Neurological Sciences (2020) 41:1865–1872
https://doi.org/10.1007/s10072-020-04292-x

ORIGINAL ARTICLE

Elevated cerebrospinal fluid homocysteine is associated with blood-brain barrier disruption in amyotrophic lateral sclerosis patients

Yonghua Wu[1] · Xu Yang[1] · Xiaogang Li[2] · Haining Wang[3] · Tiancheng Wang[1]

Received: 12 September 2019 / Accepted: 11 February 2020 / Published online: 21 February 2020

Abstract

Objectives Homocysteine (Hcy) has been shown to be relevant in the pathogenesis of amyotrophic lateral sclerosis (ALS). Although the CSF Hcy changes were explored in patients with ALS previously, the outcomes were inconsistent, and the permeability of the blood-brain barrier (BBB) may involve in the process. The aim of this study was to investigate the relationship between concentration of Hcy and BBB integrity indicated by CSF/serum albumin ratio (Q_{alb}).

Methods CSF and plasma/serum levels of Hcy, folate, and vitamin B12 and other biochemical biomarkers such as albumin, β_2-microglobulin, high sensitive-C reactive protein (hs-CRP), microalbumin, immunoglobulin G (IgG), IgM, IgA, and complement 3 and 4 were analyzed in all 31 ALS patients and 34 controls. Routine CSF analysis including cells/leukocytes count, total protein, glucose, and chlorides were also performed.

Results CSF Hcy levels (0.50 ± 0.46 vs 0.25 ± 0.27 μmol/L) and Q_{alb} (8.09 ± 3.03 vs 6.39 ± 2.21) were significantly higher in the ALS group than that in controls ($P < 0.05$). The generalized linear mixed model analysis showed that the CSF Hcy was positively correlated with Q_{alb} in ALS patients ($P < 0.05$).

Conclusions BBB permeability is increased in ALS patients. CSF Hcy level is associated with BBB integrity. Q_{alb} is a significantly independent predisposing factor for CSF Hcy.

Keywords Homocysteine · Folate · Vitamin B_{12} · Blood-brain barrier · Amyotrophic lateral sclerosis · Q-albumin

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by neuronal loss and degeneration of motor neurons in the spinal cord, motor cortex, and brain stem that leads to paralysis and even death within several years after disease onset. However, the causes and pathophysiology of sporadic ALS have not been fully elucidated [1, 2].

Homocysteine (Hcy), which is a pro-inflammatory thiol-containing redox-active endogenous amino acid, is considered to be a risk factor for vascular diseases [3, 4]. Studies demonstrated that Hcy has neurotoxicity effects via the promotion of oxidative stress [5, 6]. Increased Hcy in cerebrospinal fluid (CSF) or plasma/serum has been found in neurodegenerative disorders, such as Parkinson's disease (PD) [7, 8] and Alzheimer's disease (AD) [9, 10]. In a study of transgenic mice, Hcy has been reported to be involved in the pathogenesis of ALS [11]. Plasma/serum Hcy has also been shown to be significantly elevated in patients with ALS, especially in those with faster progression of that disease [12–15]. However, the results were inconsistent [6, 16, 17].

The structural and functional integrity of the blood-brain barrier (BBB) is important to normal brain functioning. The formation and maintenance of BBB/blood spinal cord barrier (BSCB) are accomplished through expression of tight junctions and adherens junctions connecting neighboring endothelial cells, as well as the paucity of transendothelial bulk-flow transcytosis. It was showed that BBB disruption contributes to

✉ Tiancheng Wang
tcwang@bjmu.edu.cn

[1] Department of Laboratory Medicine, Peking University Third Hospital, No.49, North Garden Road, Haidian District, Beijing 100191, China

[2] Department of Neurology, Peking University Third Hospital, Beijing, China

[3] Department of Endocrinology and Metabolism, Peking University Third Hospital, Beijing, China

early motor neuron degeneration [18] and that ALS is started by BBB impairment in rodent ALS model [19–21]. BBB breakdown was also found in SOD^{1G93A} rats, and BBB/BSCB disruption occurred before motor neuron harm in a mutant mouse model of ALS [22, 23]. Moreover, BBB/BSCB alterations with pericyte reductions and reduced levels of tight junction proteins have been revealed in the spinal cord of ALS patients [24, 25]. BBB disruption is often accompanied by the increase of oxidative damage and inflammation in the CSF of ALS patients [26].

The effects of BBB permeability on the metabolism of Hcy, folate and vitamin B_{12} in CSF, and plasma/serum in ALS are less reported. The association between CSF Hcy level and BBB in ALS patients is still unclear. In the present study, we aim to investigate whether there is a relationship between BBB permeability and CSF/plasma Hcy metabolism, in order to further evaluate the integrity of BBB on Hcy metabolism in CSF.

Methods and methods

Participants

The present study composed 31 consecutive ALS patients and 34 control subjects. The ALS patients were from those who attended the department of neurology in our hospital. Patients were diagnosed according to the revised El Escorial criteria [27], and the ALS functional rating scale (ALSFRS) score was collected [28]. Subjects were excluded if they have a history or clinical evidence of cardiac and vascular diseases, renal or hepatic insufficiencies, diabetes mellitus, thyroid dysfunction, as well as cancer. None of the patients or control subjects was taking any vitamin B-vitamins (vitamin B_2, B_6, B_{12}), folate, multivitamins, or prescribed drugs known to affect Hcy metabolism. All the participants were nonsmoker and nondrinker. The subjects with comorbidities such as cardiovascular, metabolic, and neuropsychiatric diseases were excluded form present study.

Controls were age and gender-matched patients admitted to our hospital with neurological disorders assumed to be unrelated to the Hcy level, including headache and back pain, or without any diagnosed neurologic condition.

Participants in this study underwent a comprehensive clinical investigation, including medical history, family history, and physical, neurological, and psychiatric examination.

All subjects gave their informed consent to participate in this study for diagnostic and research purposes before inclusion in this study, which was approved by the ethics committee of our hospital. The principle of the Helsinki Declaration for using human subjects was obeyed.

Samples collection

For each subject, both CSF and plasma/serum samples were obtained after a period of 12-h fasting. CSF samples were taken from the lumbar punctures. Peripheral venous blood was withdrawn into vacuum blood collection tubes (gel/clot activator or EDTA-K^{2+}) (INSEPACK®, SEKISUI, Beijing, China). Plasma or serum samples were obtained by centrifugation at 3500 rpm for 10 min to precipitate the cellular components of blood specimens. Plasma/serum and CSF samples were analyzed within 60 min after collection or stored at − 80 °C for further use.

Laboratory measurement

The concentrations of Hcy, folate, and vitamin B_{12} and β_2-microglobulin were assayed by fluorescence polarization immunoassay (FPIA), ion capture immunoassay (ICIA), and microparticle enzyme immunoassay (MEIA) using Abbott AxSYM auto analyzer. Albumin was assayed by bromocresol green (BCG) method, and microalbumin was determined by turbidimetry (Biosino Biotechnology & Science Inc., Beijing, China); the high sensitive-C reactive protein (hs-CRP) was measured with the latex agglutination assay (Shanghai Shensuo Unf Medical Diagnostic Articles Co., Ltd., Shanghai, China), all index above were measured by using Olympus AU5400 auto analyzer. The immunoglobulin (Ig) G, IgM, IgA, and complement 3 and 4 (C_3 and C_4) concentrations were measured by rate turbidimetry with the IMMAGE 800 Immunochemistry System. The routine CSF analysis including cells/leukocytes count, total protein, glucose, and chlorides was performed by microscopy, Sysmex XN analyzer, and Vitros FS5.1 analyzer, respectively.

Statistical analysis

Data normality was tested with one-sample Kolmogorov-Smirnov method. For the normal data, the Student's t test was used for mean comparison between two groups, while Mann-Whitney U nonparametric test was used for the data of non-normally distributed. The χ^2 test was used to compare the differences in frequency distribution on gender. Spearman correlation was performed for bivariate analysis. The generalized linear mixed model (GLMMs) was used to explore the possible association between Hcy of CSF and Q_{alb} in ALS patients. All reported P values were two sided, and the P values of less than 0.05 were considered the statistical significance. All statistical analysis was performed using the Statistical Package of Social Science (SPSS, version 23.0, SPSS Inc., Chicago, IL, USA). The charts were drawn with corrplot (version 0.84) and ggplot2 (version 3.1.1) packages of the R (version 3.5.3).

Results

Demographic features and levels of laboratory index

The demographic features and laboratory index in CSF and plasma of ALS patients and control subjects in the study are shown in Table 1. The minimum and the maximum age for ALS patients and controls were 22–80 and 14–73, respectively. There were no significant differences in their age and sex distributions between ALS patients and controls ($P > 0.05$).

CSF Hcy and Q_{alb} for ALS patients and controls

CSF Hcy levels (0.50 ± 0.46 vs 0.25 ± 0.27 μmol/L) and Q_{alb} (8.09 ± 3.03 vs 6.39 ± 2.21) were significantly higher in the ALS patients than those in the controls ($P < 0.05$). The median of Hcy level in CSF was 0.50 vs 0.15 μmol/L (range 1.60 vs 0.90 μmol/L) in the ALS patients and controls, respectively; the median of Q_{alb} was 7.63 vs 5.64 (range 13.44 vs 11.45) in the ALS patients and controls, respectively (Fig. 1A and Table 1). There were no significant differences in CSF folate and vitamin B_{12}, plasma Hcy, serum folate, vitamin B_{12} levels, and another laboratory index between the two groups, respectively ($P > 0.05$) (Fig. 1B and Table 1).

Relationship between laboratory index in CSF and plasma/serum of ALS patients and controls

As indicated in Fig. 2, there was a difference in the correlation pattern between the ALS patients and the control group. In the controls, CSF Hcy was positively correlated with plasma Hcy and negatively correlated with CSF folate, respectively. CSF vitamin B_{12} was positively with serum vitamin B_{12} ($P < 0.05$) (Fig. 2A). Meanwhile, in ALS patients, CSF Hcy was negatively correlated with serum folate; serum vitamin B_{12} was negatively correlated with plasma Hcy and positively correlated with CSF vitamin B_{12}, respectively ($P < 0.05$). Q_{alb} was positively correlated with plasma Hcy and negatively correlated with CSF vitamin B_{12} and serum folate, respectively ($P < 0.05$) (Fig. 2B).

Table 1 Demographic features and levels of laboratory index in ALS patients and controls

	ALS patients ($n = 31$)		Controls ($n = 34$)		P value
Variables	Mean ± SD	Median (Range)	Mean ± SD	Median (Range)	
Age (years)	55.13 ± 14.07 22, 80 (min, max)	56.00 (58.00)	47.21 ± 17.72 14, 73 (min, max)	50.50 (59.00)	0.054
Gender (male/female and %)	20/11 (64.5/35.5)		15/19 (44.12/55.88)		0.136
Disease duration (year)	1.55 ± 1.32	1.30 (3.60)	–		–
ALSFRS Score	38.27 ± 3.40	37.90 (12.70)	–		–
Comorbidities of ALS	None		–		–
Cerebrospinal fluid					
Homocysteine (μmol/L)	0.50 ± 0.46	0.50 (1.60)	0.25 ± 0.27	0.15 (0.90)	0.026
Folate (ng/mL)	14.96 ± 1.88	14.90 (8.40)	15.31 ± 1.50	15.60 (6.50)	0.432
Vitamin B_{12} (pg/mL)	98.61 ± 77.71	67.35 (287.40)	99.48 ± 89.57	53.45 (347.00)	0.999
β_2-microglobulin (μg/mL)	1087.82 ± 293.21	1058.10 (1102.50)	1105.40 ± 344.48	1128.65 (1613.00)	0.840
Immunoglobulin G (mg/dL)	6.09 ± 3.44	6.01 (6.43)	2.58 ± 1.16	2.58 (1.64)	0.234
Cells (/μL)	3.86 ± 5.29	3.00 (20.00)	3.36 ± 3.32	2.00 (10.00)	0.905
Leukocyte (/μL)	0.57 ± 0.94	0.00 (2.00)	0.86 ± 1.70	0.00 (6.00)	0.451
Total protein (mg/dL)	53.60 ± 26.53	51.95 (117.60)	72.98 ± 72.98	51.50 (281.00)	0.523
Glucose (mmol/L)	4.00 ± 1.01	3.60 (2.20)	3.62 ± 0.49	3.60 (1.20)	0.504
Chloride (mmol/L)	121.45 ± 0.37	121.45 (0.90)	124.34 ± 4.13	125.10 (8.60)	0.280
Plasma or serum					
Homocysteine (μmol/L)	10.95 ± 2.70	10.68 (10.70)	12.39 ± 2.97	11.75(10.20)	0.057
Folate (ng/mL)	7.40 ± 3.87	6.10 (16.00)	6.42 ± 2.68	5.30 (9.70)	0.676
Vitamin B_{12} (pg/mL)	945.72 ± 501.40	889.00 (2276.00)	883.93 ± 622.92	735.50 (2204.10)	0.743
β_2-microglobulin (μg/mL)	1290.43 ± 358.06	1171.00 (1351.0)	1303.68 ± 503.62	1256.75 (2944.10)	0.905
hs-CRP (mg/L)	4.05 ± 4.73	1.90 (12.74)	1.04 ± 0.94	1.02 (1.87)	0.066
Immunoglobulin G (g/L)	9.95 ± 2.47	9.30 (5.40)	12.45 ± 3.89	12.45 (5.50)	0.681
Immunoglobulin M (g/L)	1.22 ± 0.27	1.31 (0.60)	1.08 ± 0.52	1.18 (1.12)	0.651
Immunoglobulin A (g/L)	1.93 ± 0.53	2.09 (1.13)	3.32 ± 1.79	3.07 (5.00)	0.168
Complement C3 (g/L)	0.92 ± 0.25	0.88 (0.50)	0.84 ± 0.36	0.92 (0.80)	0.565
Complement C4 (g/L)	0.24 ± 0.03	0.26 (0.05)	0.14 ± 0.06	0.16 (0.14)	0.322
Q_{alb}	8.09 ± 3.03	7.63 (13.44)	6.39 ± 2.21	5.64 (11.45)	0.019

Values are presented as mean ± standard deviation and median (range). χ^2 test was used to compare the distribution of gender. The Student's t test or nonparametric test (Mann-Whitney test) were used according to the data distribution

$P<0.05$ was considered statistically significant

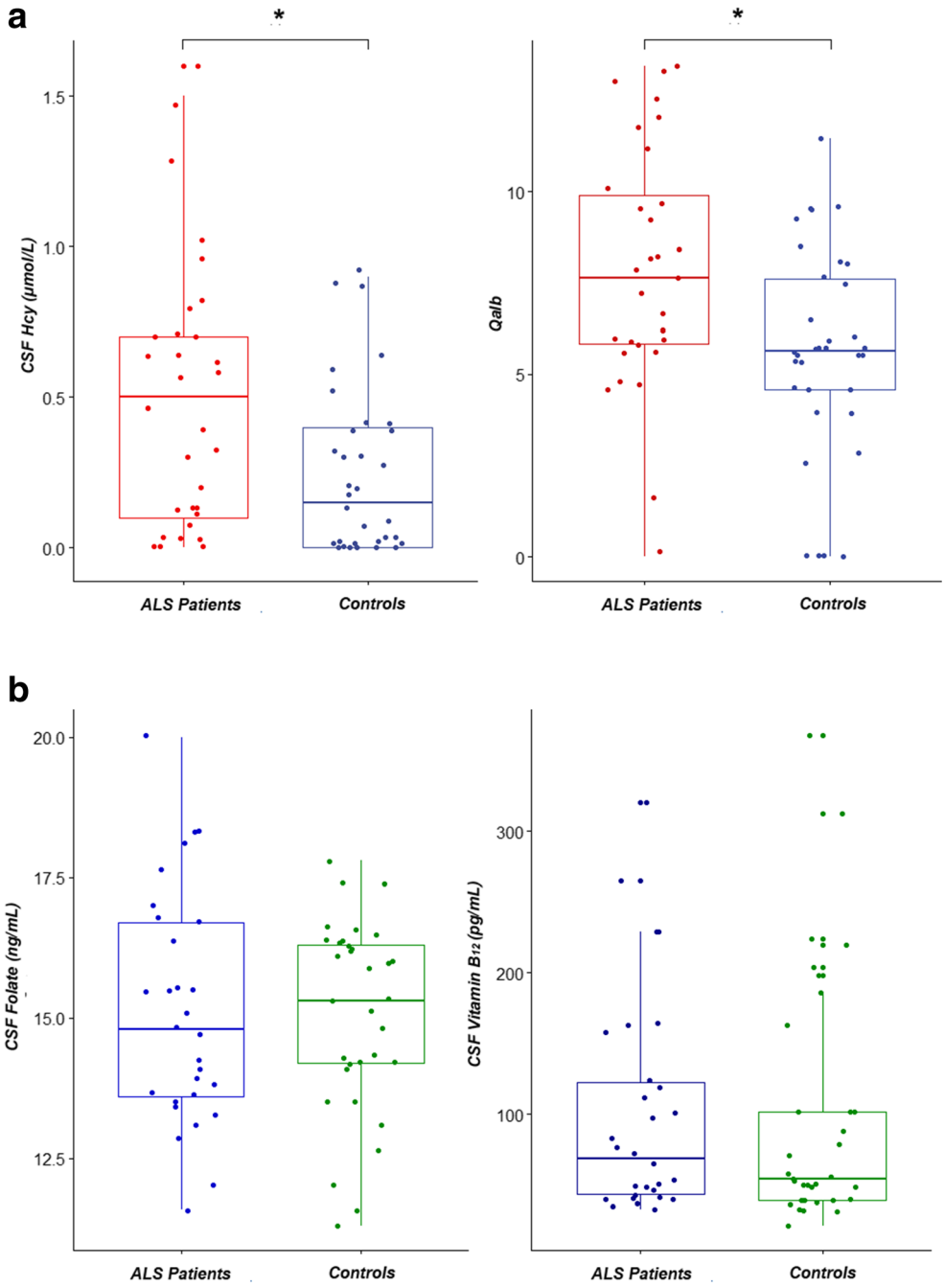

Fig. 1 The boxplot of CSF Hcy concentration and Q_{alb}, the CSF folate, and vitamin B12 concentrations respectively (1A and 1B) in ALS patients and controls. *$P<0.05$ was considered statistically significant

GLMMs analysis of CSF Hcy in ALS patients

To estimate the impact of predictor variables including age, gender, CSF vitamin B_{12}, folate and plasma Hcy, serum vitamin B_{12}, folate concentrations, as well as Q_{alb} on CSF Hcy level in ALS patients, GLMMs analysis was performed. The results showed that in ALS patients, the CSF Hcy was significantly correlated with CSF folate ($r = 0.281$, $P = 0.014$), serum folate ($r = -0.405$, $P = 0.018$), serum hs-CRP ($r = 0.214$, $P = 0.049$), CSF total protein ($r = 0.026$, $P = 0.019$), and Q_{alb} ($r = 0.159$, $P = 0.047$), respectively. Q_{alb} is an independent predisposing factor for elevated CSF Hcy in ALS patients (Table 2).

Discussion

BBB integrity is critical for maintaining homeostasis in the central nerve system (CNS) [29]. Within the neurovascular unit, the endothelial cells conform to BBB that limits the entry of potentially neurotoxic plasma components, blood cells, and pathogens into the brain [25, 30, 31]. Pericytes, which share a

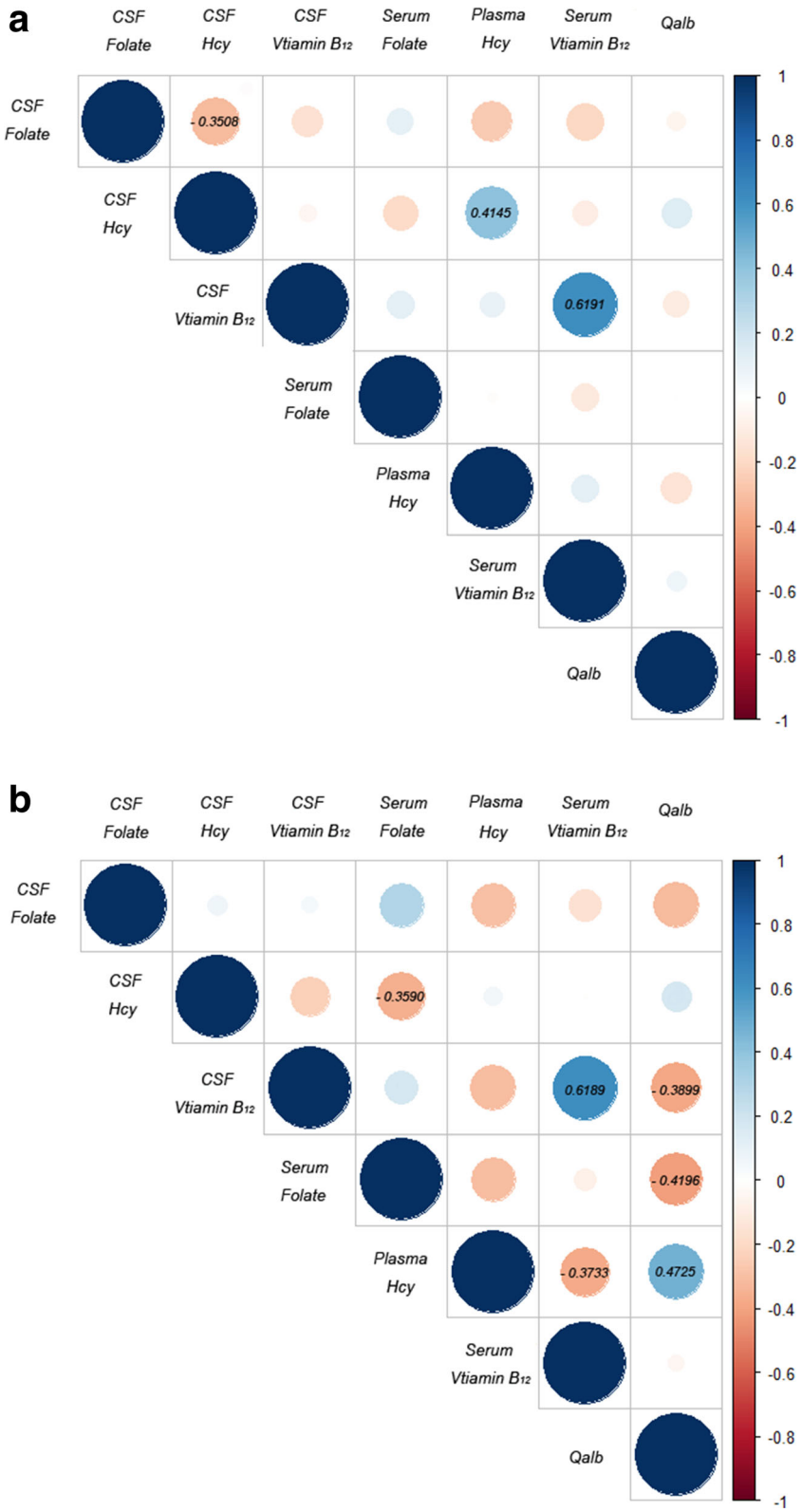

Fig. 2 Spearman correlations among CSF and plasma Hcy, serum folate, vitamin B12, as well as the Q_{alb} in controls and ALS patients, respectively (Fig. 2A and Fig. 2B). The circle in blue represented a positive correlation, and the red represented a negative correlation (for interpretation of the references to color in this figure legend). The size of the circle represented the magnitude of the correlation coefficient. Only the correlation coefficient which has statistically significant is displayed in the circle (P<0.05)

basement membrane with endothelial cells, contribute to the regulation of BBB permeability [32]. Only some substances, such as blood methionine, transfer through the BBB via a specific receptor [33]. Both the damage of the endothelial cells in the neurovascular unit and dysfunction of transporters can cause the increasing permeability of the BBB.

Table 2 GLMMs analysis of the factors correlated with CSF Hcy concentration in ALS patients

Model term	Coefficient	Std. error	*t* value	*P* value	95% CI		
Intercept	13.741	6.649	2.067	0.078	− 1.982	–	29.465
Age	− 0.030	0.016	− 1.866	0.104	− 0.069	–	0.008
Gender	− 0.479	0.252	− 1.896	0.100	− 1.076	–	0.118
Plasma homocysteine	− 0.196	0.104	− 1.886	0.101	− 0.441	–	0.050
CSF folate	0.281	0.087	3.234	0.014*	0.075	–	0.486
Serum folate	− 0.405	0.132	− 3.065	0.018*	− 0.717	–	− 0.092
CSF vitamin B_{12}	0.012	0.007	1.879	0.102	− 0.003	–	0.028
Serum vitamin B_{12}	− 0.003	0.001	− 1.788	0.117	− 0.006	–	0.001
CSF β_2-microglobulin	0.001	0.000	2.040	0.081	− 0.000	–	0.002
Serum β_2-microglobulin	− 0.000	0.000	− 0.736	0.486	− 0.001	–	0.000
CSF IgG	− 0.009	0.010	− 0.870	0.413	− 0.032	–	0.015
Serum IgG	− 0.164	0.095	− 1.740	0.125	− 0.388	–	0.059
Serum hs-CRP	0.214	0.090	2.381	0.049*	0.002	–	0.426
CSF total protein	− 0.026	0.009	− 3.018	0.019*	− 0.046	–	− 0.006
CSF glucose	0.014	0.090	0.154	0.882	− 0.198	–	0.226
CSF chlorides	− 0.074	0.036	− 2.083	0.076	− 0.159	–	0.010
Q_{alb}	0.159	0.066	2.410	0.047*	0.003	–	0.316

* $P<0.05$ was considered statistically significant

CSF/serum albumin ratio, known as the Q_{alb}, is considered to be the most common indicator and established method expressing the degree of the damage of the BBB [34, 35]. Studies in animal models and humans demonstrated that the BBB/BSCB breakdown relates to neurological deficits and other pathologies in the majority of neurodegenerative disorders such as ALS [24], AD [29], multiple sclerosis (MS) [36], and PD [32, 37]. BBB dysfunction in ALS cause venous reflux and down-regulates tight junction proteins Occludin and ZO-1, which brings to breaks in the tight junctions between endothelial cells in the veins and leads to leakage of toxic blood components into CNS tissue [38, 39]. Existing evidence has proved the injury of pericyte and endothelial cell and BBB disruption in ALS [38], and nearly half of patients with ALS were inclined to develop vascular pathology based on increased BBB permeability [17].

Hcy is a pro-inflammatory sulfur-containing redox-active endogenous amino acid formed by the demethylation of nutritional methionine. Most Hcy is remethylated to methionine which requires folate as a methyl donor and vitamin B_{12} as a cofactor. High level of Hcy can decrease cell viability significantly and cause brain damage through the mechanisms such as disturbed methylation, oxidative stress, and apoptosis [5]. Previous studies have found that increased Hcy concentration in plasma and CSF was correlated with progression of disease in ALS patients; alterations of folate and vitamin B_{12} metabolism were possibly involved in the pathophysiological process of ALS, and elevated level of Hcy in ALS was able to be lowered by folate and vitamin B_{12} treatment [12, 15, 40–42].

BBB/BSCB dysfunction and increased Hcy level in CSF/plasma play vital roles in ALS [21–23]. However, little is known about the relationship between BBB integrity and the level of Hcy, folate, vitamin B_{12} of plasma/serum, and CSF in ALS patients.

Previous studies suggested that Hcy, folate, and vitamin B_{12} levels in CSF were influenced by multiple factors including the transmembrane concentration difference, biomolecule size, as well as the permeability of BBB. Under normal physiological circumstances, Hcy, folate, and vitamin B_{12} enter the CSF from blood circulation by means of specific transporters, receptors, and channels, respectively [23]. During ALS, the injury of corresponding transporters may be inconsistent. Besides, compared with folate and vitamin B_{12}, the molecule of Hcy is relatively small. Hcy concentration in blood circulation was about 20- to 30-fold higher than that in CSF, which means there was a higher Hcy concentration gradient across the BBB. It was one of the possible reasons that Hcy of plasma could penetrate the damaged BBB and enter the CSF more easily. In the current study, CSF Hcy level was found to increase significantly in ALS patients, while for plasma Hcy level, there was no significant difference between ALS patients and control group. We also found Q_{alb} was significantly higher in ALS patients than that in controls. The bivariate Spearman correlation showed that the CSF Hcy level of ALS patients was positively correlated with Q_{alb} but without significance ($P > 0.05$, Fig. 2B). However, the correlated patterns among CSF and plasma/serum indexes of ALS patients were significantly different from that of control groups (Fig. 2A and Fig. 2B). Those changes suggested that the

Hcy metabolism in CSF of ALS patients was compromised due to BBB dysfunction. That can also explain why the Hcy level is high in the CSF of the ALS patients, but not significantly different from that in the plasma as compared with controls. Our results were different from the results of the previous study, in which Hcy level was high both in the CSF and plasma of the ALS patients [40]. The main reason may be related to the following two aspects. Different methods were used to determine the level of Hcy and other biological items; variance between methods possibly existed. Besides, CSF and plasma/serum samples were from different subjects in that study, while those samples in the present study were simultaneously collected from the same patient, biological variance, gender difference, and age may also contribute to the inconsistence.

Although Spearman correlation can reveal the preliminary relationship between the bivariate, it is difficult to rule out the influence of confounding factors. Considering the complex impact of age, gender, folate, vitamin B_{12}, etc. on CSF Hcy metabolism, we performed GLMMs analysis which has the advantage of controlling important confounding factors of study subjects and dealing with the non-normally distributed data as well as the autocorrelation problem among measured biomarkers, increased statistical power by combining information across the subjects in the study. The GLMMs analysis showed that, after adjusting for gender, age, and other variables, Q_{alb} still was an independent risk factor for increasing Hcy concentration in CSF ($P<0.05$). Those data further supported the idea that the BBB damage was an important cause of the increased CSF Hcy concentration of ALS patients.

There are some limitations to the current study. Firstly, Q_{alb} was used as an indicator of the BBB permeability. Although Q_{alb} is a classic index, there are still other potentially influencing factors such as microvascular changes that may affect the CSF flow rate [43]. Imaging techniques like dynamic contrast-enhanced magnetic resonance imaging (MRI) are recommended for further confirmation [35]. Secondly, diet habits, lifestyle, and other nutrients including vitamin B_2 and B_6 as well as some trace elements may have influences on Hcy level in blood and CSF. Thirdly, the ages of patients and controls were not perfectly matched since it is relatively difficult to have the proper controls and the matching was not predetermined in the present study. In addition, although quality control management was through all the tests, there were possibly some differences due to chance for the use of multiple tests, and multicenter testing and larger population prospective survey are still needed to further validate the results.

Conclusion

Our data suggested that there is a compromised BBB in ALS patients. Elevated Hcy concentration in CSF further aggravates the BBB damage and dysfunction. In this situation, Hcy in the blood is more likely to enter CSF, which further increases the Hcy level in CSF. The BBB plays a crucial role in Hcy metabolism process of CSF in ALS. These findings will lead to further studies about the related mechanisms and potential improved therapeutic protocol in the clinical practice.

Funding information This work was supported by grants from the National Natural Sciences Foundation of China (39970711 and 81673118). The authors are grateful to our phlebotomy staff and other collaborators for their assistance with the specimen collection.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval Ethical approval for this study was granted by the Ethical Committee of the Peking University Third hospital and Peking University Health Science Center. The principle of the Helsinki Declaration for using human subjects was obeyed. A written informed consent was obtained from all participants prior to being enrolled.

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