Elevated cerebrospinal fluid and plasma homocysteine levels in ALS

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Background: High cerebrospinal fluid (CSF) and plasma levels of homocysteine (HC) have been reported in certain neurodegenerative disorders, such as Alzheimer's, Parkinson's diseases and, recently, amyotrophic lateral sclerosis (ALS).
Objectives: To assay the CSF and plasma levels of HC in ALS patients and controls, and to evaluate the relationship between HC levels and clinical variables of the disease.
Methods: Cerebrospinal fluid from sixty-nine (M/F 1.87) and plasma from sixty-five ALS patients (M/F 1.83) were taken and stored at −80°C until use. Controls (CSF = 55; plasma = 67) were patients admitted to our hospital for neurological disorders with no known relationship to HC changes. CSF and plasma from ALS patients and controls were obtained as a necessary step of the diagnostic workup. HC levels in CSF and plasma were assayed using a high performance liquid chromatograph (HPLC) and a fluorimeter detector.
Results: The median level of total HC in the CSF of ALS patients was 0.46 μM, significantly higher than that of the controls (0.24 μM, +91.6%, $P < 0.001$). A similar trend was observed when HC was assayed in plasma (ALS, 12.4 μM vs. controls, 7.26 μM, +70.8%, $P < 0.001$). The CSF and plasma HC levels showed no relationship with the disease progression, age at onset, and the site of onset.
Conclusions: Homocysteine is a biochemical marker in ALS, and it might be related to the pathophysiology of the disease.

Introduction

Growing evidence suggests that homocysteine (HC), a putative risk factor for stroke and coronary artery disease [1,2], could play a role in the pathophysiology of several neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases [3,4].

Homocysteine shows neurotoxic properties, with a relevant stimulating effect on N-methyl-D-aspartate (NMDA) and group I metabotropic glutamate (mGlu) receptors, leading to intracellular Ca^{++} increase and reactive oxygen species generation [5]. Further, HC appears to induce mitochondrial dysfunction and apoptotic degeneration in cultured neurons [6,7].

Amongst the neurodegenerative disorders, amyotrophic lateral sclerosis (ALS) is a disease with a multifactorial etiopathogenesis, in which excitotoxicity, intracellular calcium increase, mitochondrial damage, altered axonal transport, oxidative stress and apoptosis may contribute to pathogenesis [8,9]. In this context, the contribution of HC might be relevant, as it has been shown, in a murine model of ALS (i.e. Cu,Zn-SOD1 transgenic mice), that this sulphur amino acid is cytotoxic to motor neurons, and that attenuation of its plasma levels prolongs survival [10,11]. Further, a recent report showed that plasma HC levels were significantly elevated in ALS, and in particular in those patients with a faster progression of the disease [12], suggesting that this endogenous molecule might represent a marker of neurodegeneration in this devastating motor neuron disorder.

The present work aimed to extend the recent findings on plasma HC in ALS [12] by assaying the amino acid levels in both cerebrospinal fluid (CSF) and plasma of ALS patients and controls. CSF HC has never been evaluated in ALS, and its levels might give additional information on the putative impact of this endogenous amino acid in such a devastating motor neuron disease.

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Methods

This study was approved by the ethics committee of the Department of Clinical Neurosciences, University of Palermo. All ALS patients and controls gave their informed consent to the use of their biological materials for diagnostic and research purposes before inclusion in this study.

Patients

Sixty-nine non-demented sporadic ALS patients (M/F = 1.87) underwent an extensive imaging and laboratory work-up and were diagnosed according to the revised El-Escorial/WFN criteria [13]. None was with percutaneous endoscopic gastrostomy (PEG) or under non-invasive ventilation at the time of CSF and blood drawing. Forty-six patients presented as spinal-onset (66.7%), whereas in the remaining 21 the onset was bulbar (33.3%). Disability was rated at three-months interval using the clinimetric Appel ALS rating scale (AARS) [14]. At least three evaluations with AARS were considered sufficient to establish the rate of disease progression (i.e. slow, intermediate and rapid course) [14].

The mean age (years ± SD) of the ALS patients at the time of the lumbar puncture (LP) was 62.7 ± 10.2. Controls were fifty-five patients (M/F = 1.88) who underwent a LP during their diagnostic work-up and were diagnosed with neurological disorders that are not known to affect the HC levels. The mean age of the control patients at the time of the LP was 60.1 ± 11.3. The complete clinical and demographic characteristics of the two groups are listed in Table 1. The list of the neurological diseases diagnosed in the LP control group is shown in Table 2.

Plasma samples were obtained from sixty-five ALS patients (mean age: 63.5 ± 10.4 years, M/F = 1.83) and sixty-seven controls (mean age: 58.7 ± 10.6 years, M/F = 1.25) (see Table 1 for the demographic and clinical details). Again, controls were patients with neurological disorders assumed to be unrelated to the HC levels (Table 2).

Subjects were excluded if they were taking folate or B-vitamins or drugs known to be associated with increased HC levels, or if they had diabetes, vascular diseases, hypothyroidism, renal or hepatic insufficiencies[15]. Based on the above criteria we excluded twelve eligible ALS and fifteen controls. Both ALS and controls enrolled in this study were mostly non-smokers, with the percentage of smokers below 40% in each group. Amongst the smokers, 10% of ALS and 12% of controls smoked less than 8–10 cigarettes/day (i.e. light smokers). With regard to the alcohol intake, 36% amongst the ALS patients and 44% of the controls drunk 1–2 drinks/day. In all cases, wine was the drink preferred.

Table 1 Demographic and clinical characteristics of the ALS patients and controls

	ALS	Controls
CSF group	$n = 69$	$n = 55$
All		
Age (years)	62.7 ± 10.2	60.1 ± 11.3
M/F	1.87	1.88
Bulbar-onset (33.4%, $n = 23$)		
Age (years)	63.6 ± 8.2	
Spinal-onset (66.6%, $n = 46$)		
Age (years)	62.0 ± 11.2	
Plasma group	$n = 65$	$n = 67$
All		
Age (years)	63.5 ± 10.4	58.7 ± 10.6
M/F	1.83	1.25
Bulbar-onset (29.1%, $n = 24$)		
Age (years)	65.7 ± 9.7	
Spinal-onset (70.9%, $n = 41$)		
Age (years)	62.9 ± 10.0	

Quantitative data are expressed as mean ± SD.

Table 2 List of the neurological disorders in the two control groups

	Control group	
	CSF	Plasma
Tension headache	4	14
Cervical spondylotic myelopathy	12	10
Hereditary motor-sensory polyneuropathy (CMT I and II)	9	10
Idiopathic polyneuropathy[a]	15	15
Conversion disorder or neurosis	15	18

[a]Diabetic, inflammatory, paraneoplastic, and other common causes of polyneuropathy were excluded.

Table 3 CSF and plasma homocysteine (HC) levels in ALS patients according to the site of onset or sex

	HC (μM)	
	CSF	Plasma
Bulbar-onset(B)	0.50 (0.30–0.64)	13.1 (9.7–15.4)
	$n = 23$	$n = 24$
Spinal-onset (S)	0.45 (0.35–0.46)	11.5 (9.2–14.7)
	$n = 46$	$n = 41$
Men (M)	0.45 (0.36–0.60)	12.8 (9.0–14.3)
	$n = 45$	$n = 42$
Women (W)	0.49 (0.28–0.62)	11.9 (9.5–15.1)
	$n = 24$	$n = 23$

Data are expressed as median (IQ range). All comparisons (site of onset: B vs. S; sex: M vs. W) were non significant (Mann–Whitney rank sum test).

All serum chemical and immunological tests, including folate and B12-vitamin levels, were within normal range in both ALS patients and controls (data not shown).

The CSF and plasma samples were collected between 8:00 AM and 10:00 AM. from fasted patients, labelled to ensure anonymity and stored at −80°C until analysis. These biological fluids were aliquots from samples taken from each ALS patient and disease control during their diagnostic work-up. Routine CSF analysis (i.e. leukocytes count, total protein, glucose, the CSF/serum albumin concentration ratio) was normal in each patient or control included in this study (data not shown).

Homocysteine assay

Sample preparation

Ten micro liters of a 10% (v/v) solution of MSH (β-mercaptoethanol) were added to 150 μl of CSF. The proteins were removed by centrifugation for 4 min at 11000 *g* and the sample was filtered. 20 μl of the filtrate was added to iodoacetic acid and *o*-phtalaldheyde (OPA) reagents and injected into a HPLC column (5 μm spherisorb ODS 25 cm × 0.45 cm reversed-phase). Plasma was treated in a similar manner.

Chromatography

The chromatographic system consisted of two Gilson pumps controlled by a gradient controller, a rheodyne injector and a 5 μm Spherisorb ODS reversed phase column. Detection of the OPA-derivatized total HC was achieved using a spectrofluorimeter. Excitation and emission wavelengths were set at 335 nm and 455 nm, respectively.

Mobile phase A was 0.05 M sodium acetate containing 8% acetonitrile pH 6.8 and mobile phase B was acetonitrile. The flow-rate was 1 ml/min. Separation of HC was accomplished isocratically using mobile phase A.

Statistical analysis

All analyses were done using SIGMASTAT 3.5 software package (Systat Software Inc., San José, CA, USA). Parametric data were expressed as mean ± SD and analysed by ANOVA. CSF and plasma HC levels in ALS patients and controls were expressed as median with interquartile ranges. The Mann–Whitney rank sum test evaluated the differences in median HC levels between groups. All correlations were studied using the Spearman's rank correlation coefficient. P values < 0.05 were considered significant.

Results

The concentration of CSF and plasma total HC was significantly higher in ALS patients compared to controls (Fig. 1). In CSF, the median HC level was 0.46 μM (0.36–0.68) in the ALS group, and 0.24 μM (0.21–0.27) in the control group, $P < 0.001$ (Fig. 1a). Plasma total HC was 12.4 μM (10.7–14.9) in ALS and 7.26 μM (6.6–8.3) in controls, $P < 0.001$ (Fig. 1b). In a subgroup analysis, we excluded from the controls those patients with hereditary neuropathy and polyneuropathy and found that the median levels of CSF and plasma HC were not significant different from the whole group (data not shown).

Homocysteine levels in CSF and plasma of ALS patients were apparently unrelated to the site of onset and sex (Table 3). Further, no significant relationship was found between disease severity (as measured by the AARS performed at the time of CSF drawing), age at onset and CSF HC (AARS vs. HC, $r^2 = 0.14$, $P = 0.07$; age at onset vs. HC, $r^2 = 0.07$, $P = 0.09$). In addition, a scatter plot of the HC levels in ALS CSF and plasma demonstrated no relationship between the

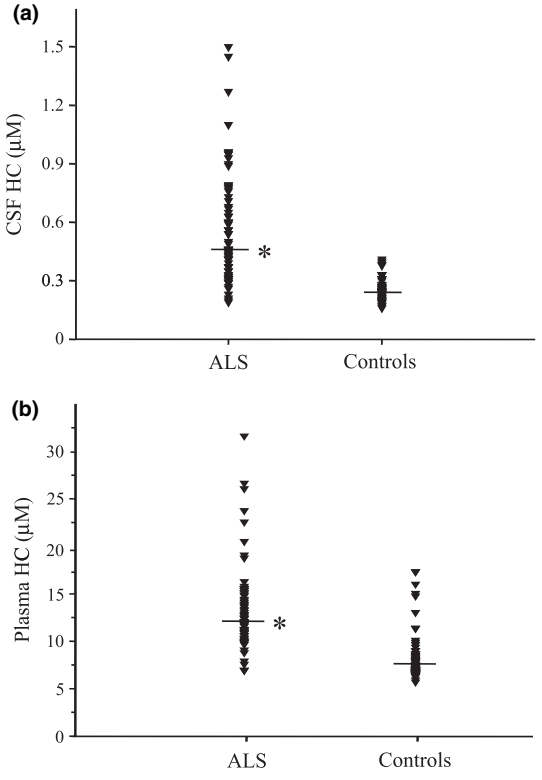

Figure 1 CSF (a) and plasma (b) homocysteine (HC) concentrations in patients with ALS and controls. Horizontal bars show the median HC value in each group. *$P = 0.001$, ALS vs. controls (Mann–Whitney rank sum test).

two variables ($r^2 = 0.002$, $P = 0.68$). This suggests that CSF HC originates directly in the central nervous system.

When plasma HC was measured, a weakly significant correlation with age at onset was found ($r^2 = 0.14$, $P = 0.04$), whereas disease severity, sex and site of onset were unrelated to the plasma HC levels (data not shown).

We then analysed the relationship between CSF HC levels and the rate of the disease progression (as measured by AARS) and found that the two variables were poorly related. In particular, the median CSF HC level was 0.45 μM (0.33–0.56) in the slowly progressing patients and 0.52 μM (0.41–0.73) in the intermediate/rapidly progressing group ($P = 0.11$, Mann–Whitney rank sum test). A similar, not significant ($P = 0.14$) trend was observed with the plasma HC (data not shown).

Discussion

Our study shows that CSF and plasma HC levels are increased in ALS, making this non-protein amino acid an interesting candidate as a biological marker for such a severe neurodegenerative disorder. HC level in ALS is apparently unrelated to age at onset, sex, site of onset, and disease progression. This is supported by a recent work that showed a small, although significant, increase of plasma HC levels in ALS patients [12].

Homocysteine is a non-protein thiol-containing amino acid, precursor of methionine and its metabolism is mainly dependent on vitamin cofactors as folate and vitamin B12. An additional, vitamin-independent re-methylation pathway relies on betaine [15]. Both N^5-methyltetrahydrofolate and betaine work as a methyl donors in a trans-methylation process that from HC leads to methionine; on its turn, methionine can be demethylated to HC.

Another metabolic pathway of the sulphur-amino acid is the trans-sulphuration process leading to the formation of cystathionine and, in the end, cysteine [15,16]. HC can, however, be also oxidized to homocysteic acid (HCA) in brain cells, whose levels increase in response to excitatory stimulation [17].

Homocysteine and its derivative HCA can be neurotoxic to brain neurons through an excitotoxic mechanism [7,18]. In particular, HCA is involved in the degeneration of spinal motor neurons in conjunction with disruption of calcium homeostasis, and HCA is cytotoxic to motor neurons derived from the SOD1 transgenic mouse model of ALS [10,18]. Further, HC immunoreactivity appears to be increased in astrocytes of symptomatic $SOD1^{G93A}$ mouse [19]. Taken together, these data suggest that HC might actually be involved in motoneuron cell death in ALS, even though there is no evidence for a pathogenic role of the amino acid in this devastating disorder.

Homocysteine is considered an independent risk factor for cardiovascular disease, including ischaemic heart disease, stroke, and peripheral vascular disease [20]. In particular, HC increases vascular endothelial growth factor (VEGF) expression, which in its turn promotes progression to atherosclerosis [21,22]. VEGF has been shown to be protective to motor neurons and its overexpression prolongs survival in $SOD1^{G93A}$ mice [23,24]. Thus, the relationship between HC, motor neurons, and ALS appear to be complex and still imperfectly defined. HC might in fact have a neuroprotective role on motor neurons, through its effect on VEGF expression [11,21,22,24].

In our study on ALS, the relatively high HC levels appeared to be independent from the vitamin B12 and folate levels, both of which were normal. Increased plasma HC levels with no changes in vitamin B12 and folate levels has also been described in multiple sclerosis [25]. Other studies have found, however, that increased HC is associated decreased folate and vitamin B12 levels [12,26].

Since the amino acids do not diffuse freely across the blood-brain barrier [27], it can be assumed that CSF HC origins directly in the central nervous system. Whether it represents a decreased catabolism or an increased synthesis has to be clarified. However, as the basal levels of HC are primarily set by the re-methylation process [28], it is likely that a reduced catabolism could be the responsible factor for the increased HC we found in our ALS patients. To this regard, methylene tetrahydrofolate reductase (MTHFR) is a key enzyme in the methylation process of HC to methionine [17]. It is therefore possible that an insufficient MTFHR activity might be involved in the increased CSF and plasma HC levels in ALS, an intriguing hypothesis that deserves further evaluation. A MTHFR polymorphism have been associated with hyper-HC in Parkinson's disease [29].

The analysis of CSF and plasma HC levels in our ALS patients showed a wide range of values, many patients showing levels of the sulphur amino acid up to two-three fold higher than the controls. Finding a wide range of CSF or plasma levels of a putative biological marker in ALS is not unusual (e.g. anti-FAS antibodies [30], neurofilaments heavy chain [31], glutamate [32]) and it can be interpreted as a indication of the high heterogeneity, on the etiopathogenetic ground, of this severe neurodegenerative disorder. However, no clear-cut association was found between high CSF and/or plasma HC levels and the different demographic or clinical variables. This suggests that other factors, most probably linked to a specific genetic susceptibility,

might be directly or indirectly responsible for the HC levels variability in ALS. It is therefore speculated that HC, in combination with other variables, might contribute to the multifactorial pathogenesis of this devastating neurodegenerative disease.

In conclusion, we found that HC is increased in CSF and plasma of ALS patients. This enhancement seems to be independent of the vitamin levels. Our data suggest that HC might represent a biological marker in ALS.

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