psychiatry. European Psychiatry. 2010; PW67 (18^{th} European Congress of Psychiatry, Munich, Germany, 2010)

Abbreviations

5HT: serotonin

5HT2A: serotonin receptor type 2A

5HTT: serotonin transporter

5HTTLPR: serotonin transporter gene-linked polymorphic region

α7 nAChR: alpha-7 nicotinic acetylcholine receptor

ACE: Addenbrooke's Cognitive Examination

AD: Alzheimer's disease

APOE: apolipoprotein E

APP: amyloid precursor protein

Aβ: amyloid-β

BACE1: β-site APP cleaving enzyme-1

BDNF: brain-derived neurotrophic factor

CALHM1: calcium homeostasis modulator 1

CHRFAM7A: fusion of the CHRNA7 exons 5-10 and FAM7A exons A-E

CHRNA7: α7 nAChR subunit

DHCR24: 24-dehydrocholesterol reductase

FAM7A: family with sequence similarity 7A

HWE: Hardy-Weinberg equilibrium

IFNG: Interferon-γ

MMSE: Mini-Mental State Exam

NINCDS/ADRDA: National Institute of Neurological and Communicative Disorders and Stroke / Alzheimer's Disease and Related Disorders Associations

OR: odds ratio

PCR: polymerase chain reaction

PGE2: prostaglandin E2

PLAG4A: cytosolic phospholipase A_2, group IVA

PSEN1: presenilin 1

PSEN2: presenilin 2

PLAU: urokinase-type plasminogen activator

PTGS2: prostaglandin endoperoxide synthase 2

Seladin1: Selective AD Indicator

SLC6A4: solute carrier family 6 member 4 gene encoding serotonin transporter

SNP: single nucleotide polymorphism

Table of contents

Introduction ..... - 4 -
I. Amyloid-β metabolism and AD ..... - 7 -
II. Cholesterol metabolism and AD ..... - 9 -
III. Neuroinflammation and AD ..... - 9 -
IV. Neuronal dysfunction and AD ..... - 10 -
Aims ..... - 13 -
Subjects and Methods ..... - 14 -
Patients and controls ..... - 14 -
Genetic analyses ..... - 15 -
Statistical analyses ..... - 18 -
Results ..... - 19 -
I. Amyloid-β metabolism-related polymorphisms ..... - 20 -
II. Cholesterol metabolism-related polymorphisms ..... - 22 -
III. Neuroinflammation-related polymorphisms ..... - 24 -
IV. Neuronal dysfunction ..... - 26 -
Discussion ..... - 31 -
I. Amyloid-β metabolism-related polymorphisms ..... - 32 -
II. Cholesterol metabolism-related polymorphisms ..... - 33 -
III. Neuroinflammation-related polymorphisms ..... - 34 -
IV. Neuronal dysfunction ..... - 35 -
V. Genetic interactions ..... - 39 -
Conclusions ..... - 40 -
Limitations ..... - 41 -
References ..... - 43 -
Appendix ..... - 49 -

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder representing the most common cause of dementia in the elderly population (Ritchie and Lovestone, 2002). Current estimates indicate, that worldwide about 25-30 million people are suffering from AD, and the number of cases will triple by 2040 due to increasing life expectancy (Ferri et al., 2005). The incidence of AD increases with age: every five years after the age of 65 the risk of developing this devastating disease approximately doubles (Minati et al., 2009). In addition to the tragedy of the patients, AD places psychological and economical burden on caregivers, and represents a major public health problem being among the most costly diseases for the society in Europe and in the United States.

The clinical manifestation of AD is characterized by progressive memory impairment and cognitive deficits. Typically, AD begins with subtle and poorly recognized failure of memory that worsens inevitably. Further symptoms include confusion, impaired social judgment, language disturbance, agitation, withdrawal, irritability and impulsivity. AD pathology is characterized by the presence of extracellular senile plaques composed of amyloid-β (Aβ) peptid and intraneuronal neurofibrillary tangles containing hyperphosphorylated tau protein (Selkoe et al., 2001). The amyloid and the tau hypotheses consider these proteins as inducers and key players of the disease. Further neuropathological features include cortical atrophy, degeneration of cholinergic basal forebrain neurons, hippocampal atrophy and enlarged ventricles.

Genes have a varied influence on developing AD, ranging from the autosomal-dominant inheritance in the familial forms (1-5% of cases) to the polygenic background in late-onset (>65 years of age) sporadic AD (≥95% of cases). In addition to the genetic component, the risk for developing AD, the age at onset and the course of the disease are influenced by several other factors including sociodemographic, life style, environment and comorbid medical conditions (Papassotiropoulos et al., 2006). Age and female sex represent risk for developing AD. Poor education, low mental ability, traumatic brain injury, stroke and history of depression can also predispose to AD.

The genetic component however, seems to be of major importance, since according to twin studies, a major part of the risk for sporadic AD is genetically determined (Gatz et al., 1997).

The amyloid precursor protein (APP) and the presenilin 1 (PSEN1) and 2 (PSEN2) genes are currently known to be implicated in the familial forms of AD (Papassotiropoulos et al., 2006). Identification and characterization of dominant mutations of these genes was instrumental for the understanding of the biological mechanisms leading to enhanced Aβ accumulation and senile plaques generation. In contrast with the familial AD, the causing factors of the Aβ accumulations and other pathological mechanisms remain mostly unclear in the sporadic form. The complex genetic model of sporadic AD suggests that several heterogeneous susceptibility sets of genes may converge on the pathological processes that underlie the disease. However, so far only the apolipoprotein E (APOE) gene has been definitively associated with the risk for AD (Brouwers et al., 2008).

APOE is involved in lipid transport and metabolism. Furthermore, it plays a specific role in the central nervous system, including neuronal development, regeneration and certain neurodegenerative processes. The polymorphism of the APOE gene determines three isoforms of APOE protein (ε2, ε3, ε4) with different conformation and lipid binding properties (Cedazo-Mínguez and Cowburn, 2001). Proportional relationship was found between the number of the inherited ε4 alleles and the risk for developing AD and the age at onset. The APOE ε4 isoform prefers very low density lipoprotein and it is less effective in cholesterol transport as compared to the other APOE isoforms (Cedazo-Mínguez and Cowburn, 2001). Membrane cholesterol modulates the cleavage of the APP protein and in the presence of the ε4 isoform the balance is shifted to the production of Aβ (Stefani and Liguri, 2009).

The amyloid cascade hypothesis has been the predominant model of molecular mechanisms underlying the pathogenesis of AD. According to this model, the fundamental cause of AD is the accumulation and aggregation of the Aβ peptide in the brain (Hardy and Selkoe, 2002). The amyloid cascade hypothesis is widely accepted, but does not explain all aspects of AD aetiology, and the major role of other pathogenic mechanisms is also presumable including inflammation, oxidative stress, lipid dysfunction, signaling deficits and neuronal dysfunction.

The genetic epidemiology of sporadic AD remains a very active area of research, since a large part of the genetic aetiology is still poorly understood and remains unresolved. The aim of our work was to contribute to this field investigating gene polymorphisms presumably involved in AD pathogenesis. The candidate gene polymorphisms in this study were selected and grouped on the basis of the processes that presumably lead to the development of AD: Aβ metabolism, cholesterol metabolism, neuroinflammation and neuronal dysfunction (Figure 1). The characteristics of the investigated gene polymorphisms are summarized in Table 1.

Table 1. Investigated polymorphisms

Gene		Chromosomal	The investigated polymorphisms		Changes at	Alleles	Functional consequences
Official symbol	Name	localization	Rs number	Position	protein level		
APOE	apolipoprotein E	19q13.2	rs429358 rs7412	exon 4 C/T exon 4 C/T	Cys112Arg Cys158Arg	ε2, ε3, ε4	ε4 allele: ↓ cholesterol transport
BACE1	beta-site APP-cleaving enzyme 1	11q23.3	rs638405	exon 5 C/G	- (Val262) Silent mutation	C, G	Not known
PLAU	plasminogen activator, urokinase	10q22.2	rs2227564	exon 6 C/T	Pro141Leu	C, T	Not known
CALHM1	calcium homeostasis modulator 1	10q24.33	rs2986017	exon 1 T/C	Leu86Pro	Leu, Pro	Leu allele: ↓ CALHM1 function
DHCR24	24-dehydrocholesterol reductase	1p33-p31.1	rs600491	intron 5 C/T	-	C, T	Not known
DHCR24	24-dehydrocholesterol reductase	1p33-p31.1	rs638944	intron 2 G/T	-	G, T	Not known
IFNG	interferon, gamma	12q14	rs2430561	intron 1 T/A	-	T, A	T allele: ↑ IFNG level
PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium-dependent)	1q25	rs10798059	intron 1 A/G	-	A1, A2	A2 allele: ↑ PLA2G4A level
PTGS2	prostaglandin-endoperoxide synthase 2 (cyclooxygenase 2)	1q25.2-q25.3	rs20417	promoter G/C	-	G, C	G allele: ↑ PTGS2 promoter activity
CHRFAM7A	CHRNA7 (cholinergic receptor, nicotinic, alpha 7, exons 5-10) and FAM7A (family with sequence similarity 7A, exons A-E) fusion	15q13.1	-	exon 6	deletion, frameshift	-2bp, wild	-2bp allele: stop codon
SLC6A4 (5HTTLPR)	solute carrier family 6, member 4 (serotonin transporter gene-linked polymorphic region)	17q11.1-q12	rs4795541	promoter	insertion/deletion	L, S	S allele: ↓ serotonin reuptake
HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	13q14-q21	rs6313	exon 1 T/C	- (Ser34) Silent mutation	T, C	C: ↓ HTR2A gene activity
BDNF	brain-derived neurotrophic factor	11p13	rs6265	exon 1 G/A	Val66Met	Val, Met	Met allele: ↓ BDNF secretion

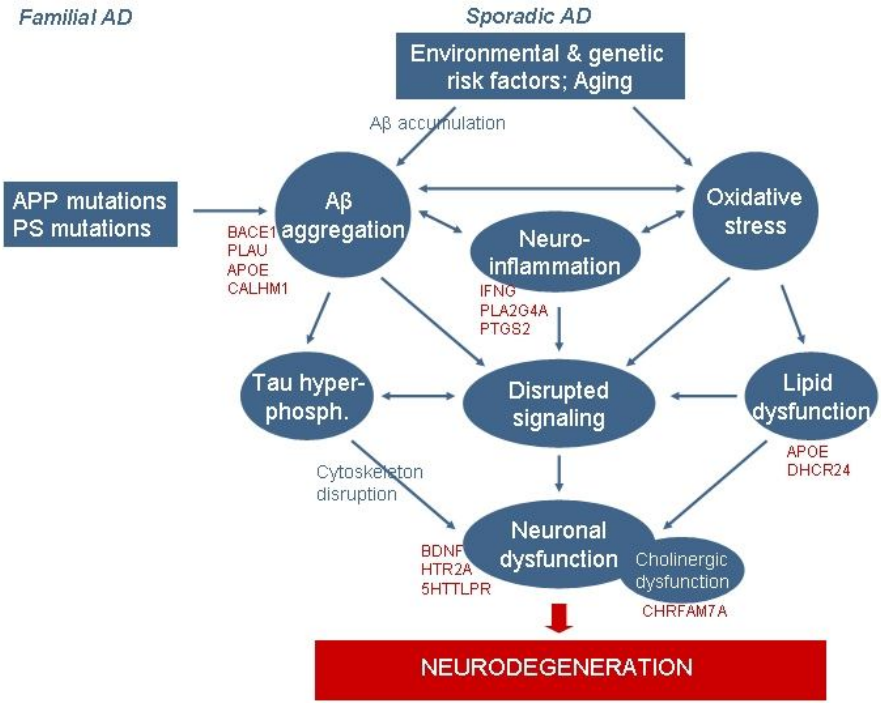

Figure 1. The pathological cascade of Alzheimer's disease (AD)

Mutations of the amyloid precursor protein (APP) and the presenilin genes (PS) cause familial forms of AD by increasing the accumulation of amyloid-β (Aβ). In the sporadic form of AD multiple environmental and genetic risk factors interact through various pathways to cause neurodegeneration. Aging is a major contributor, and the apolipoprotein E (APOE) gene is so far the only unequivocally confirmed genetic risk factor for sporadic AD. The investigated genes (shown with red) of this study were selected and grouped on the basis of the main pathological processes presumably leading to AD.

I. Amyloid-β metabolism and AD

APP is a single transmembrane polypeptide with characteristics of a cell surface receptor. The primary function of APP is unknown, but it is believed to have role during neuronal development, to be implicated in synaptic formation and repair, signaling and cell adhesion (Walsh et al., 2007). APP follows two distinct cleavage pathways competing α- and β-secretases, and both pathways are active in normal metabolism (Selkoe et al., 2001). The predominant cleavage of APP is mediated by the α-secretase generating non-amyloidogenic products. The cleavage leading to Aβ generation is mediated by β- and γ-secretases. PSEN1 and PSEN2 are part of the γ-secretase complex.

The β-site APP cleaving enzyme-1 (BACE1) gene at locus 11q23.3 encodes β-secretase, the key and rate-limiting enzyme in the cascade of Aβ formation. Genetic variations of BACE1 may act on the Aβ generation and thereby influence neurodegenerative processes leading to AD. The rs638405 synonymous polymorphism of BACE1 is a nucleotide change in

exon 5 (C786G) with no alteration at the amino acid level (Val262) (Murphy et al., 2001). The reports of the association studies on the role of BACE1 in AD are contradictory. Some studies have reported that the BACE1 C786G polymorphism influences the risk for AD, especially in those carrying the APOE ε4 allele (Clarimon et al., 2003, Kirschlint et al., 2003; Cai et al., 2005). However, other authors have not found such an association (Murphy et al., 2001; Jo et al., 2008; Todd et al., 2008). This study is the first report on BACE1 C786G polymorphism in the Hungarian population.

The PLAU gene encoding urokinase-type plasminogen activator (uPA) maps to chromosome 10q22.2, a candidate susceptibility locus in a broad AD linkage region (10q21-24) (Riemenschneider et al., 2006). The uPA serine protease converts the inactive plasminogen to the active plasmin form, and it is also capable to degrade Aβ directly. Plasmin promotes α-cleavage of APP, degrades secreted and aggregated Aβ, thereby blocks Aβ neurotoxicity (Finckh et al., 2003). The frequent single nucleotide polymorphism (SNP) of PLAU (rs2227564, C1788T) is located in exon 6, and results in an amino acid change at codon 141 (Pro141Leu). The findings of the association studies investigating the Pro141Leu polymorphism in AD are controversial, therefore further evaluations of genotypic distribution are required (Finckh et al., 2003; Myers et al., 2004; Ertekin-Taner et al., 2005; Bagnoli et al., 2005; Papassotiropoulos et al., 2005; Riemenschneider et al., 2006).

An increasing body of evidence supports the major contribution of the dysregulation of calcium homeostasis in accelerating pathological changes in AD, i.e. Aβ accumulation (Bezprozvanny and Mattson, 2008). A promising candidate gene for AD, the calcium homeostasis modulator 1 (CALHM1) has been recently identified at position 10q24.33, in the same AD linkage region (10q21-24), where the PLAU gene is also located. It is localized both in the cell and in the endoplasmatic reticulum membranes and it appears to modulate the intracellular calcium levels (Dreses-Werringloer et al., 2008).

An SNP (rs2986017) at nucleotide 257 (C/T) producing a non-conservative amino acid substitution at codon 86 (Pro86Leu) has been identified. The Leu allele has been reported to be associated with AD, and in vitro demonstrated to result in impaired CALHM1 function leading to decreased calcium permeability and reduced cytoplasmic calcium levels (Dreses-Werringloer et al., 2008). Intracellular calcium levels were reported to affect the metabolism of APP and thereby the levels of Aβ (Green et al., 2008; Tanzi et al., 2008). With regards to the hypothesis that CALHM1 polymorphism may represent a susceptibility factor for AD, recent association studies failed to support this possibility (Bertram et al., 2008; Minster et al., 2009, Sleegers et al., 2009).

II. Cholesterol metabolism and AD

Cholesterol is a necessary structural component and cell fluidity modulator of the cell membrane. Most of the CNS cholesterol is produced via local de novo synthesis. The role of cholesterol in AD is a controversial topic, but it seems that an optimal amount of cell cholesterol may be critical for brain homeostasis (Peri and Serio, 2008). The amount of cell cholesterol has a major impact on Aβ generation and cell resistance against Aβ toxicity.

The 24-dehydrocholesterol reductase (DHCR24) gene at locus 1p33-p31.1 encodes Seladin1 (Selective AD Indicator), an enzyme that is involved is cholesterol biosynthetic pathway. Seladin1 catalyses the conversion of desmosterol to cholesterol, but also has other relevant biological effects. Seladin1 confers resistance against Aβ and oxidative stress induced apoptosis by effective inhibition of caspase-3 activity, and prevention of p53 degradation (Peri and Serio, 2008). Seladin1 also affects the Aβ generation via the modulation of membrane cholesterol content.

The genetic association between Seladin1 and risk for AD was investigated by Lämsä and co-workers genotyping four SNPs of DHCR24 genes: rs638944 (intron 2 G/T), rs600491 (intron 5 C/T), rs718265 (intron 6 A/G), rs7374 (exon 9 C/T) (Lämsä et al., 2007). No significant association of single SNPs with AD risk was found, but in men the T allele of rs600491 increased the risk for AD. Haplotype analyses revealed, that from the two haplotype blocks (block 1: rs638944 and rs600491; block 2: rs718265 and rs7374), only block 1 showed significant association with risk for AD, being CG the risk haplotype (Lämsä et al., 2007).

III. Neuroinflammation and AD

An increasing body of evidence supports the major contribution of inflammatory processes in accelerating pathological changes in AD (Lukiw and Bazan, 2000). The activated microglia-driven inflammatory response resulted in an elevated release of various pro-inflammatory mediators such as cytokines and prostaglandins which may interact at multiple levels with neurodegeneration (Heneka and O'Banion, 2007). The extent of the inflammatory response can be influenced by the individual's genetic background of inflammatory mediators (Wan et al., 2008). The cytokine interferon-γ (IFNG) plays an important role in the induction of the immune-mediated inflammatory response (Blasko, 2001). In human neuroblastoma cells pre-treatment with IFNG increased the expression of cytosolic phospholipase A_2, group IVA

(PLAG4A) resulting in an elevated release of arachidonic acid and a subsequently elevated level of prostaglandins (Bate et al., 2006). The T allele of the IFNG T874A polymorphism correlates with an increased level of IFNG (Pravica et al., 2000). Association studies did not support the hypothesis that the IFN-γ T874A polymorphism may represent a risk factor for AD (Scola et al., 2003; Galimberti et al., 2004). Phospholipase A_2 is a superfamily of enzymes that include key modulators of cerebral phospholipid metabolism. The PLA2G4A catalyzes the release of arachidonic acid from membrane phospholipids. A polymorphic site for Ban*I* restriction enzyme in PLA2G4A gene is an A to G base change (Wei and Hemmings, 2004). According to our knowledge this is the first report on PLA2G4A Ban*I* polymorphism in AD.

Prostaglandin endoperoxide synthase 2 (PTGS2) is a key enzyme in prostaglandin biosynthesis converting arachidonic acid to prostaglandin E2 (PGE2). A functional polymorphism (G-765C) in the promoter region of the PTGS2 gene has been identified and significantly lower promoter activity has been reported for the C allele (Papafili et al., 2002). Abdullah and co-workers reported that the possession of the C allele is associated with decreased risk for AD (Abdullah et al., 2006). PLA2G4A and PTGS2 are involved in the same pathway of prostaglandin synthesis and both PLA2G4A and PTGS2 genes are located at the same 1q25 region, thus these two genes may interact in many ways. Since IFNG has been demonstrated to elevate PLA2G4A expression, it may also interplay in the generation of prostaglandins therefore a combined effect with PLA2G4A and PTGS2 can be postulated.

Besides its major role in cholesterol metabolism and its involvement in Aβ generation, APOE plays also an isoform-specific role in mediating brain inflammatory response. Cell culture studies reported association between the presence of APOE ε4 and increased inflammatory response (Jofre-Monseny, 2007). Mouse macrophages transfected with APOE ε4 secrete significantly more pro-inflammatory cytokines as compared to those transfected with APOE ε3 (Tsoi, 2007).

IV. Neuronal dysfunction and AD

IV.1. Cholinergic dysfunction

AD is associated with a progressive loss of cholinergic neurons and a consequent acetylcholine deficit, particularly in the temporal and parietal neocortex and hippocampus. The extensive degeneration of the cholinergic neurons in the basal nucleus of Meynert and in

the medial septal nucleus is responsible for the loss of up to 95% of the cholinergic innervation to the cortex. The reduced activity of choline acetyltransferase also contributes to the decreasing availability of acetylcholine. It is also presumable that altered activity of acetylcholine receptors determined by genetic variations can also influence the cholinergic transmission.

Alpha-7 nicotinic acetylcholine receptors (α7 nAChRs) are homopentamer, ligand-gated cationic channels. They are widely expressed in the central nervous system with high levels in the regions relevant to memory functions and involved in processing of sensory information, such as hippocampus (Weiland et al., 2000). It has been demonstrated that Aβ binds to α7 nAChR with high affinity and they both are present in senile plaques (Wang et al., 2000). Their interaction alters several neurochemical processes including Ca^{2+} homeostasis and acetylcholine release, and thereby modulates neuronal physiological functions implicated in memory processes. Chronic inhibition of cholinergic signaling by Aβ could contribute to the cognitive deficits associated with AD (Pettit et al., 2001).

The α7 nAChR subunit gene (CHRNA7) at region 15q13.1 is duplicated from exon 5 to 10 (Gault et al., 1998; Riley et al., 2002). The partially duplicated CHRNA7 and four other exons originated from Family with sequence similarity 7A (FAM7A) gene form a hybrid gene (CHRFAM7A). CHRFAM7A is not present on every human chromosome and some individuals lack one (30%) or both (5%) copies (Riley et al., 2002). A -2bp deletion polymorphism at position 497-498 in exon 6 was identified, which is specific to CHRFAM7A and does not occur in CHRNA7 (Gault et al., 1998). The -2bp deletion causes a frameshift, introducing a stop codon within exon 6 and therefore a truncation in a putative gene product. Since CHRFAM7A is reported to be expressed as mRNA, possible regulatory effects should also be considered. Liou and co-workers (2001) failed to find association between CHRFAM7A -2bp deletion polymorphism and AD, investigating a relatively low number of cases in an Asian population (Liou et al., 2001).

IV.2. Serotonergic dysfunction

History of depression, and in particular with first onset before 60 years of age, represents risk for developing AD later in life (Geerlings et al., 2008). Possible links between AD and depression may be the long term occurrence of inflammatory processes, and the involvement of serotonergic disturbances (Ownby et al., 2006). Serotonergic involvement in AD is

supported by findings including cerebrospinal fluid alteration of serotonin (5HT) and loss of synthesizing neurons and 5HT receptors in AD.

The solute carrier family 6 member 4 (SLC6A4) gene at locus 17q11.1-q12 encodes 5HT transporter (5HTT). The promoter region of the SLC6A4 gene shows a 22 bp tandem repeat polymorphism, which is designated as 5HTT gene-linked polymorphic region (5HTTLPR). The two major alleles have 14 and 16 repeats, thus differing in 44 bp, and were denoted as short (S) and long (L) alleles. The 5HTTLPR polymorphism determines dose-dependent 5HT reuptake from the synaptic cleft, the S allele is less effective. Association studies reported mainly negative results investigating the relationship between AD and 5HTTLPR (Seripa et al., 2008; Micheli et al., 2006; Grünblatt et al., 2008).

The gene HTR2A, which codes for the 5HT receptor type 2A (5HT2A) is located at 13q14–q21. The rs6313 polymorphism of HTR2A is a nucleotide change in exon 1 (T102C), and does not alter the serine at position 34 (silent mutation). This polymorphism is located near to the promoter region thereby may have some role in the regulation of gene expression. The activity of the C allele has been shown to be significantly decreased as compared to the T allele. Association studies did not support the association of HTR2A T102C and 5HTTLPR polymorphisms with AD, when they were separately analyzed (Micheli et al., 2006; Lam et al., 2004). However, Micheli and co-workers (2006) found an interaction between the HTR2A T102C and 5HTTLPR polymorphisms: HTR2A C/C and 5HTTLPR L/L genotype carriers had an increased risk for AD.

IV.3. Brain-derived neurotrophic factor

The brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors, produced by cortical neurons. Besides its general role in neurodevelopment, BDNF has important functions in the adult brain such as promoting the survival and maintaining the structural integrity of neuronal cells (Murer et al., 2001). The activity-dependent expression of BDNF plays a role in modulating synaptic changes associated with learning and memory (Tyler et al., 2002). It has been reported that patients with AD have reduced BDNF levels in the hippocampus and in the temporal cortex as compared to healthy controls (Ferrer et al., 1999).

The BDNF gene encodes a precursor peptide (pro-BDNF) which is secreted and cleaved by extracellular protease to form the mature BDNF protein (Seidah et al. 1996). An SNP at nucleotide 196 (G/A) producing a non-conservative amino acid substitution at codon

66 (Val/Met) has been identified (Ventriglia et al., 2002). Although this SNP is located in the 5' pro-BDNF sequence and does not affect the function of the mature BDNF, it has a major impact on the intracellular trafficking and regulated secretion of pro-BDNF (Egan et al., 2003). Genetic influences on BDNF secretion can lead to alterations in hippocampal activity. The BDNF Val66Met polymorphism has been found to be associated with episodic memory and hippocampal function (Hariri et al., 2003). Nevertheless, there are conflicting reports on the correlation between AD and BDNF Val66Met polymorphism (Combarros et al., 2004; Matsushita et al., 2005; Ventriglia et al., 2002; Akatsu et al., 2006).

Aims

- The aim of our investigation was to provide data on APOE polymorphism in the Hungarian population to further confirm the role of ε4 allele in AD.
- We tested the hypothesis whether the BACE1 C786G and PLAU Pro141Leu polymorphisms are associated with AD, either alone or in genetic interaction.
- With reference to the Dreses-Werringloer paper (2008) we tried to support their findings, that CALHM1 Leu86 allele can increase the risk for developing AD.
- The aim of our study was to test the hypothesis that the rs638944 and rs600491 polymorphisms of the DHCR24 gene encoding Seladin1 influence the susceptibility to AD.
- We investigated the possible role of IFNG T874A, PLA2G4A Ban*I* and PTGS2 G-765C polymorphisms in AD. Our study was undertaken to confirm the hypothesis that the above-mentioned variants of these genes, either alone or in epistasis, may represent a risk factor for AD.
- The aim of our study was to test the hypothesis that the CHRFAM7A -2bp deletion polymorphism confers predisposition to AD.
- We investigated the possible role of HTR2A T102C and 5HTTLPR polymorphisms in AD either alone or in genetic interaction.
- We tested the hypothesis that the BDNF Val66Met polymorphism influences the risk for developing AD.

Subjects and Methods

Patients and controls

A total of 495 Hungarian Caucasian subjects were enrolled in this study. The characteristics of the participants are presented in Table 2. The study included 250 patients with late onset AD recruited from the Memory Clinic of the Department of Psychiatry, University of Szeged. The diagnosis of probable AD fulfilled the criteria for Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DMS-IV) and National Institute of Neurological and Communicative Disorders and Stroke / Alzheimer's Disease and Related Disorders Associations (NINCDS/ADRDA) (McKhann et al., 1984). All AD cases were defined as sporadic since in their family history there was no first or second degree relative with dementia.

Table 2. Characteristics of the probands

	AD n=250	HC n=245
Age (years; mean ± SD)	76.8 ± 7.3	75.2 ± 6.9
Age at onset (years; mean ± SD)	72.4 ± 6.1	-
Male/female (%)	32/68	31/69
MMSE (scores; mean ± SD)	18.5 ± 5.9	29.1 ± 0.9
ACE (scores; mean ± SD)	53.5 ± 13.4	-
APOE ε4 allele carrier (%)	46.4	13.8

AD: Alzheimer's disease; HC: healthy control; MMSE: Mini-Mental State Exam; ACE: Addenbrooke's Cognitive Examination; APOE ε4 allele carrier: apolipoprotein E ε4 allele carrier (homozygous or heterozygous)

The clinical diagnosis of probable AD was supported by psychiatric and neurological examinations, basic clinical tests such as Mini-Mental State Exam (MMSE), Addenbrooke's Cognitive Examination (ACE) including Clock Drawing and Verbal Fluency tests. The AD patients all had experienced a progressive loss of cognitive functions (in more than two cognitive domains indicative of cortical dysfunction) for at least 1 year with memory loss as the most significant symptom. Brain CT or MRI images were also evaluated.

As a healthy control (HC) group we studied 245 elderly, cognitively intact, healthy individuals that were selected from visitors of patients at the Memory Clinic, Department of Psychiatry, University of Szeged. All of them were medication free and lack of any

significant illnesses and any signs of dementia. Informed consent was obtained from the subjects participated in this study and all protocols were approved by the local ethics committee.

MMSE was used as a measure of global cognitive performance. MMSE scores in the HC group were higher than 28 points (mean ± SD: 29.1 ± 0.9) and none of the probands had any verified symptoms of dementia. The mean MMSE score in the AD group was 18.5 ± 5.9 (mean ± SD) (Table 2).

Genetic analyses

Blood samples were taken by venous puncture. Genomic DNA was extracted from peripheral blood leukocytes according to a standard procedure using the Roche kit. The genetic analyses were performed by polymerase chain reaction (PCR) based methods or direct sequencing. PCR products and digested fragments were separated by agarose or polyacrilamide gel electrophoresis with ethidium bromide staining. Bands on gels were detected and documented by Quantity One 1-D Analysis software of BioRad GelDoc System.

APOE genotypes were determined by a previously described PCR-restriction fragment length polymorphism (RFLP) method with the restriction enzyme *Cfo*I (Kálmán et al., 1997). Genotyping of the BACE1 polymorphism was done by PCR amplification and enzymatic digestion with restriction enzyme *Bcl*I (Cai et al., 2005). Genotyping of the PLAU polymorphism was assessed by restriction enzyme *Alu*I with the same method described by Pesaresi et al (Pesaresi et al., 2006).

The DNA samples were genotyped for CALHM1 polymorphism through direct sequencing of a 291 bp long PCR product. The primers used in the amplification were: forward primer 5'-TCC TTC ATG AAT GGC ATC TG-3' and reverse primer 5'-CCC AGA CGA CAG GCG CGA TG-3'. The following 25 µl reaction mixture was used for the PCR amplification: 50 ng of genomic DNA, 10 pmol of each primer (Invitrogen), 1.5 mM $MgCl_2$, 200 µM dNTP mix, 1 unit Taq polymerase (BioRad).

The amplification program consisted of an initial denaturation step of 3 min at 95°C, then 5 cycles with three temperature segments: 30 sec at 95°C for denaturation; 30 sec at 70°C for annealing; 30 sec at 72°C for amplification. The next step was 25 cycles of three temperature segments: 30 sec at 95°C for denaturation; 30 sec at 61°C for annealing; 30 sec at 72°C for amplification. At last 72°C for 2 min was performed.

The PCR products were purified by ethanol precipitation and bidirectionally sequenced. Direct sequencing of the PCR products was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) on the ABI 3100 Genetic Analyzer (Applied Biosystems). Obtained sequences were analyzed using Applied Biosystem Sequencing Analysis v.3.7 software.

The primers used in the genotyping of the DHCR24 rs600491 polymorphism were: forward primer 5'-CCT CCT CAG CTT TCC TAC CC-3' and reverse primer 5'- TCC AGC TTC TGA CTC CTG GT-3'. The following 25 µl reaction mixture was used for the PCR amplification: 50 ng of genomic DNA, 10 pmol of each primer (Invitrogen), 1.5 mM $MgCl_2$, 200 µM dNTP mix, 1 unit Taq polymerase (BioRad).

The amplification program consisted of an initial denaturation step of 5 min at 94°C, then 25 cycles of three temperature segments: 30 sec at 94°C for denaturation; 30 sec at 66°C for annealing; 30 sec at 72°C for amplification. At last 72°C for 5 min was performed. After amplification, the PCR products were digested with (1 unit/5µl of PCR product) *Aci*I restriction enzyme at 37 ºC for 12 hours.

The PCR product was a 309 bp DNA fragment, which has one cleaving site for *Aci*I in case of the T allele and two cleaving sites in case of the C allele. The digested fragments of the T allele were 214 and 95 bp of length. The C allele resulted in 187, 27 and 95 bp fragments.

The DHCR24 rs638944 polymorphism was determined by allele specific primers in two parallel PCR reactions. To asses the success of the PCR amplification a generic control PCR product of 667 bp was amplified in each reaction using the following primers: forward primer 5'- CTG CTG CAC ACA GAA GGT GT -3' and reverse primer 5'- TTT GCG GTT CAC AGT ACC AA -3'.

The allele specific primers used in the genotyping of the DHCR24 rs638944 polymorphism were: T allele specific forward primer 5'- TTC TGG CTC TCG GTT AGC TAG T -3' and G allele specific forward primer 5'- CTG GCT CTC GGT TAG CTA GG -3'. The common reverse primer for the allele specific primers was the same as the one for the control forward primer. The allele specific PCR product for the DHCR24 rs638944 polymorphism was 552 bp long.

One of the two parallel multiplex PCR reactions contained the DHCR24 rs638944 G allele specific primer with the suitable reverse primer and the generic control primers. The other reaction contained the DHCR24 rs638944 T allele specific primer with the suitable reverse primers and the generic control primers.

The following 25 µl reaction mixture was used for the PCR amplification: 50 ng of genomic DNA, 10 pmol of each primer (Invitrogen), 1.5 mM $MgCl_2$, 200 µM dNTP mix, 1 unit Taq polymerase (BioRad). The amplification program consisted of an initial denaturation step of 30 sec at 95°C followed by 25 cycles of three temperature segments: 30 sec at 95°C for denaturation; 30 sec at 65°C for annealing; 30 sec at 72°C for amplification. At last 72°C for 3 min was performed.

Genotyping of the IFNG polymorphism was done by PCR amplification with allele specific primers as described by Raitala and co-workers (Raitala et al., 2005). Genotyping at PLA2G4A loci was assessed by restriction enzyme Ban*I* with the same method described by Chowdari et al (Chowdari et al., 2004). The two alleles of the PLA2G4A Ban*I* polymorphism are designated as A1 (with nucleotide A) and A2 (with nucleotide G, the Ban*I*-cut allele). Genotyping of the PTGS2 polymorphism was done by PCR amplification and enzymatic digestion with restriction enzyme Aci*I* (Papafili et al., 2002).

Genotyping of the CHRFAM7A -2bp deletion polymorphism was done by PCR amplifications, and the two bp difference between the wild and the -2bp deletion alleles was revealed by 10% polyacrilamide gel electrophoresis with ethidium bromide staining (Lai et al., 2001). We were focusing on the deletion polymorphism of the CHRFAM7A, and did not investigate the copy number, therefore we determined three groups. The genotype lacking the -2bp deletion was designated genotype 1, genotype having one copy of the -2bp allele was designated genotype 2 and genotype with two copies of the -2bp allele was designated genotype 3.

Genotyping of the 5HTTLPR polymorphism was carried out as previously described (Sundaramurthy et al., 2000). The 44 bp difference between the 5HTTLPR L and the S alleles was revealed by 2% agarose gel electrophoresis with ethidium bromide staining. The HTR2A primers used in the amplification were described by Virgos et al (2001). After amplification, the PCR products were digested with *Msp*I restriction enzyme at 37 ºC for 12 hours.

Genotyping of the BDNF Val66Met polymorphism was done by PCR amplification and enzymatic digestion with restriction enzyme *Pma*CI (Chen-Jee Houg et al., 2003).

Statistical analyses

The program SPSS 15.0 was used for all statistical analyses, and the significance level was set at $p<0.05$. Fisher's exact and Pearson's $\chi 2$ tests were used to compare gender, Hardy-Weinberg equilibrium (HWE), allele and genotype frequencies between the AD and HC groups. The mean age of the AD and HC groups was compared by using the t-test for independent samples. Analysis of variance was carried out to determine possible effect of the different genotypes on age at onset of AD. A logistic regression model was applied to test for interactions between the investigated polymorphisms and to estimate crude and adjusted odds ratios (ORs) with 95% confidence intervals (95% CI) in testing for possible associations between genotypes or alleles and the risk for AD.

Results

Statistically no significant difference in mean age or in the distribution of genders between AD and HC groups was found (p>0.05). A deviation from the HWE was detected in IFNG genotype distribution in the HC population (p=0.002), and in PLA2G4A genotype distribution in the AD group (p=0.008). All the other investigated genotype frequencies were in HWE for cases and controls, respectively (p>0.05).

As expected from many previous studies the genotypes with the APOE ε4 allele were significantly over-represented in the AD group as compared to the controls (p<0.0001). APOE genotype frequencies observed in the investigated groups are shown in Table 3. Among the patients with AD, no APOE ε2/ε2 carriers were detected and even in the HC group only four cases were found. The heterozygous ε2/ε3 genotype also occurred with low frequency in AD (AD: 4.8%, versus HC: 11.0%). The ratio of the ε3/ε4 carriers was significantly higher in AD than in HC group (AD: 34.4%, HC: 12.2%). The occurrence of the ε4 allele were significantly over-represented in AD as compared to HC (AD: 27.8%, HC: 7.1%, p<0.0001).

The crude ORs for AD conferred by APOE genotypes are presented in Figure 2. The following genotype categories were designated: **1**=ε2/ε2 and ε2/ε3; **2**=ε3/ε3; **3**=ε2/ε4 and ε3/ε4; **4**=ε4/ε4, considering ε3/ε3 genotype as the reference. The OR for AD was reduced in ε4 negative carriers of ε2 allele, and was higher in the ε4 positive individuals as compared to those with ε3/ε3 genotype. The ε4 homozygotes have significantly increased risk for AD (OR=4.29), than ε4 heterozygotes (OR=16.97), which further supports the previous results of the allele dose dependent risk of APOE ε4 allele for AD.

Stratification according to gender revealed an increased risk for AD in women carrying the ε4/ε4 genotype (OR=22.29; p=0.003) as compared to men with the ε4/ε4 genotype (OR=11.61; p=0.024). However, the same effect size was calculated for women (OR=4.05; p<0.001) and men (OR=4.81; p<0.001) carrying the ε3/ε4 genotype. In case of ε4 negative carriers of ε2 allele the OR for AD was reduced - but not significantly - both in women and in men as compared to the ε3/ε3 carriers. No statistically significant differences were found between the means of the age at onset among the AD patients with the different APOE genotypes (p>0.05).

Table 3. APOE genotype frequencies in the AD and HC groups

APOE genotypes	AD n=250	HC n=245
ε2/ε2	0 (0%)	4 (1.6%)
ε2/ε3	12 (4.8%)	27 (11.0%)
ε2/ε4	7 (2.8%)	3 (1.2%)
ε3/ε3	122 (48.8%)	180 (73.5%)
ε3/ε4	86 (34.4%)	29 (11.8%)
ε4/ε4	23 (9.2%)	2 (0.8%)

* χ^2=68.357 (5), p<0.0001

APOE: apolipoprotein E; AD: Alzheimer's disease; HC: Healthy control

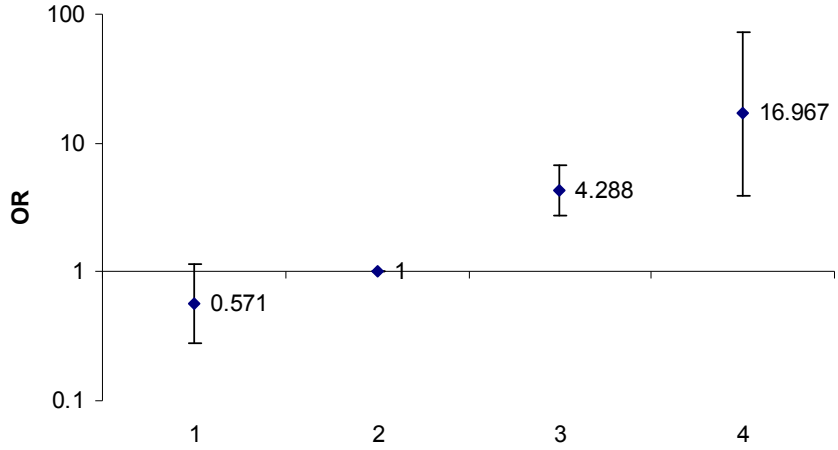

Figure 2. Odds ratios (ORs) with 95% CI for AD conferred by APOE genotypes The genotypes are ranked by the increasing risk for AD, considering ε3/ε3 genotype as reference category (OR=1). Genotypes: **1**=ε2/ε2, ε2/ε3; **2**=ε3/ε3; **3**=ε2/ε4, ε3/ε4; **4**=ε4/ε4.

I. Amyloid-β metabolism-related polymorphisms

The BACE1 and PLAU genotype frequencies observed in the examined groups are presented in Table 4. Comparison of BACE1 genotype frequencies between AD and HC groups did not reach statistically significant difference (p=0.647). The percentage of the different PLAU genotypes was similar in AD as compared to HC, and showed no statistically significant difference (p=0.964). Logistic regression analysis revealed no effect of interaction between PLAU and BACE1 genotypes (p=0.716), and there was also no interaction with APOE ε4 allele on AD risk (BACE1*APOE: p=0.648; PLAU*APOE: p=0.579).

As presented in Table 5, the CALHM1 Pro/Leu and Leu/Leu genotypes occurred with higher frequency in AD as compared to HC group, although the difference did not show statistical significance (p=0.153). Given the relatively low occurrence of the Leu/Leu genotype both in AD cases and in controls, the analysis was also conducted by presence or

absence of the Leu allele in the genotypes. The frequency of the Leu+ genotypes (Leu/Leu and Pro/Leu genotypes together) was found to be marginally significantly higher in the AD than in the HC group (AD: 53.8%, HC: 44.6%; p=0.056). The crude ORs for AD conferred by CALHM1 genotypes are presented in Figure 3. The effects of Pro/Leu or Leu/Leu genotypes on AD risk were not significant. The Leu+ genotypes had a marginally significantly increased risk for AD (OR=1.45; p=0.053) considering Pro/Pro genotype as reference category (OR=1).

Table 4. BACE1 and PLAU genotype frequencies in the AD and HC groups

Genotypes	AD n=239	HC n=201
BACE1 *		
CC	50 (20.9%)	35 (17.4%)
CG	117 (49.0%)	102 (50.7%)
GG	72 (30.1%)	64 (31.9%)
PLAU #		
CC	156 (65.4%)	129 (64.1%)
CT	78 (32.6%)	68 (33.8%)
TT	4 (2.0%)	4 (2.1%)

* χ^2=0.870 (2), p=0.647
# χ^2=0.073 (2), p=0.964
BACE1: β-site APP cleaving enzyme-1; PLAU: urokinase-type plasminogen activator; AD: Alzheimer's disease; HC: Healthy control

Table 5. CALHM1 genotype frequencies in the AD and HC groups

CALHM1 genotypes	AD n=238	HC n=202
Pro/Pro	110 (46.2%)	112 (55.4%)
Pro/Leu	101 (42.5%)	70 (34.7%)
Leu/Leu	27 (11.3%)	20 (9.9%)

χ2=3.760 (2) p=0.153
CALHM1: Calcium homeostasis modulator1; AD: Alzheimer's disease; HC: Healthy control

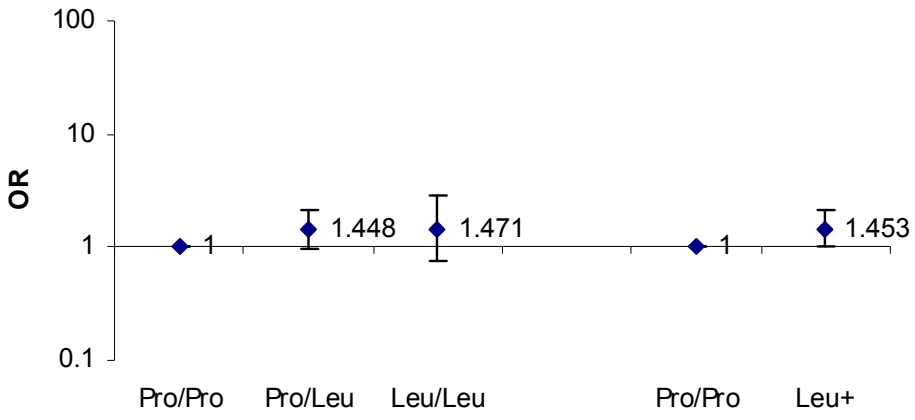

Figure 3. Odds ratios (ORs) with 95% CI for AD conferred by CALHM1 genotypes

According to the frequency data, the possession of the CALHM1 Leu and the APOE ε4 alleles was considered as possible risk factors for AD. Table 6 summarizes the frequencies and ORs for the interaction between the probable risk factors for AD. Both AD and HC groups were divided into subgroups according to the presence or absence of CALHM1 Leu and APOE ε4 alleles. The OR for the co-presence of CALHM1 Leu and APOE ε4 alleles was higher (OR=6.69; $p<0.0001$) than the ORs for the possession of only one of them (ε4 allele: OR=4.28, $p<0.0001$; Leu allele: OR=1.49, $p=0.093$). The effect of the Leu allele without the ε4 allele is not significant. The interaction between the CALHM1 Leu and APOE ε4 alleles did not contribute significantly to the logistic regression model ($p=0.913$).

Table 6. Frequencies and odds ratios (ORs) for the interaction between APOE ε4 and CALHM1 Leu alleles

APOE ε4	CALHM1 Leu	AD n=238	HC n=202	OR	95% CI	*p**
+	+	58 (24.4%)	14 (6.9%)	6.687	3.419–13.076	<0.0001
+	-	53 (22.3%)	20 (9.9%)	4.277	2.321–7.882	<0.0001
-	+	70 (29.4%)	76 (37.6%)	1.487	0.935–2.362	0.093
-	-	57 (23.9%)	92 (45.6%)		reference	

The reference category is HC. * Result of Wald statistics.
APOE ε4: Apolipoprotein E ε4 allele; CALHM1 Leu: Calcium homeostasis modulator1 Leu allele; AD: Alzheimer's disease; HC: Healthy control

Analyzing the results according to gender and age at onset, no interactions among these factors and the investigated polymorphisms (BACE1, PLAU, CALHM1) were observed ($p>0.1$). Given the co-localization of PLAU and CALHM1 genes in the 10q21-24 AD linkage region, a possible interaction between PLAU and CALHM1 on prediction of AD was assessed, but no interaction was found ($p=0.891$).

II. Cholesterol metabolism-related polymorphisms

The DHCR24 rs600491 genotype frequencies observed in the investigated groups are shown in Table 7. The DHCR24 rs600491 genotype and allele distributions did not differ significantly between the AD and HC groups ($p=0.881$ for genotypes; $p=0.845$ for alleles). Stratification according to gender however, revealed a statistically significant association between T/T genotype and AD risk in men (AD: 32.4%, HC: 16.9%; $p=0.028$), in contrast with the results in women (AD: 29.6%, HC: 33.8%; $p=0.472$).

The crude ORs for AD in men conferred by DHCR24 rs600491 genotypes are presented in Figure 4. Men with the T/T genotype had a significantly increased risk for AD (OR=4.37; p=0.009) considering C/C genotype as reference category (OR=1). The effect of the T/T genotype on AD risk was also significant when it was compared to C+ genotypes as reference category (OR=1). Logistic regression analysis revealed no interaction between the DHCR24 rs600491 and APOE polymorphisms (p=0.856).

Table 7. DHCR24 rs600491 genotype frequencies in the AD and HC groups

DHCR24 rs600491 genotypes	AD n=236	HC n=204
All*		
C/C	38 (16.1%)	35 (17.2%)
C/T	126 (53.4%)	111 (54.4%)
T/T	72 (30.5%)	58 (28.4%)
Men#		
C/C	8 (10.8%)	16 (24.6%)
C/T	42 (56.8%)	38 (58.5%)
T/T	24 (32.4%)	11 (16.9%)
Women¤		
C/C	30 (18.5%)	19 (13.7%)
C/T	84 (51.9%)	73 (52.5%)
T/T	48 (29.6%)	47 (33.8%)

*$\chi2$=0.254 (2) p=0.881
#$\chi2$=7.142 (2) p=0.028
¤$\chi2$=1.502 (2) p=0.472
DHCR24: Seladin1 (Selective AD indicator) gene; AD: Alzheimer's disease; HC: Healthy control

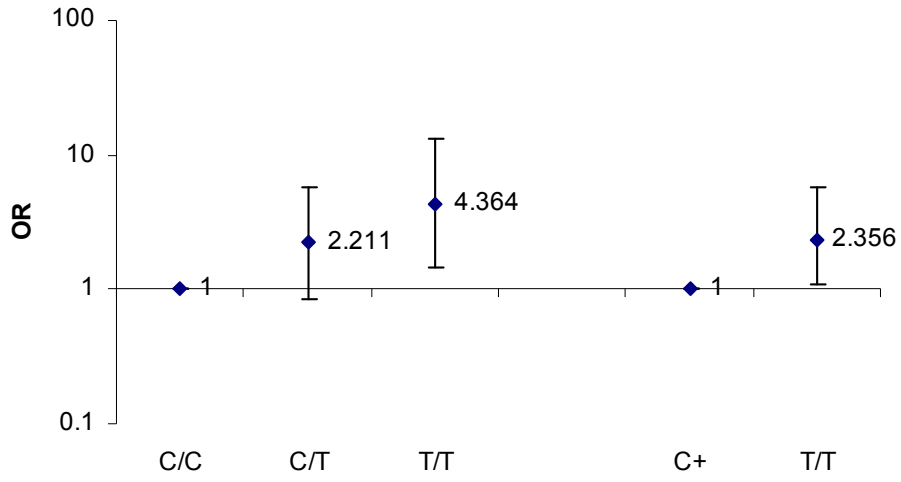

Figure 4. Odds ratios (ORs) with 95% CI for AD conferred by DHCR24 rs600491 genotypes in men

As shown in Table 8, the different DHCR24 rs638944 genotype frequencies were similar in the AD as compared to the HC group, and showed no statistically significant difference (p=0.687). Logistic regression analysis revealed no interaction between the DHCR24 rs638944 and APOE polymorphisms (p=0.795).

Table 8. DHCR24 rs638944 genotype frequencies in the AD and HC groups

DHCR24 rs638944 genotypes	AD n=236	HC n=204
G/G	88 (37.3%)	81 (39.7%)
G/T	116 (49.2%)	92 (45.1%)
T/T	32 (13.5%)	31 (15.2%)

$\chi 2$=0.752 (2) p=0.687
DHCR24: Seladin1 (Selective AD indicator) gene; AD: Alzheimer's disease; HC: Healthy control

III. Neuroinflammation-related polymorphisms

Table 9 presents the genotype distribution of the IFNG, PLA2G4A and PTGS2 polymorphisms in the investigated groups. The frequencies of the IFNG A/A genotype and the A allele were higher in AD as compared to HC group, although the differences did not reach statistical significance (p=0.174 for genotypes, p=0.139 for alleles). Comparison of PLA2G4A genotype and allele frequencies between AD and HC showed no statistically significant difference, although genotype A1/A1 occurred more frequently in AD than in HC (p=0.143 for genotypes, p=0.269 for alleles).

The PTGS2 G/G genotype was significantly over-represented in AD as compared to HC group (AD: 74.7%; HC: 59.6%), while the G/C and C/C genotypes were significantly more frequent in HC than in AD group (G/C: AD: 23.6%, HC: 33.1%; C/C: AD: 1.7%, HC: 7.3%; p<0.001). The PTGS2 allele distribution also showed statistically significant difference between cases and controls with higher G allele frequency in the AD group (AD: 86.5%, HC: 76.1%; p<0.001).

The crude ORs for AD conferred by PTGS2 genotypes are presented in Figure 5. The effect of the G/G genotype on AD risk was significantly increased (OR=5.46; p=0.003) as compared to C/C genotype as reference category (OR=1). The G/G genotype also had a significantly increased risk (OR=2.00; p<0.001) when it was compared to C+ genotypes as reference category (OR=1). Analyzing the results according to gender and age at onset, no interactions among these factors and the investigated polymorphisms (IFNG, PLA2G4A, PTGS2) were observed (p>0.1).

In accordance with the genotype frequencies, IFNG A/A, PLA2G4A A1/A1, PTGS2 G/G genotypes were considered as possible risk factors for AD. The interaction between the possible risk factors were investigated in pairs, and none of them contributed significantly to the logistic regression model (IFNG*PLA2G4A: p=0.877; IFNG*PTGS2: p=0.245; PLA2G4A*PTGS2: p=0.872). Logistic regression analysis also revealed no effect of interaction with APOE ε4 allele on AD risk (IFNG*APOE: p=0.132; PLA2G4A*APOE: p=0.733; PTGS2*APOE: p=0.482).

Table 9. IFNG, PLA2G4A and PTGS2 genotype frequencies in the AD and HC groups

Genotypes	AD n=237	HC n=245
IFNG *		
T/T	47 (19.8%)	55 (22.4%)
T/A	131 (55.3%)	146 (59.6%)
A/A	59 (24.9%)	44 (18.0%)
PLA2G4A #		
A1/A1	58 (24.5%)	43 (17.6%)
A1/A2	97 (40.9%)	116 (47.3%)
A2/A2	82 (34.6%)	86 (35.1%)
PTGS2 ¤		
G/G	177 (74.7%)	146 (59.6%)
G/C	56 (23.6%)	81 (33.1%)
C/C	4 (1.7%)	18 (7.3%)

* χ^2=3.492 (2) p=0.174
# χ^2=3.886 (2) p=0.143
¤ χ^2=16.318 (2) p<0.001
HC: Healthy control; AD: Alzheimer's disease; IFNG: interferon-γ; PLA2G4A: cytosolic phospholipase A2; PTGS2: prostaglandin endoperoxide synthase 2

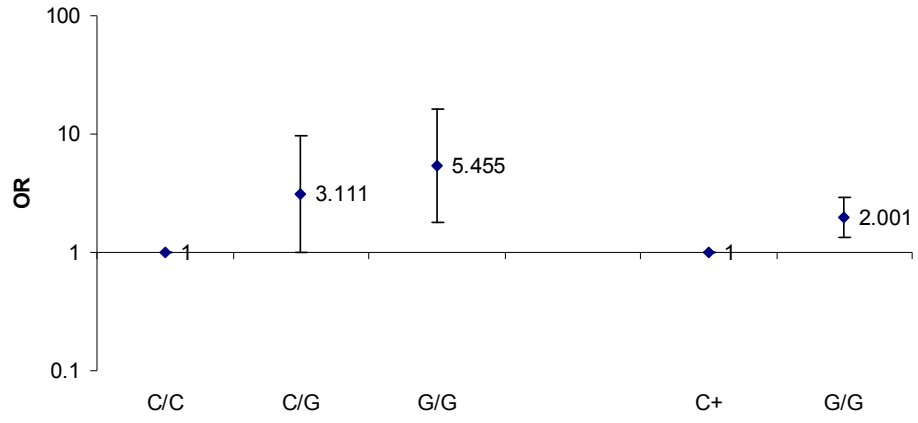

Figure 5. Odds ratios (ORs) with 95% CI for AD conferred by PTGS2 genotypes

IV. Neuronal dysfunction

IV.1. Cholinergic system-related polymorphism

CHRFAM7A genotype frequencies are presented in Table 10. Genotype 1 was significantly over-represented in AD as compared to HC (AD: 36.0%, HC: 24.0%), while the frequency of genotype 3 was significantly lower in AD, than in HC (AD: 12.6%, HC: 22.3%). The differences were statistically significant (p=0.011). The crude ORs for AD conferred by CHRFAM7A genotypes are presented in Figure 6. The CHRFAM7A genotype 1 had a significantly increased risk on AD (OR=2.66; p=0.012) as compared to genotype 3 as reference category (OR=1).

Table 10. CHRFAM7A genotype frequencies in the AD and HC groups

CHRFAM7A genotypes	AD n=175	HC n=175
1	63 (36.0%)	42 (24.0%)
2	90 (51.4%)	94 (53.7%)
3	22 (12.6%)	39 (22.3%)

χ^2=9.025 (2), p=0.011

AD: Alzheimer's disease; HC: healthy control; CHRFAM7A: partially duplicated variant of α7 nicotinic acetylcholine receptor subunit gene

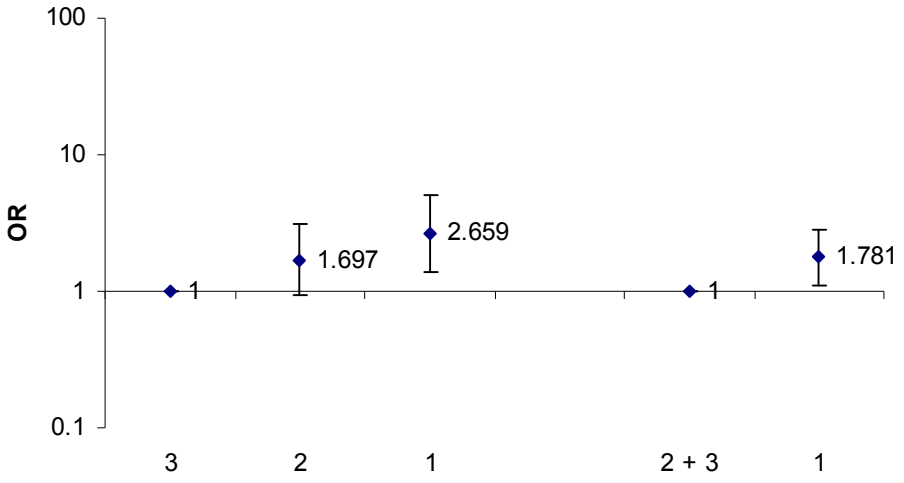

Figure 6. Odds ratios (ORs) with 95% CI for AD conferred by CHRFAM7A genotypes

According to the genotype frequencies CHRFAM7A genotype 1 and APOE ε4 allele were considered as possible risk factors for AD. Table 11 summarizes the frequencies and ORs for the interaction between APOE ε4 allele and CHRFAM7A genotype 1 in the investigated groups. Simultaneous presence of APOE ε4 allele and CHRFAM7A genotype 1

occurred more frequently in AD as compared to HC (AD: 12.6%, HC: 4.0%). The ORs for the presence of ε4 allele with the CHRFAM7A genotype 1 (OR=6.03, $p<0.001$) or without the CHRFAM7A genotype 1 (OR=6.11, $p<0.001$) were same values, therefore it is unlikely that the combination of these genetic variants would be involved in AD. Logistic regression analysis revealed no interaction between the CHRFAM7A and APOE polymorphisms ($p=0.165$).

Table 11. Frequencies and odds ratios (ORs) for the interaction between APOE ε4 allele and CHRFAM7A genotype 1

APOE ε4	CHRFAM7A genotype 1	AD n=175	HC n=175	OR	95% CI	*p**
+	+	22 (12.6%)	7 (4.0%)	6.028	2.438-14.902	<0.0001
+	-	51 (29.1%)	16 (9.1%)	6.114	3.220-11.609	<0.0001
-	+	41 (23.4%)	35 (20.0%)	2.247	1.300-3.883	0.004
-	-	61 (34.9%)	117 (66.9%)		Reference	

The reference category is HC. * Result of Wald statistics.
AD: Alzheimer's disease; HC: Healthy control; APOE ε4: Apolipoprotein E ε4 allele; CHRFAM7A: partially duplicated variant of α7 nicotinic acetylcholine receptor subunit gene

IV.2. Serotonergic system-related polymorphisms

Table 12 presents the genotype frequencies of the HTR2A and 5HTTLPR polymorphisms in the examined groups. Compared with the controls, there was a higher frequency of HTR2A C/C genotype and lower frequency of T/T and T/C genotypes in the AD group without statistically significant difference ($p=0.108$). Individuals with at least one T allele were grouped together. The analysis was also performed by the presence or absence of the T allele in the genotypes, thereby T- (C/C) and T+ (C/T and T/T) genotype categories were investigated, and a statistically significant difference was found between cases and controls ($p=0.037$). However, no significant association of HTR2A alleles and the risk for AD was observed ($p=0.251$).

The crude ORs for AD conferred by HTR2A genotypes are presented in Figure 7. The effects of C/C and C/T genotypes on AD risk were not significant considering T/T genotype as reference category (OR=1). However, as compared to the T+ genotypes as reference category (OR=1) the HTR2A C/C genotype had a significantly increased risk for AD (OR=1.53; $p=0.035$).

The frequency of the different 5HTTLPR genotypes was similar in AD as compared to HC, and there was no statistically significant difference ($p=0.883$). The 5HTTLPR allele

distribution also did not show statistically significant difference between the two investigated groups (p=0.768).

Table 12. HTR2A and 5HTTLPR genotype frequencies in the AD and HC groups

Genotypes	AD n=247	HC n=206
HTR2A*		
T/T	36 (14.6%)	34 (16.5%)
T/C	117 (47.4%)	113 (54.9%)
C/C	94 (38.0%)	59 (28.6%)
5HTTLPR#		
L/L	78 (31.6%)	64 (31.0%)
L/S	129 (52.2%)	105 (51.0%)
S/S	40 (16.2%)	37 (18.0%)

* χ^2=4.459 (2) p=0.108
# χ^2=0.250 (2) p=0.883
HC: Healthy control; AD: Alzheimer's disease; HTR2A: serotonin receptor A2; 5HTTLPR: serotonin transporter gene-linked polymorphic region

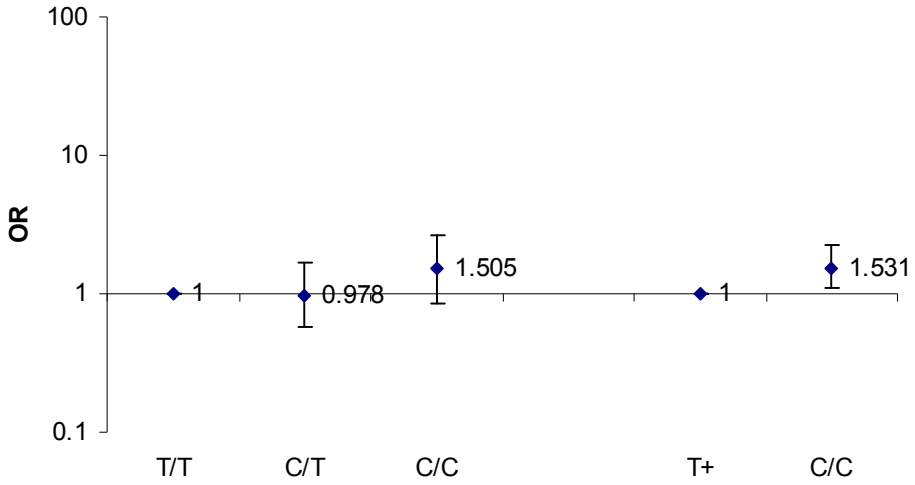

Figure 7. Odds ratios (ORs) with 95%CI for AD conferred by HTR2A genotypes

The presence of the HTR2A C/C and the 5HTTLPR L/L genotypes were considered as possible risk factors for AD. Both AD and HC groups were divided into four subgroups according to the presence or absence of the HTR2A C/C and 5HTTLPR L/L genotypes. Table 13 summarizes the frequencies and ORs for the interaction between the probable risk factors for AD. Logistic regression analysis revealed an interaction between the 5HTTLPR and the HTR2A T102C polymorphisms (p=0.032). The L/L and C/C genotype carriers had a significantly increased risk for AD (OR=2.50; p=0.016). The effect of the L/L genotype without the C/C genotype and the effect of the C/C genotype among those who do not carry the L/L genotype was not significant (OR=0.79; p=0.327; OR=1.16; p=0.524).

Table 13. Frequencies and odds ratios (ORs) for the interaction between HTR2A C/C and 5HTTLPR L/L genotypes

5HTTLPR L/L	HTR2A C/C	AD n=247	HC n=206	OR	95% CI	*p**
+	+	31 (12.6%)	11 (5.3%)	2.499	1.190–5.247	0.016
+	-	47 (19.0%)	53 (25.7%)	0.786	0.486–1.272	0.327
-	+	63 (25.5%)	48 (23.3%)	1.164	0.730–1.857	0.524
-	-	106 (42.9%)	94 (45.6%)		reference	

The reference category is HC. * Result of Wald statistics

HC: Healthy control; AD: Alzheimer's disease; HTR2A C/C: serotonin receptor A2 C/C genotype; 5HTTLPR L/L: serotonin transporter gene-linked polymorphic region L/L genotype

Stratification of HTR2A and 5HTTLPR data according to APOE ε4 status (individuals with one or two ε4 alleles vs. individuals with no ε4 allele) did not reveal interaction either between HTR2A and APOE (p=0.690) or between 5HTTLPR and APOE (p=0.859). There was also no statistically significant difference of age at onset for AD if we compared the mean ages for the different HTR2A and 5HTTLPR genotypes (p>0.05).

IV.3. Brain-derived neurotrophic factor polymorphism

The BDNF Val66Met genotype frequencies observed in the investigated groups are presented in Table 14. The frequency of the BDNF Val/Val homozygous genotype was significantly higher in AD than in the HC group (AD: 58.8%, HC: 31.7%; p<0.0001). There were also robust differences in the distribution of the Val/Met and Met/Met genotypes in AD versus HC (Val/Met: AD: 35.0%, HC: 48.2%; Met/Met: AD: 6.2%, HC: 20.1%; p<0.0001).

The crude ORs for AD conferred by BDNF genotypes are presented in Figure 8. The effect of the Val/Val genotype on AD risk was significantly increased (OR=5.97; p<0.001) as compared to Met/Met genotype as reference (OR=1). The Val/Val genotype also had a significantly increased risk (OR=3.07; p<0.001) when it was compared to Met+ genotypes as reference (OR=1). The Val allele was significantly over-represented in AD as compared to HC (AD: 76.2%, HC: 55.8%; p<0.0001).

Table 15 summarizes the frequencies and ORs for the interaction between the APOE ε4 allele and BDNF Val/Val genotype in the examined groups divided into subgroups according to the presence of the ε4 allele (one or two) and the presence of the Val/Val genotype. Genotypes containing both the ε4 allele and BDNF Val/Val genotype occurred more frequently in AD than in HC (AD: 20.6%, HC: 1.8%).

Table 14. BDNF genotype frequencies in the AD and HC groups

BDNF genotypes	AD n=160	HC n=164
Val/Val	94 (58.8%)	52 (31.7%)
Val/Met	56 (35.0%)	79 (48.2%)
Met/Met	10 (6.2%)	33 (20.1%)

χ^2=28.258 (2), p<0.0001
HC: Healthy control; AD: Alzheimer's disease; BDNF: brain-derived neurotrophic factor

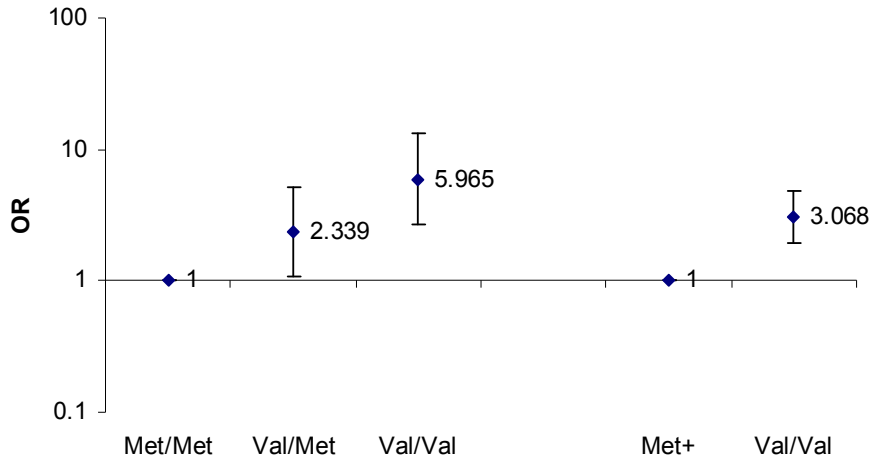

Figure 8. Odds ratios (ORs) with 95%CI for AD conferred by BDNF genotypes

The interaction between the BDNF and APOE polymorphisms did not contribute statistically significantly to the logistic regression model (p=0.135). The OR for the presence of both the ε4 allele and BDNF Val/Val genotype in the AD group was 26.05 as compared to patients with neither the ε4 nor the BDNF Val allele. Since in AD, the OR for the occurrence of the ε4 allele and BDNF Val/Val genotype was much higher than the OR for the presence of the ε4 allele without the Val/Val genotype, we propose a synergistic effect of the two SNPs on the risk for AD. Furthermore, the crude ORs for the effect of the BDNF Val/Val genotype investigating in itself (OR: 3.07) and the APOE ε4 allele investigating in itself (OR: 5.37) were remarkably lower than the adjusted OR for their combined effect (OR: 26.05).

Table 15. Frequencies and odds ratios (ORs) for the interaction between APOE ε4 and BDNF Val alleles

APOE ε4	BDNF Val/Val	AD n=160	HC n=164	OR	95% CI	p
+	+	33 (20.6%)	3 (1.8%)	26.053	7.530-90.139	<0.0001
+	-	28 (17.5%)	22 (13.4%)	3.014	1.535-5.920	0.001
-	+	61 (38.1%)	49 (29.9%)	2.948	1.729-5.029	<0.0001
-	-	38 (23.8%)	90 (54.9%)		Reference	

The reference category is HC. * Result of Wald statistics.
AD: Alzheimer's disease; HC: Healthy control; APOE ε4: Apolipoprotein E ε4 allele; BDNF Val/Val: brain-derived neurotrophic factor Val/Val genotype

Discussion

We found the expected statistically significant APOE ε4 allele elevation in AD as compared to the HC group. These results are in line with former findings for the Hungarian population (Kálmán et al., 1997; Janka et al., 2002; Juhász et al., 2005) and with results on other ethnic groups (Lung et al., 2005; Murrell et al., 2006; Sando et al., 2008). The reported ε4 allele frequencies for patients with AD show a wide range of variance from 19 to 55% even within Europe, however these are the end values and they are generally between 30 and 45%. The Caucasian population is not homogeneous and the Hungarian population is genetically different from other European ethnic groups. According to our results, the ε4 allele frequency in Hungarians is lower as compared to the average ε4 allele frequency of other Caucasian populations (Table 16). The different association studies including our study are consistent however, since significantly higher ε4 allele frequency was found in AD compared to healthy controls in each population.

Table 16. ApoE ε4 allele frequencies in AD in different populations

Population		ε4 allele frequency in AD*
Caucasian		**37%**
Benjamin, 1995	Norway	34%
Chartier-Harlin, 1994	UK	29%
Chartier-Harlin, 1994	France	24%
Kuusisto, 1994	Finland	36%
Lehtimaki, 1995	Finland	55%
Sorbi, 1994	Italy	19%
Present study	Hungary	28%
Asian		**28%**
African descent		**35%**
Hispanic		**24%**

*Data are from www.alzgene.org; AD: Alzheimer's disease

Our findings confirm the results of genetic and epidemiological studies reporting that the ε4 allele is the most important known risk factor for late-onset sporadic AD. The presence of the ε4 allele increases the risk and reduces the age at onset of dementia, in contrast to the possession of the ε2 allele that has a protective effect. Our data partly confirmed the effect of gender on AD risk, since we found an increased predisposition to AD in ε4/ε4 homozygote women as compared to ε4/ε4 homozygote men, but the same risk for AD in women carrying

the ε3/ε4 genotype as compared to men with the ε3/ε4 genotype. Our results support the dose dependent risk of APOE ε4 allele for AD, in view of the fact that we found significantly increased risk for AD in ε4 homozygotes as compared to ε4 heterozygotes (Figure 2).

APOE ε4 isoform has been demonstrated to negatively influence and intensify the biochemical disturbances of AD, including Aβ deposition, tangle formation, neuronal cell death, dysfunction of lipid homeostasis and cholinergic signaling (Cedazo-Mínguez and Cowburn, 2001). The APOE ε4 isoform associated neuropathology is most likely attributable to its conformational specificity defined by the amino acid change at residues 112 and 158 (Mahley et al., 2006). APOE ε3 has Cys-112 and Arg-158, while ε2 has Cys and ε4 has Arg at both positions. In ε4 isoform Arg-112 mediates the interaction between the two structural domains of APOE: the N-terminal domain containing the low-density lipoprotein receptor and the C-terminal domain containing the major lipid binding site (Mahley et al., 2006). The ε2 and ε3 isoforms are much less likely to undergo domain interaction than ε4. In cultured neuronal cell study exogenous APOE ε4 increased the Aβ generation more as compared to exogenous ε3, but when domain interaction was blocked within ε4, this difference was not detected between the two isoforms (Ye et al., 2005).

I. Amyloid-β metabolism-related polymorphisms

Our findings failed to support the hypothesis that BACE1 C786G and PLAU Pro141Leu polymorphisms confer susceptibility to AD, since we did not find statistically significant differences neither in genotype nor in allele distribution comparing the AD and the HC groups. We did not detect genetic interaction between the BACE1 and the APOE genotypes or between the PLAU and the APOE genotypes in the development of AD.

We also failed to support the hypothesis that CALHM1 rs2986017 polymorphism at 10q24 is associated with late onset AD, since comparing the AD and the HC groups we found no significant differences either in genotype or in allele distribution. Only a very modest, marginally significant effect of the Leu+ genotypes on AD was observed.

Dreses-Werringloer and co-workers (2008) examined this polymorphism in five Caucasian AD and control samples, and found that the Leu allele is associated with the risk for AD (Dreses-Werringloer, et al, 2008). Further studies including ours, however, did not corroborate this observation. Bertram et al (2008) investigated several independent datasets of Caucasian patients and controls and found no association between rs2986017 polymorphism

and AD (Bertram et al, 2008). The CALHM1 genotype distribution reported by Minster and co-workers (2009) looks very similar to that found by Sleegers et al (2009) and Beecham et al (2009) without any statistically significant difference comparing the case and the control groups.

There are also contradictory reports on the correlation between the CALHM1 polymorphism and the age at onset of AD. The Dreses-Werringloer study (2008) showed evidence for the association between the Leu/Leu genotype and an earlier onset of AD (Dreses-Werringloer, et al, 2008), but in another paper only a modest correlation was reported (Minster et al, 2009).

Although the effect of the CALHM1 Leu allele on AD in itself in our study was found to be small and only marginally significant, we hypothesized that in combination with the APOE ε4 allele it may increase the effect of the ε4 allele occurring on its own. We failed to detect a genetic interaction between the CALHM1 Leu allele and the APOE genotypes in the development of AD. Despite the modest correlation between the presence of the Leu allele and AD, this study does not support the involvement of CALHM1 rs2986017 polymorphism in the aetiology of AD.

II. Cholesterol metabolism-related polymorphisms

Our results indicate a gender dependent effect of DHCR24 rs600491 polymorphism on the susceptibility to AD. A statistically significant correlation was found in men between the T/T genotype and the risk for AD, whereas this association was not observed in the whole population or in women. Men with the T/T genotype had a significantly increased risk for AD as compared to those men carrying the C allele. Our findings on DHCR24 rs600491 polymorphism are in line with the study of Lämsä and co-workers (2007) that was also reported an increased risk for AD in men carrying the T/T genotype.

We failed to support the hypothesis that DHCR24 rs638944 polymorphism influences the predisposition to AD, since we did not observe statistically significant differences neither in genotype nor in allele distribution comparing the AD and the HC groups. We also failed to detect genetic interaction between the DHCR24 and the APOE genotypes in the development of AD.

III. Neuroinflammation-related polymorphisms

Our results indicate that PTGS2 G/G genotype can confer susceptibility to AD and the possession of the C allele can have a protective effect. In case of the IFNG and the PLA2G4A polymorphisms, we did not find statistically significant differences neither in genotype nor in allele distribution comparing the AD and the HC groups. We also did not detect an epistasis between the investigated polymorphisms in the development of AD.

The genotype distribution of PTGS2 in this study is very similar to that was found by Abdullah and co-workers in a Caucasian case-control sample from Florida (Abdullah et al., 2006). We confirmed their findings in a Hungarian sample. The investigated PTGS2 promoter polymorphism has functional consequence, since the C allele was reported to have a significantly lower promoter activity in human lung fibroblasts (Papafili et al., 2002). In agreement with this observation, higher PTGS2 expression and PGE2 levels were measured in monocytes from probands with G/G genotypes as compared to probands having G/C or C/C genotypes (Cipollone et al., 2004).

There are conflicting reports on PTGS2 mRNA level in AD brain, as both increased and decreased levels were reported compared to healthy brain samples (Heneka and O'Banion, 2007). PTGS2 expression seems to vary with the severity of the disease with increased PTGS2 mRNA level in the early stage of AD (Hoozemans et al., 2008). The main activity of non-steroidal anti-inflammatory drugs (NSAIDs) is to inhibit PTGS activity, the traditional NSAIDs act on both the constitutive PTGS1 and the induced PTGS2 isoforms. According to epidemiological studies the long-term use of traditional NSAIDs can reduce the risk of developing AD and can delay its onset (McGeer and McGeer, 2007). Compared with non-users, 50% lower risk of being affected by AD has been reported for long-term NSAID users (Landi et al., 2003). However, this protective effect is not yet confirmed with specific PTGS2 inhibitors, it has been reported that daily celecoxib (primarily inhibits PTGS2) use may improve cognitive performance and increase regional brain metabolism in people with age-associated memory decline (Small et al., 2008).

PTGS2 is an interesting candidate gene for AD on account of its chromosomal location and physiological function. The gene encoding PTGS2 maps to 1q25.2-q25.3, which is located in a repeatedly established AD linkage region (Liu et al., 2007; Butler et al., 2009).

Since the C/G and C/C genotypes were over-represented in the HC as compared to the AD group, the C allele appeared to reduce the risk for developing AD. Possible explanation for the protective effect of the PTGS2 C allele could be that due to its reduced promoter

activity, in the early stages of AD the PTGS2 expression in individuals with the C allele is not as high as in patients with the G/G genotype. The intensity of PTGS2 expression can influence the extent of inflammatory response, which may interact with other pathological processes in AD.

Our findings do not confirm the hypothesis that the investigated IFNG and PLA2G4A polymorphisms are associated with late onset AD. The results indicate however, that PTGS2 G-765C promoter polymorphism influences the risk for AD, and supports the involvement of PTGS2 in the aetiology of AD.

IV. Neuronal dysfunction

IV.1. Cholinergic system-related polymorphism

The CHRFAM7A genotype without the -2bp allele was significantly over-represented in AD as compared to the controls. According to our results, the partially duplicated variant of the α7 nAChR subunit gene can be a strong candidate gene for AD. It was demonstrated that CHRFAM7A are transcribed, but there is no evidence whether this transcript is translated or not (Gault et al, 1998; Riley et al, 2002). It is unlikely that this hybrid gene functions as a nicotinic receptor due to the absence of signal peptide, glycosylation site and part of the ligand-binding site encoded by exons 1-4 (Leonard and Freedman, 2006). It is possible however, that if CHRFAM7A is translated, the gene product is able to interact with α7 polypeptide, since most of the contact regions are encoded in exon 5-10 (Riley et al, 2002).

The mechanism by which the -2bp allele variant of duplicated exon 6 decreases the risk of AD remains to be established. The -2bp deletion results in a stop codon within exon 6, therefore the putative translational product will be truncated. Possible explanation could be that the wild type CHRFAM7A gene product may alter the normal assembly of the α7 nAChR which could be avoided by the truncated gene product (Riley et al, 2002). This hypothesis is also supported by the observation that the deletion of the CHRNA7 gene improves cognitive deficits and synaptic pathology in a mouse model of Alzheimer's disease (Dziewczapolski et al., 2009).

Besides AD we investigated the CHRFAM7A polymorphism in other dementias such as dementia with Lewy bodies (DLB), Pick’s disease (PiD) and vascular dementia (VD), respectively (Fehér et al., 2009a). Similar to AD, DLB and PiD are also neurodegenerative disorders associated with abnormal protein aggregations and inclusion body formation. DLB

pathology is characterized by abnormal aggregations of α-synuclein termed Lewy bodies (Neef and Walling, 2006). The term "Pick's disease" is restricted to cases showing lobar atrophy with Pick bodies composed of numerous randomly oriented hyperphosphorylated tau fibrils (Frederick, 2006), and PiD is included in the nomenclature of frontotemporal dementia (Lund and Manchester Research Groups, 1994). VD results from various cerebrovascular diseases leading to cognitive impairment which depends on the severity and location of lesions (Black, 2007).

In an experimental system, Aβ effectively induced tau phosphorylation via α7 nAChR that could be decreased by α7 nAChR antagonists. Aβ - α7 nAChR interaction activates mitogen-activated cascade protein which is a tau protein kinase (Wang et al., 2003). Although the α7 nAChR-mediated and Aβ induced tau phosphorylation seemed to be a normal reversible cellular response, the above mentioned findings suggest that α7 nAChR may be implicated in neuropathological conditions associated with abundant Aβ and/or abnormal functions of tau protein. Abnormal α7 nAChR physiology and loss of neurons expressing α7 nAChR may contribute to memory and cognition impairment (Wang et al., 2003).

The CHRFAM7A genotype without the -2bp allele was significantly over-represented in DLB and in PiD compared to healthy controls. The absence of the -2bp allele in DLB and especially in PiD occurred even more frequently than in AD. However, the small sample size for DLB and PiD can influence the results of the statistical analyses. The CHRFAM7A genotype frequencies showed no significant differences between VD and HC (Fehér et al., 2009a).

There may be a functional relationship between Aβ, α7 nAChR and tau protein, since it has been reported that Aβ - α7 nAChR interaction can activate tau phosphorylation (Wang et al., 2003). This relationship can be the common feature by which genetic variants of CHRFAM7A can influence the risk either for AD, DLB or for PiD. Aβ is the major component of senile plaques implicated in AD and DLB, and the hyperphosphorylated tau protein is the main constituent of neurofibrillary tangles in AD brains and Pick bodies occurring in PiD.

Although these findings may suggest that the CHRFAM7A -2bp deletion polymorphism can be implicated not just in AD, but in DLB and PiD as well, additional studies are required to increase number of cases, and to investigate the copy number polymorphism of CHRFAM7A. According to our findings it is unlikely that the -2bp deletion polymorphism of CHRFAM7A plays an important role in the pathogenesis of VD in the Hungarian population.

IV.2. Serotonergic system-related polymorphisms

Our results indicate that the HTR2A T102C polymorphism may influence the susceptibility to AD, especially in combination with the 5HTTLPR L/L genotype. Although we did not detect statistically significant differences in the HTR2A genotype distribution between AD and HC groups, an association was found between the C/C genotype and the risk for AD when we conducted the C/T and T/T genotypes.

However, we did not find significant correlation between AD and the HTTLPR polymorphism in itself, we hypothesized that in combination with the HTR2A C/C genotype it may increase the effect of the C/C genotype occurring on its own. Our results confirm the earlier findings of an increased frequency of the co-presence of the L/L and C/C genotypes in AD as compared to HC group (Micheli et al., 2006). We found that the simultaneous occurrence of the HTTLPR L/L and HTR2A C/C genotypes enhance the risk 2.5-fold for the development of AD. The Micheli study (2006) reported a significant eightfold higher risk for AD in case of simultaneous presence of the L/L and C/C genotypes.

These findings can be of considerable relevance given the fact that both polymorphisms are functional and both act on the same biological pathway, namely serotonergic transmission. The HTR2A gene activity of the C allele was proved to be significantly decreased as compared to the T allele, therefore in individuals with the C/C genotype less receptor may be available for 5HT than in C/T or T/T carriers. The 5HTTLPR polymorphism determines dose-dependent 5HT reuptake from the synaptic cleft, being the S allele less effective than the L allele. In summary, in case of simultaneous presence of the HTR2A C/C and the 5HTTLPR L/L genotypes, the serotonergic transmission may be less effective as compared to the other combinations of HTR2A and 5HTTLPR genotypes.

IV.3. Brain-derived neurotrophic factor polymorphism

Our results revealed that the Val66Met polymorphism of the BDNF gene may be implicated in the susceptibility to AD, since we have found that the Val/Val genotype and the Val allele occurred with significantly higher frequency in AD than in HC. Japanese and Italian studies have also reported this association (Ventriglia et al., 2002; Matsushita et al., 2005), although another Japanese and a Spanish study failed to find the same results (Combarros et al., 2004; Akatsu et al., 2006). The frequency of the Val allele in the Hungarian AD group in this study was similar to the data published for Italian and Spanish populations (Hungarian: 76%;

Italian: 78%; Spanish: 79%). While in the Japanese population, Matsushita and co-workers (2005) reported 61%, Akatsu et al (2006) reported 57% frequency for the occurrence of the Val allele in AD. In contrast to the findings in the AD group, the distribution of the Val allele among the Hungarian HC probands was closer to the results found in the Japanese HC groups [Hungarian: 56%; Japanese (Matsushita et al., 2005): 56%; Japanese (Akatsu et al., 2006): 57%; versus Italian: 70%; Spanish: 81%]. According to these findings, the BDNF Val66Met polymorphism is highly heterogeneous among the different ethnic populations, even within Europe.

BDNF is important for memory-related hippocampal processes. The functional Val66Met polymorphism of BDNF can influence the activity-dependent BDNF secretion, thus it can impact on hippocampal activity. Egan et al. (2003) reported impaired episodic memory in Met allele carrier healthy probands and found that neurons transfected with $BDNF_{Met}$ showed lower depolarization-induced secretion of BDNF. These results are consistent with another study that demonstrated reduced hippocampal activity during declarative memory processing in Met allele carrier probands (Hariri et al., 2003). According to our findings and other association studies, the Val allele is associated with AD (Combarros et al., 2004; Matsushita et al., 2005).

The decreased BDNF level in AD reported by several studies (Ferrer et al, 1999; Holsinger et al., 2000) can be the result of the neurodegenerative processes, the loss of neurons producing BDNF and therefore it can be independent of the BDNF genotype. In the early stage of AD higher BDNF level was measured than in HC (Laske et al., 2006), which can be influenced by the level of the regulated secretion of BDNF, and therefore the BDNF Val66Met genotype. A possible explanation of the contradictory observations and the role of the BDNF Val66Met polymorphism in AD need further investigation.

Besides AD we investigated the BDNF Val66Met polymorphism in DLB and PiD as well (Fehér et al, 2009b), and we found that it does not confer susceptibility to DLB. The BDNF Val66Met genotype frequencies did not differ significantly either in our, or in a Japanese study (Akatsu et al., 2006). The distribution of the Val allele in the Hungarian DLB and HC groups was similar to the Japanese data (Hungarian DLB: 52%; Japanese DLB: 60%; Hungarian HC: 56%; Japanese HC: 55%). The heterozygous Val/Met genotype occurred in PiD with statistically significantly higher frequency as compared to HC, but we have not found significant difference in the distribution of BDNF alleles. The small sample size for DLB and PiD can influence the results of the statistical analyses therefore these findings generate hypothesis and further investigations are required.

V. Genetic interactions

Increasing body of evidence supports the complex genetic model of AD, which suggests that polygenic network of susceptibility genes may underlie the disease. Since the predisposing gene variants confer only fractional risk, genetic interactions may have a major role in contributing to neurodegeneration in AD. Figure 9 summarizes and illustrates the assumed interactions between the investigated gene polymorphisms of this study. The interactions were investigated in pairs of polymorphisms, and the pairs were selected on the basis of the same chromosomal localization and/or on the involvement of the same pathogenic mechanism in AD.

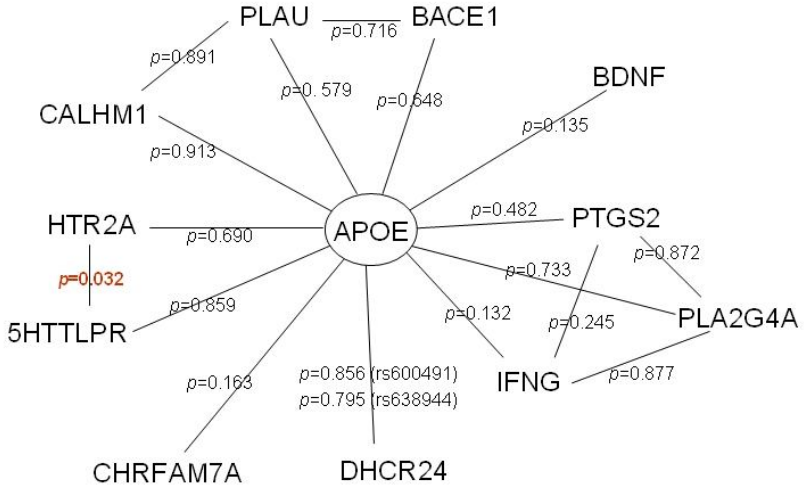

Figure 9. Interactions between the investigated polymorphisms
The interactions between the polymorphisms were investigated in pairs depicted by the lines connecting them. The *p* values came from logistic regression analyses.

The interaction was clearly demonstrable in case of the investigated serotonin-related genes. Logistic regression analysis revealed an interaction between the 5HTTLPR and the HTR2A polymorphisms. The simultaneous presence of the 5HTTLPR L/L and HTR2A C/C genotypes increase the risk for AD 2.5-fold, while the effects of L/L or C/C genotypes on AD risk analyzed separately on the risk for AD were not statistically significant.

According to the logistic regression model there is no interaction between the BDNF and APOE polymorphisms. However, considering the calculated effect sizes, it is presumable that the interaction between the BDNF and APOE polymorphisms would be statistically verifiable by increasing the number of cases. The adjusted ORs for the different BDNF and

APOE genotype combinations suggest a synergistic effect between the BDNF and APOE polymorphisms. The OR for the presence of both the APOE ε4 allele and BDNF Val/Val genotype was more than eightfold higher than the ORs for the presence of one of them.

Conclusions

- Our results confirm the previous findings that APOE ε4 allele confers an allele dose dependent risk for AD.
- We failed to detect association between BACE1 C786G, PLAU Pro141Leu and CALHM1 Leu86Pro polymorphisms and the risk for AD.
- Our findings indicate a gender dependent effect on AD risk with increased susceptibility to AD in men carrying the T/T genotype of the DHCR24 rs600491 polymorphism.
- We failed to detect association between IFNG T874A and PLA2G4A Ban*I* polymorphisms and the risk for AD.
- We found that the PTGS2 G/G genotype can confer susceptibility to AD and the possession of the C allele can have a protective effect.
- According to our results the -2bp deletion polymorphism of CHRFAM7A can be implicated in AD.
- Our study demonstrates an interaction between 5HTTLPR L/L and HTR2A C/C genotypes that seems to increase the risk for AD.
- We suggest that the BDNF Val/Val genotype in itself and in combination with the APOE ε4 allele can be risk factor for AD.
- In summery, our results support the involvement of the following genes in AD aetiology: APOE, DHCR24, PTGS2, CHRFAM7A, HTR2A in epistasis with SLC6A4 and BDNF.

Limitations

A deviation from the HWE was detected in IFNG genotype distribution in the HC and in PLA2G4A genotype distribution in the AD group. Deviation from HWE may indicate disturbing influences on the investigated group including selection, limited population size, random genetic drift or gene flow. The failure in these assumptions of HWE unlikely affects our sample, and all the other genotype distributions were in HWE for cases and controls, respectively. A probable explanation for the deviation from HWE may be an error in genotyping, therefore in case of IFNG and PLA2G4A polymorphisms the genotyping procedure and the results were double checked, and no error was found in the genotyping.

A possible limitation of our study is the relatively low number of cases that may influence the statistical analyses. In case of BACE1, PLAU, DHCR24, IFNG, PLA24A, HTR2A and HTTLPR polymorphisms the post hoc power analyses did not reveal sufficient power size (<0.8) which may lead to false negative results, therefore further investigations are required with increased number of cases.

Case-control association studies have been criticized for conflicting results that may arise from heterogeneity in sampling strategies, in the clinical profiles or ethnical background of patients compared with inappropriate group of controls incorrectly matched with patients. In our study these biases were attempted to be avoided by investigating well defined AD cases and appropriately matched (in the aspect of age, gender and ethnicity) healthy, cognitively intact control group. Misleading findings can also arise from linkage disequilibrium. If association is found with a polymorphism, the functional variant may be located either at the polymorphic site under study or at another polymorphic site somewhere else in the same gene or in a nearby gene. Thereby further investigations addressing other polymorphisms in the investigated genes are required.

An extension of our work would be the option to make use of the genetic analysis in homogeneous subgroups of AD patients defined by quantitative traits underlying disease syndromes. The genetic investigation of such endophenotypes would offer an alternative or a complement to studies of categorical disease phenotypes.

Notwithstanding, the main advances of this study are: 1.) Genotype distributions of different polymorphisms seem to depend on ethnic backgrounds, and our study (except for APOE polymorphism) are the first report on the Hungarian population in AD. 2.) Well defined AD cases were compared with appropriately matched (in the aspect of age, gender and ethnicity) healthy, cognitively intact control group. 3.) This is the first study on

PLA2G4A *Ban*I polymorphism in AD. 4.) In case of APOE, DHCR24, PTGS2, BDNF polymorphisms we could clearly confirm the previous positive findings in an independent population. 5.) An interaction between the HTR2A and the HTTLPR polymorphisms was clearly demonstrated and it was in agreement with a previous report.

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Appendix

I. Fehér Á, Juhász A, Rimanóczy Á, Kálmán J, Janka Z. Association between BDNF Val66Met Polymorphism and Alzheimer's Disease, Dementia with Lewy Bodies and Pick's Disease. Azheimer's Disease and Associated Disorders. 2009. 23(3):224-228.

II. Fehér Á, Juhász A, Rimanóczy Á, Csibri É, Kálmán J, Janka Z. Association between a genetic variant of the alpha-7 nicotinic acetylcholine receptor subunit and four types of dementia. Dementia and Geriatric Cognitive Disorders. 2009. 28(1): 56-61.

ORIGINAL ARTICLE

Association Between BDNF Val66Met Polymorphism and Alzheimer Disease, Dementia With Lewy Bodies, and Pick Disease

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Abstract: A functional polymorphism of the brain-derived neurotrophic factor (BDNF Val66Met) has been reported to affect memory-related hippocampal activity. Apolipoprotein E (ApoE) gene polymorphism is known to be associated with Alzheimer disease (AD), dementia with Lewy bodies (DLB), and Pick disease (PiD). We tested the hypothesis that BDNF Val and ApoE ε4 alleles confer susceptibility to AD, DLB, and PiD. The study included 160 AD, 34 DLB patients, 38 autopsy-confirmed PiD, and 164 age-matched healthy control (HC) probands. The frequency of the BDNF Val allele was significantly higher in AD, but there were no statistical differences in the allele distribution in PiD or in DLB as compared with HC. The Val/Met genotype occurred with statistically significantly higher frequency in PiD than in HC. The ApoE ε4 allele was significantly overrepresented in all dementias as compared with HC. Genotypes containing both ApoE ε4 and BDNF Val alleles occurred more frequently in all investigated dementias than in HC. We suggest that the presence of the BDNF Val allele in itself and in combination with the ApoE ε4 allele can be risk factors for AD, and the results indicate a synergistic effect of the 2 polymorphisms on DLB and PiD risk.

Key Words: apolipoprotein E (ApoE) polymorphism, brain-derived neurotrophic factor (BDNF) Val66Met polymorphism, Alzheimer disease, dementia with Lewy bodies, Pick disease

Alzheimer Dis Assoc Disord

Alzheimer disease (AD), dementia with Lewy bodies (DLB), and Pick disease (PiD) are neurodegenerative diseases that seem to have common features at cellular and molecular levels including protein aggregation and inclusion body formation. These inclusions probably represent an end stage of a molecular cascade.[1] AD involves 2 major kinds of protein aggregations: extracellular senile plaques containing β-amyloid and intracellular neurofibrillary tangles of the microtubular protein τ.[2] DLB is a synucleinopathy, a neurodegenerative condition associated with abnormal aggregations of α-synuclein.[3] Similar to AD, PiD is taupathy: its pathologic features imply the presence of neuronal inclusions called Pick bodies, which are composed of numerous randomly oriented τ fibrils.[4]

Received for publication June 3, 2008; accepted November 8, 2008.
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Supported by grants from Hungarian Scientific Research Fund T 046152/2004, K 60589/2006, and Hungarian Ministry of Health 198 04/2006.
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The brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors, produced by cortical neurons. Besides its general role in neurodevelopment, BDNF has important functions in the adult brain such as promoting the survival and maintaining the structural integrity of neuronal cells.[5] The activity-dependent expression of BDNF plays a role in modulating synaptic changes associated with learning and memory.[6] Several studies have suggested that the altered functioning of BDNF could be involved in the pathogenesis of neurodegenerative disorders. It has been reported that patients with AD have reduced BDNF levels in the hippocampus and in the temporal cortex compared with healthy controls (HCs).[7]

The BDNF gene encodes a precursor peptide (pro-BDNF) that is secreted and cleaved by extracellular protease to form the mature BDNF protein.[8] A single nucleotide polymorphism (SNP) at nucleotide 196 (G/A) producing a nonconservative amino acid substitution at codon 66 (Val/Met) has been identified.[9] Although this SNP is located in the 5′ pro-BDNF sequence and does not affect the function of the mature BDNF, it has a major impact on the intracellular trafficking and regulated secretion of pro-BDNF.[10] Genetic influences on BDNF secretion can lead to alterations in hippocampal activity. The BDNF Val66Met polymorphism has been found to be associated with episodic memory and hippocampal function.[11] There are conflicting reports on the correlation between AD and BDNF Val66Met. Association studies have reported that the Val allele occurs with significantly higher frequency in AD as compared with HCs.[12,13] However, other authors have not found such a correlation.[9,14]

Apolipoprotein E (ApoE) is involved in lipid transport and metabolism. In addition, it plays a specific role in the central nervous system, including neuronal development, regeneration, and certain neurodegenerative processes. The polymorphism of the ApoE gene determines 3 isoforms of ApoE protein (ε2, ε3, and ε4) with different conformation and lipid binding properties.[15] The ε3 allele is the most prevalent form, and the proportion of the 3 alleles differs between populations. ApoE ε4 allele carriers have an increased risk of several neurodegenerative disorders, but the most well known one is the association between the ε4 allele and AD.[16] There are contradictory reports on the association between the ε4 allele and DLB.[17,18] Association studies have reported that the ε4 allele is significantly overrepresented in PiD[19,20] and the number of ε4 alleles was inversely related to the onset of PiD.[19]

Both ApoE and BDNF Val66Met genotype frequencies depend on ethnic background. The Hungarian popula-

tion has been reported to be genetically different from other European ethnic groups.[21] The aim of our investigation was to provide data on ApoE and BDNF Val66Met polymorphisms in the Hungarian population. We tested the hypothesis that the ApoE ε4 and BDNF Val alleles confer susceptibility to AD and to the rare PiD and DLB, from which very few genetic data are available. Furthermore, we investigated the interaction between the ApoE ε4 and BDNF Val alleles in the above-mentioned diseases.

SUBJECTS AND METHODS

Probands were whites living in Hungary. The study included 160 AD patients from the Department of Psychiatry, Memory Clinic, University of Szeged, who met the NINCDS/ADRDA criteria for probable AD.[22] The DLB group consisted of 34 probands, who were patients of the Department of Psychiatry and Psychotherapy, Semmelweis Medical University, Budapest. The diagnosis was established according to the McKeith criteria.[23] In 20 of the DLB cases, the clinical diagnosis was verified by postmortem examination as well. The 38 PiD samples were from the tissue bank at the Department of Neuropathology, National Institute of Psychiatry and Neurology, Budapest. The clinical diagnoses were verified by postmortem examination. The 164 age and sex-matched HC probands were selected from spouses of patients at the Department of Psychiatry, Memory Clinic, University of Szeged. Informed consent was obtained from the living subjects included in this study and all protocols were approved by the local ethical committee.

Mini Mental State Examination (MMSE) scores lower than 24 were considered characteristic of dementia. The MMSE scores in the HC group were higher than 28 points (mean: 29.1 ± 0.9) and none of the probands had any verified symptoms of dementia. The mean MMSE score in the DLB group was 18.5 ± 5.8. In the AD group, it was 17.9 ± 6.1 points. For the PiD group we have no MMSE data. The characteristics of the probands are presented in Table 1.

DNA was extracted from peripheral blood leukocytes according to a standard procedure using the Roche kit. In the PiD cases, DNA was extracted from paraffin-embedded brain tissues using the EZNA kit (PeqLab Biotechnologie GmbH). It would be more correct to compare brain samples of PiD patients with brain samples of HC subjects, but it has been shown[24,25] that regardless of the origin of the sample, the genotypic concordance is at least 97%. ApoE genotyping was conducted by polymerase chain reaction as described earlier[26] Genotyping of the BDNF Val66Met polymorphism was performed by polymerase chain reaction amplification and enzymatic digestion with restriction enzyme *Pma*CI, followed by polyacrylamide gel electrophoresis with ethidium bromide staining.[27]

The ApoE and BDNF genotype and allele frequencies were compared using Pearson χ^2 test. A multinomial logistic regression model was used to test for the interaction between the ApoE ε4 and BDNF Val alleles. The significance level was set at $P < 0.05$. The program SPSS 15.0 was used for all statistical analyses.

TABLE 1. Characteristics of the Probands

	HC	AD	DLB	PiD
	n = 164	n = 160	n = 34	n = 38
Age (y)	71.7 ± 8.5	73.7 ± 9.5	76.7 ± 7.2	70.0 ± 8.3
Male/female	63/101	57/103	15/19	12/26
MMSE (scores)	29.1 ± 0.9	17.9 ± 6.1	18.5 ± 5.8	—

AD indicates Alzheimer disease; DLB, dementia with Lewy bodies; HC, healthy control; MMSE, Mini Mental State Examination; PiD, Pick disease.

RESULTS

The BDNF Val66Met genotype and allele frequencies are presented in Table 2. The frequency of the BDNF Val/Val homozygous genotype was significantly higher in AD than in the HC group (AD: 58.8%, HC: 31.7%), but it occurred in DLB and especially in PiD with a lower frequency than in the HC group (DLB: 17.7%, PiD: 5.3%). There were also robust differences in the distribution of the Val/Met and Met/Met genotypes in AD versus HC (Val/Met—AD: 35.0%, HC: 48.2%; Met/Met—AD: 6.2%, HC: 20.1%). The Val/Met genotype occurred with significantly higher frequency in PiD than in HC (PiD: 78.9%, HC: 48.2%). The Val allele was significantly overrepresented in AD, but there were no statistical differences in its occurrence either in DLB or in PiD as compared with HC (AD: 76.2%, DLB: 51.5%, PiD: 44.7%, and HC: 55.8%).

The ApoE genotype and allele distribution is shown in Table 3. Among the patients with dementia, no ApoE ε2/ε2 carriers were detected, and even in the HC group only 2 cases were found. The heterozygous ε2/ε3 genotype also occurred with low frequency in AD, DLB, and PiD (AD: 6.2%, DLB: 2.9%, and PiD: 5.3% vs. HC: 14.7%). The ε3/ε4 genotype and the ε4 allele were significantly overrepresented in all groups of dementia compared with HC (ε3/ε4 genotype—AD: 30.0%, DLB: 50.0%, PiD: 52.6%, and HC: 12.8%; ε4 allele—AD: 24.1%, DLB: 27.9%, PiD: 40.8%, and HC: 8.9%). Furthermore, the frequency of the ε3/ε4 genotype in PiD and DLB was much higher than in AD. The PiD cases showed a great difference in the distribution of the ε4 allele not just from HC, but also from the other 2 dementias.

Table 4 summarizes the frequencies and odds ratios (ORs) for the interaction between the ApoE ε4 and BDNF Val alleles in the groups of different dementias and HC

TABLE 2. BDNF Val66Met Genotype and Allele Frequencies in Different Groups

	HC	AD	DLB	PiD
	n = 164 (%)	n = 160 (%)	n = 34 (%)	n = 38 (%)
Genotype*				
Val/Val	52 (31.7)	94 (58.8)	6 (17.7)	2 (5.3)
Val/Met	79 (48.2)	56 (35.0)	23 (67.6)	30 (78.9)
Met/Met	33 (20.1)	10 (6.2)	5 (14.7)	6 (15.8)
Allele†				
Val	183 (55.8)	244 (76.2)	35 (51.5)	34 (44.7)
Met	145 (44.2)	76 (23.8)	33 (48.5)	42 (55.3)

*HC versus AD: $\chi^2(2) = 28.258$, $P < 0.0001$; HC versus DLB: $\chi^2(2) = 4.405$, $P = 0.111$; HC versus PiD: $\chi^2(2) = 13.786$, $P = 0.001$.

†HC versus AD: $\chi^2(1) = 30.163$, $P < 0.0001$; HC versus DLB: $\chi^2(1) = 0.425$, $P = 0.514$; HC versus PiD: $\chi^2(1) = 3.034$, $P = 0.082$.

AD indicates Alzheimer disease; BDNF, brain-derived neurotrophic factor; DLB, dementia with Lewy bodies; HC, healthy control; PiD, Pick disease.

TABLE 3. ApoE Genotype and Allele Frequencies in Different Groups

	HC	AD	DLB	PiD
	n = 164 (%)	n = 160 (%)	n = 34 (%)	n = 38 (%)
Genotype*				
ε2/ε2	2 (1.2)	—	—	—
ε2/ε3	24 (14.7)	10 (6.2)	1 (2.9)	2 (5.3)
ε2/ε4	—	1 (0.6)	—	7 (18.4)
ε3/ε3	113 (68.9)	87 (54.4)	15 (44.2)	7 (18.4)
ε3/ε4	21 (12.8)	48 (30.0)	17 (50.0)	20 (52.6)
ε4/ε4	4 (2.4)	14 (8.8)	1 (2.9)	2 (5.3)
Allele†				
ε2	28 (8.5)	11 (3.4)	1 (1.5)	9 (11.8)
ε3	271 (82.6)	232 (72.5)	48 (70.6)	36 (47.4)
ε4	29 (8.9)	77 (24.1)	19 (27.9)	31 (40.8)

*HC versus AD: $\chi^2(5) = 28.220$, $P < 0.0001$; HC versus DLB: $\chi^2(4) = 26.469$, $P < 0.0001$; HC versus PiD: $\chi^2(5) = 70.951$, $P < 0.0001$.

†HC versus AD: $\chi^2(2) = 32.076$, $P < 0.0001$; HC versus DLB: $\chi^2(2) = 21.803$, $P < 0.0001$; HC versus PiD: $\chi^2(2) = 53.233$, $P < 0.0001$.

AD indicates Alzheimer disease; ApoE, apolipoprotein E; DLB, dementia with Lewy bodies; HC, healthy control; PiD, Pick disease.

divided into subgroups according to the presence of the ε4 allele (1 or 2) and the presence of the Val allele (1 or 2). Genotypes containing both the ε4 and BDNF Val alleles occurred more frequently in all types of dementias than in HC. Moreover, in the PiD group, the frequency of these genotypes was outstandingly high (AD: 36.9%, DLB: 47.1%, PiD: 63.2%, and HC: 10.4%). The OR for the presence of both the ε4 and BDNF Val alleles in the AD group was 14.46 [95% confidence interval (CI): 5.10-40.98], in the DLB group was 7.84 (95% CI: 1.97-31.13), and in the PiD group was 35.29 (95% CI: 4.35-286.24), as compared with patients with neither the ε4 nor the BDNF Val alleles. As in AD, the OR 14.46 (95% CI: 5.10-40.98) for the occurrence of the ε4 and BDNF Val alleles was much higher than the OR 2.08 (95% CI: 0.47-9.29) for the presence of ε4 without the Val allele, we propose a synergistic effect of the 2 SNPs on risk for AD. In the case of DLB and PiD, we found that the OR for the presence of both alleles [DLB: OR 7.84 (1.98-31.13); PiD: OR 35.29 (4.35-286.24)] was also higher as compared with the OR for having the ε4 and lacking the Val allele [DLB: OR 2.08 (0.294-14.766); PiD: OR 15.63 (1.58-154.28)]. Therefore, we suggest that the presence of the Val allele, in combination with the ε4 allele, might play a role in the risk for DLB and PiD.

DISCUSSION

Our data revealed that the Val66Met polymorphism of the BDNF gene may be implicated in the susceptibility to AD, as we have found that the Val/Val genotype and the Val allele occurred with significantly higher frequency in AD than in HC. Japanese and Italian studies have also reported this association,[9,13] although another Japanese study and a Spanish study failed to find the same results.[12,14] The frequency of occurrence of the Val allele in the Hungarian AD group in this study was similar to the data published for Italian and Spanish populations (Hungarian: 76%; Italian: 78%; and Spanish: 79%). Although in the Japanese population, Matsushita and co-workers[13] reported 61%, Akatsu et al[14] reported 57% frequency for the occurrence of the Val allele in AD. In contrast to the findings in the AD group, the distribution of the Val allele among the Hungarian HC probands was closer to the results found in the Japanese HC groups (Hungarian: 56%; Japanese[13]: 56%; Japanese[14]: 57%; vs. Italian: 70%; Spanish: 81%). According to these findings, the BDNF Val66Met polymorphism is highly heterogeneous among the different ethnic populations, even within Europe.

BDNF is important for memory-related hippocampal processes. The functional Val66Met polymorphism of

TABLE 4. Interaction Between ApoE ε4 and BDNF Val Alleles in Different Groups

		No. Persons (Frequency %)				
ApoE ε4	BDNF Val	HC n = 164	AD n = 160	OR	95% CI	*P*
+	+	17 (10.4)	59 (36.9)	14.461	5.103-40.979	< 0.0001
+	−	8 (4.9)	4 (2.5)	2.083	0.467-9.288	0.336
−	+	114(69.5)	91 (56.8)	3.326	1.309-8.452	0.012
−	−	25 (15.2)	6 (3.8)	Reference		
		HC	**DLB**			
		n = 164	**n = 34**			
+	+	17 (10.4)	16 (47.1)	7.843	1.976-31.128	0.003
+	−	8 (4.9)	2 (5.9)	2.083	0.294-14.766	0.463
−	+	114(69.5)	13 (38.2)	0.950	0.252-3.586	0.940
−	−	25 (15.2)	3 (8.8)	Reference		
		HC	**PiD**			
		n = 164	**n = 38**			
+	+	17 (10.4)	24 (63.2)	35.294	4.352-286.241	0.001
+	−	8 (4.9)	5 (13.1)	15.625	1.582-154.279	0.019
−	+	114(69.5)	8 (21.1)	1.754	0.210-14.666	0.604
−	−	25 (15.2)	1 (2.6)	Reference		

The reference category is HC.

Model: $\chi^2(9) = 82.344$, $P < 0.0001$.

AD indicates Alzheimer disease; ApoE, apolipoprotein E; BDNF, brain-derived neurotrophic factor; CI, confidence interval; DLB, dementia with Lewy bodies; HC, healthy control; PiD, Pick disease; OR, odds ratio.

BDNF can influence the activity-dependent BDNF secretion, thus it can impact on hippocampal activity. Egan et al[10] reported impaired episodic memory in Met allele carrier healthy probands and found that neurons transfected with $BDNF_{Met}$ showed lower depolarization-induced secretion of BDNF. These results are consistent with another study that demonstrated reduced hippocampal activity during declarative memory processing in Met allele carrier probands.[11] According to our findings and other association studies, the Val allele is associated with AD.[12,13]

The decreased BDNF level in AD reported by several studies[7,28] can be the result of the neurodegenerative processes, the loss of neurons producing BDNF, and therefore, it can be independent of the BDNF genotype. In the early stage of AD, higher BDNF level was measured than in HC,[29] which can be influenced by the level of the regulated secretion of BDNF, and therefore, the BDNF Val66Met genotype. A possible explanation of the contradictory observations and the role of the BDNF Val66Met polymorphism in AD need further investigation.

According to our data, the BDNF Val66Met polymorphism does not confer susceptibility to DLB, and these results are in agreement with a Japanese study.[14] The BDNF Val66Met genotype frequencies did not differ significantly in the Japanese study, nor in this study. The distribution of the Val allele in the Hungarian DLB and HC groups was similar to the Japanese data (Hungarian DLB: 52%; Japanese DLB 60%; Hungarian HC: 56%; and Japanese HC: 55%).

This is the first report on BDNF Val66Met polymorphism in PiD. The heterozygous Val/Met genotype occurred in PiD with outstandingly high frequency, which was statistically significant compared with HC, but we have not found significant difference in the distribution of BDNF alleles. The small sample size for DLB and PiD can influence the results of the statistical analyses and can explain the wide CIs; therefore, this is a study generating hypothesis and further investigations are required.

It has been shown that $BDNF_{Met}$ could alter the intracellular distribution and activity-dependent secretion of $BDNF_{Val}$. The $BDNF_{Val}/BDNF_{Met}$ heterodimers coexpression results in decreased secretion,[10] which can be a possible explanation for our findings in PiD. Memory impairment has been observed in probands having the Val/Met genotype.[10,11] As the activity-dependent release of BDNF modulates synaptic transmission and neural plasticity,[30] this behavioral deficit may be attributable to the decreased secretion of $BDNF_{Val}/BDNF_{Met}$ heterodimers.

We found that the ApoE ε4 allele was significantly overrepresented in AD, DLB, and PiD, as compared with the HC group. These results are in line with our former findings for the AD group in the Hungarian population[26,31,32] and with results on other ethnic groups.[33–35] Despite the relatively low number of DLB and PiD patients, our findings can provide important data, as we have a found significant difference in the distribution of the ε4 allele in DLB and PiD, as compared with HC. The frequency of the ε4 allele was even higher in PiD than in AD. These findings confirm the results of genetic and epidemiologic studies that have reported that the ε4 allele is the most important known risk factor for late-onset familial and sporadic forms of AD and other dementias. The presence of the ε4 allele increases the risk and reduces the age for the onset of dementia, in contrast to the possession of the ε2 allele that has a protective effect. ApoE ε4 isoform has been demonstrated to negatively influence and intensify the biochemical disturbances of AD, including β-amyloid deposition, tangle formation, neuronal cell death, dysfunction of lipid homeostasis, and cholinergic signaling.[15]

In summary, our findings indicate that the BDNF Val allele may confer susceptibility to AD, whereas the BDNF Val/Met genotype increases the risk for PiD. Genotypes containing both the ApoE ε4 and BDNF Val alleles were overrepresented in all investigated dementias, and the comparison of the ORs for having both ε4 and Val alleles and for having only ε4 without the Val allele might reveal a possible role of these risk factors in the pathogenesis of AD, DLB, and PiD.

ACKNOWLEDGMENT

The authors thank all probands for their participation in this study.

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Original Research Article

Dementia
and Geriatric
Cognitive Disorders

Dement Geriatr Cogn Disord 613

Accepted: June 2, 2009

Association between a Genetic Variant of the Alpha-7 Nicotinic Acetylcholine Receptor Subunit and Four Types of Dementia

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Key Words

Apolipoprotein E · α7 nicotinic acetylcholine receptor · Alzheimer's disease · Dementia with Lewy bodies · Pick's disease · Vascular dementia

Abstract

We tested the hypothesis whether the partially duplicated variant of α7 nicotinic acetylcholine receptor subunit gene (CHRFAM7A) 2-bp deletion (–2 bp) polymorphism and apolipoprotein E (ApoE) ε4 allele confer susceptibility to Alzheimer's disease (AD), dementia with Lewy bodies (DLB), Pick's disease (PiD) and vascular dementia (VD). The study included 175 AD, 35 DLB patients, 38 PiD, 96 VD and 175 healthy control (HC) probands. The CHRFAM7A genotype without the –2 bp allele was significantly over-represented in AD ($p = 0.011$), DLB ($p = 0.001$) and PiD ($p < 0.0001$) compared to HC, but there were no statistical differences in VD ($p = 0.407$) compared to HC. We confirmed again that the ApoE ε4 allele is a risk factor for dementias. The –2 bp polymorphism of CHRFAM7A can be implicated in AD, DLB and PiD. However, it is unlikely that it plays an important role in the pathogenesis of VD.

Introduction

Alzheimer's disease (AD), dementia with Lewy bodies (DLB) and Pick's disease (PiD) are neurodegenerative disorders associated with abnormal protein aggregation and inclusion body formation. AD pathology is characterized by the presence of senile plaques composed of amyloid-β_{42} ($A\beta_{42}$) and neurofibrillary tangles containing hyperphosphorylated tau protein [1]. DLB is a neurodegenerative condition associated with abnormal aggregation of α-synuclein, termed Lewy bodies [2]. The term 'Pick's disease' is restricted to cases showing lobar atrophy with Pick bodies, composed of numerous randomly oriented hyperphosphorylated tau fibrils [3], and PiD is included in the nomenclature of frontotemporal dementia [4]. Vascular dementia (VD) results from various cerebrovascular diseases leading to cognitive impairment which depends on the severity and location of lesions [5].

Alpha-7 nicotinic acetylcholine receptors (α7 nAChRs) are homopentamer, ligand-gated cationic channels. They are widely expressed in the central nervous system with high levels in the regions relevant to memory functions and involved in processing of sensory information, such as the hippocampus [6]. It has been demonstrated that

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$A\beta_{42}$ binds to α7 nAChR with high affinity and they both are present in senile plaques [7]. Their interaction alters several neurochemical processes, including Ca^{2+} homeostasis and acetylcholine release, and thereby modulates neuronal physiological functions implicated in memory processes. Chronic inhibition of cholinergic signaling by $A\beta_{42}$ could contribute to the cognitive deficits associated with AD [8]. In an experimental system, $A\beta_{42}$ effectively induced tau phosphorylation via α7 nAChR that could be decreased by α7 nAChR antagonists. $A\beta_{42}$–α7 nAChR interaction activates mitogen-activated cascade protein, which is a tau protein kinase [9]. Although the α7 nAChR-mediated and $A\beta_{42}$-induced tau phosphorylation seemed to be a normal reversible cellular response, the above mentioned findings suggest that α7 nAChR may be implicated in neuropathological conditions associated with abundant $A\beta_{42}$ and/or abnormal functions of tau protein. Abnormal α7 nAChR physiology and loss of neurons expressing α7 nAChR may contribute to memory and cognition impairment [9].

The α7 nAChR subunit gene (CHRNA7) is located in the highly duplicated 15q13–q14 region implicated in several neuropsychiatric disorders, including schizophrenia and bipolar affective disorder. The CHRNA7 gene is duplicated from exon 5 to 10 with >99% identity at the nucleotide level [10, 11]. The partially duplicated CHRNA7 and 4 novel exons D–A originated from the FAM7A gene form a hybrid gene (CHRFAM7A). CHRFAM7A is not present on every human chromosome and some individuals lack 1 (30%) or both (5%) copies [11]. A 2 bp deletion (–2 bp) polymorphism at position 497–498 in exon 6 was identified, which is specific to CHRFAM7A and does not occur in CHRNA7 [10]. The –2 bp deletion causes a frameshift, introducing a stop codon within exon 6, and therefore a truncation in a putative gene product. Since CHRFAM7A is reported to be expressed as mRNA, possible regulatory effects should also be considered. Recent findings of Flomen et al. [12] suggested that CHRFAM7A exists in 2 orientations (either direct or inverted orientation relative to CHRNA7) and this inversion polymorphism is in a linkage disequilibrium with the –2 bp polymorphism.

Apolipoprotein E (apoE) is involved in lipid transport and metabolism. In addition, it plays specific roles in the central nervous system including neuronal development, regeneration and certain neurodegenerative processes. Polymorphism of the ApoE gene located on chromosome 19 determines 3 isoforms (ε2, ε3, ε4) with different conformation and lipid-binding properties [13]. The ε3 and ε2 isoforms bind primarily to high-density lipoprotein, while ε4 prefers very-low-density lipoprotein and is less effective in cholesterol transport. ApoE ε4 increases the risk and reduces the age of the onset in AD [14]. Furthermore, ε4 carriers have an increased risk of other dementias, such as DLB and PiD [15, 16].

Liou and co-workers (2001) failed to find association between the CHRFAM7A –2 bp polymorphism and AD, investigating a relatively low number of cases in an Asian population [17]. According to our knowledge, the –2 bp polymorphism of CHRFAM7A has not been studied in any other dementias. The aim of our study was to provide data about ApoE and CHRFAM7A –2 bp polymorphisms in different dementias in the Hungarian Caucasian population. We tested the hypothesis whether the ApoE ε4 and the CHRFAM7A wild-type alleles confer susceptibility to dementias, while the presence of the CHRFAM7A –2 bp allele can have a protective effect against AD, VD and the rare-occurring DLB and PiD, from which very few genetic association data are available. In addition, we investigated the interaction between the ApoE and CHRFAM7A polymorphisms in the above mentioned diseases.

Materials and Methods

Probands were all Caucasians living in Hungary. The study included 175 AD patients from the Memory Clinic, Department of Psychiatry, University of Szeged, who met the NINCDS/ADRDA criteria for probable AD [18]. The clinical diagnosis of probable AD was based on the history of the probands, psychiatric and neurological examination, basic clinical tests such as the Mini-Mental State Examination (MMSE) and the Hachinsky, Clock Drawing and Verbal fluency scores, as well as brain CT or MRI imaging. Probands all had experienced a progressive loss of cognitive functions (in more than 2 cognitive domains indicative of cortical dysfunction) for at least 1 year with memory loss as the most significant symptom. The CT or MRI images for AD patients revealed generalized or temporal lobe atrophy. However, cerebrospinal amyloid and tau levels were not evaluated.

Twenty DLB cases were selected retrospectively on the basis of the postmortem neuropathological examination. In these cases, the Consortium on DLB International Workshop's neuropathological criteria of DLB was considered [19]. The remaining 15 probable DLB probands were patients of the Semmelweis Medical University, Department of Psychiatry and Psychotherapy, Budapest. Their clinical diagnoses were established according to the McKeith criteria [20]. None of them had confirmed autopsy diagnoses since all of them are still alive.

The 38 PiD cases were all carefully selected autopsy cases from the tissue bank at the Department of Neuropathology, National Institute of Psychiatry and Neurology, Budapest. The clinical diagnoses were verified by postmortem examination. Besides the marked frontotemporal atrophy, sections from different parts of the cerebral cortex and basal ganglia were stained with cresyl vio-

Table 1. Characteristics of the probands

	HC (n = 175)	AD (n = 175)	DLB (n = 35)	PiD (n = 38)	VD (n = 96)
Age, years	71.7 ± 8.5	74.0 ± 9.2	76.7 ± 7.2	70.0 ± 8.3	75.3 ± 9.8
Male/female	77/98	59/116	15/20	12/26	37/59
MMSE, scores	29.1 ± 0.9	17.9 ± 6.1	18.5 ± 5.8	–	19.7 ± 5.7

Table 2. CHRFAM7A genotype frequencies in different groups

Genotype*	HC (n = 175)	AD (n = 175)	DLB (n = 35)	PiD (n = 38)	VD (n = 96)
1	42 (24.0)	63 (36.0)	19 (54.3)	29 (76.3)	30 (31.2)
2	94 (53.7)	90 (51.4)	9 (25.7)	8 (21.1)	45 (46.9)
3	39 (22.3)	22 (12.6)	7 (20.0)	1 (2.6)	21 (21.9)

* HC versus AD: $\chi^2(2) = 9.025$, p = 0.011; HC versus DLB = Fisher's exact test: p = 0.001; HC versus PiD: Fisher's exact test: p < 0.0001; HC versus VD: $\chi^2(2) = 1.797$, p = 0.407.

Percentages are shown in parentheses.

let and with the Bielschowsky silver method; Pick cells and Pick bodies were seen.

The 96 patients of VD were clinically diagnosed as probable VD cases according to the criteria of ICD-10 [21], and the NINDS-AIREN International Workshop's diagnostic criteria for research studies [22]. All VD patients had small-vessel disease, characterized by subcortical multiple lacunar infarcts around the lateral ventricles in both hemispheres, revealed by brain MRI evaluation. Other types of VD cases with cortical or strategic infarcts were not involved in the study.

The 175 age-matched healthy control (HC) probands were selected from spouses of patients at the Memory Clinic, Department of Psychiatry, University of Szeged. HCs were medication-free, lacked any significant medical illnesses and had Hachinsky scores <4. Informed consent was obtained from the living subjects included in this study and all protocols were approved by the local ethical committee.

MMSE scores <24 were considered characteristic of dementia. MMSE scores in the HC group were always >28 points (mean: 29.1 ± 0.9) and none had verified symptoms of dementia. For the PiD group, we have no MMSE data. The MMSE mean score in the AD group was 17.9 ± 6.1, in the DLB group, it was 18.5 ± 5.8, and in the VD group, it was 19.7 ± 5.7. The characteristics of the probands are presented in table 1.

DNA was extracted from peripheral blood leukocytes according to a standard Roche procedure kit. In the PiD cases, DNA was extracted from paraffin-embedded brain tissues by E.Z.N.A. kit (PeqLab Biotechnologie GmbH, Erlangen, Germany). ApoE genotyping was made by PCR as described earlier [23]. Genotyping of the CHRFAM7A –2 bp polymorphism was performed using PCR amplifications, followed by polyacrylamide gel electrophoresis with ethidium bromide staining [24]. We focused on the deletion polymorphism of the CHRFAM7A and did not investigate the copy number or the inversion polymorphisms; therefore, we determined 3 groups. The genotype lacking the 2 bp deletion was designated genotype 1, the genotype having 1 copy of the –2 bp allele was designated genotype 2, and the genotype with 2 copies of the –2 bp allele was designated genotype 3.

The ApoE and the CHRFAM7A genotype frequencies were compared with the Fisher's exact and the Pearson χ^2 tests. A multinomial logistic regression model was used to test for the interaction between the ApoE ε4 allele and CHRFAM7A wild genotype (without the –2 bp allele). The significance level was set at $p < 0.01$. SPSS 15.0 was used for all computer statistical analyses.

Results

CHRFAM7A genotype frequencies are presented in table 2. Genotype 1 was significantly over-represented in AD, DLB and PiD compared to HC. Moreover, the frequency of genotype 1 in PiD was outstandingly high (AD: 36.0%, DLB: 54.3%, PiD: 76.3%, HC: 24.0%). The frequency of genotype 3 was significantly lower in AD and in PiD than in the HC group (AD: 12.6%, PiD: 2.6%, HC: 22.3%), but it occurred in DLB and in VD with similar frequency compared to the HC group (DLB: 20.0%, VD: 21.9%). The differences were statistically significant in the DLB (p = 0.001) and PiD (p < 0.0001) groups, and marginally significant in the AD (p = 0.011) group compared to HC, as we set the significance level to 0.01. Comparison of the CHRFAM7A genotype frequencies between VD and HC showed no statistically significant difference (p = 0.407), although genotype 1 occurred more frequently in the VD group than in the HC group (VD: 31.2%, HC: 24.0%).

The distribution of the ApoE genotypes and alleles are presented in table 3. Among the patients with dementia, no ApoE ε2/ε2 carriers were detected and even in the HC group, only 2 cases were found. The ratio of the ε3/ε4 carriers was significantly higher in all groups of dementia than in the HC group; furthermore, in the DLB and PiD groups, it was outstandingly high (AD: 32.0%, DLB:

Table 3. ApoE genotype and allele frequencies in different groups

	HC n = 175	AD n = 175	DLB n = 35	PiD n = 38	VD n = 96
Genotype*					
ε2/ε2	2 (1.1)	–	–	–	–
ε2/ε3	25 (14.3)	11 (6.3)	1 (2.9)	2 (5.3)	10 (10.4)
ε2/ε4	–	3 (1.7)	–	7 (18.4)	3 (3.1)
ε3/ε3	125 (71.4)	91 (52.0)	16 (45.7)	7 (18.4)	58 (60.4)
ε3/ε4	19 (10.9)	56 (32.0)	17 (48.5)	20 (52.6)	23 (24.0)
ε4/ε4	4 (2.3)	14 (8.0)	1 (2.9)	2 (5.3)	2 (2.1)
Allele**					
ε2	29 (8.3)	14 (4.0)	1 (1.4)	9 (11.8)	13 (6.8)
ε3	294 (84.0)	249 (71.1)	50 (71.4)	36 (47.4)	149 (77.6)
ε4	27 (7.7)	87 (24.9)	19 (27.2)	31 (40.8)	30 (15.6)

* HC versus AD: $\chi^2(5) = 39.605$, $p < 0.0001$; HC versus DLB: Fisher's exact test: $p < 0.0001$; HC versus PiD: Fisher's exact test: $p < 0.0001$; HC versus VD: $\chi^2(5) = 15.275$, $p = 0.009$.

** HC versus AD: $\chi^2(2) = 40.541$, $p < 0.0001$; HC versus DLB: Fisher's exact test: $p < 0.0001$; HC versus PiD: Fisher's exact test: $p < 0.0001$; HC versus VD: $\chi^2(2) = 8.365$, $p = 0.015$.

Percentages are shown in parentheses.

Table 4. Interaction between ApoE ε4 allele and CHRFAM7A genotype 1 in the different groups

ApoE ε4	CHRFAM7A genotype 1	HC (n = 175)	AD (n = 175)	OR	95% CI	p*
+	+	7 (4.0%)	22 (12.6%)	6.028	2.438–14.902	<0.0001
+	–	16 (9.1%)	51 (29.1%)	6.114	3.220–11.609	<0.0001
–	+	35 (20.0%)	41 (23.4%)	2.247	1.300–3.883	0.004
–	–	117 (66.9%)	61 (34.9%)		reference	
		HC (n = 175)	DLB (n = 35)			
+	+	7 (4.0%)	9 (25.7%)	21.490	6.170–74.848	<0.0001
+	–	16 (9.1%)	9 (25.7%)	9.402	3.076–28.740	<0.0001
–	+	35 (20.0%)	10 (28.6%)	4.776	1.693–13.471	0.003
–	–	117 (66.9%)	7 (20.0%)		reference	
		HC (n = 175)	PiD n = 38			
+	+	7 (4.0%)	21 (55.2%)	351.000	41.043–3001.733	<0.0001
+	–	16 (9.1%)	8 (21.1%)	58.500	6.859–498.957	<0.0001
–	+	35 (20.0%)	8 (21.1%)	26.743	3.233–221.216	0.002
–	-	117 (66.9%)	1 (2.6%)		reference	
		HC (n = 175)	VD (n = 96)			
+	+	7 (4.0%)	10 (10.4%)	3.482	1.252–9.682	0.170
+	–	16 (9.1%)	18 (18.8%)	2.742	1.292–5.820	0.009
–	+	35 (20.0%)	20 (20.8%)	1.393	0.732–2.652	0.313
–	–	117 (66.9%)	48 (50.0%)		reference	

The reference category is HC. Model: $\chi^2(12) = 122.352$, $p < 0.0001$.

* Wald χ^2 test.

48.5%, PiD: 52.6%, VD: 24.0%, HC: 10.9%). The ε4 allele was significantly over-represented in AD, DLB and PiD compared to HC (AD: 24.9%, $p < 0.0001$; DLB: 27.2%, $p < 0.0001$; PiD: 40.8%, $p < 0.0001$). Comparison of ε4 allele frequencies between VD and HC showed a marginally significant difference (VD: 15.6%, HC: 7.7%, $p = 0.015$). The frequency of the occurrence of the ε4 allele in PiD cases was extremely high, not only compared to HC, but also to other dementias.

The genotype frequencies of both ApoE and CHRFAM7A polymorphisms in the PiD group differed significantly from the genotype distribution in the AD and VD groups (CHRFAM7A genotypes: PiD vs. AD: $p < 0.0001$; PiD vs. VD: $p < 0.0001$, and ApoE genotypes: PiD vs. AD: $p < 0.0001$; PiD vs. VD: $p < 0.0001$).

Table 4 summarizes the frequencies and odds ratios (OR) for the interaction between ApoE ε4 allele and CHRFAM7A genotype 1 in different groups. Simultaneous presence of ApoE ε4 allele and CHRFAM7A genotype 1 occurred more frequently in all types of dementia compared to HC (AD: 12.6%, DLB: 25.7%, PiD: 55.2%, VD: 10.4%, HC: 4.0%).

The ORs for the presence of ε4 allele with or without the CHRFAM7A genotype 1 were similar in AD and VD (table 4); therefore, it is unlikely that the combination of these genetic variants would be involved in AD or VD. In DLB and PiD, however, the OR for the presence of both risk factors was much higher than the OR for the possession of ε4 allele without CHRFAM7A genotype 1 (table 4), suggesting that the simultaneous occurrence of ε4 allele and CHRFAM7A genotype 1 can enhance the risk for the development of DLB and PiD.

Discussion

Our major finding was that the CHRFAM7A genotype without the –2 bp allele was significantly over-represented in AD, DLB and PiD compared to HC and we further confirmed that the ApoE ε4 allele is a risk factor for dementias. The simultaneous occurrence of these 2 risk factors was over-represented in the DLB and PiD groups.

The CHRFAM7A genotype 1 occurred with significantly higher frequency in the AD, DLB and PiD groups than in the HC group. The absence of the –2 bp allele in the DLB group and especially in the PiD group occurred even more frequently than in the AD group; however, the small sample size for DLB and PiD can affect the results of the statistical analyses. The CHRFAM7A genotype frequencies showed no significant differences between the VD and HC groups. According to these results, the partially duplicated variant of the α7 nAChR subunit gene, expressed together with the 4 exons from the FAM7A gene, can be a strong candidate gene for neurodegenerative disorders associated with abnormal protein aggregations. There may be a functional relationship between $A\beta_{42}$, α7 nAChR and tau protein, since it has been reported that an $A\beta_{42}$–α7 nAChR interaction can activate tau phosphorylation [9]. This relationship can be the common feature by which genetic variants of CHRFAM7A can influence the risk either for AD, DLB or PiD. $A\beta_{42}$ is the major component of senile plaques implicated in AD and DLB, and the hyperphosphorylated tau protein is the main constituent of neurofibrillary tangles in AD brains and Pick bodies occurring in PiD.

The duplicated α7 nAChR exons (CHRNA7 5–10) and the 4 novel exons (FAM7A D–A) are transcribed together, but there is no evidence whether this transcript is translated or not [10, 11]. It is unlikely that this hybrid gene functions as a nicotinic receptor due to the absence of signal peptide, glycosylation site and part of the ligand-binding site encoded by exons 1–4 [25]. It is possible, however, that if CHRFAM7A is translated, the gene product is able to interact with α7 polypeptide since most of the contact regions are encoded in exons 5–10 [11]. The mechanisms by which the –2 bp allele variant of duplicated exon 6 decrease the risk of dementias remain to be established. The 2 bp deletion results in a stop codon within exon 6; therefore, the putative translational product will be truncated. A possible explanation could be that the wild-type CHRFAM7A gene product may alter the normal assembly of the α7 nAChR, which could be avoided by the truncated gene product [11].

Our study demonstrates the association of the ApoE ε4 allele with AD, DLB, PiD and VD. These results in the AD and VD groups are in line with our former findings in the Hungarian population [23, 26–28] and with results on other ethnic groups [29, 30]. However, in the case of VD, no correlation between the ε4 allele and VD has also been reported [31, 32]. The apparently contradictory data can be explained by the lack of clear-cut diagnostic criteria and great clinical heterogeneity of this disorder. Although in this study a relatively low number of DLB and PiD probands were included, our findings can provide important data, since we have found a statistically significant difference in the occurrence of the ε4 allele in DLB and in PiD compared to HC. The frequency of the ε4 allele was even higher in PiD than in AD, which is in agreement with our former result [16].

There are some limitations of this study, however. The small sample size of the DLB and PiD groups may influence the results of the statistical analyses and can explain the wide confidence intervals. The different diagnostic standards (clinical diagnoses for AD and VD, postmortem for most of the DLB and all of the PiD subjects) may also influence the interpretability of data. Another limitation of this study may be that only the deletion polymorphism of CHRFAM7A was investigated. Therefore, this study can be considered as a hypothesis-generating work and further investigations are required.

Although our results may suggest that the absence of the –2 bp allele of CHRFAM7A can be implicated not just in AD, but in DLB and PiD as well, additional studies may be required to increase the number of DLB and PiD cases, and to investigate the copy number polymorphism of CHRFAM7A. Nevertheless, according to our findings, it is unlikely that the –2 bp polymorphism of CHRFAM7A plays an important role in the pathogenesis of VD.

Acknowledgements

The authors wish to thank all probands for their participation in this study. This work was supported by grants from Hungarian Scientific Research Fund T 046152/2004, K 60589/2006 and Hungarian Ministry of Health 198 04/2006.

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