MULTIPLE SCLEROSIS TO DATE: DIAGNOSIS, EPIDEMIOLOGY, NEW ASPECTS OF THE PATHOMECHANISM AND THE THERAPY

Ph.D. thesis
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>5-HT</td>
<td>Serotonin</td>
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<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DA</td>
<td>Dopamine</td>
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<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
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<tr>
<td>E</td>
<td>Epinephrine</td>
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<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalitis</td>
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<tr>
<td>EDSS</td>
<td>Expanded disability status scale</td>
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<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
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<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
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<tr>
<td>HPA</td>
<td>Hypothalamus-pituitary gland-adrenal medulla</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin-G</td>
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<tr>
<td>INF-β</td>
<td>Interferon-β</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>L-DOPA</td>
<td>L-hydroxy-phenylalanine</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
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<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase-9</td>
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<tr>
<td>MMPG</td>
<td>Methoxy-hydroxyphenyl glycol</td>
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<tr>
<td>MP</td>
<td>Methylprednisolone</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>NE</td>
<td>Norepinephrine</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>NO</td>
<td>Nitric oxid</td>
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<tr>
<td>OCB</td>
<td>Oligoclonal band</td>
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<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>R-R</td>
<td>Relapsing-remitting</td>
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<tr>
<td>SH groups</td>
<td>Sulphydryl groups</td>
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<tr>
<td>VEP</td>
<td>Visual evoked potential</td>
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<tr>
<td>VMA</td>
<td>Vanilmandelic acid</td>
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Summary

Multiple sclerosis (MS) is an inflammatory demyelinating disease of unknown origin. It is the most common neurological disease among young Caucasian adults, primarily in women. The risk factors for MS include genetic and environmental influences. The diagnosis of MS is mainly clinical, based on the symptoms and clinical course of the disease. The different diagnostic tools may confirm this clinical diagnosis. In consequence of recent clinical and scientific results relating to the diagnosis and therapy of MS, epidemiological studies to determine the prevalence of the disease have come into the focus of interest.

The diagnostic criteria postulated by Poser et al (1) necessitate clinical, laboratory and cerebrospinal fluid (CSF) analyses to establish the definitive diagnosis of MS.

During the analysis of the cerebrospinal fluid (CSF), the most important task is to identify the humoral immune response. The Charcot Foundation recommended the isoelectric focusing technique as the most sensitive, essential test method (2).

In 1961, Lehoczky and Halasi first determined the Hungarian prevalence of MS, as 20/100,000 (see the review by Pálffy et al. (12)). In 1983, Pálffy et al. (12) reported the prevalence in Baranya County as 37/100,000. Unfortunately, their patients did not undergo MRI examination.

Since the Poser criteria system and MRI have become popular in daily practice, the newly introduced therapies require the determination of the prevalence, and clinical forms of MS, and the functional status of MS patients.

With the availability of improved genetic epidemiological tools and statistical methodology, it has become clear that MS is a complex condition with genetic epidemiology very similar to that of a number of other organ-specific autoimmune diseases. The susceptibility to the illness is determined by a number of largely uncharacterized genes and environmental factors. However, it is not easy to see any analogy or to postulate any particular mechanism whereby these factors exert their effects. It is therefore useful to make a careful study of the over- and under-expression of the responses to environmental exposures (13-16).

The current hypothesis of the pathogenesis of MS suggests that the primary peripheral activation of autoreactive T helper-1 lymphocytes precede the recognition of central nervous system (CNS) auto-antigens. These T cells proliferate, secrete cytokines and cross the blood-brain barrier (BBB) to find their antigens in the CNS, where they cause further inflammatory damage. It has been hypothesized that relapsing-remitting (RR) MS is driven by a systematic antigen presentation and that chronic progressive MS depends on the CNS presentation of antigens.

We postulated that the deactivation of the immune system after an MS relapse (remission) could be mediated by catecholamines.

Mickel (32) proposed that a lipid peroxidation disturbance caused by free radicals is involved in the breakdown of the myelin sheath. Since then, several studies have demonstrated the role of increased free radical production and/or a decreased antioxidant defense in the CNS as causal factors of MS (33-36). Increased lipid peroxide levels have been observed both in the CSF and in the blood of MS patients (34,37). Catalase, superoxide dismutase, glutathione peroxidase and glucose-6-phosphate
dehydrogenase, which may protect cell membranes from peroxidative reactions, display different activities in the erythrocytes of patients and controls (37-43).

We examined the balance between the plasma concentrations of lipid peroxides and blood nonenzymatic antioxidants (glutathione, alpha-tocopherol, retinol, plasma SH groups and uric acid) in relation to the clinical state of MS patients. Furthermore, we were interested in establishing whether interferon-β (INF-β) therapy, which has been shown to induce positive clinical results in MS (48), has any effect on the tested parameters.

In the last 10 years immunomodulant therapy has been introduced for MS. The first representatives of this group are the β-interferons. The IFN are proteins belonging in the cytokine family. They are integral parts of the immune process exerting immunomodulatory, antiviral and antiproliferative effects. IFN-β-1b (Betaferon) was the first medicament, which proved to decrease the number of exacerbations by 34% in relapsing-remitting and relapsing-progressive MS in a multicenter, double-blind placebo-controlled trial (48).

We used isoelectric focusing to evaluate the OCBs in the CSF of clinically definitive MS patients. Oligoclonal bands presented in 91% of MS patients.

Our study indicated that the prevalence in Csongrád County is 62/100,000. On the prevalence day, 130 of 248 patients lived in Szeged. The incidence of MS in the city of Szeged was 5/100,000 in 1997 and 6/100,000 in 1998. There were 66 (27%) males and 182 (73%) females giving a male/female ratio of 1:2.75. The clinical forms of MS were as follows: 15% benign form, 54% relapsing-remitting form, 20% secondary chronic progressive form and 11% primary chronic progressive form. The Expanded Disability Status Scale (EDSS) score in the benign form was 0-3 points, and the average duration of the disease was 27 years. Sixty per cent of the relapsing-remitting MS patients had EDSS scores of 0-4 points and 33% had EDSS scores of 4.5-6.5 points. Fifty-six per cent of the secondary chronic progressive MS patients had EDSS scores of 4-6.5 points, while 44% were confined to wheelchairs or bed-ridden (EDSS scores > 7).

We report on 3 affected sisters, whose parents had no neurological or autoimmune disease in their medical history. Since the familial environment does not necessarily lead to the disease, a genetic mutation of the sisters might be possible. Although the parents and grandparents were healthy, a common viral infection in childhood or some other environmental factor cannot be excluded, as a triggering factor of the disease. The crude risk of MS in the Northern European population is 1:600. Accordingly the case of these affected sisters might be interesting and their further follow-up, with encoding of the natural over- or under-expression might lead to a better understanding of MS (95).

The norepinephrine content in the peripheral blood lymphocytes is significantly lower in MS patients than in healthy individuals, but in the early stage of the disease, and hence in first attack patients the epinephrine content is higher. With regard to the fact that the lymphocytes in relapse have a higher β-receptor density, new means of early intervention in the pathogenesis of MS at the lymphocyte level may be possible. These data suggest a connection between the peripheral blood lymphocyte catecholamine content and the course of the disease, and may contribute to a better understanding of
the pathogenesis of MS. They may also suggest a new therapeutic approach through recognition of the role played by the lymphocytes in this disease.

Our present study has provided evidence of peroxidative reactions in MS patients during exacerbation, and supports the role of oxidative stress in the pathomechanism of the disease.

The results of our self-control clinical study of INF-β-1b in the treatment of MS patients during a 3-year follow-up period indicated a 77% reduction in the relapse rate, a 75% reduction in the methylprednisolone need and an 84% reduction in the number of days of hospitalization. The long-term importance of immunomodulants as concerns the relapse rate and the progression of MS were confirmed by the findings of phase IV clinical trials and every-day practice.
1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of unknown origin. It is the most common neurological disease among young Caucasian adults, primarily in women. The risk factors for MS include genetic and environmental influences. The diagnosis of MS is mainly clinical, based on the symptoms and clinical course of the disease. The different diagnostic tools may confirm this clinical diagnosis. From the early 1990s, new diagnostic protocols were developed following the general use of MRI. The diagnostic criteria of MS developed by Poser et al. (1) in 1983, were initially a scientific protocol, but in a few years became part of the daily routine. The Charcot Foundation in collaboration with 12 accredited European CSF laboratories determined the essential, optional and additional tests for the CSF-diagnosis of MS in 1994 (2). In 1996, Lublin and Reingold developed the standard nomenclature for the clinical forms of MS. New therapeutic approaches were introduced in relapsing-remitting MS from the middle of the 1990s.

In consequence of the new clinical and scientific results relating to the diagnosis and therapy of MS, to determine the prevalence of the disease, epidemiological studies came into the focus of interest.

1.1. CSF in the diagnosis of MS

The diagnostic criteria postulated by Poser et al. (1) necessitate clinical, laboratory and CSF analyses to establish the definitive diagnosis of MS.

During the analysis of the CSF, the most important task is to identify the humoral immune response. The Charcot Foundation recommended the isoelectric focusing technique as the most sensitive, essential test method (2).

Optional tests could be as follows:
- determination of the blood-CSF barrier function
- detection of intrathecal immunglobulin-G (IgG) production
- measurement of CSF cells

Additional tests:
- measurement of IgM
- measurement of IgA
- determination of free kappa and lambda light chains
- measurement of myelin basic protein and determination of virus antibody levels

1.2. Epidemiology of MS

Prevalence

Dean (4) determined the prevalence of MS within the European continental zone as 30-80/100,000. The occurrence was found to be related to the geographical distribution, migration and a genetic contribution (5,6). In research on the etiologic factors of MS, the occurrence and clinical forms of the disease have been examined in different population groups, such as mestizos, Indians, etc. (7,8). A distinction between low, medium and high-risk factor areas can be made on the basis of geographical lines of latitude. However, the new prevalence tests reveal that in both low and medium-risk factor areas there can in fact be an enhanced occurrence (4,9,10). There are both pro and contra arguments for
a north-south gradient distribution (4,11). In 1961, Lehoczky and Halasi first determined the Hungarian prevalence of MS, which was found to be 20/100,000 (see the review by Pálffy et al (15)). In 1983, Pálffy et al. (12) reported the prevalence in Baranya County as 37/100,000. Unfortunately, their patients did not undergo MRI examination.

Since the Poser criteria system and MRI have become popular in daily practice, and the newly introduced therapies require determining the prevalence- and clinical forms of MS, and the functional status of MS patients.

1.3. New aspect of the pathomechanism

1.3.1. Etiology: genetic and/or environmental factors?

With the availability improved genetic epidemiological tools and statistical methodology, it has become clear that MS is a complex condition with genetic epidemiology very similar to that of a number of other organ-specific autoimmune diseases. The susceptibility to the illness is determined by a number of largely uncharacterized genes and environmental factors. However, it is not easy to see any analogy or to postulate any particular mechanism whereby these factors exert their effects. It is therefore useful to make a careful study of the over and under-expression of the responses to environmental exposures (13-16). It has become evident that the parents or siblings of MS patients often carry the trait of the disease, though without any neurological symptom. A shared childhood and adolescence seems to have little impact on the risk of development of MS in siblings; the sequence of birth of affected individuals is more important than the year of onset (13,15,17,18). We have encountered an unusual occurrence where all of 3 sisters were suffering from MS, whereas their parents and grandparents displayed no trait of MS.

1.3.2. Involvement of the sympathoadrenergic mechanism in MS

Current hypotheses on the pathogenesis of MS suggest that the primary peripheral activation of autoreactive T helper-1 lymphocytes precede the recognition of CNS auto-antigens. These T cells proliferate, secrete cytokines and cross the blood-brain barrier (BBB) to find their antigens in the CNS where they cause further inflammatory damage. It has been hypothesized that relapsing-remitting (RR) MS is driven by a systematic antigen presentation and that chronic progressive MS depends on the CNS presentation of antigen (19). Studies involving experimental models of MS demonstrate the importance of the lymphocytes and sympathoadrenergic mechanisms (20). As an immune privileged site, the brain is not totally separated from the immune system, as thought earlier. The CNS is connected to the deep cervical lymphatic nodes and shares messengers with the immune system. One group of these common transmitters is the catecholamines. Immunocompetent cells have been shown to contain and produce catecholamines, serotonin, melatonin and acetylcholine (21-25).

Lymphocytes have a cellular uptake mechanism, but are also capable of the endogenous synthesis of dopamine (DA) and norepinephrine (NE). Additionally, they are able to store and degrade catecholamines and possibly to regulate their own activity via an autocrine loop (26).

The catecholamines secreted by the sympathetic nervous system predominantly act on human T cells of the CD8+, CD28- (suppressor) subset (27). This subset has the highest β-adrenergic receptor density.
NE stimulates, while noradrenergic denervation diminishes the Th1 responses (cellular immunity). Humoral immunity is also affected, perhaps via additional signaling to B cells, NE favoring IgM responses and noradrenergic denervation favoring a shift from IgM to IgG responses (28).

The regulation of lymphocyte functions by catecholamines could prove to be an important part of immune deactivation in the nervous system. Studies on human neutrophils and peripheral blood mononuclear cells demonstrated a catecholamines lifecycle in these cells, suggesting the presence of autoregulatory adrenergic mechanisms (29-31).

We hypothesized that the deactivation of the immune system after the MS relapse (remission) could be mediated by catecholamines.

1.3.3. The role of antioxidants in the blood

Mickel (32) proposed that a lipid peroxidation disturbance caused by free radicals is involved in the breakdown of the myelin sheath. Since then, a number of studies have demonstrated the role of increased free radical production and/or a decreased antioxidant defense in the CNS as causal factors of MS (33-36). Increased lipid peroxide levels have been observed both in the CSF and in the blood of MS patients (34,37). Catalase, superoxide dismutase, glutathione peroxidase and glucose-6-phosphate dehydrogenase, which may protect cell membranes from peroxidative reactions, display different activities in the erythrocytes of patients and controls (37-43). The primary defense of blood against reactive oxygen species is the glutathione redox system of the erythrocytes. In addition to protecting the host cell, erythrocyte glutathione (GSH) can efficiently defend other tissues (44,45). The protective mechanism involving GSH results in an increased formation and subsequent translocation into the plasma of oxidized glutathione (GSSG). The plasma GSSG provides a sensitive index of the whole-body oxidative stress (46,47). Other radical-scavenging antioxidants in the blood include plasma free sulfhydryl groups (SH groups), alpha-tocopherol, retinol, and uric acid. These scavenger molecules function individually at their own sites but may also act cooperatively or in a synergistic way to afford appropriate protection against oxidant attacks.

We examined the balance between the plasma concentration of lipid peroxides and blood nonenzymatic antioxidants (GSH, alpha-tocopherol, retinol, plasma SH groups and uric acid) in relation to the clinical state of MS patients. Furthermore, we were interested in establishing whether β-INF therapy, which has been shown to induce positive clinical results in MS (48), has any effect on the tested parameters.

1.4. New therapeutic approaches

In the last 10 years, immunomodulant therapy has been introduced for MS. The first representatives of this group are the β-INF (48). The IFNs are proteins belonging in the cytokines family. They are integral parts of the immune process exerting immunomodulatory, antiviral and antiproliferative effects. The IFNs are classified into three major groups: alpha, beta and gamma. IFN-alpha is produced by B-lymphocytes, natural killer (NK) cells and macrophages. IFN-β is produced by fibroblasts; epithelial cells, monocytes and macrophages, while IFN-gamma is synthesized by T-lymphocytes and NK cells (49,50).
Recombinant IFN-β-1b is expressed as a non-glycosylated protein in E. coli. It differs in structure from human IFN by 2 amino acids and the lack of glycosylation (51). IFN-β-1b decreases lymphocyte migration (52) directly, since there are significant changes in the MRI images even at the beginning of the therapy. Another research group found that IFN-β-1b hinders lymphocyte migration to the CNS by decreasing the activity of the enzyme matrix metalloproteinase-9 (MMP-9) (52). There is evidence of a close relation of IFN-β-1b and the adhesion cascade that also prevents activated lymphocytes from entering the CNS. The increase in the level of soluble cell adhesion molecules correlated with a decrease in the number of contrast enhancing MRI lesion (53).

IFN-β-1b acts on several levels of the immune system. It down-regulates the level of antigen-presentation by activating T-cells. It has an antiproliferative effect and regulates the Th1-Th2 balance. Finally, on the cytokines level it decreases the amount of INF-gamma and increases the amounts of IL-10 and TGF-β (54,55). IFN-β-1b (Betaferon) was the first medicament, which proved to decrease the number of exacerbations, by 34% in relapsing-remitting and relapsing-progressive MS in a multicenter, double blind placebo-controlled trial (48). As compared to the placebo arm, the rate of patients free from exacerbations was 100% higher. The time to the first exacerbation and the number of days of hospitalization decreased, and the active MRI lesions decreased by 40% (56). As concerns the neurological condition of the patients, there was no significant difference between the placebo arm and the treatment arm; however, there was a trend to an improvement in the treatment arm (48). In our phase IV clinical trial, we provide data relating to 31 patients with a 1 and a 3-year follow-up.

2. Methods

2.1. Quantitative and qualitative analysis of CSF proteins

In 1996, we examined 37 clinically definitive MS patients (1). The mean age of the patients was 37.1 (20-67 years), and the male/female ratio was 1:3. Lumbar punctures were made in the L3/L4 or L4/L5 interspace. Quantitative and qualitative analysis of CSF and serum proteins were made (2). Following the macroscopic evaluation of the CSF, we measured the number of cells in a Fuchs-Rosenthal chamber. Quantitative analysis of the CSF and serum proteins was carried out by laser immune-nephelometry (57). We determined the IgG, IgA, IgM and albumin (Alb) contents in the serum and CSF.

The following formula was used for the quantitative determination of intrathecal IgG production:

\[
\text{IgG index} = \frac{(\text{CSF-IgG/S-IgG})}{(\text{CSF-Alb/S-Alb})}
\]

The concentrations of Alb and IgG, IgM and IgA in the CSF were expressed in mg/l and those in the serum in g/l. (59).

The qualitative analyses of the CSF and serum proteins were performed by agarose gel electrophoresis and isoelectric focusing (60,61).

2.2. Prevalence of MS in the city of Szeged and Csongrád County

The epidemiological study was carried out in our MS Outpatient Unit, the only facility that has dealt with the medical care of MS in the city of Szeged during the last 35 years. This Outpatient Unit specializes in the care and nursing of the MS patients in the South-Hungarian region. In our first
epidemiological study in the city of Szeged, between 1990 and 1997 (62), we checked the medical records of all general practitioners, neurological and ophthalmologic departments and social homes in the city. On the basis of these medical records (onset of disease and clinical course), patients with probable MS were registered and examined by our outpatient unit. The diagnoses were established by using the diagnostic criteria of Poser et al. (1). For all patients, we performed brain MRI, and in some patients additionally optic nerve and/or spinal cord MRI and analysis of the CSF. Oligoclonal bands (OCBs) were determined by isoelectric focusing, and laser-nephelometry analysis was used for the quantitative determination of proteins to calculate the Link index (2). In all cases of CSF analysis, we obtained the informed consent of the patients. The criteria for the A2 subgroup were 2 clinically proven lesions and positive MRI imaging. The criteria for the B subgroups were at least 1 lesion (B1) or 2 lesions (B2) proven clinically plus positive MRI imaging, positive OCBs in the CSF, and a positive Link index (2).

The degree of physical disability was determined by using the Kurtzke expanded disability status scale (EDSS) (63).

The outpatient unit has had an MS Register since 1996, with up-to-date records on the Szeged MS patient population. We examine patients every 3 months in order to determine the neurological status and the EDSS score. In the event of relapses, we perform an extra neurological examination and, if necessary, admit the patients to hospital. After discharge, a follow-up visit is scheduled for 2-4 weeks later and we recheck the neurological status and the EDSS score.

If a patient moves from the city or dies, we updated the register. At the end of the year, we calculate the yearly incidence of the disease.

On the basis of the methods used in this register, we have performed a wider survey in Csongrád County as a whole. According to the files of the Hungarian Central Statistical Office, the city of Szeged (the county town of Csongrád County) has 198,686 inhabitants, while Csongrád County has an additional 222,506 inhabitants. In the present study, 400,128 inhabitants were taken as representative sample: the population of a small city with 21,064 residents and 12 MS patients were excluded but this did not modify our results. The above method was applied to record and diagnose MS patients among 201,442 inhabitants of Csongrád County.

We chose 1 July 1999 as the prevalence day. We determined the prevalence of the disease and the male/female ratio. We estimated the following parameters as concerns the clinical forms of the disease: the distribution of the patients; the average age at the onset of the disease; the average duration of the disease; the average age on the prevalence day and the EDSS score based on the most recent examination (1 April -1 July 1999).

2.3. New aspects of the pathomechanism
2.3.1. Etiology: genetic and/or environmental factors?

We diagnosed 3 sisters with new-onset MS. These cases suggested the role of genetic or environmental factors in the etiology. The sisters were diagnosed both clinically and by laboratory methods (MRI,
2.3.2. Involvement of the sympathoadrenergic mechanism

**Patients and controls**

Totals of 58 patients were examined and were found to have clinically definitive and laboratory-supported definitive MS according to the Poser criteria (1). In the patient group, 10 subjects were laboratory-supported definitive (first-attack) patients, while 48 exhibited relapsing-remitting course MS. Both the CSF findings (OCBs with isoelectric focusing electrophoresis) and the MRI examinations (several periventricular T2-weighted lesions) of the first-attack patients supported the diagnosis of MS. All the relapsing-remitting patients were in remission, none of them had received steroid therapy within 30 days and none of them were on tricyclic antidepressants, cardiac drugs or amantadine. The neurological conditions of the patients were expressed as Kurtzke EDSS scores (63). Healthy individuals (n=19) served as controls. The ethical committee of Albert Szent-Györgyi Medical School (886/1998) had approved the study. For statistical analysis patient's subgroups were formed according to a. / the clinical course of the disease: first-attack (10 cases), or relapsing-remitting (48 cases); b. / the EDSS score: EDSS score < 4.0 (49 cases) or > 4.0 (9 cases); c. / duration of the disease: < 5 years (30 cases) or > 5 years (28 cases); d. / time since last relapse: relapse within 6 months (19 cases) and more than 6 months (39 cases).

**Preparation of lymphocytes**

Peripheral vein blood samples (12 ml) were prepared by centrifugation at 2,500xg for 10 min. The lymphocytes were isolated by centrifugation on a Lymphoprep® (Nycomed Pharma, Oslo, Norway) density gradient and, after washing and centrifugation steps, kept at -80°C until analysis. The lymphocytes were extracted by adding 25 μl perchloric acid (containing 1 mM NaEDTA and 1 mM \( \text{Na}_2\text{SO}_3 \)) to the pellet and ultrasonicated on ice for 2 min using a MSE Soniprep 150 probe. After centrifugation (30 min, 4°C, 35,000xg) the supernatant was frozen and stored at -80°C until analysis. The pellet was used for spectrophotometric protein quantitation with the bicinchoninic acid protein assay reagent (Pierce Chemical Company, USA).

**Capillary electrophoresis with electrochemical detection**

The capillary electrophoretic system used was described in detail earlier (21,22,29). Briefly, a buffer-filled fused silica capillary (Polymicro Technologies, Phoenix, USA) measuring 10 μm in I.D. and 65 cm in length was placed between two buffer reservoirs. High voltage was applied at the injection end, and the reservoir containing the detecting end was held at ground potential. Electrokinetic injection was used for all sample introductions, procedures for 5s at 30 kV; the sample volume was approximately 600pl. The easily oxidized analytes were detected in the amperometric mode with a two-electrode configuration, using optimized end-column detection (26). A Carbon-fiber microelectrode was inserted into the end of the electrophoresis capillary and held at 0.8 V versus a sodium-saturated calomel electrode. Reagents: 2-(N-morpholino) ethanosulfonic acid (MES), serotonin (5-HT), norepinephrine (NE), epinephrine (E), dopamine (DA), L-hydroxyphenylalanine (L-DOPA), VMA, MHPG, HVA and...
dihydroxyphenylacetic acid (DOPAC) were obtained from Sigma and used in the form received. The electrophoresis buffer was 25 mM MES adjusted to pH 5.65 with NaOH. Calibration standards were prepared as 10mM stock solutions in perchloric acid and diluted to the desired concentration in electrophoresis buffer. Hydrofluoric acid was obtained as a 40% aqueous solution from Aldrich, and used for the etching of the detector end of the capillary. Catecholamine levels of lymphocytes were quantified by direct comparison with the standard electropherograms run before and after the patient sample. The catecholamine content of the lymphocytes is given in fmol/µg protein. Detection limits were determined (for DA, NE 0.13 fmol/µg protein, for E 0.37 fmol/µg protein, and for DOPAC 0.11 fmol/µg protein) and estimated at twice the peak-to-peak noise level by extrapolation from plots of peak area versus concentration. Between the series of runs, the capillary was flushed with 0.1 M NaOH to refresh the inner capillary surface and to maintain reproducible separation conditions. For a more detailed description of the method see Bergquist et al. (21).

Statistical analysis
Kruskall-Wallis test (SPSS 7.5 for Windows) was performed for statistical analysis to compare the catecholamine levels in the healthy controls and the subgroups of MS patients, followed by the Mann-Whitney U test for pair-wise comparisons to examine the differences between the patients and the healthy controls. The Kruskal-Wallis test was also used for the statistical analysis of the differences between the healthy controls and the different MS subgroups (regarding the EDSS score, the medication and the duration).

2.3.3. The role of antioxidants in the blood
Patients
Twenty-five patients with relapsing-remitting MS were included in the study. The definitive diagnosis of MS was confirmed according to the clinical and laboratory diagnostic criteria of the Poser Committee (1). While MRI is regularly used in the diagnosis, for this study the patients were rated by a standardized neurological examination. As ongoing disease activity has been observed on serial MRI scans in clinically stable patients, MRI was suggested to provide a more complete measure of disease activity than clinical evaluation alone. However, Smith et al (64) observed a significant association between the periods of clinical worsening and the MRI findings, including increases in the total number, the number of new lesions, and the total area of gadolinium enhancement. Their finding of a significant correlation between increased MRI activity and a more active clinical disease has been confirmed by more recent studies (65-68).

The patients were divided into the following three groups on the basis of their neurological findings: a./ the exacerbation group, i.e. MS patients during an attack; b./ the remission group, i.e. MS patients during an attack-free, stable period; and c./ the remission + INF group, i.e. MS patients without clinical activity under long-term β-INF treatment (Betaferon, 8M I.U. subcutaneously every second day for the past 2 months). The patients had not received either steroid therapy, or vitamin supplementation during the 3 month preceding the investigation, and none were smokers. In the exacerbation group, none of the
patients had had more than 2 relapses and the average time between the appearance of new neurological signs and blood collection was 3 days. Individuals with lower back pain served as neurological controls. Blood was always collected during fasting in the morning hours. Values affected by age or sex [malone dialdehyde-bis-(diethyl acetal)] (MDA), retinol, alpha-tocopherol, cholesterol, triglyceride and uric acid] were adjusted accordingly.

Plasma lipid peroxides were assayed by the method of Wong et al (69), and were expressed in terms of MDA.

The concentrations of GSH+GSSG and oxidized glutathione in the whole blood hemolysate were measured by combining previously accepted standard methods (70).

The concentration of plasma-free SH groups were determined spectrophotometrically at 412 nm (71).

The protein contents of plasma samples were measured using the method of Lowry et al. (72).

Heparinized plasma samples stored at −70 °C were used for the analysis of retinol and alpha-tocopherol, which were determined by using the method of Catignani and Bieri (73). The concentration of alpha-tocopherol was expressed with reference to plasma cholesterol plus triglyceride (74).

The concentration of uric acid was measured in trichloracetic acid extracts of blood by the method of Harkness et al. (75). Quantitative determination was made by HPLC.

The study was approved by the Human Investigation Review Board of the University, and informed consent was obtained from each patient participating in the study.

Statistical analysis
All data are expressed as means ± standard error. One-way analysis of variance was followed by the least-significant-difference test to determine significant differences between groups. A p value of < 0.05 was considered statistically significant.

2.4. New therapeutic approaches
In Hungary, IFN-β-1b was approved for the treatment of MS in 1996. Patients were enrolled in the clinical trial between July and December 1996 according to the guidelines of the American Academy of Neurology (50) (Table 1.).

Table 1. Guidelines of the American Academy of Neurology for the treatment of MS patients with IFN-β-1b.

<table>
<thead>
<tr>
<th>1. Relapsing-remitting or relapsing-progressive form of MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Clinically and laboratory definitive or clinically definitive MS according to the Poser criteria</td>
</tr>
<tr>
<td>3. Functional status scale: EDSS 0-5,5</td>
</tr>
<tr>
<td>4. Age: 18-50 years</td>
</tr>
<tr>
<td>5. Two relapses as a minimum in the last 2 years before treatment</td>
</tr>
</tbody>
</table>

Thirsty six patients were randomized in line with the above-mentioned criteria. However, the results relate to 31 patients because of the dropouts occurring during the 3 years. All the patients were in the laboratory-supported definite MS category according to the Poser criteria (1). In the original group of
randomized patients there were 26 females and 10 males. 34 patients had the relapsing-remitting and 2 had the relapsing-progressive form of the disease. Of the patients completing the study, 22 were females and 9 males.

The patients injected themselves subcutaneously with 8M IU IFN-β-1b every other day. EDSS scores were determined at the beginning of the therapy, then monthly and in the case of exacerbations before and after high-dose steroid treatment. We controlled the laboratory parameters (routine urine and blood tests, glucose, kidney and liver functional tests) monthly for 3 months, and then every third month. Side effects perceived by the patients or the medical staff was registered monthly. In the event of an exacerbation, we determined the EDSS score before and after the steroid treatment and 1 month later. We calculated the steroid needs and recorded the number of days of hospitalization. We compared the relapse-rates for the 2 years prior to treatment (1995-96) and for the 3 years of IFN-β-1b treatment, the EDSS score, the duration of hospitalization and the steroid needs for remission in 1995-96 and in the 3 years of therapy.

Statistical analysis was performed by one-way ANOVA analysis followed by pair-wise Bonferroni comparison.

3. Results

3.1. CSF in the diagnosis of MS

Intrathecal IgG synthesis was detected in 28 of 37 clinically definitive MS patients according to the Poser criteria (1) by laser nephelometry (57,58). Table 2. Presents the detailed data.

Table 2. Quantitative analysis relating to intrathecal immunoglobulin synthesis

<table>
<thead>
<tr>
<th>No. of cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG index ↑</td>
<td>28/37 (76)</td>
</tr>
<tr>
<td>IgM index ↑</td>
<td>12/28</td>
</tr>
<tr>
<td>IgA index ↑</td>
<td>6/28</td>
</tr>
<tr>
<td>Normal IgG, IgM and IgA Index</td>
<td>7/37 (19)</td>
</tr>
<tr>
<td>Blood-CSF barrier damage</td>
<td>2/37 (5)</td>
</tr>
</tbody>
</table>

During the qualitative analysis of CSF and serum proteins by agarose-gel electrophoresis (60), OCBs were determined in 25 cases. However, the isoelectric focusing test (2,59) revealed OCBs in 34 cases (Table 3).

Table 3. Results of qualitative analysis

<table>
<thead>
<tr>
<th>Agarose-gel electrophoresis (%)</th>
<th>Isoelectric focusing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OGP</td>
<td>25/37 (68)</td>
</tr>
<tr>
<td>Normal protein distribution</td>
<td>8/37 (21)</td>
</tr>
<tr>
<td>Other electropherogram</td>
<td>4/37 (11)</td>
</tr>
</tbody>
</table>
3.2. Epidemiology of MS

We found 248 MS patients alive on the prevalence day in Csongrád County. Two hundred and thirty-eight patients were in the laboratory-supported definite MS category. According to the Poser (1) criteria, 30 patients were in the B2 and 208 patients in the B1 category. Ten patients were in the clinically definitive Poser A2 MS category (with negative CSF OCBs and Link index). The prevalence in Csongrád County was found to be 62/100,000. On the prevalence day, 130 of 248 patients lived in Szeged. The MS register revealed that 26 new MS patients were diagnosed in the city of Szeged between 1 January 1997 and 1 July 1999. In this period, 5 patients died 12 patients moved from Szeged to the county and 9 moved from the county. The incidence of MS in the city of Szeged was 5/100,000 in 1997 and 6/100,000 in 1998. Between 1 January and 1 July 1999, we diagnosed 4 new cases. There were 66 (27%) males and 182 (73%) females, giving a male/female ratio of 1:2.75.

The clinical forms of MS were as follows: 15% benign form, 54% relapsing-remitting form, 20% secondary chronic progressive form and 11% primary chronic progressive form (Table 4). The EDSS score in the benign form was 0-3 points, and the average duration of the disease was 27 years. Sixty per cent of the relapsing-remitting MS patients had EDSS scores of 0-4 points and 33% had EDSS scores of 4.5-6.5 points. Fifty-six per cent of secondary chronic progressive MS patients had EDSS scores 4-6.5 points, while 44% were confined to wheelchairs or bed-ridden (EDSS scores ≥7), (Table 5).

Table 4. Patient characteristics in the various clinical forms

<table>
<thead>
<tr>
<th></th>
<th>Benign</th>
<th>Relapsing-remitting</th>
<th>Secondary chronic progressive</th>
<th>Primary chronic progressive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>37 (15%)</td>
<td>137 (54%)</td>
<td>48 (20%)</td>
<td>26 (11%)</td>
</tr>
<tr>
<td>Mean age at onset of the disease (years)</td>
<td>28</td>
<td>28</td>
<td>30</td>
<td>52</td>
</tr>
<tr>
<td>Range</td>
<td>16-41</td>
<td>16-40</td>
<td>13-47</td>
<td>42-62</td>
</tr>
<tr>
<td>Mean years on the prevalence day</td>
<td>55</td>
<td>36</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>Range</td>
<td>38-72</td>
<td>18-54</td>
<td>39-69</td>
<td>49-68</td>
</tr>
<tr>
<td>Average years duration of the disease</td>
<td>27</td>
<td>8</td>
<td>29</td>
<td>7</td>
</tr>
<tr>
<td>Range</td>
<td>10-34</td>
<td>0-15</td>
<td>5-47</td>
<td>2-13</td>
</tr>
</tbody>
</table>
### Table 5. Distribution of patients according to clinical forms and EDSS score

<table>
<thead>
<tr>
<th>Benign form</th>
<th>Relapsing-remitting form</th>
<th>Secondary chronic progressive form</th>
<th>Primary chronic progressive form</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDSS</td>
<td>N</td>
<td>%</td>
<td>EDSS</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>27</td>
<td>0-4</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>13</td>
<td>4.5-6.5</td>
</tr>
<tr>
<td>1.5</td>
<td>1</td>
<td>4</td>
<td>≥7</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>30</td>
<td>4.5-6.5</td>
</tr>
<tr>
<td>2.5</td>
<td>5</td>
<td>13</td>
<td>4.5-6.5</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>13</td>
<td>4.5-6.5</td>
</tr>
</tbody>
</table>

### 3.3. New aspects of the pathomechanism

#### 3.3.1. Etiology: genetic and/or environmental factors?

The symptoms of Patient I (G. J. born on 30 January 1973) began in 1993 with lower-limb weakness and paraesthesia. The neurological condition showed the following symptoms: paraparesis, muscle strength 4/5, tendon reflexes in the lower limbs +3 and paraesthesia. The CT of the brain and the evoked potentials (EMG, ENG and SSEP) were negative. The condition of the patient improved spontaneously in 2 weeks, and accordingly no further examinations were performed. Two months after her second labor in March 1998, she was admitted because of limb weakness, nystagmus and ataxia. The neurological symptoms were: grade I horizontal nystagmus, mild trunk ataxia, hemiparesis on the left side, muscle strength 4/5, tendon reflexes +4, and a positive Babinski reflex on both sides. EDSS score: 3 points (63). The MRI of the brain corresponded to demyelination (Fig.1).

**CSF findings**

- Cytology: 5/µl lymphocytes
- Total protein: 0.208 g/l
- Albumin index: 3.9 x 10⁻³
- IgG index: 1.31
- Local intrathecal synthesis: 52%

**Electrophoresis:** OCBs (Fig. 2).

For quantitative analysis laser-nephelometry, and for qualitative analysis isoelectric focusing and immunoblotting were used. The degree of local intrathecal synthesis was calculated according to the Reiber formula (57). The somatosensory evoked potentials (n.medianus, n.ulnaris, n.tibialis and n.peroneus) revealed central myelin damage. The VEP showed extended latency on both sides. After the examination, the patient received high-dose methylprednisolone (MP) therapy: 1 g MP intravenously for 3 days, followed by 1 mg/body weight/day MP orally for 11 days (76). Remission was achieved after the MP therapy. The patient was symptom-free (EDSS score: 0). In July 1998, after her second relapse, she received megadose MP therapy and achieved total remission.
Patient II (G.O., born on 22 January 1974); a sister of patient I. exhibited left-side optic neuritis at the initial examinations in February 1998. The visus at admission was 0.1 on the left side. The neurological condition was negative. The MRI findings on the head and optic nerve pointed to demyelination (Fig. 1).

CSF findings
- Cytology: 5/μl lymphocytes
- Total protein: 0.335 g/l
- Albumin index: 4.5 x 10^{-3}
- IgG index: 1.25
- Local intrathecal synthesis: 46%

OCBs were detected by electrophoresis (Fig. 2). VEP: extended latency on the left side P100: 154 msec, on the right side P100: 98 msec. After the examination, the patient received megadose MP therapy; the visus on the left side improved to 1.

Patient III (G.A., born on 8 September 1976); also a sister of patient I. was examined because of paraesthesia in all the extremities and vertigo. The only symptom in her neurological condition was the paraesthesia. EDSS score: 1 point. The MRI of the brain corresponded to demyelination (Fig. 1).

CSF findings
- Cytology: 4/μl lymphocytes
- Total protein: 0.315 g/l
- Albumin index: 4.6 x 10^{-3}
- IgG index: 2.20
- Local intrathecal synthesis: 70%

OCBs were detected by electrophoresis (Fig. 2). VEP: extended latency on the left side P100: 142 msec, on the right side P100: 138 msec. In consequence of her negative neurological condition, despite the subjective complaints, she did not receive MP therapy.

According to the Poser (1) diagnostic criteria, all three sisters had definitive MS: Patient I had the relapsing-remitting form and Patients II and III had first attack MS. The neurological condition and the MRI examination of the parents were negative. We excluded previous neurological disease, and systematically taken medication during recent decades. All 4 living grandparents were healthy, as concerns the neurological findings.
Figure 1. The MRI of the brain corresponded to demyelination
3.3.2. Involvement of the sympathoadrenergic mechanism in MS

The electrophoretic mobilities of the major peaks in the electropherogram corresponded to the calculated electrophoretic mobilities of DA, NE, E, uric acid and dihydroxyphenylacetic acid (DOPAC) (Fig. 3). We excluded the serotonin (5-HT), methoxy-hydroxyphenyl glycol (MHPG), vanilmandelic acid (VMA) and ascorbic acid data because their levels were often under the detection limit (MHPG was detectable in 7/19 controls and in 19/67 MS patients and VMA in 5/19 and 27/67 cases respectively). We also excluded L-hydroxyphenylalanine (L-DOPA) since it is a neutral molecule and has the same electrophoretic mobility as all other neutrals, thereby leading to difficulties with the quantification. The levels of catecholamines are presented in Table 6.
Table 6. Catecholamine contents of peripheral blood lymphocytes in healthy individuals and various subgroups of MS patients

<table>
<thead>
<tr>
<th>Test group</th>
<th>No. of subjects</th>
<th>DA</th>
<th>NE</th>
<th>E</th>
<th>DOPAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy individuals</td>
<td>19</td>
<td>1.39±0.32</td>
<td>1.59±0.60</td>
<td>*50.0</td>
<td>*43.8</td>
</tr>
<tr>
<td>RR (first-attack MS)</td>
<td>10</td>
<td>1.89±0.85</td>
<td>0.24±0.10a</td>
<td>*30.0b</td>
<td>*62.9c</td>
</tr>
<tr>
<td>RR (MS in remission)</td>
<td>48</td>
<td>1.68±0.33</td>
<td>0.48±0.11a</td>
<td>*36.0d</td>
<td>*40.0</td>
</tr>
</tbody>
</table>

Values given as mean±SEM fmol/µg protein and as *mean ranks.

* Significant difference between the first-attack and relapsing-remitting MS patients and healthy the controls with the Kruskal-Wallis test, p=0.027
b Significant difference between the first-attack and the relapsing-remitting MS patients and the healthy controls with the Kruskal-Wallis test, p=0.028
c Significant difference between the first-attack MS patients and the healthy controls with the Mann-Whitney U test, p=0.035
d Significant difference between the relapsing-remitting MS patients and the healthy controls with Mann-Whitney U test, p=0.017
e Significant difference between the first-attack and the relapsing-remitting MS patients with the Mann-Whitney U test, p=0.008

Healthy controls versus first-attack and relapsing-remitting MS patients

When the MS patient subgroup and the healthy individuals were compared, significantly lower levels of NE (Kruskal-Wallis test, p=0.027) and higher levels of epinephrine (Kruskal Wallis test, p=0.028) were found in the lymphocytes. Pair wise comparisons with the Mann-Whitney U test showed that the relapsing-remitting MS (p=0.017) and the first-attack MS patients (p=0.035) had lower levels of intracellular NE than in the healthy controls (Table 6). The E content of the lymphocytes in the first-attack MS patients was higher than those in the relapsing-remitting MS group (p=0.008) and the controls (p=0.056).

Differences between healthy controls and the MS subgroups regarding EDSS Scores, duration of the disease and medication

Both the MS patients with a short disease duration (n=30, mean ± SEM: 378 ± 90 fmol/µg) and those with a longer disease duration (n=28, mean ± SEM: 453 ± 154 fmol/µg) displayed lower intracellular NE levels (Mann-Whitney U test, p=0.033) as compared with the control group (n=19, mean ± SEM: 1594 ± 599 fmol/µg). The lymphocytes of both the patients in a better neurological condition (n=49, mean ± SEM: 368 ± 64 fmol/µg) and those with an EDSS score >4 (n=9, mean ± SEM: 807 ± 516 fmol/µg) contained less NE (p=0.036) than the cells of the controls (n=19, mean ± SEM: 1594 ± 599 fmol/µg). The administration of anxiolytics did not exert any significant effect on the catecholamine levels of the lymphocytes. Slight, non-significant differences in the NE contents (p=0.061) of the lymphocytes were found between the group without immunomodulating medication (n=42, mean ±
SEM: 332 ± 56 fmol/µg), those receiving INF-β-1b treatment (n=9, mean ± SEM: 450 ± 235 fmol/µg),
those receiving glatiramer acetate treatment (n=7, mean ± SEM: 1039 ± 649 fmol/µg) and the controls
(n=19, mean ± SEM: 1594 ± 599 fmol/µg).

3.3.3. The role of antioxidants in the blood

The plasma concentration of MDA was 38% higher in the exacerbation group than in the control group
(Table 7), but this rise was not statistically significant. The blood GSSG concentration was
significantly higher (p<0.005) both in patients with exacerbation than in all other groups (Fig. 4). The
blood GSH level was higher (p<0.001) both in patients with exacerbation and in patients in remission
than in the controls (Fig. 5).

The level of plasma-free SH groups were significantly higher (p<0.001) during exacerbation than in the
other groups (Fig. 6).

No significant difference in retinol level appeared between the tested patients and the controls (Table
7.). Both the plasma alpha-tocopherol concentration (Table 7) and the ratio of alpha-tocopherol to
cholesterol plus triglyceride (Fig. 7) were significantly lower (p<0.001) during exacerbation. INF-β
increased the plasma alpha-tocopherol level (p<0.001), but not the lipid-corrected alpha-tocopherol
level as compared to the controls. The cholesterol and triglyceride levels did not differ significantly
between the groups (Table 7).

There was no significant difference in plasma uric acid between the patients and the controls.

Table 7. Plasma concentrations of MDA, protein, retinol, alpha-tocopherol, triglyceride,
cholesterol, and uric acid in controls and MS patients

<table>
<thead>
<tr>
<th></th>
<th>Control group (n=9)</th>
<th>Exacerbation group (n=7)</th>
<th>Remission group (n=12)</th>
<th>Remission+INF group (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (µmol/l)</td>
<td>1.32±0.15</td>
<td>1.82±0.21</td>
<td>1.66±0.15</td>
<td>1.47±0.15</td>
</tr>
<tr>
<td>Protein (g/l)</td>
<td>68.44±2.63</td>
<td>63.91±1.81</td>
<td>65.56±1.67</td>
<td>67.17±2.22</td>
</tr>
<tr>
<td>Retinol (µmol/l)</td>
<td>2.02±0.55</td>
<td>2.00±0.50</td>
<td>2.68±0.38</td>
<td>2.58±0.34</td>
</tr>
<tr>
<td>Alpha-tocopherol (µmol/l)</td>
<td>29.50±2.33</td>
<td>15.65±2.58</td>
<td>34.88±2.26</td>
<td>39.88±4.23</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.09±0.12</td>
<td>0.84±0.11</td>
<td>1.22±0.13</td>
<td>1.34±0.11</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.16±0.30</td>
<td>5.18±0.61</td>
<td>5.98±0.34</td>
<td>5.86±0.38</td>
</tr>
<tr>
<td>Uric acid (µmol/l)</td>
<td>247±17</td>
<td>251±22</td>
<td>238±19</td>
<td>240±21</td>
</tr>
</tbody>
</table>

* p<0.001, exacerbation group vs. all the other groups

** p<0.001, remission +INF group vs. control and exacerbation groups
Figure 4. Plasma GSSG concentration in the controls and the MS patients. In the exacerbation group, the plasma GSSG concentration was significantly higher than in all other groups (*p<0.05)

Figure 5. Plasma GSH concentration in the controls and the MS patients. The GSH level was significantly higher in the patients with exacerbation and in the patients in remission than in the controls (**p<0.01)
Figure 6. Plasma SH group concentration in the controls and the MS patients. The patients with exacerbation had a significantly higher concentration of SH groups than all other groups (**p<0.01)

Figure 7. Ratio of plasma alpha-tocopherol to cholesterol plus triglyceride in the controls and the MS patients. The patients with exacerbation had a significantly lower ratio than all other groups (***(p<0.001)
3.4. New therapeutic approaches

Dropouts: From the 36 patients, 31 completed the study. The data on the 5 dropouts were as follows: One female patient had constant high fever, and diffuse muscle pain occurred and remained unchanged in the first 5 months of the treatment. Because of squeezing chest pain, she was taken to the Intensive Care Unit with the suspicion of myocardial infarction. IFN-β-1b therapy was stopped on the first day of observation. ECG, cardiologic ultrasonography and enzyme levels excluded the possibility of myocardial infarction. After the discontinuation of IFN-β-1b, her fever stopped. On the second day of intensive observation, she became tetraparetic. This symptom was regarded as a new relapse of the disease. Following high-dose steroid treatment, the patient remitted and her muscle pain ceased. After this, we discontinued further IFN-β-1b therapy at the patient's request because of the side effects. In the second year of the therapy, another female patient died; this was unrelated to her MS. In one case, pregnancy was the cause of the dropout after one and a half years. In 2 other cases (one male and one female patient), non-compliance was the cause of the dropout in the second year of the treatment.

Clinical results. The data on the 31 completed patients were as follows: the mean age at baseline was 37 ± 8 years, the mean EDSS score at baseline was 1.8 ± 1.2 and the mean duration of the disease at baseline was 4 ± 4 years (range 3-18).

The time to the first exacerbation after the beginning of treatment was 312 ± 309 days and the progression index was 0.86. In the 2 years before the treatment, the 31 patients had altogether 80 relapses. In the 3 years of treatment, the total number of relapses was 36 (Table 8). Before treatment, the annual relapse rate was 1.5 ± 3.1 (total number of relapses: 80), while as a result of the treatment it decreased to 0.3 ± 3.7, i.e. the IFN-β-1b treatment reduced the relapse rate by 77% as compared to the auto control (one-way ANOVA, p<0.05) (Fig. 8). The proportion of relapse-free patients was about 32% (10/31), 39% of the patients had 1 relapse (12/31), 16% had 2 relapses (5/31), 7% had 3 exacerbations (2/31) and 3% had 6 or 8 relapses, respectively (1/31).

The mean EDSS score at baseline was 1.8 ± 1.2 and after 3 years of treatment it was 2.2 ± 1.4, but the difference did not attain statistical significance (p=0.368). In 1995-96, before the start of therapy, the 31 patients spent a total of 628.5 days on average in hospital annually. In the 3 years of IFN-β-1b, the duration of hospitalization decreased by 87% (one-way ANOVA, p<0.05) (Fig. 9). In the 2 years preceding IFN-β-1b therapy, 211.5 g of methylprednisolone was needed to treat the relapses of the patients. IFN-β-1b therapy reduced this methylprednisolone need by 80% (p<0.05) (Fig.10).

Side effects: At the beginning of the therapy, subfebrility or fever was experienced on the days of injection in 30 patients. These symptoms ceased by the third month in 24 patients, but became permanent in 6 patients. In 30 patients, painful erythema appeared that gradually decreased in intensity by the third month; in 3 patients, necrosis evolved at the injection site in the second year of the treatment. As a result of the injection, small, painless erythema occurred sporadically throughout the 3 years, but this symptom was well tolerated by the patients as compared to the initial reactions. In the first month, 10 patients had flu-like symptoms, and 3 patients complained of frequent tension headaches. In 3 patients, emotional lability and imperative crying attacks appeared, which reacted well to antidepressants and ceased after 3 months. No patient needed prolonged antidepressant treatment. In
the course of the treatment, a decreased leukocyte count and elevated liver enzymes were observed in 6 patients, but these changes were not clinically relevant.

Table 8. Differences observed during the 3 years of IFN-β-1b treatment

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of relapses</td>
<td>30</td>
<td>50</td>
<td>14</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>Steroid needs (g)</td>
<td>135</td>
<td>218</td>
<td>54</td>
<td>52</td>
<td>24</td>
</tr>
<tr>
<td>Relapse rate*</td>
<td>1,0</td>
<td>1,6</td>
<td>0,4</td>
<td>0,4</td>
<td>0,2</td>
</tr>
</tbody>
</table>

* Relapse rate = annual relapse rate/number of patients

Number of patients: n=31

Figure 8. Number of relapses/year

Figure 9. Duration of hospitalization before and during IFN-β-1b treatment
4. Discussion

4.1. CSF in the diagnosis of MS

The Charcot Foundation recommends the isoelectric focusing technique as the most sensitive, essential test method in the qualitative analysis of the CSF and serum proteins (2). In clinically definitive MS patients, the sensitivity of OCB detection was 91% by isoelectric focusing, but only 68% measured by agarose-electrophoresis. Link and Tibbling (58) were able to identify OCBs in 98% of patients with definitive MS. In a study of 2043 patients, Laterre et al. (77) demonstrated 2 or more OCBs in 87% of MS patients. Mehta and Patrick (78) and Chu et al. (79) detected OCBs in 95% of MS patients. Our results corresponded with the international data. We utilize this method in our epidemiological studies and in the daily routine MS diagnostic.

4.2. Epidemiology of MS

Dean (4) determined the prevalence of MS within continental Europe as 30-80/100,000. The occurrence was found to be related to the geographical distribution, migration and a genetic contribution (6,80). A distinction between low, medium and high-risk factor areas can be made on the basis of geographical lines of latitude. However, the new prevalence tests reveal that in both low and medium-risk factor areas, the rate can in fact be higher (81-87). There are both pro and con arguments for a north-south gradient distribution (88). Rothwell and Charton (89) found a high prevalence rate of MS in South-east Scotland, suggesting that the Scottish population as a whole has a genetic susceptibility to the disease.

In 1983, Pálffy et al. (12) reported the prevalence in Baranya County as 37/100,000. In 1997, the prevalence was 65/100,000 in the city of Szeged (62); in 1999 it was 62/100,000 in Csongrád County, including the population of Szeged. The prevalence of MS for the 400,000 inhabitants of Csongrád County is nearly the same as the previous prevalence data from Szeged and prevalence other communities in the same geographical region.

The male/female ratio in Szeged was to be found 1:3, which differs from the international data (3), because the ratio of females was higher by 8.5% than that of males. The mean age was lower by 5.5 years in the city than in the county. In Csongrád County, this ratio was 1:2.75, which is comparable to the international data.
In Szeged, the distribution of the patients by clinical forms of the disease was 5% benign form, 80% relapsing-remitting form, 4% secondary chronic progressive form and 11% primary chronic progressive form.

In Csongrád County, we found 15% benign form, 54% relapsing-remitting form, 20% secondary chronic progressive form and 11% primary chronic progressive form for the patients with MS.

The proportion of secondary chronic progressive MS patients in the county was higher (20%), than in the city of Szeged (4%). Most of these MS patients were found in homes for the aged in the county. Because of the progression of the disease and the worsening social status these chronic progressive MS patients moved to smaller, colonies from the city. Therefore, the MS population in the city is younger than that in the county (difference in average age 5.5 years). In the current and a previous epidemiological study in this region the ratio of the primary chronic progressive MS was 11%, which is equal to the international data.

The average duration of the disease in the benign form of MS was 27 years, and the EDSS score was between 0 and 3. These data fit the criteria of benign MS.

Sixty per cent of relapsing-remitting MS patients were capable of normal activities; only 7% were confined to a wheelchair and required help. Of the relapsing-remitting patients, 21% were treated with INF-β-1b or glatiramer acetate to modify the EDSS score.

Of the secondary chronic progressive MS patients, 56% had and EDSS score between 4 and 6.5, while 44% were confined to a wheelchair and required help.

In the larger population of Csongrád County (a more representative population than that in Szeged) the distribution of the patients by the clinical forms of the disease was comparable to the international results (3).

To finance the new and expensive therapeutic approaches (INF or glatiramer acetate) the healthcare system needs the most exact data possible about the prevalence of MS and the EDSS classification of the disease by clinical forms. The sample in the current epidemiological study represents 4% of the Hungarian population, which could be enough to estimate the national expenditure.

4.3. New aspects of the pathomechanism
4.3.1. Etiology: genetic and/or environmental factors?

Adoption studies suggest that the familial risk of MS susceptibility is influenced by genetic rather than by environmental factors (6,90,91). The influence of environmental factors is difficult to exclude, since twins and siblings generally share the same environment. For a differentiation between the influence of genetic and environmental factors, populations of adopted children and their parents have been used with success. A comprehensive Canadian study of recurrence showed a lifetime risk of 0.2% for the entire population, which increased to 3% in other first-degree relatives (relative risk 20) and 1% in second and third-degree relatives (relative risk 5.5) (92). Comparison studies from the United Kingdom confirmed higher recurrence rates for sisters (4.4%) and brothers (3.2%) compared with parents (2.8%) and offspring (1.8%). The reduction in risk varies from 2.8% in first-degree relatives to 1% and 0.9% in second and third-degree relatives, as compared with the background age-adjusted risk in this area.
population of 0.3% (93). Previously published data indicated that the family risk ranges from 300-fold for monozygotic twins to 20-40-fold for biological first-degree relatives over the general population prevalence of 0.1% (6). The prevalence of familial MS in first-degree relatives is 5-10%, while that in monozygotic twins is 20-30% (94). These findings support the role of genetic factors in MS. The role of environmental factors is greater in mono- than in dizygotic twins than in first-degree relatives, which leads to confusion concerning the etiology (90). Adoption studies suggest that the familial environment plays a role in the development of MS, while the frequency of the disease in non-biological relatives is equal to that in the normal population (6).

According to these findings, the familial occurrence of MS is genetically determined. We report here on 3 affected sisters, whose parents had no neurological or autoimmune disease in their medical history. Since the familial environment does not necessarily lead to the disease, a genetic mutation of the sisters might be possible. Although the parents and grandparents were healthy, we cannot exclude a common viral infection in their childhood nor some other environmental factor, as a triggering factor of the disease. The crude risk of MS in the Northern-European population is 1:600. Accordingly the case of these affected sisters might be interesting and their further follow-up, with encoding of the natural over- or under-expression might lead to a better understanding of MS (95).

4.3.2. Involvement of the sympathoadrenergic mechanism in MS

Modern analytical tools such as capillary electrophoresis techniques allow the detection of intracellular catecholamine levels and give an insight into their regulation of lymphocyte differentiation, proliferation and apoptosis. The increased β-adrenergic receptor density on the lymphocytes of MS patients in relapse suggests an involvement of lymphocytes and catecholamines in the pathogenesis of the disease. A general problem in MS research is that the phenomena observed can either be secondary to the disease progress with no causality, or reflect mechanisms of importance for the disease.

Scattered reports suggest a role for low molecular weight neurotransmitters in the pathogenesis of MS. Elevation of the levels of NE by using antidepressants and L-DOPA have been found to affect the symptoms of MS (96). Maprotilin and lofepramin enhance the levels of NE in the synapses (97). Seventy-five per cent of MS patients treated with L-DOPA experienced an improvement after 1-2 month (96). Numerous studies have revealed that NE may regulate early immune events such as antigen localization, presentation, B-cell activation, inhibition of T-suppressor cell activation and of both Th1 and Th2-cell functions (98,99). NE may also suppress the normal immune response (100). Elevated levels of NE have been observed in the CSF, but not in the blood of MS patients (101). It has been hypothesized that there is a deficiency of NE in the nerve terminals in MS, similar to the DA deficiency in Parkinson disease patients. This hypothesis is supported by the fact that near the fourth ventricle lies the locus ceruleus, a NE-mediated part of the brain regarded as a "stress center". Lower levels of NE in MS could possibly explain the reduced awareness and memory function, difficulties with micturition and cerebellar symptoms, which are the opposite of the "fight or flight" reactions (96). Recent MRI and neuropathological findings suggest an early axonal damage in MS that could be prognostic for the further disease progression (102-105). If the neurons are damaged, there could be an ungoverned release of catecholamines and high local concentrations in the area of the lesion. The
lymphocytes in the region may be exposed to these high concentrations and at high intracellular levels by an initial uptake (as may be possible in the first-attack form).

We found changed intracellular catecholamine levels in the peripheral blood mononuclear cells (PBMCs) of MS patients. The changes reflect the whole PBMC population and probably only a small proportion of them are directly involved in the CNS pathogenesis. However, if the effect of immune regulation in MS is more systemic, this could be measured in the periphery. Normally, just a few leukocytes are present in the CSF and the collection of these cells would be very difficult and demand single-cell analysis. After considering these problems, we concentrated on collecting PBMCs. The inclusion criteria for first-attack patients were several T2-weighted lesions on the brain MRI and positive CSF findings (OCBs, elevated IgG levels in the CSF, and positive IgG immunoblots). In the city of Szeged, the incidence of MS in 1996 was 7/100,000/year (62). Because of the low number of first-attack MS patients, it was difficult to add more data to this group.

Catecholamines also affect the NK cell function through the β-adrenergic receptors (106). Activated lymphocytes have increased numbers of muscarinic and nicotinic receptors (107). A number of reports suggest involvement of the catecholaminergic system in MS. A 2-fold increase in β-receptor density was found on the PBMCs during relapse in relapsing-remitting MS patients and during secondary chronic progression, while the levels of NE and E in the plasma were similar to the control levels (108). From patients with chronic progressive MS, an increased number of β-adrenergic receptors were found on CD8+ T cells. In contrast, patients with stable MS and those with relapsing-remitting form disease before, during or after attacks had unchanged receptor densities (27). The plasma E levels in the samples drawn from patients in the supine or upright position were similar in chronic progressive MS to those for normal individuals, but the supine plasma NE levels were higher in chronic progressive MS (107). In a recent study, the percentages of T and B cells in the peripheral blood from MS patients in relapse, with viral inflammatory or with non-inflammatory neurological disease, were similar (110). Various cell surface molecules on peripheral blood CD4+ T cells and disease activity by MRI examination were monitored in relapse and in remission, but no differences and no correlation to disease activity could be found (111). No differences in plasma DA-β hydroxylase activity have been reported between healthy individuals and MS patients (either in relapse and remission). (112). The synthesis of catecholamines in the lymphocytes is under nicotinic control, and acetylcholine might regulate catecholamine synthesis through activation of the rate-limiting enzyme tyrosine hydroxylase (113). We have not encountered any other data on differences in the enzyme activity related to the catecholamine metabolism in the lymphocytes or peripheral blood of MS patients.

We observed higher intracellular levels of E in first-attack MS patients and the lymphocytes express primarily β-adrenergic receptors. Thus, we can propose the following hypothesis, presented schematically in Fig.11. An increased level of E activates the lymphocytes; they cross the BBB and find their antigens. This process is followed by the production of cytokines, which either results in an inflammatory process or acts as the major compartment in the relapse process. A relapse-increased β-receptor density on the lymphocytes has been described, lending support to our hypothesis (108). It is
not clear whether the lymphocytes merely mirror the state of the disease, reflecting the altered hypothalamus-pituitary gland-adrenal medulla (HPA) axis function and drain the catecholamines from the plasma, or are active participants, eliminating the catecholamines by uptake and degradation, or releasing them into the MS plaque. The lower level of NE in the peripheral blood lymphocytes of relapsing-remitting MS patients in remission could be due to the β-adrenergic receptor down-regulation after a bout or to the degradation of the catecholamines. Remission may be due to a general down-regulation of the immune response by immunologically non-specific mechanisms, such as the endogenous secretion of corticosteroids. Later in the disease process, a negative feedback suppresses the production of the catecholamines, resulting in a decreased catecholamine content of the peripheral blood lymphocytes during remission. This may explain why relapsing-remitting MS patients in remission may have lower levels of catecholamines such as NE and also account for the neuroimmunological entity of the relapse.

Higher catecholamine levels in the peripheral blood lymphocytes might prevent relapses. Catecholamines have a relatively short duration of action, which could be triggered by widespread activation, except when the levels are chronically changed. One of the risk factors for autoimmunity is the low NE level in MS patients, which reflects the low activity of the HPA axis.

Relapses can be induced by infection, stress, or an elevated level of E, which activates the lymphocytes, resulting in turn in activation of the disease. After nicotinic activation of the lymphocytes and intracellular NE and L-DOPA production occurs (113). The catecholamine levels may play an important regulatory role, especially in relapsing-remitting MS patients when the β-receptors on the lymphocytes are increased. This needs to be further investigated before any strong conclusions may be drawn.

MS patients have a significantly lower NE content in their peripheral blood lymphocytes than that for healthy individuals, but in the early stage of the disease, and hence in first-attack patients the E content is higher. With regard to the fact that the lymphocytes in relapse have a higher β-receptor density, new means of early intervention in the pathogenesis of MS at the lymphocyte level may be possible. These data suggest a connection between the peripheral blood lymphocytes catecholamine content and the course of the disease, and may contribute to a better understanding of the pathogenesis of MS. They may also suggest a new therapeutic approach through recognition of the role played by lymphocytes in this disease.
4.3.3. The role of antioxidants in the blood

The oxidation of GSH i.e. the elevation in the GSSG concentration and the simultaneous decrease in alpha-tocopherol level in the blood of MS patients, provided evidence of an increased generation of reactive oxygen species (ROS) in the active phase of the disease. However, the formation of lipid peroxides was not significantly enhanced during exacerbation.

Both GSH and alpha-tocopherol are potent inhibitors of lipid peroxidation. While, the role of GSH in this process is primarily, if not solely, to prevent the initiation of radical formation, alpha-tocopherol inhibits the propagation of chain reactions (114). The slight rise in the MDA concentration, in contrast with the marked changes in the GSSG and alpha-tocopherol levels, in the patients in relapse suggests that free radical generation is counteracted to a significant extent by the antioxidant defense mechanisms. It is to be noted that the studied relapse period was the first or second relapse for these patients, and that they were at the beginning of exacerbation at the time of the investigation.

The literature data concerning the plasma MDA level in MS are controversial: both significant and also, considerable but not significant elevations, or even normal values have been reported (34,37,42,115). The differences between these results are perhaps, due to differences in the current phase of the disease studied (e.g. the number of exacerbation, or the beginning or end of the relapse or remission periods).
In addition to the GSSG concentration, the GSH level was also significantly increased in the blood of the patients with exacerbation. The increase in GSH under conditions of continuous oxidant generation is likely to be a compensatory mechanism, which defends the cells from further oxidant injuries (116). Hunter et al. (117) reported that MS erythrocytes are less susceptible to hydrogen peroxide-induced lipid peroxidation in vivo. They suggested that the levels of intracellular GSH or another endogenous antioxidant are elevated in MS erythrocytes, and this suggestion accords well with our results. In contrast, Jensen and Clausen (40) found a low glutathione peroxidase activity and a low GSH concentration in MS erythrocytes. They studied clinically stable patients, but this could not be the reason for the inconsistent results, as the GSH level was also increased during remission in our study.

Erythrocyte GSH has also been suggested to be an important component of the inter-organ GSH homeostasis. Dass et al. (118) proposed a model, which includes a substantial output of GSH by the kidney and the liver red blood cells, and the extraction of GSH from these cells by those tissues that have previously been identified as sites of GSH utilization, including the lung, heart, gut and brain (119). According to this model, the elevated level of GSH in the peripheral blood of MS patients observed in the present study may reflect enhanced transportation and bioavailability of this tripeptide and its constituent amino acids to the peripheral tissues, including the CNS.

The increased level of SH groups in the plasma during exacerbation is most probably related to the high GSH content of the erythrocytes. The mediating vehicle between these two environments may be the membrane SH groups (120,121).

The changes observed in the blood of MS patients indicate an enhanced generation of ROS. However, it is not clear how these changes at the periphery are related to the pathology in the CNS. Free radicals are thought to play a major role in destruction of the myelin sheath. Activated macrophages are able to produce both nitric oxide (NO) and superoxide anion (122). While NO itself displays low toxicity, and superoxide also is relatively inert, the interaction of these two radicals results in formation of the powerful oxidant peroxynitrite (123). NO and superoxide can also release iron from ferritin (124,125). Ferritin, an iron-storage protein, is present in almost all cell types, including astrocytes, macrophages, oligodendrocytes and microglia (126). The iron released from the protein bond may react with superoxide anion and hydrogen peroxide to generate hydroxyl radical. Peroxynitrite and the highly reactive hydroxyl radical oxidize DNA and proteins and initiate lipid peroxidation, which in turn may lead to demyelination and neuronal damage. Free radicals may also contribute to damage of the BBB, which is an early event of MS lesions (127).

The role of NO in the pathomechanism of MS is supported by the observation that the levels of inducible nitric oxide synthase (iNOS) messenger RNA are markedly higher in MS brains than in normal controls (33). Strong inducible and constitutive NOS immunoreactivity has been found in macrophages distributed within regions of active demyelination (128). The presence of nitrotyrosine residues has also been demonstrated in the brain of MS patients (127).

The free radicals may be involved in the induction and presumably perpetuation of the disease as well. Experimental autoimmune encephalitis (EAE) is the rodent model for MS. Repeated injection of a scavenger of oxygen radicals starting at the time of EAE induction delayed the onset and markedly reduced the severity of the disease (129). Furthermore, all treated mice completely recovered after 40
days. In another study, the use of an inhibitor of iNOS induction and scavengers of NO and peroxinitrite exerted significant therapeutic effects (130). Treatment with high doses of uric acid (scavenger of peroxynitrite) virtually prevented clinical symptoms of EAE.

Therapeutic agents, which can scavenge or prevent radical formation should also be of significant benefit to the patients. INF-β, with is efficacious treatment for MS, has been shown to inhibit iNOS activity and suppress endogenous NO production (131,132). In the present study INF-β induced a significant elevation in the plasma alpha-tocopherol level as compared to the controls. However, this can be attributed at most only partially to the decreased free radical production and concomitant-vitamin sparing effect of the drug. The plasma concentration of alpha-tocopherol is strongly correlated with plasma lipids, which exert carrier functions for the lipophilic antioxidant in the blood (133), and the lipid-corrected alpha-tocopherol values were not markedly enhanced.

In conclusion, the present study provides evidence of peroxidative reactions in MS patients during exacerbation, and supports the role of oxidative stress in the pathomechanism of the disease.

4.4 New therapeutic approaches

According to the clinical trials during the last 10 years, there is a new strategy in the treatment of relapsing-remitting MS. The therapy can be divided into two parts: the treatment of acute exacerbations and decreasing the activity of the disease. Four drugs are available today for decreasing the activity of the disease. These are various INF-β (la-lb), and glatiramer acetate (46,58,134,135). Our data indicated that IFN-β-1b treatment decreased the relapse rate by 77%, not merely by 34% as in a placebo-controlled trial (46). In a 6-year follow-up of another immunomodulant therapy (glatiramer acetate), the relapse rate was decreased by 72% (136). In a Swiss 1-year study the relapse rate was found to decrease by 49% in 30 patients treated with IFN-β-1b (137). In the trial by Lienert et al. (138) 47% of the patients were relapse-free after 2-years of treatment with IFN-β-1b, and the relapse rate decreased from 1.90 to 1.06, the EDSS score of patients became worse in 20%, improved in 7%, and was unchanged in 73% of the patient. Haas et al. (139) published data on 151 patients. After 1 year of treatment 43.7% of the patients were relapse-free and the relapse rate decreased from 1.4 to 1.0. In the study by Granieri et al. (140) the relapse rate based on the 2 years before treatment was 1.1, while it decreased after 1 year of treatment to 0.83. No data are available referring to a 3-year follow-up similar to our trial. The 77% decrease in relapse rate after 3 years of treatment (apart from the effectiveness of the therapy) is due to the screening of the patients, the low baseline EDSS score, the relatively short duration of the disease (4-5 years), the small number of patients and the open trial. We did not find any side effects different from those already published (52). There was no significant improvement in the EDSS score, which is also in line with published data (48). Multicenter, double-blind clinical trials indicate that IFN-β-1b treatment decreases the number of days of hospitalization and the steroid needs (48,56). However, no numerical data are available. In our study, the number of days of hospitalization decreased by 87%, and the methylprednisolone need decreased by 80%. IFN-β-1b treatment administered in time is able to slow down the complicated immune process responsible for the damage of the myelin sheath and finally the diverse neurological impairment. The long-term importance of
immunomodulants on the relapse rate and the progression of the disease is confirmed by phase IV clinical trials and everyday practice.

5. Conclusions
1. We used the isoelectric focusing to evaluate the OCBs in the CSF of clinically definitive MS patients. OCBs presented in 91% of the MS patients.
2. We determined the prevalence of MS in the population of Csongrád County. With EDDS, we determined the functional status of the patients in the different clinical forms of the disease.
3.1. Through evaluation of the catecholamine contents of the lymphocytes, we found that the peripheral blood lymphocyte level of NE was significantly higher in the first-attack MS patients, than in the controls. However, the NE levels were significantly lower in the relapsing-remitting patients in remission. The catecholamines are known to be able to affect the lymphocyte activity, both by stimulation and by immunosuppression. Our results suggest that the catecholamines are important regulators of lymphocyte activation in MS.
3.2. Nonezymatic antioxidants in the blood were also studied. The changes in these antioxidants suggest increased free radical production and consumption of the scavenger molecules during the active phase of the disease.
4. Our self-control clinical study of INF-β-1b in the treatment of MS patients during a 3-year follow-up period demonstrated a 77% reduction in the relapse rate, a 75% reduction in methylprednisolone need and an 84% reduction in the number of days of hospitalization.
The long-term importance of immunomodulants on the relapse rate and the progression of MS were confirmed by phase IV clinical trials and everyday practice.

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