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Serum cystatin C and anti-*N*-methyl-D-aspartate receptor encephalitis

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Background: Cystatin C (CysC) is associated with many neurodegenerative disorders and autoimmune diseases, but its relationship with anti-*N*-Methyl-D-aspartate receptor (anti-NMDAR) encephalitis is unknown.

Methods: Serum levels of CysC were determined in 66 patients with anti-NMDAR encephalitis and 115 healthy controls. Of the 66 patients, 30 had a follow-up evaluation at 3 months after admission. Association of CysC with anti-NMDAR encephalitis and its clinical parameters were evaluated in the patients.

Results: The serum levels of CysC were significantly lower in patients with anti-NMDAR encephalitis than in controls (0.70 ± 0.13 vs 0.83 ± 0.17 mg/mL, $P < .001$). Disease severity and disease duration were significantly associated with CysC levels. Furthermore, a follow-up evaluation revealed that after treatment anti-NMDAR encephalitis patients had significantly increased serum CysC levels ($P < .001$) and significantly decreased modified Rankin Scale (mRS) scores ($P < .001$) compared with before treatment. In addition, a significant negative correlation was observed between the change in CysC levels and the change in mRS scores ($r = -.700$, $P < .001$).

Conclusion: Our results show that the serum levels of CysC are associated with anti-NMDAR encephalitis and its clinical parameters and that the changes in CysC levels correlate with therapeutic effect. Therefore, our findings provide new insights into the association between serum CysC and anti-NMDAR encephalitis.

KEYWORDS

anti-*N*-Methyl-D-aspartate receptor encephalitis, autoimmune disorders, Cystatin C

1 | INTRODUCTION

Cystatin C (CysC) is a non-glycosylated 13 kDa basic protein, consisting of 120 amino acids. It is a secreted cysteine inhibitor encoded by the CST3 (*Cst3*) gene, which is located on the short arm of chromosome 20.[1] CysC belongs to the cystatin superfamily of cysteine proteinase inhibitors that modulate protein degradation and cell-matrix interactions.[2] CysC is produced by nearly all cells and is secreted into the extracellular space, blood and cerebrospinal fluid (CSF). It is commonly used as a biomarker of renal function[3] and is a risk factor and strong predictor of cardiovascular disease.[4,5] Moreover, CysC is thought to play a major role in modulating immune cell activation and inflammation-driven cell death and counteracts the action of cathepsins, which are a family of lysosomal proteins released by activated microglia/macrophages.[6] In addition, recent studies have shown that CysC can protect neuronal cells from cell death through activating the autophagy pathway.[7,8]

Therefore, CysC may exert multiple functions in the pathogenesis and progression of immunological and neurological diseases. Indeed, a significant association of CysC with autoimmune diseases, such as systemic lupus erythematosus (SLE)[9] and chronic arthritis,[10] has been reported. Furthermore, CysC is associated with a variety of

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neurological diseases, including inflammatory neurological diseases, such as Guillain-Barre syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP) and multiple sclerosis (MS)[11,12] and neurodegenerative disorders, such as Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD).[7,13-16]

Anti-*N*-methyl-D-aspartate receptor (NMDAR) encephalitis is a rapid-onset, immune-mediated disorder that is often associated with psychosis, seizures, various movement disorders and autonomic system disturbances.[17] It develops through the action of immunoglobulin G (NMDAR antibodies) against the GluN1 subunit of the NMDAR.[18] Anti-NMDAR encephalitis is a severe and rare disorder that can affect patients of all ages with or without tumours in various regions, such as ovarian teratomas[19,20] Multiple studies have shown that immune cells, including B cells and T cells, are involved in the pathogenesis of anti-NMDAR encephalitis.[21,22]

However, the importance of CysC in anti-NMDAR encephalitis is unknown. Here, we analysed serum CysC levels in anti-NMDAR encephalitis patients and investigated the associations between the serum CysC levels and clinical parameters in these patients.

2 | METHODS

2.1 | Patients and controls

Sixty-six patients with anti-NMDAR encephalitis were recruited in the Department of Neurology, the Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China. The 115 healthy controls were selected from the Medical Examination Centre of the Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. All patients with anti-NMDAR encephalitis were hospitalized from July 2014 to August 2017 and diagnosed according to the standards defined by the criteria of Dalmau et al[17,23]: 1. The presence of one or more of the six major groups of symptoms: (i) Abnormal (psychiatric) behaviour or cognitive dysfunction; (ii) Speech dysfunction (pressured speech, verbal reduction, mutism); (iii) Seizures; (iv) Movement disorder, dyskinesias or rigidity/abnormal postures; (v) Decreased level of consciousness; (vi) Autonomic dysfunction or central hypoventilation. 2. Antibodies (IgG anti-GluN1 antibodies) testing in CSF were positive. 3. Reasonable exclusion of other disorders. In this study, no subject presented with hypertension, cardiopathy, diabetes or hepatic/renal dysfunction. In addition, none of the subjects presented abnormal levels of the prostate carcinoma-related mediators, prostate-specific antigen (PSA), carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP).

Cerebrospinal fluid (CSF) samples from anti-NMDAR encephalitis patients were analysed by cell-based indirect immunostaining using a commercially available kit (EUROIMMUN Medizinische Labordiagnostika, Lübeck, Germany) to detect IgG antibodies against NMDAR, according to the manufacturer's instructions. Brain magnetic resonance imaging (MRI) and CSF examinations were reviewed. All patients were screened with computed tomography (CT) or MRI or B ultrasound at least once for systemic tumours. Each patient's neurological status was assessed using the modified Rankin Scale (mRS).[24]

Treatments included first-line immunotherapy, second-line immunotherapy and tumour removal. First-line immunotherapies included the use of steroids, intravenous immunoglobulins alone or combined; second-line immunotherapy included rituximab, azathioprine or cyclophosphamide treatment alone or combined. We recorded the initial treatment as a failure if no sustained improvement occurred within 1 month of initiation of immunotherapy or tumour removal and if the mRS score remained at 4 or higher.

2.2 | Ethics statement and consent to participate

This research was approved by the ethics committee of the Third Affiliated Hospital of Sun Yat-sen University. Informed written consent was obtained from the patients or their representatives. Clinical evaluation was performed by an experienced neurologist who was blinded to the treatment and serum testing results of the patients. The authors of the study performed the analysis of serum samples and were blinded regarding clinical evaluation and treatment of the patients.

2.3 | Biochemical assays

Venous blood was collected for the measurement of serum CysC concentrations in the morning after an overnight fast and isolated by centrifugation at 3000 rpm for 10 minutes within 1 hour of sample collection. The separated serum was stored at −80°C before evaluation. The serum CysC levels were measured within 4 hours using a Clinical Analyzer 7180-ISE (Hitachi High-Technologies, Tokyo, Japan), and the normal range of serum CysC values was 0.55-1.55 mg/L in the Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China.

2.4 | Follow-up evaluations

Thirty anti-NMDAR encephalitis patients were followed up at 3 months after treatment. Patients who were followed up received repeated assessments of mRS scores and serum CysC levels in the Third Affiliated Hospital of Sun Yat-Sen University.

2.5 | Statistical analysis

All statistical analyses were performed using SPSS 16.0 software (SPSS Inc, Chicago, IL, USA). All continuous variables are presented as the mean ± standard deviation if the data were normally distributed or as medians (min, max) if the data were not normally distributed. To assess the significance of differences between groups, the Student's *t* test was applied when the data were normally distributed, while Mann-Whitney *U* tests were performed when the data were not normally distributed. Pearson's correlation and Spearman's rank correlation coefficients were used to evaluate correlations between different clinical parameters for normal and non-normal data, respectively. Pearson's correlations were used to evaluate the correlations between serum CysC levels and age. Spearman's correlations were used to evaluate the correlations between serum CysC levels and mRS scores, disease duration, CSF WBC, TP, GLU and CL. *P* values < .05 were considered statistically significant.

3 | RESULTS

3.1 | Demographic and clinical features of patients and healthy controls

Table 1 shows a total of 66 anti-NMDAR encephalitis patients (mean age, 28.4 years; female:male = 37:29) and 115 healthy controls (mean age, 29.6 years; female:male = 63:52). The median disease duration, median mRS score, median CSF WBC and TP in the anti-NMDAR encephalitis patients were 30 days (range 2-170 days), 4.0 (range 1-5), 8×10^6 (range 0-844 × 10^6) and 0.3 g/L (range 0.06-2.85 g/L), respectively. Sixteen of 66 patients (24.2%) had tumours, including ovarian teratoma (n = 12, 18.2%), ovarian cysts (n = 3, 4.5%) and colon carcinoma (n = 1, 1.5%). Twenty-two of 66 patients (33.3%) had comorbidities, including anaemia (n = 3, 4.5%), gallbladder polyps (n = 3, 4.5%), gallstone (n = 2, 3.0%), chronic cholecystitis (n = 4, 6.1%), Hashimoto thyroiditis (n = 2, 3.0%) and other (n = 8, 1 lumbar disc herniation, 1 arachnoid cyst, 1 favism, 1 thyroid cyst, 1 parotitis, 1 cervical spondylosis, 1 chronic nasosinusitis, 1 haemorrhoids). Forty-nine patients (74.2%) received first-line treatment, 17 (25.8%) received combined first- and second-line treatments, and 14 (21.2%) received tumour removal treatment. Sixteen patients (24.2%) needed neurological intensive care unit (NICU) support, and 8 patients (12.1%) had mechanical ventilation.

As showed in Table 2, proportion of females was 56.1% (37/66), and children (age < 18 years) was 25.8% (17/66). The mean serum CysC levels of female anti-NMDAR encephalitis were 0.68 (range 0.45-0.98) mg/L, and the mean CysC levels of children were 0.69 (range 0.56-0.91) mg/L.

3.2 | Comparison of serum CysC levels between patients with anti-NMDAR encephalitis and healthy controls

We determined the levels of serum CysC in all patients with anti-NMDAR encephalitis and in the healthy controls. The levels of serum CysC in the patients (0.70 ± 0.13 mg/mL) were significantly lower than in the healthy controls (0.83 ± 0.17 mg/mL) ($P < .001$) (Figure 1A). To investigate whether the difference was gender dependent, we performed a stratified analysis based on gender. The stratified analysis showed that the serum CysC levels in male and female patients were significantly lower than in male and female controls, respectively (Figure 1B).

3.3 | Relationship between CysC levels and the clinical and immunological characteristics of anti-NMDAR encephalitis

We next investigated the relationship between CysC levels and the immunological characteristics of anti-NMDAR encephalitis. To evaluate the association of CysC with clinical features, we subdivided the anti-NMDAR encephalitis patients into subgroups according to sex, age, mRS, disease duration, brain MRI, tumour presence and response to therapy and compared the levels of CsyC within the groups. As summarized in Table 2, patients with mRS scores < 4 had significantly

TABLE 1 Demographic features of patients with anti-NMDAR encephalitis and healthy controls

	Anti-NMDAR encephalitis (n = 66)	Healthy controls (n = 115)
Age onset (y, mean ± SD)	28.4 ± 14.9	29.6 ± 14.6
Gender (male: female)	29:37	52:63
Disease duration (d, median, range)	30 (2-720)	–
CSF anti-NMDAR Abs positive (n, %)	66 (100)	–
CysC level (mg/L, mean ± SD, range)	0.70 ± 0.13 (0.45-0.98)	0.83 ± 0.17 (0.48-1.32)
mRS (median, range)	4.0 (1.0-5.0)	–
CSF routine		
CSF WBC (×10^6, median, range)	8 (0-844)	–
CSF TP (g/L, median, range)	0.3 (0.06-2.85)	–
CSF Glu (m mol/L, median, range)	3.31 (1.81-6.23)	–
CSF CL (m mol/L, median, range)	123.0 (6.43-132.2)	–
With tumour (n, %)	16 (24.2)	–
Ovarian teratoma	12 (18.2)	–
Ovarian cysts	3 (4.5)	–
Colon carcinoma	1 (1.5)	–
Comorbidities (n, %)		
Anaemia	3 (4.5)	–
Gallbladder polyps	3 (4.5)	–
Gallstone	2 (3.0)	–
Chronic cholecystitis	4 (6.1)	–
Hashimoto thyroiditis	2 (3.0)	–
Other	8 (12.1)	–
Symptom onset (n, %)		
Prodromal symptoms (fever, headache)	22 (33.3)	–
Psychiatric symptoms	37 (56.1)	–
Memory deficits	7 (10.6)	–
Speech disturbances	9 (13.6)	–
Seizures	26 (39.4)	–
Movement disorders	17 (25.8)	–
Loss of consciousness	14 (21.2)	–
Sleep disorder	9 (13.6)	–
Treatment (n, %)		–
First-line treatment	49 (74.2)	–
First-line combined with second-line treatment	17 (25.8)	–
Tumour removal	14 (21.2)	–
With NICU support (n, %)	16 (24.2)	–
With mechanical ventilation (n, %)	8 (12.1)	–

Anti-NMDAR, anti-*N*-Methyl-D-aspartate receptor; CysC, cystatin C; mRS, modified Rankin Scale; CSF, cerebrospinal fluid; Ab, antibody; WBC, white blood cells; TP, total protein; Glu, glucose; CL, chlorine; SD, standard deviation; NICU, neurological intensive care unit.
Other included 1 lumbar disc herniation, 1 arachnoid cyst, 1 favism, 1 thyroid cyst, 1 parotitis, 1 cervical spondylosis, 1 chronic nasosinusitis, 1 haemorrhoids.

TABLE 2 Serum CysC levels in patients with anti-NMDAR encephalitis

Variables	Mean ± SD (mg/L)	Range (min-max, mg/L)	*P* Value
Sex			
Male (n = 29)	0.73 ± 0.12	0.55-0.98	
Female (n = 37)	0.68 ± 0.14	0.45-0.98	.216
Age			
<18 y (n = 17)	0.69 ± 0.10	0.56-0.91	
≥18 y (n = 49)	0.70 ± 0.14	0.45-0.98	.895
mRS			
<4 (n = 30)	0.76 ± 0.12	0.61-0.98	
≥4 (n = 36)	0.65 ± 0.12	0.45-0.96	<.001
Disease duration (d)			
>30 (n = 27)	0.75 ± 0.13	0.57-0.98	
≤30 (n = 39)	0.67 ± 0.12	0.45-0.92	.016
Brain MRI			
Normal (n = 31)	0.68 ± 0.13	0.45-0.96	
Abnormal (n = 35)	0.72 ± 0.13	0.49-0.98	.223
Tumour			
With (n = 16)	0.66 ± 0.13	0.45-0.96	
Without (n = 50)	0.72 ± 0.13	0.49-0.98	.095
Response to therapy			
Favourable (n = 51)	0.72 ± 0.13	0.45-0.98	
Limited (n = 15)	0.66 ± 0.13	89.0-345.0	.112

Anti-NMDAR, anti-*N*-Methyl-D-aspartate receptor; UA, uric acid; mRS, modified Rankin Scale; MRI, magnetic resonance imaging; SD, standard deviation.

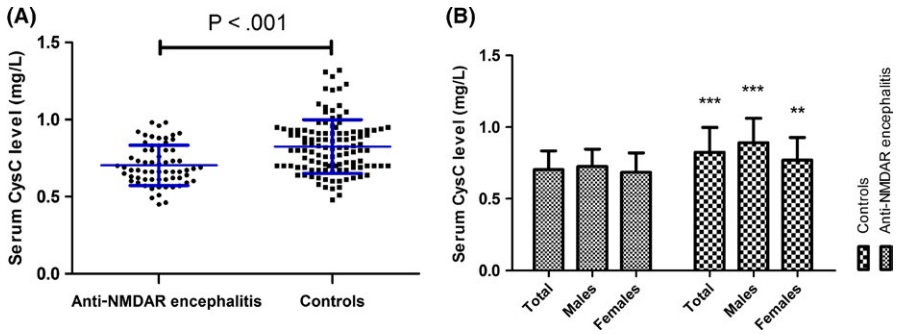

FIGURE 1 Comparison of serum CysC levels between anti-NMDAR encephalitis patients and healthy control subjects (controls). (A) Comparison of CysC levels between the anti-NMDAR encephalitis patients (n = 66) and controls (n = 115). (B) Comparison of CysC levels between the anti-NMDAR encephalitis patients and controls according to gender. Anti-NMDAR encephalitis patients (male) vs controls (male), ***P < .001; anti-NMDAR encephalitis patients (female) vs controls (female), **P < .01

higher serum CysC levels than those with mRS scores ≥ 4 (P < .001). In addition, a significant association was also observed between CysC and disease duration, where serum CysC levels in patients with disease duration ≤ 30 days were significantly lower than in patients with disease duration > 30 days (P = .016).

We also evaluated the correlation between CysC levels and clinical characteristics, as well as the CSF parameters of anti-NMDAR encephalitis. Depending on the data distribution, either Pearson's or Spearman's correlations were used to evaluate the correlations between serum CysC levels and clinical variables. As shown in Table 3, the serum levels of CysC significantly correlated with mRS scores (r = −.423, P < .001) and disease duration (r = .316, P = .01), while no significant correlation was observed between CysC levels and the other variables. To eliminate the influence of gender, we divided the patients and healthy subjects into two groups (females/males) and performed the correlation analysis. The negative correlation between CysC levels and mRS scores was observed for both males (r = −.521, P = .004) and females (r = −.385, P = .019). However, the positive correlation between CysC and disease duration was observed only for males (r = .390, P = .037).

TABLE 3 Correlation coefficients generated between serum CysC levels and the clinical characteristics and CSF parameters in patients with anti-NMDAR encephalitis

	Total		Male		Female	
	r	*P*	*r*	*P*	*r*	*P*
Age	.174	.161	.095	.623	.202	.230
mRS	−.423**	<.001	−.521**	.004	−.385*	.019
Disease duration	.316**	.010	.390*	.037	.130	.444
CSF WBC	−.133	.304	−.195	.321	−.166	.349
CSF TP	.029	.822	−.042	.830	−.017	.923
CSF GLU	.047	.714	.046	.816	−.034	.849
CSF CL	.041	.750	−.057	.774	.168	.342

For all abbreviation definitions, see footnotes to Table 1.
Pearson's correlations were used to evaluate the correlations between serum CysC and age. Spearman's correlations were used to evaluate the correlations between serum CysC and mRS scores, disease duration, CSF WBC, TP, GLU and CL.
*P < .05, **P < .01.

TABLE 4 Demographics of the 30 anti-NMDAR encephalitis with 3-mo follow-up

	Anti-NMDAR encephalitis (n = 30)		
	Before treatment	After treatment	*P* value
CSF anti-NMDAR Abs positive(n, %)	30 (100)	18 (60)	–
Sex (male:female)	16:14	16:14	–
Age onset (median, range, y)	26.5 (12-64)	26.5 (12-64)	–
Disease duration (median, range, d)	25 (5-600)	115 (95-690)	–
mRS (median, range)	4 (1-5)	2 (0-5)	<.001
CysC level (mg/L, median, range)	0.67 (0.45-0.98)	0.82 (0.49-1.38)	<.001

Anti-NMDAR, anti-N-Methyl-D-aspartate receptor; CSF, cerebrospinal fluid; Ab, antibody; CysC, cystatin C; mRS, modified Rankin Scale.

3.4 | Follow-up evaluation of serum CysC levels in anti-NMDAR encephalitis patients after treatment

As our results demonstrated that the serum levels of CysC are associated with anti-NMDAR encephalitis and its disease severity, we further investigated whether the serum levels of CysC change during the treatment. The serum levels of CysC and mRS scores were determined in 30 anti-NMDAR encephalitis patients (male: female 16:14; median age onset 26.5 years) before and at 3 months after treatment (Table 4 and Figure 2). As shown in Figure 2A, the mRS scores were significantly decreased after treatment compared with before treatment (median, 2 vs 4, P < .001), suggesting the treatment was effective. Interestingly, following anti-NMDAR encephalitis treatment, the serum levels of CysC were significantly increased (0.85 ± 0.18 vs 0.69 ± 0.12 mg/L, P < .001) (Figure 2B). A correlation analysis showed a negative correlation between changes in the serum levels of CysC and changes in the mRS scores (r = −.700, P < .001) (Figure 2C).

4 | DISCUSSION

In the present study, we investigated the relationship between the serum levels of CysC and anti-NMDAR encephalitis. To the best of our knowledge, this is the first study to analyse serum CysC levels in patients with anti-NMDAR encephalitis.

Our results demonstrated that the serum levels of CysC in anti-NMDAR encephalitis patients were significantly lower compared with healthy controls, suggesting an association of CysC with the disease. Furthermore, we also found that serum CysC levels were associated with disease severity and disease duration, that is patients with a worse prognosis or shorter disease duration, who may suffer from more inflammatory oxidative injury, had lower serum CysC levels. Thus, these results suggest that the lower serum CysC levels observed in anti-NMDAR encephalitis patients may be associated with less anti-inflammatory action and less neuroprotection. Both clinical and experimental evidence from previous studies indicates that CysC is functionally associated with neurological and inflammatory diseases. Clinical studies have shown that CysC plays a role in anti-inflammation[25-27] and exerts neuroprotective effects against many neurological diseases, including ALS,[7] AD,[13] PD[14] and subarachnoid haemorrhage (SAH).[8] In addition, multiple lines of experimental evidence support an anti-inflammatory and neuroprotective role for CysC. CysC deficiency exacerbated the clinical symptoms and neuropathologies in a mouse model of an inherited neurodegenerative disorder (progressive myoclonic epilepsy type 1 mice), including motor coordination disorder, cerebellar atrophy, neuronal loss in the cerebellum and cerebral cortex and gliosis.[28]

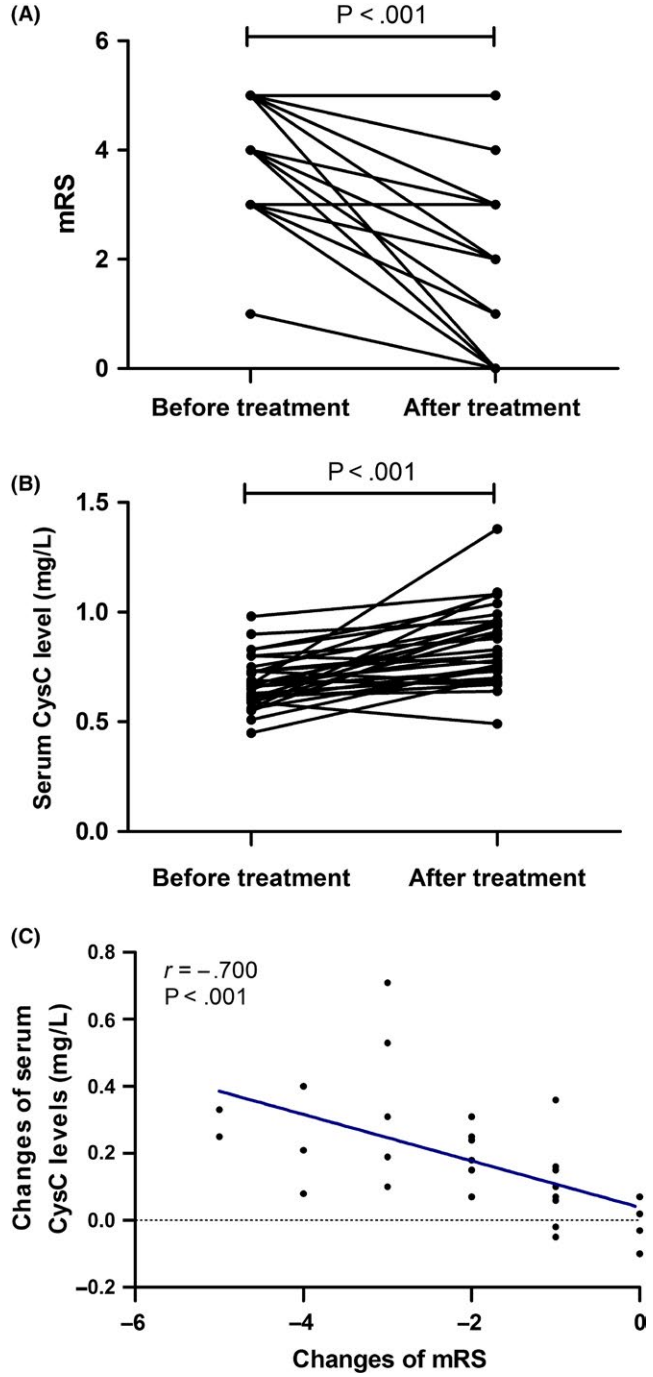

FIGURE 2 Changes in serum levels of CysC in anti-NMDAR encephalitis patients during treatment. (A) Improvement in mRS scores from initial admission to follow-up evaluation 3 mo after treatment. (B) Changes in serum CysC levels from initial admission to follow-up evaluation 3 mo after treatment. (C) Relationship between the changes in mRS scores and the changes in serum CysC levels after treatment ($r = -.700$, $P < .001$). Cys C, Cystatin C; mRS, modified Rankin Scale; n = 30

The lack of CysC has been reported to primarily affect priming of the immune system and enhance experimental arthritis in vivo.[29] It has also been shown that CysC deficiency leads to an enhanced activation of dendritic cells as antigen-presenting cells both in vivo and ex vivo.[29] Anti-NMDAR encephalitis is an immune-mediated inflammatory disorder. Immune cells such as B cells and T cells are important effectors and regulators of immune responses, inflammation in anti-NMDAR encephalitis.[22,30-32] Although the mechanism behind the association between low serum CysC levels and anti-NMDAR encephalitis is unknown, it suggests a role for CysC in the pathogenesis of anti-NMDAR encephalitis. We speculate that low serum CysC levels may contribute to an imbalance in the immune system, less protection against inflammatory oxidative damage and neuronal cell injury in anti-NMDAR encephalitis. However, further studies are required to clarify the underlying mechanisms.

Interestingly, our results demonstrated that the serum levels of CysC in anti-NMDAR encephalitis significantly increased after treatment. Moreover, there was a significant negative correlation between the changes in the serum levels of CysC and the changes in mRS scores. The reasons why serum CysC levels were elevated after treatment are unknown. First, we speculate that elevated serum CysC levels may be associated with reduced CNS inflammatory oxidative damage after treatment. Alternatively, higher levels of CysC after treatment could exert more protection against inflammatory responses and neuronal cell injury in anti-NMDAR encephalitis. Second, the difference between serum CysC levels before and after treatment might be also explained by the treatment such as corticoids. These data suggest that CysC may be a therapeutic candidate that could potentially prevent brain damage in anti-NMDAR encephalitis patients.

In the present study, the percentage of male patients (44%) was higher than the results reported by Dalmau et al (males 9%)[33] and Irani et al (males 30%).[34] The aetiology of anti-NMDAR encephalitis is unclear. Lots of studies have shown that anti-NMDAR encephalitis may be triggered by tumours[20,35,36] or virus infection.[37-39] Therefore, we speculated that the disproportion may be associated with the different aetiology of the disease, or different sample size, race of the patients. However, further studies will be required to clarify this hypothesis.

We acknowledge that there are some limitations in our study. First, the sample size of patients was small (n = 66 patients). Second, as CSF CysC levels in normal conditions are five times higher than in serum,[40] it would be more appropriate to assay CysC levels from CSF and serum in order to obtain a more congruent correlation as previous paper.[16,41] However, we did not test the CysC levels in the CSF of anti-NMDAR encephalitis patients. Third, CysC is encoded by the CST3 (*Cst3*) gene; however, we did not investigate the CST3 gene of anti-NMDAR encephalitis patients.

In conclusion, our results show that serum levels of CysC are associated with anti-NMDAR encephalitis and its disease severity. Our results support an anti-inflammatory and neuroprotective role for CysC in anti-NMDAR encephalitis, which sheds new light on the pathogenesis of the disease.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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