Association of Pain with Plasma C5a in Patients with Neuromyelitis Optica Spectrum Disorders During Remission

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Objective: To investigate the association of pain with plasma C5a levels and other related inflammatory cytokines in neuromyelitis optica spectrum disorders (NMOSD) patients during remission.

Participants and Methods: NMOSD patients (n = 87) and healthy controls (HC; n = 44) were consecutively recruited between January 2017 and April 2018. Plasma complement 5 (C5), C5a, interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-1β levels were detected. Visual Analogue Scale (VAS), ID pain scale, 24-item Hamilton Depression Scale (HAMD), Multiple Sclerosis Impact Scale (MSIS-29), and Kurtzke Expanded Disability Status Scale (EDSS) were used to evaluate the degree and types of pain, the existence of depression and anxiety, and the life quality and disability status of patients. Binary logistic regression equation was used to assess the association of pain with plasma C5a levels.

Results: Among the 87 NMOSD patients, 40 complained of pain that in 67.5% (27/40) of cases had a neuropathic component (ID pain ≥2). Plasma C5a, IL-6, TNF-α, and IL-1β levels were significantly elevated in NMOSD patients than in HC. Plasma C5 levels were negatively correlated with the time from sampling to the last relapse or disease onset. NMOSD patients with pain had higher plasma C5a levels, and they suffered from a higher disability, more anxiety, and worse life quality compared to those patients without pain. In NMOSD patients with pain, there were not significant differences between plasma levels of C5, C5a, IL-6, TNF-α, or IL-1β, regardless of neuropathic pain or not. Binary logistic regression showed that the OR of plasma C5a level was 1.002, with gender and EDSS score were identified as independent factors associated with pain in NMOSD.

Conclusion: NMOSD patients during remission had elevated C5a and related inflammatory cytokines levels in peripheral blood. Elevated C5a may have a unique role in the pathogenesis of pain in NMOSD patients.

Keywords: neuromyelitis optica spectrum disorders, remission, pain, C5a

Introduction

Neuromyelitis optica spectrum disorders (NMOSD) is a rare autoimmune inflammatory demyelinating disease of the central nervous system (CNS) characterized by recurrent inflammation of the optic nerve, spinal cord, and specific brain areas. Compared to multiple sclerosis (MS) that primarily targets myelin, NMOSD induces irreparable neuronal cell death which leads to more severe disability and a poorer prognosis. The clinical presentation of NMOSD includes severe episodes of optic neuritis that may lead to loss of vision, transverse myelitis causing paraplegia or paralysis, and sometimes brainstem encephalitis causing intractable vomiting or hiccupps. In addition, NMOSD causes severe, persistent pain which is more prevalent (83.8%–91%) than in MS (~47%) and most other neurological diseases. Many NMOSD patients experience pain that severely affects their quality of life and is often refractory to treatment.

Aquaporin-4 antibody (AQP4- immunoglobulin [IgG]) is a highly specific antibody involved in the disease pathogenesis of NMOSD, which can be used to distinguish NMOSD from MS. Animal experiments showed that immunoglobulin...
G from aquaporin-4-autoantibody-positive neuromyelitis optica (NMO) patients did not activate mouse complement and was not pathogenic when injected into mouse brain. However, co-injection of immunoglobulin G from NMO patients with human complement produced NMO-like lesions in mice, suggesting that human complement plays a key role in the pathogenesis of NMOSD. Complement 5 (C5) is an activated complement protein common to all complement activation pathways. One of its cleavage products, C5a, is the most effective complement-derived inflammatory mediator. Several lines of evidence have shown that C5a plays a pivotal role in neuropathic pain mechanisms; however, its association with pain in NMOSD remains unclear. Therefore, in this study, we investigated the association of pain with plasma levels of C5a and other related inflammatory factors in patients with NMOSD during remission.

**Participants and Methods**

**Participants**

Patients with NMOSD and healthy controls (HC) were consecutively recruited from the Beijing Tiantan Hospital between January 2017 and April 2018. The diagnosis of NMOSD was confirmed according to the 2015 revised international criteria. All patients were in remission (had remained relapse-free for over a 1-month period) and had no other autoimmune comorbidities at the time of blood collection. In NMOSD patients, clinical relapses were defined as new neurologic symptoms lasting at least 24 hours and accompanied by new neurologic findings, occurring 30 days after the previous attack. Acute phase was defined as within one month of onset or relapse of NMOSD. Age- and sex-matched volunteers without immune disorders were recruited as HC. Infections were ruled out based on complete blood count testing in all participants. Clinical records of participants were collected, including sex, age at sampling, disease duration since the first onset, and relapse times; the mean annualized relapse rate (ARR, times/year) was calculated accordingly.

**Measurement Instruments and Evaluation**

Visual analogue scale (VAS) and ID pain scale were used for evaluating the degree of pain and screening of neuropathic pain (ID pain score ≥ 2). In addition, 24-item Hamilton Depression Scale (HAMD) and 14-item Hamilton Anxiety Scale (HAMA) were used to evaluate the existence of depression and anxiety; a total score of HAMD ≥ 8 indicated depression and HAMA ≥ 7 indicated anxiety. Also, Multiple Sclerosis Impact Scale (MSIS-29) was used to evaluate the impact of disease on the life quality of patients. The total score of MSIS-29 ranged between 29 and 145, with a higher score indicating lower life quality. Kurtzke Expanded Disability Status Scale (EDSS) was used to evaluate the disability status of patients.

**Assay for Plasma C5, C5a, IL-6, TNF-α, and IL-1β Levels**

Peripheral blood was obtained from each participant. All blood samples were collected at 9:00 a.m. After collection into a 4-mL disposable BD Vacutainer® containing ethylene diamine tetraacetic acid, plasma samples were separated at 2000 × g for 10 min within 3 h and stored in aliquots at −80°C until further analysis. All testing was performed in a blinded manner with respect to diagnosis or clinical presentations. Plasma C5, C5a, interleukin (IL)−6, tumor necrosis factor (TNF)-α, and IL-1β levels were assayed using MILLIPLEX® map human High Sensitivity Cytokine/Chemokine Panels (Cat. HCMP1MAG, HCYTOMAG) (Merck KGaA, Darmstadt, Germany) according to the manufacturer’s instructions.

**Statistical Analysis**

Statistical analysis was conducted using SPSS 22.0 (International Business Machines Corporation, Chicago, IL, USA). After normality test, data with non-Gaussian distributions were analyzed using the Mann–Whitney U-test for two groups. Normally distributed data were processed using Student’s t-test or analysis of variance. Demographic features of NMOSD patients with and without pain, including gender, age, age at disease onset, disease duration, ARR, relapse times, etc., were compared. To clarify whether plasma C5a was correlated with pain risk in NMOSD, binary logistic regression was conducted after adjusting for the above demographic features. Odds ratios (ORs) and 95% confidence intervals (CIs) for pain and plasma C5a were also estimated. A P-value of <0.05 was considered to be statistically significant.
Results
Demographics and Disease Characteristics
A total of 87 patients with NMOSD and 44 HC were enrolled in the current study. Among the 87 NMOSD patients, 40 complained of pain that in 67.5% (27/40) of cases had a neuropathic component (ID pain ≥2). The demographics and disease characteristics of the participants are presented in Table 1. NMOSD patients with pain showed significantly higher EDSS and MSIS-29 scores, as well as anxiety rates than those without pain (all P values < 0.01). There were no significant differences in proportions of depression in NMOSD patients with pain and without pain.

Plasma C5, C5a, IL-6, TNF-α, and IL-1β Levels in NMOSD Patients and HC
There was no significant difference (P=0.10) between plasma C5 levels in patients with NMOSD and HC. However, plasma C5a levels in NMOSD patients was significantly higher than in HC (P<0.000) (Table 2).

Next, we examined the levels of plasma IL-6, TNF-α, and IL-1β, whose productions may be induced by C5a. The data showed that plasma levels of all the cytokines mentioned above were significantly elevated in NMOSD patients than in HC (P=0.035, 0.019, and 0.012, respectively) (Table 2).

Table 3 shows the Spearman correlations among plasma C5a and IL-6, TNF-α, and IL-1β in NMOSD patients during remission. Briefly, plasma IL-6 and IL-1β levels were positively correlated with plasma C5a levels.

Influences of the Time from Sampling to the Last Relapse or Disease Onset on Plasma Levels of C5, C5a, IL-6, TNF-α, and IL-1β in NMOSD Patients During Remission
The time from sampling to the last relapse or disease onset was calculated and the correlations of C5, C5a, and inflammatory cytokines with it were analyzed. The results showed that the time from sampling to the last relapse or disease onset of NMOSD patients was 198.75 ± 20.61 (days, mean ± SE), ranged from 31 to 1192 days. Spearman’s rank correlation analysis showed that plasma C5 levels were negatively correlated with the time interval (P=−0.016, r=−0.258). Plasma levels of C5a, IL-6, TNF-α, and IL-1β had no correlation with the time interval (P=0.729, 0.285, 0.188, and 0.913, respectively).

Plasma Levels of C5, C5a, IL-6, TNF-α, and IL-1β in NMOSD Patients with Pain and without Pain, and in Those with Different Type of Pain
There were no significant differences in plasma C5 levels between NMOSD patients with and without pain (P=0.698). Nevertheless, NNMOSD patients with pain had higher plasma C5a levels than those patients without pain (P=0.008). In

Table 1 Demographic Characteristics of Participants in the Study

| Index                     | All Participants |          | P values |          |          |          |          |
|---------------------------|------------------|----------|----------|----------|----------|----------|----------|
|                           | HC (n=44)        | NMOSD (n=87) | P        | NMOSD (n=87) | P        | NMOSD (n=87) | P        |
| Gender, female/male       | 34/10            | 78/9     | 0.057    | 38/2     | 40/7     | 0.247    |
| Age (years, mean ± SE)    | 35.02±1.26       | 39.70±1.40 | 0.421   | 41.95±1.83 | 37.79±2.00 | 0.133    |
| Age at onset (years, mean ± SE) | –          | 36.69±1.39 | –       | 38.88±1.86 | 34.83±2.02 | 0.149    |
| Duration of disease (years, mean ± SE) | –          | 3.01±0.48  | –       | 3.13±0.81  | 2.90±0.57  | 0.480    |
| ARR (times/year, mean ± SE) | –          | 0.85±0.11  | –       | 0.90±0.18  | 0.80±0.14  | 0.626    |
| Relapse times (mean ± SE)  | –          | 2.00±0.45  | –       | 2.08±0.67  | 1.90±0.59  | 0.327    |
| VAS                       | –          | 2.28±0.31  | –       | 4.95±0.34  | –        | –        |
| EDSS                      | –          | 3.84±0.18  | –       | 4.33±0.26  | 3.44±0.24  | 0.007    |
| MSIS-29                   | –          | 60.82±2.69 | –       | 73.63±4.23 | 49.91±2.56 | 0.000    |
| HAMA (score≥7)            | –          | 36/51     | –       | 23/17     | 13/34     | 0.005    |
| HAMD (score≥8)            | –          | 44/43     | –       | 24/16     | 20/27     | 0.105    |

Abbreviations: HC, healthy control; NMOSD, neuromyelitis optica spectrum disorders; SE, standard error; VAS, visual analogue scale; EDSS, expanded disability status scale; MSIS, multiple sclerosis impact scale; HAMA, Hamilton anxiety scale; HAMD, Hamilton depression scale.
addition, no significant differences were found between plasma levels of IL-6, TNF-α, or IL-1β in NMOSD patients with pain and without pain (P=0.190, 0.123, and 0.528, respectively) (Table 2).

In NMOSD patients with pain, there were not significant differences between plasma levels of C5, C5a, IL-6, TNF-α, or IL-1β, regardless of neuropathic pain or not (all P>0.05) (Table 2).

### Association of C5a Level with Pain in Patients with NMOSD During Remission

Binary logistic regression analysis showed that the plasma C5a level was significantly correlated with pain risk in patients with NMOSD (P = 0.007). All models were adjusted for gender, age, disease duration, relapse times, and EDSS score, which were included in univariate analysis and clinical practice (Table 4). Gender and EDSS score were identified as independent factors associated with pain in NMOSD (P = 0.047 and 0.005, respectively).

### Discussion

Severe episodes of painful optic neuritis and longitudinally extensive transverse myelitis are the most typical clinical syndromes of NMOSD. In addition, some limited forms of encephalic lesions and coexisting autoimmune disorders are

| Variable | B    | S.E. | OR value | P value | 95% CI        |
|----------|------|------|----------|---------|---------------|
| C5a      | 0.002| 0.174| 1.002    | 0.007   | 1.001~1.004   |
| Gender   | 1.851| 0.933| 6.365    | 0.047   | 1.022~39.627  |
| EDSS score | 0.492| 0.174| 1.636    | 0.005   | 1.163~2.303   |
| Constant | −7.862| 2.415| 0.000    | 0.001   |               |

**Abbreviation:** EDSS, expanded disability status scale.
encompassed in NMOSD. Pain, which is highly prevalent in patients with NMOSD, can greatly impair quality of life. In the present study, 46% of the patients with NMOSD during remission complained of current pain. They suffered from a higher disability, greater anxiety, and worse life quality.

Next, we investigated the association of pain with plasma levels of C5a and other related inflammatory factors in NMOSD patients during remission. These patients had significantly higher plasma C5a levels compared to HC. What’s more, the inflammatory cytokines IL-6 and IL-1β plasma levels were also elevated and positively correlated with the C5a level. Also, 46% of NMOSD patients in the current study experienced ongoing pain, and further binary logistic regression analysis showed that pain was positively correlated with the C5a level, with gender and EDSS score were identified as independent factors.

AQP4-IgGs are present in 65 to 88% of patients with NMOSD. AQP4-IgG triggers the complement cascade, which leads to inflammation and the formation of the membrane attack complex. Recent studies have suggested that the C5 inhibitor eculizumab, which inhibits its cleavage into C5a and C5b and the subsequent neurologic damage, significantly reduced relapse risk compared to placebo in patients with AQP4-IgG-positive NMOSD. As one of the C5 cleavage products, C5a is an efficient proinflammatory factor activated its cell membrane receptors, C5aR, mainly distributed in neutrophils, astrocytes, and microglia. Increased concentration of C5a had been found in the cerebrospinal fluid (CSF) of patients with active NMOSD, and the CSF-C5a levels were correlated with the severity of exacerbation. In CNS, C5a is associated with impaired BBB integrity in a neuroinflammatory setting where C5a affects both endothelial and astroglial cells. The inflammatory response induced by C5a can promote the production of C5a, form a positive feedback cycle, promote the expansion of NMO lesions, and prolong the inflammatory process. These humoral and cellular immune responses induced by C5a are consistent with the results of NMO neuropathology in human and animal models. C5a/C5aR signaling can also induce the production of proinflammatory cytokines (TNF, IL-1β, and IL-6) in vitro and in vivo. Higher levels of IL-6 were seen in CSF and serum of patients with NMO attack. Our results suggested that although NMOSD patients during remission seemed to be in a stable condition, there were still abnormally high levels of C5a and related inflammatory cytokines and disordered inflammatory environment in peripheral blood.

There are two types of pain being most characteristic in patients with NMOSD: evoked pain most often caused by painful tonic muscle spasms, and ongoing neuropathic pain. What’s more, pain due to excess loading and pain as a side effect of immunotherapy and in the context of comorbidities are also not rare in NMOSD patients. Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as a chronic pain state initiated or caused by a lesion or disease of the central or peripheral somatosensory nervous system. It may result from different stimuli such as physical trauma, infection, inflammation, metabolic abnormalities, vascular abnormalities, neurotoxins (including chemotherapeutic agents), radiation, surgery procedures or autoimmune disease that induce pathological changes in the tissue and nerves. Long and centrally situated inflammatory lesions in the spinal cord provide a credible explanation for most pain incidence. In the present study, about 68% of NMOSD patients experiencing pain with a neuropathic component (ID pain ≥2). Previous studies suggested that C5a and its cell membrane receptor, C5aR, participate in acute and chronic pain pathophysiological mechanisms.

Emerging evidences suggest that C5a has potent nociceptive activity in several models of inflammatory and neuropathic pain. Inflammatory pain is mainly triggered by the interaction between inflammatory mediators in the damaged tissue, such as prostaglandins, sympathetic amines and kinins and their receptors expressed in the primary nociceptive neuron membranes, which leads to peripheral sensitization. The peripheral pro-nociceptive action of these cytokines (TNF, CXCL1/2 chemokines and IL-1β) during inflammatory pain partially dependent on neutrophil recruitment. C5a/ C5aR has been involved in the development of several inflammatory events, including edema, leukocyte recruitment and activation and production of proinflammatory cytokines, which drive inflammatory and neuropathic pain. C5a could also mediate peripheral sensitization during inflammatory pain through direct action on C5aR-expressing nociceptors. Glial cell activation is involved in the induction and maintenance of neuropathic pain, such as satellite glial cells in the dorsal root ganglia (DRGs) and spinal and supraspinal microglial cells and astrocytes. Upregulated levels of C5a and C5aR were found in spinal cord microglia in animals subjected to spared nerve injury (SNI), a model of neuropathic pain. The expression of C5aR and C5 also increased in the spinal cord after nerve injury, and intrathecal administration of C5a induces...
cold allodynia in a dose-dependent manner. Nerve injury-induced allodynia is ameliorated in C5-deficient mice and in mice intrathecally treated with C5aR antagonist (PMX-53), further supporting the role of C5a/Ca5R signaling in the genesis of neuropathic pain.\textsuperscript{12,13} Although it is clear that spinal C5a/C5aR signaling accounts for the genesis of the neuropathic pain process, the mechanisms involved are not fully understood.

However, to the best of our knowledge, no studies reported on the relationship between C5a levels in peripheral blood and pain in patients with NMOSD during remission. Direct administration of C5a elicits mechanical and thermal hypersensitivity in rodents, and both genetic deletion and pharmacologic antagonism of C5aR produce analgesia in murine models of inflammatory, arthritic, postsurgical, and neuropathic pain.\textsuperscript{11,37,38} In this study, plasma levels of C5a, IL-6, TNF-α, and IL-1β were significantly higher in NMOSD patients during remission than healthy controls; however, only plasma C5a level showed elevated in patients with pain, which suggested that C5a may play a unique role in the pathogenesis of pain in NMOSD, especially during remission. In addition, only plasma C5 levels were negatively correlated with the time interval. Since plasma C5 levels in patients with NMOSD showed no significant difference with that in HC, we believed that the inclusion time from the acute phase did not influence the results significantly in our study.

A few limitations to our study should be mentioned. Types of pain in NMOSD patients including painful tonic muscle spasms, neuropathic pain, and sometimes pain due to excess loading and pain as a side effect of immunotherapy and in the context of comorbidities. We did not conduct detailed subgroup analysis for pain types due to a small sample. Moreover, as this was only an observational study, a longitudinal study is needed to be performed to figure out the association of pain with plasma C5a level in patients with NMOSD during remission and attack.

**Conclusion**
Collectively, the findings of this study showed that NMOSD patients during remission may have unregulated C5a and related-inflammatory cytokines levels in peripheral blood; however, higher plasma C5a level in patients with pain suggesting that C5a may play a unique role in the pathogenesis of pain in NMOSD.

**Abbreviations**
NMOSD, neuromyelitis optica spectrum disorders; MS, multiple sclerosis; NMO, neuromyelitis optica; CNS, central nervous system; C5, complement 5; IL, interleukin; TNF, tumor necrosis factor; VAS, Visual Analogue Scale; HAMD, Hamilton Depression Scale; HAMA, Hamilton Anxiety Scale; MSIS, Multiple Sclerosis Impact Scale; EDSS, Kurtzke Expanded Disability Status Scale; AQP4, Aquaporin-4; HC, healthy controls; ARR, annualized relapse rate; OR, odds ratio; CI, confidence interval; SE, standard error; CSF, cerebrospinal fluid; C5aR, C5a receptor; DRG, dorsal root ganglions; SNI, spared nerve injury.

**Ethics Approval and Informed Consent**
The study was approved by the Ethics Committee of Beijing Tiantan Hospital, Capital Medical University, China. Prior to participation, all participants provided written informed consent.

**Author Contributions**
All authors contributed substantially to conception and design, data analysis, drafting or revising the article. All authors agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be responsible for all aspects of the work.

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**Disclosure**
The authors declare that they have no competing interests in this work.
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