Genetic background of neurological diseases

Ph.D. Thesis

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<th>Definition</th>
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<tbody>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>ADEM</td>
<td>Acute-disseminated encephalomyelitis</td>
</tr>
<tr>
<td>AHL</td>
<td>Acute haemorrhagic leucoencephalitis</td>
</tr>
<tr>
<td>PML</td>
<td>Progressive multifocal leucoencephalopathy</td>
</tr>
<tr>
<td>CPM</td>
<td>Central pontin myelinolysis</td>
</tr>
<tr>
<td>EPM</td>
<td>Extrapontin myelinolysis</td>
</tr>
<tr>
<td>LA</td>
<td>Leukoaraiosis</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylene tetrahydrofolate reductase</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>AQP-4</td>
<td>Aquaporin-4</td>
</tr>
<tr>
<td>CADASIL</td>
<td>Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy</td>
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<tr>
<td>NMSS</td>
<td>National Multiple Sclerosis Society</td>
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<tr>
<td>RR</td>
<td>Relapsing-remitting</td>
</tr>
<tr>
<td>SP</td>
<td>Secondary progressive</td>
</tr>
<tr>
<td>PP</td>
<td>Primary progressive</td>
</tr>
<tr>
<td>PR</td>
<td>Progressive relapsing</td>
</tr>
<tr>
<td>CIS</td>
<td>Clinically isolated syndrome</td>
</tr>
<tr>
<td>EDSS</td>
<td>Expanded Disability Status Scale</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td><strong>AMPs</strong></td>
<td>Antimicrobial peptides</td>
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<td>---------------</td>
<td>------------------------</td>
</tr>
<tr>
<td><strong>hBDs</strong></td>
<td>Human β-defensins</td>
</tr>
<tr>
<td><strong>HBD1, HBD2</strong></td>
<td>Human beta defensin-1, -2</td>
</tr>
<tr>
<td><strong>UTR</strong></td>
<td>Untranslated region</td>
</tr>
<tr>
<td><strong>PCR</strong></td>
<td>Polymerase chain reaction</td>
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<tr>
<td><strong>RT-PCR</strong></td>
<td>Real time polymerase chain reaction</td>
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<tr>
<td><strong>CT</strong></td>
<td>Threshold cycle</td>
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<tr>
<td><strong>ELISA</strong></td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td><strong>SNPs</strong></td>
<td>Single nucleotide polymorphisms</td>
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<tr>
<td><strong>CNPs</strong></td>
<td>Copy number polymorphisms</td>
</tr>
<tr>
<td><strong>CNVs</strong></td>
<td>Copy number variations</td>
</tr>
<tr>
<td><strong>AD</strong></td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td><strong>Abeta</strong></td>
<td>Amyloid β</td>
</tr>
<tr>
<td><strong>eFAD</strong></td>
<td>Early onset familial AD</td>
</tr>
<tr>
<td><strong>LOAD</strong></td>
<td>Late-onset AD</td>
</tr>
<tr>
<td><strong>SPECT</strong></td>
<td>Single photon emission computed tomography</td>
</tr>
<tr>
<td><strong>DSM-IV</strong></td>
<td>Fourth edition of the Diagnostic and Statistical Manual of Mental Disorders</td>
</tr>
<tr>
<td><strong>NINCDS-ADRDA</strong></td>
<td></td>
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<tr>
<td><strong>MMSE</strong></td>
<td>Mini-Mental State Exam</td>
</tr>
<tr>
<td><strong>ADAS-Cog</strong></td>
<td>AD Assessment Scale – Cognitive Subscale</td>
</tr>
<tr>
<td><strong>CDT</strong></td>
<td>Clock Drawing Test</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

1.1 Definition of the demyelinating disorders

Demyelinating diseases are characterized by the loss of the normal myelin development and integrity. Demyelination may attack axons in the central and peripheral nervous system and it creates a variety of neurological disorders and syndromes.

1.1.1 Classification of the CNS demyelination

The classification of the CNS demyelination is complicated. However, it can be classified according to the pathogenesis of the disease. Inflammatory demyelination, demyelination due to viral inflection, demyelination caused by acquired metabolic derangements, hypoxic–ischaemic form and demyelination caused by focal compression are the principal categories (Table 1 below).

<table>
<thead>
<tr>
<th>INFLAMMATORY DEMYELINATION</th>
<th>Multiple sclerosis (MS)</th>
</tr>
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<tbody>
<tr>
<td>Classical MS</td>
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<tr>
<td>Acute (Marburg-type) MS</td>
<td></td>
</tr>
<tr>
<td>Neuromyelitis optica (Devic’s disease)</td>
<td></td>
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<tr>
<td>Concentric sclerosis (Baló’s sclerosis)</td>
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<tr>
<td>Acute-disseminated encephalomyelitis (ADEM)</td>
<td></td>
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<tr>
<td>Acute haemorrhagic leucoencephalitis (AHL)</td>
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<table>
<thead>
<tr>
<th>VIRIAL DEMYELINATION</th>
<th>Progressive multifocal leucoencephalopathy (PML)</th>
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<tr>
<td>Other viral demyelinating diseases involved in HIV infection</td>
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</table>

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<tr>
<th>ACQUIRED METABOLIC DEMYELINATION</th>
<th>Central pontin myelinolysis (CPM)</th>
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<tr>
<td></td>
<td>Extrapontin myelinolysis (EPM)</td>
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<table>
<thead>
<tr>
<th>HYPOXIC- ISCHAEMIC DEMYELINATION</th>
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<table>
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<tr>
<th>COMPRESSION- INDUCED DEMYELINATION</th>
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Table 1. Classification of CNS demyelination (according to Love)
1.1.2 Pathogenesis of the ischemic demyelination

The ischemic demyelinating disorder, also known as age-related white matter lesion of the brain, is a common clinical phenomenon. It is the most frequently seen lesion in neuroimaging scans. Ischemic demyelinization is called Leukoaraiosis (LA) in neuroimaging terms. Here, LA refers to diffuse areas of hyperintensity in T2-weighed magnetic resonance imaging (MRI) scans and it involves white matter. One quarter of people over the age of 65 are affected to some degree by white matter changes. Moreover, LA is definitely associated with cognitive decline, slowness of mental processing (dementia) and a poor quality of life. It is a slowly developing illness, associated with various clinical risk factors and endogenous genetic factors.

Two major mechanisms have been proposed for it. The first is the chronic endothelial dysfunction. The increased levels of circulating markers of endothelial cell activation or dysfunction such as ICAM-1 and thrombomodulin were found in leukoaraiosis, and ICAM-1 levels correlated with the progression of LA. The second is the elevated blood–brain barrier permeability, which results in the leakage of plasma components into the vessel wall and surrounding brain parenchyma. Another hypothesis states that cerebral hypoperfusion may cause an energy deficit in the glia cells and neurons. Chronic hypoxia may be presumed to primarily damage the function of the mitochondria.

Aging, hypertension, diabetes mellitus, a prior stroke event, and cardiac diseases are the main vascular risk factors for LA. The temporal and occipital border zones are the most frequent regions of LA, because the superficial and deep perforans are end arterioles. The two systems do not connect, but only meet in a junctional zone around the lateral ventricles.

The key genetic factors of LA pathogenesis are the methylene tetrahydrofolate reductase (MTHFR) C677T variant, the angiotensin-converting enzyme (ACE) I/D and the apolipoprotein E (APOE) 2 or 4 polymorphisms. The homozygous MTHFR 677TT variant is associated with an elevated serum homocysteine level, which has an unfavourable effect on vasoregulation. The increased plasma homocysteine level will result in endothelial dysfunction. Homocysteine directly damages the vascular matrix because it affects the biosynthetic and biochemical functions of the vascular cell. Endothelial-derived nitric oxide production is also caused by homocysteine. LA is positively correlated with the plasma homocysteine level, so it is inversely correlated with the plasma folate level. The MTHFR A1298C polymorphism is also a common genetic variant and it is thought to be a vascular risk factor. The ACE I/D polymorphism is an important genetic factor in the renin-angiotensin system, and it has regulatory roles in the cardiovascular system. Alongside this,
the homozygous ACE D/D polymorphism has an unfavourable effect on the vasoregulatory system. 22 Also, the incidence of the ACE D/D genotype might adversely affect vasoconstriction, vascular walls undermine thickening, reducing the proliferation of smooth muscle cells and cause a stroke. 16 The APOE 2 or 4 allele is also shown to give the rise to LA. 23 The APOE 4 allele leads to a reduced neuroregeneration ability, and the cytoskeleton can become unstable and the glia cells may become more vulnerable. 24 Despite this, the APOE 4 genotype is supposed to be a risk factor for ischemic brain injury or stroke. 25 In the presence of the APOE 2 allele, the cytoskeleton might become too rigid and this may be harmful during the prolonged hypoperfusion of the brain 9, and it may decrease the range of mechanical and chemical flexibility of the glial cytoskeleton. 9 The homozygous MTHFR 677TT mutation in combination with the homozygous ACE D/D genotype has been associated with a higher risk of LA. 15 Furthermore, the MTHFR 677TT variant and the ACE D/D polymorphism contribute to small-vessel disease or vasoregulation impairment. These two genotypes in combination with the APOE 4 or 2 allele have also been reported to give rise to LA. 23 A new finding is the role of the aquaporin-4 (AQP-4) genotypes in LA. 26 AQP-4 is the major water channel in the brain and the spinal cord 27, and it aids the movement of water between the blood and brain, and between the brain and the cerebrospinal fluid. 28 AQP-4 is alleged to be involved in the etiology of neuromyelitis optica 29, cerebral oedema 30, sudden infant death syndrome 31 and migraine 32. The CT/TT genotypes of the AQP-4 rs2075575 polymorphism could be associated with cerebral small-vessel disease and these genotypes may be a significant promoting factor for LA. 26

The CADASIL study, which investigated the cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, is caused by mutations in the notch-3 gene. The deposition of the extracellular portion of notch 3 in the arterial wall of both cerebral and systemic arteries may be identified adjacent to granular osmiophilic material, a deposition of unknown composition. 33 This should be investigated further.

1.1.3 The pathogenesis of the Multiple sclerosis (MS)

MS is a chronic immune-mediated inflammatory disease of the central nervous system (CNS) that typically commences in the third or fourth decade of life. Demyelination and subsequent axonal damage is characteristic in MS. 34 35 The main pathological symptoms of the disease are the destruction of the myelin sheets of nerve fibres, the relative sparsity of the axons, and the infiltration of inflammatory cells in a perivascular surrounding. 36 37 The prevalence of MS in different regions of the world ranges from 15/100,000 to 250/100,000. 38
In 1996, the US National Multiple Sclerosis Society (NMSS) Advisory Committee provided standardized definitions for MS clinical courses; namely relapsing-remitting (RR), secondary progressive (SP), primary progressive (PP) and progressive relapsing (PR). In 2013, the clinically isolated syndrome (CIS) form was added and the PR form has been eliminated by the Committee (see figures 2 and 3 below).

**Figure 2. Relapsing-remitting MS (Lublin, 2013)**

CIS is a well-defined syndrome that includes optic neuritis, cerebellar dysfunction and partial myelitis, and it forms a part of the RR MS spectrum.

**Figure 3. Progressive MS (Lublin, 2013)**
The original diagnostic criteria for MS were based on clinical features suggestive of CNS demyelination. However, there are other different criteria used for the diagnosis of MS as well. The oldest is the Schumacker committee criteria (1965) and the most recent is the modified McDonald criteria (2010) (Figure 4 below).  

| Schumacker criteria (1965) | 1. Clinical sign of problem in CNS  
| 2. Evidence of two or more areas of CNS involvement  
| 3. Evidence of white matter involvement  
| 4. One of these: Two or more relapses (each lasting ≥24 h and separated by at least 1 month) or progression (slow or stepwise)  
| 5. Patient should be between 19 and 50 yrs old at time of examination  
| 6. No better explanation for patient's symptoms and signs  
| Poser's criteria (1983) | 1. CDMS (clinically definite MS): two or more attacks (relapses), with clinical (neurological dysfunction demonstrable by neurological examination) or paraplastic evidence (demonstrable by any test) of the existence of a non-clinical lesion in the CNS. At least one event should be of clinical evidence.  
| 2. LSDMS (laboratory supported definite MS): At least one attack and oligoclonal bands, i.e., two attacks (occurrence of a symptom of neurological dysfunction for more than 24 h), and one evidence (clinical or paraplastic): i.e., one attack and two clinical evidences; iii. one attack, one clinical and one paraplastic evidence.  
| 3. CPMS (clinically probable MS): One attack, i.e., two attacks and one clinical evidence; ii, one attack and two clinical evidences; iii, one attack, one clinical and one paraplastic evidence.  
| 4. LSPPS (laboratory supported probable MS): Two attacks with no other evidence.  
| McDonald criteria (2001) | Clinical presentation  
* 2 or more attacks (relapses)  
* 2 or more objective clinical lesions  
* 1 objective clinical lesion  
* MRI  
* or a positive CSF and 2 or more MRI lesions consistent with MS  
* or further clinical attack involving different site  
* or second clinical attack  
* or positive CSF and 2 or more MRI lesions consistent with MS and  
* MRI  
* or second clinical attack  
Insidious neurological progression suggestive of MS (primary progressive MS)  
One year of disease progression (retrospectively or prospectively determined) and two of the following:  
a. Positive brain MRI (nine T2 lesions or more or two T2 lesions with positive VEP)  
b. Positive spinal cord MRI (two focal T2 lesions)  
c. Positive CSF  
Modified McDonald criteria (2010)  
* 2 or more attacks  
* 1 objective clinical lesion  
* MRI  
* or positive CSF and 2 or more MRI lesions consistent with MS  
* or further clinical attack involving different site  
* or second clinical attack  
* or positive CSF and 2 or more MRI lesions consistent with MS and  
* MRI  
* or second clinical attack  
Insidious neurological progression suggestive of MS (primary progressive MS)  
One year of disease progression (retrospectively or prospectively determined) and two or three of the following:  
1. Evidence of D2 in the brain based on 1 or more T2 lesions in the  
MS characteristic regions (periventricular, juxtacortical, or infratentorial)  
2. Evidence of D2 in the spinal cord based on 2 or more T2 lesions in the cord  
3. Positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index)  

Figure 4. Various diagnostic criteria for MS (Karussis, 2013)
The Expanded Disability Status Scale (EDSS) is a method for quantifying disability in MS patients (0.0: Normal neurological status and 10.0: Death due to MS). EDSS steps 1.0 to 4.5 refer to patients with MS who are fully ambulatory, while EDSS steps 5.0 to 9.5 are defined in the terms of ambulation impairment.  

The pathogenesis in MS is not clear. It is a multifactorial disease, where environmental, genetic and infectious factors play important roles in its pathogenesis.  

The inflammatory process is anticipated in MS pathogenesis, which is propagated by an autoimmune cascade. Four types of immunopathological mechanisms have been identified in MS lesions. Type I is T-cell mediated. In this way, demyelination is induced by macrophages either directly or by macrophage toxins. The most common pathology of MS lesions is type II, where demyelination is caused by specific antibodies and intense activity of the complement system. Type III is known as distal oligodendropathy, where degenerative changes occur in distal processes and they are followed by apoptosis. Type IV involves primary oligodendrocyte damage followed by secondary demyelination.  

The geographical situation (high risk in northern Europe, in northern US, Canada, in southern Australia and in New-Zealand), vitamin D deficiency, smoking habits and infections (Epstein-Barr virus) are the most important environmental risk factors in MS.  

There are many genetic polymorphisms that can influence the pathogenesis of MS. The cytokines and their receptor genes (IL-1β, IL-2, IL-10, TNF-α, -β), chemokines and their receptor genes (CCL-5, -3, -4), the cytotoxic T lymphocyte associated-4 (CTLA-4) gene, APOE, genes associated with apoptosis, MHC classes I and II genes (HLA-DRB1* 1501- DQA1* 0102-DQB1* 0602 (DR15)) and the T-cell receptor (TCR) gene polymorphisms are the most serious, and these things were described in genome-wide association studies (GWAS). Viral infections (such as the Epstein-Barr virus and human herpes virus 6) are suspected of being a trigger for MS.  

1.2 Alzheimer’s disease  

Alzheimer’s disease (AD) is a progressive neurodegenerative disease and it is the leading cause of cognitive and behavioural impairment in our society. AD is pathologically characterised by the observed accumulation and deposition of amyloid β (A β) peptides and the appearance of neuronal inclusion of abnormally phosphorylated tau in the brain, especially in the hippocampus and cerebral cortex. Gene mutations in early onset familial AD (eFAD) may cause the production of the longer Aβ42 variant that preferentially accumulates in plaques. In sporadic or late-onset AD (LOAD), this commences after the age of 65 years.
Many genetical and environmental factors may contribute to the pathogenesis of LOAD, and it affects about 95% of sufferers. Several genes are implicated as a risk factor, but it is at present not known how they alter amyloid levels. 48 Five percent of elderly persons over 65 years suffer from AD and this percentage for those over 85 years increases to 30%. By 2040, it is expected that 81 million people will suffer from AD. 49

AD is multifactorial and heterogeneous disorder (Iqbal & Iqbal), and recent studies indicate that some epigenetic or environmental effects are risk factors to AD. 50 It has also been postulated that bacterial or viral infection in the brain may play an initiating role in amyloid plaque formation and the development of AD. 51 Various animal studies have demonstrated that several pathogens like Chlamydia pneumoniae, Herpes simplex, Escherichia coli, and Cryptococcus neoformans play a significant role in the development of amyloid plaque formation, and bacterial or viral CNS pathogens may speed up the development of AD. 52 Amyloidogenesis and most of the changes seen in AD, such as inflammation, brain cell atrophy, immunological aberrations, altered gene expression and cognitive deficits are also known to be a result of microbial infection. 53

New scientific evidence appears to strengthen the view that the initiating event in AD is related to the abnormal processing of the beta-amyloid (Aβ) peptide, ultimately leading to the formation of Aβ plaques in the brain. 51 However, the cause of the amyloid - β (Aβ) imbalance is still an open question in the pathogenesis of AD.

1.3 Genetic polymorphisms

1.3.1 Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) are single base pair positions in genomic DNA. By definition, the allele frequency of SNP variants is less than 1%. SNPs could be bi-, tri-, or tetra-allelic polymorphisms. 54 The frequency of single base differences in two equivalent chromosomes is 1/1000 basis pair (bp). 55 56 57 The rate of nucleotide difference between two randomly chosen chromosomes is an index called nucleotide diversity. 58 Here, 1/1000 bp means that there is on average a 0.1% chance of any base being heterozygous in an individual. 54 SNPs may also occur on coding and non-coding regions of the genome. 54 In the coding regions of the genes, they play an important role of the known recessively or dominantly inherited monogenic disorders. SNPs in the regulatory regions of genes might influence the risk of common disease, such as the apolipoprotein E and the factor V Leiden mutations. 59 60 Most SNPs are located in non-coding regions and these have an unknown
effect on the phenotype of an individual. These kinds of SNPs are routinely applied in population and evolutionary studies. 61 62

1.3.2 Copy number polymorphisms

Copy number polymorphisms (CNPs) or copy number variations (CNVs) are defined as a DNA segment which is 1 kb or larger and they are present with a variable copy number relative to a reference genome. 63 CNPs may be simple in structure, such as tandem duplication, and they may contain complex benefits or losses of homologous sequences at multiple sites in the genome. 64 CNPs influence gene expression, phenotypic variation and adaptation. 65 66 CNPs may cause diseases such as microdeletion and microduplication disorders 67 68 and increase the risk of contracting complex diseases. 69

1.4 Associated polymorphisms with leukoaraiosis and MS

1.4.1 Leukoaraiosis and MTHFR A1298C variant

The methylene tetrahydrofolate reductase (MTHFR) enzyme is essential for DNA synthesis and methylation. Gene polymorphisms of the enzyme increase the risk for birth defects, different types of cancers and neurological diseases. 70 MTHFR is a regulatory enzyme in folate metabolism and catalyses the conversion of 5,10-methylenetetrahydrofolate to 5- methyltetrahydrofolate. 71 The MTHFR gene is located at 1p36.3. 72 The MTHFR gene has two SNPs (C677T and A1298C), which are associated with reduced enzyme activity and arise from an elevated plasma homocysteine level. Hyperhomocysteinemia is a key vascular risk factor as well. 71

The principal genetic factors of LA pathogenesis are the methylene tetrahydrofolate reductase (MTHFR) C677T variant, the angiotensin-converting enzyme (ACE) I/D and the apolipoprotein E (APOE) 2 or 4 polymorphisms. 15 16 The MTHFR C677T polymorphism in combination with the ACE D allele is associated with a higher risk for LA. 15 MTHFR A1298C has been postulated as a vascular risk factor, and it can increase the risk of predisposition to LA or modify the development of LA. 21

1.4.2 Leukoaraiosis and mitochondria

Chronic hypoperfusion seems to play a crucial role in the development of vascular demyelinisation. The neurons and the glia cells have an energy deficit due to the ischemia. The function of the mitochondria (MIT) may be damaged during the chronic hypoperfusion period by the intracellular electrochemical changes (changes in the normal polarisation of the
glia cells and neurons, reversions in the intracellular pH and cation concentrations). The damaged MIT is unable to carry out the well-organized production of energy for the cytosol. Despite this, mutations of the mitochondrial DNA or tRNA trigger a quality malfunction in the affected mitochondria. The absolute number of mitochondria is in all likelihood essential for the neuron and glia cells. In clinical studies, direct data cannot be readily obtained. However, the number of mitochondria in other tissues, which are functionally continually connected with brain tissue, may be informative. The white blood cells, for example, circulate and communicate with the brain tissue all the time. Their energetic storage may be important for the maintenance of the brain, since they act as a scavenger of the free radicals; and they also undermine the integrity of the neurovascular system of the brain. The white blood cells, vascular cells and brain tissue all most likely act in unison. However, there is almost no data regarding the segregation of the number of mitochondria among the different cell types. The absolute number of mitochondria in the white blood cells may provide an indication of the absolute number of mitochondria in the given individual.

With this in mind, we examined whether the number of mitochondria in the white blood cells are associated with the development of LA. Besides genetic heterogeneity of the mitochondrial DNA, there exist two big types of mitochondrial DNA; namely one with D-loop sequences and one without them. The former is called mitochondrial DNA (mDNA), while the latter is actually a mitochondrial DNA with a large and common deletion of 123-1256 bp (dmDNA). This deleted mitochondrial DNA appears to malfunction in some unknown way.

1.4.3 Multiple sclerosis and human beta defensins

Defensins are the largest family of antimicrobial peptides (AMPs). Defensins and defensin-like proteins are quasi universal participants in host defence against infection. Defensins are small cysteine-rich peptides that can be classified as either α-defensin or β-defensin, depending upon the arrangement of six critical cysteine residues. Defensins work actively against Gram-positive and –negative bacteria, viruses (adenovirus, herpes virus, influenza virus, cytomegalovirus, HIV and fungi. The most frequently studied antimicrobial peptides are the human β-defensins (hBDs). The β-defensins comprise the largest group, with around 40 members encoded in the human genome. The majority of studies to date have focused on HBD1 and HBD2. Human β-defensins are produced by epithelial cells of the skin, gut, respiratory and urogenital tissue. HBD1 is also expressed by leukocytes. HBD1 has been observed in human astrocytes and microglia cells. Furthermore, epithelial cells, the
expression of HBD2, has been detected in human monocytes, macrophages, and dendritic cells. Astrocytes may provide alternative ways of local HBD2 synthesis in the brain. The generally synthesized defensins as AMPs may protect the brain against bacterial or viral infections and defensins, and have immunomodulatory functions.

Defensin genes have been mapped to 8p22-p23. In this case, two types of genetic polymorphisms have been identified in genes encoding defensins, copy number polymorphisms (HBD2 encoded by the gene DEFB4) and single nucleotide polymorphisms (HBD1 encoded by the gene DEFB1). Three frequent SNPs at positions G-20A (rs11362), C-44G (rs 1800972) and G-52A (rs1799946) in the 5′-untranslated region (UTR) of DEFB1 have been described.

The β-defensins are found to be impaired in many inflammatory diseases, including Crohn's disease, psoriasis, pulmonary inflammation, and periodontal disease. β-defensins have been hypothesised to play a role in the etiology of neurodegeneration with a focus on traumatic brain injury, which is a risk factor for AD. The role of HBDs genes as potential modifiers in MS had not been hypothesised previously.

1.4.4 Alzheimer’s disease and human α- and β- defensins

New findings strengthen the hypothesis that the initiating event in Alzheimer's disease (AD) is related to the abnormal processing of the beta-amyloid (Aβ) peptide, ultimately leading to the formation of Aβ plaques in the brain. However, the precise cause of the amyloid - β (Aβ) imbalance is still an unresolved question in the pathogenesis of AD. Recently, a hypothesis was put forward that brain infections with bacteria or viruses may play an initiating role in amyloid plaque formation and the development of AD. This had led some scientists to take more seriously the potential contribution of pathogenic microbes to ageing and AD. In particular, most of the changes observed in AD, such as inflammation, brain cell atrophy, immunological aberrations, amyloidogenesis, altered gene expression and cognitive deficits are also viewed as a consequence of microbial infection. The inflammatory response to an infection may indirectly lead to an upregulation of amyloid production, thereby initiating the amyloid cascade. It is suspected that neuropathological alterations might be associated with abnormal expression and the regulatory function of antimicrobial peptides (AMPs), including defensins. It is worth mentioning that AMPs in general are able to cross the blood-brain barrier (BBB). Apart from their natural antimicrobial effects, AMPs also trigger immunostimulatory effects, including the upregulation of TNF-α, IL-8, and chemoattraction. An overproduction of defensins as AMPS might be a consequence of
previous infections, or even the alteration of the microbiome. This is why we hypothesise that, based on existing literature, an upregulation of the production of defensins as AMPs might trigger the aggregation of amyloid in the brain. Human β-defensins have been hypothesised to play a role in the aetiology of neurodegeneration with a focus on chronic inflammation-associated brain injury, which is a risk factor for AD. 

Human α-defensins include human neutrophil peptide 1-4 (HNP1-4) and intestinal human defensins (HD-5 and HD-6) produced by Paneth cells. Besides the antimicrobial effects, alpha defensins display chemotactic activity and induce proinflammatory cytokines. Neutrophil granulocytes are considered to be the main cellular origin of α-defensins here; and HNP 1-3 comprise 30%-50% of the granule proteins. HNPs may be released into the extracellular milieu following granulocyte activation as a consequence of degranulation, leakage, cell death, and lysis during inflammation.
2. AIMS OF THE STUDY

An investigation of various genetic polymorphisms in leukoaraiosis and multiple sclerosis, which have a multifactorial origins.

This is involved examining:

- The methylene tetrahydrofolate reductase (MTHFR) A1298C genetic variant in patients with leukoaraiosis;

- The absolute number of mitochondria in patients with leukoaraiosis;

- The role of human beta-defensins-1 and -2 (HBD1, HBD2) in MS: the relevance of the SNPs of the DEFB1 gene and the copy number polymorphism of the DEFB4 genes in multiple sclerosis.

- The role of defensin β-2 and α defensins (HNP 1-3) in Alzheimer’s disease.
3. MATERIALS AND METHODS

3.1 Patients and controls

Control subjects and patients gave their informed consent to their participation in our studies and the local ethics committees (Human Investigation Review Board of Pándy Kálmnán County Hospital in Gyula, Hungary, Human Investigation Review Board at the University of Szeged) approved it. All controls and patients were of Hungarian ethnic origin and resident in Hungary.

3.1.1 MTHFR A1298C variant and the absolute number of mitochondria in the patient group with leukoaraiosis and controls

Leukoaraiosis was defined by Fazekas et al. as irregular periventricular hyperintensities extending into the deep white matter in T2 weighted MRI scans (grade 3) and deep white matter hyperintense signals with initial indications of confluence of the foci or with large confluent areas in the T2 weighted MRI scans (grade 2-3).

The MTHFR A1298C study population comprised 198 LA patients with a mean age of 64.9 ± 9.23 years (105 women and 93 men). The patients were diagnosed and recruited from the Department of Neurology of the Pándy Kálmnán County Hospital in Gyula. In contrast, the controls consisted of 235 neuroimaging alteration free subjects (127 women and 108 men, age 54.7 ± 12.8 years). They were randomly selected from the Pándy Kálmnán County Hospital practice register subject to the proviso of having negative brain MRI scans. The clinical risk factors were registered in Table 2.

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>LA group (n=198)</th>
<th>Control group (n=235)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (females/males)</td>
<td>105/93</td>
<td>127/108</td>
</tr>
<tr>
<td>Age (years)</td>
<td>64.9±9.23</td>
<td>54.7±12.8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.3±2.3</td>
<td>24.6±1.98</td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>5.41±1.32</td>
<td>5.12±1.34</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.45±1.59</td>
<td>1.38±0.96</td>
</tr>
<tr>
<td>Hypertension</td>
<td>69.7 %</td>
<td>18.7 %</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>15.2 %</td>
<td>13.2 %</td>
</tr>
<tr>
<td>Smokers</td>
<td>15.2 %</td>
<td>11.9 %</td>
</tr>
</tbody>
</table>
Drinkers | 6.1 % | 4.3 %
Ishaemic heart disease | 10.1 % | 6%

Table 2. Clinical and laboratory data on patients and control subjects

The mitochondria study group consisted of 234 LA patients (123 women and 111 men, age 71.6 ± 10.8 years) and 123 MRI alteration – free controls (65 women and 58 men, age 59.4 ± 8.62 years). Once again, the patients and controls were recruited from the Pándy Kálmán County Hospital in Gyula, Hungary. The relevant clinical data is listed in Table 3 below.

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>LA group (n= 234)</th>
<th>Control group (n=123)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (females/males)</td>
<td>123/111</td>
<td>65/58</td>
</tr>
<tr>
<td>Age (years)</td>
<td>71.6±10.8</td>
<td>59.4±8.62</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6±2.33</td>
<td>24.4±3.42</td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>5.91±1.24</td>
<td>5.52±1.56</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.48±0.91</td>
<td>1.50±0.82</td>
</tr>
<tr>
<td>Hypertension</td>
<td>46.6 %</td>
<td>22.0 %</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>17.9 %</td>
<td>4.88 %</td>
</tr>
<tr>
<td>Smokers</td>
<td>29.9 %</td>
<td>28.5 %</td>
</tr>
<tr>
<td>Drinkers</td>
<td>12.0 %</td>
<td>9.0 %</td>
</tr>
</tbody>
</table>

Table 3. Characteristics of patients and control subjects

3.1.2 Human beta defensins in patients group with multiple sclerosis and controls

The study included 250 MS patients. The population in question contained 250 RR (relapsing remitting) and 53 SP (secondary progressive) persons with a mean age of 44.23 ± 13.02 years. Patients were diagnosed and registered at the Department of Neurology at the University of Szeged and Department of Neurology of the Pándy Kálmán County Hospital in Gyula. Here, the criterion was applied in the diagnosis of clinically definitive MS. The RR type was described as the presence of exacerbations followed by complete or partial remissions. The SP form was defined as the presence of a gradual onset of the disability lasting six months or more, with or without relapses, in subjects who - before the progressive
phase - had exhibited an RR course. The clinical classification was also supported by the MRI criteria. MRI protocols were established in accordance with the European Concerted Action Guidelines. The expanded disability status scale score (EDSS) for the study population ranged from 0 to 8 (mean, 3.37 ± 1.94) with a disorder duration of less than 1-20 years (mean, 8.83 ± 2.16 years). All patients gave their informed consent to participate in the study, and the local ethics committees – Human Investigation Review Board at the University of Szeged Albert Szent-Györgyi Medical and Pharmaceutical Center and the Human Investigation Review Board of Pándy Kálmán County Hospital in Gyula – gave prior approval to the study. The control patients comprised 232 age and gender-matched healthy blood donors, while the control group was selected from blood donors at the Regional Center of the Hungarian National Blood Transfusion Service in Szeged, Hungary. Statistical data concerning the MS patients and controls is given in Table 4 below.

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Relapsing-remitting MS (n= 197)</th>
<th>Secondary progressive MS (n=53)</th>
<th>Overall MS (n=250)</th>
<th>Controls (n=232)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (males/females)</td>
<td>49/1148</td>
<td>16/37</td>
<td>65/185</td>
<td>168/64</td>
</tr>
<tr>
<td>Age (years, means, SD)</td>
<td>45.7±14.5</td>
<td>56.75±12.68</td>
<td>44.23±13.02</td>
<td>46.8±11.98</td>
</tr>
<tr>
<td>Disease duration (years, means, SD)</td>
<td>10.36±10.02</td>
<td>7.3±4.7</td>
<td>8.83±2.16</td>
<td>Not relevant</td>
</tr>
<tr>
<td>EDDS score (means, SD)</td>
<td>2.36±1.48</td>
<td>5.5±1.39</td>
<td>3.37±1.94</td>
<td>Not relevant</td>
</tr>
</tbody>
</table>

Table 4. Statistics for the MS patients and controls

### 3.1.3 Human α- and β- defensin in Alzheimer’s group and controls

The study included 206 AD patients (69 men and 137 women, the average age and standard deviation (S.D.) was 76.42 ± 4.21 years, and the average onset was at 65 years of age. A clinical diagnosis of AD was established by an initial evaluation via careful history taking (personal and family histories), neurological and psychiatric examinations, along with the assessment of psychometric tests to confirm cognitive impairment. In addition, a brain CT scan or MRI was performed in each case, and in some cases SPECT was performed to rule out other possible neurological disorders. Routine laboratory processing involving the determination of thyroid hormone levels was also carried out. Each participants fulfilled
criteria outlined in the Fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV, 1994) and seemed to have AD based on the criteria of NINCDS–ADRDA. The cognitive evaluation of AD patients was performed using the AD Assessment Scale – Cognitive Subscale (ADAS-Cog), the Mini-Mental State Exam (MMSE) and the Clock Drawing Test (CDT). To exclude pseudodementia caused by depression, a patient’s mood was scored via the Beck Depression Inventory, which might be too restrictive, but only patients that had fewer than 12 BDI scores were included in the study. The control group comprised 250 age and sex matched healthy volunteers (92 men and 158 women, average age ± S.D. 72.69 ± 6.82 years) with normal cognition (MMSE score higher than 28) recruited from the Regional Center of the Hungarian National Blood Transfusion Service and the Department of Neurology at the University of Szeged (2010 January - December 2014).

All the cases and controls were of Hungarian ethnic origin. Informed consent was obtained from all the patients or responsible guardian in the case of incapacity and controls. When conducting our experiments with the patients, we followed the guidelines of the Patient Right’s Protection Act of our institutions and international laws. The local Ethics Committee of the Hungarian Investigation Review Board also gave prior approval of the study.

3.2 Genotyping procedures

3.2.1 DNA isolation

Genomic DNA was extracted from 200 µl of peripheral whole blood anticoagulated with EDTA. Here, two kinds of leukocyte DNA extraction were applied. One type of DNA isolation was the desalting method devised by Miller et al. The other method was DNA extraction with the High Pure PCR Template Preparation Kit (Roche Diagnostic GmbH, Mannheim, Germany, Cat.No: 1796828) and the manufacturer’s instructions were adhered to. The DNA concentration was measured with a Qubit fluorometer (Invitrogen, Carlsbad, USA). Afterwards, the genomic DNA was then stored at -20 °C until needed.

3.2.2 Determination of MTHFR A1298C variant in leukoaraiosis

The genetic variants of MTHFR A677T and A1298C were identified by using a LightCycler probe system. The amplification mix contained the following ingredients: 1 µl of sample DNA, 1 µl buffer (Light Cycler DNA Master Hybridization Probes 10X Buffer, Roche), 3 mmol/l MgCl2, 2% dimethyl-sulphoxide, primers and probes listed in Table 5, with
a total volume of 10 µl per single tube reaction. The assay conditions were 90 sec at 94 °C, and 40 cycles of 94 °C for 10 sec, and 50 °C for 10 sec and the elongation (72 °C for 10 sec). The next step was a melting point analysis in the F2 channel (640 nm) of the MTHFR A677T variant. The melting point of the A677T mutant allele was 67 °C and the wild type was 69 °C (Figure 5). The A1298C polymorphism was measured in the F3 channel at 705 nm. Here, the melting points of the wild type and the mutant allele were 54 °C and 58 °C, respectively (Figure 6).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Notation</th>
<th>Concentration (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MTHFR C677T mutation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTHFR-for</td>
<td>TGAGG AGA AGG TGT CXT GCg ggA</td>
<td>X=LC-Red640</td>
<td>0.1</td>
</tr>
<tr>
<td>MTHFR-rev</td>
<td>AGA Cg gTg Cgg TgA gAg Tg</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>MTHFR-probe</td>
<td>AgC AgC gTg ATg ATg AAA TCg gCT CC-Fluorescein</td>
<td>3′Fluorescein</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>MTHFR A1298C mutation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTHFR2-for</td>
<td>CTT Tgg ggA gCT gAA ggA CTA CTA C</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>MTHFR2-rev</td>
<td>CAC TTT gTg ACC ATT CCg gTT Tg</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>MTHFR2-probe</td>
<td>CTT TCT TCA CTg gTC AgC-Fluorescein</td>
<td>3′Fluorescein</td>
<td>0.1</td>
</tr>
<tr>
<td>MTHFR2-anchor</td>
<td>LCRd705-CCT CCC CCC ACA TCT TCA gC-Phosphate</td>
<td>5′LCRed705 3′Phosphate</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 5. Primers and probes of the MTHFR C677T and A1298C mutations
Figure 5. Examination of MTHFR C677T variants. The dotted line represents CC677, the continuous line represents C677T, and the dashed line represents 677TT.

Figure 6. Examination of MTHFR A1298C variants. The dotted line represents AA1298, the continuous line represents A1298C, and the dashed line represents 1298CC.

3.2.3 Determination of the absolute number of mitochondria per cell in leukocyte cells

The TaqMan real-time PCR assay, specially designed for the amplification of mitochondrial (m) and deleted mitochondrial (dm) DNA, was carried out using a specific set
of primers and probes (mDNA forward 5'-ATG GCC AAC CTC CTA CTC CTC ATT-3'; reverse 5'-TTA TGG CGT CAG CGA AGG GTT GTA-3' and a HEX-labeled probe 5'-[HEX]CGC AAT GGC AT T CCT AAT GCT TAC CG[TAM]; dmDNA forward 5'-ACA CAA ACT ACC ACC TTT GGC AGC-3'; reverse 5'-TTC GAG TGC TAT AGG CGC TTG TCA-3' and a FAM-labeled probe 5'-[6FAM]ACG AGG AAT ACC TTT CCT CAC AGG TTT CT[TAM]). Quantitative mt and dmt DNA amplification data were normalised to GAPDH [ROX-labelled GAPDH (Sigma-Aldrich®)] as an internal reference gene, which was coamplified simultaneously in a single-tube assay. The primers and probes were all obtained from Sigma-Aldrich®. Thermal cycling was performed on a Bio-Rad CFX96TM Real-Time PCR system. Here, amplification reactions (10 µl each) were performed in duplicate with 1 µl of template DNA. The amplification mix contained the following ingredients: 5 µl of iQTM Multiplex Powermix (Bio-Rad, USA), 0.4 µl of each primer, 0.1 µl of each fluorogenic probe, 1.3 µl of RNase and DNase-free water (Fermentas, Vilnius, Lithuania), and 1 µl of sample DNA, in a total volume of 10 µl per single tube reaction. The assay conditions were 3 min at 95 °C, and 44 cycles of 95 °C for 10 sec and 60 °C for 45 sec. The quantification was evaluated using the comparative CT (Threshold Cycle) method. 103

3.2.4 Determination of the DEFB1 SNPs in multiple sclerosis

Human β-defensin 1 (DEFB1) – Genotyping was carried out using Custom TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). Fluorogenic minor groove binder probes were utilised for each case using the dyes-6-carboxy-fluorescein FAM (excitation 494 nm) and VIC (excitation 538 nm): DEFB1 polymorphisms: c.-20G > A (rs11362) Applied Biosystems code c_11636793_20, DEFB1 c.-44C > G (rs1800972) c_11636794_10, and DEFB1 c.—52G>A (rs1799946) c_11636795_20. Thermal cycling was performed on a Bio Rad CFX96™ Real-Time PCR system. The amplification mix contained the following ingredients: 6 µl of Maxima® Probe/ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania), 0.3 µl of primer-probe mix, 5.1 µl of RNase and DNase-free water (Fermentas, Vilnius, Lithuania), and 1 µl of sample DNA, in a total volume of 11.4 µl per single tube reaction. The assay conditions were 10 min at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each 96-well plate contained 93 samples of an unknown genotype and three reactions with reagents, but no DNA. DNase-free water was used as the non-template control. The initial and post assay analyses were performed using the Bio Rad CFX96™ Real-Time system Precision Melt Analysis™ software package.
3.2.5 Determination of the DEFB4 gene copy number in multiple sclerosis

The determination of the DEFB4 Gene Copy Number – A TaqMan real-time PCR assay, specifically employed for the amplification of genomic DEFB4, was performed using a specific set of amplification primers (forward 5’-TGAAGCTCCCAGCCATCAG-3’; reverse 5’-TATTTCCCTGCCCATCTCA-3’ and a VIC-labelled probe 5’- VIC-ATCTCCTCTTTCTCGTCC-MGB). Quantitative DEFB4 amplification values were normalised to ABL [FAM-labelled albumin (Applied Biosystems, Cat. No. 4331182)] as an internal reference gene, which was added to each single tube. The primers and probe of DEFB4 were designed using Primer Express 3.0 (Applied Biosystems). The probes were purchased from Applied Biosystems, while the primers were obtained from Invitrogen (Carlsbad, CA, USA). Real-time PCR was carried out using the Bio Rad CFX96TM Real-Time PCR system. The amplifications were performed in triplicate with the Maxima® Probe /ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania), 20 ng of template DNA, 300 nM per primer and 200 nM of each fluorogenic probe. The steps of thermal cycling were 10 min at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The quantification was evaluated using the comparative CT (Threshold Cycle) method. 103

3.2.6 The determination of the DEFB4 gene copy number in AD patients

A TaqMan real-time PCR assay, specially designed for the amplification of genomic DEFB4, was performed as described previously. 104 Put briefly, quantitative DEFB4 amplification data was normalised to ABL [FAM-labelled albumin (Applied Biosystems, Cat. No. 4331182)] as a standard reference gene that is actually present only in 2 copies in the genome. The reference gene was used as an internal standard 105 and added to every single tube. Once again, the quantification was evaluated by applying the comparative CT (Threshold Cycle) method. 103

3.3 ELISA procedures

3.3.1 Plasma levels of HBD2 with Capture ELISA in multiple sclerosis

ELISA of hBD2 (Alpha Diagnostic San Antonio, TX, USA) was used to test for the occurrence of the human β-defensin 2 peptide in the plasma of controls and patients, based on the manufacturer’s recommendations. Here, the detection limit of the hBD2 ELISA Kit was 0.8 pg hBD2 protein/ml.
3.3.2 Human cerebrospinal fluid (CF) collection for defensin ELISA in AD

The CF samples used in this study were derived from patients undergoing a lumbar puncture in the L4–L5 vertebral interspace. All the procedures were conducted in the morning and all the samples (12 mL) of CF taken from each patient and control subjects were collected into polypropylene tubes. These samples were then transferred to the laboratory at -20 °C within 1-2 hours, and each CSF sample was divided into aliquot and frozen to minimise any metabolic damage. A routine laboratory investigation was performed and the remainder of the samples were stored at -80 °C until needed.

Ethical permission for lumbar puncture was also obtained from the Ethics Committee of the University of Szeged, Hungary, where written informed consent was required for all probands (permit no. 184/2012).

3.3.3 ELISA of human β-defensin 2 (hBD2) in AD

ELISA of hBD2 (Alpha Diagnostic San Antonio, TX, USA) was used to test for the occurrence of the human β-defensin 2 peptide in the sera and in the CF of controls and patients, based on the manufacturer’s recommendations. Here, the detection limit of the hBD2 ELISA Kit was 0.8 pg hBD2 protein/ml.

3.3.4 An assay of the HNP 1-3 concentrations in AD

The HNP1-3 concentrations in sera and in the CF were determined by ELISA (Hycult-Biotech HK324, Uden, The Netherlands) based on the recommendations of the manufacturer.

3.4 Statistical analysis

3.4.1 A statistical analysis of the MTHFR A1298C variant in leuкоaraiosis

The clinical data was expressed as means ± SD, where appropriate. The differences between the clinical parameters in the stroke group and the controls were assessed via the χ2-test or the Mann–Whitney test, where appropriate. As a first step, we analysed whether the MTHFR A1298C variant was a risk factor for LA. Since MTHFR C677T was not found to be an independent risk factor for LA in our earlier study, an analysis of the distribution of the C677T variant was not our primary goal. However, as a second step, we investigated whether the presence of the C677T variant had a modifying effect if it occurred in combination with the MTHFR A1298C variant. The frequencies of the genetic variants were then compared with the χ2-test. After performing a univariate statistical analysis, we carried out logistic regression calculations in order to discover differences between the LA subjects and the
controls. Logistic regression analyses were performed using the statistical package SYSTAT 10 (Chicago, USA) for Windows.

3.4.2 A statistical analysis of absolute mitochondrial number in laukoaraiosis

The number of mDNA and dmDNA per white blood cell in the LA group were compared with those in the control group using the two-paired T test. In order to make the comparison more sensitive biologically, the mathematical difference between the number of mDNA and dmDNA per cell was divided by the sum of the number of mDNA and dmDNA per cell in the same individual. This calculated ratio gave the weight of uncompensated dmDNA per cell, which was called the K ratio. In the equilibrium state, \( K = \text{number of mDNA} - \text{number of dmDNA} / \text{the number of mDNA + number of dmDNA} \). The K values were compared statistically between the LA and control groups by means of the two-paired T-test. An univariate statistical analysis was followed by a logistic regression comparison involving the age, hypertension, diabetes mellitus (for the LA and control groups) and K values.

3.4.3 A statistical analysis of human beta defensins in multiple sclerosis

The significance of the genotype frequency was analysed via the chi-square test and the Fischer test. Here, the level of significance was \( p < 0.05 \). The genotype frequencies of SNPs were tested for deviation from the Hardy-Weinberg equilibrium via the chi-square test, with one degree of freedom. Plasma levels of human \( \beta \)-defensin 2 were expressed as medians with the Mann-Whitney test. The group means were then discriminated via the ANOVA test and then Bonferroni’s multiple comparison tests was applied. The GraphPad prism 5 statistical program was utilised in all the statistical calculations (GraphPad Software Inc. San Diego, CA, USA).

3.4.5 A statistical analysis of \( \alpha \)- and \( \beta \)-defensins in Alzheimer’s disease

The significance of the genotype frequency was analysed via the Mann-Whitney test. Here, the level of significance was \( p < 0.05 \). The levels of human \( \beta \)-defensin 2 and HNP 1-3 in the sera and in the cerebrospinal fluids (CF) were expressed as mean ± SEM, and the significance was determined using the Student unpaired t-test. The GraphPad Prism 5 statistical program was again used to perform all the statistical calculations (GraphPad Software Inc. San Diego, CA, USA).
4. RESULTS

4.1 MTHFR A1298C variant in leukoaraiosis

The presence of the 1298C variant (heterozygous A1298C or homozygous 1298CC) was found to be an independent risk factor for LA (54.1% vs 43.8%; adjusted OR, 1.6; 95% confidence interval, 1.2–2.5; p <0.034). The presence of the heterozygous T677C or A1298C variants did not increase the risk of contracting LA if they occurred by themselves. However, the clustering of both heterogeneous variants in the same person created a slight risk of LA (4% vs 0.4%; adjusted OR, 79.5; 95% confidence interval, 1.2–98.5; p <0.008; Table 6). Data concerning the combinations of the two variants are given in tables 7 and 8 below.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>LA (n= 198)</th>
<th>Controls (n=235)</th>
<th>P</th>
<th>Crude OD, 95 % confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR A1298C + C677T</td>
<td>8 (4%)</td>
<td>1 (0.4%)</td>
<td>&lt;0.008</td>
<td>79.5 (1.22-98.5)</td>
</tr>
</tbody>
</table>

Table 6. Synergistic interaction between the heterozygous MTHFR A1298C and C677T genotypes

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>MTHFR 1298AA</th>
<th>MTHFR A1298C</th>
<th>MTHFR 1298CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR CC677</td>
<td>36</td>
<td>43</td>
<td>15</td>
</tr>
<tr>
<td>MTHFR C677T</td>
<td>71</td>
<td>41</td>
<td>1</td>
</tr>
<tr>
<td>MTHFR 677TT</td>
<td>25</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 7. Distribution of the different genotypes among the control subjects

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>MTHFR 1298AA</th>
<th>MTHFR A1298C</th>
<th>MTHFR 1298CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR CC677</td>
<td>23</td>
<td>32</td>
<td>15</td>
</tr>
<tr>
<td>MTHFR C677T</td>
<td>46</td>
<td>49</td>
<td>3</td>
</tr>
<tr>
<td>MTHFR 677TT</td>
<td>22</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 8. Distribution of the different genotypes among the LA patients
4.2 Absolute number of the mithoconria per cell in leukocyte cells in leukoaraiosis

The frequency of hypertension in the LA group was higher than that in the controls. Interestingly, the mDNA content and dmDNA content did not differ significantly between the LA and control groups. After performing a logistic regression analysis, we found that the calculated value of K for the LA group was significantly lower than that for the controls. These logistic regression analyses were carried out in order to learn the differences between the LA and controls with regard to the age, hypertension and diabetes mellitus. In this way, we were able to minimise the confounding effects of these clinical factors, and their association with the reduced number of mitochondria (LA: K 0.37, 95% CI 0.05; controls: K 0.48, 95% CI 0.076; p<0.001).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>LA group (n=234)</th>
<th>Controls (n=123)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of non-deleted MIT per cell</td>
<td>5.5±0.45</td>
<td>5.4±0.5</td>
</tr>
<tr>
<td>(means±2SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of deleted MIT per cell</td>
<td>3.45±0.5</td>
<td>3.5±0.5</td>
</tr>
<tr>
<td>(means±2SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*K</td>
<td>0.37±0.03</td>
<td>0.48±0.003**</td>
</tr>
</tbody>
</table>

* Non-deleted mitochondrial DNA content per cell minus deleted mitochondrial DNA content per cell divided by the sum of the contents of deleted and non-deleted mitochondrial DNA per cell [K = (mDNA — dmDNA) / (mDNA + dmDNA)]

** p<0.001: the leukoaraiosis group was compared with the control group by means of the two-paired T-test.

Table 9. Distributions of the number of deleted and non-deleted mitochondrial DNA per cell in the leukoaraiosis and control groups

4.3 Human β–defensin 1 genotyping in MS

*DEFB1 c.-20G<A, DEFB1 c.-44C>G, and DEFB1 c.-52G>A polymorphisms*

The total numbers of MS patients in these genotyping experiments was 250 and the control group contained 200 healthy members. The prevalence of the three genotypes of DEFB1 and the controls subjects were found to be in accordance with the Hardy-Weinberg equilibrium (DEFB1 c.-20G>A p= 0.677 and in the control group p= 0.912; DEFB1 c.-
44C>G p= 0.999 and in the controls p= 0.723; DEFB1 c.-52G>A p= 0.657 and in the control group p= 0.253, respectively).

The genotypic distributions of DEFB1 c.-20G<A, DEFB1 c.-44C>G, and DEFB1 c.-52G>A polymorphisms are listed in tables 10, 11 and 12 below.

<table>
<thead>
<tr>
<th></th>
<th>GG (%)</th>
<th>GA (%)</th>
<th>AA (%)</th>
<th>χ² test*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with MS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=250)</td>
<td>78 (31)</td>
<td>131 (53)</td>
<td>41 (16)</td>
<td>0.4106</td>
</tr>
<tr>
<td>Controls (n=200)</td>
<td>62 (31)</td>
<td>96 (48)</td>
<td>42 (21)</td>
<td></td>
</tr>
</tbody>
</table>

*chi square test vs. controls

Table 10. Distribution of DEFB1 c.-20G>A genotypes in MS patients

<table>
<thead>
<tr>
<th></th>
<th>CC (%)</th>
<th>CG (%)</th>
<th>GG (%)</th>
<th>χ² test*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with MS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=250)</td>
<td>160 (64++)</td>
<td>80 (32)</td>
<td>10 (4)+</td>
<td>0.0002</td>
</tr>
<tr>
<td>Controls (n=200)</td>
<td>90 (45)</td>
<td>92 (46)</td>
<td>18 (9)</td>
<td></td>
</tr>
</tbody>
</table>

* chi square test vs. controls
+ Fischer test: p= 0.0317; OR= 2.384; CI= 1.074-5.288
++ Fischer test: p<0.0001; OR= 0.465; CI= 0.318-0.680

Table 11. Distribution of DEFB1 c.-44C>G genotypes in MS patients

<table>
<thead>
<tr>
<th></th>
<th>GG (%)</th>
<th>GA (%)</th>
<th>AA (%)</th>
<th>χ² test*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with MS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=250)</td>
<td>100 (40)</td>
<td>111 (44)</td>
<td>39 (16)</td>
<td>0.766</td>
</tr>
<tr>
<td>Controls (n=200)</td>
<td>80 (40)</td>
<td>84 (42)</td>
<td>36 (18)</td>
<td></td>
</tr>
</tbody>
</table>

*chi square test vs. controls

Table 12. Distribution of DEFB1 c.-52G>A genotypes in MS patients

In the genotype distribution no significant difference was found between the DEFB1 c.-20G>A and the controls (P= 0.4106). Similarly, in the genotypes no significant differences were found between the DEFB1 c.-52G>A and the healthy subjects (P= 0.766). In the case of the DEFB1 c.-44C>G polymorphism, in the genotype distribution there was significant difference between the patient group and the control group (p= 0.0002; Table 11 and Figure 7).
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Figure 7. Distribution of DEFB1 c.-44C>G genotypes in MS patients and controls. Using the Fischer test, both the frequency of GG (*P= 0.0317; OR= 2.384; CI= 1.074-5.288) and the CC genotypes (**p<0.0001; OR= 0.465; CI= 0.318-0.680) vs. controls were found to be significantly different.

4.4 Determination of the DEFB4 gene copy number in MS

The measurement of CN was performed in 250 patients in the MS group and in 232 healthy controls. Here, the range of copy numbers was found to be between 2 and 8 per genome in the control group. Of these, four copies were the median number of the healthy controls. The proportions of control individuals who carried the median (4), more than median (>4), or less than the median (<4) number of copies were 39%, 33% and 28%, respectively. For the patients with multiple sclerosis, the frequency distribution of the subgroups was different from that of the control group (p= 0.002, Figure 2). Among the patients with MS, 43% had a lower (<4) copy number compared to that for the control subjects. The frequency of a CN >4 was only 27% among the patents with MS and 33% among the controls. As we can seen when the copy numbers were higher than 4 there was no statistical difference between MS patients and control subjects. However, there was a lower frequency among patients (27 %) than in the control subjects (33%) (see Figure 8).
Figure 8. Distribution of DEFB4 gene CN in MS patients and controls. Applying the Fischer test, the frequency of CN <4 between the MS group and the control subjects was found to be significantly different (*p= 0.0005; OR= 0.507; CI= 0.3464 to 0.7413).

4.5 Plasma levels of hBD2

Samples of patients with MS and controls with known CNs of DEFB4 were used to perform ELISA for hBD2 in order to determine whether the plasma levels of hBD2 varied relative to the CN variation of the DEFB4 gene. The levels of human β-defensin 2 in MS patients (n= 80) were significantly lower overall than those in the control subjects (n=50). Here, the median levels of hBD2 in the patients were 150.6 ± 12.71 pg/ml vs 262.1 ± 23.82 pg/ml in the control group, with p<0.0001; Figure 9). The circulating level of human β-defensin 2 has a close correlation with the CN in the control group. And, similar to the controls, the plasma levels were found to be significantly lower in the CN<4 group (135.3 ± 25.49 pg/ml), compared to patients with copy numbers of 4 (173.7 ± 44.09 pg/ml). The defensin levels, however, were not elevated in patients with CN>4 (148.9 ± 31.21 pg/ml) (see Figure 8).
Figure 9. The median levels of hBD2 in the patients (**150.6 ± 12.71 pg/ml) and in the controls (262.1 ± 23.82 pg/ml). The horizontal lines represent medians. Here, p < 0.0001 based on the Mann-Whitney test.

Figure 10. Plasma levels of hBD2 in MS and in control group with DEFB4 copy numbers of <4, 4, >4. The values are means and SDs of the results for 50 samples in the control group and 80 samples in MS patients. The differences among group means were determined using the ANOVA test (difference between patients and controls with p < 0.0001) followed by
Bonferroni’s multiple comparison test ** CN<4 MS patients vs controls with p < 0.001, ** CN=4 MS patients vs controls p < 0.001, *** CN>4 MS group vs control patients p < 0.001.

4.6 Copy number (CN) polymorphism of DEFB4 in AD

The measurement of CN was performed in 206 patients in the AD group and in 250 subjects in the control group. Here, the median CN in the controls was 4, the 75% percentile was 5, and the 25% percentile was 3.4. In the AD patients the median was 5, with a 75% percentile of 6, and with a 25% percentile of 4, with p<0.001 using the Mann-Whitney test (see Figure 11).

![Figure 11](image_url)

Figure 11. CN polymorphism of DEFB4 in Alzheimer’s disease

4.7 Serum levels of hBD2 in AD

In a pilot study, the levels of human β-defensin 2 in the sera of 52 patients with AD and in 45 subjects in the control group were measured. Significantly higher levels of hBD2 were found in AD patients than those in the control group (see Figure 12). The median levels were 265.5 pg/ml in AD vs. 169.4 pg/ml in controls, respectively (p < 0.01).
4.8 Levels of hBD2 in cerebrospinal fluids (CF) in AD

Though relatively lower concentrations of human β-defensin 2 were detected in the CF of the study group than in their serum levels, the difference between the AD and control group was significant. Once again, patients with AD (n= 43) exhibited higher hBD2 concentrations in their CF as than those in the control subjects (n = 40); 8.6 pg/ml vs 1.201 pg/ml in the control group (p < 0.001; see Figure 13).
4.9 Serum levels of HNP1-3 (α-defensin) in AD

Serum concentrations of α-defensin (HNP1-3) in 43 patients with AD were measured and compared with those of 40 subjects in the control group. Although, there was a high individual variation in the serum levels of α-defensin, significant differences were actually observed between the healthy subjects and AD patients. The median serum concentrations of AD patients were about 147.3 ng/ml, which were significantly higher than those in controls, namely 122.3 ng/ml (p < 0.05; see Figure 14).

![Figure 14. Serum levels of HNP1-3 in Alzheimer’s disease](image)

4.10 Levels of HNP1-3 in cerebrospinal fluid in AD

The concentrations of HNP 1-3 in the CF of 52 AD patients were measured and compared with 40 control CF samples. As can be seen, higher concentrations of HNP 1-3 were measured in the CF of AD patients (median concentration 85.16 ng/ml) than in the CF of those in the control group (1.23 ng/ml, p<0.001; see Figure 15).
Figure 15. CF levels of HNP1-3 in patients with Alzheimer’s disease
5. DISCUSSION

5.1 The MTHFR A1298C variant in leucoaraiosis

Previous studies demonstrated that an elevated serum homocysteine level may be associated with LA. Also, an elevated serum homocysteine level is presumed to be associated with an endothelial dysfunction or microangiopathy. The genetic studies relating to the MTHFR C677T variant suggest that, although the 677T variant is unfavourable because it raises the serum homocysteine level, it does not increase the risk of contracting LA if it is present on its own. We also demonstrated earlier that the 677T variant in combination with other genetic variants increases the risk of contracting LA.

The present study reveals that a person carrying either a heterozygous A1298C or a homozygous 1298CC variant is at a higher risk of contracting LA than one carrying neither of them. This genetic variant has not been examined so far in any LA case–control study. Our findings therefore suggest for the first time that the A1298C variant may be more important than the C677T variant in the evolution of LA. We also found that the heterozygous C677T and A1298C variants do not pose a risk of contracting LA if they are present by themselves. However, their combination in the same person leads to a marked risk of contracting LA. Here, although the number of patients displaying the combination of the two heterozygous MTHFR variants was low, the significance level relating to the approximately tenfold increase in the unfavourable combination in the LA group compared with that in the control group suggests that there is a definite link. At present the exact cause of this synergistic effect is not known. However, two possible explanations readily emerge. They are: (1) the two variants can potentially increase the serum homocysteine level in an additive manner and (2) the co-occurrence of the two unfavourable MTHFR genetic variants may influence the regulation of the enzyme. A properly balanced regulation of the MTHFR may be a key factor that can define the daily shifts in the serum homocysteine level. The clustering of the A1298C and C677T variants might give rise to an unfavourable regulatory nature in the dynamically changing activity of the MTHFR. Then the presence of the two heterozygous variants might result in a significantly unfavourable phenotype of the conformation of the enzyme protein.

5.2 The absolute number of mitochondria per cell in leukocyte cells in leucoaraiosis

The basic contents for mDNA and dmDNA were found to be statistically the same in the LA group and control group, and the K value was significantly lower for the LA group than that for the control group. This suggested that there was a larger proportion of dmDNA
present. Having dmDNA may possibly lead to a mitochondrial malfunction in the following way: a, lower energy production; b, a lower free radical scavenging capacity; c, a lower rate of adaptation to the prevailing demand for energy production; d, a narrower range in the adjustment to the prevailing energy demand; e, a lower metabolic function capacity in general; f, a greater extent of free radical production; and g, a general malfunction of the mitochondrial genetic regulation.

No genetic or biochemical data is available to suggest which of these postulated mechanisms actually exist, but a lower and narrower energy capacity appears probable as the main pathomechanism behind LA. It was demonstrated earlier at the molecular level that LA can arise from a very slight, but chronic level of hypoxia, which may be caused by various environmental and genetic susceptibility factors. 106 Our present findings agree with the earlier ones that uncoupling protein genetic variants play a role in the development of LA. 107 The uncoupling proteins govern the electro-chemical gradient between the inner and outer spaces of the mitochondria 107, this gradient being essential for the energy production of the mitochondria.

If dmDNA is associated with any kind of biochemical malfunction, an uncompensated and larger proportion of dmDNA in the cells may be unfavourable from an energetic viewpoint. Our results appear to indicate that the lower the difference between the contents of mDNA (which compensates for malfunctions of the dmDNA) and dmDNA, the larger the risk of contracting LA in a given individual. Overall, the results of our study suggest that the ratio of the dmDNA content and mDNA content may play a significant role in the pathogenesis of LA. These results also point to the need for new approaches for the examination of mitochondrial contents in other common brain disorders.

Limitations of the study
1. The numbers of mitochondria in the affected brain tissues could not be examined, as this study was a clinical one in a human patient population. Brain biopsies would not have been ethical; hence we were unable to identify associations between the numbers of mitochondria in different human tissues.
2. Although we found no apparent change in the number of mitochondria in a small cohort of study subjects over several weeks (which involved several turnovers of the mitochondria in the white blood cells), insufficient scientific data is available concerning the stability of the
absolute numbers of mitochondria in the different tissues. This should be clarified in future studies.

3. In this study, no investigations were carried out to identify the properties of the normal functioning of the mitochondria with deletion DNAs. However, these limitations do not greatly affect the present results, since they are not directly associated with the findings. Moreover, they really should be viewed as open scientific questions, that should be addressed in future investigations.

4. Although the logistic regression statistical method has greatly decreased the confounding effects of the clinical factors such as age, hypertension and diabetes mellitus, the results need to be confirmed using a larger population group.

5.3 Genetic polymorphisms of human β-defensins in patients with multiple sclerosis

In our present study, an association between human β-defensins and multiple sclerosis was found. By investigating three SNPs of DEFB1, the distributions of the C-44G genotypes were found to be different between patients with MS and those in the healthy control group, while the frequency of the GG genotype was significantly higher in the control population. This suggests that the presence of the G allele most likely leads to strengthened HBD1 antimicrobial activity, which is less frequent among patients with MS. The G allele of C-44G SNP generates a putative binding site for nuclear factor κB (NF-κB) and in all likelihood induces overexpression. The proposed effect of this SNP could partly explain why the GG genotype was considered to be a protective genotype in atopic dermatitis and also a contributory factor in the susceptibility to Candida colonisation in diabetic patients. By contrast, in these studies, subjects carrying the CC genotype at the -44 locus site of the gene were at a greater risk of becoming infected. It was recently suggested that the C allele of DEFB1 C-44G SNP probably abrogates NF-κB-dependent DEFB1 up regulation.

The above findings are consistent with our present observation that the GG phenotype might also play a protective role in MS, and vice versa, and the higher frequency of the CC genotype might be connected with a lower expression of human defensin β-1. Among the 250 patients with MS, only 9 (4%) had GG homoyzgote and 62% of the patients had CC homozygote, in contrast with 45% of the control group that had CC homozygote. These observations appear to emphasise the importance of DEFB1 polymorphisms in MS. Similarly, the production of the inducible hBD-2 is lower in MS patients. It is suggested that the significantly lower frequency of the copy number of DEFB2 might be one of the reasons for the decreased levels of circulating hBD2 in the blood samples of patients with MS.
When the association between the copy numbers and the plasma levels of hBD2 was investigated, a correlation between the ELISA results and copy number genotypes was found in the control group, but not in the groups of patients with MS. Moreover, the low hBD2 levels correlated nicely with the low frequency of copy numbers (i.e. <4 copy) in the control group, but not in the groups of patients with MS. The low hBD2 levels correlated nicely with the low frequency of copy numbers (i.e. <4 copy), but in the patients with copy numbers >4, the plasma levels of hBD2 did not seem to be elevated. We suppose that other factors not yet defined might be responsible for the low levels of hBD2 even in the case of higher copy numbers. We hypothesise that abnormalities in the production and the function of human defensin-β might be connected with an altered microbiome in MS, as suggested in a recent study. 88

While it is unclear whether enteric microbiota affects human MS, a higher proportion of MS patients exhibited antibody responses against gastrointestinal antigens that those in with healthy control subjects. This might indicate an altered gut microbiome and immune status. 113

In addition, as β defensins can be produced not only by epithelial cells, but also by astrocytes, and microglia cells 74 79, their importance in the central nervous system (CNS) needs to be taken into account. Human defensin-β might function as an initial line of defence within the CNS either as an antimicrobial, or an immunomodulator, or both. 88 What is more, these defensins may also be neuroprotective through their ability to inhibit cellular apoptosis in the CNS. 88

The extreme low frequency of the GG genotype of the C-44G SNP of DEFB1, the high frequency of the low copy number (<4) of DEFB2, and the significantly decreased plasma levels of hBD2 in patients highlight the importance of human defensin-β levels in MS patients.

Further studies are necessary to elucidate the precise way the impaired function of human defensin-β influences the pathomechanism of multiple sclerosis.

5.4 Association between human defensin β-2 and AD, and between human defensin-α (HNP 1-3) and AD

Higher concentrations of the inducible hBD-2 were found in the cerebrospinal fluid and in the sera of AD patients. It is suggested that the significantly higher frequency of the copy number of DEFB4, encoding hBD2, might be one of the reasons for the increased levels of circulating hBD2 in the blood samples and in the CF of patients with MS.
As β defensins can be produced not only by epithelial cells, but also by astrocytes, and microglia cells their importance in the CNS should be taken into consideration in future studies. Human defensin-β might function as an initial line of defence within the CNS either as an antimicrobial, or an immunomodulator, or both.

In addition, the contribution of the microbiota to AD pathogenesis was recently investigated and it supports the hypothesis of a microbiome-brain axis. Here, microbiome means the collective genomes of total microbiota. Recent studies have also begun to clarify the degree of involvement of microbiome in AD pathogenesis. It was found that the composition of the human microbiome and exposure to pathogens varies with age, diet, lifestyle and biological environment. Studies indicate that incidence of AD and microbiome exposure and complexity vary greatly in different human populations. Here, the participation of defensins in AD should not be neglected.

An elevated hBD expression in the CF suggests that brain synthesises antimicrobial peptides and they circulate throughout the ventricular system and protect the CNS against microbial infection. Choroid plexus regulates immune functions between peripheral and brain circulation, and it is well documented in AD neuropathology (epithelial cell atrophy, impaired secretory and transporter functions, reduced amyloid β clearance). The dysfunction of the choroid plexus may give rise to neuropathological and inflammatory processes. In vitro results suggest that astrocytes may be responsible for local hBD-2 synthesis in the brain. Astrocytes and microglia are important in the cerebral neuroinflammatory response, and in vitro they express hBD-1 and -2; and they may modulate adaptive immunity. Williams et al. found a significant elevation of the hBD-1 mRNA level in the choroid plexus, and increased protein level in hippocampal neurons of an AD brain. The increased hBD-1 expression within an AD brain may be a protective response to inflammatory stimuli and potential modulator of the host’s innate immune response within the CNS.

It has been suggested that chronic infections might be initial events in AD pathogenesis, which can lead to persistent inflammatory stimuli. The inflammatory response thereafter may indirectly lead to the upregulation of amyloid β production. It may well be that the induction of defensins is also involved in the amyloid development. Many antimicrobial peptides exhibit structural characteristics including β-sheet conformation similar to amyloid β that contribute to olygomeration. It should also be mentioned here that the existence of the oligomerization of monomeric hBD-2 has now been demonstrated.
Not only were the inducible hBD-2 elevated in the cerebrospinal fluid and in the sera of AD patients, but the levels of HNP1-3 were also higher both in the sera and in the CF. Our findings are in accordance with recent data by Watt et al. in 2015, who reported that peripheral α-defensins are elevated in Alzheimer’s disease. However, no measurements of the defensins in the CF were included in their study. While copy number polymorphism of the DEFB4 gene has been reported to influence the production of hBD2, the secretion of HNP 1-3 however seems to be independent of the copy number of the DEFA gene. Hence we did not investigate it here.

The present study supports the view of the potential role for antimicrobial peptides like human α and β defensins in AD pathology; they are pathogen targeting agents in brain infections involving AD. Whether the elevated levels of defensins are a consequence of inflammation, or they themselves induce neurodegeneration and amyloid formation is currently unknown. Further investigations are therefore necessary to elucidate the regulatory functions of defensins in the pathomechanism of AD.
SUMMARY AND NEW RESULTS

- Our results revealed an association between the A1298C polymorphism of the MTHFR gene and leukoaraiosis. A person carrying either a heterozygous A1298C or a homozygous 1298CC variant is at a higher risk of contracting LA than one carrying neither of them.

- Our study suggests that the ratio of the dmDNA content and mDNA content may be of great significance in the pathogenesis of LA. The findings here suggest the need for a new perspective when we investigate the mitochondrial contents in other common brain disorders.

- The extremely low frequency of the GG genotype of the C-44G SNP of DEFB1, the high frequency of the low copy number (<4) of DEFB2, and the significantly decreased plasma levels of hBD2 in patients all highlight the importance of the role of human defensin-β in MS.

- Human α and β defensins in AD pathology, as pathogen targeting agents, play a role in brain infections related to AD. Whether the elevated levels of defensins are a consequence of inflammation, or they themselves induce neurodegeneration and amyloid formation, is currently not known.
ACKNOWLEDGEMENTS

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Lastly, I wish to thank my family and close friends for all the love, patience and encouragement they have given me in the course of my academic studies in Szeged.
REFERENCES


APPENDIX
Evaluation of the MTHFR A1298C Variant in Leukoaraiosis

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Abstract Vascular demyelinization of the white matter of the brain is referred to as leukoaraiosis (LA). This very frequent entity is associated with a cognitive decline, thereby resulting in a deteriorating quality of life. Besides poorly controlled hypertension and aging, its development is reported to be associated with an elevated serum homocysteine level. Although the methylenetetrahydrofolate reductase (MTHFR) C677T genetic variant is associated with an elevated serum homocysteine level, it has not been proved to be an independent risk factor for LA. The aim of the present study was to examine whether the MTHFR A1298C genetic variant, which is also believed to be unfavorable, is associated with the presence of LA. The clinical and genetic data on 198 LA patients and 235 neuroimaging alteration-free controls were analyzed. The presence of the A1298C or the 1298CC variant was calculated to be a risk factor for LA, as compared with the absence of both of them. The clustering of the heterozygous A1298C and C677T variants was proved to involve the risk of LA. Our results suggest that the MTHFR A1298C variant confers an independent genetic risk of LA, and this pathological role may be amplified by the MTHFR C677T variant.

Keywords Leukoaraiosis · Genetics · Risk factors · Genetic variants · MTHFR

Introduction

Leukoaraiosis (LA), a neuroimaging term, can be defined as a condition accompanied by hyperintensive signals in T2-weighted magnetic resonance imaging (MRI) scans of the white matter of the brain (Pantoni and Garcia 1997; Pantoni 2002). LA is presumed to be a very frequent entity: one quarter of people over the age of 65 are estimated to carry some degree of LA (van Gijn 1998). Chronic hypoxia and vascular microcirculation disturbances are presumed to lie behind LA (Markus et al. 2000; Szolnoki et al. 2007). LA brings about far-reaching health problems in that it is definitely associated with a cognitive decline or dementia (Yao et al. 1992; Ylikoski et al. 1993; Wardlaw et al. 2003; Pantoni et al. 2006; Inzitari et al. 2007) and the resultant poor quality of life.

LA is a slowly developing entity, associated with a number of environmental and genetic factors. Hypertension and aging are among the most important risk factors. A chronic endothelial dysfunction or blood–brain barrier damage, which can result from various clinical and genetic factors, may be key causative factor of LA (Hassan et al. 2003; Szolnoki 2007). Besides environmental stress and anatomic alterations such as hypertension and a decreased density of the brain microvasculature, the complex interaction of a number of...
genetic susceptibility factors may be presumed behind LA (Moody et al. 2004; Brown et al. 2007; Khan et al. 2007). These genetic factors do not appear to exert a major effect on LA but predispose to or modify its development (Fornage et al. 2007).

An elevated serum homocysteine level has been reported to be associated with LA (Clarke et al. 2000; Naka et al. 2006; Censori et al. 2007). In addition to a daily intake of folic acid, the serum homocysteine level can also be modified by the functioning of the methylenetetrahydrofolate reductase (MTHFR). MTHFR C677T polymorphism has been demonstrated to be associated with LA if it is present in combination with the angiotensin-converting enzyme D allele (Szolnoki et al. 2001). Its presence alone, however, was not proved to result in LA (Szolnoki et al. 2001, 2007). A second common genetic variant in the MTHFR gene (A1298C) has also been postulated as an unfavorable vascular risk factor (Szolnoki et al. 2006).

In this context, the aims of the present study were to examine whether the MTHFR A1298C genetic variant is associated with LA and whether there is an unfavorable synergistic effect between the unfavorable MTHFR 677T allele and the MTHFR A1298C variants as regards the frequency of LA.

Methods

Subjects

The 198 study subjects were the same as in our previous LA study (Szolnoki et al. 2001). The patient selection and clinical check-up were described earlier (Szolnoki et al. 2001). LA was defined by Fazekas et al. (1987) as irregular periventricular hyperintensities extending into the deep white matter in T2-weighted MRI scans (periventricular hyperintensities of grade 3) and deep white matter hyperintense signals with initial indications of confluence of the foci or with large confluent areas in the T2-weighted MRI scans (deep white matter hyperintense signals of grade 2–3).
The controls consisted of 235 neuroimaging alteration-free subjects. They were randomly selected from our practice register with the requirement of negative brain MRI scans. The study was approved by the local ethical committee.

DNA Analysis

Genomic DNA was extracted from 200 μl of peripheral blood anticoagulated with EDTA by the desalting method (Miller et al. 1988). The genetic variants of MTHFR A677T and A1298C were identified by a LightCycler probe system, as described earlier (Szolnoki et al. 2006). LightCycler examinations are illustrated in Figs. 1 and 2.

Assessment of Clinical Data

The smoking and drinking habits and the presence of hypertension or diabetes mellitus were recorded in all groups. The serum cholesterol and serum triglyceride levels were also measured and analyzed as important clinical parameters. Hypertension was diagnosed when the blood pressure repeatedly exceeded 130 mmHg systolic and/or 85 mmHg diastolic or when the patient was taking antihypertensive medication. Diabetes mellitus was diagnosed when the glucose level was at least 7.78 mM in a fasting state and/or at least 11.11 mM 2 h after a meal or a 75-g oral glucose load according to the World Health Organization criteria (WHO 1985). Ischemic heart disease was diagnosed when a history of angina pectoris or acute myocardial infarction was present or if there was ECG evidence of coronary heart disease.

Patients were classified as smokers if they had ever smoked more than five cigarettes per day for at least a year. Patients were considered to be moderately heavy drinkers if they drank 40 g of alcohol or more per day. The body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters.

Statistics

The clinical data were expressed as means ± SD where appropriate. The differences between the clinical parameters in the stroke group and the controls were assessed by using the $\chi^2$-test or the Mann–Whitney test where appropriate. The BMI body mass index

\* $p<0.001$; \** $p<0.0005$

The controls consisted of 235 neuroimaging alteration-free subjects. They were randomly selected from our practice register with the requirement of negative brain MRI scans.

The study was approved by the local ethical committee.

DNA Analysis

Genomic DNA was extracted from 200 μl of peripheral blood anticoagulated with EDTA by the desalting method (Miller et al. 1988). The genetic variants of MTHFR A677T and A1298C were identified by a LightCycler probe system, as described earlier (Szolnoki et al. 2006). LightCycler examinations are illustrated in Figs. 1 and 2.

Table 1: Major clinical and laboratory data on patients and control subjects

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Leukoaraiosis group (n=198)</th>
<th>Control group (n=235)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, females/males</td>
<td>105/93</td>
<td>127/108</td>
</tr>
<tr>
<td>Age, years</td>
<td>64.9±9.23*</td>
<td>54.7±12.8</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.3±2.3</td>
<td>24.6±1.98</td>
</tr>
<tr>
<td>Cholesterol, mM</td>
<td>5.41±1.32</td>
<td>5.12±1.34</td>
</tr>
<tr>
<td>Triglycerides, mM</td>
<td>1.45±1.59</td>
<td>1.38±0.96</td>
</tr>
<tr>
<td>Hypertension</td>
<td>69.7%**</td>
<td>18.7%</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>15.2%</td>
<td>13.2%</td>
</tr>
<tr>
<td>Smokers</td>
<td>15.2%</td>
<td>11.9%</td>
</tr>
<tr>
<td>Drinkers</td>
<td>6.1%</td>
<td>4.3%</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>10.1%</td>
<td>6%</td>
</tr>
</tbody>
</table>

The overall stroke group was compared with the control group by the $\chi^2$-test or the Mann–Whitney test where appropriate.

BMI body mass index

* $p<0.001$; ** $p<0.0005$

Table 2: Distribution of the different genotypes among the participants

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Leukoaraiosis, n=198</th>
<th>Controls, n=235</th>
<th>$p$</th>
<th>Adjusted OR*</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR AA1298</td>
<td>91 (46%)</td>
<td>132 (56.2%)</td>
<td>&lt;0.034</td>
<td>0.63</td>
<td>0.42–0.82</td>
</tr>
<tr>
<td>MTHFR A1298C</td>
<td>89 (44.9%)</td>
<td>85 (36.2%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MTHFR 1298CC</td>
<td>18 (9.1%)</td>
<td>18 (7.6%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MTHFR A1298C+1298CC</td>
<td>107 (54.1%)</td>
<td>103 (43.8%)</td>
<td>&lt;0.034</td>
<td>1.6</td>
<td>1.2–2.5</td>
</tr>
<tr>
<td>MTHFR CC677</td>
<td>70 (35.4%)</td>
<td>94 (40%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MTHFR C677T</td>
<td>98 (49.5%)</td>
<td>113 (48%)</td>
<td>NS</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>MTHFR 677TT</td>
<td>30 (15.1%)</td>
<td>28 (12%)</td>
<td>NS</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

The stroke groups were compared with the controls by the $\chi^2$-test.

NS not significant, NR not relevant

* Adjusted OR was calculated after adjustment for differences in age and hypertension between controls and leukoaraiosis groups
was in combination with the MTHFR A1298C variant. The frequencies of the genetic variants were compared with the χ²-test.

After univariate statistical analysis, we also carried out logistic regression calculation in order to adjust the differences in significant clinical risk factors (age and hypertension) between the LA subjects and the controls. Logistic regression analyses were performed with the statistical package SYSTAT 10 (Chicago, USA) for Windows.

**Results**

The clinical parameters are presented in Table 1. The presence of the 1298C variant (heterozygous A1298C or homozygous 1298CC) was found to be an independent risk factor for LA (54.1% vs 43.8%; adjusted OR, 1.6; 95% confidence interval, 1.2–2.5; p<0.034). Similarly as in our previous papers, the MTHFR T677C variant was not calculated to be a risk factor for LA. The distribution of the different genetic variants is given in Table 2.

The presence of the heterozygous T677C or A1298C variants did not predispose to LA if they occurred alone. However, the clustering of both heterogeneous variants in the same person meant a significant moderate risk of LA (4% vs 0.4%; adjusted OR, 79.5; 95% confidence interval, 1.2–98.5; p<0.008; Table 3). Data relating to the combinations of the two variants are to be found in Tables 4 and 5.

**Discussion**

Previous studies and the clinical data demonstrated that an elevated serum homocysteine level can be associated with LA. An elevated serum homocysteine level is presumed to be associated with an endothelial dysfunction or microangiopathy (Szolnoki 2007). The genetic studies relating to the MTHFR C677T variant suggest that, although the 677T variant is unfavorable by increasing the serum homocysteine level, it does not imply a significant risk of LA if it is present alone. We earlier demonstrated that the 677T variant in combination with other genetic variants may confer a risk of LA (Szolnoki et al. 2001).

The present study reveals that a person carrying either a heterozygous A1298C or a homozygous 1298CC variant is at a higher risk of LA relative to one carrying neither of them. This genetic variant has not been examined so far in any LA case–control study. Our findings therefore suggest for the first time that the A1298C variant may be more important than the C677T variant in the evolution of LA. We also found that the heterozygous C677T and A1298C variants do not pose a risk of LA if they are alone. However, their combination in the same person leads to a marked risk of LA. Although the number of patients displaying the combination of the two heterozygous MTHFR variants was low, the significance level relating to the approximately tenfold more frequent occurrence of the unfavorable combination in the LA group as compared with that in the control group suggests a valid association.

The exact cause of this synergistic effect is not known. Two possible explanations immediately emerge: (1) the two variants can potentially increase the serum homocysteine level in an additive manner and (2) the co-occurrence of the two unfavorable MTHFR genetic variants may influence the regulation of the enzyme. A properly balanced regulation of the MTHFR may be a key factor which can define the daily shifts in the serum homocysteine level. The clustering of the A1298C and C677T variants might give rise to an unfavorable regulatory nature in the dynamically changing activity of the MTHFR. The presence of the two heterozygous variants might result in a significantly unfavorable phenotype at the level of the conformation of the enzyme protein.

### Table 3

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Leukoaraiosis, N=198</th>
<th>Controls, N=235</th>
<th>P</th>
<th>Crude OR, 95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR A1298C + C677T</td>
<td>8 (4%)</td>
<td>1 (0.4%)</td>
<td>&lt;0.008</td>
<td>79.5 (1.22–98.5)</td>
</tr>
</tbody>
</table>

The stroke groups were compared with the controls by the χ²-test.

### Table 4

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>MTHFR 1298AA</th>
<th>MTHFR A1298C</th>
<th>MTHFR 1298CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR CC677</td>
<td>36</td>
<td>43</td>
<td>15</td>
</tr>
<tr>
<td>MTHFR C677T</td>
<td>71</td>
<td>41</td>
<td>1</td>
</tr>
<tr>
<td>MTHFR 677TT</td>
<td>25</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

### Table 5

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>MTHFR 1298AA</th>
<th>MTHFR A1298C</th>
<th>MTHFR 1298CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR CC677</td>
<td>23</td>
<td>32</td>
<td>15</td>
</tr>
<tr>
<td>MTHFR C677T</td>
<td>46</td>
<td>49</td>
<td>3</td>
</tr>
<tr>
<td>MTHFR 677TT</td>
<td>22</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>
Both theories are difficult to check on. The once-measured serum homocysteine level does not reveal the nature of its daily fluctuations. LA develops during a very long period. In this respect, either the average of the serum homocysteine level or the nature of the changes in the serum homocysteine appears to be of great importance. The protein activity or regulation theory can be solved only with the help of proteomics.

Following are the strengths of the study: (1) The control subjects were neuroimaging alteration-free, which is highly important because one quarter of the population over the age of 65 may be carriers of LA without clinical complaints, and such subjects may well confound the valid difference between the LA group and true controls. This problem may have a particular impact as regards minor genetic variants such as MTHFR A1298C and (2) the logistic regression approach clearly proved that the A1298C variant is an independent risk factor for LA. A univariate statistical approach may lead to false conclusions in view of the confounding effects of hypertension and age.

The limitations of the study are as follows: (1) The interaction between the MTHFR A1298C and C677T variants requires confirmation with regard to the low number of combinations of the two genetic variants and (2) the presence of other modifying genetic variants, e.g., in the angiotensin-converting enzyme, may change the association.

As this paper is the first relating to this association in LA, the findings demand confirmation for independent studies.

References


II.
Aims – Recent studies have started to elucidate the contribution of microbiome to the pathogenesis of multiple sclerosis (MS). It is also supposed, that neuropathological alterations might be associated with abnormal expression and regulatory function of antimicrobial peptides (AMPs), including defensins. It is in our interest to investigate the relevance of the single nucleotide polymorphisms (SNPs) of the DEFB1 gene and the copy number polymorphism of the DEFB4 genes in MS.

Methods – DEFB1 polymorphisms: c.-20G > A (rs11362), DEFB1 c.-44C > G (rs1800972), DEFB1 c.-52G>A (rs1799946), and the DEFB4 gene copy number were investigated in 250 MS patients. The control patients comprised 232 age- and gender-matched healthy blood donors. The occurrence of the human β-defensin 2 peptide (hBD2) in the plasma of controls and patients was determined by ELISA.

Results – The DEFB1 c.-44C>G polymorphism the GG protective genotype was much less frequent among patients than among the controls. A higher frequency of a lower (<4) copy number of the DEFB4 gene was observed in the patients with MS as compared with the controls (43% vs. 28%, respectively). The median levels of the circulating hBD2 in the patients were 150.6±12.71 pg/ml vs. 262.1±23.82 pg/ml in the control group (p<0.0001). Our results suggest that β-defensins play role in the development of MS.

Keywords: human β-defensin; copy number; polymorphism; multiple sclerosis

Célkitűzés – Az újabb vizsgálati eredmények kezdenek rávilágítani a mikrobiom szerepére a sclerosis multiplex patogenezisében. Azt is feltételezik, hogy a neuropatológiai változások összefüggésben állhatnak az antimikrobiális peptidek (AMP), közöttük a defensinek rendellenes expressziójával és szabályozó funkciójával. Vizsgáltuk a DEFB1 gén egynukleotidos polimorfizmusának és a DEFB4 gén köpiaszám-polimorfizmúsának a jelentőségét sclerosis multiplexben.

Módszerek – A DEFB1-polimorfizmusokat – c.-20G > A (rs11362), DEFB1 c.-44C > G (rs1800972), DEFB1 c.-52G>A (rs1799946), és a DEFB4-gén köpiaszámát vizsgáltuk 250, sclerosis multiplexben szenvedő beteg esetében. Kontrollcsoportonként 232, életkor és nem szerint illesztett egészséges véradó szolgált. ELISA-val határoztuk meg a humán β-defensin 2 peptid (hBD2) előfordulását a kontrollok és a betegek plazmájában.

Eredmények – A DEFB1 c.-44C>G GG protektív genotípus ritkábban fordult elő a betegek, mint a kontrollok csoportjában. A DEFB4-gén kisebb (<4) köpiaszámának a nagyobb frekvenciáját figyeltük meg a betegek, mint a kontrollok esetében (43% vs. 28%). A keringő hBD2 szintjének médiájára a betegek esetében 150,6±12,71 pg/ml, a kontrollcsoportban 262,1±23,82 pg/ml volt (p<0,0001). Az eredményeink szerint a β-defensinek szerepet játszanak a sclerosis multiplex kialakulásában.

Keywords: human β-defensin; copy number; polymorphism; multiple sclerosis

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www.elitmed.hu
Multiple sclerosis (MS) is a devastating neu- roinflammatory disorder of the brain and the spinal cord. MS pathogenesis involves inflammation, neurodegeneration and demyelination. The main pathological symptoms of the disease are the destruction of the myelin sheaths of nerve fibers, the relative sparing of the axons, and the infiltration of inflammatory cells in a perivascular surrounding. Although the well-defined autoimmune activities of the variant types against the central nervous system (CNS) are of great importance in the process of the disease, the pathomechanism and the direct causative factors have not yet been explained. The disease is defined by combination of exogenous factors and genetic background.

The contribution of the microbiota to MS pathogenesis has recently been investigated. Viral infections, such as Epstein-Barr virus, or human herpesvirus 6 has been suspected as trigger for MS. Accumulating informations strengthen the concept of microbiome-brain axis. Microbiome refers to the collective genomes of total microbiota. Recent studies have started to elucidate the contribution of microbiome to MS pathogenesis, which is being investigated in EAE models work. In addition, the recent trend of research has focused on the role of natural antimicrobial peptides in CNS disorders. It is supposed, that neuropathological alterations might be associated with abnormal expression and regulatory function of antimicrobial peptides (AMPs), including defensins.

Defensins and defensin-like proteins are fairly universal participants in host defence against infection. Defensins are small cysteine-rich peptides that can be classified as either α-defensin or β-defensin, depending upon the arrangement of six critical cysteine residues. Defensins have activity against Gram-positive and -negative bacteria, viruses (adenovirus, herpes virus, influenza virus, cytomegalovirus, HIV), and fungi, with minimal inhibitory concentrations in the μg ml⁻¹ range. The most frequently studied antimicrobial peptides are the human β-defensins (hBDs). The β-defensins are directly antimicrobial, and that microbial pathogens and pathogen induced cytokines stimulate their expression. The expression of hBDs can be stimulated by interleukin-1α (IL-1α), IL-1β, tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), IL-18. Then again, β-defensins induce the production of different chemokines and cytokines such as macrophage inflammatory protein 3-α (MIP-3α), interferon inducible protein 10, IL-1, IL-6, IL-10 and TNF-α. The β-defensins comprise the largest group, with around 40 members encoded in the human genome, but most studies have focused on HBD1 and HBD2. The expression of HBD1 is generally essential, the level of HBD2 is raised by proinflammatory cytokines and bacteria. Human β-defensins are produced by epithelial cells of the skin, gut, respiratory and urogenital tissue. HBD1 is also expressed by leukocytes. HBD1 mRNA has been observed in human astrocytes and microglia cells. Furthermore, epithelial cells, the expression of HBD2 has been detected in human monocytes, macrophages, and dendritic cells. Astrocytes may be other ways of local HBD2 synthesis in the brain. The generally synthesized defensins, as antimicrobial peptides (AMPs) may protect the brain against bacterial or viral infections. It is noteworthy, however, that the antimicrobial function of AMPs might actually secondary to their immunomodulatory properties.

The level of defensin expression varies among individuals and it has been suggested that this variation is due to genetic differences in the genes encoding defensins. Defensin genes have been mapped to 8p22-p23. Two types of genetic polymorphisms have been identified in genes encoding defensins, copy number polymorphisms and single nucleotide polymorphisms (SNPs). Human defensin beta-1 (HBD-1) encoded by the gene DEFBI, in which several SNPs (single nucleotide polymorphisms) have been characterized. Three frequent SNPs at positions G-20A (rs11362), C-44G (rs 1800972) and G-52A (rs1799946) in the 5′-untranslated region (UTR) of DEFBI were described. The untranslated variants influence HBD-1 expression or function.

The β-defensins are found to be impaired in many inflammatory diseases, including Crohn’s disease, psoriasis, pulmonary inflammation, and periodontal disease. β-defensins have been hypothesized to play a role in the aetiology of neurodegeneration with a focus on traumatic brain injury, a risk factor for Alzheimer’s disease (AD). A role of HBDs genes as potential modifiers in multiple sclerosis (MS) has not been hypothesized previously. It has been published that commensal bacteria within the gut can directly control the development of EAE, the experimental model of human multiple sclerosis by a combination of regulatory and antiinflammatory cell populations. It can be speculated, that the induction of defensins might also be involved in this process. Therefore, it is in our interest to investigate the relevance of the SNPs of the DEFB1 gene and the copy number polymorphism of the DEFB4 genes in MS.
Materials and methods

PATIENTS

The study included 250 MS patients. The examined population contained 197 RR (relapsing remitting) and 53 SP (secondary progressive) persons with a mean age of 44.23±13.02 years. Patients were diagnosed and collected from the Department of Neurology at the University of Szeged and Department of Neurology of Pándy Country Hospital in Gyula. The criterion was a diagnosis of clinically definitive MS. The RR type was described as the presence of exacerbations followed by complete or partial remissions. The SP form was defined as the presence of a slow deterioration of the disability lasting for more than six months, with or without relapses, in subjects who - before the progressive phase - had exhibited a RR course. The clinical classification was supported by the MRI criteria. MRI protocols were materialized in accordance with the European Concerted Action Guidelines. The expanded disability status scale score (EDSS) for the study population ranged from 0 to 8 (mean, 3.37±1.94) with a disorder duration of less than 1-20 years (mean, 8.83±2.16 years). All patients have given informed consent to their participation in this study, and the local ethics committees – Human Investigation Review Board at the University of Szeged Albert Szent-Györgyi Medical and Pharmaceutical Centre and the Human Investigation Review Board of Pándy Country Hospital, Gyula – have given prior approval to the study.

The control patients comprised 232 age and gender-matched healthy blood donors. The control group was selected from blood donors at the Regional Centre of the Hungarian National Blood Transfusion Service, Szeged Hungary. The characteristics of MS patients and controls are presented in Table 1.

DNA ISOLATION

Genomic DNA was extracted from 200 µl of peripheral blood anticoagulated with EDTA by the manufacturer’s instructions (High Pure PCR Template Preparation Kit; Roche Diagnostic GmbH, Mannheim, Germany, Cat.no: 1796828). The genomic DNA was stored at -20 °C until further use.

GENOTYPING

Human β-defensin 1 (DEFB1) – Genotyping was carried out Custom TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). Fluorogenic minor groove binder probes were used for each case using the dyes-6-carboxy-fluorescein FAM (excitation 494 nm) and VIC (excitation 538 nm): DEFB1 polymorphisms: c.-20G > A (rs11362), DEFB1 c.-44C > G (rs1800972), and DEFB1 c.—52G>A (rs1799946). Thermal cycling was performed on Bio Rad CFX96™ Real-Time PCR system. The amplification mix contains the following ingredients: 6 µl of Maxima® Probe/ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania), 0.3 µl of primer-probe mix, 5.1 µl of RNase and DNase-free water (Fermentas, Vilnius, Lithuania), and 1 µl of sample DNA, in a total volume of 11.4 µl per single tube reaction. Assay conditions were 10 min at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each 96-well plate contained 93 samples of an unknown genotype and three reactions with reagents but no DNA. DNase-free water was used as non-template control. Initial and post assay analyses were performed using the Bio Rad CFX96™ Real-Time system Precision Melt Analysis™ Software.

Determination of DEFB4 Gene Copy Number – A TaqMan real-time PCR assay, specifically for

Table 1. Characteristics of patients and controls

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Relapsing-remitting MS, N=197</th>
<th>Secondary progressive MS, N=53</th>
<th>Overall MS, N=250</th>
<th>Controls, N=232</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients (male/female)</td>
<td>49/148</td>
<td>16/37</td>
<td>65/185</td>
<td>168/64</td>
</tr>
<tr>
<td>Age (years) (means, SD)</td>
<td>45.7±14.5</td>
<td>56.75±12.68</td>
<td>44.23±13.02</td>
<td>46.8±11.98</td>
</tr>
<tr>
<td>Disease duration (years) (means, SD)</td>
<td>10.36±10.02</td>
<td>7.3±4.7</td>
<td>8.83±2.16</td>
<td>Not relevant</td>
</tr>
<tr>
<td>EDDS score (means, SD)</td>
<td>2.36±1.48</td>
<td>5.5±1.39</td>
<td>3.37±1.94</td>
<td>Not relevant</td>
</tr>
</tbody>
</table>
amplification of genomic DEFB4, was materialized by using a specific set of amplification primers (forward 5’-TGAAGCTCCCAGCCATCAG-3’; reverse 5’-TATTTCCCTGGCCCATCTCA-3’ and a VIC-labeled probe 5’-VIC- ATCTCCTCTTCTCGTTCC-MGB). Quantitative DEFB4 amplification data were normalized to ABL [FAM-lebeled albumin (Applied Biosystems, Cat. No. 4331182)] as an internal reference gene, which was added to each single tube. DEFB4’s primers and probe were designed using Primer Express 3.0 (Applied Biosystems). Probes were purchased from Applied Biosystems, and primers were obtained from Invitrogen (Carlsbad, CA, USA). Real-time PCR was carried out the Bio Rad CFX96TM Real-Time PCR system. Amplifications were performed in triplicate with Maxima® Probe /ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania), 20 ng of template DNA, 300 nM per primer, 200 nM of each fluorogenic probe. Steps of thermal cycling were 10 min at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Quantification was evaluated by the comparative CT (Threshold Cycle) method33.

ELISA OF HUMAN B-DEFENSIN 2

ELISA of human β-defensin 2 (hBD2) (Alpha Diagnostic San Antonio, TX, USA) was used to test for the occurrence of the human β-defensin 2 peptide in the plasma of controls and patients, on the basis of manufacturer’s instructions. The detection limit of the hBD2 ELISA Kit was 0.8 pg hBD2 protein/ml.

STATISTICAL ANALYSIS

The significance of the genotype frequency was analysed by the chi-square test and the Fisher test. The rate of significance was P<0.05. The genotype frequencies of SNPs were tested for deviation from the Hardy-Weinberg equilibrium by the chi-square test, with one degree of freedom. Plasma levels of human β-defensin 2 are expressed as medians with the Mann-Whitney test. The group means were discriminated by ANOVA test and followed by Bonferroni’s multiple comparison test.

The GraphPad prism 5 statistical program was used to all statistical calculations (GraphPad Software Inc. San Diego, CA, USA).

Results

DEFB1 C.-20G>A, DEFB1 C.-44C>G, AND DEFB1 C.-52G>A POLYMORPHISMS

The total number of MS patients in these genotyping experiments was 250 and the control group contained 200 healthy members. The three genotypes of DEFB1 and the controls patients were in accordance with the Hardy-Weinberg equilibrium (DEFB1 c.-20G>A P=0.677 and in the control group P=0.912; DEFB1 c.-44C>G P=0.999 and in the controls P=0.723; DEFB1 c.-52G>A P=0.657 and in the control group P=0.253 respectively).

The genotypic distributions of DEFB1 c.-20G>A, DEFB1 c.-44C>G, and DEFB1 c.-52G>A polymorphisms are presented in Tables 2–4.

There was no significant difference in genotype distribution between the DEFB1 c.-20G>A and the controls (P=0.4106). Similarly, no significant differences in genotypes between the DEFB1 c.-52G>A and the healthy patients (P=0.766). In the case of the DEFB1 c.-44C>G polymorphism there was significant difference in genotypes distribution between the patients and the control group (P=0.0002; Table 3, and Figure 1.).
The determination of copy number (CN) was performed in 250 patients in the MS group and in 232 healthy controls. The range of copy numbers was between two and eight per genome in the control group. Four copies were the median number of the healthy controls. The proportions of control individuals who carried the median (4), more than median (>4), or less than the median (<4) number of copies were 39%, 33%, 28%, respectively. In the patients with multiple sclerosis, the frequency distribution of the subgroups was different from the of the control group (P=0.002, Figure 2). Among the patients with MS, 43% had a lower (<4) copy number compared to the controls. The frequency of a CN >4 was only 27% among the patients with MS and 33% among the controls. As suggested, the copy numbers were higher than 4, the difference between MS patients and controls was not statistically significant, however, there was a lower frequency among patients (27%) than in the controls (33%) (Figure 2).

PLASMA LEVELS OF HBD2

ELISA for hBD2 was performed from samples of patients with MS and controls with known CNs of DEFB4, to determine whether the plasma levels of hBD2 varied with respect to the CN variation of the DEFB4 gene. The level of human β-defensin 2 in MS patients (n=80) were significantly lower altogether than it was in the controls (n=50). The median levels of hBD2 in the patients were 150.6±12.71 pg/ml vs 262.1±23.82 pg/ml in the control group, p<0.0001 (Figure 3). The circulating levels of human β-defensin 2 correlated well with the CN in the control group. Similarly to the controls, the plasma levels were found to be significantly lower in the <4 CN group (135.3±25.49 pg/ml), compared to patients with copy numbers of 4 (173.7±44.09 pg/ml). The defensin levels however were not elevated in patients with CN higher than 4 (148.9±31.21 pg/ml) (Figure 4).

Discussion

In our present study, an association between human β-defensins and multiple sclerosis has been found. By investigating three SNPs of DEFB1, the distributions of the C-44G genotypes were different between patients with MS and healthy controls,
while the frequency of the GG genotype was significantly higher in the control population. This indicates that the presence of G allele probably leads to strengthened HBD1 antimicrobial activity, which is less frequent among patients with MS. The G allele of C-44G SNP generates a putative binding site for nuclear factor κB (NF-κB) and very likely induces overexpression. The proposed effect of this SNP could partially explain why the GG genotype was considered to be a protective genotype in atopic dermatitis and also in susceptibility to Candida colonization in diabetic patients. Conversely, in these studies, subjects carrying the CC genotype at the -44 locus site of the gene were at a greater risk of acquiring infection. It has been recently suggested, that the C allele of DEFB1 C-44G SNP probably abrogates NF-κB-dependent DEFB1 up regulation.

These data are consistent with our present observation that the GG phenotype could also be protective in MS, and vice versa, the higher frequency of CC genotype might be connected with lower expression of human defensin β-1. Among the 250 patients with MS only 9 (4%) were GG homozygotes, and 62% of the patients were CC homozygotes, as compared with 45% of CC homozygotes in the control group. These observations draw attention to the importance of DEFB1 polymorphisms in MS.

Similarly, the production of the inducible hBD-2 is lower in MS patients. We suggest that the significantly lower frequency of the copy number of DEFB2 might be one of the reasons of the decreased levels of circulating hBD2 in the blood samples of patients.

When the association between the copy numbers and the plasma levels of hBD2 was investigated, a correlation between the ELISA results and copy number genotypes was found in the control group, but not in the groups of patients with MS. The low hBD2 levels correlated well with the low frequency of copy numbers (i.e. <4 copy) in the control group, but not in the groups of patients with MS. The low hBD2 levels correlated well with the low frequency of copy numbers (i.e. <4 copy), but in the patients with copy numbers >4, the plasma levels of hBD2 did not seem to be elevated. We suppose, that perhaps yet undefined other factors might be responsible for the low levels of hBD2 even in the case of higher copy numbers. We hypothesize that abnormalities in the production and the function of human defensin-β might be connected to an altered microbiome in MS, as it has been suggested recently.

While it is unclear whether enteric microbiota affect human MS, a higher percentage of MS patients exhibited antibody responses against gastrointestinal antigens in contrast to healthy control, which could indicate altered gut microbiome and immune status.

In addition, β defensins can be produced not only by epithelial cells, but also by astrocytes, and microglia cells, therefore their local importance in the central nervous system (CNS) has to be taken into consideration. Human defensin-β might function as an initial line of defence within the CNS either as an antimicrobial, or an immunomodulator, or both. In addition, these defensins may be also neuroprotective through their ability to inhibit cellular apoptosis in CNS.

The extreme low frequency of GG genotype of the C-44G SNP of DEFB1, the high frequency of the low copy number (<4) of DEFB2, and the significantly decreased plasma levels of hBD2 in patients underline the importance of human defensin-β in MS.

Further studies are necessary to elucidate the exact mechanism on how the impaired function of human defensin-β influences the pathomechanism of multiple sclerosis.

ACKNOWLEDGEMENT
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**Figure 4. Plasma levels of hBD2 in MS and in control group with DEFB4 copy numbers of <4, 4, >4.** Data are means and SD of the results on 50 samples in control group and 80 in MS patients. Differences between group means were determined with ANOVA test (difference between patients and controls P<0.0001) followed by Bonferroni’s multiple comparison test *CN<4 MS patients vs. controls P<0.001, **CN=4 MS patients vs. controls P<0.001, ***CN>4 MS group vs. control patients P<0.001.
REFERENCES

III.
Decreased Number of Mitochondria in Leukoaraiosis

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Background and Aims. Leukoaraiosis (LA), one of the most frequent causes of an age-associated cognitive decline, can be associated with a poor quality of life, leading overall to far-reaching public health problems. Chronic hypoxia of the white matter of the brain may be a factor triggering this entity. LA may develop as a consequence of chronically insufficient cellular energy production and the accumulation of free radicals.

Methods. In this context, after hypothesizing that the number of healthy mitochondria can be crucial in this complex process, a case-control LA study was carried out in which we analyzed the numbers of deleted and non-deleted mitochondria (the common D-loop deletion) per white blood cell. A total of 234 patients with LA and 123 MRI alteration-free subjects served as a control group.

Results. Interestingly, it emerged that the ratio of deleted relative to non-deleted mitochondria is strongly associated with the risk of LA. The calculated K ratio in the LA group was significantly lower than the K ratio in the controls (LA: K 0.37 95% CI 0.05; controls: K 0.48, 95% CI 0.076, p < 0.001).

Conclusions. Our study suggests that the ratio of the dmDNA and mDNA can be of great importance in the pathogenesis of LA. © 2015 IMSS. Published by Elsevier Inc.

Key Words: Leukoaraiosis, Mitochondria, Energy production, White matter damage.

Introduction

Leukoaraiosis (LA) is originally a neuroimaging term and refers to hypodensities in CT scans and hyper-intense signals in T2-weighed MRI scans. Although LA is a neuroimaging term, it relates to a complex clinical entity (1–3). LA has been reported to be associated with a cognitive decline and a slowing of mental processing, which can be manifested as a slowness of speech or understanding (4–6). LA is considered to be a vascular demyelinating process (7,8). Although it results from factors such as aging and hypertension (9), development of LA may be associated with a number of several genetic susceptibility factors (10–15).

These genetic and other risk factors suggest that chronic hypoxia might trigger intracellular biochemical events, which result in LA (16,17). Accordingly, we hypothesized that a key role in the demyelination process of LA may be played by chronically insufficient energy production (18). Hence, in addition to certain biochemical features, the number of mitochondria, the main energy-supplying organelles in mammalian cells (19), may be determinstic for the fate of neurons and glia cells. Mitochondrial mutations can affect different cell types in different ways; this is reflected in mitochondria-associated genetic disorders such as MELAS (20). In such cases, mutations of the mitochondrial DNA or tRNA bring about a malfunction in the affected mitochondria (21). However, virtually no data are available as concerns the number of the mitochondria...
themselves, which must be an important feature from an energetic aspect.

Although the numbers of mitochondria are probably essential as concerns the neurons and glia cells (17), these data cannot easily be examined in clinical studies. However, the numbers of mitochondria in other tissues, which are functionally in constant contact with the brain tissue, may be informative. The white blood cells, for example, constantly circulate and communicate with the brain tissue. Their energy store may be important for the maintenance of the brain because they act as free radical scavengers and also check on the integrity of the neurovascular system of the brain (22). The white blood cells, vascular cells and brain tissue most likely behave as a functional unit. However, very few data have been published as regards the numbers of mitochondria among the different cell types. The number of mitochondria in the white blood cells may be an indicator of the overall number of mitochondria in the given individual.

In this context, we examined whether there is an association between the number of mitochondria in the white blood cells and the development of LA. Besides the genetic heterogeneity of the mitochondrial DNA (23), there are two large types of mitochondrial DNA: one with D-loop sequences and one without them. The former is known as mitochondrial DNA (mDNA) and the latter as mitochondrial DNA with a large and common deletion of 123-1256 bp (dmDNA). This latter DNA might be considered to malfunction in some way (24,25). In our present study we identified the copy number per white blood cell for both mDNA and dmDNA.

### Patients and Methods

#### Study Population

A total of 234 patients with LA were recruited after a clinical scrutiny. LA was diagnosed on the basis of MRI scans. Patients with degrees of I or II ical scrutiny. LA was diagnosed on the basis of MRI scans. A total of 234 patients with LA were recruited after a clinical valuation of mDNA and dmDNA. This latter DNA might be considered to malfunction in some way (24,25). In our present study we identified the copy number per white blood cell for both mDNA and dmDNA.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>TaqMan signal dyes</th>
<th>Conc. (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH for</td>
<td>5'-TCC AGT ATG ATT CCA CCC ATG GCA-3'</td>
<td>-</td>
<td>0.4</td>
</tr>
<tr>
<td>GAPDH rev</td>
<td>5'-TTC TAC ATG GTG GTG AAG ACG CCA-3'</td>
<td>-</td>
<td>0.4</td>
</tr>
<tr>
<td>GAPDH probe</td>
<td>5'-TTC CAT GGC ACC ATC AAG GCT GAG AA-3'</td>
<td>ROX/BHQ2</td>
<td>0.2</td>
</tr>
<tr>
<td>mtDNA for</td>
<td>5'-ATG GCC AAC CTC CTA CTC CTC ATT-3</td>
<td>-</td>
<td>0.4</td>
</tr>
<tr>
<td>mtDNA rev</td>
<td>5'-TTA TGG CGT CAG CGA AGG GTT GTA-3'</td>
<td>-</td>
<td>0.4</td>
</tr>
<tr>
<td>mtDNA probe</td>
<td>5'-CGC AAT GCC ATT CCT AAT GCT TAC CG-3'</td>
<td>HEX/TAMRA</td>
<td>0.1</td>
</tr>
<tr>
<td>dmtDNA for</td>
<td>5'-ACA CAA ACT ACC ACC 'TTT GGC AGC-3'</td>
<td>-</td>
<td>0.4</td>
</tr>
<tr>
<td>dmtDNA rev</td>
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<td>-</td>
<td>0.4</td>
</tr>
<tr>
<td>dmtDNA probe</td>
<td>5'-CCA AAG ACC ACA TCA TCG AAA CCG CA-3'</td>
<td>FAM/TAMRA</td>
<td>0.1</td>
</tr>
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</table>
Table 2. Characteristics of patients and control subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Leukoaraiosis n = 234</th>
<th>Controls n = 123</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (females, males)</td>
<td>123, 111</td>
<td>65, 58</td>
</tr>
<tr>
<td>Age, years</td>
<td>71.6 ± 10.8</td>
<td>59.4 ± 8.62</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.6 ± 2.33</td>
<td>24.4 ± 3.42</td>
</tr>
<tr>
<td>Cholesterol, mmol</td>
<td>5.91 ± 1.24</td>
<td>5.52 ± 1.56</td>
</tr>
<tr>
<td>Triglycerides, mmol/C6</td>
<td>1.48 ± 0.91</td>
<td>1.50 ± 0.82</td>
</tr>
<tr>
<td>Hypertension</td>
<td>46.6%*</td>
<td>22.0%</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>17.9%*</td>
<td>4.88%</td>
</tr>
<tr>
<td>Smokers</td>
<td>29.9%</td>
<td>28.5%</td>
</tr>
<tr>
<td>Drinkers</td>
<td>12.0%</td>
<td>9.0%</td>
</tr>
</tbody>
</table>

*p < 0.05; the leukoaraiosis group was compared with the control group by means of the χ² test or Mann-Whitney test.

per single-tube reaction. The assay conditions were 3 min at 95°C and 44 cycles of 95°C for 10 sec and 60°C for 45 sec. Quantification was evaluated by the comparative CT (threshold cycle) method (29).

Because no data were available concerning the stability of the number of mitochondria in white blood cells, the numbers of mitochondria in five patients and five controls were determined three times with a minimum interval of 1 month between blood drawings in the same subject. We found merely minimal number differences between the different blood samples in the same subject (data not shown).

Statistical Analysis

Clinical variables in the LA and control groups are listed and compared statistically in Table 2.

mDNA and dmDNA contents per white blood cell in the LA group were compared with those in the control group by means of the two-paired $T$-test (Table 3). In order to make the comparison biologically more sensitive, the mathematical difference between the mDNA and mdDNA contents per cell were derived by the sum of mDNA and dmDNA contents per cell in the same individual. This calculated ratio (K) indicated the weight of uncompensated dmDNA per cell:

$$K = \frac{(\text{mDNA content} - \text{dmDNA content})}{(\text{mDNA content} + \text{dmDNA content})}.$$

K values for each of the LA patients and the controls were compared statistically between the LA and control groups by means of the two-paired $T$-test (Table 3).

Univariate statistical analysis was followed by a logistic regression comparison involving the age, hypertension and diabetes mellitus (due to the statistical difference between the LA and control groups) in addition to the calculated values of K.

Results

The frequency of hypertension in the LA group was higher than that in the controls (Table 2). mDNA and dmDNA content did not differ statistically between the LA and control groups (Table 3).

The calculated value of K for the LA group was significantly lower than that for the controls after the used logistic regression analysis. This logistic regression analysis was carried out for the differences between the LA and controls with regards to the age, hypertension and diabetes mellitus. In this way, we could diminish the confounding effects of these clinical factors because they could also be associated with the reduced number of mitochondria (LA: K 0.37, 95% CI 0.05; controls: K 0.48, 95% CI 0.076; $p < 0.001$).

Discussion

Although the basic mDNA and dmDNA contents were statistically the same in the LA and control groups, the value of K was significantly lower for the LA group than that for the control group. This was a mathematical indicator of a larger proportion of dmDNA. dmDNA can potentially result in a mitochondrial malfunction in the following ways: (a) lower energy production; (b) a lower free radical scavenging capacity; (c) a lower rate of adaptation to the prevailing demand for energy production; (d) a narrower range in the adjustment to the prevailing energy demand; (e) a lower of metabolic function capacity in general; (f) a larger extent of free radical production; and (g) a general malfunction of the mitochondrial genetic regulation.

No genetic and biochemical data are available to suggest which of these postulated mechanisms actually exist, but a lower and narrower energy capacity appears probable as the main pathomechanism behind LA. It was demonstrated earlier at a molecular level that LA can result from a very slight, but chronic, level of hypoxia, which can be caused by various environmental and genetic susceptibility factors.
Our present findings are in accord with the earlier findings that uncoupling protein genetic variants play roles in the development of LA (30). The uncoupling proteins govern the electrochemical gradient between the inner and outer spaces of the mitochondria (31), this gradient being essential for the energy production of the mitochondria. If dmDNA is associated with any kind of biochemical malfunction, an uncompensated and larger proportion of dmDNA in the cells can be unfavorable from an energetic aspect.

Our results revealed that the lower the difference between the contents of mDNA (which compensates malfunctions of the dmDNA) and dmDNA the larger the risk of LA in the given individual.

In conclusion, our study suggests that the ratio of dmDNA and mDNA contents can be of great importance in the pathogenesis of LA. These results indicate the need for new approaches for examination of mitochondrial contents in other common brain disorders.

**Study Limitations**

The numbers of mitochondria in the affected brain tissues could not be examined because this was a clinical study carried out in a human patient population. Brain biopsies would not have been ethical; thus, it remained to identify associations between the numbers of mitochondria in different human tissues. Although we found no apparent change in the number of mitochondria in a small cohort of study subjects during several weeks (which involved several turnovers of the mitochondria in the white blood cells), insufficient scientific data are available concerning the stability of the numbers of mitochondria in the different tissues. This should be clarified.

No examinations were carried out in this study to identify the features of the malfunctioning of the mitochondria with deletion DNAs. However, these limitations do not greatly affect the present results because they are not obviously associated with the findings. Moreover, these postulated limitations are rather scientific questions, which should be considered in subsequent examinations.

Albeit the logistic regression statistical method has greatly decreased the confounding effects of the clinical factors such as age, hypertension (32,33) and diabetes mellitus (34), the results need to be confirmed by a larger population study.

**References**

IV.
Relevance of defensin β-2 and α defensins (HNP1-3) in Alzheimer’s disease

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ABSTRACT

The DEFB4 gene copy numbers were investigated in 206 AD patients and in 250 controls. The levels of the human defensin β–2 (hBD2) and α-defensins (HNP 1-3) in the sera and in the cerebrospinal fluid (CSF) of the patients and the controls were determined.

Higher copy numbers of the DEFB4 gene was observed in AD patients as compared with the controls. The levels of hBD-2 and HNP 1-3 were significantly elevated in the sera and in the CSF of the AD patients.

These data suggest that both defensin β–2 and α-defensins have potential role in the development of AD.

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1. Introduction

Initiating event in AD is related to the abnormal processing of beta-amyloid (Aβ), ultimately leading to the formation of Aβ plaques in the brain (Jack et al., 2010). It was recently postulated that brain infections involving bacteria or viruses may play an initiating role in amyloid plaque formation and the development of AD (Hill et al., 2014; Maheshwari and Esluck, 2015; Mawanda and Wallace, 2013). Persistent subclinical CNS infections have been reported for AD patients, caused by various pathogens such as Chlamydia pneumoniae, Helicobacter pylori, Herpes simplex virusi, spirochetes or even fungi (Shima et al., 2010; Löveheim et al., 2015; Miklossy 2011; Alonso et al., 2014).

It is presumed, that neuropathological alterations might be associated with abnormal expression and regulatory function of antimicrobial peptides (AMPs), including defensins; (Foster and McVey Neufeld, 2013; Williams et al., 2012). Human β-defensins have been suggested to play a role in chronic inflammation-associated brain injury, a risk factor for AD. In this respect, the literature reports (Williams et al., 2012; Welling et al., 2015) lead us to hypothesize that upregulation of the production of defensins might trigger the aggregation of amyloid in the brain.

Human α-defensins include human neutrophil peptide 1-4 (HNP1-4) and intestinal human defensins (HD-5 and HD-6), produced by Paneth cells. Human β-defensins make up another family of antimicrobial peptides (Ganz, 2003; Pazgier et al., 2006). Besides their antibacterial and antiviral effects, defensins exert numerous immunological effects (Oppenheim and Yang, 2005; Yang et al., 2002). While the expression of human defensin beta-1 (hBD1) is generally constitutive, the levels of human defensin beta-2 (hBD2) are inducible by proinflammatory cytokines and bacteria.

Defensin genes have been mapped to 8p22-p23 (Linzmeier and Ganz, 2005). A role of the DEFB4 gene in determining human β-defensin-2 (hBD2) as potential modifier in AD has not been hypothesized previously.

We therefore considered it of interest to investigate the relevance of the copy number (CN) polymorphism of the DEFB4 genes in AD. We additionally investigated the levels of human β-defensin-2 (hBD 2) in the cerebrospinal fluid (CSF) and the sera of the patients with AD. The study was supplemented with the measurement of human α-defensin (Human Neutrophil Peptide 1-3; HNP1-3) in the circulation and in the CSF.

2. Materials and methods

2.1. Patients and controls

A total of 206 AD patients 69 men and 137 women, average age and standard deviation (S.D.) 76.42 ± 4.21 years, (minimum age at onset was 65 years) were enrolled in the study. The clinical diagnosis of AD was based on neurological and psychiatric examinations, together with the assessment of psychometric tests to
confirm cognitive impairment. Additionally, a brain CT scan or MRI was performed in each case. All participants fulfilled criteria of NINCDS–ADRDA (McKhann et al., 1984; Dubois et al., 2007). The cognitive evaluation of AD patients was based on the AD Assessment Scale – Cognitive Subscale (ADAS–Cog) (Rosen et al., 1984; Pálkasi et al., 2012), the Mini-Mental State Exam (MMSE) (Folstein et al., 1975; Janka et al., 1988), and the Clock Drawing Test (CDT) (Kálmán et al., 1995). To exclude pseudodementia caused by depression, mood was scored via the Beck Depression Inventory (Beck et al., 1961), which might be a limitation (Wagle et al., 2000), but patients with less than 12 BDI scores have only been included to the study.

The control group comprised 250 healthy volunteers (92 men and 158 women, average age ± S.D. 72.69 ± 6.82 years with normal cognition (MMSE score higher than 28). All cases and controls were of Hungarian ethnic origin. Informed consent was obtained from all patients or responsible guardian in case of incapacity and controls. All patients were treated in accordance with the Patient Right’s Protection Act of our institutions and according to international guidelines.

The local Ethics Committee of the Hungarian Investigation Review Board gave prior approval to the study.

2.2. Determination of DEFB4 gene copy number (CN)

Genomic DNA was extracted from peripheral blood anticoagulated with EDTA in accordance with the manufacturer’s instructions (High Pure PCR Template Preparation Kit; Roche Diagnostic GmbH, Mannheim, Germany, Cat. no: 1796828). A TaqMan real-time PCR assay, specifically for amplification of genomic DEFB4, was performed as described previously (Tiszlavicz et al., 2012). Briefly, quantitative DEFB4 amplification data were normalized to ABL [FAM-labeled albumin (Applied Biosystems, Cat. no. 4331182)], as a standard reference gene considered to be present in only 2 copies in the genome. The reference gene was used as an internal standard (Bentley et al., 2010) added to each single tube. Quantification was evaluated by the comparative CT (Threshold Cycle) method (Szilagyi et al., 2006).

2.3. Determination of human β-defensin 2 (hBD2) and HNP1-3 concentrations

ELISA of hBD2 (Alpha Diagnostic San Antonio, TX, USA) was used to test for the occurrence of the human β-defensin 2 peptide in the sera and in CSF of the controls and patients, on the basis of manufacturer’s instructions. The HNP1-3 concentrations in the sera and CSF were determined by ELISA (HyCult-Biotech HK324, Uden, The Netherlands) according to the instructions of the manufacturer.

2.4. Statistical analysis

The significance of the differences was analyzed by the Mann-Whitney test. The GraphPad prism 5 statistical program was used for all statistical calculations (GraphPad Software Inc. San Diego, CA, USA).

3. Results

3.1. Copy number (CN) polymorphism of DEFB4

The determination of CN was performed in 206 patients in the AD group and in 250 controls. The median CN in the controls was 4, the 75% percentile 5, and the 25% percentile was 3. In the AD

![](https://example.com/fig1.png)
patients the median was 5, with a 75% percentile of 6, and with a 25% percentile of 4; p < 0.001 with the Mann-Whitney test (Fig. 1 inset).

3.2. Levels of hBD2 in sera and in cerebrospinal fluids (CSF)

The levels of human β-defensin 2 were determined in the sera of 52 AD patients and 45 controls. Significantly higher levels of hBD2 were measured in AD patients than in the controls (Fig. 1a). The median levels were 265.5 pg/ml in AD vs. 169.9 pg/ml respectively (p < 0.01). The median levels of β-defensin 2 in the CSF of 43 AD patients were 8.6 pg/ml vs 1.201 pg/ml in the control group (n = 40), p < 0.001 Fig. 1b.

3.3. Levels of HNP1-3 (α-defensins) in sera and in cerebrospinal fluids (CSF)

The median serum concentrations of α-defensin in 43 AD patients were 147.3 ng/ml, significantly higher than that in 40 controls: 122.3 ng/ml (p < 0.05) Fig. 1c. The median concentration of α-defensin in the CSF of 52 AD patients was significantly higher: 85.16 ng/ml than that in 40 controls: 1.23 ng/ml (p < 0.001) Fig. 1d.

4. Discussion

It has been postulated, that chronic infections might be initial events in the pathogenesis of AD, which can give rise to persistent inflammatory stimuli. The inflammatory response thereafter may indirectly lead to the upregulation of amyloid β production (Welling et al., 2015). Not only the levels of the inducible hBD2 were measured in AD patients than in the controls (Fig. 1a), but also those of HNP1-3. Our data are in accordance with recent studies (Welling et al., 2015). Not only the levels of the inducible hBD2 were measured in AD patients than in the controls (Fig. 1a). The median levels were 265.5 pg/ml in AD vs. 169.9 pg/ml respectively (p < 0.01). The levels of β-defensin 2 in the CSF of 43 AD patients were 8.6 pg/ml vs 1.201 pg/ml in the control group (n = 40), p < 0.001 Fig. 1b.

Further investigations are necessary to elucidate the regulatory functions of defensins in the pathomechanism of AD.

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