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ORIGINAL COMMUNICATION

Hyperhomocysteinemia is associated with cognitive impairment in multiple sclerosis

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Abstract Hyperhomocysteinemia (HHcy) has been associated with cognitive impairment in various neurological diseases. Cognitive impairment occurs early in multiple sclerosis (MS). Conflicting data have been reported regarding plasma total homocysteine (tHcy) levels in MS patients, and the impact of HHcy on cognitive impairment in MS is not known. This study investigated whether plasma total homocysteine levels are increased in MS and if HHcy is associated with cognitive impairment in MS. We compared tHcy levels in 94 patients with MS and 53 healthy age-matched controls. We used a neuropsychological test battery that included the Raven's Coloured Progressive Matrices, the Visual Search Test, the Trail Making Test A and B, the Immediate and Delayed Recall of a Short Story, the 30 Paired Word Associates, the Rey-Osterrieth Complex Figure Test, and the Semantic and Verbal Fluency Tests. Clinical (sex, age, type of MS, relapse, disease duration, coexisting disease, smoking habit, and physical disability) and laboratory variables (HHcy, low serum levels of folate and vit.B12, MTHFR genotype) were evaluated for their ability to predict cognitive impairment. The mean tHcy was higher in patients (13.19 μmol/L, SD5.58) than in controls (9.81 μmol/L, SD2.53; $p < 0.001$). Univariate analysis determined the following factors to be associated with cognitive impairment: higher age at observation, chronic progressive course of disease, longer disease duration, moderate or severe physical disability, and frequency of HHcy. With multivariate regression analysis, there remained a significant association only between frequency of HHcy and cognitive impairment (β 0.262, $p = 0.01$). We conclude that tHcy levels are increased in MS and that HHcy is associated with cognitive impairment in this disease.

Key words cognitive performance · homocysteine · multiple sclerosis · neuropsychology

Introduction

Cognitive impairment occurs early in multiple sclerosis (MS) and affects 30–70 % of patients [1, 2]. Differences in the nature and extent of cognitive dysfunction in MS are related to a variety of factors including clinical course, duration of illness, disability, treatment, and lesion loads demonstrated by brain magnetic resonance imaging (MRI) [1–4].

Homocysteine (Hcy) is a sulfur-containing metabolite of methionine that can become elevated in the plasma

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as a result of genetic or nutrient-related disturbances [5, 6]. Recent studies investigated the link between hyperhomocysteinemia (HHcy) and the risk of dementia [7, 8] and cognitive impairment in older adults [9, 10].

Studies comparing plasma total homocysteine (tHcy) concentrations between MS patients and controls have yielded conflicting findings [11–13]. The relationship between HHcy and cognitive impairment in MS is unknown. The aim of this study was twofold. First, we sought to test the hypothesis that patients with MS have higher tHcy levels than healthy controls. Second, we sought to determine whether HHcy is associated with cognitive impairment in MS.

Methods

Patients and controls

One hundred thirty-seven consecutive patients referred to our Neurology Unit in the last 2 years were considered for enrollment. Inclusion criteria were diagnosis of MS according to the revised McDonald's criteria [14] and no corticosteroid treatment for at least 3 months. Patients in relapse were admitted, and corticosteroid treatment was initiated soon after completion of the study. Exclusion criteria were vitamin B12 or folate supplementation within the preceding year, presence of psychiatric illness, including severe depression and treatment with neuroleptic, antidepressant, or antiepileptic drugs. The presence or absence of depression was determined using the Beck Depression Inventory [15].

Ninety-four of 137 patients (52 women, 42 men; age 20–69 years, median 36 years) matched the inclusion or exclusion criteria and entered the study. The clinical types of MS were: relapsing-remitting (n = 77 patients, 81.9 %) and chronic progressive (n = 17, 18.1 %; n = 15 secondary progressive MS, n = 2 primary progressive MS). All patients underwent cerebral and cervical MRI examination after triple-dose gadolinium-diethylenetriaminepentaacetic acid within the 7 days preceding or following tHcy determination. At the time of the study, 17 of 94 patients (18.1 %) had MRI evidence of relapse. The mean educational level was 9.18 ± 4 years (range = 5–18 years, median = 8 years). The duration of illness (difference between age at examination and age at the first clinical manifestation) ranged between 1.2 months and 41.12 years (mean 10.2 ± 8.9 years). Twenty-five of 94 patients (26.6 %) were habitual smokers. Coexisting disease was present in 7 of 94 patients (7.4 %) and included essential hypertension (n = 4), deep vein thrombosis (n = 1), ischemic cardiomyopathy (n = 1), and diabetes mellitus (n = 3). Physical disability was rated using Kurtzke's Expanded Disability Status Scale (EDSS) score [16], and the patients were separated into two groups according to an EDSS cut-off value of 3.5. Sixty-five patients (69.1 %) had an EDSS score ≤ 3.5, and 29 (30.9 %) had a score > 3.5.

Healthy volunteers (blood donors) were recruited from the Thrombosis and Hemostasis Centre (Azienda Ospedaliera, Reggio Cal.). None of the controls were treated with vitamin B12 or folate supplementation. All subjects (patients and controls) gave informed consent before inclusion in the study.

Neuropsychological assessment

The neuropsychological evaluation was performed in the week preceding or following tHcy determination. The evaluation required approximately 2 hours and was performed in two sessions. To minimize the effect of fatigue, periods of rest were provided during testing. The evaluation was performed using the Raven's Coloured Progressive Matrices [17], the Visual Search Test [18], the Trail Making Test A and B [19], the Immediate and Delayed Recall of a Short Story [18], the 30 Paired Word Associates [20], the Rey-Osterrieth Complex Figure Test [21], the Semantic Verbal Fluency Test [18], and the Phonemic Verbal Fluency Test [22]. Results were compared with Italian published norms [17–22]. Individual test performance was considered abnormal when it was two or more SDs below the control mean. To analyze differences in cognitive dysfunction, we divided patients into three subgroups based on the number of tests failed: unimpaired (0–2 failed tests), mildly impaired (3–5 failed tests), and moderately impaired (> 5 failed tests).

Laboratory assessments

Blood samples were assessed for tHcy, serum vitamin B12, and serum folate. All blood samples were obtained from fasting subjects from 8:00 to 9:00 a.m. Blood was collected from patients and controls by the same personnel, in the same setting, at the Thrombosis and Hemostasis's Centre. Plasma samples were stored at –80 °C until analysis. Plasma was obtained by centrifuging whole blood at 3000×g for 20 min at 4 °C within 2 hours after collection. tHcy in plasma was measured with a microplate enzyme immunoassay (BioRad Laboratories, Inc, Hercules, CA, USA) [23]. HHcy was defined as tHcy ≥ 15 µmol/L. Serum folate and vitamin B12 levels were assayed in 24 patients and 35 controls using a commercially available kit (Vitros Immunodiagnostic System; Ortho-Clinical Diagnostics, Milan, Italy). The C677T mutation of the methylene tetrahydrofolate reductase (MTHFR) gene was examined in 57 patients by PCR-RFLP of DNA samples [24] using the enzyme Hinf I.

Statistical analysis

Statistical comparisons involving binary variables were performed using 2-way tables for the Fisher exact test and multi-way tables for the Pearson chi-square test. The nonparametric Kruskal-Wallis and Mann-Whitney U test were used to analyze differences between two or more groups. The Spearman rank correlation coefficient (rho) was used to assess the strength of the straight-line association between the variables. Since the dependent variable was restricted to more than two categories, linear regression, which estimates the coefficients of the linear equation involving one or more independent variables that best predict the value of the dependent variable, was used for multivariate analysis. The strength of the association between each independent variable and the dependent variable was expressed as the standardized regression coefficient beta. The variables with probability values < 0.10 were eligible for inclusion as predictor variables in the multivariate regression analysis. We constructed a series of multiple regression models of increasing complexity (models #1–4, Table 4) in which we included all of the variables that were significantly associated with cognitive impairment at the univariate analysis. A $p < 0.05$ was considered significant for all statistical calculations. All statistical calculations were performed using the statistical package SPSS for Windows, release 11.5, 2002 (SPSS UK, Working, Surrey, United Kingdom).

Results

Participant demographics are shown in Table 1. The two groups had comparable age, sex, and smoking habits.

tHcy levels

The mean plasma tHcy level was significantly higher among MS patients (13.19 µmol/L) than controls

Table 1 Participant demographics and results of laboratory tests

	Patients	Healthy controls	p
Number of subjects	94	53	
Sex (F/M)	52/42	28/25	NS
Age (years), mean (SD)	36.63 (10.36)	37.15 (12.06)	NS
Smoking habit	25 (26.6 %)	13 (24.5 %)	NS
Plasma tHcy (μmol/L), mean (SD)	13.19 (5.58)	9.81 (2.53)	< 0.001
Frequency of HHcy	33 (35.10 %)	2 (3.8 %)	< 0.0001
Serum folate* (nmol/L), mean ± SD (range)	14.3 ± 12.7 (3.0–52)	13.8 ± 8.2 (3.8–42)	NS
Frequency of low serum folate*	6	3	NS
Serum vitamin B12* (pmol/L), mean ± SD (range)	273 ± 112 (98–578)	291 ± 109 (119–532)	NS
Frequency of low serum vitamin B12*	0	0	NS

* Determination performed in 24 patients and 35 controls
F female; *HHcy* hyperhomocysteine; *M* male; *SD* standard deviation; *NS* nonsignificant; *tHcy* total homocysteine

(9.81 μmol/L, $p < 0.001$, Table 1). The frequency of pathological values was also significantly higher in patients versus controls (MS: 33/94, 35.10 %; controls: 2/53, 3.8 %; $p < 0.0001$). There were no significant differences in mean levels or pathological values of serum vitamin B12 or serum folate (Table 1).

Variables associated with cognitive impairment

Cognitive impairment was present in 34/94 patients (36.17 %). It was mild in 24/94 (25.53 %) and moderate in 10/94 (10.63 %). The MTHFR genotype was C/C in 22, C/T in 25, and T/T in 10 patients. Levels of plasma tHcy significantly correlated with the number of failed cognitive tests (Fig. 1A) and were significantly higher among cases with a moderately impaired cognitive function (Fig. 1B). Univariate analysis (Table 2) determined that the following variables were significantly associated with cognitive impairment: higher age at observation, chronic progressive course, longer duration of disease, moderate or severe disability (EDSS > 3.5), higher plasma tHcy levels, and frequency of HHcy. Sex, relapse, coexisting disease, smoking habit, educational level, frequency of low serum folate, and MTHFR genotype did not significantly correlate with cognitive impairment. Multivariate regression analysis with the variables age at observation, chronic progressive course, duration of disease, EDSS > 3.5, and frequency of HHcy, determined only frequency of HHcy to be significantly associated with cognitive dysfunction (Table 4; beta 0.262, $p = 0.01$).

Cognitive impairment in patients with HHcy

Compared to MS patients with normal tHcy levels, patients with HHcy had abnormalities in nonverbal reasoning (Raven's Progressive Matrices), visual attention (visual search test, Trail Making Test A and B), visual-spatial memory (recall on the Rey-Osterrieth Complex

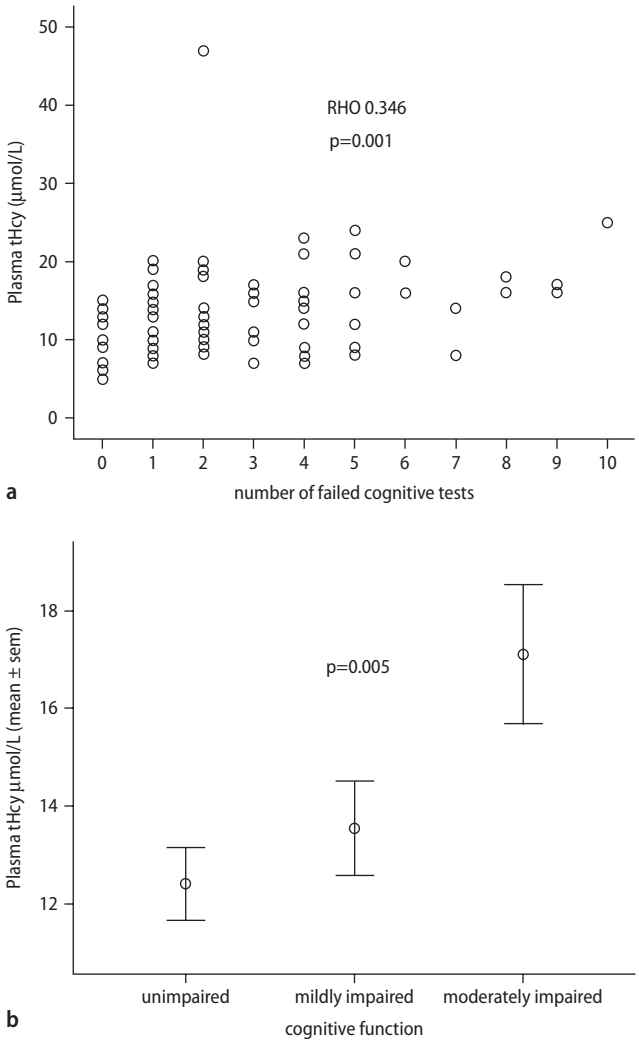

Fig. 1 Relationship between levels of total plasma homocysteine and cognitive performance. **A** Spearman correlation coefficient (rho). Significant positive correlation between levels of total plasma homocysteine (tHcy) and failed cognitive tests. **B** Mann-Whitney U test. Significantly higher levels of tHcy in patients with moderately impaired cognitive function

Table 2 Univariate analysis of risk factors of cognitive dysfunction in 91 MS patients

Variables	Unimpaired (n = 60)	Mildly impaired (n = 24)	Moderately impaired (n = 10)	p
Male sex, n (%)	24 (40)	11 (45.8)	7 (77.8)	NS
Age in years, mean ± SD	34.7 ± 8.5	37.2 ± 12.2	47.0 ± 10.4	0.018
Educational level; mean ± SD	9.0 ± 4.0	8.9 ± 3.9	9.0 ± 4.3	NS
Chronic progressive course, n (%)	8 (13.3)	2 (8.3)	7 (70.0)	< 0.0001
Relapse, n (%)	10 (16.7)	5 (20.8)	2 (20.0)	NS
Disease duration in years, mean ± SD	8.0 ± 7.0	11.9 ± 10.3	18.9 ± 10.6	0.002
Coexisting disease, n (%)	3 (5.0)	3 (12.5)	1 (10.0)	NS
Smoking, n (%)	15 (25.0)	7 (29.2)	3 (30.0)	NS
EDSS > 3.5, n (%)	13 (21.7)	9 (37.5)	7 (70.0)	0.007
Plasma tHcy (μmol/L), mean ± SD	12.4 ± 5.8	13.5 ± 4.8	17.1 ± 4.5	0.005
HHcy, n (%)	14 (23.3)	11 (45.8)	8 (80.0)	0.001
Low folate*, n (%)	4 (26.7)	2 (25.0)	0 (0)	NS
MTHFR** genotype C/T or T/T, n (%)	21 (63.6)	12 (66.7)	2 (33.3)	NS

* Determination performed in 24 patients; ** Determination performed in 57 patients
EDSS Kurtzke's Expanded Disability Status Scale; *HHcy* hyperhomocysteinemic status; *MTHFR* methylene tetrahydrofolate reductase; *NS* not significant; *SD* standard deviation; *tHcy* plasma total homocysteine

Figure Test), and visual-spatial ability (copy ability on Rey-Osterrieth Complex Figure Test). In contrast, verbal fluency (phonemic and semantic verbal fluency) and verbal memory (short story and 30 paired word associates) were not significantly compromised in the HHcy group (Table 3).

Table 3 Relationship between homocysteine status and cognitive performance

Cognitive tests	Patients		p
	Normal tHcy	Hyper tHcy	
Raven's Coloured Progressive Matrices			
normal/abnormal	59/2	24/9	0.001
Visual search test			
normal/abnormal	59/2	25/8	0.003
Trail Making Test A			
normal/abnormal	42/19	14/19	0.016
Trail Making Test B			
normal/abnormal	41/20	14/19	0.028
Short story			
normal/abnormal	43/18	22/11	0.816
30 paired word associates			
normal/abnormal	56/5	22/11	0.003
Rey-Osterrieth Complex Figure Test (recall)			
normal/abnormal	31/30	9/24	0.031
Rey-Osterrieth Complex Figure Test (copy)			
normal/abnormal	55/6	24/9	0.039
Semantic verbal fluency			
normal/abnormal	59/2	29/4	0.179
Phonemic verbal fluency			
normal/abnormal	54/7	26/7	0.234

tHcy total plasmatic homocysteine; *NS* not significant

Discussion

In agreement with previous reports [12–13], we found that patients with MS had increased plasma tHcy levels. This is in contrast to another study [11] that found no significant differences with regard to serum Hcy between MS patients and controls. However, this difference in findings may be explained by the smaller sample size in the former study (38 patients and 20 healthy blood donors). Homocysteine is produced during methionine metabolism, and is removed either by conversion to cysteine (with vitamin B6 as a cofactor) or by remethylation to methionine (with vitamin B12 and folate as cofactors). Deficiency of vitamin B6, B12, and folate may cause HHcy. We found that patients and controls had equivalent levels of vitamin B12 and folate. Although we did not determine levels of vitamin B6, other authors [13] demonstrated no significant differences in the plasma levels of vitamin B6 between MS patients and controls. Collectively, these data suggest that HHcy may be caused by increased production rather than decreased removal of Hcy.

In previous studies, HHcy was shown to be a risk factor for dementia and Alzheimer's disease [3] and was associated with worse cognitive performance and cognitive decline in nondemented elderly people [5, 6]. We showed that HHcy is significantly associated with cognitive impairment and that there was a significant positive correlation between plasma tHcy levels and cognitive impairment in MS patients. Increased tHcy levels have been associated with depression, reduced memory function, and reduced constructional ability in nondemented elderly subjects [6, 25]. A previous pilot study [26] examined the correlation between tHcy levels and

Table 4 Multiple regression models associated with cognitive function

Covariates	Dependent variable: cognitive function				
	Unadjusted	Model 1	Model 2	Model 3	Model 4
HHcy	(β = 0.379), P < 0.0001	(β = 0.311), P = 0.002	(β = 0.290), P = 0.004	(β = 0.264), P = 0.008	(β = 2.262), P = 0.010
Age	…	(β = 0.255), P = 0.010	(β = 0.09), P = 0.5	(β = 0.07), P = 0.6	(β = 0.06), P = 0.6
Duration of disease	…	…	(β = 0.233), P = 0.086	(β = 0.197), P = 0.15	(β = 0.198), P = 0.15
Chronic progressive course	…	…	…	(β = 0.141), P = 0.18	(β = 0.128), P = 0.3
EDSS	…	…	…	…	(β = 0.02), P = 0.8

Data are reported as standardized regression coefficients (β) and P value. *EDSS* Kurtzke's Expanded Disability Status Scale; *HHcy* hyperhomocysteinemic status

clinical functioning in MS patients. Results showed a positive correlation between tHcy levels and the EDSS and a negative correlation with the MS Functional Composite test (MSFC), indicating worse functioning in patients with HHcy. In addition, there was a negative correlation between tHcy levels and cognition subtests of the MSFC, suggesting a relationship between HHcy and cognitive impairment. These results should be interpreted with some caution since these authors did not correct for educational level, which has recently been demonstrated to affect the cognitive performance of MS patients [27]. We found no significant differences in the educational level between patients whose cognition was unimpaired, mildly impaired, and severely impaired.

In MS patients, primary language functions, immediate and implicit memory, and verbal intellectual skills seem to be preserved, whereas information processing abilities, complex visuospatial tasks, conceptual reasoning, sustained attention, and working memory are often impaired [28]. In our study, the cognitive battery included tests of attention, but processing speed and working memory assessment were not fully covered, so working memory was not adequately explored. Nevertheless, we found that the most severely affected functions in patients with HHcy were nonverbal reasoning, visual attention, visual-spatial memory and visual-spatial ability. Verbal fluency and verbal memory were not significantly different between patients with HHcy and normal tHcy. These cognitive dysfunctions seem to be different from those observed in subcortical dementia and nondemented older adults and may be explained on the basis of a "multiple disconnection syndrome" [28].

In a previous longitudinal study [7] in which tHcy levels were not evaluated, the extent of cognitive impairment in MS was associated with age, disease course, and disability. In our study, the association between HHcy and cognitive impairment appeared to be independent of age, progressive disease course, and degree of physical disability, suggesting a direct relationship between HHcy and cognitive dysfunction. We also considered functional mutations of the MTHFR gene, which have been associated with increased levels of tHcy, as a possible independent factor determining cognitive impairment. However, our findings do not support a role for functional mutation of the MTHFR gene and CI in MS. Nonetheless, it must be emphasized that the cross-sectional nature of our analysis does not allow us to infer a causality link between HHcy and cognitive impairment in MS, and future prospective cohort studies to test such a causal relationship are warranted.

Studies investigating possible predictors of cognitive impairment in MS have focused on MRI measurements as indicators of neuropathological involvement; such studies have shown consistent correlations between cognitive impairment and brain lesion load or brain atrophy [29]. HHcy is associated with increased risk of vascular disease. It could provoke "silent" subcortical lacunar infarcts, increase the white-matter lesion load in MS patients, and provoke additional cognitive impairment. On the other hand, it has been hypothesized that Hcy produces cognitive impairment in the elderly via a direct neurotoxic effect [6], and a significant association has been demonstrated between HHcy and cortical atrophy and dementia [30]. We did not investigate the correlations between MRI data and cognitive impairment and tHcy levels. Thus, we are unable to confirm or refute these hypotheses, and further studies are needed to clarify this issue. As an alternative hypothesis, HHcy should be considered as an epi-phenomenon secondary to the degenerative process in MS as well as in dementia and normal ageing.

In conclusion, HHcy is independently associated with cognitive impairment in MS. Although physicians treat cases with HHcy, further prospective and intervention studies are needed to determine whether lowering the plasma Hcy level can reduce the risk of cognitive decline and dementia in MS.

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