PAPER

Plasma homocysteine levels in multiple sclerosis

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Background: There is evidence that homocysteine contributes to various neurodegenerative disorders, and elevated plasma homocysteine levels have been observed in patients with multiple sclerosis (MS).

Objective: To investigate if and why plasma homocysteine levels are increased in MS, and whether they play a role in the disease course.

Methods: We compared plasma levels of homocysteine in 88 patients with MS and 57 healthy controls. In the MS group, 28 had a benign course, 37 were secondary progressive, and 23 primary progressive. To explore the underlying mechanisms, we measured serum levels of vitamins B₆ and B₁₂, folate, interleukin (IL)-12, tumour necrosis factor (TNF)- α , leukocyte nitric oxide production, and plasma diene conjugate levels (measure of oxidative stress).

Results: Mean (SD) plasma homocysteine concentration was higher in patients (13.8 (4.9) μ mol/l) than in controls (10.1 (2.5) μ mol/l; $p < 0.0001$). However, there were no significant differences in homocysteine levels between the three clinical subgroups of MS. Serum concentrations of vitamin B₆, vitamin B₁₂, and folate were not different between patients with MS and controls. In the MS group, there were no correlations between plasma homocysteine levels and the serum concentrations of IL-12 or TNF- α , leukocyte nitric oxide production, or plasma diene conjugate levels.

Conclusions: Elevated plasma homocysteine occurs in both benign and progressive disease courses of MS, and seems unrelated to immune activation, oxidative stress, or a deficiency in vitamin B₆, vitamin B₁₂, or folate.

Deficiencies in vitamin B₆, vitamin B₁₂, or folate are associated with elevated plasma levels of homocysteine. In the search for a possible relationship between vitamin B₁₂ deficiency and multiple sclerosis (MS), two studies found elevated plasma homocysteine levels in patients with MS.^{1,2} The frequency of biologically relevant vitamin B₁₂ deficiency in MS is low,^{2,3} and it is uncertain whether this provides a satisfactory explanation for the raised plasma homocysteine concentrations. A recent report studying oxidative stress found elevated plasma homocysteine levels in patients with secondary progressive MS compared with healthy controls,⁴ but another group reported no significant difference in plasma homocysteine concentrations between patients with MS and controls.⁵

The nervous system may be particularly sensitive to extracellular homocysteine, as it promotes excitotoxicity via stimulation of *N*-methyl-D-aspartate receptors, and damages neuronal DNA, thereby triggering apoptosis.^{6,7} Increased levels of homocysteine may play a role in the pathogenesis of Alzheimer's disease, through both vascular and degenerative mechanisms, and hasten the onset and progression of Parkinson's disease.^{8,9} High homocysteine resulting from poor reconversion to methionine is related to low methionine availability. Methionine is an important methyl group donor in many biochemical processes. Hypomethylation of myelin basic protein (MBP)-arginine decreases the hydrophobicity of MBP and could give rise to less stable myelin structures and enhance degeneration of the myelin sheath.¹⁰

The aim of this study was twofold: firstly, to test the hypothesis that patients with progressive MS have higher plasma homocysteine levels than those with a benign course, and secondly, to find out whether elevated plasma homocysteine in MS is caused by low blood vitamin status (folate, vitamin B₆, and vitamin B₁₂), circulating inflammatory cytokines (interleukin IL)-12 and tumour necrosis factor (TNF)- α , or oxidative stress (nitric oxide production by peripheral blood leukocytes, and diene conjugate levels in plasma).

METHODS

The study was approved by the medical ethics committee of the University Hospital, Groningen. All subjects (88 patients with MS and 57 healthy controls) gave their informed consent before inclusion in the study.

Patients

All patients met the criteria of MS.^{11,12} Seventeen patients were using beta interferon. No other immunomodulatory or immunosuppressive drugs were used by any of the patients. Relapse or the use of corticosteroids within the past 3 months was an exclusion criterion. Patients with MS were subdivided into three groups: relapsing MS with a benign course having an Expanded Disability Status Scale (EDSS) score ≤ 3 despite at least 10 years of disease duration ($n = 28$),¹³ secondary progressive MS ($n = 37$), and primary progressive MS ($n = 23$). The term secondary progressive MS was used for patients who had switched from a relapsing remitting disorder to a progressive downhill course that could still be accompanied by some overlapping relapses, and the term primary progressive MS was used for patients in whom the disease was characterised by a progressive course from the onset, without superimposed exacerbations.¹⁴

Tests

Blood samples were drawn through an intravenous cannula in the forearm.

Homocysteine, vitamin B₆, folate, vitamin B₁₂

Plasma total homocysteine (that is, the sum of both reduced and oxidised forms) was determined using an immunochemical method (ImX; Abbott Laboratories, Zwolle, the Netherlands). Vitamin B₆ was measured as pyridoxal-5'-phosphate in EDTA-anti-coagulated whole blood by high

Abbreviations: EDSS, Expanded Disability Status Scale; IL, interleukin; MBP, myelin basic protein; MS, multiple sclerosis; TNF, tumour necrosis factor

performance liquid chromatography. Serum vitamin B₁₂ and folate were analysed using an immunofluorometric technique (Autodelfia, Wallay Oy, Germany).

Inflammatory cytokines

Serum levels of TNF- α and IL-12 were measured using commercial ELISA kits (R&D Systems Inc., Minneapolis, MN, USA). We used IL-12p70 ELISA, which recognises specifically the biologically active IL-12 heterodimer without cross-reactivity with the individual subunits of the dimer (p35 and p40).

Leukocyte nitric oxide production

Nitrite determination is an index of nitric oxide production. Leucocytes were isolated as described before, and nitrite levels in leukocyte supernatants were quantified by a spectrophotometric assay based on the Griess reaction.¹⁵ Protein content was measured using the method of Lowry *et al.*¹⁶

Diene conjugate levels

Diene conjugates are formed by the molecular rearrangement of the double bonds in polyunsaturated fatty acid residues of lipids, and are an index of lipid peroxidation. Plasma (0.5 ml) was added to 2 ml of a 1:1 heptane-isopropanol solution. The mixture was cooled until phase separation occurred, then 0.5 ml of the heptane phase, which contains the lipid oxidation products, was aspirated and 2 ml of distilled ethanol was added. Absorbance of this solution was measured at 232 nm and diene conjugate content was calculated using a standard curve.

Statistical analysis

Categorical data were compared using the χ^2 test, and continuous data between two groups compared using the Mann-Whitney U test. Differences between the MS subgroups and controls were assessed using the Kruskal-Wallis test. Only when these results were significant were comparisons between specific groups performed, using Dunn's multiple comparisons test. The Spearman correlation analysis test was performed for correlation studies. Significance for all tests was set at $p = 0.05$.

RESULTS

Demographic data of the controls and patients with MS are depicted in table 1.

Homocysteine

The mean (SD) plasma homocysteine concentration was higher in the MS population than in the control group (13.8 (4.9) v 10.1 (2.5) $\mu\text{mol/l}$; $p < 0.0001$) (fig 1). Compared with healthy controls, each of the three MS subgroups had more individuals with homocysteine levels $>15 \mu\text{mol/l}$ (table 2). No differences were found in plasma homocysteine concentrations between the three MS subgroups ($p = 0.56$). Plasma homocysteine concentrations were not different between users and non-users of beta interferon ($p = 0.27$).

Vitamin B₆, folate, vitamin B₁₂

No differences were found in the concentrations of folate, vitamin B₆, and vitamin B₁₂ between the three MS groups and controls (table 2). After correction for serum folate, serum vitamin B₁₂, blood vitamin B₆, and serum creatinine concentrations, the difference in plasma homocysteine concentration between the whole MS group and the control group was $4.5 \mu\text{mol/l}$ (95% confidence interval 2.7 to $6.2 \mu\text{mol/l}$; $p < 0.0005$).

Inflammatory cytokines, nitric oxide, and diene conjugates

These were only measured in the patients with MS. Mean (SEM) levels were: serum IL-12, 18.5 (2.3) pg/ml; serum TNF- α , 2.86 (0.19) pg/ml; nitric oxide in leukocytes supernatants, 11.6 (1.0) nmol/mg protein; plasma diene conjugates, 2.49 (0.11) pmol/mg. There was no correlation between plasma homocysteine and serum levels of either IL-12 ($p = 0.32$) or TNF- α ($p = 0.08$), leukocyte nitrite concentrations ($p = 0.48$), or plasma diene conjugate levels ($p = 0.44$).

DISCUSSION

In agreement with previous studies, we found that plasma homocysteine levels in subjects with MS (not related to relapse) are increased.^{1,2} We further showed that increased homocysteine levels did not differ between MS individuals with a benign and those with a progressive disease course. This suggests that, in contrast to the situation suspected in

Figure 1 Box plot presentation of plasma homocysteine concentrations in patients with MS and controls.

Table 1 Demographic data of the study groups

	Relapsing MS with benign course	Secondary progressive MS	Primary progressive MS	Healthy controls
No. of patients	28	37	23	57
Age in years, mean (SD)	48.50 (10.06)	50.21 (9.34)	51.83 (8.39)	50.86 (12.80)
Sex (F/M)	19/9	24/13	16/7	31/26
Disease duration in years, mean (SD)	20.54 (8.04)	20.37 (8.22)	12.26 (4.71)	–
EDSS, median (range)	2.0 (0.0 to 3.0)	7.0 (4.5 to 8.5)	6.0 (2.2 to 8.0)	–

Table 2 Homocysteine, vitamin B₆, vitamin B₁₂, and folate levels

	Relapsing MS with benign course	Secondary progressive MS	Primary progressive MS	Healthy controls	p
Plasma homocysteine in $\mu\text{mol/l}$, mean (SD)	12.9 (3.9)*	14.9 (5.9)†	13.1 (3.8)*	10.1 (2.5)	<0.0001
Plasma homocysteine >15 $\mu\text{mol/l}$, n (%)	9 (32%)	12 (32%)	8 (35%)	2 (4%)	<0.0005
Whole blood vitamin B ₆ in nmol/l, median (range)	66 (34 to 261)	64 (26 to 370)	49 (15 to 560)	58 (30 to 406)	0.33
Serum vitamin B ₁₂ in pmol/l, median (range)	260 (117 to 510)	246 (111 to 632)	265 (131 to 657)	297 (143 to 928)	0.15
Serum folate in nmol/l, median (range)	12 (4 to 41)	10 (3 to 48)	12 (4 to 59)	12 (7 to 51)	0.24

Dunn's multiple comparison test: * $p < 0.01$ v controls; † $p < 0.001$ v controls.

some neurodegenerative diseases,^{8,9} homocysteine is not implicated in disease severity and progression of MS.

Homocysteine arises during methionine metabolism, and is removed either by conversion to cysteine or by remethylation to methionine. The former enzymatic reaction requires vitamin B₆ as cofactor, and the latter vitamin B₁₂ and folate.¹⁷ Deficiency of any of these vitamins can be a cause of elevated plasma homocysteine. Folate, vitamin B₆, and vitamin B₁₂ are essential for the proliferation of immunocompetent cells, and high turnover rate may be encountered in disorders that are associated with long lasting immune activation.^{18–20} However, compared with healthy controls, levels of vitamin B₆, vitamin B₁₂, and folate were not different in patients with MS. In addition, after correction for these vitamins there was still a highly significant difference of 4.5 $\mu\text{mol/l}$ in plasma homocysteine concentration between patients with MS and controls, suggesting that increased plasma homocysteine levels may be caused by increased production rather than decreased removal.¹⁷

Homocysteine, a sulphur amino acid, undergoes rapid auto-oxidation in plasma, with production of hydrogen peroxide and peroxynitrite in the presence of nitric oxide.²¹ Several studies have found increased levels of nitric oxide metabolites in the serum of patients with MS.^{22–26} Besler and Çomoglu reported an inverse correlation between plasma homocysteine levels and total antioxidant capacity.⁴ We did not find a correlation between plasma homocysteine levels and oxidative stress as measured by plasma diene conjugate levels and leukocyte nitric oxide production. Furthermore, there was no association between the concentration of plasma homocysteine and serum levels of IL-12 and TNF- α . IL-12 and TNF- α are major proinflammatory cytokines, which reflect immune activation in MS.²⁷ We can only speculate on the possible mechanism underlying the elevated homocysteine levels in MS. Homocysteine is also produced and secreted by astrocytes.²⁸ In MS there is activation of astrocytes and disruption of the blood brain barrier, which may explain why the astroglial protein S100B is elevated in serum of patients with MS.²⁹ Thus, it may be possible that astrocyte derived homocysteine contributes to the elevated plasma homocysteine levels in MS.

Moderately raised plasma homocysteine levels have been associated with an increased risk of atherosclerosis and thrombosis,³⁰ and could account for atherothrombotic vascular complications observed in patients with rheumatoid arthritis, inflammatory bowel disease, and psoriasis.^{31–33} Although this has not been well studied, there are a few older reports suggesting an increased occurrence of vascular comorbidity in patients with MS.^{34,35} It would be interesting to investigate whether elevated plasma homocysteine levels in patients with MS are a potential threat for concomitant vascular disease, and whether they can and should be lowered by using vitamin supplements.

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Competing interests: none

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