NEW CEREBROSPINAL FLUID RESEARCH TO UNCOVER MECHANISMS DRIVING NEUROLOGICAL AND PSYCHIATRIC DISEASES

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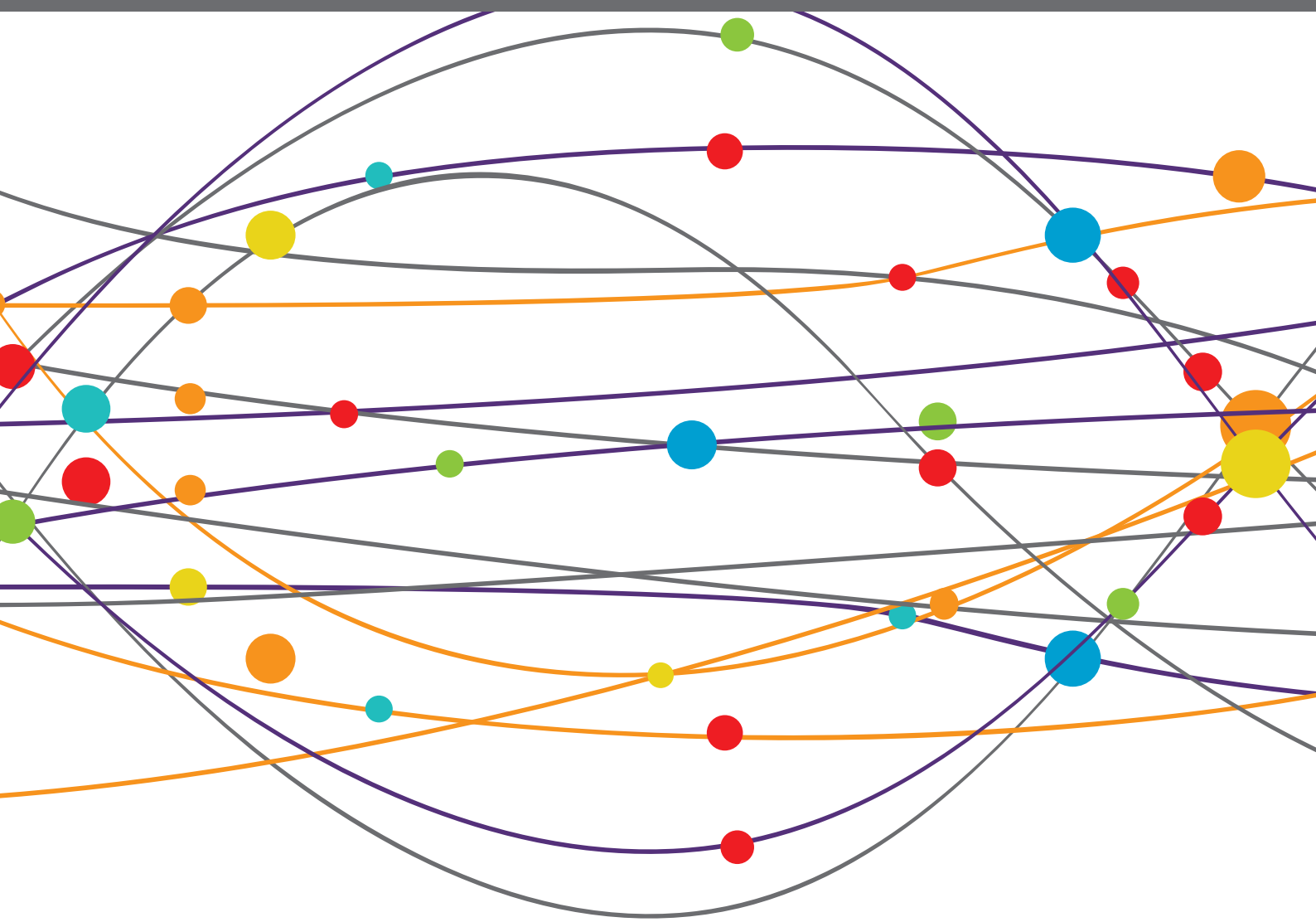

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NEW CEREBROSPINAL FLUID RESEARCH TO UNCOVER MECHANISMS DRIVING NEUROLOGICAL AND PSYCHIATRIC DISEASES

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ORIGINAL RESEARCH
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Immune Cell Activation in the Cerebrospinal Fluid of Patients With Parkinson's Disease

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Background: Parkinson's disease (PD) is a common neurodegenerative disorder. The contribution of the immune system to its pathogenesis remains incompletely understood.

Methods: In this study, we performed comprehensive immune cell profiling in the cerebrospinal fluid (CSF) and peripheral blood (PB) of PD patients. Ten PD patients were diagnosed according to brain bank criteria and underwent detailed clinical examination, magnetic resonance imaging, PB and CSF immune cell profiling by multiparameter flow cytometry, and cytokine and chemokine measurements by bead-based arrays. Thirteen healthy elderly volunteers served as control population.

Results: The proportions of activated T-lymphocytes and non-classical monocytes in the CSF were increased in patients with PD compared to the control group. In accordance, we found increased levels of the pro-inflammatory cytokines IL-2, IL-6 and TNFα and of the monocyte chemoattractant protein 1 (MCP-1) in the CSF of the included PD patients.

Conclusions: Our data provide novel evidence for a response of the innate and adaptive immune system in the central nervous system of patients with PD.

Keywords: Parkinson's disease, cerebrospinal fluid, immune cells, T lymphocytes, monocytes

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INTRODUCTION

Parkinson's disease (PD) represents the most common neurodegenerative disorder following Alzheimer's disease (1). PD prevalence and incidence increase exponentially with age and peak beyond the age of 80 years (2). Clinically, PD is defined by the presence of classical parkinsonian motor symptoms, accompanied by a variety of non-motor features (3–5).

PD is a slowly progressive neurodegenerative disorder characterized by an early prominent death of dopaminergic neurons in the substantia nigra (6). Affected neurons display cytoplasmic accumulation of proteinaceous aggregates called Lewy bodies, which are mainly composed of α-synuclein and ubiquitin (6). PD results from a complicated interplay of genetic and environmental factors (1). Accumulating research provides evidence for a prominent response of the innate and adaptive immune system in PD (7, 8). In humans, neuropathological studies demonstrated the presence of activated microglia, the resident macrophages of the central nervous system (CNS), in the substantia nigra and other affected brain regions. Additional post-mortem studies found

infiltration of the substantia nigra by T lymphocytes, the presence of activated astrocytes, and increased parenchymal cytokine levels (9–13). Functional brain imaging studies detected significant microglial activation in various regions of the CNS (14–16). In particular, PD patients with dementia revealed widespread cortical microglial activation in addition to the subcortical changes (17, 18). Patients with PD have increased cerebrospinal fluid (CSF) levels of inflammatory chemo- and cytokines, including tumor necrosis factor α (TNFα) and interleukin (IL)-6 (12, 19, 20). Genetic studies identified several polymorphisms in genes that are involved in inflammatory processes, such as *TNF*, *IL1B*, *CD14,* and *TREM2*, as risk factors for PD (21, 22). Moreover, genome wide association studies revealed *HLA-DRB5* as another susceptibility locus (23).

CSF was shown to reflect many biochemical and cellular events within the brain parenchyma. It is easily accessible in clinical practice and routinely obtained for a variety of diagnostic purposes. Immunophenotyping of CSF cells may be useful to gain insight into the pathophysiology of CNS disorders, and sharpen diagnostic accuracy or estimation of individual prognosis (24).

In this study, we aimed to determine the immune cell profile in the CSF of patients with PD by using multiparameter flow cytometry. We found an intrathecal increase of non-classical monocyte and activated T-lymphocyte proportions in the PD group compared to healthy elderly controls. Correspondingly, we detected increased concentrations of pro-inflammatory cytokines and the chemokine MCP-1 in the CSF of PD patients. Together, these data provide new lines of evidence for a role of both innate and adaptive immune responses in human PD.

METHODS

Protocol Approval, Registration, and Patient Consent

All patients were recruited from the movement disorder unit at the Department of Neurology, University Hospital Münster, Germany. All participants in this study gave written informed consent. The study was approved by the local ethics committee (2014-624-f-S).

Participants and Study Population

We included 10 PD patients that presented to our movement disorder clinic. Patients were diagnosed according to the UK Brain Bank criteria. Exclusion criteria for this study were concomitant autoimmune diseases, anti-inflammatory co-medication (e.g., cytotoxic agents, steroids, non-steroid analgesia), evidence of an acute systemic inflammatory process at the time of CSF withdrawal (elevated erythrocyte sedimentation rate above 25 mm/h, C-reactive protein above 0.5 mg/dL, or leukocytes above $11 \times 10^3/\mu L$), or blood-tinged CSF. Patient characteristics are described in detail in **Table 1**. The CSF control group consisted of 13 healthy elderly participants with a normal neurologic examination. There was no statistical difference regarding their age. CSF was collected during spinal anesthesia before hip replacement and yielded, in all cases, normal cell counts and protein levels.

TABLE 1 | Demographics and clinical characteristics of study participants.

Demographics	PD ($n = 10$), CTRL ($n = 13$)
Age, range [y] (controls)	79, 69–82 (68, 50–79)
Gender [female, %] (controls)	20 (80)
CLINICAL CHARACTERISTICS	
PD type [hypokinetic-rigid/mixed, %]	50/50
Disease duration [years]	3 ± 3.96
Hoehn and Yahr stage (25)	3 ± 0.75
L-Dopa/equivalent dose [mg/d] (26)	612.5 ± 324.38
MDS-UPDRS-ON III score (27)	16 ± 6.25
MoCA score (28)	23.5 ± 9.30
CSF-ANALYSIS	
CSF protein [mg/l] (controls)	553 ± 271 (368 ± 94)

Numbers represent the mean ± standard deviation. y, years; disease duration, time between the first reported symptoms and the diagnosis; CTRL, healthy elderly controls.

Multiparameter Flow Cytometry and CSF Analysis

Multiparameter flow cytometry of immune cells in PB and CSF samples was done as described previously (24, 29). During lumbar puncture CSF was sampled into polypropylene tubes. All CSF samples were processed in <20 min. Cells were isolated from CSF by centrifugation (15 min, 290 g, 4°C) and subsequent incubation in VersaLyse buffer (Beckman Coulter, Germany). PB samples were collected in EDTA monovettes and cells were isolated by using VersaLyse buffer. For immunostainings, the following fluorochrome-conjugated antibodies were used: CD14-FITC, CD138-PE, HLA-DR-ECD, CD3-PC5.5, CD56-PC7, CD4-APC, CD19-APC-Alexafluor700, CD16-APC-Alexafluor750, CD8-PacificBlue, and CD45-KromeOrange (all from Beckman-Coulter). Data acquisition was performed with a Navios flow cytometer (Beckman-Coulter). Gating strategy for Leukocytes and Monocytes is described and illustrated in **Supplementary Figure 1**.

Quantification of Cytokines and Chemokines in the Serum and Cerebrospinal Fluid

CSF was sampled, and supernatants were obtained by centrifugation as described above. The CSF supernatants were then stored at −20°C until analysis of cytokines (IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, IFNy, TNFa) and chemokines (CCL11, CCL17, CCL20, CXCL1, CXCL5, CXCL9, CXCL11, IL-8, IP-10, MCP-1, MIP-1a, MIP-1b, RANTES) using a bead-based cytokine array (LEGENDplex; BioLegend) according to the manufacturer's instructions.

Statistics

Statistical analysis was performed using Graphpad Prism 6. All data are reported as mean ± standard deviation, and the pre-chosen significance level for all confirmatory tests was $p < 0.05$. Flow cytometry data were analyzed by using the Mann-Whitney test, presuming a non-Gaussian distribution.

RESULTS

The Immune Cell Profile of Patients With PD

Multicolor flow cytometry did not detect significant differences in the proportions or absolute numbers of granulocytes, monocytes, and lymphocytes in the CSF of patients with PD compared to healthy elderly controls (**Figure 1**, and data not shown). Interestingly, subpopulation analysis of innate immune cells revealed an intrathecal shift in cell proportions from classical monocytes (defined as $CD14^+/CD16^-$) to non-classical monocytes ($CD14^+/CD16^+$; **Figure 1**). This intrathecal shift was not reflected in the PB (**Figure 1**). Adaptive immune cell subgroup analysis demonstrated no differences in the levels of B lymphocytes (data not shown), but an increase in the fractions of both total T lymphocytes and activated (defined by HLA-DR expression) T lymphocytes in the CSF (**Figure 2**). The CD4/CD8 T lymphocyte ratio remained unchanged, but specifically $CD8^+$ T lymphocytes showed a larger fraction of HLA-DR activated cells (**Figure 2**). Both $CD4^+$ and $CD8^+$ T lymphocyte activation was also increased in the PB (**Figure 2**). However, we detected no significant alterations in the absolute cell numbers of monocyte or T lymphocyte subsets (data not shown).

The Cytokine and Chemokine Profile of Patients With PD

Using a bead-based cytokine array, we detected no significant differences in cytokine (IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, IFNy, TNFa) and chemokine (CCL11, CCL17, CCL20, CXCL1, CXCL5, CXCL9, CXCL11, IL-8, IP-10, MCP-1, MIP-1a, MIP-1b, RANTES) levels in the serum (data not shown) of PD. Interestingly, we found an increase of the pro-inflammatory cytokines IL-2, IL-6, and TNFα as well as of the pro-migratory chemokine MCP-1 (monocyte chemoattractant protein-1) in the CSF of PD patients, whereas anti-inflammatory IL-9 was decreased (**Figure 3** A+B, only detected cytokines and chemokines shown).

DISCUSSION

In summary, phenotyping of CSF immune cells by multiparameter flow cytometry in patients with PD revealed a strong phenotypical shift of intrathecal monocytes and an increased percentage of activated T lymphocytes. In accordance, the levels of pro-inflammatory cytokines and MCP-1 were higher in the CSF of PD patients.

Evidence for a response of the innate immune system in the disease course of PD has been reported previously by neuropathological and functional brain imaging studies, genetics and CSF cytokine profiling assays (22). The hallmark response of innate immunity had been increased microglia activation and microglia recruitment to affected brain areas (13). Our results showing a strong shift from classical ($CD14^+CD16^-$) to non-classical ($CD14^+CD16^+$) monocytes within the intrathecal compartment represent a novel line of evidence for a response of innate immunity within the CNS. In contrast to a previous study that reported a significant increase in the proportion of PB classical monocytes in patients with PD (30), we found no significant differences in the proportion of peripheral monocyte subtypes. Monocytes originate from myeloid precursors in the bone marrow and are divided into two major subpopulations, the classical $CD14^+CD16^-$ and the non-classical $CD14^+CD16^+$ monocytes (31). Classical monocytes are highly plastic and, upon recruitment to inflamed tissue, modify their phenotype according to the requirements of the specific microenvironment. They can differentiate into macrophages and are involved in tissue maintenance, pathogen clearance and induction of adaptive immune responses. Non-classical monocytes are thought to patrol along blood vessels and to be involved in tissue homeostasis and local regeneration, however recent reports describe them as the primary inflammatory monocyte subtype with properties for antigen presentation (32, 33). Together, our

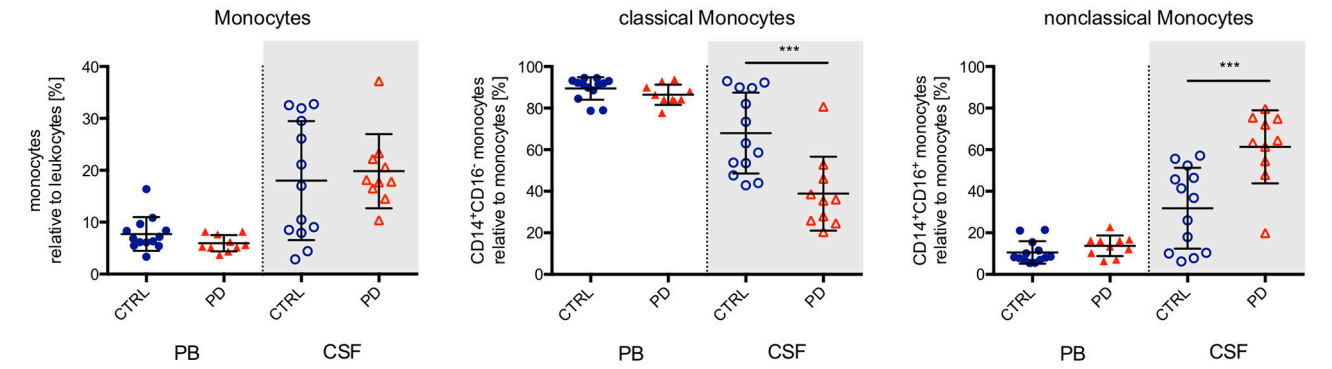

FIGURE 1 | Monocytes in the peripheral blood and cerebrospinal fluid. The proportion of monocytes in relation to total leukocytes was determined in the peripheral blood (PB, closed symbols, white background) and cerebrospinal fluid (CSF, open symbols, gray background) from healthy elderly controls ($n = 13$; blue circles) and PD patients ($n = 10$; red triangles). While the proportion of total monocytes did not differ between study groups, monocyte subgroup analysis based on expression of the surface marker CD16 demonstrated a highly significant decrease of classical monocytes ($CD14^+CD16^-$) and a concomitant highly significant increase of non-classical monocytes ($CD14^+CD16^+$). For statistical analysis, the Mann-Whitney test was performed (***$p < 0.001$).

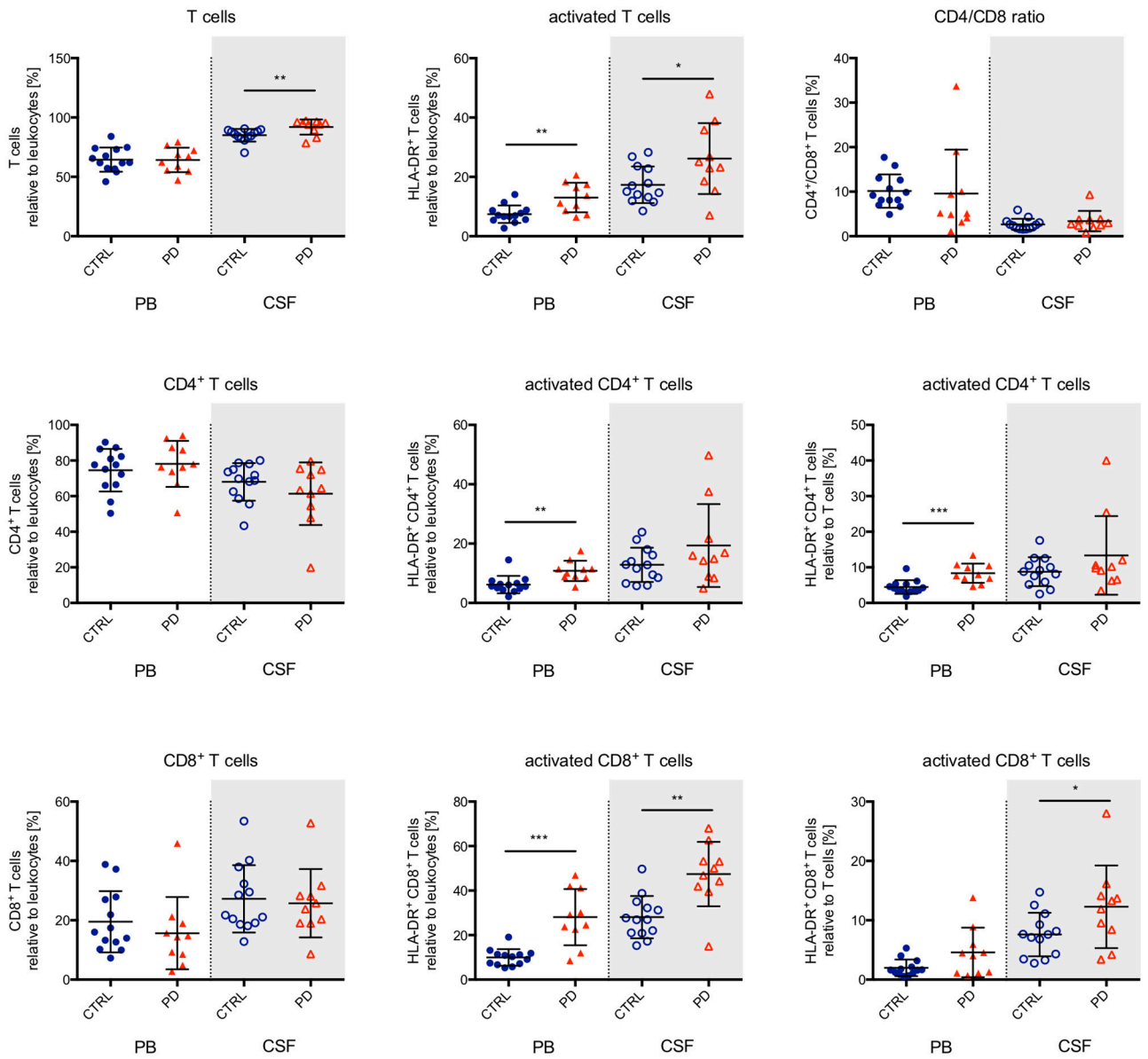

FIGURE 2 | Lymphocytes in the peripheral blood and cerebrospinal fluid. The proportion of T lymphocytes in relation to total leukocytes was determined in the peripheral blood (PB, closed symbols, white background) and cerebrospinal fluid (CSF, open symbols, gray background) from healthy elderly controls ($n = 13$; blue circles) and PD patients ($n = 10$; red triangles). We found a significant increase of total and activated T lymphocytes in the CSF. CD4 and CD8 expression did not differ between study groups, but specifically CD8+ T lymphocytes displayed a larger fraction of HLA-DR expressing activated cells, both in the PB and CSF. For statistical analysis, the Mann-Whitney test was performed (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

data showing a strong increase in the proportion of CSF, but not PB, non-classical monocytes in patients with PD and previous evidence of increased classical monocytes in the PB of patients with PD suggest the occurrence of specific intrathecal monocyte activation. Thus, it is tempting to speculate that in PD classical monocytes are recruited from the periphery across the blood-liquor barrier to give rise to non-classical monocytes within the CSF.

Changes in the chemokine profile have been reported in PD patients in different stages of the disease (34). Interestingly, we found increased levels of MCP-1 (CCL-2) in the CSF of PD patients. The MCP-1 receptor CCR2 is expressed on monocytes and MCP-1/CCR2 signaling is involved in the regulation of migration and infiltration of monocytes into host tissues (35). Increased levels of MCP-1 had been previously described in both serum and CSF samples of PD patients, and higher levels of MCP-1 correlated with manifestation of cognitive impairment and depression (30, 36, 37). However, only classical monocytes express high levels of CCR2. Therefore, the shift in monocyte subsets might be related (1) to an enhanced evasion

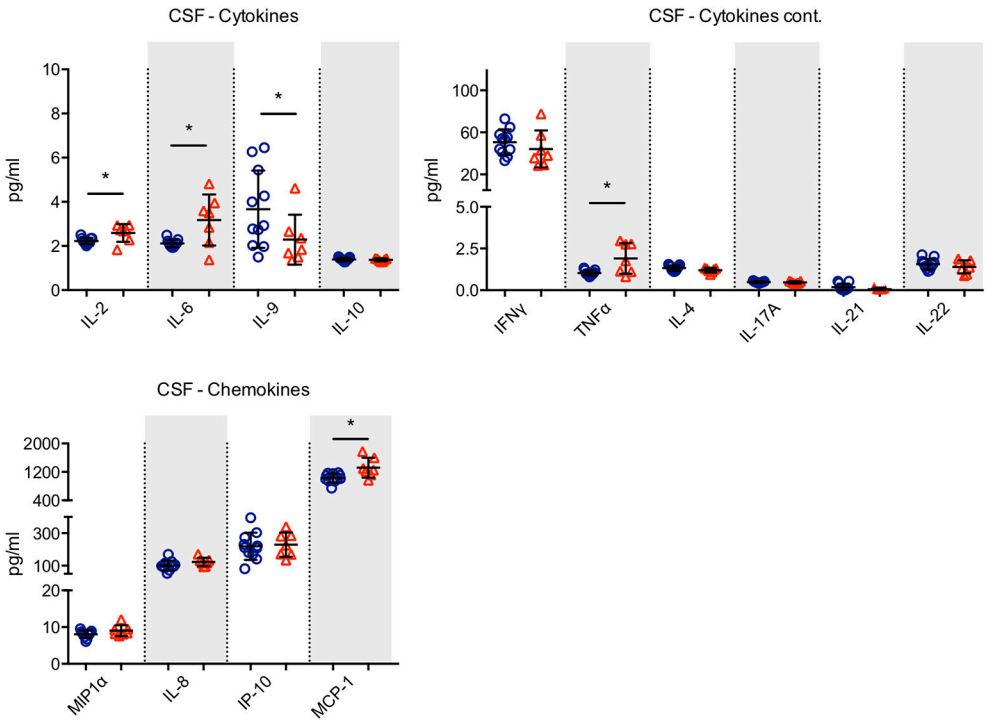

FIGURE 3 | Cytokine and chemokine production in the cerebrospinal fluid. The concentrations of cytokines and chemokines were determined in the cerebrospinal fluid from healthy elderly controls ($n = 11$; blue circles) and PD patients ($n = 7$; red triangles) by a bead-based cytokine array. We found a significant increase of the pro-inflammatory cytokines IL-2, IL-6, and TNFα as well as of the pro-migratory chemokine MCP-1 in the CSF of PD patients, whereas anti-inflammatory IL-9 was decreased. For statistical analysis, the Mann-Whitney test was performed (*$p < 0.05$).

of classical monocytes into the brain tissue or (2) classical monocytes increasingly developing into non-classical monocytes. We found no evidence for a peripheral expansion of non-classical monocytes and their subsequent migration into the CNS since the specific monocyte subtype composition was confined to the CSF compartment. Monocytes invading the CNS might develop into macrophages within the brain parenchyma, thus strengthening the innate arm of the CNS immune system (30, 38).

In addition to the well-established response of innate immunity, more recent evidence has suggested a role for the adaptive immune system in PD. Genome-wide association studies have established an association of PD with alleles of the major histocompatibility complex (MHC) (23). Previous PB immune cell phenotyping had demonstrated increased LRRK2 levels in peripheral lymphocytes, which are involved in the regulation of T cell activation and division (39). Neuropathological studies have reported T cell infiltration into the substantia nigra (13). Interestingly, quantitative analysis demonstrated a substantial accumulation of $CD8^+$ T lymphocytes and to a lesser degree of $CD4^+$ T lymphocytes within the substantia nigra, although animal models suggested a more enigmatic functional role of the latter in the disease process (10). Our data support the neuropathological post-mortem results and provide the first direct *in vivo* evidence in man showing increased invasion of T lymphocytes into the intrathecal compartment and their functional activation. Recently, an elegant series of immunological studies led to the current concept of the contribution of T lymphocytes to the pathogenesis of PD: degenerating dopaminergic neurons present modified α-synuclein-derived peptides via MHC class I molecules on their surface and release them to the extracellular space, where they activate microglia, and drain into the periphery outside the CNS. In lymph nodes, they activate antigen-presenting cells, which present the α-synuclein fragments via MHC II surface molecules leading to T lymphocyte activation. The latter infiltrate the CNS and accumulate at sites of inflammation, where they are re-stimulated by α-synuclein-presenting neurons and microglia. This may result in exacerbated inflammation, oxidative stress, and neuronal injury (7, 10, 40, 41). Our data contribute to these findings by providing direct *in vivo* evidence for activated T lymphocytes in the intrathecal compartment.

In accordance to previous studies, we found increased levels of the pro-inflammatory cytokines IL-2, IL-6, and TNFα (12, 20). However, we were not able to detect a cytokine pattern indicating the enrichment of a specific T-helper (T_H) subtype. Interestingly, IL-9 was decreased in PD patients, a pleiotropic cytokine with supposed regulatory effects in CNS autoimmunity (42). Low patient numbers and the assays' detection limits might affect our results or explain differences between them and previous

studies. Concomitant autoimmune diseases, anti-inflammatory co-medication, or evidence of an acute inflammatory process at the time of CSF withdrawal were exclusion criteria for patients in the present study. However, we cannot exclude the possibility of unknown comorbidities that may affect results in the presented patient cohort.

In conclusion, our results demonstrate a shift of monocyte subsets and activation of T lymphocytes in the CSF of PD patients. Although it remains unclear whether such alterations play a primary or secondary role in neurodegeneration, our results provide a new tier of evidence for activation of both innate and adaptive immune responses in the disease course of PD. Moreover, dysregulated immune cells may represent interesting molecular targets accessible as biomarkers for the identification of disease-associated neuroinflammatory processes and amenable to therapeutic intervention. Such intervention could have positive clinical effects and potentially modify the disease course. Future studies are necessary to corroborate our findings. Correlation analysis based on larger cohorts will be necessary to link CSF immune cell responses to PD disease stages and other clinical and paraclinical parameters. Additional cellular surface markers and translational research will be required to unveil the cascade of events leading to altered CSF monocyte and lymphocyte phenotypes and define their origins and exact cellular identities.

AUTHOR CONTRIBUTIONS

JBS conceived, organized and executed the research project and reviewed the manuscript. MP conceived the research project, performed data analysis and wrote the first draft of the manuscript. GM helped to conceive the study and reviewed the manuscript. CCG performed data analysis and reviewed the manuscript. HW helped to conceive the study and reviewed the manuscript. SGM helped to conceive the study and reviewed the manuscript. TR conceived the research project, performed data analysis and reviewed the manuscript. TW conceived and supervised the research project and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2018.01081/full#supplementary-material

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BRIEF RESEARCH REPORT
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Cell-Free Mitochondrial DNA in the CSF: A Potential Prognostic Biomarker of Anti-NMDAR Encephalitis

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Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is an autoimmune inflammatory brain disease that can develop a variety of neuropsychiatric presentations. However, the underlying nature of its inflammatory neuronal injury remains unclear. Mitochondrial DNA (mtDNA) is recently regarded as a damage-associated molecular pattern molecule (DAMP) that can initiate an inflammatory response. In the presenting study, we aimed to evaluate the levels of cell-free mtDNA in cerebrospinal fluid (CSF) of patients with anti-NMDAR encephalitis and to determine a potential role of cell-free mtDNA in the prognosis of anti-NMDAR encephalitis. A total of 33 patients with NMDAR encephalitis and 17 patients with other non-inflammatory disorders as controls were included in this study. The CSF levels of cell-free mtDNA were measured by quantitative polymerase chain reaction (qPCR). Cytokines including interleukin (IL)-6, IL-10, and tumor necrosis factor alpha (TNF-α) were measured by ELISA. The modified Rankin scale (mRS) score was evaluated for neurologic disabilities. Our data showed that the CSF levels of cell-free mtDNA and inflammation-associated cytokines were significantly higher in the patients with anti-NMDAR encephalitis compared with those in controls. Positive correlations were detected between the CSF levels of cell-free mtDNA and mRS scores of patients with anti-NMDAR encephalitis at both their admission and 6-month follow up. These findings suggest that the CSF level of cell-free mtDNA reflects the underlying neuroinflammatory process in patients with anti-NMDAR encephalitis and correlates with their clinical mRS scores. Therefore, cell-free mtDNA may be a potential prognostic biomarker for anti-NMDAR encephalitis.

Keywords: anti-NMDAR encephalitis, cerebrospinal fluid, cell-free mitochondrial DNA, cytokines, IL-6, IL-10, TNF-α, modified rankin scale

INTRODUCTION

Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is a newly recognized acute autoimmune encephalitis caused by anti-neuronal autoantibodies, which affects mainly children and young adult females (1, 2). Its typical clinical manifestations include a progressive development of neurologic and psychiatric symptoms, including abnormal movements, seizures, impaired memory, and behavior disorders (2, 3). Anti-NMDAR encephalitis is usually associated with teratoma and often secondary to CNS infection caused by viruses or parasites (4, 5). Some studies have suggested that anti-NMDAR antibodies initiate the innate immune response and promote sterile neuroinflammation which leads to the lesioning of brain tissues (6, 7). However, one of the major challenges in the management of anti-NMDAR encephalitis is to identify biomarkers with diagnostic and prognosis predicting value.

Mitochondrial DNA (mtDNA) is a damage-associated molecular pattern molecule (DAMP) that can trigger activation of the human innate immune system and initiate an inflammatory response (8, 9). Usually, the basal level of CSF cell-free mtDNA stays low and reflects the normal turnover of mtDNA in the brain, whereas elevated CSF levels of mtDNA were reported in patients with traumatic brain injury and multiple sclerosis. This indicates that mtDNA plays a mediatory role in sterile inflammatory responses (10, 11). Thus, it is crucial to evaluate the profile of cell-free mtDNA in anti-NMDAR encephalitis and its potential relationship with the pathophysiology of this disease. In this study, we investigated the cell-free mtDNA profile in the CSF of patients with anti-NMDAR encephalitis vs. other non-inflammatory neurological disorders including peripheral nerve disease and hysteria. We further examined possible associations of their cell-free mtDNA profiles with the modified Rankin Scale (mRS) score and their cytokine profiles, including IL-6, IL-10, and TNF-α (12).

MATERIALS AND METHODS

Patients and Controls

We recruited a total of 33 patients from the Department of Neurology, Nanfang Hospital, Southern Medical University, according to inclusion criteria based on the revised anti-NMDAR encephalitis diagnosis criteria of Graus et al. published in 2016 (2, 13). Specifically, all anti-NMDAR encephalitis patients were treatment-naïve, and their CSF was sampled at acute onset with confirmation of positive antibodies against the NR1 subunit of the NMDAR by cell-based analysis and negative for viral DNA and other pathogens. All the patients' CSF were negative for the detection of tuberculosis, cryptococcus, and some common viruses by PCR. All patients with anti-NMDAR encephalitis were treated with first-line treatment or combined second-line treatment (1). The control group consisted of a cohort of 17 age- and gender-matched patients with confirmed other non-inflammatory neurological disorders. Patients were tested negative for CSF antibodies against the NR1 subunit of the NMDAR. This study was conducted with the approval (NFEC-2018-095) of the Ethics Committee of Nanfang Hospital, Southern Medical University. Written informed consent was obtained from all patients for this study.

CSF Collection and DNA Preparation

All 33 patients and the 17 control individuals were subjected to lumbar puncture for CSF analysis within 3 days of their admission; 15 of the 33 patients with anti-NMDAR encephalitis received another lumbar puncture for CSF re-evaluation at 6 months follow-up after discharge. CSF samples were processed within 30 min of collection and centrifuged at 1,000 g for 10 min. The CSF supernatants were then transferred to polypropylene tubes and stored at −80°C. DNA was extracted from supernatants using the QIAmp DNA Mini Kit (Qiagen GmbH).

Quantitation of CSF Cell-Free mtDNA

The assessment of concentration of mtDNA was performed by quantitative polymerase chain reaction (qPCR) (**Supplementary Material**) (11). Each measurement consisted of biological duplicates and technical triplicates; the samples from anti-NMDAR encephalitis patients and control individuals were randomized to avoid batch effects.

Determination of Inflammatory Cytokine Levels

The levels of inflammation-associated cytokines were quantified using Sandwich ELISA kits [Bender MedSystems GmbH (IL-6 and IL-10) (Vienna, Austria) and Cusabio (TNF-α) (Wuhan, China)] according to the manufacturers' instructions (**Supplementary Material**).

Evaluation of mRS

The mRS score was evaluated for neurologic disabilities (14). All 33 patients with anti-NMDAR encephalitis were evaluated for mRS scores at the times of their admission, while 15 patients received re-evaluation for mRS scores at their 6-month follow-up.

Statistical Analysis

Data were expressed as mean ± SD or the median (range). Statistical analyses were performed using SPSS version 20.0 (IBM Corp, Armonk, NY, USA). Independent-samples non-parametric tests were performed to compare the levels of CSF cell-free mtDNA or inflammatory cytokines between patients and controls. Paired *t*-tests were performed to compare parameters in the 15 patients at their admission vs. follow-up. Correlations among the quantitative parameters were evaluated using Pearson's test; correlations between mRS scores and quantitative parameters were assessed with Spearman's test. A $p < 0.05$ was regarded as statistically significant.

RESULTS

Demographic and Clinical Features of Anti-NMDAR Encephalitis Patients

The demographic data and clinical features of patients ($n = 33$) and controls ($n = 17$) are shown in **Table 1**. All patients were

TABLE 1 | Clinic manifestations and characteristics of anti-NMDAR encephalitis and controls.

	NMDAR ($n = 33$)	Control ($n = 17$)
Gender (male/female)	14/19	8/9
Age (years, mean)	34.8 ± 17.6	35.0 ± 15.3
PSYCHIATRIC AND NEUROLOGIC SYMPTOMS		
Fever	19 (58%)	–
Disorders of memory, behavior, and cognition	28 (85%)	–
Seizures	21 (64%)	–
Autonomic disturbances	13 (39%)	–
Disturbance of consciousness	20 (61%)	–
Abnormal movements	16 (48%)	–
Abnormal electroencephalogram	25 (76%)	–
Ovarian teratoma	2 (6%)	–
CSF ASSESSMENT		
White blood cell count($\times 10^6$/L, median)	3 (0, 17.5)	0 (0, 0)**
Protein (g/L, median)	0.28 (0.16, 0.58)	0.32 (0.21, 0.38)
IL-6(pg/ml, median)	7.30 (3.85, 13.40)	2.90 (2.60, 3.85)***
IL-10(pg/ml, median)	4.91 (3.64, 5.79)	0.91 (0.00, 1.13)***
TNF-α(pg/ml, median)	6.03 (4.33, 11.34)	1.23 (0.19, 2.30)***
mRS SCORES		
Maximum mRS scores	4 (4, 5)	–
6-months mRS scores	3 (2, 3.5)	–
Anti-NMDAR antibody	33	0

$^{**}p < 0.01$; $^{***}p < 0.001$.

confirmed by positive detection of anti-NMDAR autoantibodies in their CSF. Psychiatric symptoms (85%), electroencephalogram (EEG) abnormality (76%), and seizure onset (64%) were the most common clinical presentations in the patients with anti-NMDAR encephalitis. The other symptoms include fever, autonomic disturbances, disturbance of consciousness, abnormal movements, and so on. These patients with fever were neither identified with clues of bacterial infection nor increased levels of C reactive protein or procalcitonin. Notably, compared with their peak mRS scores at admission, the mRS scores at 6-month follow up in the 15 follow-up patients were significantly lower (paired t-test, $p < 0.001$), indicating the effectiveness of treatment.

Cell-Free mtDNA and Inflammatory Cytokines in the CSF of Anti-NMDAR Encephalitis Patients

To investigate the role of cell-free mtDNA in anti-NMDAR encephalitis, we evaluated the CSF levels of cell-free mtDNA in these patients ($n = 33$) and controls ($n = 17$) using a qPCR assay. As shown in **Figure 1A**, the copy number of CSF cell-free mtDNA was significantly elevated in anti-NMDAR encephalitis patients at the acute stage (258.2 copies/10 μl: 148.7, 461.3) compared to controls (73.6 copies/10 μl; 51.8, 95.1) ($p < 0.001$). To further evaluate the role of humoral immunity in anti-NMDAR encephalitis, we measured the CSF levels of inflammation-related cytokines, IL-6, IL-10, and TNF-α by ELISA. The ELISA data showed that the levels of pro-inflammatory cytokines IL-6 and TNF-α, as well as the anti-inflammatory cytokine IL-10, were significantly higher in patients with anti-NMDAR encephalitis compared with controls ($p < 0.001$, <0.001, <0.001, respectively) (**Table 1**, **Figures 1B–D**).

Furthermore, the receiver operating characteristic (ROC) curve analysis of cell-free mtDNA as a diagnostic biomarker of anti-NMDAR encephalitis showed that the sensitivity was 100% and the specificity was 76.5% when 91.55 copies/10 μl was selected as the cut-off value. Area under the ROC curve (AUC^{ROC}) was 0.964 (95% CI: 0.919–1.010, $p < 0.001$) (**Figure 1E**).

Altered CSF Levels of Cell-Free mtDNA and Inflammatory Cytokines in the Course of the Disease

The levels of CSF cell-free mtDNA in patients dropped significantly from 318.6 ± 196.7 copies/10 μl (peak) to 212.2 ± 129.8 copies/10 μl at 6-month follow up ($p = 0.003$), while the latter was still higher compared to controls ($p < 0.001$). The levels of CSF IL-6, IL-10, and TNF-α were also significantly reduced at 6-month follow up compared to that at the acute stage of anti-NMDAR encephalitis ($p = 0.005$, 0.003, <0.001, respectively).

The Relationship Between CSF Levels of Cell-Free mtDNA and Inflammatory Cytokines in Anti-NMDAR Encephalitis

Correlation analysis was performed to demonstrate changes in the DAMP molecule, cell-free mtDNA with humoral immunity sponsors, e.g., IL-6, IL-10, and TNF-α. The analysis showed that there was a statistically significant correlation of CSF cell-free mtDNA with IL-6 ($r = 0.307$, $p = 0.041$), but neither with IL-10 ($r = -0.085$, $p = 0.319$) nor TNF-α ($r = -0.050$, $p = 0.391$) in the acute stage of anti-NMDAR encephalitis.

Relationship Between CSF Levels of Cell-Free mtDNA and Clinical Outcome in Anti-NMDAR Encephalitis

The relationships between clinical outcome, mRS scores, and CSF levels of cell-free mtDNA, IL-6, IL-10, and TNF-α in patients with anti-NMDAR encephalitis were evaluated. There were significant positive correlations between CSF levels of cell-free mtDNA and mRS scores at both the acute stage ($r = 0.744$, $p = 0.000$) and the 6-month follow up ($r = 0.576$, $p = 0.000$, **Figure 1F**). However, no significant correlation was shown between the CSF levels of cell-free mtDNA and three cytokines IL-6, IL-10, and TNF-α, or clinical outcome.

DISCUSSION

In this cross-sectional study, we report for the first time the identification of cell-free mtDNA as a potential biomarker of mitochondrial damage in anti-NMDAR encephalitis. Our data demonstrating significantly increased CSF cell-free mtDNA suggests that cell-free mtDNA may participate in the

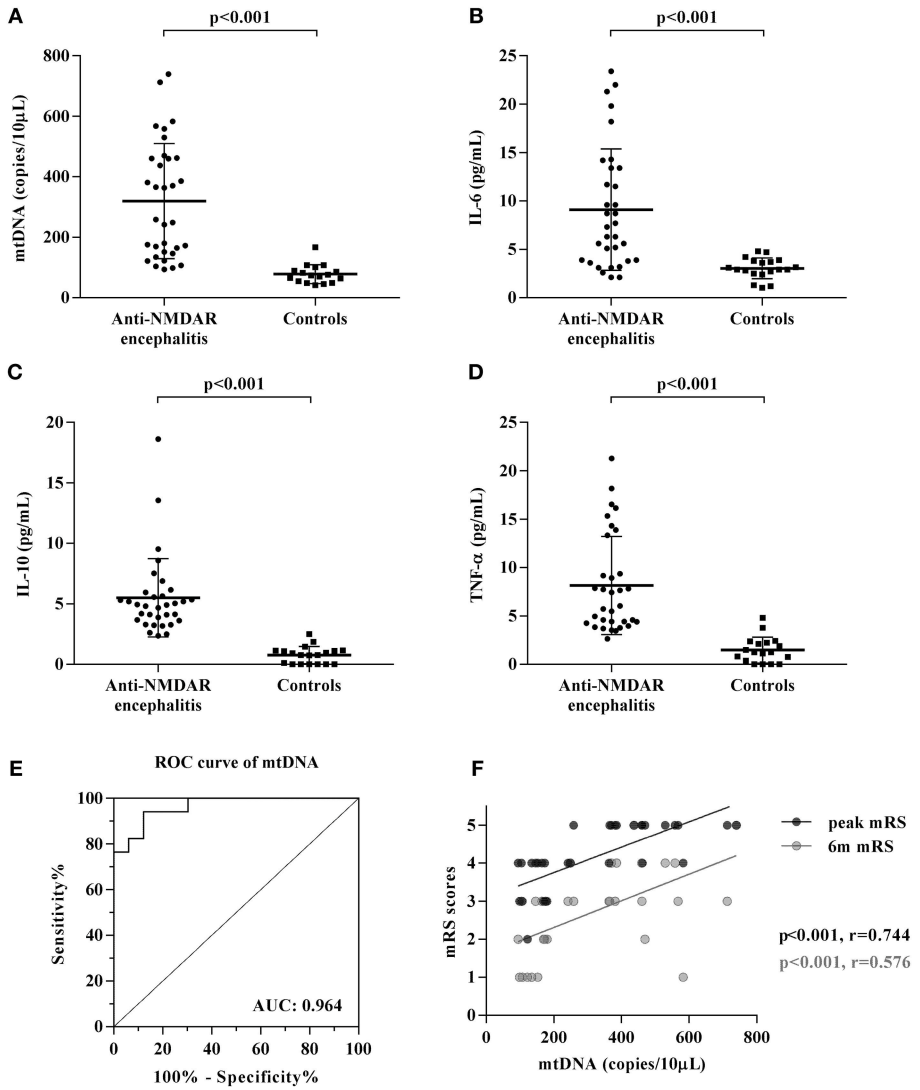

FIGURE 1 | Changes of CSF mtDNA and inflammatory cytokines in anti-NMDAR encephalitis**. (A)** The q-PCR assessment of CSF levels of mtDNA showed elevated cell-free mtDNA copies in patients with anti-NMDAR encephalitis (NMDAR) vs. controls (CTL), while the levels of inflammation-related cytokines, IL-6 **(B)**, IL-10 **(C)**, and TNF-α **(D)** were also significantly changed in anti-NMDAR encephalitis. The *p*-values were indicated within figures. ROC analysis showed that when the cut-off value was set as 91.55 copies/10 μL, the sensitivity of CSF cell-free mtDNA test was 100% and the specificity was 76.5%, and the area under ROC curve (AUC^{ROC}) was 0.964 (95% CI: 0.919–1.010, $p < 0.0001$) **(E)**. Potential correlations were analyzed between the patient' mRS scores acquired at their peak presentations (peak mRS) and 6-month follow-up (6 m mRS) to their corresponding CSF levels of cell-free mtDNA **(F)**.

pathogenesis of this disease, which in turn could reflect the severity of neurological impairment.

Both the innate and acquired immune system play a role in the pathogenesis of anti-NMDAR encephalitis (6). The innate immune system recognizes endogenous damage-associated molecular pattern (DAMP) molecules and exogenous pathogen-associated molecular pattern (PAMP) molecules, which will initiate a non-infectious or a pathogen-induced inflammatory response, correspondingly (15). Since mitochondria are evolutionarily derived from bacteria by endosymbiosis and therefore bear bacterial molecular motifs (15), mtDNA is considered an activator of the innate immune system. The mtDNA is a DAMP molecule that can initiate antimicrobial responses and inflammatory pathology (16) in various situations, such as shock, injury, infection, and cancer (15, 17–19). Cell-free mtDNA can be recognized by pattern-recognition receptors (PRRs), usually the toll-like receptor-9 (TLR-9) (8, 15) and result in increased expressions of inflammatory cytokines and pro-inflammatory molecules (9, 18, 20).

The anti-inflammatory cytokine IL-6 is increased in neuronal and CNS autoimmune diseases, such as neuromyelitis optica and multiple sclerosis (21, 22). Mechanistically, IL-6 stimulates B-cell differentiation, promotes the survival of plasmablasts, and enhances the intrathecal production of anti-NMDAR antibodies

(12, 23, 24). Here, we demonstrated that the CSF level of IL-6 was significantly increased and positively correlated with CSF mtDNA levels in anti-NMDAR encephalitis. However, the other two inflammatory cytokines, IL-10 and TNF-α lack correlation with changes of CSF mtDNA though they also increased in the patients with anti-NMDAR encephalitis. These findings suggest that while cytokines may play an essential role in the pathogenesis of anti-NMDAR encephalitis, IL-6 may specifically act as a downstream molecule of the mtDNA mediated immune response.

Since a positive prognosis of anti-NMDAR encephalitis is closely linked to early treatment, early diagnosis of this disease is crucial. CSF biomarkers that have a strong correlation with the prognosis of anti-NMDAR encephalitis would be particularly useful in the assessment of the severity and evaluation of recovery for patients. In this study, we demonstrated that the CSF levels of cell-free mtDNA positively correlated with both the maximum and 6-month follow up mRS scores. This suggests that a higher level of CSF cell-free mtDNA may indicate a more severe clinical presentation and worse prognosis of anti-NMDAR encephalitis. Therefore, changes in CSF cell-free mtDNA, combined with changes in anti-NMDAR antibody titer, may become a promising indicator for prognosis predicting and monitoring in anti-NMDAR encephalitis.

CSF cytotoxicity has been thought to be a cause of certain neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) (25). Transfering the CSF of ALS pathients to rat cerebral ventricle could provoke changes similar to those found in the disease (26). Though the pathogenesis of anti-NMDAR encephalitis is not all clear, tumor is though to be associated with anti-NMDAR encephalitis. CNS infections were also thought as immunological triggers in some cases, especially in relapse post-herpes simplex virus encephalitis (4, 27). In the present study, all the patients' CSF were negative for the detection of tuberculosis, cryptococcus, and some common viruses by PCR. The correlation between mitochondrial DNA and infection needs to be clarified in further studies.

Yet, this result has some limitations: the control group comprised subjects with non-inflammatory CNS diseases. It may be better to include a control cohort with viral encephalitis since it is initiated by PAMP, which usually mimics presentations of anti-NMDAR encephalitis. Future studies should include a control group of subjects with PAMP related encephalitis.

CONCLUSION

This report details, for the first time, the role of CSF cell-free mtDNA in anti-NMDAR encephalitis. Our findings suggest that CSF cell-free mtDNA may be implicated in the pathogenesis of anti-NMDAR encephalitis. In addition, CSF cell-free mtDNA may also act as a prognostic biomarker as it correlated positively with mRS scores and indicated that higher levels of CSF cell-free mtDNA were associated with more severe clinical presentations and a worse prognosis.

AVAILABILITY OF DATA AND MATERIAL

We declare that materials described in this manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participants' confidentiality.

AUTHOR CONTRIBUTIONS

H-HW, H-YS, TJ, and JZ co-conceived this study and designed the experiments. YP, SP, DZ, and XZ collected the CSF samples and clinical data. YP, DZ, and H-HW performed the experiments and analyzed the data. YP, H-HW, and H-YS wrote the manuscript and prepared the table, figures. YP, JZ, and H-HW revised the article. All authors read and approved the final manuscript and agreed to submit it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00103/full#supplementary-material

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Immune Cell Profiling of the Cerebrospinal Fluid Provides Pathogenetic Insights Into Inflammatory Neuropathies

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Objective: Utilize immune cell profiles in the cerebrospinal fluid (CSF) to advance the understanding and potentially support the diagnosis of inflammatory neuropathies.

Methods: We analyzed CSF cell flow cytometry data of patients with definite Guillain-Barré syndrome (GBS, $n = 26$) and chronic inflammatory demyelinating polyneuropathy (CIDP, $n = 32$) based on established diagnostic criteria in comparison to controls with relapsing-remitting multiple sclerosis (RRMS, $n = 49$) and idiopathic intracranial hypertension (IIH, $n = 63$).

Results: Flow cytometry revealed disease-specific changes of CSF cell composition with a significant increase of NKT cells and CD8+ T cells in CIDP, NK cells in GBS, and B cells and plasma cells in MS in comparison to IIH controls. Principal component analysis demonstrated distinct CSF immune cells pattern in inflammatory neuropathies vs. RRMS. Systematic receiver operator curve (ROC) analysis identified NKT cells as the best parameter to distinguish GBS from CIDP. Composite scores combing several of the CSF parameters differentiated inflammatory neuropathies from IIH and GBS from CIDP with high confidence. Applying a novel dimension reduction technique, we observed an intra-disease heterogeneity of inflammatory neuropathies.

Conclusion: Inflammatory neuropathies display disease- and subtype-specific alterations of CSF cell composition. The increase of NKT cells and CD8+ T cells in CIDP and NK cells in GBS, suggests a central role of cytotoxic cell types in inflammatory neuropathies varying between acute and chronic subtypes. Composite scores constructed from multi-dimensional CSF parameters establish potential novel diagnostic tools. Intra-disease heterogeneity suggests distinct disease mechanisms in subgroups of inflammatory neuropathies.

Keywords: inflammatory neuropathies, Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, immune cell profile, cerebrospinal fluid, flow cytometry

INTRODUCTION

Guillain-Barré syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy (CIDP) are the most frequent of the heterogeneous group of immune-mediated neuropathies. Both can cause considerable and often permanent disability (1). The diagnosis of GBS and CIDP can remain challenging despite the existence of diagnostic criteria for GBS (2) and CIDP (3). This is in part due to (1) atypical clinical presentations (4), (2) technical difficulties in nerve conduction studies (5), and (3) low specificity of diagnostic criteria (6). Diagnosis is further impeded by the overlap between recurrent GBS and relapsing CIDP courses. Therefore, better mechanistically understanding and distinguishing the heterogeneity of inflammatory neuropathies would be of considerable clinical relevance, especially in the light of differential treatment requirements (7).

In contrast to the vast expansion of knowledge in CNS autoimmunity, comparably little progress has been made in the understanding of PNS autoimmunity; especially regarding the immunological factors differentiating acute from chronic immune neuropathies. Using CSF, some of the few available studies found a specific cytokine profile in the CSF of CIDP patients (8, 9). Other studies reported changes of Th17 cells (10) and NK cells (11). Certain T helper cell populations are elevated in the CSF in GBS (12) and in a corresponding animal model (13). Despite these scattered observations, a comprehensive immune cell profiling of the CSF has not been performed.

We here performed a systematic retrospective analysis of flow cytometry profiling of CSF leukocytes, in patients with definite GBS and CIDP in comparison to relapsing-remitting multiple sclerosis (RRMS) and idiopathic intracranial hypertension (IIH) to aid the understanding and diagnosis of inflammatory neuropathies.

METHODS

Patients

In our center, all CSF samples obtained during regular working hours are routinely analyzed by flow cytometry. We retrospectively searched files of patients who had been admitted to the Department of Neurology at the University Hospital Münster between years 2012 and 2018 for the ICD-10 diagnosis codes G61.0, G61.8, G35.1, and G93.2 and who had received lumbar puncture (LP) for routine CSF analysis and CSF cell flow cytometry. In total, we identified 26 patients with GBS, 32 patients with CIDP, 49 patients with RRMS, serving as a reference group with known inflammatory CSF changes, and 63 patients with IIH, serving as a control group with mostly normal CSF (14). Only GBS patients who fulfilled Brighton criteria (2) level 1 were admitted to the study. Only CIDP patients who had a definite CIDP according to the EFNS/PNS diagnostic criteria (3) were included in the study. In addition, Hughes disability score (0—healthy, 1—minor signs of neuropathy, but capable of manual work, 2—able to walk with aid of stick, but incapable of manual work, 3—able to walk with support, 4—confined to bed or chairbound, 5—requiring assisted ventilation, 6—death) (15) and the modified Rankin scale (mRS) were determined on day of admission. For the disease severity analysis the mRS was dichotomized to classify mildly (mRS 1–2) and severely (mRS 3–6) affected patients. Patients with RRMS were selected according to the 2017 revision of the McDonald criteria (16). Patients who had been treated with alemtuzumab, ocrelizumab, cladribine, or rituximab in the last year or with fingolimod, natalizumab, mitoxantrone, cyclophosphamide, methotrexate, ciclosporin A in the last 3 months were excluded from all cohorts. Current treatments with interferons, glatiramer acetate, dimethyl fumarate, azathioprine, steroids, or intravenous immunoglobulins were accepted.

CSF Flow Cytometry

LPs were performed under sterile conditions using 20G Sprotte Canulae (Pajunk Medical). All samples were pseudonymized at collection. CSF was transported to further processing as quickly as possible and centrifuged at 300 g for 15 min. The supernatant was removed and CSF cells were stained for flow cytometry as described previously (17–19). Briefly, CSF flow cytometry was performed using a Navious flow cytometer (Beckman Coulter). Cells were incubated in VersaLyse buffer and stained using the following anti-human antibodies (Beckman Coulter; clone names indicated): CD3 (UCHT1); CD4 (13B8.2); CD8 (B9.11); CD14 (RMO52); CD16 (3G8); CD19 (J3-119); CD45 (J.33); CD56 (C218); CD138 (B-A38); HLA-DR (Immu-357). Gating was first by forward scatter (FSC)/sideward scatter (SSC) and subsequently on CD45+ cells and the percentage of cell populations was assessed for further analysis. In detail, cell populations were defined as follows: *CD45 cells*: %CD45+ of all events, l*ymphocytes*: % of cells gated as lymphocytes by FSC/SSC of CD45+ cells, *monocytes*: % of cells gated as monocytes by FSC/SSC of CD45+ cells, *T cells*: %CD3+CD56- of lymphocytes, *CD4 cells*: %CD4+ of T cells, *CD8 cells*: %CD8+ of T cells, *CD4CD8 cells*: %CD4+CD8+ of T cells, *CD4/CD8 ratio*: %CD4+ of T cells / %CD8+ of T cells, *HLA-DR T cells*: %HLA-DR+ of T cells, *HLA-DR CD4 T cells*: %HLA-DR+ of CD4 cells, *HLA-DR CD8 T cells*: %HLA-DR+ of CD8 cells, *NK cells*: %CD56+CD3- of lymphocytes, *NKT cells*: %CD56+CD3+ of lymphocytes, *HLA-DR NK cells*: %HLA-DR+ of NK cells, *CD56dim CD16+ NK cells*: %CD56dimCD16+ of NK cells, *CD56bright CD16- NK cells*: %CD56brightCD16- of NK cells, *B cells*: %CD19+ of lymphocytes, *plasma cells*: %CD138+ of lymphocytes, *classical monocytes*: %CD14+CD16-/dim of monocytes, *non-classical monocytes*: %CD14+/lowCD16+ of monocytes (**Supplementary Figure 1**). CSF protein concentration, albumin, IgG, IgA, and IgM levels in the CSF were analyzed using nephelometry. A Reiber scheme was created for each Ig and we evaluated the presence of a BBB disruption or an intrathecal Ig synthesis. We used isoelectric focusing followed by silver nitrate staining to detect oligoclonal bands (OCBs). Of note, OCBs, BBB disruption, and intrathecal Ig synthesis are dichotomous parameters, while all other parameters are continuous.

Statistical Analysis

Statistical analysis of the data was performed using *R* version 3.5.1. The statistical significance of the data was determined using either the chi-squared test for comparing frequencies,

the Mann-Whitney U-test for comparing two groups or the Kruskal-Wallis test with the Dunn test as a *post hoc* test when performing multiple comparisons. Correction for multiple testing was performed by Benjamini-Hochberg's false discovery rate correction. A $p < 0.05$ was considered statistically significant. Clustered heatmaps were created with the R package pheatmap. First of all, the mean of each parameter was calculated categorized by disease. To improve comparability, the results were scaled and centered by subtracting the column means from their corresponding column and dividing the columns by their standard deviations. Hierarchical clustering of rows was performed with complete linkage clustering and Euclidean distance measure. Correlation matrix was calculated with Spearman's rank correlation coefficient and data were hierarchically clustered with complete linkage and Euclidean distance measure. To reduce dataset dimensionality and detect patters of CSF data, principal component analysis (PCA) was performed with the R package factoextra treating each patient as one datapoint. Furthermore, to visualize our complex data we used a recently published dimension reduction technique, the uniform manifold approximation and projection for dimension reduction (UMAP) (20), which represents a further development of the t-Distributed stochastic neighbor embedding algorithm (t-SNE) (21). To investigate the most suitable parameters for distinguishing between patients with CIDP, GBS, RRMS, and IIH, receiver operating characteristics (ROC) analysis was performed with the R package pROC (22). A ROC analysis allows systematically evaluating the sensitivity and specificity of a test and returns area under the curve (AUC) values. An AUC of 0.5 represents an uninformative classifier, while an AUC of 1 indicates perfect performance (23). When multiple predictors were used for ROC analysis, we performed a generalized linear model with logistic regression by adding multiple parameters in advance. The optimal number of parameters was determined by the Bayesian information criteria. The composite scores were selected by the regsubsets function of the R-package leaps using exhaustive search. The 95% confidence interval was calculated using De Long test.

Standard Protocol Approvals, Registrations, and Patient Consents

The study was conducted according to the declaration of Helsinki and approved by the local ethical committee (AZ 2018-563-f-S).

RESULTS

Patient Characteristics and Validation of the Approach

First, we characterized the patient cohorts. Patients with IIH and RRMS were younger and more often female than patients with GBS and CIDP and the lag between onset of symptoms in GBS was shorter than in CIDP (**Table 1**) (24–27). The percentage of non-treated patients in CIDP and GBS was comparable (**Supplementary Figure 3, Table 1**). The most common therapy in both groups was intravenous immunoglobulins (**Supplementary Figure 3**, **Table 1**). We identified 4 out of 32 CIDP patients that were initially misdiagnosed as GBS because of a rapid-onset with consecutive chronic course. All were later correctly classified as CIDP patients (**Table 1**). As expected, RRMS patients showed mildly elevated cell counts in CSF as well as increased proportions of intrathecal immunoglobulin (Ig) synthesis, and presence of oligoclonal bands (OCBs) (**Figures 1A,C**) (28). In contrast, CSF protein and blood-brain barrier (BBB) disruption were significantly increased in GBS and CIDP patients (**Figure 1C**).

TABLE 1 | Demographics and basic CSF characteristics of the patients.

	CIDP	GBS	IIH	RRMS
Number of patients	32 (4 A-CIDP)	26	63	49
Age (median with range)	58 (18–78)	59 (18–83)	32 (18–76)	33 (18–55)
Female (number/percent)	6/18.7%	12/46.2%	50/79.4%	33/67.3%
Male (number/percent)	26/81.3%	14/53.8%	13/20.6%	16/32.7%
CSF cells (median with range) /µl	1 (0–12)	1 (0–37)	1 (0–5)	4 (0–58)
CSF protein (median with range) mg/l	1075 (358–4640)	972 (308–3690)	345 (115–823)	431 (152–705)
BBBD (number/percent)	31/96.8%	23/88.5%	7/11.1%	8/16.3%
Intrathecal Ig synthesis (number/percent)	1/1.6%	0/0 %	1/1.6%	30/61.2%
OCBs (number/percent)	4/12.5%	2/7.7%	1/1.6%	43/87.8%
Hughes Score (median with range)	2 (1–4)** Mean: 2.1	3 (1–5)** Mean: 3.1		
Modified Rankin Scale (median with range)	3 (1–4)* Mean: 2.5	4 (1–5)* Mean: 3.6		
Non-treated patients in the last 3 months (number/percent)	18/56.2%	15/57.7%		
Therapy with IVIGs in the last 3 months (number/percent of treated patients)	8/57.1%	6/54.5%		
Time between onset of symptoms and sampling (median) days	485	10		

*A-CIDP, Acute onset CIDP; BBBD, blood-brain barrier disruption; CIDP, chronic inflammatory demyelinating polyneuropathy; GBS, Guillain-Barré syndrome; IIH, idiopathic intracranial hypertension; Ig, immunoglobulin; OCB, oligoclonal band; IVIG, intravenous immunoglobulin; RRMS, relapsing remitting multiple sclerosis *$p < 0.05$, **$p < 0.01$ (calculated by Mann-Whitney U-test).*

We next collected and systematically analyzed multi-dimensional flow cytometry data of CSF cells that are routinely obtained in our center together with standard CSF parameters. **Supplementary Figure 2** displays representative flow cytometry data of each diagnosis. As described (29), CSF in RRMS patients showed an expansion of plasma cells and B cells and increased frequencies of OCBs and intrathecal Ig synthesis as indicators of intrathecal monoclonal B cell responses (**Figures 1A,B**). Classical monocytes and non-classical monocytes, which were recently shown to play a pivotal role in the pathophysiology of MS (30), were also expanded in MS. This served as a positive control that our approach indeed replicates known inflammatory changes.

Disease- and Subtype-Specific CSF Alterations in Inflammatory Neuropathies

In GBS and CIDP, CSF exhibited known disease-associated changes including increased protein concentration and BBB disruption (i.e., "cytoalbuminologic dissociation"), with no differences between GBS and CIDP (**Figures 1A,C**). Non-specific indicators of lymphocyte activation (i.e., HLA-DR+ T cell populations) were also increased in line with the inflammatory etiology of both diseases. Similar changes have been previously described in various neurological diseases (17, 31). Notably, CSF of GBS patients displayed a disease-specific increase of NK cells (**Figures 1A,C**). The CSF of CIDP patients showed some overlapping, but also distinct changes and featured an increase of NKT cells and CD8+ T cells (**Figures 1A,C**). Inflammatory neuropathies thus exhibit a disease- and subtype-specific pattern of CSF cell abnormalities indicating a relevance of cytotoxic immune responses in both diseases that is distinct between subtypes.

We next aimed to understand the interdependence of individual CSF parameters and therefore performed a correlation analysis (**Figure 1B**). We found that some parameters formed two distinct and apparently co-regulated modules while the remaining parameters showed no apparent inter-relation. One module was best described as representing leukocyte activation (i.e., HLA-DR+ populations) and more widely included elevation of CNS protein, BBB disruption, NK cells, CD8+ T cells, and NKT cells and could thus be assigned to inflammatory neuropathies. The second module was best described as B cell-related (e.g., B cells, plasma cells, OCB, intrathecal immunoglobulin synthesis) and therefore matched the immune cell profile of RRMS (**Figure 1B**). As expected, given their reciprocal relationship, the proportion of CD4+ and CD8+ T cells and classical and non-classical monocytes were each negatively correlated. CSF parameters thus form mechanistically related clusters.

Disability Increases With CSF Indicators of Inflammation

We sought to find CSF parameters that correlate with the severity of the disease, as defined by the Hughes disability score (HDS) and the modified Rankin scale (mRS). Interestingly, there was a significant positive correlation between HLA-DR+CD4+ cells and HDS/mRS (Spearman correlation coefficient = 0.4/0.42, p = 0.041/0.033) and between non-classical monocytes and HDS/mRS (Spearman correlation coefficient = 0.51/0.49, p = 0.0075/0.01) in GBS patients (**Supplementary Figure 6A**). In CIDP patients, we detected a significant positive correlation between CSF protein concentration and HDS/mRS (Spearman correlation coefficient = 0.37/0.39, p = 0.039/0.027) (**Supplementary Figure 6B**). This indicates that markers of inflammation positively correlate with disease severity.

Distinct Disease-Specific Immune Cell Profiles

To aid the understanding of our complex dataset, we performed principal component analysis (PCA) regarding patients as multi-dimensional data points (**Figure 2A**). PCA illustrated that RRMS, IIH and CIDP/GBS patients each formed distinct clusters of CSF profiles, which were significantly different with non-overlapping confidence interval (**Figure 2A**). In contrast, CIDP and GBS showed some overlap. Next, we aimed to understand which parameters controlled PCA clustering of diseases. Principal component (PC) 2 determined the difference between RRMS vs. IIH, with the main contributors being parameters of the B cell lineage (plasma cells, B cells, OCBs, and intrathecal Ig synthesis) (**Figure 2B**). In contrast, PC1 determined the difference between GBS/CIDP vs. IIH and its main contributors were activated and non-activated CD8+ T cells, as well as NKT cells (**Figure 2B**). The main loadings of PC1 and PC2 thus corresponded well to known disease-specific alterations in MS and again supported T/NK cell-driven pathology in GBS/CIDP. This illustrates the applicability of dimensionality reduction techniques to understanding clinical datasets.

Differentiating Inflammatory Neuropathies With CSF Parameters

Next, we sought to systematically test our findings for potential diagnostic value. The area under the curve (AUC) in a ROC analysis can be used to measure the quality of a diagnostic test ranging from acceptable (AUC 0.7–0.8) through excellent (AUC 0.8–0.9) to outstanding (AUC >0.9) (32). We thereby tested which individual CSF parameter best differentiated inflammatory neuropathies from controls and GBS from CIDP. As expected, CSF protein (AUC 0.95) and BBB disruption (AUC 0.894) distinguished GBS from IIH patients, followed by activated and non-activated T cells, but also NK cells (AUC 0.73) (**Figure 3A**, **Supplementary Table 1**). The parameters discriminating CIDP from IIH were again CSF protein (AUC 0.97) and BBB disruption (AUC 0.94) and activated and non-activated T cells (**Figure 3B**, **Supplementary Table 2**).

Next, we searched for parameters distinguishing GBS from CIDP and found that NKT cells were the best parameter (AUC 0.76) differentiating CIDP and GBS followed by classical and intermediate monocytes (**Figure 3C**, **Supplementary Table 3**). CSF protein concentration and BBB disruption did not discriminate well between CIDP and GBS since they were elevated in both diseases. We thus identify a first potential

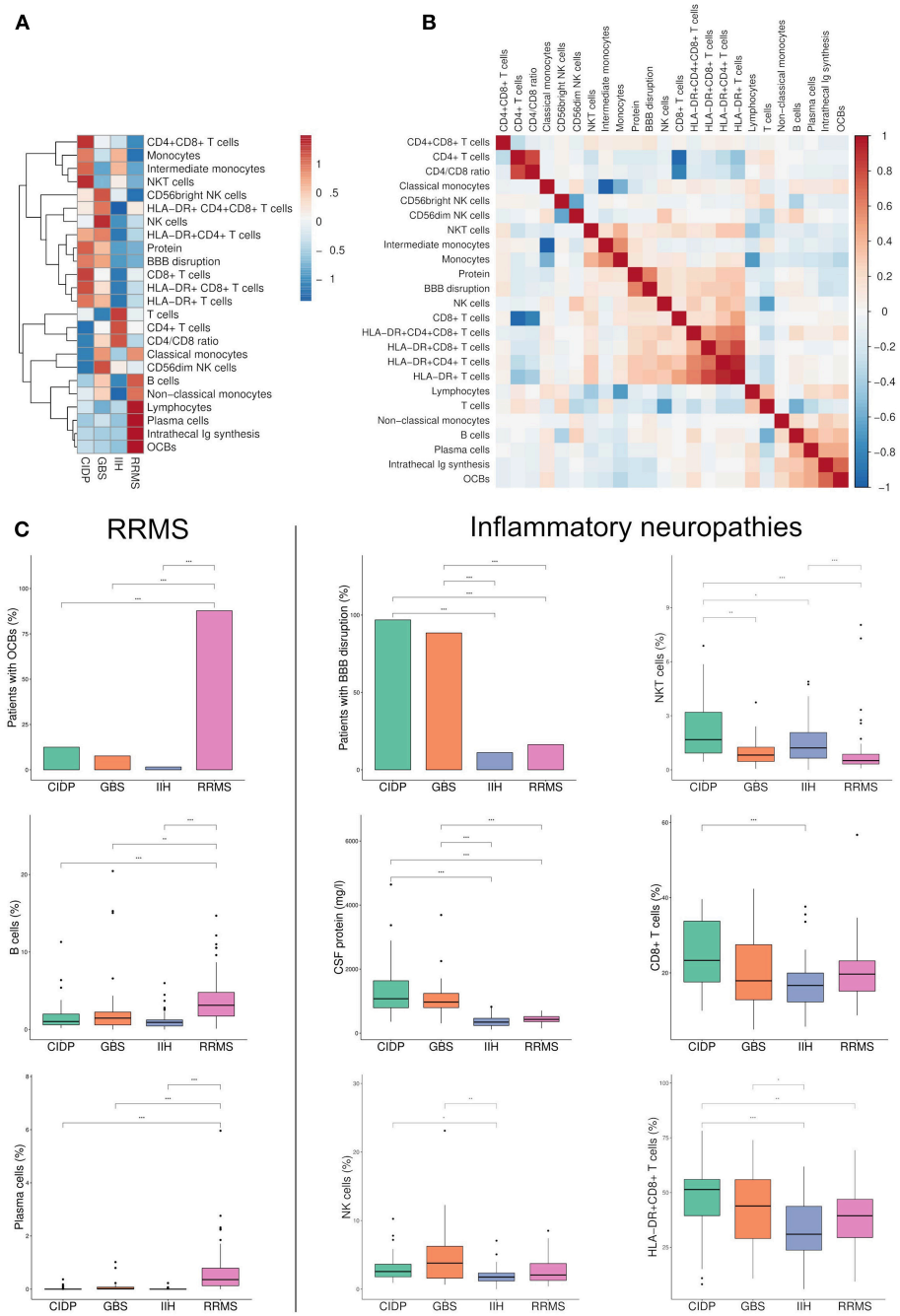

FIGURE 1 | Flow cytometry identifies new parameters to discriminate between immune-mediated neuropathies. **(A)** Heatmap depicting row mean of each CSF parameter per row calculated for chronic inflammatory demyelinating neuropathy (CIDP), Guillain-Barré syndrome (GBS), relapsing-remitting multiple sclerosis (RRMS) and idiopathic intracranial hypertension (IIH). The means were scaled and centered for each row by subtracting the column means from their corresponding column and dividing the columns by their standard deviations. Next, hierarchical clustering was performed with complete linkage method and Euclidean distance measure and visualized in a heatmap. **(B)** A correlation matrix of the investigated parameters was calculated with Spearman's rank correlation coefficient. Correlations coefficients were clustered hierarchically with the single linkage method and Euclidean distance measure. The correlation coefficients are colored according to the value. Positive correlations are displayed in red, negative correlations are colored in blue. **(C)** Box plots and bar plots of selected CSF parameters categorized by diagnosis. RRMS-related markers are shown on the left, markers related to inflammatory neuropathies are displayed on the right. Boxes indicate the lower quartile, median, and upper quartile with whiskers extending to the furthest value within 1.5 times the interquartile range of the box. Outliers are identified individually. The statistical significance of the results was determined using Kruskal-Wallis test and the Dunn test as a *post hoc* test. Correction for multiple testing was performed by Benjamini-Hochberg's false discovery rate correction. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. BBB, blood-brain barrier; OCBs, oligoclonal bands; Ig, immunoglobulin.

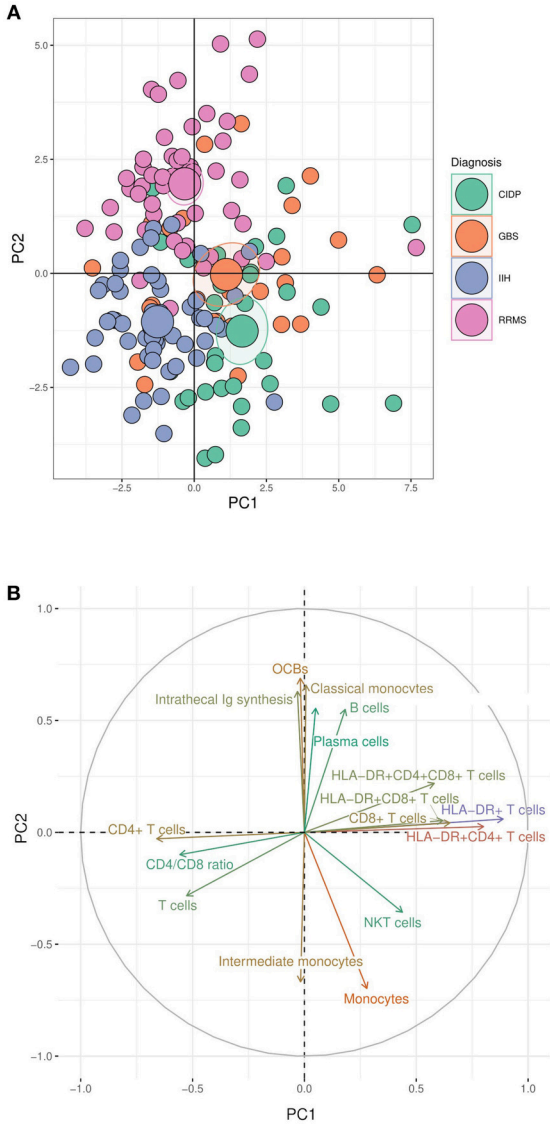

FIGURE 2 | Dimensionality reduction reveals distinct immune cell profiles of inflammatory neuropathies. **(A)** Principal component (PC) analysis (PCA) was performed using data from patients with chronic inflammatory demyelinating polyneuropathy (CIDP), Guillain-Barré syndrome (GBS), relapsing-remitting multiple sclerosis (RRMS), and idiopathic intracranial hypertension (IIH). Each circle represents a single patient; larger circles represent the group mean. The ellipses around each group mean point represent the confidence intervals. **(B)** The top 16 parameters contributing to PC1 and PC2 in the PCA are depicted. A long distance between a variable and the origin indicates a major influence of the corresponding variable on the PC. OCBs, oligoclonal bands; Ig, immunoglobulin.

surrogate parameter distinguishing CIDP from GBS—a clinically relevant, but difficult differentiation.

Composite Scores of CSF Parameters

We then speculated that combining individual parameters could improve the discriminatory ability of multi-parametric CSF analysis and therefore calculated summed composite scores. Models were determined computationally with the maximal number of parameters limited to four (see Methods for details). These summed composite scores improved diagnostic accuracy slightly for the comparisons of GBS against IIH (composite AUC 0.999 vs. single AUC 0.954) (**Figure 4A**) and for CIDP against IIH (composite AUC 0.994 vs. single AUC 0.974) (**Figure 4B**). Overall, the diagnosis of inflammatory neuropathies thus mostly relied on CSF protein and additional parameters provided minor benefit. However, the differentiation between CIDP and GBS was different. Adding classical monocytes, T cells, activated NK cells and intrathecal Ig synthesis improved the AUC from 0.759 to 0.872 (**Figure 4C**). Flow cytometry-based composite scores might thus help differentiating subtypes of inflammatory neuropathies in the future.

Intra-Disease Heterogeneity of Inflammatory Neuropathies

To further detect disease-specific CSF patterns, we performed a dimensionality reduction technique named uniform manifold approximation and projection for dimension reduction (UMAP) (20). In contrast to PCA, UMAP is non-linear and therefore less prone to outliers. This analysis identified two apparent subgroups of patients (**Figure 5A**) and thereby a surprising intra-disease heterogeneity of immune-mediated neuropathies. We classified inflammatory neuropathy patients based on the UMAP plot into group A, which formed a homogeneous cluster of CIDP and GBS patients, and group B, which showed considerable overlap with RRMS and IIH patients (**Figure 5A**). In comparison to group B, all patients from group A showed an elevated CSF protein concentration. Furthermore, group A displayed an increase of non-classical and intermediate monocytes in CIDP and an elevation of HLA-DR+CD4+CD8+ T cells in GBS (**Figure 5B**). Of note, this segregation was not driven by diagnostic certainty or treatment as the proportion of therapy-naïve patients was very similar in CIDP (59.1 % group A vs. 60% group B; chi-squared $p = 1$) and in GBS patients (57.1% group A vs. 58.3% group B; chi-squared $p = 0.98$) (**Figure 5B**). Based on these CSF surrogates, inflammatory neuropathy patients thus subset into two groups characterized by high CSF protein and raised non-classical monocytes in CIDP and elevated activated double positive T cells in GBS (group A) vs. low protein and overlap with control patients (group B). These findings suggest distinct disease mechanisms in subgroups of inflammatory neuropathies.

DISCUSSION

We here use multi-parametric CSF analysis combined with novel analytical approaches to identify disease- and subtype-specific changes in inflammatory neuropathies. Both GBS and CIDP show activation and elevation of cytotoxic immune cells in the CSF compartment that will form the basis for future mechanistic studies. In contrast, multiple sclerosis exhibits known signs of intrathecal B cell responses. The elevation of NKT cells and CD8+ T cells in CIDP and NK cells in GBS suggests a pivotal role of these cytotoxic cells in the pathophysiology of inflammatory neuropathies and could constitute a novel therapeutic target. We systematically evaluate these newly identified parameters

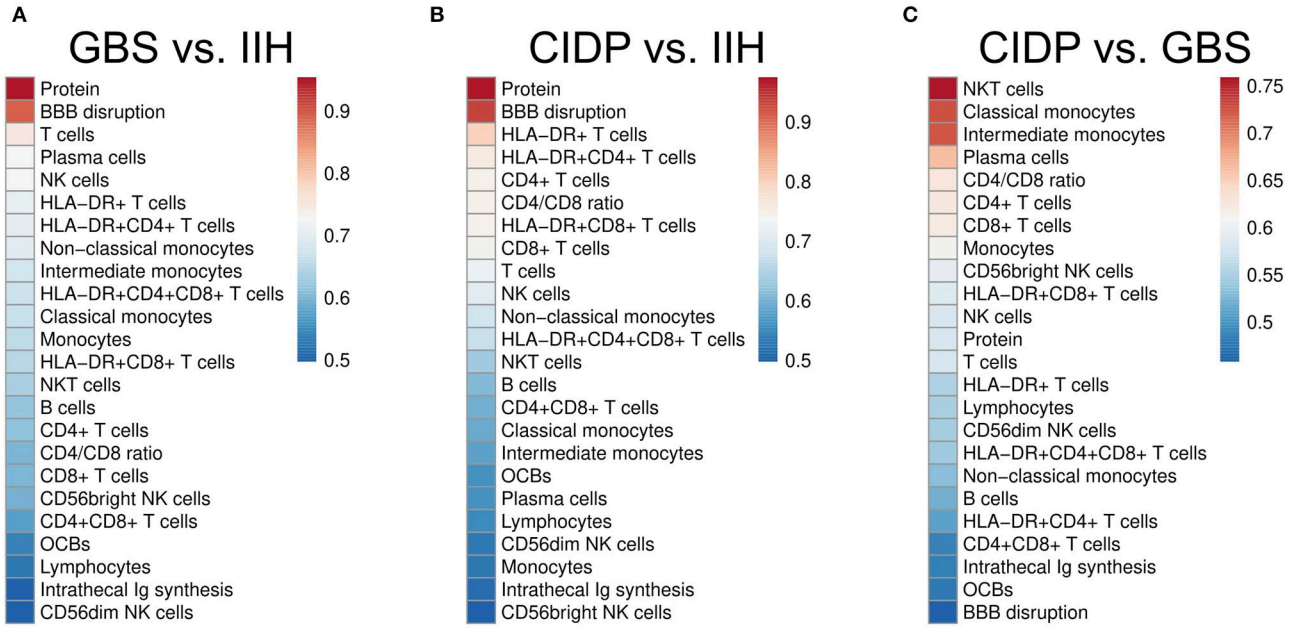

FIGURE 3 | CSF parameters differentiate inflammatory neuropathies with good confidence. Receiver operating characteristics (ROC) analysis was performed for each CSF parameter in different subsets. Heatmaps depict the color-coded values of the area under the curve (AUC) of the ROC analyses of different subsets sorted by size. **(A)** Guillain–Barré syndrome (GBS) vs. idiopathic intracranial hypertension (IIH). **(B)** Chronic inflammatory demyelinating neuropathy (CIDP) vs. IIH. **(C)** CIDP vs. GBS. BBB, blood-brain barrier; OCBs, oligoclonal bands; Ig, immunoglobulin. Please note the different color scales.

for diagnostic value and identify NKT cells as a first potential surrogate parameter, distinguishing GBS from CIDP with moderate accuracy. Constructing a novel composite score further improved the ability to distinguish GBS form CIDP. Our findings thus suggest diagnostic options in inflammatory neuropathies by immune cell profiling of the CSF in the future.

The understanding of the heterogeneity and pathophysiology of PNS autoimmunity remains limited. Our observations of elevated CD8+ T cells is in line with recent studies, which suggested a key role of CD8+ T cells in CIDP (33, 34). In GBS, increased levels of T cells, NK cells and macrophages were found in peripheral nerves of an animal GBS model (13). Accordingly, we now found elevated levels of NK cells and activated T cells in GBS suggesting that peripheral nerve and other immune compartments may communicate. At first glance, treated GBS patients differ in their immune cell profile compared to non-treated GBS patients (**Supplementary Figure 4**). However, the broad NK signal in the full cohort becomes more restricted to the CD56bright subset of NK cells in untreated GBS patients suggesting a more specific subset expansion. In addition, treatment and disease severity are mutual confounders as severely affected patients are more likely to be treated (**Supplementary Figure 5B**). In fact, immune cell profiles of severely affected GBS patients were similar to treated GBS patients (**Supplementary Figures 5A,C**). We thus speculate that differences between treated and non-treated GBS patients are mainly due to disease severity. We observed positive correlations between GBS disability and NK cells and activated T cells highlighting their important pathophysiological role. We are the first to observe expansion of NKT cells specifically in CIDP, but not in GBS. Of note, this cell immune profile was equally observed in treated and non-treated CIDP patients indicating that the observed immune cell pattern characterizes CIDP irrespective of previous treatments. NKT cells represent an innate-like T cell subset that express an invariant chain of the T cell receptor and recognize peptide antigens by CD1d (35). Depending on the tissue, NKT cells play either protective (36) or pathological roles (37) in various diseases. For example, NKT cells were described to protect mice from EAE, the mouse model of MS (38). NKT are usually subdivided in classical and non-classical NKT cells that probably represent functionally distinct cells types (39). A deeper analysis of NKT cells may thus reveal a more detailed understanding of the pathogenetic role of NKT cells in inflammatory neuropathies. The addition of humoral markers, such as sCD21 (40), sCD27 (40), TACI (41), and YKL-40 (42), may help to distinguish MS subtypes and such proteomics-based approaches of CSF could also be relevant for identifying novel mechanisms in inflammatory neuropathies. Further research on the functional role of NKT cells in CIDP will be required to potentially identify a promising target for immune therapy (35).

Despite well-defined diagnostic criteria, immune-mediated neuropathies are often misdiagnosed. In a recent study, the reliance on subjective perception and liberal electrophysiologic interpretation were identified as common diagnostic errors (43). Therefore, objective parameters to correctly diagnose

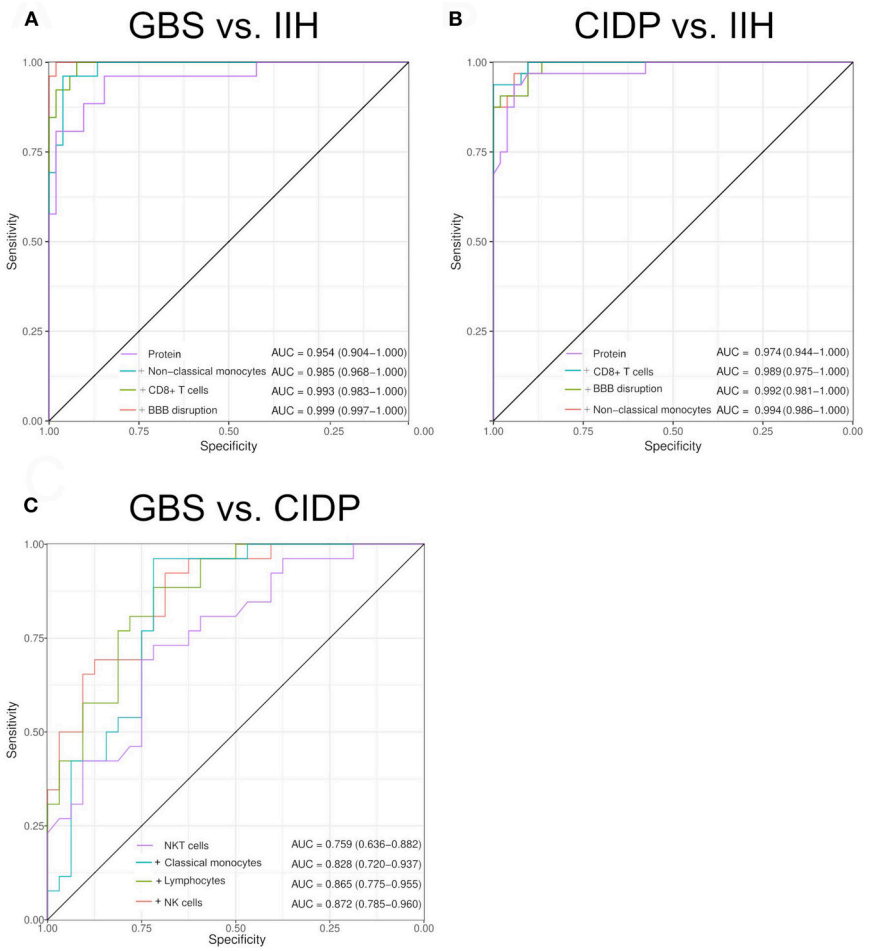

FIGURE 4 | Composite scores of multiple CSF parameters allow differentiating inflammatory neuropathies with high sensitivity and specificity. Two to five CSF parameters were combined by a generalized linear model with logistic regression. Receiver operating characteristics (ROC) analysis was performed. We increased the number of parameters sequentially. The model selection was performed by an exhaustive search. The maximal number of parameters was determined by the Bayesian information criteria. The individual parameters that form the composite score and the resulting AUC are given in the respective figure. The confidence was calculated using De Long test and is indicated in brackets. **(A)** Guillain–Barré syndrome (GBS) vs. idiopathic intracranial hypertension (IIH). **(B)** Chronic inflammatory demyelinating neuropathy (CIDP) vs. IIH. **(C)** CIDP vs. GBS. BBB, blood-brain barrier.

immune-mediated neuropathies are of high importance. Especially, differentiating GBS from CIDP is of high clinical relevance since treatment options are different (44) and a first episode of relapsing CIDP may easily be confused with GBS initially (45, 46).

Our study has clear limitations due to its retrospective study design and comparably small patient cohort. However, recruiting rare inflammatory neuropathy patients for an analytical method that requires fragile CSF cells to be analyzed immediately with extensive technical equipment is very challenging in a prospective study design. To the best of our knowledge, our study therefore constitutes the first comprehensive flow cytometry characterization of CSF cells in inflammatory neuropathies and articulates new mechanistic hypotheses. It will be important to evaluate our single parameters and composite scores evaluated here against other neuropathy controls in a more real-world clinical scenario with higher sample sizes. Hereafter, scores could help diagnosing inflammatory neuropathies in the future.

Furthermore, our data suggest a previously unknown intra-disease heterogeneity of inflammatory neuropathies that is driven by CSF protein, specific players of innate immunity in CIDP and activated T cells in GBS. It is tempting to speculate that these different groups differ in pathophysiology and may require different therapeutic approaches.

In summary, our cellular immune profiling flow cytometry of CSF cells adds to the understanding of divergent pathogenetic traits between GBS and CIDP paving the way for subsequent mechanistic studies. Furthermore, our composites scores represent potential tools in the diagnosis of immune-mediated neuropathies that are objective and can be easily standardized.

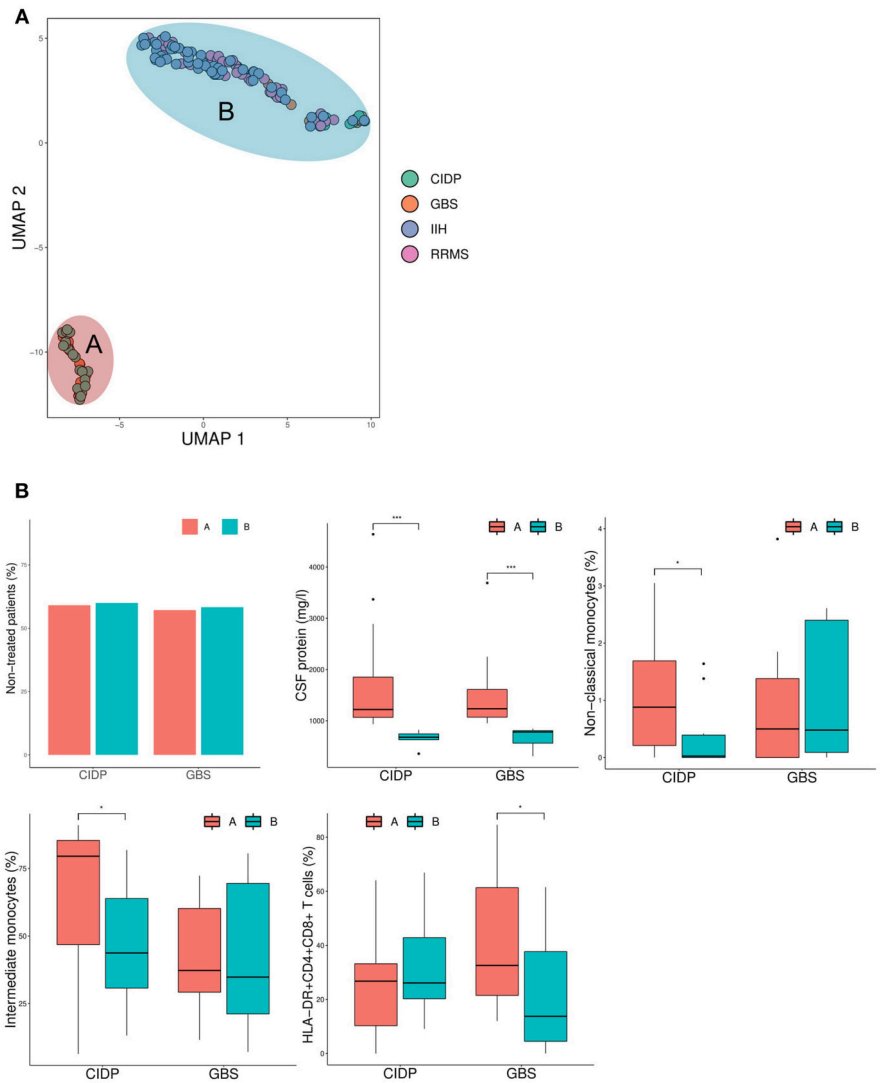

FIGURE 5 | Inflammatory neuropathies show intra-disease heterogeneity characterized by CSF protein and monocytes. **(A)** The uniform manifold approximation and projection for dimension reduction (UMAP) (20), a recently published dimension reduction technique, was performed. Each circle represents a single patient and diagnosis is color coded. Based on the results of UMAP, patients were divided into group A and B as denoted. **(B)** Box plots show the CSF parameters that were significantly different between group A and group B. Boxes indicate the lower quartile, median, and upper quartile with whiskers extending to the furthest value within 1.5 times the interquartile range of the box. Outliers are identified individually. The statistical significance of the results was determined using the Mann-Whitney U-test for continuous variables (CSF protein, non-classical monocytes, intermediate monocytes, HLA-DR+CD4+CD8+ T cells) and the chi-squared test for the dichotomous variable (therapy). $^{*}p < 0.05$, $^{***}p < 0.001$. CIDP, chronic inflammatory demyelinating neuropathy; GBS, Guillain-Barré syndrome; RRMS, relapsing-remitting multiple sclerosis; IIH, idiopathic intracranial hypertension.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

MH performed data acquisition and data analysis with statistical analysis and drafted the manuscript. AS-M, TB, JW, and TR contributed to data acquisition and analysis. SM, LK, CG, and HW revised the manuscript and co-supervised the project. GMzH conceptualized the project, revised the manuscript, and supervised the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00515/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Glial Activation Markers in CSF and Serum From Patients With Primary Progressive Multiple Sclerosis: Potential of Serum GFAP as Disease Severity Marker?

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Background: In progressive multiple sclerosis (MS), glial activation is thought to be a relevant mechanism of disability progression. Therefore, *in vivo* assessment of the glial cell activity is, in the emerging treatment era of primary progressive MS (PPMS), more important than ever.

Objectives: To test the association of cerebrospinal fluid (CSF) and serum markers of glial activation in PPMS patients; including glial fibrillary acidic protein (GFAP), chitinase-3-like protein 1 (CHI3L1), soluble variant of triggering receptor expressed on myeloid cells 2 (sTREM2), and marker of neuroaxonal damage (Neurofilament light chain, NfL) as well as clinical severity.

Methods: CSF and serum samples from PPMS patients were collected in the MS-centers at Universities of Freiburg (n = 49), Ulm (n = 27), Muenster (n = 11), and Rostock (n = 6). sTREM2 and CHI3L1 levels were measured using the previously reported ELISA assays, while NfL and GFAP were measured using SIMOA assays. Clinical data included age, gender, disease duration, treatment status, and Expanded Disability Status Scale (EDSS).

Results: 93 CSF samples and 71 matching serum samples were analyzed. The median age of patients was 49 years and disease duration 4.5 years. $GFAP_{serum}$ correlated with EDSS after correction for age ($\beta = 0.3$, $p = 0.001$). Furthermore, EDSS was higher in patients with a $GFAP_{serum}$ level $\geq$ 151.7 pg/ml compared to patients with $GFAP_{serum}$ below this cut-off (5.5 vs. 4.0, $p = 0.009$). Other markers did not correlate with the clinical severity. Moreover, we found a correlation between NfL_{CSF} and $GFAP_{CSF}$, sTREM2 and

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CHI3L1 ($\rho = 0.4$ for $GFAP_{CSF}$ and sTREM2, $\rho = 0.3$ for CHI3L1, $p < 0.01$ for sTREM2 and CHI3L1 and <0.001 for $GFAP_{CSF}$). CHI3L1 did not correlate with $GFAP_{CSF}$ but with sTREM2 ($\rho = 0.4$, $p < 0.01$).

Discussion: The correlation between the glial activation markers in CSF with the markers of neuroaxonal demise supports the notion of the glial involvement in PPMS. The positive correlation between $GFAP_{CSF}$ with disease duration and $GFAP_{serum}$ with the clinical severity of the disease may highlight a particular role of the astrocytes in PPMS and mark the potential of $GFAP_{serum}$ as a disease severity marker.

Keywords: SiMoA, GFAP, PPMS, glial activation, progressive multiple sclerosis, neurofilaments, CHI3L1, sTREM2

INTRODUCTION

The pathophysiology of primary progressive multiple sclerosis (PPMS) is complex and involves various mechanisms including inflammatory triggered demyelination, activation of B and T lymphocytes, mitochondrial dysfunction, and iron accumulation (1). However, the glial activation is considered to play a decisive role in the progression of neuroaxonal demise (2–4). While clinical and many radiological parameters can detect the final pathway of those different pathophysiological processes (progression of clinical disability, new MRI-lesions etc.), several other aspects such as role, extent and contribution of the various pathophysiological mechanisms remain widely unexplored. A biomarker-based approach may offer a unique window to assess such disease processes *in vivo* (5, 6). Over the last years, the level and clinical meaning of different biomarkers in CSF like glial fibrillary acidic protein (GFAP) as a marker for astrocytic activation (7–16), chitinase 3 like 1 protein (CHI3L1) (13, 14, 17–19) and soluble triggering receptor expressed on myeloid cells 2 (sTREM2) for microglial activation (20–23) and neurofilaments light chain (NfL) for neuroaxonal damage were reported. The single molecular assay (SIMOA) enables the detection of the ultra-low concentration of some of those biomarkers in serum (16, 24, 25). We previously showed that GFAP in serum correlates with the Expanded Disability Status Scale (EDSS) specifically in PPMS but not in patients with a relapsing-remitting disease course (16). Similar results were reported later from other groups (26). In this study, we aim to reproduce these findings in a large cohort of PPMS patients and to explore the clinical meaning of the other glial activation markers in PPMS.

METHODS

Patient Selection

CSF and serum samples from patients with PPMS were collected from the University Hospitals of Freiburg, Ulm, Muenster, and Rostock. The patients were admitted or seen within the Outpatient Departments between 2010 and 2018. In all patients, the diagnosis has been revised according to the McDonald criteria from 2017 (27) after careful exclusion of relevant differential diagnoses. The lumbar puncture was performed as a part of the diagnostic workup. The clinical severity was measured by assessing the Expanded Disability Status Scale (EDSS), Multiple Sclerosis Severity Score (MSSS) as well as the Age-related Multiple Sclerosis Severity Score (ARMSS) as reported recently (28).

CSF and Serum Sample Processing

A standardized protocol for CSF and serum collection was applied as previously recommended (29). Biosamples from patients were stored according to the predefined standard operating procedure (SOPs) at a local biobank at minus 80°C. Later they were transferred for measurement on dry ice to the biobank of the coordinating center in Ulm for further analysis. Hemolytic CSF specimens were excluded. From some patients, only CSF samples were available, with no matching serum samples.

Assessments of the Biomarkers

GFAP and NfL in CSF and serum were measured using Simoa assays (GFAP Discovery kits and NfL Early Access assays, Quanterix Corporation). CHI3L1 was measured using the commercial ELISA-Kits (Human Chitinase 3-like 1 Quantikine ELISA Kit DC3L10, R&D Systems). sTREM2 was measured using the previously reported ELISA using the MSD Platform (21). Samples were diluted, as recommended by the manufacturer, and concentrations were calculated using the corresponding standard curve. The intra-assay coefficient of variation (CV) was assessed by measuring a QC of serum and CSF sample in 5 replicates with a CV below 10% was obtained, whereas a CV of lower than 10% had to be achieved for a valid analysis. We did not find an influence of up to 5 freeze-thaw cycles on the investigated biomarkers, except for GFAP in CSF. Here, the concentration decreased by over 50% after within 2 freeze-thaw cycles. Therefore, GFAP CSF levels between centers were compared, and exceedingly low values were excluded from the analysis.

To compare potentially pathological serum biomarker levels, we determined a cut-off in a group of 20 patients with other non-inflammatory neurological diseases we previously published (16). As the concentration of serum GFAP in a normal or healthy population is not described, we used the 90th percentile to determine a cut-off value for further analysis. This yielded a cut-off value of 151.7 pg/ml for serum GFAP. A cut-off value for serum NfL of 16 pg/ml was suggested recently (30).

TABLE 1 | Clinical characteristics of the included subjects.

	Median (25–75 percentile), $n = 93$
Age	49 years (44–57)
Gender ♀:♂	1.1:1
Disease duration in years	4.5 (2–12)
Expanded disability status scale (EDSS) at the time of lumbar puncture (LP)	4.5 (3.5–6.5)
Multiple sclerosis severity score (MSSS)	8.1 (6.4–9.1)
Age-related multiple sclerosis severity score (ARMSS)	6.2 (4.6–7.9)

TABLE 2 | Concentrations of the assessed biomarkers in CSF and serum.

	Median (25–75 percentile)
Cerebrospinal fluid glial fibrillary acidic protein ($GFAP_{CSF}$) in pg/ml ($n = 76$)	7,820 (5,050–1,1165)
Serum glial fibrillary acidic protein ($GFAP_{serum}$) in pg/ml ($n = 71$)	126.0 (104.5–174.0)
Cerebrospinal fluid neurofilaments light chain (NfL_{CSF}) in pg/ml ($n = 93$)	1230.8 (840–2,125)
Serum neurofilaments light chain (NfL_{serum}) in pg/ml ($n = 71$)	18.5 (12.3–25.9)
Cerebrospinal fluid chitinase 3 like 1 protein (CHI3L1) in ng/ml ($n = 93$)	210.8 (138.5–291.0)
Cerebrospinal fluid soluble triggering receptor expressed on myeloid cells 2 (sTREM2) in ng/ml ($n = 87$)	3.1 (2.3–4.4)

Statistical Analysis

All statistical tests were performed using SPSS® Statistics version 25 (IBM Corporation). The Shapiro-Wilk test was used to examine the distribution of the data. Mann-Whitney *U* test and Kruskal-Wallis test were used to compare medians in skewed distributed parameters. A multiple linear regression model and univariant general linear model was applied to account for a possible confounding bias caused by the strong correlation between GFAP levels and age. The Spearman's rho test was used to test for correlations. A p-value ≤ 0.05 was considered as statistically significant. Figures were made using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Clinical Characteristics

A Total of 93 CSF and 71 matching serum samples were collected. The summary of the clinical characteristics is mentioned in **Table 1**, and the concentration of the different CSF and serum biomarkers are shown in **Table 2**. Fifty-five patients did not receive any disease modifying treatment at and before the time of sample collection. Thirty-eight patients were on a treatment: Mitoxantrone ($n = 19$), three monthly pulse-steroid ($n = 11$), Rituximab ($n = 5$), cyclosporine ($n = 2$), and Interferon beta 1a ($n = 1$).

Comparison Between Centers

Despite having similar patients characteristics (age, gender distribution, disease duration, and disease severity as assessed by EDSS), $GFAP_{CSF}$ levels differ between the centers; values form Muenster and Rostock were significantly lower than those from Ulm and Freiburg, whereas sTREM2 levels were lower only in the samples form Rostock compared to all other centers (**Figure 1**). Thus, in the following analysis, we excluded the $GFAP_{CSF}$ from Muenster and Rostock ($n = 17$), and sTREM2 measurements form Rostock ($n = 6$). Concentrations of CHI3L1, NfL_{CSF}, NfL_{serum}, and $GFAP_{serum}$ in the samples of Muenster and Rostock were included in the statistical analysis.

Clinical Aspects

None of the biomarkers correlated with the age except $GFAP_{serum}$ and NfL_{serum} ($\rho = 0.4$ and 0.3, $p = 0.005$ and 0.014, respectively) (**Figure 2**). Gender and treatment status did not influence the levels any of the tested markers neither in CSF nor in serum (data not shown).

Of all the assessed markers (in CSF and in serum), only $GFAP_{CSF}$ correlated with disease duration ($\rho = 0.3$, $p = 0.014$). None of the CSF markers correlated with disease severity.

Regarding the serum markers, we found a moderate correlation between $GFAP_{serum}$ and EDSS ($\rho = 0.4$, $p = 0.004$), which remained significant after adjusting by the effect of age ($\beta = 0.3$, $p = 0.001$) (**Figure 3**). NfL_{serum} did not correlate with any of the disease severity parameters (**Figure 3**). No significant correlations were found between $GFAP_{serum}$ and MSSS or ARMSS (data not shown).

To further confirm the association of $GFAP_{serum}$ with the EDSS, we compared the EDSS values with $GFAP_{serum}$ and NfL_{serum} levels higher or lower than the cut-off that was determined as described beforehand. Here, 63% of our PPMS patients had $GFAP_{serum}$ above this cut-off of 151.7 pg/ml ($n = 45$). Moreover, they had a significantly higher median EDSS than patients below this cut-off (5.5 vs. 4.0, $p = 0.009$, **Figure 4**). No differences were found for this comparison for NfL_{serum} (cut-off 16 pg/ml, 4.5 vs. 4.5, $p = 0.16$, **Figure 4**). There was a significant age difference for the grouping by NfL_{serum} but not for $GFAP_{serum}$ ($p = 0.01$ and 0.47, respectively, data not shown).

Considering $NfL_{serum} \geq 16$ pg/ml as a cut-off value for active diseases, PPMS patients with $NfL_{serum} \geq 16$ pg/ml ($n = 44$) have higher median concentration of $GFAP_{serum}$ than those with $NfL_{serum} < 16$ pg/ml ($n = 27$) (131.0 vs. 114.5 pg/ml, $p = 0.037$ after correction for age, **Figure 5**).

Correlation Between CSF and Serum Parameters

We found a moderate correlation between GFAP levels in CSF and serum ($\rho = 0.4$, $p = 0.001$), and strong correlation between levels of NfL in CSF and serum ($\rho = 0.6$, $p < 0.001$).

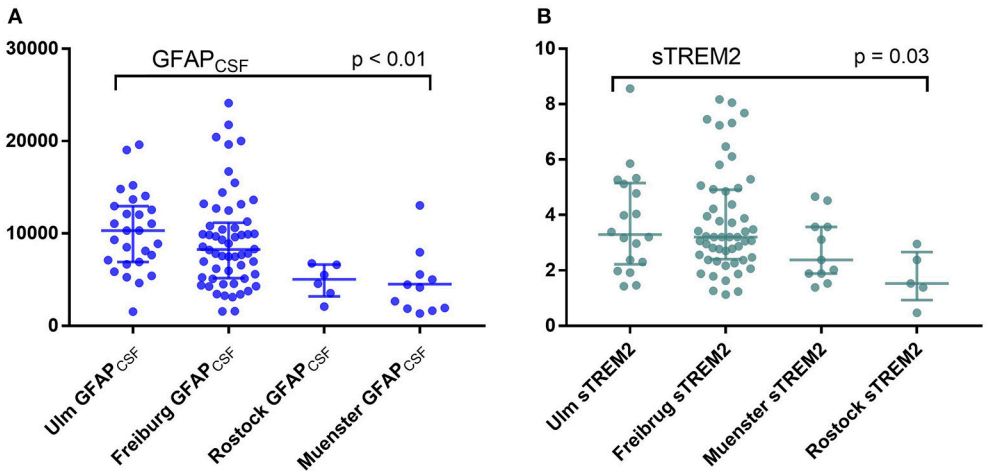

FIGURE 1 | Comparison between the levels of **(A)** glial fibrillary acidic protein (GFAP) and **(B)** soluble triggering receptor expressed on myeloid cells 2 (sTREM2) in cerebrospinal fluid (CSF) between the four participating centers (Kruskal-Wallis test).

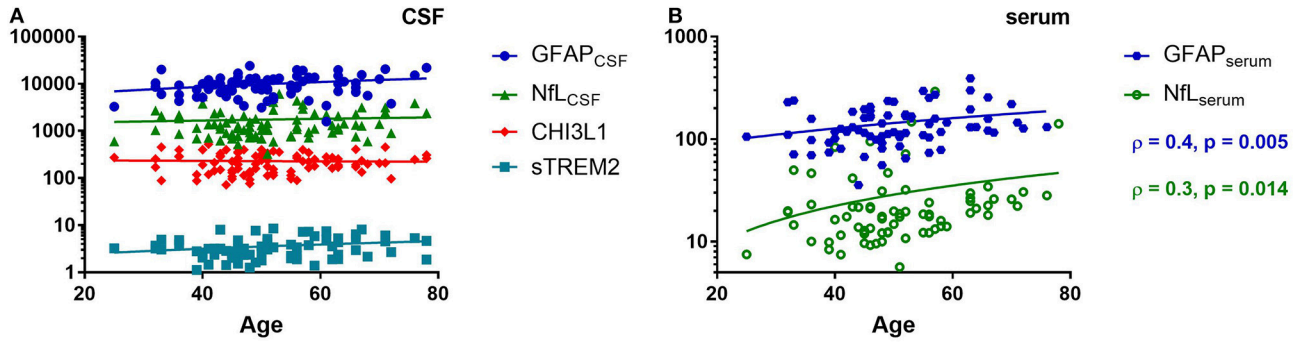

FIGURE 2 | Spearman correlation of various cerebrospinal fluid (CSF) **(A)** and serum **(B)** biomarkers with the age of the patients. Glial fibrillary acidic protein (GFAP), neurofilaments light chain (NfL), chitinase 3 like 1 (CHI3L1), soluble triggering receptor expressed on myeloid cells 2 (sTREM2), ρ (Spearman rho).

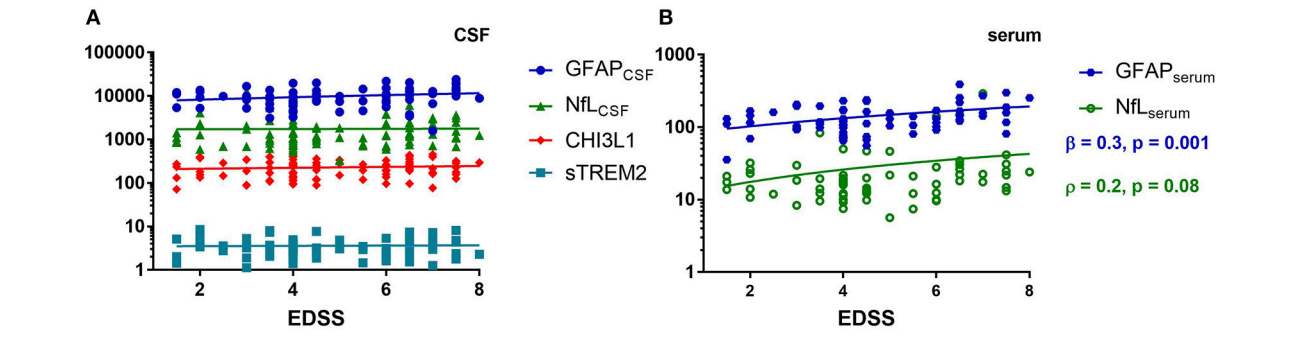

FIGURE 3 | Spearman correlation of cerebrospinal fluid (CSF) **(A)** and serum **(B)** biomarkers with the clinical severity as measured by the expanded disability status scale (EDSS). Glial fibrillary acidic protein (GFAP), neurofilaments light chain (NfL), chitinase 3 like 1 (CHI3L1), soluble triggering receptor expressed on myeloid cells 2 (sTREM2), ρ (Spearman rho), β (standardized coefficient) of multiple linear regression, age as a covariant.

In CSF, NfL correlated with sTREM2 ($\rho = 0.4$, $p < 0.01$,) CHI3L1 ($\rho = 0.3$, $p < 0.01$) and GFAP ($\rho = 0.4$, $p < 0.001$, **Table 3** and **Figure 6**). Moreover, CHI3L1 correlated with sTREM2 ($\rho = 0.4$, $p < 0.01$, **Figure 7**) but not with GFAP. On the other hand, GFAP did not correlate with sTREM2.

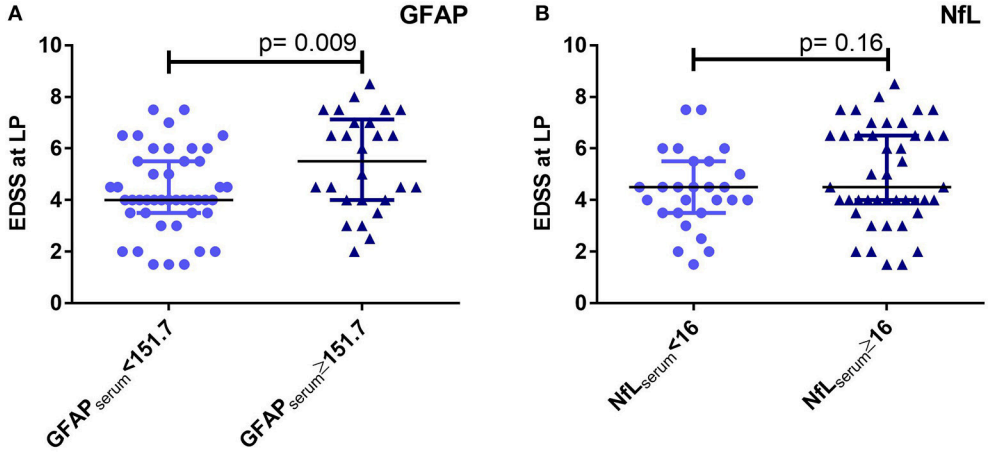

FIGURE 4 | Comparison of expanded disability status scale (EDSS) values for primary progressive multiple sclerosis (PPMS) patients below and above cut-offs of 151.7 pg/ml for glial fibrillary acidic protein (GFAP) **(A)** and 16 pg/ml for neurofilaments light chain (NfL) **(B)** in serum (Mann-Whitney *U* test), respectively. There was a significant age difference for the grouping by NfL but not for GFAP (Mann-Whitney *U* test, $p = 0.01$ and 0.47, respectively). LP, lumbar puncture.

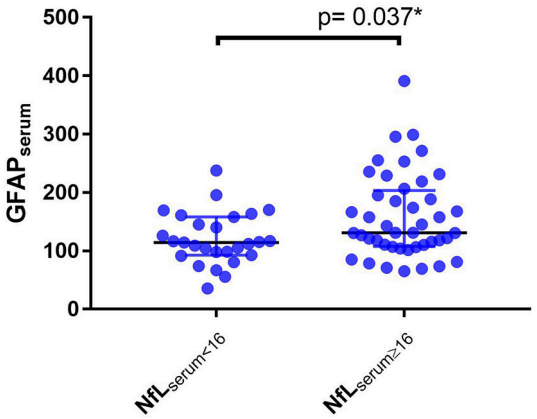

FIGURE 5 | Level of serum glial fibrillary acidic protein ($GFAP_{serum}$) in PPMS patients with serum neurofilaments light chain levels equal to or higher than 16 pg/ml ($NfL_{serum} \geq 16$) or lower the 16 pg/ml ($NfL_{serum} < 16$), *Univariant general linear model corrected for age.

TABLE 3 | Correlations between various CSF biomarkers.

Marker	NfL_{CSF}	$GFAP_{CSF}$	CHI3L1
sTREM2	0.4**	n.s.	0.4**
CHI3L1	0.3**	n.s.	
$GFAP_{CSF}$	0.4***		

*GFAP, glial fibrillary acidic protein; NfL, neurofilaments light chain; CHI3L1, chitinase 3 like 1; sTREM2, soluble triggering receptor expressed on myeloid cells 2. **$p < 0.01$, ***$p < 0.001$.*

DISCUSSION

Assessment of various biomarkers reflecting different pathophysiological processes involved in the disease progression and the downstream-treatment effect is becoming increasingly important due to the emerging treatment options for progressive MS. Our study evaluates the levels of astroglial activation (GFAP), microglial activation (CHI3L1 and sTREM2) and neuroaxonal damage (NfL) in CSF and serum of a multicentric cohort of PPMS-Patients.

The glial activation is a putative cornerstone in the progression of neurodegeneration in PPMS (1). The astrocytes involvement in MS generally is a double-edged sword; while the glial scar formation may protect the tissue from further damage, it might prevent the remyelination in MS (31). The activation of astrocytes is an early event in the development of MS lesions with the release of cytokines like CCL-2, CXCL-12, MMPs, TGFβ, IL-1, IL-6, IL-10, IL-12, IL-15, IL-23, and IL-27 leading to dysfunction of the blood-brain barrier (BBB) as well as recruitment of innate and adaptive immune cells (32). Furthermore, the A1 subtype of astrocytes is a potent killer of neurons and oligodendrocytes in EAE models and was reported in acute and chronic MS lesions (33).

Nevertheless, the astrocytes in progressive MS play a more specific role in the maintenance of the local inflammation; in models of chronic EAE, levels of lactosylceramide (LacCer) synthesized by β-1,4-galactosyltransferase 6 (B4GALT6) from the activated astrocytes were elevated in the EAE-lesions. Suppression of B4GALT6-activity reduces local inflammation, microglial activation and monocytes recruitment and subsequently the resulting neurodegeneration (34).

Correlation between $GFAP_{CSF}$ and clinical severity were inconsistent among previous studies using the standard ELISA assay (9, 12–15). In a previous study from one of our centers, we reported a moderate correlation between levels of GFAP in serum, but not in CSF, with disease severity scores (16). Our current multicentric cohort validated our results showing a consistent correlation between $GFAP_{serum}$ with EDSS score, even after correction for the age of the patients. In accordance with those results, patients with $GFAP_{serum}$ above the

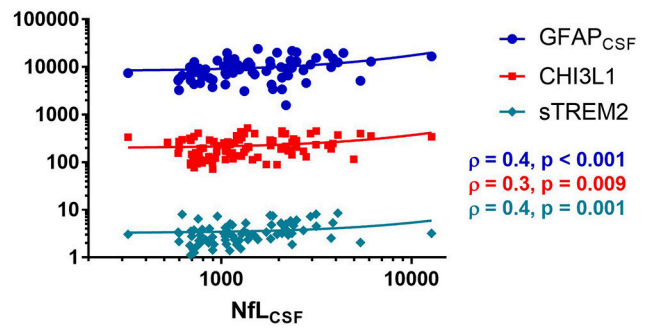

FIGURE 6 | Spearman correlation between neurofilaments light chain (NfL), glial fibrillary acidic protein (GFAP), chitinase 3 like 1 (CHI3L1), and soluble triggering receptor expressed on myeloid cells 2 (sTREM2), ρ (Spearman rho).

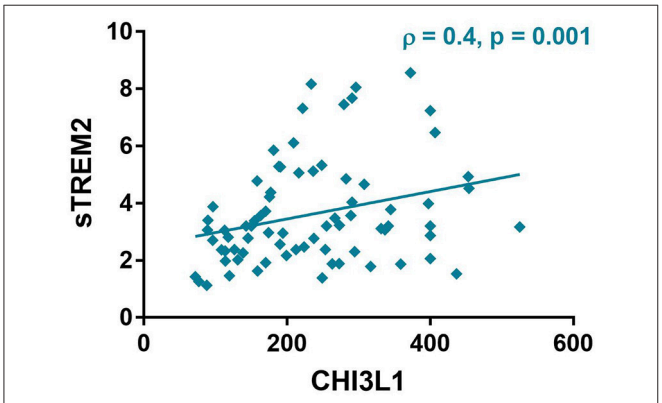

FIGURE 7 | Spearman correlation between chitinase 3 like 1 (CHI3L1) and soluble triggering receptor expressed on myeloid cells 2 (sTREM2), ρ (Spearman rho).

proposed cut-off of 151.7 pg/ml based on our previous results, had more severe disease. Furthermore, levels with GFAP$_{serum}$ were higher in patients having NfL$_{serum}$ ≥ 16, a recently proposed cut-off value for higher activity and worse disease prognosis (30). Nevertheless, this NfL cut-off value was proposed according to pooled results from various studies with relapsing-remitting MS patients. Thus, its value in PPMS has yet to be validated.

Why GFAP$_{serum}$ might reflect the disease activity better than GFAP$_{CSF}$ is still not entirely explained. As previously suggested from our group, the enhanced expression of GFAP in the activated astrocytes end feet in the predominantly perivascular MS lesions might be directly drained into the blood compartment and not into the CSF space (16). Supporting data were reported in mouse models of EAE (35). Moreover, the GFAP, as well as other markers, might be transported to blood directly via the glymphatic system as shown recently in murine models of traumatic brain injury (36).

While a recent study suggested lower levels of GFAP$_{serum}$ in relapsing MS patients under treatment (26), the concentration of GFAP$_{serum}$ did not differ according to treatment status in our cohort. This can be explained by the fact, that none of the above-mentioned treatments were proven effective in PPMS. This observation regarding levels of GFAP$_{serum}$, as well as other markers including the NfL$_{serum}$, might underscore the ineffectiveness of those treatments in PPMS.

The validation of those results in our larger multicentric cohort might highlight the clinical meaning of levels of GFAP in serum as a possible serum marker in PPMS patients.

Like the astrocytes, the activated microglial release different cytokines (IL-1, Il-6, and TNF-α) as well as NO and ROS leading to exacerbation of inflammatory cascade, to attraction of inflammatory cells from blood and also to mitochondrial dysfunction, a significant mechanism in the disease progression in PMS (2, 3, 37, 38). Moreover, microglial activation in PMS appears to be diffusely prevalent not only in MS lesions but also in normal-appearing white matter (NAWM) forming the so-called microglial nodules. In the less inflammatory cortical lesions, active demyelination can be found in close proximity with the microglia (3).

Data regarding sTREM2 in PPMS are scarce with two studies reported concentration in CSF in twenty-one and in three PPMS patients, respectively (20, 22). In accordance with previous reports, no correlation was found between sTREM2 and EDSS or MSSS. Yet, sTREM2 correlated with NfL in CSF, which might highlight the role of microglia in the neuroaxonal demise in PPMS.

The meaning of CHI3L1 appears to be controversial; some reports consider it as a marker of astrocytic activation (18, 39), whereas other studies count it a marker of active microglial cells (40). The correlation between CHI3L1 and sTREM2 found in our patients is equivocal; it may underscore the microglial origin of CHI3L1 or might reflect the crosstalk between the microglia and astrocytes in PPMS (33) leaving the question regarding the origin of CHI3L1 in MS brains unanswered.

The prognostic value of CHI3L1 is prominent in CIS and RRMS patients (13, 17, 41–44) and to a lesser extent in SPMS (19). CHI3L1 in CSF did not correlate with the clinical parameters in PPMS patients in the above-mentioned studies. Consistent with the various histopathological studies, all measured glial activation markers in CSF correlated with the neuroaxonal demise as assessed by the NfL, but not with the clinical severity scores. This paradox might be due to some methodological limitations of the assays, the need of more specific markers or due to the limited ability of the applied clinical scores to reflect the extent of the neurodegeneration in PPMS. Indeed, the shortness of EDSS to reflect all aspects of the disease progression in PPMS is a lesson learned from the various negative clinical trials in PPMS (45). The upper arm function and the subtle cognitive deficits are underrepresented in the EDSS (46). Furthermore, the EDSS scores were mostly based on the walking distance reported by the patients, which might lead to an incorrect evaluation of the EDSS score (47).

Another limitation of our study is the missing detailed magnetic resonance imaging data (MRI). However, the main aim of our study was to explore the clinical meaning of the glial activation markers.

Although the concentration of most of the measured biomarkers did not vary between centers, GFAP and sTREM2 were notably lower in two of the four centers. As this is a retrospective study, we were not able to completely capture all pre-analytical procedures. To avoid potential bias of pre-analytical procedures, we did the comparison of analyte levels per center and excluded statistically low values. The excluded samples represent a minority in our cohort (17/93 for GFAP and 6/93 for sTREM2), and their exclusion had no statistical impact on the above-mentioned results (data not shown).

A point to be considered while interpreting our results is the protective role of glial cells following neuronal injury. Microglial and astrocytic activation were reported upon axonal degeneration and contributes to tissue repair and limitation of the inflammatory activity (48–50). Our markers reflect the glial activation generally, but not necessarily their pathological role. Complementary data from more specific markers of subsets of microglia or astrocytes could be helpful to understand their pathogenetic role.

In summary, we analyzed in this study various markers of glial activation in CSF and serum and evaluated their correlation with neuroaxonal damage markers and disease severity measures. The correlation between the microglial and astrocytic activation markers in CSF with the markers of neuroaxonal demise (NfL) may underscore the glial involvement in the neurodegeneration in PPMS. The positive correlation between GFAP in serum with the clinical severity of the disease may highlight the potential of $GFAP_{serum}$ as a disease progression marker. It his highly desirable to confirm this finding in a prospectively collected study cohort and compare it to standardized acquired MRI data. Additionally, the determination of GFAP serum levels in a large group of healthy controls might help to further differentiate between age related normal and abnormal GFAP levels.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study was reviewed by the ethics committee of the University of Ulm, and all experimental protocols were approved (approval number 270/17). Our study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki. Written informed consent was obtained from all patients participating in this study.

AUTHOR CONTRIBUTIONS

AA, HT, and AH: study concept. AA and TH: data acquisition, data analysis, and interpretation. AH, EM-R, and MS-C: biomarkers assessment. AA: drafting of the manuscript. HT, SR, MO, CH, UZ, and SM: study supervision and critical revision. All authors critically reviewed and approved the manuscript.

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Neurofilament Light Chain as a Biomarker in Multiple Sclerosis

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Due to the unpredictable course and heterogenous treatment response in multiple sclerosis (MS), there is a clear need for biomarkers that reflect disease activity in the clinical follow-up of these patients. Neurofilaments are neuron-specific components of the cytoskeleton that can be assayed in different body compartments. They have been explored as potential biomarkers for many years. Neurofilament light chain (NF-L) appears the most promising biomarker in MS patients, and there is now little doubt that NF-L should have a role in the follow-up of MS patients. Newer assays and techniques for NF-L detection available in serum samples confirms the usefulness of NF-L as a biomarker. Nevertheless, there is still a need for prospective studies, and studies to determine clinical useful cut-off values. This review evaluates the strengths and weaknesses of NF-L as a biomarker in patients with MS.

Keywords: neurofilament light (NF-L), biomarker, multiple scleorsis (MS), serum, cerebrospinal Fluid—CSF, axonal damage

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NEUROFILAMENTS—AN INTRODUCTION

Neurofilaments are cytoskeletal components of neurons that are particularly abundant in axons. Their functions include provision of structural support and maintaining size, shape, and caliber of the axons (1). Neurofilaments belong to the intermediate filaments family, and the triplet comprises three subunits; neurofilament light chain (NF-L), neurofilament medium (NF-M) and neurofilament heavy (NF-H). The nomenclature-light (∼68 kDa), -medium (∼145 kDa),- and heavy (∼200 kDa) refers to the molecular weight of the filaments (2, 3).

Following axonal damage in the central nervous system (CNS), neurofilament proteins released into cerebrospinal fluid (CSF) provide an indication of axonal damage and neuronal death. The most extensively subtype studied in this context is NF-L.

Given that neurofilaments are found in the cytoplasm of neurons, all diseases that lead to neuronal and axonal damage can increase the CSF-levels of these proteins. In animal studies, NF-L levels have been used as a marker of axonal damage for decades (4). In humans, neurofilaments were first used as markers of neuronal damage in a study of 12 patients with amyotrophic lateral sclerosis (ALS) and 11 patients with Alzheimer's disease (5).

Subsequently, higher than control levels of neurofilaments were found in the CSF of 60 patients with relapsing-remitting multiple sclerosis (RRMS) (1), suggesting these proteins could also be used as a biomarker of MS disease activity. The field of neurofilament research is rapidly expanding and neurofilament levels are under investigation as markers of disease activity and progression in a number of different neurological conditions including stroke (6), ALS (7), frontotemporal

dementia (8), and MS (9). Recently, it has also been suggested that NF-L can be associated with paraneoplastic CNS disorders (10), and peripheral nervous system disorders (11).

Biomarkers of disease activity or disability progression are an unmet need in several neurological conditions. A good and reliable biomarker should say something about the stage of the disease, the prognosis and the response to treatment. A biomarker does not, however, need to be disease specific. It is now clear that elevated NF-L occurs in several neurological disorders and levels depend on age (9, 12). Nevertheless, increased NF-L level appears to reflect ongoing neuronal damage, irrespective of the underlying pathology, making it a potentially interesting biomarker (9).

This review will focus on strengths and weakness of NF-L as a biomarker in the clinical management of MS.

MULTIPLE SCLEROSIS—NEUROFILAMENTS IN CEREBROSPINAL FLUID AND SERUM

MS is an inflammatory demyelinating disease of the CNS, usually characterized by relapsing episodes of neurological dysfunction and gradual, progressive decline. Biomarkers reflecting ongoing neuronal damage are therefore of great value in order to characterize the stage of disease, the prognosis and treatment response.

The presence of neurofilament proteins in CSF has been the subject of intense study since the finding of elevated NF-L levels in patients with RRMS (1).

Neurofilament heavy chain has also been investigated as a potential biomarker in MS and levels appear to correlate with Expanded Disability Status Scale (EDSS) in patients with RRMS and clinically isolated syndrome (CIS) ($n = 102$), and also increased in patients with ongoing relapse ($n = 61$) (13). However, comparison of NF-H and NF-L, suggests that NF-L discriminates better between MS (and CIS) and controls (14).

Until recently, the greater majority of studies have focused on detecting these proteins in CSF. Since CSF sampling requires a semi-invasive lumbar puncture, a search for other ways of accessing this clinically useful biomarker has focused on other body fluids. Thus, while lumbar puncture provides a direct approach to the CNS, access to blood is less invasive and better tolerated by patients. Further, as neurofilaments are neuron specific, the finding of these proteins in serum reflects leakage or diffusion through the blood-brain barrier (BBB). NF-L can also reach the blood through CSF drainage into venous blood (15).

Following neuronal biomarkers in blood faces several problems, including patient specific differences in the degree of protein leakage through the BBB (12) and the need for sufficiently sensitive methods of detection. The single-molecule enzyme-linked immunosorbent assay (ELISA) called single molecule array (Simoa) (https://www.quanterix.com) can detect very low concentrations of single enzyme-labeled proteins (16). In fact, this powerful new technique is 126- and 25-fold more sensitive than regular ELISA or electrochemiluminescence, respectively (15).

A strong correlation was detected in paired serum and CSF samples from 373 participants, (286 with MS, 45 with other neurological conditions, and 42 healthy controls) ($r = 0.62$) (17). The important fact that serum NF-L levels correlate with CSF levels have been confirmed in several studies (9, 18, 19). Levels in serum and plasma have also been found to correlate (12). However, although strong positive association is found between CSF and serum NF-L levels, the levels in serum have been evaluated to be a 42-fold lower than in CSF (9).

NEUROFILAMENTS AS A PROGNOSTIC BIOMARKER IN ON, CIS, AND RIS

Patients with either optic neuritis (ON), CIS or radiological isolated syndrome (RIS) are all at risk of developing MS. Identifying those who will convert would be of great importance as one could more aggressively start early treatment. Studies looking at NF-L as a predictor of conversion have provided inconsistent results. In one study investigating multiple biomarkers in ON patients ($n = 56$), increased NF-L levels were found with increasing time from onset to CSF sampling; however, there was no correlation between NF-L and severity of symptoms or Gd-enhanced lesions on MRI (20). Increasing levels of NF-L is interesting in this context as this may reflect the presence of a "silent" axonal damage, and thus neurodegeneration. Subsequently, studies have shown that NF-L could potentially predict conversion to MS after ON ($n = 86$) (21). In addition, CSF NF-L predicted not only visual outcome after ON, but also seemed to have potential as a biomarker for incomplete remission ($n = 47$) (22).

In a retrospective cohort of 68 patients diagnosed with CIS, the levels of CSF-NF-L were significantly higher in patients who later developed MS. However, NF-L was found only to be a weak risk factor for converting to MS compared to oligoclonal bands, and T2 lesions on MRI (23). Another study with a small sample size (CIS patients = 38) sought to evaluate both NF-L and N-acetylaspartate as potential biomarkers. The investigators found higher CSF NF-L levels in both CIS-patients and those in the early stages of MS compared to healthy controls. NF-L levels were also related to conversion from CIS to RRMS (24). This was also the case in a cohort of 109 CIS-patients where converters had higher NF-L CSF levels (25), and in a study of patients with RIS where CSF-NF-L levels were an independent risk factor for later conversion to CIS ($n = 75$) (26). A smaller 2-year follow-up study of CIS patients ($n = 19$), showed higher CSF-NF-L in the converters than non-converters, and NF-L levels further classified 84% of the patients correctly in terms of conversion/non-conversion (27).

In contrast to the above studies, an investigation of 47 patients with CIS confirmed higher levels of CSF NF-L compared to controls, found no difference in NF-L levels between converters and non-converters (28). Likewise, equal CSF NF-L levels were detected in 39 CIS patients, independent of converters or non-converters (29). Yet another study found higher levels of serum-NF-L in CIS-patients compared to controls ($n = 92$), but no difference between what was defined as fast converters to clinical definite MS ($n = 100$) and non-converters ($n = 98$) (30).

Overall, NF-L appears useful as a biomarker and as a predictor of outcome in the early clinical stages prior to definite MS. Although the findings are not completely consistent across studies, this does not invalidate NF-L as a biomarker as the differences could be explained by alterations in diagnostic criteria for CIS, and study design of retrospective studies of relatively small study populations.

NEUROFILAMENTS AND CLINICAL, RADIOLOGICAL, AND PROGNOSTIC FEATURES IN MS

Studies comparing MS patients to healthy controls show that there is a general increase in NF-L levels in patients, and a positive correlation with relapses. Levels of CSF NF-L were almost 10 times higher in MS patients with exacerbations ($n = 66$) than healthy controls ($n = 50$) (31), underlining the correlation between axonal damage and relapses.

When looking at disease progression, correlation of NF-L with EDSS is not always found (31–33). This can, however, usually be explained by small changes in scores over time, small patient populations, limited sensitivity and intra- and inter-rater challenges of the scale. A small study indicated that baseline CSF NF-L levels were higher in those patients that experienced EDSS progression after 5 and 10 years, and was significantly associated with conversion to SPMS (34). The NEDA (no evidence of disease activity)-classification, which comprises EDSS, relapses and MRI changes has also been correlated in various degrees with NF-L (27, 35).

In RRMS and progressive MS patients, the levels of NF-L are higher in the presence of disease activity ($n = 82$) (17). Higher CSF NF-L levels compared to controls were found in primary progressive MS (PPMS), but there was no significant difference between PPMS ($n = 21$) and secondary progressive MS (SPMS) ($n = 10$) (36). In cohort of 99 patients with RRMS, high levels of CSF NF-L were associated with worse outcome and conversion to SPMS (37).

The most objective findings in the clinical follow-up of MS patients are changes in MRI, often evaluated on the basis of new or enlarged T2-lesions, or T1gadolinium-enhanced lesions (Gd-lesions). The studies of radiological changes have also shown more consistent correlations with NF-L levels, and several studies have reported a predictive value in NF-L in regards to one-going lesions, and prior to lesions (9, 17, 27, 32, 38, 39).

A study of two different cohorts, one cross-sectional ($n = 142$) and one longitudinal ($n = 246$) showed generally higher NF-L levels in patients vs. healthy controls, a correlation with presence of relapses, worsening EDSS and MRI lesion activity [both T2-lesions and Gd-lesions (9)]. In another follow-up study of 39 patients, CSF NF-L correctly predicted NEDA-status after 2 years in 85% of cases (27). Both serum and CSF NF-L levels have been found higher during relapses, and with Gd-lesions (17). Further confirmation of these findings came from studies showing positive correlation between serum NF-L and number of Gd-enhanced lesions ($n = 25$) (38), and correlation with new or enlarged T2-lesions and the ability to predict new Gd-lesions ($n = 85$) (32).

In a cohort of 25 natalizumab-treated patients followed for 3 years, CSF-NF-L at baseline correlated with percentage brain volume change (39), and in a retrospective CIS cohort ($n = 41$) NF-L correlated with changes in gray matter on MRI, and an inverse correlation was detected between degree of MRI normalization and NF-L (40).

The radiological correlations with NF-L indicates the usefulness of NF-L as a marker of ongoing brain damage in MS. NF-L is probably the most promising new biomarker to be used in clinical practice for evaluating disease activity.

NEUROFILAMENTS AND TREATMENT RESPONSE

Independent of the type of disease-modifying treatment, the majority of investigators have detected an inverse correlation between NF-L levels and treatment. Lower levels of NF-L were found in treated patients compared to treatment-naïve individuals ($n = 21$) (38), and it has also been shown that NF-L levels fall in follow-up studies of disease-modifying treatment vs. no treatment (9). CSF and serum-NF-L levels are stable in treatment-naïve patients ($n = 10$) or when shifted to similar efficacy ($n = 20$), but fall when patients are shifted to drugs with higher efficacy ($n = 68$) or when drugs are started in the treatment-naïve ($n = 50$) (17). Serum NF-L levels fall after initiation of interferon-beta ($n = 85$) (32), and plasma NF-L levels fell by 34% after 12 months of fingolimod treatment (shifted from interferon or glatiramer acetate), ($n = 243$) (12).

In a cohort of 92 MS patients started on natalizumab, CSF collected at baseline and after 6 or 12 months showed a significant fall in NF-L, to similar levels as healthy controls, independent of relapses in the months before treatment startup (41). These findings have been confirmed in other studies (33), and also in fingolimod-treated patients vs. placebo in CSF ($n = 36$) (42). In one cohort of 75 patients with clinical stable RRMS, patients were switched from first-line injectable treatment to rituximab and followed with CSF-NFL at baseline, at month 12 and month 24 ($n = 65$). NF-L levels decreased significantly 12 months after therapy shift, and as clinical and radiological signs from relapse approached at 24 months, NF-L also increased, but not significantly, indicating NF-L as a marker of treatment response (43).

In a study of 59 MS patients on either interferon-beta ($n = 33$) or natalizumab ($n = 19$), or without treatment ($n = 7$), CSF NF-L levels were lower in both treatment groups, but the natalizumab-group was not significantly different from healthy controls, and the interferon-beta NF-L levels were still significantly higher than in the natalizumab-group (44).

In 35 patients with progressive MS, CSF NF-L levels were evaluated after 12–24 months of mitoxantrone or rituximab treatment and showed a significant decrease. For the patients on disease modifying agents at baseline, the NF-L values were already lower than the untreated group (45).

There is now clear evidence that NF-L is a good biomarker for treatment response in MS, especially for high efficacy drugs. This is probably due to the better prevention of brain damage in these treatments, underlining the role of NF-L as a marker of neuronal and axonal damage.

NEUROFILAMENTS IN GENERAL—WHAT NEXT AND HOW TO USE NF-L IN CLINICAL PRACTICE?

The majority of studies that have specifically looked at NF-L have not been performed prospectively, but retrospectively on samples taken at varying time points in relation to clinical symptoms. Further, study populations have often been small. Recently, a meta-analysis of results from 15 studies verified a significant increase of NF-L in MS patients compared to controls (46). Still, prospective studies of much larger cohorts focusing on NF-L and collecting baseline and follow-up data are necessary.

Before taking the Simoa-assay in to clinical use it is necessary to compare the results of the assay between different centers (15). Determining age-dependent cut-off values are also necessary.

A recent study aimed to estimate percentile curves for healthy controls across different age group to be used as reference (9). In serum samples from 246 healthy controls (median age 44.3 years), the median serum NF-L was 22.9 pg/ml (9). Of these, 87 controls had 1-year follow-up serum sample, and the median NF-L level increased with 1.8%, fitting with the observed positive age-association seen in the whole cohort, where the increase was 2.2% for each additional year (9). The median NF-L value found in the healthy controls is in accordance with healthy controls in a heterogenous group of studies summarized in **Table 1**. Taken together, it seems that a serum NF-L value of 16–20 pg/ml is normal in healthy individuals, with age being an increasing factor.

Interestingly, MS is not the neurological disorder that gives the highest values of NF-L (**Table 1**). Both ALS (7, 47, 48) and Creutzfeldt-Jacobs disease (54) are examples of disorders that gives especially high NF-L levels. These disorders are characterized by neurodegeneration, and little inflammation, which suggests that increased NF-L levels in active MS patients, not only reflects inflammation, but also an ongoing neurodegeneration.

NF-L cannot be used as a specific diagnostic tool in MS but, combining NF-L with other biomarkers is of value. In one cross-sectional study of 271 patients with clinical features of suspected MS onset, the combination of CSF NF-L and intrathecal immunoglobin G production had a sensitivity of 97% for detecting RRMS patients (55).

Despite not being a MS-specific biomarker, NF-L does appear to be a useful marker for disease activity and treatment response. There is good evidence that NF-L has a role in everyday clinical follow-up of MS patients, particularly as a marker of subclinical activity in RRMS (17, 32).

The question now is how to best use NF-L in clinical practice. A recent study showed that the early serum NF-L levels in newly diagnosed MS can potentially predict lesion load and brain atrophy on MRI after 10 years (56). This leads us to speculate if one should be more aggressive in treating patients with high serum NF-L levels at time of diagnosis, which, brings us to the need of cut-off values to determine high, medium and low NF-L levels.

In the follow-up of MS patients, NF-L cut-off values may be important, i.e., does the level in healthy people have a significance, or should we solely use the intra-individual value? We would suggest the latter. MS patients should be monitored

TABLE 1 | Serum NF-L levels in a heterogenous group of healthy controls.

Examined disorder	HC (*n*)	HC age	HC serum NF-L (pg/ml)	Patient serum NF-L (pg/ml)	Assay	References
ALS	50	55	16.2	125	Simoa	(47)
ALS	12	47	17	255/196 (early/late symptomatic phase)	Simoa	(7)
ALS	19	33	7.5	>54.5	ECL	(48)
AD	12	86	29	42	Simoa	(49)
AD dementia	193	76	34.7	AD dementia 51.0 MCI 42.8	Simoa (plasma)	(18)
FTD	28	65	19.6	77.9	Simoa	(50)
FTD	73	NA	3.5	31.5	ECL	(8)
CIS	92	35	7.9	24.1/19.3(Converter/NC)	ECL	(30)
MS	22	32	11	17	Simoa	(35)
MS	42	28	10.5	16.9/23 (RRMS/PMS)	Simoa	(17)
PN	25	NA	6.91	31.5	Simoa	(11)
PSP	12	70	17.5	31	Simoa	(51)
Traumatic brain injury	35	31	13	> 90	Simoa	(52)
Concussion	142	NA	8.47	NA	Simoa	(53)

HC, healthy controls; ALS, amyotrophic lateral sclerosis; AD, Alzheimer's disease; MCI, mild cognitive impairment; FTD, frontotemporal dementia; ECL, electrochemiluminescence; CIS, clinically isolated syndrome; NC, non-converters; PN, peripheral neuropathies; PSP, progressive supranuclear palsy.

with serum levels of NF-L with a time-interval of perhaps 3–6 months depending of the course of the disease. If the serum level of NF-L increases, the clinical activity should also be evaluated by MRI. In this way, unnecessary routine MRI screening can be avoided and disease activity more reliably detected. In a 2-year follow-up study of RRMS patients, an individual increase of 10 pg/ml in serum NF-L levels gave increased risk of new T1 gadolinium-enhanced lesions, and new T2 lesions (32). This increase was higher than the expected age-related increase in yearly follow-up, and might therefore indicate a level to use as an individual cut-off. What is less clear however, is whether a similar decrease in NF-L levels of 10 pg/ml is of any clinical interest. Using NF-L rate of change as a marker for disease progression is in terms with a recent study in dominant inherited Alzheimer's disease, where the rate of change in NF-L levels was associated with degree of cortical thinning on MRI (19).

In conclusion it is our opinion that serum NF-L measurements should systematically be used as prognostic biomarker to monitor MS patients for progression, disease activity, and treatment efficacy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Psychiatry

CASE REPORT
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Systemic Lupus Erythematosus With Isolated Psychiatric Symptoms and Antinuclear Antibody Detection in the Cerebrospinal Fluid

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Background: Organic psychiatric disorders can be caused by immunological disorders, such as autoimmune encephalitis or systemic lupus erythematosus (SLE). SLE can affect most organs, as well as the central nervous system (CNS). In this paper, we describe a patient with an isolated psychiatric syndrome in the context of SLE and discuss the role of antibody detection in the cerebrospinal fluid (CSF).

Case presentation: The 22-year-old German male high school graduate presented with obsessive–compulsive and schizophreniform symptoms. He first experienced obsessive–compulsive symptoms at the age of 14. At the age of 19, his obsessive thoughts, hallucinations, diffuse anxiety, depressed mood, severe dizziness, and suicidal ideation became severe and did not respond to neuroleptic or antidepressant treatment. Due to increased antinuclear antibodies (ANAs) with anti-nucleosome specificity in serum and CSF, complement activation, multiple bilateral white matter lesions, and inflammatory CSF alterations, we classified the complex syndrome as an isolated psychiatric variant of SLE. Immunosuppressive treatment with two times high-dose steroids, methotrexate, and hydroxychloroquine led to a slow but convincing improvement.

Conclusion: Some patients with psychiatric syndromes and increased ANA titers may suffer from psychiatric variants of SLE, even if the American College of Rheumatology criteria for SLE are not met. Whether the psychiatric symptoms in our patient represent a prodromal stage with the later manifestation of full-blown SLE or a subtype of SLE with isolated CNS involvement remains unclear. Regardless, early diagnosis and initiation of immunosuppressive treatment are essential steps in preventing further disease progression and organ damage. Intrathecal ANAs with extractable nuclear antigen differentiation may be a more sensitive marker of CNS involvement compared with serum analyses alone.

Keywords: systemic lupus erythematosus, neuropsychiatric systemic lupus erythematosus, schizophrenia, obsessive-compulsive disorder (OCD), psychosis

BACKGROUND

Organic psychiatric disorders might be of immunological, infectious, epileptic, neurodegenerative, traumatic, metabolic, or vascular origins (1, 2). In recent years, limbic or nonlimbic autoimmune encephalitis received increased interest because each can mimic primary psychiatric and neuropsychiatric disorders (2–4). Most of these disorders are associated with autoantibodies (abs) directed against antigens on the cell surface or in the intracellular compartment (5). Hashimoto thyroiditis and rheumatic disorders, such as systemic lupus erythematosus (SLE), can also be associated with psychiatric involvement that allows for successful immunomodulatory treatment approaches (6–9).

SLE is a prototypic systemic autoimmune disease that can affect the central nervous system (CNS) as well as the rest of the body, including joints, skin, kidneys, heart, lungs, blood vessels, or the hematopoietic system. The typical age for mostly women to become ill is between 16 and 55 years (10); the peak age in females is between 45 and 69 years, while that in males is between 40 and 89 years (11). SLE is characterized by the presence of antinuclear abs (ANAs); these abs can affect different cell types, which explains the diversity of symptoms. The reference standard for the diagnosis of this extremely heterogeneous multisystemic disease is still clinical diagnosis by an SLE expert. The main reason for the use of SLE classification criteria is to ensure a consistent definition of SLE, especially for clinical trials and surveillance. The most commonly used criteria are those established by the American College of Rheumatology (ACR) in 1982 and revised in 1997 (12, 13; https://www.rheumatology.org/Practice-Quality/Clinical-Support/Criteria/ACR-Endorsed-Criteria; see **Box 1A**). If 4 or more of the 11 criteria, including at least 1 immunological and 1 clinical criterion, are present simultaneously or serially at any point in time, SLE should be highly suspected. Although the ACR's 1997 criteria have generally performed well, concerns have been raised regarding the limited sensitivity of these criteria. Neuropsychiatric conditions, for example, might be underrepresented in the criteria. Up to 75% develop neuropsychiatric symptoms, such as cognitive dysfunction, seizures, and other psychiatric syndromes, such as mood disorders, anxiety, or psychosis (14). A subgroup of SLE patients mainly presents with manifestations involving the central, peripheral, or autonomic nervous system. These are referred to as neuropsychiatric SLE (NPSLE) (15). In 1999, the ACR published 19 case definitions of NPSLE to facilitate diagnosis and ensure comparability across clinical studies. Twelve definitions described syndromes associated with CNS involvement (15) (**Box 1B**).

Two distinct pathogenic mechanisms, tissue inflammation and thrombotic–ischemic events, have been recognized thus far. Increased ANA serum titers were detected in >95% of patients (16); however, older studies showed even lower ANA positivity (17). Positive dsDNA abs were found at a rate of 37–80% (16), and they were interpreted as a predictor of disease exacerbation (18). The metanalytic data showed that anti-nucleosome abs have comparable specificity but higher sensitivity than anti-dsDNA abs do for the diagnosis of SLE (19). Cerebrospinal fluid (CSF) pleocytosis was found in 30%, and oligoclonal bands (OCBs) were detected in 25–42% of cases (20). In the cerebral magnetic resonance imaging (cMRI), small focal hyperintensities, mainly subcortical frontoparietal or periventricular, can be detected in 15–60% of the patients. Electroencephalography (EEG) generally shows unspecific slowing (21). NPSLE treatment can be implemented with corticosteroids alone or in combination with other immunosuppressive drugs, including cyclophosphamide for remission induction or azathioprine for maintenance therapy (22). Antimalarial drugs (e.g., hydroxychloroquine) have been suggested for the prevention of NPSLE in SLE patients (23).

BOX 1 | Criteria for (neuropsychiatric) systemic lupus erythematosus.

A: Criteria for systemic lupus erythematosus of the American College of Rheumatology (12,13; https://www.rheumatology.org/Practice-Quality/Clinical-Support/Criteria/ACR-Endorsed-Criteria)

1. Malar rash; 2. discoid rash; 3. photosensitivity; 4. oral ulcers; 5. arthritis; 6. serositis; 7. renal involvement; 8. neurological involvement (seizures or psychosis); 9. hematological involvement (hemolytic anemia, leucopenia, lymphopenia, thrombocytopenia); 10. immunological changes (anti-dsDNA antibodies, anti-Sm antibodies, antiphospholipid antibodies, and 11. antinuclear antibodies.

B: Case definitions of neuropsychiatric systemic lupus erythematosus (15):

1. Aseptic meningitis; 2. cerebrovascular disease; 3. demyelinating syndrome; 4. headaches (migraines and benign intracranial hypertension); 5. movement disorders (chorea); 6. myelopathy; 7. seizure disorders; 8. acute confusional states; 9. anxiety disorders; 10. cognitive dysfunction; 11. mood disorders; and 12. psychosis.

CASE PRESENTATION

We present the case of a 22-year-old German male high school graduate with a complex psychiatric syndrome including obsessive–compulsive, schizophreniform, and derealization phenotypes. In May 2016, at age 19, there was a sudden exacerbation of these syndromes. At age 14, he first experienced obsessive–compulsive symptoms (i.e., obsessive aggressive thoughts and compulsive avoidance acts). However, he recognized that the obsessional thoughts were a product of his own mind, and these symptoms were well compensated for at the time, enabling him to live a mostly normal life. After his final examinations at school, he consumed cannabis five times. He then experienced an exacerbation of his obsessive–compulsive symptoms and suffered from more severe obsessional thoughts, including the idea that he could injure other people and himself. Furthermore, he experienced involuntary obscene thoughts. At the time, he fought such thoughts and continued to recognize that the obsessional thoughts and impulses were a product of his own mind. He also suffered from hallucinatory symptoms, such as auditory hallucinations (i.e., hearing voices) and optical distortions (i.e., the shape of leaves on the ground appearing distorted). He developed diffuse anxiety and agitation and described extreme dizziness,

as if he had drunk "five beers." Because of his depressed mood and obsessive–compulsive symptoms, he experienced suicidal ideation and complained of difficulty falling asleep and reduced energy levels, especially in the morning.

Due to the severity of these symptoms, he was first hospitalized at age 19. He received pharmacological treatment with selective serotonin reuptake inhibitors for the obsessive–compulsive symptoms (citalopram up to 40 mg/day), neuroleptics for the schizophreniform symptoms (olanzapine up to 20 mg/day, risperidone up to 5 mg/day, and aripiprazole up to 7.5 mg/day; higher doses led to an increase in inner restlessness), carbamazepine for neuronal network stabilization up to 500 mg/day, and an anticholinergic agent because of extrapyramidal side effects (biperiden up to 4 mg/day) over a period of 7 months without significant improvement. Lorazepam (up to 2 mg/day) led to a transient reduction in anxiety. Seven months after symptom onset (December 2016, under treatment with aripiprazole 7.5 mg/day and carbamazepine 500 mg/day), his mental state still revealed obsessive thoughts, depressed mood, and diffuse anxiety. Moreover, he suffered from attention and concentration deficits (**Figure 1**, t0), inner restlessness, signs of derealization (with altered, slowed, and delayed perception of his environment), and severe dizziness (still comparable with having drunk five beers). The obsessive–compulsive thoughts were still present and very difficult to manage.

Developmental, Somatic, and Family History

There were no *in utero* or birth complications, febrile convulsions, seizures, inflammatory brain diseases, or cerebral contusions in the patient's history. When entering primary school, he showed subsyndromal symptoms of inattention and motor hyperactivity. Nevertheless, he finished high school successfully and his further somatic history was unremarkable. He occasionally consumed alcohol and illegal drugs (nitrous oxide three times and cannabis five times), but there was no history of severe substance abuse. The family history showed that his grandmother suffered from depression, and his mother was diagnosed with insulin-dependent diabetes mellitus. There were no known rheumatic diseases in the family history.

Investigations

The neurological examination was normal throughout the course of the disease. Initially, the CSF analyses (3 months after exacerbation, August 2016) showed positive CSF-specific OCBs. Five months after the first steroid pulse treatment (December 2016), the patient's state deteriorated (May 2017). At that time, CSF analysis showed a mild pleocytosis (white blood cell count = 14/µl; reference <5/µl). The initial immunological screening 6 months after exacerbation in November 2016 revealed only a weak positive ANA in the indirect immunofluorescence assay. Another 6 months later (1 year after exacerbation, May 2017), we found clearly increased ANA titers in both serum and CSF (serum: titer = 800 IU; CSF: titer <100 IU) with anti-nucleosome specificity, which was also detectable in serum and CSF. At that time, we also detected decreased levels of complement component C4 and slightly increased C3d serum concentrations as indicators for increased complement activation.

Testing for rheumatoid factors, antiphospholipid abs, lupus anticoagulant, antineutrophil cytoplasmic abs, and a broad set of antineuronal and anti-thyroid abs was negative. In the

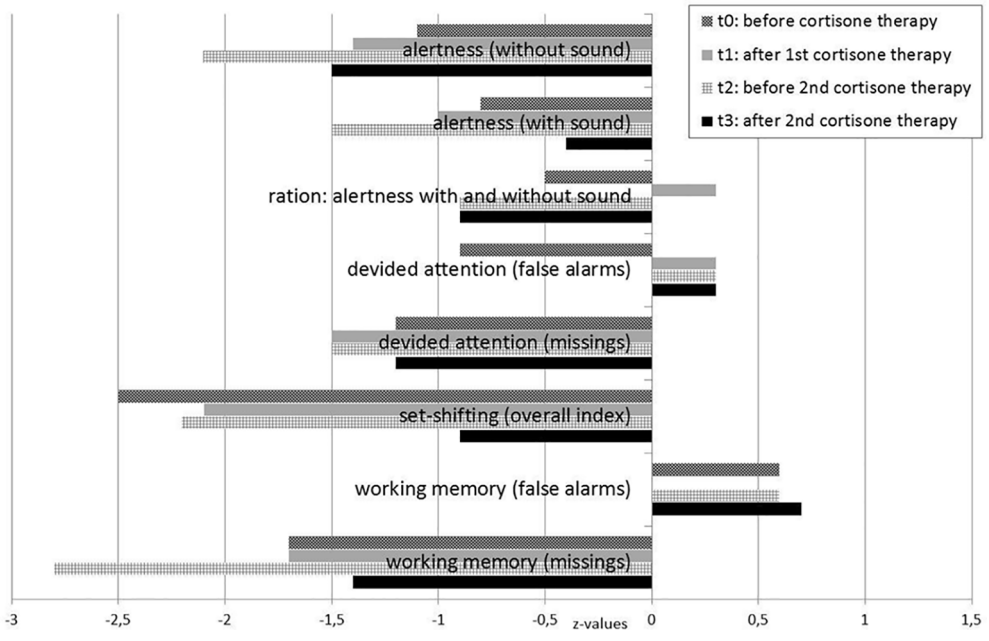

FIGURE 1 | Test for attentional performance during the course of the disease.

cMRI, multiple diffuse periventricular white matter lesions were apparent in repeated examinations throughout the course (**Figure 2**). The lesions were stable. Furthermore, there was a slightly enlarged adenohypophysis not yet affecting the chiasma opticum. The hormone screening did not detect any pathological hormone activity. The fluorodeoxyglucose positron emission tomography was normal. Repeated EEGs exhibited intermittent slowing (**Table 1**). The neuropsychological test of attentional performances showed severe deficits in alertness, divided attention, set shifting, and working memory (**Figure 1**, t0). There were no further clinical, systemic SLE signs such as skin or inner organ involvement.

Differential Diagnostic Considerations

Focusing on the symptoms our patient developed during his youth, a diagnosis of obsessive–compulsive disorder with obsessive thoughts could be considered. The fact that disease symptoms exacerbated shortly after the consumption of cannabis could point to an acute episode of drug-induced psychosis. If the auditory and optical hallucinations together with signs of derealization persisted over time, paranoid hallucinatory schizophrenia could be a plausible classification, though only if all organic signs were considered pathogenetically irrelevant. The initial organic findings (positive CSF-specific OCBs, disseminated white matter lesions, no antineuronal abs, unspecific rheumatological findings) were interpreted as post-inflammatory changes. Finally, the increased ANAs with anti-nucleosome specificity in CSF and serum, serum complement activation, CSF pleocytosis, OCBs, and cognitive and psychiatric disturbances cumulated to the final diagnosis of NPSLE. Although the patient did not completely fulfill the ACR criteria for SLE, the diagnosis was established. Other supporting biomarkers, such as anti-ribosomal P protein and anti-C1q abs, as well as the direct Coombs test, were unremarkable. Autoimmune encephalitis would also be conceivable for differential diagnosis (4). However, the established antineuronal abs were negative in serum and CSF.

Immunosuppressive Treatment and Course of the Disease

Following initial assessment and based on the therapy resistance and inflammatory CSF changes with CSF-specific OCBs, we performed a high-dose corticosteroid pulse therapy with a daily dose of 1,000 mg methylprednisolone administered intravenously for five consecutive days in the context of continued treatment with aripiprazole and carbamazepine in December 2016, which was 7 months after symptom exacerbation. Following the steroid treatment, the patient's mood, cognitive deficits, motivation, and dizziness improved. Moreover, his obsessive–compulsive symptoms were reduced. Methylprednisolone was subsequently continued orally and tapered over approximately 5 weeks (37 days). Except for steroid acne, no relevant adverse events became evident. After this immunosuppressive treatment, the patient was able to begin a volunteer social year, and treatment with aripiprazole and carbamazepine was continued. However, he still had obsessive–compulsive symptoms and cognitive deficits and felt dizzy (comparable with having drunk two or three beers). Clinically, we did not believe that these symptoms were side effects of carbamazepine because the symptoms were already reported prior to the treatment with carbamazepine, and blood concentrations were within the reference level. Neuropsychological testing showed no relevant improvement (**Figure 1**, t1). Over the next 4 months, his mental condition worsened again.

In May 2017, 1 year after his initial symptoms exacerbated, we repeated the diagnostic workup. The patient showed mild pleocytosis in the CSF and laboratory findings compatible with SLE (**Table 1**). We performed a second steroid pulse therapy with 500 mg/day methylprednisolone for five consecutive days with oral tapering over 8 weeks. At the same time, carbamazepine was stopped, and aripiprazole was reduced to 5 mg/day. As maintenance treatment, the patient received methotrexate (up to 17.5 mg/week) orally in combination with folic acid. Due to the threat of azoospermia, cyclophosphamide, which normally shows good results for NPSLE, was not initially used. The CSF white blood cell count normalized, and the cMRI showed that

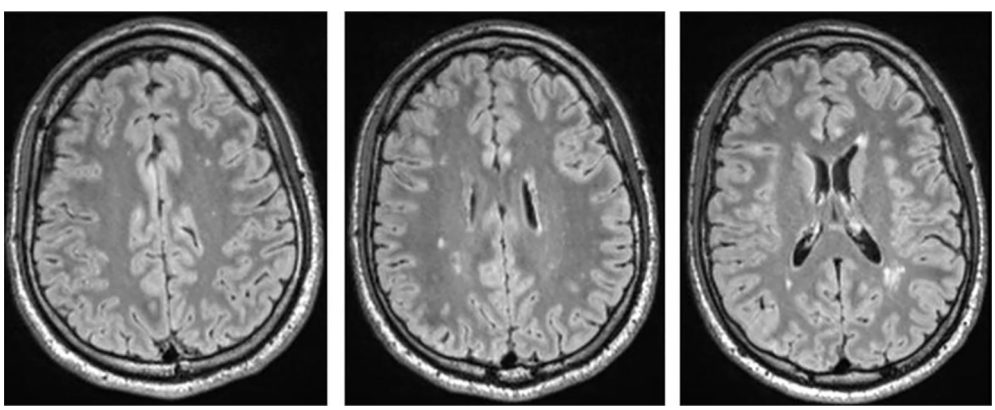

FIGURE 2 | T2w fluid-attenuated inversion recovery (FLAIR) cerebral magnetic resonance imaging (cMRI) shows multiple disseminated dotted bilateral periventricular to subcortical white matter lesions. Shown are images of the first cMRI performed 6 months after symptom exacerbation in November 2016.

TABLE 1 | Overview of diagnostic findings.

Serum basic diagnostics and blood count	• **Repeatedly normal renal, and thyroid values.** • Repeatedly no relevant alterations in peripheral differential blood cell count were observed (except twice increased leucocyte count during steroid treatment). No lymphocytopenia was found.
Rheumatological testing	• 11/2016 (6 months after symptom exacerbation): **Immunofluorescence test for antinuclear abs (ANAs) was weakly positive.** Normal values for CH50 (*89%, reference area 65–115%*), C3 (*1.24 g/L; reference area 0.90–1.80 g/L*), C4 (*0.23 g/L; reference area 0.10–0.40 g/L*), C3d (*8.40 mg/L; reference value <9 mg/L*). Antineutrophil cytoplasmic (ANCA) and anti-β2 glycoprotein I IgM and IgG antibodies were not increased (APAs); anti-dsDNA and anti-SM abs were negative. • 05/2017 (12 months after first exacerbation): **Increased ANAs with anti-nucleosome specificity** (*serum: titer of 1:800; CSF: titer <1:100; reference value <1:50*). **Slightly increased C3d concentration** (*12.90 mg/L; reference value <9 mg/L*). **Slightly decreased C4** (*0.09 g/L; reference area 0.10–0.40 g/L*). ANCA, APAs, anti-dsDNA, and anti-SM abs were still negative. The lupus anticoagulant test was negative. • 01/2019 (32 months after first exacerbation): Anti-ribosomal P protein and anti-C1q antibodies in the serum were negative. Direct Coombs test results were unremarkable.
Serum autoantibody analyses	• Repeatedly no increased thyroid abs (against thyroglobulin, thyroid peroxidase, and thyroid-stimulating hormone receptor). • Repeatedly (08/16; 05/17) no abs against intracellular onconeural or intracellular synaptic antigens (Yo, Ri, Hu, CV2/CRMP5, Ma1/2, SOX1, GAD65, amphiphysin). • Aquaporin abs negative (5/2017)
Urine	• Repeatedly no signs of proteinuria (except slightly increased protein concentration 06/2017 (*17 mg/dL, reference <15 mg/dL*), no detection of red blood cells • Normal protein/creatinine ratio (06/2017).
Cerebrospinal fluid analyses	• 8/2016 (3 months after symptom exacerbation): Normal WBC count (2/µl; reference < 5/µl); normal protein concentration: 426 mg/L*; reference <450 mg/L;* normal albumin quotient: 4.7; *age-dependent reference* $<6.5\times10^{-3}$; **detection of two CSF specific OCBs**, IgG index not increased (*0.57; reference ≤0.7*). Abs against neuronal cell surface antigens [NMDA-R, AMPA-R, GABA-B-R, VGKC-complex (LGI1, Caspr2)] were negative. • 05/2017 (12 months after first exacerbation): **increased WBC count** (14/µl; reference <5/µl); **increased protein concentration: 460 mg/L**; *reference <450 mg/L*; normal albumin quotient: 4.5; *age-dependent reference* $< 6.5\times10^{-3}$. **Only one CSF specific OCB**, IgG index not increased (0.54; reference ≦0.7). Antibodies against neuronal cell surface antigens (NMDA-R, AMPA-R, GABA-B-R, VGKC-complex [LGI1, Caspr2]) were negative. • 7/2017 (14 months after first exacerbation): Normal WBC count (2/µl; reference <5/µl); normal protein concentration: 393 mg/L; *reference <450 mg/L;* normal albumin quotient: 4.3; *age-dependent reference* $<6.5\times10^{-3}$). **Two CSF specific OCBs**, IgG index not increased (0.59; reference ≦0.7). • Negative MRZ reaction.
Cerebral magnetic resonance imaging (7/2016, 11/2016, 5/2017, 7/2017)	• 2, 6, 12, and 14 months after first exacerbation: Repeatedly multiple bilateral diffuse periventricular white matter lesions without contrast enhancement and with stable lesions; no indication for acute ischemia or intracranial hemorrhage; deformed and steadily increasing adenohypophysis not yet affecting the optic chiasm.
Electroencephalography (8/2016, 11/2016, 5/2017)	• Initially normal alpha EEG (3 months after first exacerbation), in the course of the disease (occipital accentuated), sporadic intermittent rhythmic theta activity (6 months after first exacerbation) and repeated slow delta activity, partly with generalization (12 months after first exacerbation). No epileptic discharges.
Electrophysiological testing (8/2016)	• 3 months after first exacerbation: Somatosensory evoked potentials of the median nerve, the ulnar nerve, and the tibial nerve (in each case on both sides) were normal, as well as visual evoked potential responses.
FDG-PET (12/2016)	• 7 months after first exacerbation (WBC count was normal during that time): Age-appropriate utilization of glucose; no characteristic pattern of a neurodegenerative or inflammatory central nervous system disease.
Hormone screening (7/2017, 14 months after first exacerbation, performed because of enlarged adenohypophysis)	In the glucose suppression test, a suppression of GH to values • <1 ng/ml was observed over time (suppression works adequately, which speaks against acromegaly). • ACTH test was unremarkable (which speaks against adrenocortical insufficiency). • No signs of hypopituitarism. • No other hormone overproduction (normal prolactin, etc).

Abs, antibodies; ACR, American College of Rheumatology; CSF, cerebrospinal fluid; IU, international unit; Yo, initials of the first patient; Ri, initials of the first patient; Hu, initials of the first patient; CRMP, anti-collapsin response-mediator protein; SOX1, sex-determining region Y (SRY)-box protein 1; GAD, glutamic acid decarboxylase; WBC, white blood cells; OCBs, oligoclonal bands; NMDA-R, N-methyl-D-aspartate receptor; AMPA-R, amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid-receptor; GABA-B-R, gamma-aminobutyric-acid-B-receptor; VGKC voltage-gated potassium channel; LGI1, leucine-rich glioma inactivated-1 protein; Caspr2, contactin-associated protein 2; EEG, electroencephalography; FDG-PET, fluorodeoxyglucose positron emission tomography; GH, growth hormone; ACTH, adrenocorticotropic hormone.

the multiple diffuse periventricular to subcortical white matter lesions remained stable. Neuropsychological testing revealed an improvement in alertness, mental flexibility, and working memory [**Figure 1**, t2 (before) versus t3 (after) the second steroid pulse treatment]. Two months later (August 2017, 15 months after his symptoms first exacerbated), we added the antimalarial drug hydroxychloroquine (200 mg/day). The maintenance therapy consisting of methotrexate (17.5 mg/week), hydroxychloroquine (200 mg/day), and aripiprazole (5 mg/day) resulted in a slow but substantial improvement of inner restlessness, cognitive deficits, derealization symptoms, and dizziness. Obsessive–compulsive symptoms disappeared completely. Over several months, he still felt dizzy, like "having drunk one beer." Sixteen months later (December 2018), the patient was with without dizziness ("zero beers"), aripiprazole treatment was stopped in the meantime, and he enrolled in a vocational training program. Here, he was

able to attend vocational school, but on long working days, he cognitively reached his limits.

DISCUSSION

In this paper, we present the case of a male patient with a severe psychiatric variant of NPSLE suffering from obsessive–compulsive and schizophreniform symptoms who responded slowly but very well to immunological treatment.

Diagnostic Considerations

Initially, the cMRI and CSF showed signs of inflammatory CNS involvement (i.e., disseminated periventricular white matter lesions and isolated OCBs in the CSF). At that time, the ANA immunofluorescence test was only weakly positive. In view of the organic findings, the other differential diagnostic considerations mentioned above (e.g., drug-induced disorder, obsessive–compulsive disorder, or schizophreniform syndrome) were rejected. Six months after the first assessment, the ANA titers had clearly increased, with anti-nucleosome specificity found in the CSF and serum. Along with the complement alterations, the inflammatory changes in the CSF, the cMRI and EEG alterations, as well as the clinical manifestation with psychosis, we diagnosed NPSLE. Although the ACR 1997 classification criteria for SLE were not completely fulfilled, we deemed it justified to diagnose NPSLE in this patient. The strongest indicators were as follows: A) the presence of ANAs in a young male patient, which is unusual and raises suspicion for SLE; B) the detection of anti-nucleosome abs, which have the same specificity and even higher sensitivity than anti-dsDNA abs for SLE (19); C) complement activation as an indicator for disease activity; and D) the clinical presentation with psychiatric symptoms and corresponding alterations in CSF diagnostics, cMRI, and EEG.

Neuropsychiatric manifestations usually occur early in the course of SLE, and in some patients, neuropsychiatric symptoms remain the only clinical manifestations of SLE. This case demonstrates the difference between classification and diagnosis. Classification defines a homogenous group of patients for a research purpose with suboptimal sensitivity, whereas diagnosis focuses on the individual patient's therapy and prognosis and diagnosis should also lead to the treatment of patients who do not meet the classification due to non-100% sensitivity but who should still receive treatment (24). There are limitations of the most widely used ACR 1997 classification (but not diagnostic) criteria for SLE. While photosensitivity and skin and mucous membrane involvement seems to be overrepresented, other conditions, such as neuropsychiatric involvement, might be underrepresented. Furthermore, important immunological tests, such as complement fractions, anti-b2-glycoprotein, or anti-nucleosome abs, have not been considered thus far. While our patient fulfilled only 2 (i.e., psychosis and the presence of ANA) of the possible 11 ACR criteria, he would have fulfilled 3 (i.e., psychosis, the presence of ANA, and lowered C4 levels) of the recently proposed Systemic Lupus International Collaborating Clinics (SLICC) 2012 SLE classification criteria set (25). According to the SLICC 2012 criteria, the classification of SLE also requires at least four criteria with at least one clinical and one laboratory criteria, but this case is a good example of the relevance of the composition of the applied classification criteria set.

The European League Against Rheumatism and ACR are aware of this situation, and therefore, new SLE classification standards based on weighted criteria and a continuous probability scale are currently being developed. Recent studies investigated whether the detection of certain abs (e.g., increased ANA titers) in combination with symptoms manifested in one organ system (i.e., incomplete clinical pictures of SLE) may point to a prodromal stage of SLE (26, 27). Several papers described cases with initially insufficient numbers of diagnostic findings to fulfill the ACR criteria for SLE, for example, cases with initial manifestations of the gastrointestinal tract that later developed into full-blown SLE (28, 29). There are also many cases of SLE with the presence of ANA and isolated kidney involvement with glomerulonephritis. Despite not fulfilling the ACR classification criteria, the diagnosis can be made in cases of biopsy-proven lupus nephritis. Mack et al. published a case report of a woman who suffered from a wide spectrum of psychiatric symptoms that had relapsed several times over the 25-year disease duration. Throughout the entire period, several findings indicated an immunological process as a cause of her illness, but it took approximately 23 years after first suspecting an autoimmune disorder before the diagnosis of (NP)SLE was made (9). Given this background, we conclude that an isolated psychiatric variant of SLE might well be plausible in our patient. Whether the psychiatric symptoms in our patient represent a prodromal stage with the later manifestation of full-blown SLE or a subtype of SLE with isolated CNS involvement remains unclear. Because of the autoimmune pathogenesis of this systemic disease, the continuation of the immunosuppressive maintenance therapy should prevent further disease progression and organ damage. Nevertheless, even if the patient does not currently fulfill the criteria for SLE, early diagnosis is possible, and initiating immunosuppressive treatment is essential. Therefore, it is important to consider that isolated psychiatric variants of SLE may occur without other organ manifestations.

Role of Cerebrospinal Fluid Antibody Detection

In our case, we detected ANAs with anti-nucleosome specificity not only in the serum but also in the CSF, which supported the hypothesis that the CNS is affected. Even if the CSF examinations are not established, one could hypothesize that the short-term cannabis use may have led to a temporary blood–brain barrier dysfunction and allowed the ANAs' passage to the CNS. This could explain the rapid deterioration after cannabis consumption. Besides direct ab effects, increased interferon signaling might lead to reactive microglia and therefore could lead to neuronal damage and loss of synapses (30). Nevertheless, the precise pathophysiology of immunological mechanisms in affected brain tissue and thereby the resulting neuropsychiatric symptoms have not yet been fully clarified (31).

However, if the detection of ANAs in the CSF is a marker of CNS involvement, this would be of immense help in clinical

practice. ANAs detected in serum alone are too unspecific to function as a marker for CNS involvement in clinical practice. They were found with similar prevalence rates in patients with schizophrenia and in controls in earlier studies (32). Higher rates of serum ANAs might also be due to drug-induced ANA titers, which are often observed in psychiatric patients (33). Therefore, the serum findings' significance remains unclear in individual patients with psychiatric disorders. CSF analyses could help diagnose an autoimmune connective tissue disease with CNS involvement when detecting increased intrathecal ANA titers and other inflammatory alterations, such as increased white blood cell counts and CSF-specific OCBs. A metanalysis showed a higher rate of positive titers for antineuronal abs in the CSF of patients with NPSLE compared with SLE patients (34). In classical patients with NPSLE, an intrathecal ab synthesis was previously shown (35). Anti-SSA abs were found in CSF of patients with SLE (36) and Sjögren's syndrome earlier (37). Future studies should investigate the sensitivity and specificity of intrathecal ANA synthesis with ENA specification in psychiatric variants of SLE.

CONCLUSION

Our case clearly illustrates the relevance of this issue for clinical psychiatry. Had we rejected the SLE diagnosis based on the existing classification criteria, we would not have chosen the immune-modulatory treatment approach, which proved to be extremely successful in the long run in our patient. Intrathecal ANAs with extractable nuclear antigen differentiation may be a more sensitive marker of CNS involvement than serological testing alone is.

ETHICS STATEMENT

The patient has given his signed written informed consent for this case report, including the presented images, to be published.

AUTHOR CONTRIBUTIONS

DE, LT, NV, and RD treated the patient. EL, VM, and DE performed the data research: EL and DE summarized the case report, and VM performed a literature search. EL, VM, and DE wrote the paper. KE performed the cMRI analyses. RD and BB performed the EEG and CSF analyses and neurological interpretation. NV and US performed the rheumatological analyses, clinical interpretation and therapy suggestion. AR performed the neuropsychological testing. BF, SM, PS, and KN supported the interpretation of diagnostic findings. All authors were critically involved in the theoretical discussion and composition of the manuscript. All authors read and approved the final version of the manuscript.

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Investigation of Oligoclonal IgG Bands in Tear Fluid of Multiple Sclerosis Patients

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Background: Oligoclonal IgG bands (OCB) in the cerebrospinal fluid (CSF) represent a typical marker for inflammation in multiple sclerosis (MS) patients and have a predictive and diagnostic value in patients with a first suspected demyelinating event. The detection in tears remains controversial but some reports suggested a replacement of CSF analysis by OCB detection in tears. We aimed to investigate the value of OCB detection in tears systematically in patients with MS.

Methods: Tears of 59 patients with suspected or diagnosed MS were collected with Schirmer filter paper strips. Tear IgG was purified by affinity chromatography with protein G. After isoelectric focusing in polyacrylamide gels OCB detection was performed with direct silver staining. Paired triplets of CSF, serum, and tears were analyzed. For comparison purposes we additionally used other tear collection methods (flush procedure and plastic capillary tubes) or detection techniques (Immunoblotting). Clinical and paraclinical parameters are provided.

Results: IgG collection in tears was most reliable by using Schirmer strips. Thirteen patients had to be excluded due to insufficient sample material. Tear specific proteins that interfered with OCB detection were successfully eliminated by IgG purification. The concordance of OCB in tears and CSF of all investigated MS patients was 39% with a high rate of only marginal pattern in tears. Five patients demonstrated restricted bands in tears, neither detectable in CSF nor serum. Occurrence of OCB in tears was significantly associated with pathological visual evoked potentials ($P = 0.0094$) and a history of optic neuritis ($P = 0.0258$).

Conclusion: Due to the limited concordance, high rate of samples with insufficient material, and the unknown origin of tear IgG we cannot recommend that tear OCB detection may replace CSF OCB detection in MS patients. The detection of unique OCB in tears might offer new insights in ophthalmological diseases.

Keywords: multiple sclerois, oligoclonal band (OCB), tears, tear fluid, cerebrospinal fluid, CSF, MS

INTRODUCTION

Oligoclonal IgG bands (OCB) detected in and restricted to the cerebrospinal fluid (CSF) are an immunological hallmark found in almost all patients diagnosed with multiple sclerosis (MS) (1, 2). The discovery of OCB dates back to the year 1959/1960, in which Lowenthal and his colleagues were able to detect a subdivided gamma globulin fraction in the CSF of various neuroinflammatory diseases for the first time (3, 4). The presence of OCB indicates a local B-cell response in the context of an inflammatory disease of the central nervous system (CNS). In MS patients, the detection of OCB is often the only way to identify intrathecal IgG synthesis. Its determination in patients with a first episode of neurological symptoms suggestive for a demyelinating disease gained in importance as a result of the 2017 revisions of the McDonald criteria (5). In addition, OCB in CSF are an important parameter for the risk assessment of the development of clinically definite multiple sclerosis when MS-typical symptoms occurred for the first time without fulfilling all diagnostic criteria for MS (6–9).

OCB detection in tears of MS patients was first demonstrated by Coyle and Sibony (10, 11). In their first study they found OCB in tears in 11 of 12 MS patients mostly with optic neuritis which were absent in normal controls (10). Possibly due to the poor resolution achieved by electrophoretic separation of proteins they were not able to distinguish the bands between tears and serum. Moreover, this study lacked a comparison with CSF. In their second study performed with isoelectric focusing and silver staining, 14 of 21 MS patients showed OCB in tear fluid (11). Clear distinction to serum was made in 18 MS patients, with two-thirds showing isolated OCB in tears. Only six tear samples were compared with CSF demonstrating dissimilar band patterns.

Three independent following studies with a total of 187 patients (84 MS patients, 65 other diseases, 23 healthy controls) using immunoperoxidase staining were not able to reproduce these results (12–14). First, Mavra et al. demonstrated one patient with neurosarcoidosis with OCB restricted to tears and CSF. All others, especially 28 patients with MS and 4 with optic neuritis, showed no OCB in tears (12). Second, Liedtke et al. were able to detect OCB in tears in 3 of 38 MS patients (13). Precise data if these samples had matched bands in CSF or serum are missing. Third, Martino et al. revealed one MS patient with unique OCB in tears with no correspondence in the paired CSF and serum (14). All other 17 patients with MS and 17 other neurological patients displayed no OCB in tears.

The most recent studies, all published by the same group, revealed a concordance rate of 73% (27 of 37 patients) and 80% (48 of 60 patients), respectively, for the presence or absence of OCB in tears and CSF in MS patients (15, 16). Interestingly, surprisingly low 81% (30 patients) or 75% (45 patients), respectively, of all MS patients had positive OCB in CSF. The concordance rate between CSF and tears in patients with positive OCB in CSF (CSF^{OCB+}) was 22 of 30 patients (73%) and 43 of 45 patients (96%), respectively. The same group analyzed tears of 69 patients with clinically isolated syndrome (CIS) with a concordance of 78% (54 of 69 patients) for OCB status in tears and CSF OCB. The concordance rate between CSF and tears in CSF^{OCB+} patients was 29 of 44 patients (66%) (17). This study was criticized because of methodological shortcomings (18). Another investigation from this group in 42 patients with radiologically isolated syndrome (RIS) showed—similar to their second MS study—in all CSF^{OCB+} patients a concordance rate of OCB between tears and CSF of 96% (21 of 22 patients) (19). All studies investigating OCB in tears, their methodology and results are summarized in **Table 1**.

In summary, two independent research groups showed a relevant proportion of isolated bands in paired tear/CSF samples, whereas three independent research groups were unable to confirm these results. The authors of most recent positive studies suggested to partially replace CSF OCB detection by tear OCB detection in MS, CIS, and RIS patients (16, 17, 19). Because of the invasive character of a lumbar puncture tear collection might be a promising non-invasive tool to detect OCB in patients who decline a lumbar puncture, undergo follow-up analysis, or have anatomical or medical reasons why lumbar puncture is not possible. The aim of this study was to prove the reliable detectability of OCB in tears of MS patients by isoelectric focusing, silver staining and tear IgG purification.

PATIENTS AND METHODS

Patient Characteristics

Patients with suspected or diagnosed multiple sclerosis were recruited at the Department of Neurology at Hannover Medical School, Germany. The study was approved by the local ethics committee (No. 7218) and all patients gave written consent before enrollment. To minimize the risk of artificial results patients with infectious eye disease or treatment with tear-reducing drugs were excluded from the study. In total, 119 tear samples were collected from 59 different patients with suspected or diagnosed MS. Final diagnosis of MS/CIS was made in 28 patients (**Table 2**) according to the McDonald criteria (2017) (5).

Sample Collection and Preanalytical Preparation

CSF was obtained by lumbar puncture and immediately analyzed in the neurochemistry laboratory of the Department of Neurology as reported previously (22). Successful demonstration of OCB in tears from MS patients in the most recent reports relied on collection of tears with Schirmer filter paper strips (15–17, 19). This method was mainly used in our study. Tears were collected from the conjunctival sac of the lateral inferior eyelid until the Schirmer strip was completely wetted or for a maximum of 5 min (23). Time and wetting length were recorded. The unwet parts were cut off and the wet parts were instantly placed separately in small plastic vials on ice. Tears were separated from the paper strips by centrifugation (60 s, 12 100 g, temperature: 20°C). In order to increase tear extraction from the Schirmer strip, it was moistened with 50 µL Ringer's solution and centrifuged again under the same conditions after 1 min. The samples were stored at −80°C until further analysis. In addition to the sampling with Schirmer test we also used a "flush" procedure

TABLE 1 | Studies investigating OCB in tears.

Study	Country	Study population	Methodology (collection technique, gel type, separation technique, IgG visualization, stimulation of tearing)	Results
Coyle and Sibony (10)	USA	12 MS 20 controls	Glass capillary tubes, SDS-polyacrylamide gel, electrophoresis, silver staining, stimulation by onions/aromatic ammonia	OCB in tears from the involved eye in 4 patients with acute optic neuritis faint OCB in tears in 7 patients, 5 of them with history of optic neuritis no OCB in tears of 13 controls, no CSF-matching done
Coyle et al. (11)	USA	24 MS 20 OD 15 controls	Glass capillary tubes, agarose gel, IEF, silver staining, stimulation by onions/aromatic ammonia	OCB in tears in 14 of 21 MS patients ("most not present in serum") OCB in tears in 1 of 15 not neurological patients also present in serum (type 4) no OCB in tears of 11 controls, CSF OCB data for 6 MS patients
Mavra et al. (12)	UK	28 MS 4 ON 30 OD	Glass capillary tubes, agarose gel, IEF, immunoperoxidase staining, stimulation by onions	no OCB in tears of any MS/ON patient OCB in tears in 1 of 30 other patients (type 2; neurosarcoidosis) CSF data for all but 8 patients
Liedtke et al. (13)	Germany	38 MS 14 OD 23 controls	*Schirmer strip or capillary tubes, polyacrylamide gel, IEF, immunoperoxidase staining, stimulation by ammonia vapor in case of capillary tubes	no OCB in tears in 35 of 38 MS patients no OCB in tears in 0 of 13 other patients no OCB in tears in 19 of 21 controls only 17 cases with paired CSF and serum samples, not clearly assigned
Martino et al. (14)	Italy	18 MS 17 OD	Glass capillary tubes, agarose gel, IEF, immunoperoxidase staining, stimulation by warm air flow	no OCB in tears in 16 of 18 MS patients (94% CSF^{OCB+}), 1 MS patient with unique OCB in tears, 1 MS patient with OCB in tears also present in serum (type 4) OCB in tears in 3 of 17 other patients also present in serum (type 4)
Forzy et al. (15)	France	66 MS 55 OD	Schirmer strip, agarose gel, IEF, silver staining, no stimulation	27 of 37 MS patients with same result for OCB in tears and CSF (81 % CSF^{OCB+}), 29 MS patients without CSF-matching
Devos et al. (16)	France	63 MS 52 OD 13 OIND	Schirmer strip, agarose gel, IEF, silver staining, no stimulation	48 of 60 MS patients with same result for OCB in tears and CSF (75% CSF^{OCB+}) 44 of 50 OD patients with same result for OCB in tears and CSF (8% CSF^{OCB+}) 10 of 13 OIND patients with same result for OCB in tears and CSF (31% CSF^{OCB+}) (exclusion of 5 patients because of positive OCB in serum)
Calais et al. (17)	France	82 CIS	Schirmer strip, agarose gel, IEF, immunoperoxidase staining, no stimulation	54 of 69 CIS patients with same result for OCB in tears and CSF (64% CSF^{OCB+}) (exclusion of 13 patients because of sample dilution)
Lebrun et al. (19)	France	45 RIS	Schirmer strip, agarose gel, IEF, immunoperoxidase staining, no stimulation	41 of 42 RIS patients with same result for OCB in tears and CSF (52% CSF^{OCB+}) (exclusion of 3 patients because of insufficient material)

*MS, multiple sclerosis; ON, optic neuritis; OD, other disease or condition; OIND, other inflammatory neurological disease; CIS, clinically isolated syndrome; RIS, radiologically isolated syndrome; IEF, isoelectric focusing; CSF^{OCB+}, evidence of OCB in CSF without corresponding OCB in paired serum. In (10–13) only incomplete or missing data on paired triplets of tears, CSF and serum exists. In (10–15) clear data to patient dropout because of missing material is lacking. *Liedtke et al. refer to another publication for collection technique and stimulation of tearing describing two different methods (20). Type 4 defines a negative OCB pattern (21).*

(24). Irrigation of the ocular surface with 50 µl 0.9% saline yields a higher volume and is more comfortable to the patient. Plastic capillary tubes were used for collection of tears in a few patients. No stimulation of tearing was provoked in order to avoid artificial changes of the tears. In some patients suffering from a sicca syndrome it was impossible to gather enough fluid for the investigation.

Sample Measures

In all samples total protein content was determined by the Coomassie Blue method (25). Serum and CSF were adjusted to 20 mg/l IgG and placed side by side with an appropriately diluted tear sample of the same patient. Isoelectric focusing was performed on polyacrylamide gels (pH 6–10). Direct silver staining as well as immunoblotting were applied for the detection

of OCB. Following the recommendations of the European consensus on CSF analysis in MS positive OCB are defined as pattern type 2 or type 3 (21). CSF with more than 1 but <4 CSF restricted OCB is defined as type 2a or type 3a. Negative OCB are defined as pattern type 1 or type 4. The same nomenclature referring to serum was used for OCB in tears.

Tear IgG Purification

In contrast to CSF, tears contain a high number of basic proteins (lactoferrin, lysozyme, cystatin C) in high concentrations (26). These proteins interfere with OCB detection both in direct silver staining and in immunoblotting by masking a considerable portion of the migration path. Removal of the alkaline tear proteins and isolation of pure IgG was achieved by prior affinity chromatography with protein G (Protein G Mag Sepharose®, GE Healthcare, UK).

Statistics

Results were analyzed with GraphPad Prism 5.02 (GraphPad Software, USA). Fisher's exact test was used to measure the independence of two categorical variables. This test offers an exact test result even for small sample sizes. $P < 0.05$ were considered as statistically significant.

TABLE 2 | Patient characteristics and CSF OCB status.

Group of diseases		Patients n	Age, years range (median)	Sex f:m (percentage)	Positive CSF OCB n (percentage)
All		59	18–69 (37)	41: 18 (70: 30)	37 (63)
MS	RRMS	22	18–57 (35)	14: 8 (64: 36)	21 (96)
	SPMS	2	55; 61	2: 0 (100: 0)	2 (100)
CIS		4	20 – 55 (39)	3: 1 (75: 25)	3 (75)
OIND	Autoimmune (a)	11	26–69 (38)	9: 2 (82: 18)	10 (91)
	Infectious (i)	2	32; 52	1: 1 (50: 50)	1 (50)
OI		4	26–39 (37)	4: 1 (100: 0)	0 (0)
Control		14	19–68 (42)	8: 6 (57: 43)	0 (0)

RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; CIS, clinically isolated syndrome; OINDa/i, other inflammatory neurological disease [relapsing opticus neuritis, neuromyelitis optica, myelitis, MOG positive encephalomyelitis, (primary) angiitis of the central nervous system, papillitis, viral meningitis]; OI, (possible) subclinical or clinical ocular inflammation; Control group [spinal muscular atrophy, amyotrophic lateral sclerosis, myopathy, unspecific paresthesia / hypesthesia / vertigo / muscular cramps / (back) pain / gait abnormality]. Positive OCB are defined as pattern type 2/2a or type 3/3a.

RESULTS

In most tears collected with either the flush or the capillary tube method the concentration of IgG was below the detection level (**Figure 1**, lane 5 and 7). Tear collection with the Schirmer filter paper strips yielded better results and thus the reported results are based on tear samples gained by Schirmer strips.

From the 59 recruited patients, 13 patients (22%) were excluded because of a lack of sufficient material. The tears of 3 patients were used for immunoblotting. Of the remaining 43 patients, 12 (28%) had OCB in CSF and tears, in 14 patients (32%) OCB were only detectable in CSF, and 12 patients (28%) showed OCB neither in CSF nor in tears (for details see **Table 3A**). Interestingly, in 5 patients (12%) we found positive OCB in tears without OCB detection in CSF. Of note 10 of 17 patients with positive OCB in tears showed only a marginal OCB pattern (type 2a or 3a). One of these patients diagnosed with relapsing-remitting MS with a characteristic positive OCB pattern in CSF (type 2) and few detectable OCB in tears (type 2a) is shown in **Figure 1**. Of the 3 samples stained by immunoblotting we were

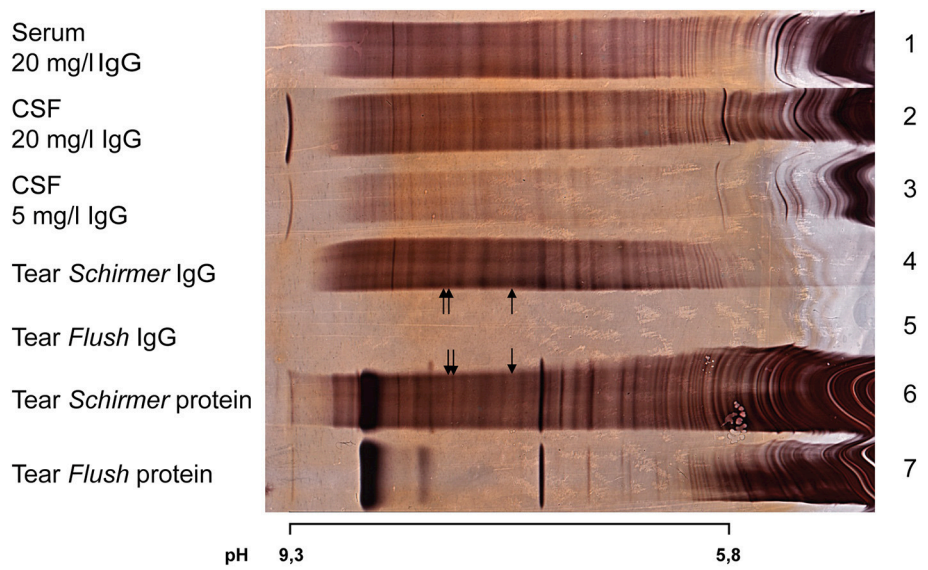

FIGURE 1 | Silver staining after isoelectric focusing on polyacrylamide gel of serum, CSF, and tear fluid (Schirmer or Flush method) of a patient diagnosed with relapsing-remitting MS. Arrows indicate positive OCB in tears. In the lanes with "tear Schirmer/Flush IgG" the IgG was isolated by protein G affinity purification prior to electrophoresis, while in the lanes "tear Schirmer/Flush protein" the unpurified sample was used.

TABLE 3A | OCB results and clinical data of all recruited patients.

Disease	Pat.	OCB		Remarks	Patient characterization (clinical data)							
	#	CSF	tears		Age	Sex	Symptoms at time of investigation	IM	ID	EDSS	Previous neurological medication	Current neurological medication
RRMS	1	**2**	n. d.		22	f	Hypesthesia, paresis	01/2012	01/2012	3.0	GLAT, DMF, NAT	ALZ
	2	**2**	**2**		46	f	Paresis	11/2015	12/2015	3.5	DMF	DMF
	3	**2**	**2a**	External CSF	32	m	Hypesthesia, paresis	2009	2011	1.5	INF, DMF	ALZ
	4	**2**	1		49	m	Paresis	1999	1999	6.5	AZA, INF, GLAT, NAT, FTY, DAC	none
	5	**2**	**2a**		36	m	Optic neuritis (left)	08/2017	08/2017	1.5	none	none
	6	**2**	**2a**		27	m	Optic neuritis (left)	06/2017	08/2017	1.0	none	none
	7	**2**	1		26	f	Hypesthesia, paresis	11/2014	12/2014	3.0	DMF	ALZ
	8	**2**	n. d.	Not enough material	57	f	Optic neuritis (right)	1981	09/2017	3.0	none	none
	9	**2**	n. d.	Not enough material	47	f	Hypesthesia, paresthesia, paresis	2013	09/2015	1.0	none	DMF
	10	**2**	1		43	f	Trigeminal neuralgia	10/2017	11/2017	1.0	none	none
	11	**2**	1		36	f	Optic neuritis (links)	09/2015	07/2016	1.0	INF	DMF
	12	**2**	**2**		44	m	Facial palsy	1998	2014	2.5	DMF, INF, FTY	ALZ
	13	**2**	**2**	CFS during natalizumab	20	f	Hypesthesia	12/2012	01/2013	3.0	GLAT, FTY, NAT	ALZ
	14	**2**	1		33	f	Paresis	2010	2010	4.0	INF, GLAT, NAT, DAC	none
	15	**2**	**2a**		37	m	Optic neuritis (right)	04/2018	04/2018	2.0	none	none
	16	**2**	1		33	f	Optic neuritis (left)	04/2018	04/2018	2.0	none	none
	17	1	**2a**	External CSF, discrepant	29	f	Optic neuritis (left)	06/2015	02/2019	1	none	none
	18	**2**	1		30	m	Paresthesia	12/2016	07/2017	1.0	none	none
	19	**2**	1		18	f	Paresthesia	12/2016	07/2017	1.0	none	none
	20	**2**	inc.	Blot	28	m	Paresthesia	08/2017	09/2017	1.0	none	none
	21	**2**	1	Tears pooled from 2 days	50	f	Diplopia	09/2017	09/2017	1.0	none	none
	22	**2**	1		45	f	Optic neuritis (right)	09/2017	09/2017	1.0	none	none
SPMS	1	**2**	n. d.	Not enough material	61	f	Paresis, bladder dysfunction	1983	1983	7.5	Mitoxantrone	IVMP
	2	**2**	n. d.	Not enough material	55	f	Paresis, bladder dysfunction	03/2000	03/2000	6.0	GLAT, NAT	Mitoxantrone
CIS	1	4	4		53	f	Optic neuritis (left)	07/2017	07/2017	3.0	none	none
	2	**2**	n. d.	Not enough material	24	f	Optic neuritis (right)	08/2017	08/2017	3.0	none	none
	3	**2**	n. d.	Not enough material	55	f	Optic neuritis (right)	08/2017	08/2017	3.0	none	none
	4	**2**	inc.	Blot	20	m	Optic neuritis (right)	08/2017	08/2017	1.0	none	none
OINDa	1	**3**	**3a**		51	f	Vertigo, paresthesia	06/2017	07/2017	2.0	none	none
	2	**3**	4		69	f	Hypesthesia, paresis, bladder dysf.	07/2017	07/2017	5.0	none	none
	3	**2**	1		26	m	Hypesthesia, paresis	06/2017	08/2017	n. a.	none	none
	4	**2a**	1		26	f	Hypesthesia	08/2017	n. a.	n. a.	none	none
	5	1	inc.	Blot	45	f	Hypesthesia, paresthesia	05/2017	08/2017	1.5	none	none
	6	**2**	n. d.	Not enough material	29	f	Optic neuritis (right)	11/2017	11/2017	1.0	none	none
	7	**2**	**2**	Replapsing ON	31	f	Optic neuritis (right)	05/2017	06/2017	1.0	none	Rituximab
	8	**2**	**2a**		49	f	Paresthesia	02/2018	n. a.	n. a.	none	none

(Continued)

TABLE 3A | Continued

Disease	Pat.	OCB		Remarks	Patient characterization (clinical data)							
	#	CSF	tears		Age	Sex	Symptoms at time of investigation	IM	ID	EDSS	Previous neurological medication	Current neurological medication
	9	**3**	n. d.	Not enough material	42	f	Headache, hypesthesia	2016	n. a.	n. a.	none	none
	10	**3**	**3**		38	m	Unspecific	03/2018	n. a.	n. a.	none	none
	11	**2**	**2**		27	f	Seizure	03/2019	n. a.	n. a.	none	none
OINDi	1	4	1		52	f	Loss of vision	09/2017	n. a.	n. a.	none	none
	2	**2**	1		32	m	Paresthesia	03/2018	n. a.	n.a	none	none
OI	1	1	**2a**	Discrepant	36	f	Optic neuritis (right)	05/2014	05/2014	3.0	none	none
	2	1	**2**	Discrepant, relapsing Zoster	37	f	Paresthesia	2010	n. a.	n. a.	none	none
	3	4	**3a**	Discrepant	39	f	Hypesthesia	12/2017	n. a.	n. a.	none	none
	4	4	**3a**	Discrepant, Zoster V1	26	f	Painful skin changes	03/2019	n. a.	n. a.	none	none
Control	1	1	n. d.	Not enough material	46	f	Aura	08/2017	n. a.	n. a.	none	none
	2	4	n. d.	Not enough material	47	m	Unspecific gait abnormality	2015	n. a.	n. a.	none	none
	3	1	1		48	f	Paresis	2015	n. a.	n. a.	none	none
	4	1	1		27	f	Unspecific vertigo	03/2019	n. a.	n. a.	none	none
	5	1	1		50	f	Muscular cramps	2017	n. a.	n. a.	none	none
	6	1	1		34	m	Pain	02/2019	03/2019	n. a.	none	none
	7	4	n. d.	Not enough material	49	m	Back pain	2012	n. a.	n. a.	none	none
	8	1	1		37	f	Hypesthesia	03/2019	n. a.	n. a.	none	none
	9	1	1		55	f	Paresthesia	03/2019	n. a.	n. a.	none	none
	10	1	1		36	f	Paresis	1992	1992	n. a.	none	Nusinersen
	11	1	1		68	m	Paresis	2017	03/2019	n. a.	none	none
	12	1	n. d.	Not enough material	19	m	Paresis	2016	2016	n. a.	none	Nusinersen
	13	1	1		22	m	Paresis	1998	1998	n. a.	none	Nusinersen
	14	1	1		23	f	Hypesthesia	03/2019	n. a.	n. a.	none	none

EDSS, expanded disability status scale; f, female; ID, initial diagnosis; IM, initial clinical manifestation; m, male; n. a., not applicable; ON, optic neuritis; MS medication: Alemtuzumab (ALZ), Azathioprine (AZA), Daclizumab (DAC), Dimethyl Fumarate (DMF), Fingolimod (FTY), Glatiramer Acetate (GLAT), Interferon-Beta (INF), intravenous methylprednisolone (IVMP), Natalizumab (NAT). Positive OCB are defined as pattern type 2/2a or type 3/3a. Negative OCB are defined as pattern type 1 or type 4. Bold entries indicate a pathology.

able to verify oligoclonal IgG in CSF but had inconclusive results in tears (**Table 3A**).

Figure 2 demonstrates the characteristic problem of OCB detection in unprepared tears: A relevant cathodic part of the gel is covered by tear specific proteins (see **Figure 2**, lane 6). IgG purification by affinity chromatography with protein G eliminates these alkaline proteins (see **Figure 2**, lane 1). The great advantage of this approach is that the silver stain could be used directly for the demonstration of IgG without interference from other proteins.

Figure 3 illustrates a patient with unique OCB in tears suffering from unspecific complaints.

Pathological visual evoked potentials showed a highly significant association to the occurrence of OCB in tears ($P = 0.0094$). In addition, a history of optic neuritis was significantly associated with OCB in tears ($P = 0.0258$). Other clinical or paraclinical factors had no influence on the occurrence of OCB in tears (CSF cell count: $P = 1.0$; blood-brain barrier dysfunction: $P = 0.0608$; cell profile: $P = 1.0$; MRZ reaction: $P = 1.0$; magnetic resonance imaging of the brain: $P = 1.0$; magnetic resonance imaging of the spinal cord: $P = 0.6699$; sex: $P = 0.7357$; for details see **Tables 3A,B** and **Figures 4A,B**). Neither the age of all patients nor the EDSS of MS patients influenced the occurrence of OCB in respective tears (**Figures 4C,D**). Contradicting, one patient suffering from optic neuritis without visual evoked potential alterations but matching magnetic resonance imaging and very recently diagnosed with MS showed OCB in tears but not in CSF (for details see **Table 3B**, Pat. # 17, RRMS disease group). However, since the OCB investigation was carried out in an external laboratory, it cannot be excluded that the lack of OCB in CSF is due to insufficient sensitivity of OCB detection.

Interestingly, all patients with OCB exclusively in tears had normal visual evoked potentials and CSF parameters (cell count,

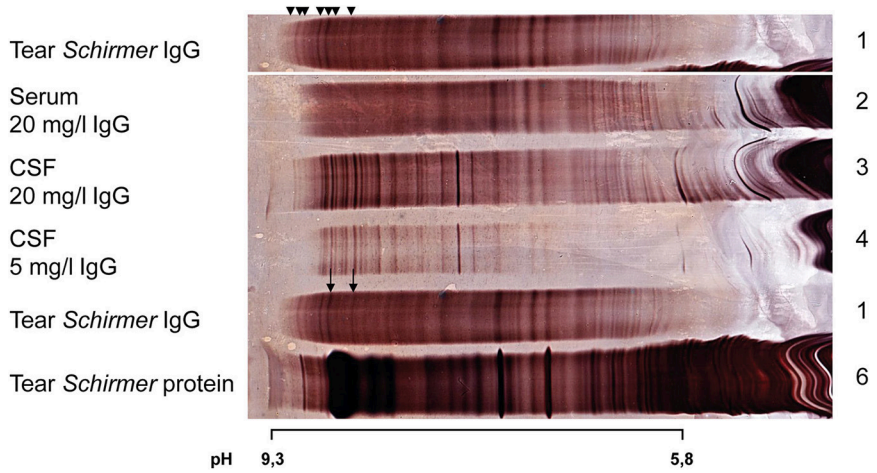

FIGURE 2 | Silver staining after isoelectric focusing on polyacrylamide gel of serum, CSF, and tear fluid (Schirmer method) of a patient diagnosed with MOG-IgG positive encephalomyelitis. For a better overview lane 1 (Tear *Schirmer* IgG) is duplicated. Arrowheads indicate bands in tear fluid, at least 3 of which were unique bands in tears. Arrows indicate 2 positive OCB in tears comigrating with CSF OCB. The comparison between lane 1 (sample with purified IgG) and lane 6 (unpurified sample) illustrates the interference of tear specific protein with OCB in the cathodic section of the gel.

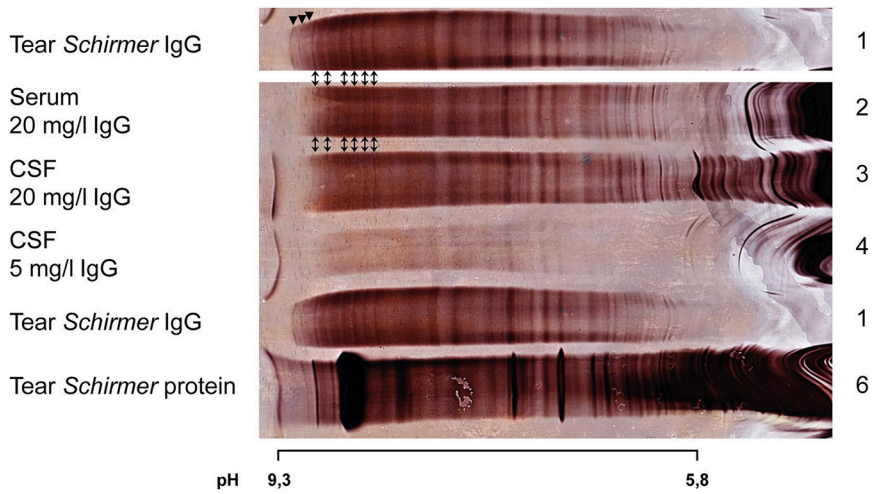

FIGURE 3 | Silver staining after isoelectric focusing on polyacrylamide gel of serum, CSF, and tear fluid (Schirmer method) of a patient suffering from unspecific paresthesia. For a better overview lane 1 (Tear *Schirmer* IgG) is duplicated. Arrowheads indicate unique bands in tears. Double arrows indicate 6 identic OCB in paired tears, serum, and CSF.

blood-brain barrier function, cell profile, MRZ reaction). Only one patient showed a pathological magnetic resonance imaging (for details see **Tables 3A,B**). One other patient had a clinically detectable ocular infection.

The sensitivity and specificity for OCB detection in tears for MS/CIS diagnosis is 41 and 50%, respectively. Nevertheless, as in CSF, there is a statistically significant difference in the occurrence of OCB in tears between the control group and MS patients ($P = 0.0302$).

DISCUSSION

This study likewise detected oligoclonal IgG in tear fluid. Compared to the positive studies of one research group with high agreement rates between CSF and tears of all CSF^{OCB+} patients [Forzy et al. (15): 73%; Devos et al. (16): 96%; Calais et al. (17): 66%; Lebrun et al. (19): 96%], our study found a significantly lower detection rate of OCB in tear fluid: 7 out of 18 (39%) analyzable MS patients (96% CSF^{OCB+}) or 12 out of 26 analyzable CSF^{OCB+} patients (46%) had a concordance of OCB between cerebrospinal and tear fluid. The concordance for OCB absence or presence in all analyzable patients was 56% (24 of 43 patients). It is important to remember that in 59% of all samples with positive OCB in tears only a marginal OCB pattern was present.

How can these different study results be explained? All previous studies that investigated OCB in tear fluid differed considerably in the methodology used to (a) obtain tear fluid and

TABLE 3B | OCB results and paraclinical data of all recruited patients.

Disease	Pat.	OCB		Patient characterization (paraclinical data)							
	#	CSF	tears	CC	Q-alb	CSF cell profile	MRZ	cMRI	sMRI	VEP	ever ON
RRMS	1	**2**	n. d.	**20**	-	Normal	**pos.**	**Multiple T2-h. lesions**	**Multiple T2-h. lesions**	n. d.	**yes**
	2	**2**	**2**	3.7	5.0	Normal	neg.	**Multiple T2-h. lesions including the left optic nerve**	normal	**Prolonged latencies left**	**yes**
	3	**2**	**2a**	n. d.	n. d.	n. d.	n. d.	**Multiple T2-h. lesions**	**Multiple T2-h. lesions**	**Prolonged latencies right**	**yes**
	4	**2**	1	0.3	5.79	Normal	n. d.	**Multiple T2-h. lesions**	**Multiple T2-h. lesions**	Normal	no
	5	**2**	**2a**	**7.3**	4.35	**Plasma cells**	neg.	**Multiple T2-h. lesions**	n. d.	**Decrease in left amplitude**	**yes**
	6	**2**	**2a**	**8.7**	5.0	**Plasma cells**	neg.	**T2-h. lesion with CE of the left optic nerve**	**Some T2-h. lesions**	**Prolonged latencies left**	**yes**
	7	**2**	1	0.3	4,9	Normal	neg.	**Multiple T2-h. lesions**	**Multiple T2-h. lesions**	Normal	**yes**
	8	**2**	n. d.	4.7	3.62	**act. monocytes**	**pos.**	**Multiple T2-h. lesions**	n. d.	**Decrease in left amplitude**	**yes**
	9	**2**	n. d.	**34**	4,18	**Plasma cells**	**pos.**	**Multiple T2-h. lesions**	n. d.	n. d.	no
	10	**2**	1	1.0	4.24	Normal	neg.	**Multiple T2-h. lesions, one with CE**		Normal	no
	11	**2**	1	2.3	2.20	**Plasma cells**	**pos.**	**Multiple T2-h. lesions including the left optic nerve**	n. d.	**Prolonged latencies left**	**yes**
	12	**2**	**2**	3.7	**11.97**	Normal	neg.	**Multiple T2-h. lesions, one with CE**	Normal	n. d.	no
	13	**2**	**2**	**98.0**	4.66	**act. lymphocytes**	**pos.**	**Multiple T2-h. lesions**	**Multiple T2-h. lesions**	**Prolonged latencies left**	**yes**
	14	**2**	1	1.0	6.01	Normal	neg.	**Multiple T2-h. lesions**	**LETM, CE**	**Prolonged latencies left**	**yes**
	15	**2**	**2a**	**22.0**	5.14	**Plasma cells**	**pos.**	**Multiple T2-h. lesions including right optic nerve**	Normal	**Prolonged latencies right**	**yes**
	16	**2**	1	**11.3**	4.62	**Plasma cells**	neg.	**Multiple T2-h. lesions including left optic nerve**	Normal	Normal	**yes**
	17	1	**2a**	4.0	5.20	normal	n. d.	**Some T2-h. lesions including left optic nerve**	**One T2-h. lesions**	Normal	yes
	18	**2**	1	3.0	3.93	Normal	**pos.**	**Multiple T2-h. lesions**	n. d.	Normal	no
	19	**2**	1	**29**	2.14	**Plasma cells**	neg.	**Some T2-h. lesions**	n. d.	Normal	no
	20	**2**	inc.	2.7	5.20	Normal	neg.	**One T2-h. lesion**	**One T2-h. lesions with CE**	n. d.	no
	21	**2**	1	2.7	5.26	**act. lymphocytes**	**pos.**	**Some T2-h. lesions**	n. d.	n. d.	no
	22	**2**	1	**5.0**	2.30	**Plasma cells**	neg.	**Multiple T2-h. lesions including both optic nerves**	n. d.	**Prolonged latencies right**	**yes**
SPMS	1	**2**	n. d.	**8.0**	n. d.	**Plasma cells**	neg.	n. d.	n. d.	n. d.	n. d.
	2	**2**	n. d.	3	n. d.	n. d.	n. d.	**Multiple T2-h. lesions**	n. d.	**Prolonged latencies right**	**yes**
CIS	1	4	4	0.3	3.69	Normal	neg.	Normal	Normal	**Prolonged latencies left**	**yes**
	2	**2**	n. d.	**20.3**	3.49	**Plasma cells**	**pos.**	**T2-h. lesion with CE of the right optic nerve**	n. d.	**Cortical signal loss right**	**yes**
	3	**2**	n. d.	**61.3**	4.89	**act. lymphocytes**	neg.	**T2-h. lesion of the right optic nerve**	n. d.	**Cortical signal loss right**	**yes**
	4	**2**	inc.	**10.3**	3.08	**Plasma cells**	**pos.**	**T2-h. lesions with CE of the right optic nerve**	n. d.	**Prolonged latencies right**	**yes**

(Continued)

TABLE 3B | Continued

Disease	Pat.	OCB		Patient characterization (paraclinical data)							
	#	CSF	tears	CC	Q-alb	CSF cell profile	MRZ	cMRI	sMRI	VEP	ever ON
OINDa	1	**3**	**3a**	**111**	**13.5**	**act. lymphocytes**	neg.	**Multiple T2-h. lesions**	**Multiple T2-h. lesions**	**Prolonged latencies right**	**yes**
	2	**3**	4	**9.3**	**14.90**	**Plasma cells**	n. d.	**Multiple T2-h. lesions**	**LETM, CE**	n. d.	no
	3	**2**	1	**9.7**	4.56	**Plasma cells**	neg.	**DWI hyperintensities**	n. d.	n. d.	no
	4	**2a**	1	3.3	3.72	**act. lymphocytes**	neg.	Normal	n. d.	Normal	no
	5	1	inc.	1.3	5,67	Normal	neg.	Unspecific	**One T2-h. lesions**	n. d.	no
	6	**2**	n. d.	**10.3**	4.49	**act. lymphocytes**	neg.	**Multiple T2-h. lesions**	**Multiple T2-h. lesions with CE**	n. d.	**yes**
	7	**2**	**2**	4.0	**7.0**	**Plasma cells**	neg.	**Multiple T2-h. lesions**	Normal	**Prolonged latencies right**	**yes**
	8	**2**	**2a**	1.3	3.67	Normal	n. d.	Normal	n. d.	n. d.	no
	9	**3**	n. d.	1.3	4.15	**Siderophages**	neg.	Unspecific	n. d.	n. d.	no
	10	**3**	**3**	**48.0**	**6.95**	**Plasma cells**	neg.	Unspecific	n. d.	n. d.	no
	11	**2**	**2**	1.7	2.81	Normal	neg.	Unspecific	n. d.	n. d.	no
OINDi	1	4	1	**13.3**	5.50	Normal	neg.	Unspecific	n. d.	Normal	no
	2	**2**	1	**13.7**	4.22	**Plasma cells**	n. d.	Unspecific	n. d.	n. d.	no
OI	1	1	**2a**	1.0	4.17	Normal	neg.	Normal	Normal	n. d.	**yes**
	2	1	**2**	1.0	2.76	Normal	neg.	Normal	n. d.	n. d.	no
	3	4	**3a**	3.3	5.35	Normal	neg.	Unspecific	n. d.	n. d.	no
	4	4	**3a**	3.0	4.19	Normal	n. d.	Normal	n. d.	n. d.	no
Control	1	1	n. d.	2.0	**9.39**	Normal	neg.	**Some T2-h. lesions**	n. d.	Normal	no
	2	4	n. d.	2.0	6.00	Normal	neg.	Unspecific	n. d.	n. d.	no
	3	1	1	0.7	2.89	Normal	n. d.	n. d.	n. d.	n. d.	no
	4	1	1	0.7	2.96	Normal	neg.	Normal	n. d.	n. d.	no
	5	1	1	0.7	2.87	Normal	n. d.	n. d.	Normal	n. d.	no
	6	1	1	2.7	4.01	Normal	n. d.	n. d.	n. d.	n. d.	no
	7	4	n. d.	3.7	**7.48**	Normal	n. d.	n. d.	Unspecific	n. d.	no
	8	1	1	0.7	2.81	Normal	n. d.	Normal	Normal	Normal	no
	9	1	1	1.7	7.53	Normal	n. d.	Unspecific	Normal	n. d.	no
	10	1	1	0.7	4.32	Normal	n. d.	n. d.	n. d.	n. d.	no
	11	1	1	1.0	6.0	Normal	n. d.	Unspecific	Normal	n. d.	no
	12	1	n. d.	3.0	4.26	Unspecific	n. d.	n. d.	n. d.	n. d.	no
	13	1	1	2.3	5.64	Normal	n. d.	Normal	n. d.	n. d.	no
	14	1	1	**31.7**	4.01	Normal	n. d.	Normal	Normal	Normal	no

act., activated; CE, contrast enhancement; CC, CSF cell count per µl; cMRI, cerebral magnetic resonance imaging; inc., inconclusive; LETM, longitudinally extensive transverse myelitis; MRZ, intrathecal polyspecific antiviral immune response (positive MRZ reaction: 2 of 3 antibody indices against measles, rubella and/or varicella zoster virus are positive); n. d., not done/no data; ON, optic neuritis; Q-alb, quotient of albumin × 10^{-3} (marker of blood-brain barrier dysfunction); sMRI, spinal magnetic resonance imaging; T2-h. lesion, T2-hyperintense lesion; VEP, visual evoked potentials. Positive OCB are defined as pattern type 2/2a or type 3/3a. Negative OCB are defined as pattern type 1 or type 4. Bold entries indicate a pathology.

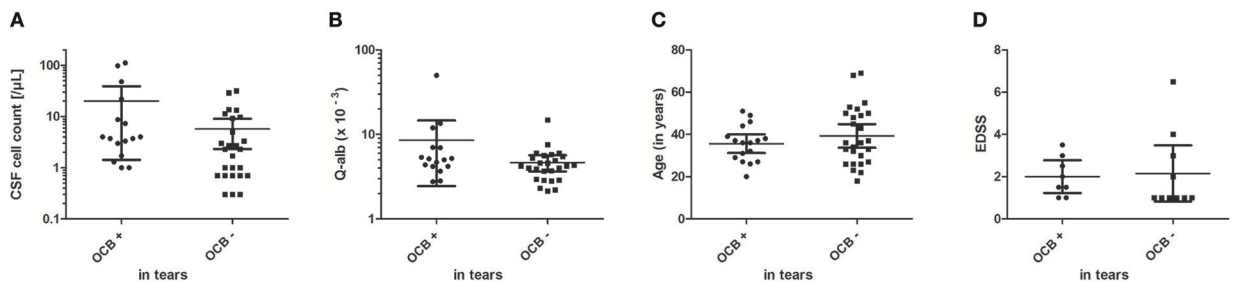

FIGURE 4 | Influence of CSF cell count **(A)**, Q-alb **(B)** and age **(C)** of all patients and EDSS **(D)** of all MS patients on the occurrence of OCB in tear fluid. For better visualization, a logarithmic illustration was used in **(A)** and **(B)**. The mean with 95% confidence interval is shown.

(b) detect OCB (**Table 1**). Tear collection was performed using either glass capillary tubes or Schirmer strips. Tear production was partly stimulated by onions, ammonia, or warm air flow. Protein separation was performed on agarose or polyacrylamide gels by electrophoresis or isoelectric focusing. IgG was visualized by silver staining or immunoblotting. In the positive studies the detection of OCB in tear fluid, however, was achieved with all the different above mentioned procedures. In our study tear collection by Schirmer strips appeared to be the best reproducible collection technique whereas other collection methods (i.e., capillary with and/without flush) provided insufficient material for detailed analysis.

As a possible explanation for the discrepancy between the study results, the duration of tear collection was considered responsible in the most recent studies. To avoid dilution by reflex secretion, a maximum duration of 1 min for tear collection was suggested (15–17, 19). However, in these studies no threshold was mentioned concerning the required minimal running distance of tears on Schirmer strips. None of our patients had a lacrimation that would wet a Schirmer strip (35 mm) within 1 min. Only five patients (8%) moistened the complete Schirmer strip within 2 min. For a reliable usability of the sample, our study showed that the minimum running distance should be between 4 and 8 mm. Nevertheless, reliable results were only achieved from ∼10 mm upwards. Accordingly, with the prerequisite of a maximum tear collection time of 1 min, hardly any patients could have been included in our study. In addition, in our study 13 patients with positive OCB in tears had a collection time of 5 min, which argues against the hypothesis of reflex dilution. An excessive dilution of the tear sample due to a longer collection time is also opposed by an average tear volume of 20 μl in our study. Devos and co-workers used a maximum of 30 μl (16).

Another relevant factor which interferes with tear OCB detection are tear specific protein bands. Accordingly, for a reliable analysis of OCB in tears an isolation of tear IgG by affinity chromatography is suggested. All previous studies analyzed crude tears. This certainly had an impact on the results.

If we propose that OCB in CSF should also be detectable in tears, in our study 14 of 26 CSF^{OCB+} patients (54%) and 10 of 17 MS patients (59%) would have "false negative" OCB in tears. Even in the CIS-study by Calais and co-workers, 15 of 44 CIS patients (34%) would have "false negative" tear results. Considering these fragile results and the high dropout rate of 22% of patients with poor tear production, which diminishes the practical applicability, we cannot recommend that tear OCB detection may replace CSF OCB detection. Moreover, one should also consider the gain in information due to CSF the differential diagnosis of MS and MS mimics (27).

The trigger for the production of OCB in tear fluid is not yet known. Our result of unique bands in tears suggests that in some cases there might be an independent local mucosal immune response. This aspect is supported by the second work by Coyle et al. who detected an independent OCB pattern in all six samples compared to serum and CSF (11). Moreover, in the studies by Martino et al. (14) one patient and by Devos et al. (16) four patients with unique OCB in tears without corresponding bands in serum or CSF were detected (14, 16). Both, the patient with zoster ophthalmicus (for details see **Table 3A**, Patient #4, OI disease group), and the patient with optic neuritis without CSF OCB (for details see **Table 3A**, Patient #17, RRMS disease group) suggest that ocular inflammation may lead to the presence of OCB in tears. This is supported by the significant relationship between visual evoked potential alterations and tear OCB positivity. Possibly OCB may develop in infectious or autoimmunological eye diseases in tear fluid. It would be worthwhile to test this hypothesis in a larger study with patients with acute and chronic eye diseases, as this could result in a possible new diagnostic tool for eye diseases and might provide new insights in the pathophysiology of eye diseases. Besides that, the occurrence of unique OCB in tears raises the controversy as how many of positive tear OCB results in MS patients are due to the chronic inflammatory CNS disease or to a present or previous inflammatory eye disease. Since the pattern between OCB in tears and CSF often do not coincide completely a different origin might be suggested.

In conclusion, OCB are detectable in tears. The high dropout rate of patients, the low concordance rate of OCB detection between CSF and tears in our study, and the ambiguous results in previous studies limit the application in daily clinical practice. Interestingly in 5 patients OCB were positive in tears but lacking in CSF. OCB detection in tears might be a useful test for ophthalmological diseases and differential diagnosis.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the ethics committee, Hannover Medical School. The protocol was approved by the local ethics committee (No. 7218), Hannover Medical School.

AUTHOR CONTRIBUTIONS

MH, LB, K-WS, SA, SG, and TS obtained the samples. MH, UW, LB performed the experiments. MH, UW, and MS conceived and designed the study. MH, UW, LB, K-WS, SA, SG, TS, and MS analyzed the data. MH, UW, TS, and MS wrote the paper. PS collected new samples, analyzed data and helped to extend the study according to the suggestion of the reviewers. All authors contributed to manuscript revision, read, and approved the submitted version.

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
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Higher CSF Levels of NLRP3 Inflammasome Is Associated With Poor Prognosis of Anti-N-methyl-D-Aspartate Receptor Encephalitis

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Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is accepted as an autoimmune disorder of the central nervous system (CNS). NLR family pyrin domain containing 3 (NLRP3) inflammasome, a potent innate inflammatory mediator, can activate IL-1β and induce the migration of T helper cell into CNS. However, the possible role of NLRP3 inflammasome in the pathogenesis of anti-NMDAR encephalitis remains unclear. This study aims to determine the levels of NLRP3 and related cytokines (IL-1β, IL-6, and IL-17) in the cerebrospinal fluid (CSF) of anti-NMDAR encephalitis patients and to assess whether NLRP3 influences the clinical outcomes of this disease. Twenty-five patients with anti-NMDAR encephalitis, 12 viral meningoencephalitis patients and 26 controls with non-inflammatory neurological diseases were recruited. CSF NLRP3 inflammasome, IL-1β, IL-6, and IL-17 were measured by enzyme-linked immunosorbent assay. Thirteen out of 25 patients were re-examed for the concentrations of NLRP3 and cytokines 6 months later. Our results showed significant increases of CSF NLRP3 inflammasome, IL-1β, IL-6, and IL-17 in anti-NMDAR encephalitis patients. There were positive correlations between CSF NLRP3 inflammasome and cytokines in anti-NMDAR encephalitis patients. There was also a positive correlation between maximum modified Rankin Scale (mRS) scores and CSF NLRP3 inflammasome in anti-NMDAR encephalitis patients. During follow-up, the decrease of mRS was positively correlated with the decrease of CSF NLRP3 inflammasomes. These results suggested that the level of CSF NLRP3 inflammasome could represent the severity of anti-NMDAR encephalitis and the reduction of CSF NLRP3 inflammasome could act as an indicator for the prognosis of this disease.

Keywords: anti-NMDAR encephalitis, neuro-inflammation, cytokine, NLRP3, modified Rankin Scale

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INTRODUCTION

As an autoimmune disorder, anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis predominantly affects young females (1). Its incidence is approximated at 5–10 per 100,000 people per year (2, 3). The typical clinical manifestation presents as psychiatric and neurologic symptoms, such as seizures, movements disorders, autonomic dysfunction, and cognition dysfunction (4, 5). Anti-NMDAR encephalitis may be associated with teratoma and partly secondary to central nervous system (CNS) infections such as herpes simplex virus or parasite (6, 7). Although B and T leukocytes have been proposed to be involved in anti-NMDAR encephalitis, the immunopathogenesis of this disease remains obscure. We have reported several inflammatory cytokines were increased in serum or cerebrospinal fluid (CSF) of anti-NMDAR encephalitis (8–10).

The inflammasomes are cytosolic multi-protein complexes that initiate the activation of caspases-1 and subsequently the cleavage of pro-inflammatory cytokines interleukin (IL)-1β and IL-18 (11). Four different receptors have been shown to form inflammasomes, three of which, NLRP1, NLRP3, and NLRC4, belong to the Nod-like receptor (NLR) family of proteins. The fourth one, AIM2, belongs to the hematopoietic interferon-inducible nuclear (HIN) protein family (12). NLRP3 inflammasome is the most extensively studied but also the most elusive one. In response to a number of physical and chemical triggers, such as bacterial infection, extracellular ATP, glucose, and cholesterin, NLRP3 activates innate immune system and leads to tissue damage (13). Some studies have reported that NLRP3 inflammasome is of great important in the pathogenesis of some central nervous disease such as stroke, epilepsy, Alzheimer's disease (AD), and Parkinson's disease (PD) (14–16). But CSF levels of NLRP3 inflammasome in anti-NMDAR encephalitis patients has not been determined.

Previous experiments have confirmed that T helper (Th) cells might support B cell function (17, 18) and that CSF IL-6 and IL-17 were elevated in anti-NMDAR encephalitis patients (19, 20). As neuroinflammation is suspected to play an important role in the severity of anti-NMDAR encephalitis, we examined the CSF levels of NLRP3 inflammasome and relevant inflammatory cytokines in patients with anti-NMDAR encephalitis, viral meningoencephalitis and non-inflammatory CNS disease. In this study, we aimed to find out the correlation between clinical outcomes and NLRP3 inflammasome, and to investigate whether NLRP3 can be used as a diagnostic and prognostic factor for anti-NMDA receptor encephalitis in clinical practice.

MATERIALS AND METHODS

Patients and Controls

We recruited 25 patients with anti-NMDAR encephalitis, 12 patients with viral meningoencephalitis (VM) and 26 controls with non-inflammatory neurological diseases from the Department of Neurology, Nanfang Hospital, Southern Medical University. The revised anti-NMDAR encephalitis diagnosis criteria of 2016 were used as the inclusion criteria for the anti-NMDAR encephalitis group (21). All CSF samples of anti-NMDAR encephalitis patients were positive for NR1 subunit of anti-NMDAR antibodies by cell-based analysis (5) and negative for viral DNA or other pathogens. All anti-NMDAR encephalitis patients were treated with plasmapheresis, intravenous immunoglobulin or high dose of methylprednisolone. Only one patient stayed in hospital more than 6 months, and the other 24 patients successfully discharged 1∼2 month after treatment. The VM group patients were made a definite diagnosis by detecting viral DNA such as herpes simplex virus, cytomegalovirus, and varicella-zoster virus in their CSF by PCR. The controls group consisted of patients with other CNS non-inflammatory disorders, including cerebral vascular disease and abnormal movements. Both the VM and control groups were negative for NR1 subunit of anti-NMDAR antibodies in CSF. The clinic manifestations and baseline characteristics of anti-NMDAR encephalitis patients ($n = 25$), VM patients ($n = 12$), and controls ($n = 26$) were shown in **Table 1**. Patients with non-inflammatory neurological disease were used as controls, including 6 cases of alzheimer's disease, 12 cases of parkinson's disease and 8 cases of normal pressure hydrocephalus. The etiologies of viral meningoencephalitis including 4 cases of herpesvirus, 2 cases of Epstein Barr virus, 3 cases of varicella zoster virus, and 3 cases of unknown etiology but responsive to antiviral therapy. In the cohort of anti-NMDAR encephalitis patients, disorders of memory, behavior, and cognition (88%) and seizure (72%) were the most common clinic manifestations.

This study was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University. Written informed consent was obtained from each participant after an explanation of the purpose and procedures of this study.

TABLE 1 | The clinic manifestations and baseline characteristics of anti-NMDAR encephalitis VM and controls.

	NMDAR ($n = 25$)	VM ($n = 12$)	Control ($n = 26$)
Gender (male/female)	11/14	7/5	13/13
Age (years)	35.5 ± 19.7	34.5 ± 15.8	38.8 ± 16.2
Psychiatric and neurologic symptoms			
Fever	14 (56%)	8 (67%)	–
Disorders of memory, behavior, and cognition	22 (88%)	4 (33%)***	–
Seizures	18 (72%)	3 (25%)**	–
Autonomic disturbances	10 (40%)	2 (17%)	–
Disturbance of consciousness	15 (60%)	6 (50%)	–
Abnormal movements	11 (44%)	2 (17%)	–
ovarian teratoma	3 (12%)	0 (0%)	–
CSF white blood cell count ($\times 10^6$/L, median)	4(1,18)	3 (0.58)	0 (0, 0)***
CSF protein(g/L, median)	0.30 (0.17, 0.76)	0.25 (0.18, 0.94)	0.28 (0.19, 0.40)
Maximum mRS scores	4, (4,5)	-	-
6 months' mRS scores after the disease onset	3, (2,3)	-	-
Anti-NMDAR antibody	25	0	0

$^{**}p < 0.01$, $^{***}p < 0.001$.

Determination of CSF NLRP3 Inflammasomes and Other Inflammatory Cytokine Levels

After the patients were hospitalized, CSF samples were obtained immediately and centrifuged at 1,000 g for 10 min. The supernatant was then transferred into a polypropylene tubes and stored at −80°C until the assays were performed. All procedures were completed in 60 min. Commercial Sandwich Enzyme-linked immunosorbent assay (ELISA) kits were used to detect the levels of CSF NLRP3 inflammasomes (CSB-E15885h, Cusabio, Wuhan, china), IL-1β, IL-6, and IL-17 (Bender MedSystems GmbH, Vienna, Austria). The assays were performed according to the manufacturers' instructions. All standards and samples were measured in duplicate and CSF samples were measured undiluted. Optical densities were determined on a Microplate Reader (BMG LabTech).

Clinic and Follow-Up Evaluation

The modified Rankin Scale (mRS) scores were used to evaluate the clinical neurologic disabilities. All patients with anti-NMDAR encephalitis were evaluated for mRS scores at the times of acute stage and 6 months after onset. And the former was defined as peak mRS scores, the latter was taken as the remission scores. Through follow-up, 13 out of 25 patients were re-examed for these four profiles in 3–6 months later. The other 12 patients did not come back to our hospital, or refused lumbar puncture once more. The change of NLRP3 (δNLRP3) and of mRS (δmRS) were calculated according to the following formulas: δNLRP3 = NLRP3 (acute stage) – NLRP3 (remission stage); δmRS = mRS (acute stage)—mRS (remission stage).

Statistical Analysis

SPSS version 20.0 (IBM Corp, Armonk, NY, USA) was used for data analyzation. Data were presented as mean (±standard deviation) or median (interquartile range). Kruskal-Wallis test was used for the comparison between groups. Paired Wilcoxon tests were used to compare these profiles before and after treatment in the 13 patients who were re-evaluated during follow-up. Correlations between the profiles were evaluated using Pearson's test or Spearman's test. p-value < 0.05 was considered statistically significant.

RESULT

Increased Levels of NLRP3 Inflammasomes and Inflammatory Cytokines in CSF of Patients With Anti-NMDAR Encephalitis

In the present study, CSF NLRP3 inflammasomes, IL-1β, IL-6, and IL-17 were detected in anti-NMDAR encephalitis patients and two control groups and the data were shown in **Table 1** and **Figure 1**. The CSF levels of NLRP3 inflammasomes, IL-1β, IL-6, and IL-17 inflammasome was notably increased in anti-NMDAR encephalitis patients in acute stage compared with controls ($p < 0.001$, $p = 0.031$, $p = 0.001$, $p < 0.001$, respectively). The NLRP3 inflammasomes, and IL-17 also were found to be markedly higher in VM patients than in controls ($p = 0.0012$, $p = 0.015$; respectively). In the 13 followed-up patients with anti-NMDAR encephalitis, the levels of CSF NLRP3 inflammasome, IL-1β, IL-6, and IL-17 were decreased in remission stage compared with that in acute stage ($p = 0.012$, $p = 0.005$, $p = 0.013$, $p = 0.001$, respectively).

Relationship Between CSF NLRP3 and Inflammatory Cytokines

In each group, we detected correlation between levels of CSF NLRP3 inflammasome and the other three interleukins. In patients with anti-NMDAR encephalitis, positive correlations were found between NLRP3 and IL-1β, IL-6, or IL-17 ($p < 0.001$, $r = 0.676$; $p < 0.001$, $r = 0.690$; $p = 0.006$, $r = 0.492$, respectively). NLRP3 was also positively correlated with IL-1β and IL-6 in the VM group. No correlations were found between NLRP3 and cytokines in the control group of non-inflammatory neurological diseases.

Relationship Between Clinical Outcomes and CSF NLRP3 or Inflammatory Cytokines in Anti-NMDAR Encephalitis

We also detected a positive correlation between peak mRS score and the level of CSF NLRP3 inflammasome in anti-NMDAR encephalitis patients ($p = 0.016$, $r = 0.429$, **Figure 1E**). Peak mRS score was positively correlated with IL-6 ($p = 0.005$, $r = 0.504$) and IL-17 ($p = 0.023$, $r = 0.403$). In the 13 re-examed anti-NMDAR encephalitis patients, we analyzed the correlation between the reduction of mRS score and the decrease of NLRP3 inflammasome, IL-1β, IL-6, and IL-17. There was a positive correlation between δmRS and the decrease of NLRP3 inflammasome ($p = 0.013$, $r = 0.641$, **Figure 1F**), but not IL-1β, IL-6, or IL-17.

DISCUSSION

It is still a challenge for clinicians to accurately diagnose CNS inflammatory diseases. Anti-NMDAR encephalitis is an inflammatory disease mediated by anti-neuronal antibody in the CNS. Patients presenting with characteristic progressive neuropsychiatric symptoms and non-specific evidence of CNS inflammation are usually diagnosed as anti-NMDAR encephalitis (22). Most importantly, since that early treatment contributed to a better prognosis, it's crucial to recognize anti-NMDAR encephalitis timely (23). Here, we sought to prove the diagnostic and prognostic value of NLRP3 inflammasome in anti-NMDAR encephalitis.

Some interesting studies have explored the relationship between NMDAR and NLRP3 inflammasomes, but the results are still controversial. In the murine model of NMDA-induced retinal excitotoxicity, activation of NMDAR can prime the NLRP3 inflammasome in a transcription-dependent manner (24). But in the models of acute hepatitis and pancreatitis, activation of NMDAR finally down-regulates NLRP3 inflammasomes via a β-arrestin-2 NF-kβ and JNK pathway (25). Under the stimulation of pathogen-associated

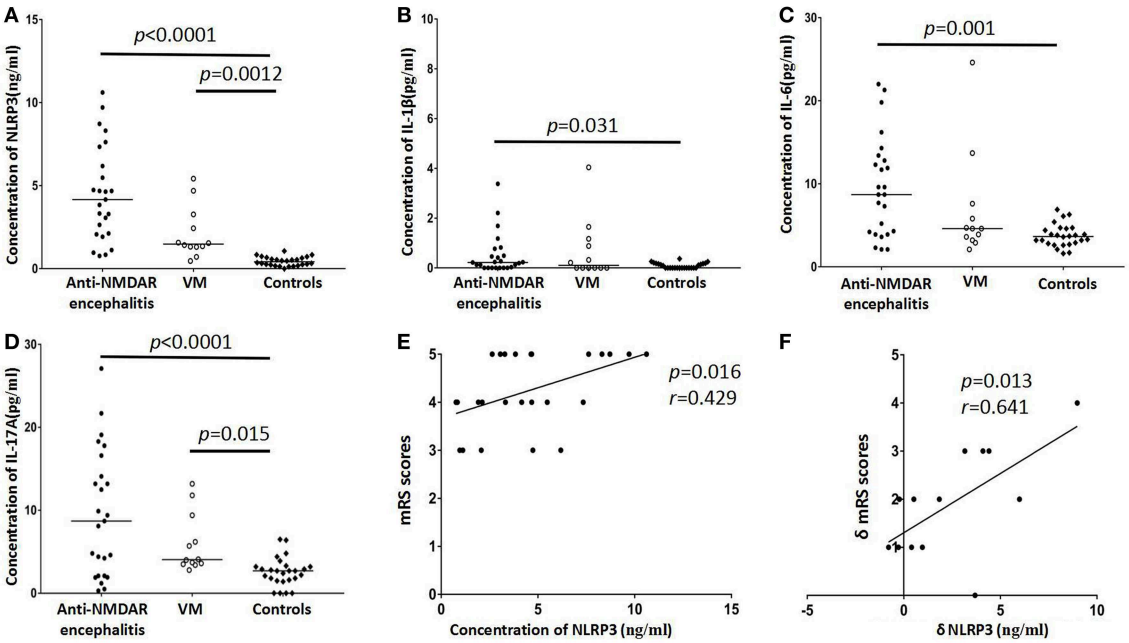

FIGURE 1 | Distribution of CSF NLRP3 inflammasomes and cytokines levels in anti-NMDAR encephalitis patients and controls groups [viral meningoencephalitis (VM) and non-inflammatory neurological diseases (Controls)]. Higher levels of CSF NLRP3 inflammasomes were found in anti-NMDAR encephalitis and VM patients **(A)**. CSF IL-1β levels of anti-NMDAR encephalitis patients had significant difference with controls **(B)**. CSF levels of IL-6 showed elevated in patients with anti-NMDAR encephalitis **(C)**. The levels of IL-17 were also significantly changed in anti-NMDAR encephalitis and VM patients **(D)**. The correlation between mRS score and CSF NLRP3 inflammasomes in anti-NMDAR encephalitis patients in acute stage **(E)**. The correlation between the δmRS and the decrease of CSF NLRP3 inflammasomes in patients with anti-NMDAR encephalitis in follow-up period **(F)**. The *p*-values and *r* values were indicated within figures. (mRS, modified Rankin Scale; VM, viral meningoencephalitis).

molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), NLRP3 inflammasome activates caspase-1, which subsequently leads to mutation IL-1β through the cleavage of pro-IL-1β and finally results in cascade inflammatory response (26). In the present study, we detected a significant increase of CSF NLRP3 inflammasomes and its downstream cytokines in anti-NMDAR encephalitis patients in acute stage, and a decrease in their respective levels during the remission stage. It suggested that NLRP3 inflammasomes might participate in the pathogenesis of anti-NMDAR encephalitis. In this disease, activated NLRP3 inflammasomes amplifies the inflammatory cascade response by activating downstream IL-1β and other inflammatory cytokines, resulting in brain damage, and neuropsychiatric symptoms. B cells have already been confirmed to take part in anti-NMDAR encephalitis (27). However, the mechanism concerning their entry into the CNS compartment, or the blood-brain barrier, is unclear. Some studies in other CNS autoimmune disease such as experimental autoimmune encephalomyelitis (EAE), have demonstrated that NLRP3 inflammasome promotes neuroinflammation by inducing the migration of Th1 and Th17 cell to CNS (28, 29). Mature IL-1β, which can be indirectly activated by NLRP3 inflammasome, was proved to be important in Th17 differentiation (30). Th17 cells produce IL-17 and IL-6, two pro-inflammatory cytokines that could regulate Th17/Treg (regulatory T cells) balance and were increased in antibody-mediated CNS disorders, such as neuromyelitis optical (NMO) (31, 32). Our study also found the increase of IL-6 and IL-17 in anti-NMDAR encephalitis patients, and there were positive correlations between the CSF NLRP3 inflammasome and IL-6 or IL-17. Both suggested that IL-17/IL-6 co-activation in anti-NMDAR encephalitis might be of great importance in the pathogenesis of anti-NMDAR encephalitis.

Many researches have demonstrated that the NLRP3 gene knockout animal could develop less neuroinflammation of CNS (26, 29). The NLRP3 inflammasome has been considered as the common cardinal pathology mediators in these diseases. Some drugs and chemicals such as anakinra, protein kinase A (PKA), minocycline and dimethyl sulfoxide have been proved to attenuate neuroinflammation by targeting pathways upstream and downstream of NLRP3 inflammasome signaling (33), which offer potential new therapeutics for these CNS autoimmune diseases, including anti-NMDAR encephalitis (11). Our study also revealed the increased CSF NLRP3 inflammasome as an indicator for the severity of anti-NMDAR encephalitis, so the future researches should focus on the attenuating effects of these potential therapeutical drugs in anti-NMDAR encephalitis *in vitro* or *in vivo*.

CONCLUSION

In this study, we found significant increases of CSF NLRP3 inflammasome, IL-1β, IL-6, and IL-17 in anti-NMDAR

encephalitis patients in acute stage compared with patients with VM as well as non-inflammatory disease controls. Moreover, in anti-NMDAR encephalitis patients, we found a positive correlation between peak mRS and CSF NLRP3 inflammasome, and the same with δmRS and the decrease of NLRP3 inflammasome. Our study suggested that the CSF levels of NLRP3 inflammasomes reflect the underlying neuroinflammatory processes in patients with anti-NMDAR encephalitis, which can act as an indicator for the severity and prognosis of anti-NMDAR encephalitis.

ETHICS STATEMENT

The study was conducted with the approval of the Ethics Committee of Nanfang Hospital, Southern Medical University (NFEC-2018-095).

AUTHOR CONTRIBUTIONS

HW conceptualized the study and designed the experiments. H-YS and SuP participated in the research design. BL, DZ, ZW, TJ, H-YS, and ShP collected the CSF samples and clinical data. YP analyzed the data and wrote the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Primary Angiitis of the Central Nervous System: New Potential Imaging Techniques and Biomarkers in Blood and Cerebrospinal Fluid

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Primary angiitis of the central nervous system (PACNS) is an inflammatory brain disease affecting the medium and small vessels of the CNS. Although recent data of patients with PACNS have advanced the understanding of the disease, the diagnosis remains challenging. Clinical presentation of PACNS is broad and unspecific and the majority of the diagnostic approaches are hallmarked by a low specificity. Thus, PACNS is commonly misdiagnosed. In addition, due to its potential aggressive course which may be altered by an adequate immunosuppressive treatment, delineation from other vasculopathies and PACNS mimics is crucial. New diagnostic tools and biomarkers which increase specificity and facilitate the diagnosis for patients with suspected PACNS are highly desirable. This short review summarizes the current procedures within the diagnostic process and aims to illustrate its difficulties and challenges. Furthermore, it highlights emerging biomarkers in the cerebrospinal fluid and peripheral venous blood as well as novel potential imaging tools that may corroborate the diagnosis. With new imaging techniques and a panel of biomarkers the certainty of the diagnosis may be increased and diagnostic processes more accelerated in the future.

Keywords: PACNS, biomarker, vasculitis, cerebrospinal fluid, peripheral venous blood, imaging, circulating endothelial cells

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INTRODUCTION

Primary angiitis of the central nervous system (PACNS) is a poorly understood disease which is restricted to the small- and medium-sized vessels of the brain and spinal cord. It is a rare disease with an estimated incidence of 2.4 cases per 100,000 person-years and an equal frequency in women and men (1). All age groups may be affected, whereas 50% are 37–59 years of age. Of note, PACNS is also a rare cause of stroke and remains an important differential diagnosis in younger (<45 years) adults (2.2%) (2, 3). Diagnosis is extremely hampered by the paucity of specific clinical symptoms and by the low specificity of the diagnostic approaches. Thus, establishing a reliable diagnosis remains challenging and PACNS is often misdiagnosed. In addition, two different subtypes of the disease have been reported, the small (SVV) and medium vessel variant (MVV), with different diagnostic phenotypes which increases the diagnostic challenge. While the MVV shows remarkable vascular irregularities in angiography procedures, current angiography techniques do not have a sufficient resolution to detect vessel abnormalities if the disease is limited to a small-vessel involvement. Moreover, since aggressive immunosuppressive therapy is mandatory to avert the

often fatal course of the disease, exclusion from other etiologies is crucial. The aim of this short review is to give an overview of the current diagnostic procedures in PACNS and emphasize its difficulties. It further highlights new potential biomarkers in the cerebrospinal fluid (CSF) and peripheral venous blood as well as imaging markers that may increase the diagnostic accuracy.

CURRENT DIAGNOSTIC APPROACHES

The clinical presentation of PACNS is broad and unspecific. Thus, the average time to diagnosis may be several months (4). The symptoms vary from cognitive dysfunction, subacute, and progressive headache, seizures to focal neurological symptoms (5). Amongst others, the differential diagnosis includes e.g., hemorrhagic or ischemic stroke, neoplasia, encephalitis, dementia, migraine, multiple sclerosis. One of the most important differential diagnosis is the reversible cerebral vasoconstriction syndrome (RCVS) which bears the closest resemblance to PACNS with similar abnormalities in angiography. Recent studies provided new insights in diagnosing and distinguishing RCVS from PACNS by taking into account the patients' variables on admission (clinical symptoms, initial brain imaging etc.) (6, 7). However, diagnosis and distinction from this non-inflammatory vasculopathy is still difficult. In 1988, Calabrese and Mallek (8) proposed diagnostic criteria for PACNS which include a history or presence of a neurological deficit unexplained by any other cause after a thorough examination, evidence of vasculitis either by histopathology or angiography with characteristic changes of vasculitis and exclusion of a systemic vasculitis or any other condition to which the angiographic changes can be secondary. Birnbaum et al. (9) modified these criteria and differentiated between a "probable" and "definite" diagnosis. While definite PACNS is defined by histopathological evidence, all other cases are categorized as suspected PACNS. These revised criteria aimed at preventing inadequate and uncontrolled application of immunosuppressive treatment. To date, the diagnostic workup encompasses extensive laboratory examinations including an immunological screening and CSF analysis, MRI, angiography, and histopathology. Most of the tests are done to rule out differential diagnosis, such as systemic vasculitis (autoimmune or infectious), non-inflammatory vasculopathies (e.g., RCVS), or malignant disorders (e.g., lymphoproliferative diseases). Most of the patients (80–90%) show a pleocytosis (about 10–20 cells/ml) and/or increased protein levels (about 120 mg/ml) (4). However, these abnormalities in CSF are unspecific and, of note, an initial normal CSF does not exclude the diagnosis (10). In addition, there is currently no evidence of differences in CSF findings between the two disease variants. For brain imaging in PACNS, MRI remains the modality of choice. Fluid attenuated inversion recovery (FLAIR)—imaging, T1- and T2-weighted imaging, diffusion-weighted imaging (DWI), susceptibility-weighted imaging (SWI), time–of-flight MR angiography (TOF) and contrast medium-enhanced imaging are required sequences. Most of the patients with PACNS (90–100%) show abnormal MRI findings (9, 11). Typical changes include diffuse white and gray matter changes, contrast medium enhanced parenchymal lesions, leptomeningeal enhancement (12), parenchymal hemorrhages as well as microbleeds (13, 14). Tumor-like mass lesions have also been reported (15). Even a generalized atrophy in the MRI scan can be a sign of PACNS (16). Furthermore, MR angiography (MRA) can be applied. While in patients with SVV brain vessels in MRA are commonly unremarkable, vessel abnormalities are likely to be detected in MVV. Typical irregularities comprise peripheral alternating stenosis/occlusions and dilatation as well as vessel wall thickening in different vascular territories. However, none of these findings are specific, but a normal MRI scan in combination with a normal CSF result rule out the diagnosis of PACNS (17, 18). The current gold standard for vessel imaging in PACNS is digital subtraction angiography (DSA). Its reported overall sensitivity varies between 40 and 90% (4, 19, 20). This high range is most likely explained by the different subtypes of the disease (21, 22). Whereas, DSA may reveal remarkable vascular irregularities in patients with a predominant MVV, it shows unconspicious vessels in SVV. Currently, DSA has only a resolution for vessels $>500\,\mu m$ in diameter (23), thus, the SVV is typically "angiography-negative." In addition, despite having a higher sensitivity in distal vessels and the posterior circulation (24) compared to MRA, the DSA provides information on changes in vessel contours only and not on the underlying pathological process (25). In particular, distinguishing non-inflammatory vasculopathies (e.g., RCVS or artherosclerosis) remains a challenge. Hence, DSA also has a low specificity. To date, brain biopsy is the only eligible technique to establish a definite diagnosis of PACNS. An optimal biopsy should contain samples from the dura, leptomeninges, cortex and white matter (26). However, the sensitivity of brain biopsy is also low (54–83%) (20, 21, 27). This broad range may be explained by the different case series including SVV and MVV. Notably, about one fourth of all autopsy-documented cases of PACNS were false-negative (28). In particular, in patients with MVV histopathology is frequently false-negative, probably due to abnormalities of medium-sized vessels that are rather detectable by angiography but not covered by brain biopsy (22). The rate of positive results can be increased by targeting MRI positive lesions (18). Three histopathological patterns are known in PACNS so far: granulomatous (58%), lymphocytic (28%) and necrotizing (14%), but they often overlap. The amyloid-ß angiitis (ABRA) with ß-amyloid depositions in the brain vessel walls is considered to be a subset of the granulomatous pattern. To date, no clear difference in clinical symptoms, aggressiveness of the disease, or treatment response between these patterns has been reported.

Due to the variable clinical presentation of PACNS, the lack of specific diagnostics and its differences between SVV and MVV (diverse DSA/MRA results, diverse biopsy results if derived from the right frontal lobe) a definite diagnosis is still a challenge. Additional diagnostic approaches are required to increase certainty. Thus, in the following we focus on potential, novel diagnostic imaging techniques and biomarkers in the CSF and blood to diagnose PACNS.

EMERGING NEW DIAGNOSTIC BIOMARKERS

Interleukin-17 in the Cerebrospinal Fluid

A promising marker for PACNS in the CSF is interleukin-17 (IL-17). IL-17 is a pro-inflammatory cytokine and a potent mediator in cellular immunity. It plays a pivotal role in the pathogenesis of systemic vasculitis, in particular, antineutrophil cytoplasmatic antibodies (ANCA)-associated vasculitis (AAV), or giant cell arteritis (29). Previously, we reported that IL-17 produced by CD4+-T-cells in the CSF was elevated in patients with PACNS (sensitivity 73%, specificity 100%) (**Figure 1A**) (30). Elevated IL-17 levels were persistent in patients with active PACNS and patients in remission, thus indicating IL-17 being a more specific biomarker of cerebral vasculitis than the cell count or protein elevation in the CSF and being crucial also in the pathogenesis of PACNS. These findings merit the attempt of making IL-17 a target for novel therapeutic interventions. Interestingly, humanized anti-IL-17 antibodies as a treatment approach were already shown to induce clinically relevant responses in patients with psoriasis and rheumatoid arthritis (31). However, the promising results of IL-17 analyses in PACNS need to be verified in larger patient cohorts.

Amyloid-beta A4 Protein (APP) in the Cerebrospinal Fluid

Mass-spectrometry-based techniques allow the assessment of a significant fraction of the proteome in biofluids without a pre-selection of target proteins. Ruland et al. reported a significantly lower abundance of proteins in the CSF of patients with PACNS (32). In particular, reduced APP concentrations were detected, a protein indicative for nervous system damage or pathology. Based on these findings the authors speculate that this protein may serve as a surrogate marker of brain injury in cerebral vasculitis. Further studies will have to validate these findings.

Circulating Endothelial Cells in the Peripheral Venous Blood

Circulating endothelial cells (CECs) are known as markers of endothelial damage (33, 34). Inflammatory or non-inflammatory endothelial damage or mechanical injury can lead to the detachment of endothelial cells from the vessel wall. Thereafter, CECs can easily be detected in the blood by immunomagnetic isolation (**Figure 1B**) or flow cytometry (35). Preliminary studies of AAV showed increased CEC levels in patients with an active disease (36) whereas the number of CECs decreased under successful immunosuppressive treatment. Likewise, lower numbers of CECs were detected in patients with non-inflammatory vascular diseases, such as stroke and myocardial infarction (34, 37, 38). Of note, similar results were observed in patients with PACNS. We demonstrated that CECs were significantly elevated in patients with active PACNS while being decreased under successful immunosuppressive medication, in healthy controls and patients with stroke and cerebrovascular risk factors (39). Hence, CECs may potentially contribute to the diagnosis in biopsy negative cases and monitor the success of immunosuppressive treatment. However, although being a promising biomarker, studies with larger patient numbers are required to verify these results.

High-Resolution Magnetic Resonance Vessel Wall Imaging (HR-MRI)

Commonly used imaging modalities fail to distinguish between inflammatory and non-inflammatory vasculopathies. Preliminary studies demonstrated the possibility of outlining the vessel wall by contrast enhancement (40–42). In enhanced MR imaging of the vessel walls ("dark blood imaging") the signal of the blood is suppressed and discrimination of the vessel wall from the lumen is increased (**Figure 2**). This HR-MRI can be performed as T1-weighted, T2-weighted or proton density-weighted sequences (43). This technique has already been used to distinguish PACNS from other vasculopathies (40, 42). In more detail, in PACNS a predominantly smooth, concentric, and long-segment wall thickening with strong enhancement and a perivascular edema were observed (44). Arteriosclerotic plaques exhibit a more eccentric, irregular, and short-segment wall thickening without perivascular edema and only mild enhancement depending on composition and activity of the plaque (25, 42). However, the distinction might still be difficult and this MRI sequence might rather help to differentiate PACNS from vasculopathies of young adults such as RCVS (41, 44). Notably, HR-MRI is merely capable of detecting the MVV with its current capacity of vessel resolution. However, this imaging technique is quickly developing and might soon lead to an improved discrimination between the MVV of PACNS and other intracranial vasculopathies (45). Of note, with higher resolutions it will be able to detect changes even in small vessels. Given that treatment can also affect the imaging findings, e.g., less enhancement under immunosuppressive or antiviral therapy (43, 46), it might also have the potential to monitor disease activity, which would make it extremely valuable for clinical trials (43, 47).

POTENTIAL NEW DIAGNOSTIC BIOMARKERS

Endothelial Progenitor Cells in the Peripheral Venous Blood

Another potential biomarker in PACNS are the endothelial progenitor cells (EPCs). In contrast to CECs, EPCs are considered to play a pivotal role in vascular regeneration and endothelial renewal (34). They are bone marrow-derived cells and originate from hematopoietic progenitor cells. EPCs are believed to be mobilized by various factors (e.g., vascular endothelial growth factor, VEGF), migrate to the site of endothelial injury, integrate in the endothelial cell layer and differentiate to mature endothelial cells (48). Interestingly, an inverse relationship of CECs and EPCs in patients with systemic vasculitis was previously reported (49). CEC values were highly elevated immediately after endothelial injury and decreased over time. In contrast, the EPC counts were low at time of endothelial injury and increased in the equal time course. Notably, similar results were observed in patients with Kawasaki disease, acute

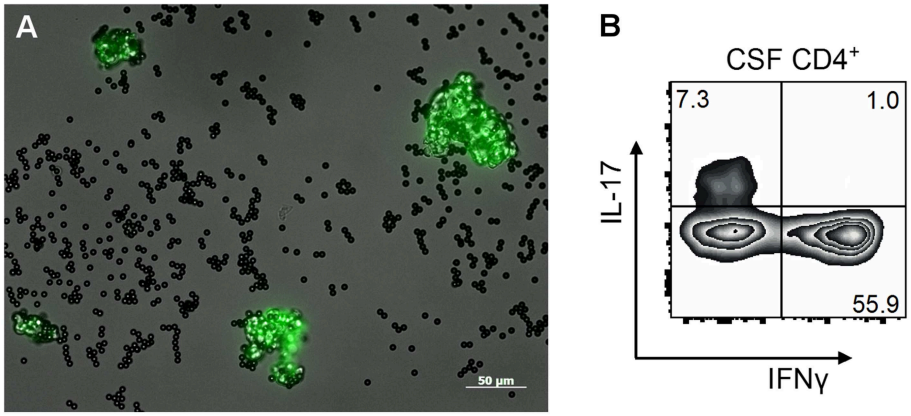

FIGURE 1 | (A) Morphology of circulating endothelial cells (CEC, green) with Dynabeads™ (black) attached after immunomagnetic isolation from peripheral blood of a patient with PACNS displayed under a fluorescence microscope. CECs were isolated by CD 146-coated Dynabeads™ and with a fluorescence-coated (FITC) lectin (*Ulex europaeus agglutinin 1*, UEA-1) in an endothelial cell specific double-staining method. Afterwards CECs were enumerated in a counting chamber using a fluorescence microscope. **(B)** FACS plot of expanded T helper cells from a patient with biopsy proven PACNS. CSF cells were expanded in the presence of feeder cells, IL-2 und PHA for 2 weeks and an intracellular cytokine staining for IL-17 and IFNγ was performed.

coronary syndrome and ischemic stroke (50–52). Hence, EPCs are presumed to be a marker for vascular or endothelial repair in inflammatory and non-inflammatory vasculopathies. However, EPCs have heretofore not been investigated, but these findings warrant the analysis of EPCs in PACNS.

Von Willebrand Factor Antigen in the Peripheral Venous Blood

The von Willebrand factor antigen (vWF-Ag) is a plasma protein which is mainly synthesized from endothelial cells. Its function includes platelet aggregation and adhesion. If endothelium is damaged, e.g., by inflammation, increased levels of vWF-Ag may then be detected in blood. In fact, vWF-Ag was previously suggested as a potential biomarker in patients with systemic vasculitis. In patients suffering from Behçet disease and AAV, vWF-Ag levels were increased compared to controls (53–55). However, these elevated levels were observed in an active stage of disease and remained increased under remission. In contrast, in patients with Granulomatosis with Polyangiitis and Kawasaki disease vWF-Ag levels correlated well with disease activity (56, 57). Likewise, a recent study demonstrated that vWF-Ag may be a marker for disease activity in childhood PACNS (58). Elevated levels were detected in active disease and normalized under successful treatment. These results justify further analyses in adult PACNS.

High Resolution Digital Subtraction Angiography

High resolution digital subtraction angiography including a 3D rotation technique provides a visualization of smaller vessels than with commonly used angiography techniques. A recent study demonstrated that this tool can display small branches (e.g., perforators) of the basilar artery (59). Fukuda et al. (60) described a fusion technique using two three-dimensional DSA images with precise information of the angio-architecture of

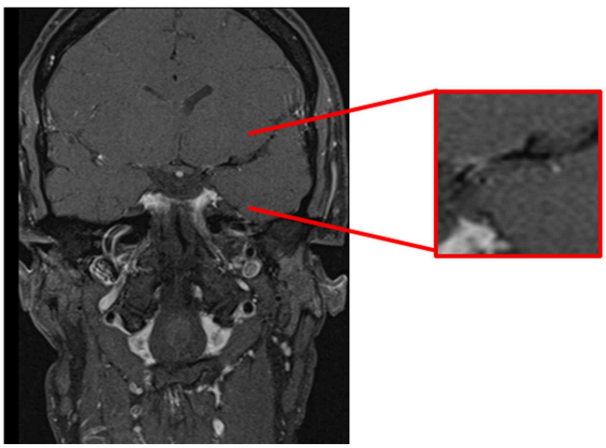

FIGURE 2 | Cranial MRI, coronal T1-weighted-(dark-blood) sequence demonstrating contrast enhancement of the vessel wall in the first segment of the left middle cerebral artery.

arterial-venous malformations. The exact structure and location of the fistula, feeders, and drainers with high spatial resolution were obtained. Another novel technique to visualize small vessels beyond the spatial resolution of commonly used imaging modalities is the time-resolved 3D rotational angiography (4D DSA) (61). In particular, for proximal stenosis of the middle cerebral artery local collateral networks can be displayed and microangio-architecture in dural arteriovenous fistulas (dAVF) or arteriovenous malformations (AVM) can be visualized (62). Notably, 4D DSA might reduce the radiation and contrast agent dose and can be less time consuming compared to commonly used methods. In summary, novel imaging techniques in DSA could potentially provide more detailed information on smaller cerebral vessels due to higher spatial resolution. These imaging sequences were applied to studies of AVMs, dAVFs and

aneurysms so far. They might also be applied to the SVV of PACNS to further increase the diagnostic specificity.

Positron Emission Tomography (PET)

Fluor-desoxy-glucose (FDG)-PET is a functional imaging technique in nuclear medicine. It uses FDG as the radiopharmaceutical and depicts metabolic activity in the body by visualization of glucose uptake in tissues. Hence, inflamed vessel walls can be uncovered. In fact, in patients with biopsy proven giant cell arteriitis, FDG-PET was successfully applied and showed a high sensitivity and specificity (63). In addition, Novikov et al. demonstrated that PET can be a valuable diagnostic tool in patients with medium vessel involvement (64) and, thus, may contribute to depict inflammation of the vessel walls in patients with inconclusive diagnostic results (65). Of note, FDG-PET was previously applied to follow up and monitor disease activity in vasculitis (66, 67). However, small-vessel involvement cannot be detected by this imaging modality (68).

CONCLUSION

This short review summarizes current diagnostic procedures applied to primary CNS vasculitis and aims at illustrating the difficulties and challenges in diagnosing PACNS. It further presents novel emerging and potential biomarkers which might facilitate the diagnostic process in the future. New biofluid and imaging markers may increase the certainty of the diagnosis. Biomarkers in the CSF (IL-17, APP) and peripheral venous blood (CEC, EPC, vWF-Ag) seem to be promising approaches, although potential differences in results between SVV and MVV remain to be determined. The ultimate goal would be to identify a panel of markers that might increase the diagnostic accuracy in PACNS in the future. In addition, novel potential imaging techniques, e.g., PET or different high resolution DSA-based techniques, can visualize smaller vessels or inflammatory vessel walls and, therefore, might support the diagnostic work. Data on these diagnostic tools are currently preliminary and, thus, need to be verified in larger patient cohorts or still need to be investigated in adult patients with PACNS. However, they might advance the diagnostic workflow in the future and aid to an accelerated diagnosis in patients lacking histological evidence, hence, in patients with suspected disease. They are promising diagnostic approaches that justify future attempts of investigation.

AUTHOR CONTRIBUTIONS

MD-C: substantial contribution to the conception and design of the work; acquisition, analysis and interpretation of data for the work; drafting the work and revising it critically for important intellectual content. SS: interpretation of data for the work; revising it critically for important intellectual content. VH: analysis and interpretation of data for the work; revising it critically for important intellectual content. CG: analysis and interpretation of data for the work; drafting of the work and revising it critically for important intellectual content. GT: analysis and interpretation of data for the work; drafting of the work and revising it critically for important intellectual content. TM: conception and design of the work; analysis and interpretation of data for the work; drafting of the work and revising it critically for important intellectual content. All authors provide approval for publication of the content. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Single-Cell High-Throughput Technologies in Cerebrospinal Fluid Research and Diagnostics

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High-throughput single-cell technologies have recently emerged as essential tools in biomedical research with great potential for clinical pathology when studying liquid and solid biopsies. We provide an update on current single-cell methods in cerebrospinal fluid research and diagnostics, focusing on high-throughput cell-type specific proteomic and genomic technologies. Proteomic methods comprising flow cytometry and mass cytometry as well as genomic approaches including immune cell repertoire and single-cell transcriptomic studies are critically reviewed and future directions discussed.

Keywords: cerebrospinal fluid (CSF), RNA sequencing (RNAseq), repertoire sequencing, single cell gene expression, mass spectrometry, flow cytometry

INTRODUCTION

Since its inception by Heinrich Quincke (1), lumbar punctures and cerebrospinal fluid (CSF) analyses have become invaluable diagnostic tools in the clinical care of neurological patients. Early-on, microscopic examination of CSF cells was included in the work-up and facilitated the diagnosis of inflammatory and tumorous diseases of the central nervous system (CNS). Quincke subclassified CSF cells into leukocytes, red blood cells and epithelial cells (2). Routine work-ups include cell counts and detailed microscopic examinations with cells spun onto glass slides and characterized by May–Gruenwald–Giemsa stain allowing differentiation of red blood cells, lymphocytes, monocytes, granulocytes, and detection of malignant cells (**Figure 1**). Red blood cells and leukocytes can be further assessed for activated cellular states (plasmablasts, activated macrophages), and associated with certain diseases (erythrophages, siderophages, lipophages) (3). Introduction of labeled antibodies against cell-specific antigens in the 1960s allowed detailed analysis on slide-bound CSF cells by immunofluorescence and enzyme-linked immunocytochemistry (4–6). However, traditional microscopic assessment exhibits several limitations: (i) microscopic examinations are supervised, investigator-biased, and must be carried

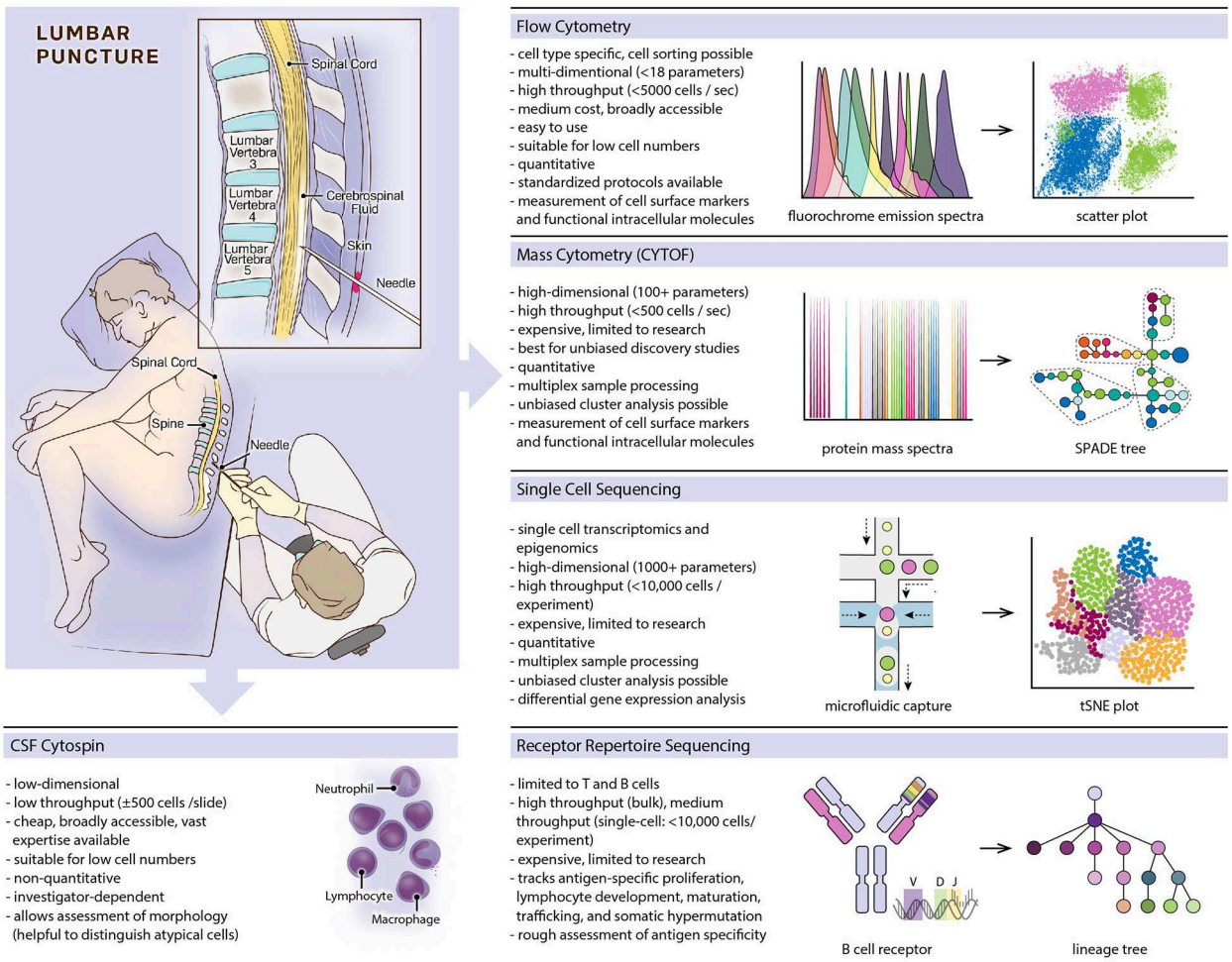

FIGURE 1 | Overview of featured single-cell methods, listing prominent characteristics of each method and providing schematic depictions of methodological workflows and typical data visualizations.

out by experienced personnel; (ii) throughput is low as specimens are spun separately on single slides; (iii) sensitivity is low, in particular for rare cell populations; (iv) quantitative analyses are challenging (**Figure 1**).

Here, we focus on next-generation high-throughput technologies allowing cell-type specific analyses with high accuracy in a fast and quantitative manner. Currently, most methods are used in research requiring expensive equipment and experience in sample preparation and computational data analysis. Focused efforts are necessary to translate findings from exploratory research into clinical practice, making those high-throughput methods broadly accessible. Obstacles include low cell counts and a short life span of CSF cells, posing challenges for biobanking. Study inclusion, sample collection, quality check (e.g., blood cell contamination, RNA integrity), and sample processing must be done quickly according to standardized protocols. Hence, we advocate for including novel single-cell technologies in future studies enabling their use on a broader scale and thereby increasing the relevance of CSF cytology in clinical settings.

Profiling CSF Cell Heterogeneity by High-Throughput Flow Cytometry

Multicolor flow cytometry was developed in the 1960s (7), became available for broader use in the 1970s, and revolutionized immunological research, biomarker development, and clinical diagnostics (8, 9) (**Figure 1**). Fluorescently labeled cells get excited by multiple lasers, and the detection of fluorochrome emissions allows a multi-parameter cell-type specific characterization. Modern cytometers can detect up to 18 fluorochromes in parallel and process several thousand cells per second. In addition to cell surface markers, intracellular molecules can be detected, revealing functional cellular states like influx of ions, expression levels of transcription factors, phosphorylation states, and cytokine levels (10, 11). Fluorescence-activated cell sorting partitions cell populations for downstream analyses including RNA sequencing and cell culture assays.

Flow cytometry has been implemented for detailed cell analyses including T cell counts in HIV, immunophenotyping in immunodeficiencies, hematological malignancies, and during

stem cell transplantation (12, 13). Flow cytometric CSF analysis still lags behind due to high variabilities in cell populations, lack of disease-specific cell markers, and low cell counts in many neurological diseases. False-negative results are common in disorders with only subtle increase in cell numbers, but higher sensitivities can be achieved by increasing CSF volumes and repeated measurements (14, 15). CSF cell populations in healthy individuals are relatively uniform, however, differ significantly from cell distributions in blood (16–18). T cells are the most abundant cell type in the CSF, the CD4/CD8 ratio is skewed toward $CD4^+$ (CSF: 3 vs. blood: 2.1), and $CCR7^+$ central memory T helper subtypes are the dominating phenotype (∼90% of the CD3+CD4+ T cell population), suggesting an important role in immune surveillance of the CNS under healthy conditions (17); granulocyte, B and NK cell counts are low (<1%) (19).

Most validated disease-specific flow cytometry panels are currently used in primary CNS lymphomas supplementing microscopic cytology and adding a high positive (92%), however, low negative predictive value (52%) (15, 20). So far, studies have not determined predictive values of CSF flow cytometry in non-malignant diseases preventing its use in routine diagnostics of neuroinflammatory, neurodegenerative, and neurovascular disorders. However, many exploratory studies have described disease-specific features, and more advanced granular flow panels will help establish flow cytometry as a valid diagnostic tool.

Elevated CD4/CD8 ratios have been described in stroke, Guillain-Barré syndrome and multiple sclerosis (MS) and low CD4/CD8 ratios in HIV. B cells and activated plasmablasts are elevated in infectious conditions including HIV and Lyme disease as well-autoimmune diseases like MS (21, 22), while monocyte counts are low in these diseases but elevated in glioblastoma patients (23, 24). Notably, NK cells have been reported to be elevated in patients with viral meningitis (24). Several studies on inflammatory diseases have used flow cytometry for more precise phenotypical profiling of T cell subsets, such as $CD8^+$ cytotoxic or γδ-TCR-positive T cells (25–29), and NK cells (17, 19, 30), and some studies could correlate findings to treatment responses or disease progression (31, 32). CSF cells in primary neurodegenerative diseases are less well-studied (vs. proteins such as neurofilaments, tau, and amyloid that are enriched in the CSF) with some studies suggesting leukocyte activation in neurodegenerative disorders. For example, $CD8^+$HLA-DR^+ activated T cells correlate with neurocognitive decline in patients with Alzheimer's disease (33). Despite ample evidence that an active immune response contributes to neuronal damage after ischemic stroke, CSF flow cytometry seems to be of limited value in CNS ischemia. A larger flow cytometry study recently reported a slight increase in cell numbers without differences in cell distributions when compared to healthy individuals, irrespective of stroke size and location (34). In summary, exploratory studies have defined flow cytometry panels for several neurological diseases. CSF flow cytometry is particularly valuable in oncological diseases, followed by inflammatory and infectious disorders. Correlations between cell status and clinical outcome can provide meaningful support for neurological diagnosis and patient care. Defining additional granular marker panels will likely increase its relevance and justify a broader use in CSF diagnostics in the future.

Characterizing CSF Cell Subsets by High-Dimensional Mass Cytometry

Mass cytometry (cytometry by time of flight, CYTOF) is related to flow cytometry but uses metal ion labels instead of fluorochromes. Individual cells vaporize in inductively coupled argon plasma with metal ions getting ionized and introduced into a time-of-flight (TOF) mass spectrometer allowing to distinguish isotopes by a single atomic mass units (35). With virtually no overlap between mass spectra, multidimensional data acquisition of more than 100 parameters per cell is possible (usually in the range of 30 to 60) allowing a throughput of up to 500 cells per second (**Figure 1**). Data deconvolution algorithms provide solutions for dimensionality reduction and clustering. Common methods include principal component analysis (PCA) (36), t-distributed stochastic neighbor embedding (t-SNE) (37), uniform manifold approximation and projection (UMAP) (38), spanning-tree progression analysis of density-normalized events (SPADE) (36) and cluster identification, characterization, and regression (CITRUS) (39). With the expansion of simultaneously detected parameters, cell characterization is possible at an unprecedented granular level, and intracellular molecular labeling further enables dynamic monitoring of functional markers that add mechanistic insight to descriptive cellular states (40, 41), and even simultaneous measurement of specific RNA and protein expression levels in single cells being possible (42).

CYTOF has been utilized to map the cellular landscape of neuronal, glial and immune cells in rodent brains. For example, CD44 was identified as a potential marker for infiltrating leukocytes, border-associated macrophages could be distinguished from microglia and dendritic cells, and a new $CD317^+MHCII^+CD39^+CD86^+$ microglia subset was identified in neurodegenerative and inflammatory models (41, 43, 44). Protocols to dissociate and measure tumor cells and tumor infiltrating leukocytes from glioma have been tested, and larger CYTOF studies from human brain tumor tissue can be expected soon (45). Recently, peripheral blood mononuclear cell (PBMC) populations of glioblastoma and narcolepsy patients have been studied by CYTOF (40, 46) and highlighted the role of immune cells. However, due to low cell counts and freeze-storing challenges, CYTOF studies have not yet been performed on CSF. CYTOF is more expensive and challenging than flow cytometry with computational expertise necessary to evaluate high dimensional data. Currently, it is still an research tool, but the myriad of investigated parameters can be condensed to a focused set of cellular markers to be adopted for flow cytometry and used to design cell-specific therapies.

Understanding CSF Lymphocyte Diversity by Immune Repertoire Sequencing

B and T cell receptors (BCR and TCR) exhibit unique genetic characteristics that can serve as natural markers of the adaptive immune system. BCR and TCR are specialized cell surface receptors on B and T lymphocytes, respectively, determining

adaptive immune responses and immune memory (**Figure 1**). Soluble BCRs are secreted as immunoglobulins, which opsonize free antigens and activate complement factors as well as innate immune cells. Cross-ligation of the membrane-bound BCR by antigens triggers B cell activation and proliferation. T cells detect specific antigens via TCRs when presented on major histocompatibility complexes (MHC) by antigen-presenting cells. During lymphocyte development, the genes coding for each lymphocyte's BCR and TCR rearrange and mutate, resulting in an astounding diversity of 10^{13}-10^{18} possible BCRs and TCRs (47–49), although the realized lymphocyte repertoire of an individual is several magnitudes smaller (50). High diversity is needed to defend against a vast number of possible pathogens. B cells (but not T cells) continue to mutate their BCR upon B cell activation, striving to further increase affinity to its cognate antigen in a process called somatic hypermutation. BCR and TCR gene signatures are unique to each lymphocyte and passed on to descendant cells. The entirety of a person's BCR and TCR sequences comprises the immune repertoire, which can be studied using DNA or mRNA next-generation sequencing methods (51–53). Repertoire analysis is challenging because high sequence variabilities complicate alignments to germline sequences. It therefore requires rigorous validation to differentiate mutations from sequencing errors. As each lymphocyte carries one unique receptor sequence, single-cell conclusions can be drawn even from bulk-sequencing experiments. However, single-cell sequencing is needed to describe a receptor in its entirety, as each receptor consists of two hetero-dimerizing protein chains (53). Direct inference of an antigen from the receptor sequence is currently not possible, however, new methods allow clustering TCRs with similar antigen-specificities based on predicted structures of antigen binding sites (54). Repertoires provide valuable information about lymphocyte development and maturation, somatic hypermutation, lymphocyte trafficking (55, 56), and malignant transformations (57). Hence, several studies have suggested the use of repertoires as disease-specific biomarkers in MS, CNS lymphomas, and other neurologic diseases (58–60).

B cell repertoire sequencing has recently attracted major attention in MS when clinical trials using B cell depleting therapies showed enormous efficacy (61, 62). Studies comparing CSF, blood, lymph nodes, and meningeal B cell follicles have suggested that B cells mature in secondary lymphoid organs and traffic across the blood brain barrier as switched memory B cells and plasmablasts (55, 56, 63). Other repertoire studies demonstrated overrepresentation of heavy chain V gene family 4 (VH4) in the CSF of MS patients, likely as a result of chronic antigen-specific B cell activation and proliferation. Specific VH4 genes together with a set of characteristic mutations were proposed as an experimental biomarker for MS (58, 60, 64, 65). Besides MS (66–69), BCR and TCR repertoire sequencing of CSF lymphocytes have been performed to tackle similar questions in other neuroimmune diseases including NMDA and LGI1-antibody positive encephalitis (70, 71), Rasmussen encephalitis (72) and glioma (73). However, larger studies are needed to recommend CSF repertoire sequencing to be used in clinical neuroimmunology.

Dissecting CSF Cellular and Molecular Heterogeneity by Single-Cell Genomics

Single-cell sequencing has emerged rapidly over the last years and provides multi-dimensional and high-throughput possibilities to study cell-type specific diversity based on cellular transcriptomes (**Figure 1**). Plate-bound (several 100 cells per experiment), droplet-bound, and multifluidic-based (several thousand cells per experiment) methods provide sequencing depths of ∼1,000 to ∼6,500 genes per cell (74, 75). Single-cell RNA-sequencing (scRNA-seq) can be performed using both fresh cell suspensions from liquid and solid tissue samples (76–79) as well as isolated nuclei from frozen material with well-preserved RNA (80–82). scRNA-seq allows studying the entire transcriptome in an unbiased manner, dissecting both cellular diversity and molecular transcriptomic changes in individual cells. This becomes an extremely powerful tool when identifying disease-related cell populations or performing repeated sampling during the course of a disease.

scRNA-seq had great influence on immunological research by enabling the identification of specific immune cell subtypes and fostering our understanding of cellular diversity and cell-type specific regulation patterns (78, 79, 83–85). Recently, elegant computational algorithms have successfully inferred BCR and TCR repertoires from scRNA-seq data (86–88). While single-cell genomic methods have been successfully applied to solid tissues using animal models and human pathologies including glioma and MS (82, 89–91), it yet has only been the subject of very few scRNA-studies focusing on HIV (92) and MS (93) suggesting the presence of disease-specific myeloid (HIV) and T follicular helper cell (MS) subtypes in the CSF.

A broader availability and a wider use of scRNA-seq have so far been impeded by high costs of reagents and the need for computational expertise to run standardized high-performance analyses. Also, due to the relatively low cell number in non-infectious CSF preparations, high-throughput scRNA-seq technologies have been restricted to solid tissue or liquid biopsies like blood, where high cell numbers are available. Novel multiplex approaches, however, can significantly reduce costs and overcome challenges related to low CSF cell number input by barcoding and pooling cells from different individuals to be distinguished in retrospect during data analysis. In an effort to improve multiplex approaches, natural genomic variations, such as single-nucleotide polymorphisms (SNPs), can be exploited to exclude droplets containing more than one cell based on their inter-individual genomic signature (94). Another multiplex assay uses lipid-tagged indices to identify cells from different individuals and applies this method to single-cell preparations (95).

In addition, single-cell epigenetic technologies were recently developed that sequence the open chromatin landscape of individual cells. These methods, which include ChIP-seq (chromatin immunoprecipitation DNA-sequencing) and ATAC-seq (assay for transposase-accessible chromatin using

sequencing) (96, 97), have become powerful tools to profile immune and tumor cell subsets in health and disease, in particular when used with other methods like single-cell repertoire sequencing (98, 99). Combinations of single-cell genomic methods in conjunction with high-throughput multiplex strategies will change biomedical research dramatically in the near future and help dissect cellular heterogeneity and cell-type specific gene regulation and expression in an unprecedented way (100).

CONCLUSION AND FUTURE DIRECTIONS

We introduce four high-throughput multi-parameter technologies and advocate for their implementation in CSF cell diagnostics to gain a deeper understanding of cellular, proteomic, and transcriptomic changes on a single-cell level. The unparalleled depth of these methods allows researchers to describe precise cellular landscapes of organ systems in health and disease, characterize specific cell subsets in vast detail, perform network analyses in complex cellular systems, and suggest new cellular biomarkers for pathologies (41, 85, 101). Currently, only flow cytometry has been introduced in routine clinical CSF diagnostics. However, its relevance is often limited and larger datasets with standardized protocols are needed to maximize its contribution to CSF diagnostics. Mass cytometry, repertoire sequencing, and single-cell transcriptomics/epigenomics are still experimental methods, ideally suited to gain detailed unbiased overviews and to provide critical insight into disease mechanisms. Large high-dimensional datasets derived from these methods need to be condensed to focused marker sets that can be measured routinely. Notably, additional single-cell technologies have been explored including genomic sequencing (102), single-cell metabolomics (103), and single-cell proteomics (104). Single-cell methods should be implemented in future clinical trials as they can add valuable mechanistic insight, and neurologists will have to monitor the maturation of these technologies in the near future as they promise to revolutionize cellular CSF diagnostics.

AUTHOR CONTRIBUTIONS

TL, A-KP, and LS conceptualized, wrote, and revised the manuscript. IM and MP contributed to writing the manuscript.

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The Influence of Blood Contamination on Cerebrospinal Fluid Diagnostics

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Background: Blood contamination due to traumatic lumbar puncture presents a diagnostic pitfall in cerebrospinal fluid (CSF) analysis. It is controversially discussed if phagocytosis of erythrocytes which can be found in the CSF after subarachnoid hemorrhage can also develop *in vitro* in the presence of artificial blood contamination. Furthermore, there is no consensus about the acceptable amount of artificial blood contamination on CSF protein results.

Methods: Two measurement series were performed in order to investigate the role of artificial blood contamination on the possible development of erythrophages and siderophages in the CSF: (1) blood contamination was simulated *in vitro* by adding blood into the CSF. (2) CSF was investigated when blood contamination occurred during a traumatic lumbar puncture. In both types of experiments, CSF including blood was incubated for 24 h and for 72 h at room temperature or at 4°C. In the third measurement series, the effects of artificial blood contamination on CSF protein results were investigated. Blood contamination was simulated *in vitro* by adding different amounts of blood ending up with five different samples containing erythrocyte counts of 2,500, 5,000, 7,500, 10,000, and 20,000 per μl CSF.

Results: Cytological examination revealed no evidence of erythrophages or siderophages *in vitro*. In contrast, already a low blood contamination (2,500 erythrocytes/μl CSF) led to false pathological results of total protein and albumin. Along with increasing amounts of blood, the frequency of false pathological protein results increased. A blood contamination of 5,000 erythrocytes/μl CSF resulted in a false positive intrathecal IgM production in nearly every fifth patient. In contrast, blood contamination with 5,000 erythrocytes/μl CSF was the acceptable amount of blood which did not lead to a false positive intrathecal synthesis of IgG and IgA.

Conclusion: Erythrophages and siderophages do not develop *in vitro*. An extensive diagnostic work up for the source of blood in the CSF should be performed when

erythrophages or siderophages are found in the CSF. The contamination of CSF with increasing volume of blood resulted in falsely elevated CSF protein concentrations. Hence, the amount of blood contamination has to be taken into consideration when interpreting CSF protein measurement results.

Keywords: cerebrospinal fluid, blood contamination, erythrophages, siderophages, intrathecal immunoglobulin synthesis, antibody index, diagnostic pitfalls

INTRODUCTION

Cerebrospinal fluid (CSF) analysis is an important routine procedure if a central nervous system (CNS) infection or subarachnoid bleeding is suspected (1). In 7% of the patients with subarachnoid hemorrhage brain imaging does not detect blood, and thus, CSF analysis is required to verify the clinical diagnosis (2–6). However, artificial bleeding induced by lumbar puncture can cause peripheral blood contamination in the CSF sample leading to false-positive results (7). In order to distinguish artificial blood contamination from subarachnoid hemorrhage, counting erythrocytes in a number of consecutive tubes, centrifuging the specimen and evaluating the supernatant for xanthochromia, spectrometry for oxyhemoglobin, bilirubin determination, or ferritin measurement is performed (1, 8). However, a diagnostic gap remains (8). Cytological examination represents another important method when a subarachnoid bleeding is suspected (1). Bleedings within the CSF compartment induce activation of macrophages which phagocyte erythrocytes and then are called erythrophages (1). Erythrophages are considered to occur ∼12–18 h after the bleeding event (9–11). These macrophages form haemosiderin storage after 36–48 h and are then called siderophages (10, 11). It is controversially discussed if erythrophages and siderophages exclusively occur in the CSF of patients with subarachnoid hemorrhage. It has been suggested that in some cases erythrophages and siderophages might develop *in vitro* in the CSF in the presence of artificial blood contamination and thus are not specific for subarachnoid bleeding (12). However, a recent study investigated a short-time (7 h) of incubation of CSF with blood but could not find any evidence of erythrophage development *in vitro* (13).

Another diagnostic problem is the analysis of CSF immunoglobulins (IgM, IgA, and IgG) in patients with artificially contaminated CSF. The evaluation of intrathecal immunoglobulin synthesis is the result of IgM, IgA, and IgG CSF/serum quotients to albumin CSF/serum quotients as described previously (14).

To date, there is no consensus about the acceptable amount of artificial blood contamination on CSF results. Therefore, we investigated the influence of artificial CSF contamination with different amounts of blood spiked into the CSF on the development of erythrophages and siderophages and on routine CSF results including total protein measurements, blood-CSF-barrier-function, intrathecal synthesis of immunoglobulins IgM, IgA, and IgG, and virus specific intrathecal antibody synthesis.

Abbreviations: CSF, cerebrospinal fluid; CNS, central nervous system; OCB, CSF-specific oligoclonal bands; Alb, CSF-serum albumin quotients; AI, antibody index.

METHODS

Experimental Design

Two different measurement series were performed in order to investigate the role of artificial blood contamination on the possible development of erythrophages and siderophages in the CSF. In the first experiment, blood contamination was simulated *in vitro* by adding blood from the same patient into CSF samples after routine lumbar puncture. Before the blood was added, the CSF samples were cytological controlled to be free of erythrocytes. In the second experiment, CSF samples were investigated if blood contamination occurred by a traumatic lumbar puncture. Traumatic lumbar puncture was defined by the evidence of erythrocytes in cytological examination. In both experiments, CSF including blood was incubated for 24 and 72 h at room temperature and at 4°C with the aim to imitate the standard clinical practice.

In a third measurement series, the effects of artificial blood contamination on CSF protein measurements were investigated. Blood contamination was imitated *in vitro* by adding different amounts of blood into CSF samples after routine lumbar puncture.

Series 1: Artificial Blood Contamination *in vitro* and Cytological Examination

In this part of the study, CSF samples contaminated with blood were generated by adding patient's EDTA blood into the CSF. Three different blood concentrations were selected: (a) 2 µl blood was incubated with 2 ml CSF (1 µl blood/1 ml CSF: mean: 3,506 ± 1,334 erythrocytes per ml CSF, range: 1,829–6,400); (b) 1 µl blood was incubated with 2 ml CSF (0.5 µl blood/1 ml CSF: mean: 2,705 ± 1,077 erythrocytes per ml CSF, range: 1,493–4,512); (c) 1 µl blood was incubated with 3 ml CSF (0.33 µl blood/1 ml CSF: mean: 1,391 ± 367 erythrocytes per ml CSF, range: 893–2,048). The mean erythrocyte count for all three concentrations was 2,541 ± 1,306 erythrocytes per ml CSF, (range 893–6,400) and 4.6 ± 0.57 million erythrocytes per µl blood (range 2.9–5.6). The CSF sample was then divided in two 1 ml samples of which one sample was stored at room temperature and the other sample was incubated at 4°C for 24 h. Cytological examination for cell type distribution with special focus on erythrophages and siderophages was then performed by two independent experienced cytologists.

These samples originated from 25 adult patients who underwent lumbar puncture for routine diagnosis. The gender

distribution was 48% females to 52% males. The median age was 49 years (range 23–81). Patients were diagnosed with autoimmune diseases ($n = 10$), neurodegenerative diseases ($n = 5$), infectious diseases ($n = 4$), neoplastic diseases ($n = 3$), and idiopathic causes such as headache ($n = 3$).

In order to investigate the effects of 72 h incubation additional experiments including new CSF samples were performed. CSF samples from 11 patients were spiked with different blood concentrations in analogy to samples that were incubated for 24 h: (a) 2 µl blood was incubated with 2 ml CSF (1 µl blood/1 ml CSF: mean: 4,720 ± 2,243 erythrocytes per ml CSF, range: 2,325–9,045); (b) 1 µl blood was incubated with 2 ml CSF (0.5 µl blood/1 ml CSF: mean: 3,194 ± 1,934 erythrocytes per ml CSF, range: 1,280–7,253); (c) 1 µl blood was incubated with 3 ml CSF (0.33 µl blood/1 ml CSF: mean: 1,947 ± 1,324 erythrocytes per ml CSF, range: 576–5,120). The mean erythrocyte count for all three concentrations in CSF was 3,287 ± 2,324 (range 576–9,045) and 4.7 ± 0.90 million erythrocytes per µl blood (range 2.7–5.8). The CSF sample was then divided in two 1 ml samples of which one sample was stored at room temperature and the other sample incubated at 4°C for 72 h. The gender distribution was 45% females to 55% males. The median age was 45 years (range 24–69). Patients were diagnosed with autoimmune diseases ($n = 6$), neurodegenerative diseases ($n = 2$), infectious diseases ($n = 2$), and idiopathic causes (one patient with bell's palsy).

Series 2: Cytological Examination of CSF Samples With Traumatic Blood Contamination

CSF samples contaminated with blood by a traumatic lumbar puncture were used in this part of the study. A mean erythrocyte count of 3,262 ± 10,153 /µl CSF (range: 20–71,680) and 4.8 ± 0.51 million erythrocytes per µl blood (range: 3.5–5.6) was found. The CSF was again divided in two 1 ml samples of which one sample was stored at room temperature and the other sample was incubated at 4°C for 24 h. Cytological examination was then performed in analogy to the examination with artificial blood contamination *in vitro*.

This part of the study included 50 adult patients who underwent lumbar puncture for routine diagnosis. The gender distribution was 46% females and 54% males. The median age was 54 years (range 19–89). Patients were diagnosed with autoimmune diseases ($n = 29$), neurodegenerative diseases ($n = 9$), seizures ($n = 4$), an infectious disease ($n = 1$), neoplastic diseases ($n = 2$), and idiopathic causes such as headache ($n = 5$).

In order to investigate the effects of 72 h incubation additional experiments including new CSF samples were performed. CSF samples originated from 10 patients contaminated with blood by traumatic lumbar puncture were divided in two 1 ml samples and stored at room temperature and incubated at 4°C for 72 h. The mean erythrocyte count was 2,532 ± 5,750 /µl CSF (range: 56–18,787) and 4.1 ± 0.75 million erythrocytes per µl blood (range 2.6–5.0). The gender distribution was 70% females and 30% males. The median age was 64 years (range 28–83). Patients were diagnosed with neurodegenerative diseases ($n = 4$), infectious diseases ($n = 2$), idiopathic causes such as headache ($n = 2$), neoplastic diseases ($n = 1$), and autoimmune diseases ($n = 1$).

Series 3: The Effects of CSF Blood Contamination on CSF Protein Diagnostics

In this part of the study, CSF samples contaminated with blood were generated by adding patient's blood into the CSF samples. In order to simulate different degrees of blood contamination, each CSF sample was divided into five portions and inoculated with corresponding patient's blood to obtain samples with 2,500 erythrocytes/µl, 5,000 erythrocytes/µl, 7,500 erythrocytes/µl, 10,000 erythrocytes/µl, and 20,000 erythrocytes/µl. Each sample of CSF and serum underwent routine protein analytical procedures. The following values were investigated: total protein, albumin quotient, intrathecal synthesis of IgM, IgA, and IgG, and virus specific antibody synthesis to measles, rubella, and varicella zoster.

This part of the study comprised 25 adult patients who underwent lumbar puncture for routine diagnosis. The gender distribution was 48% females to 52% males. The median age was 67 years (range 21–89). CSF was used from patients with no clinical or CSF evidence of an inflammatory CNS disease (CSF cell count within the normal range, no evidence of an intrathecal immunoglobulin production in CSF routine diagnostics, CSF oligoclonal band negative). Patients were diagnosed with idiopathic causes such as headache ($n = 12$), neurodegenerative diseases ($n = 10$), peripheral neuropathy ($n = 2$), and a neoplastic disease ($n = 1$).

CSF Analytical Procedures

CSF and corresponding serum samples underwent standard diagnostic procedures in the Neurochemistry Laboratory of the Department of Neurology (15). CSF leukocytes were counted manually with a Fuchs-Rosenthal counting chamber. CSF cell count ≥ 5 cells/µl was considered elevated. For cytological examinations, CSF samples were centrifuged for slide preparation. After a Pappenheim staining a combination of May-Grünwald (Merck, Darmstadt, Germany) and Giemsa staining (Sigma-Aldrich, St. Louis, USA) cells were identified under a light microscope at x250–400 magnification (16). At least 100 cells were differentiated on each slide.

Total protein in CSF was determined by a Bradford dye-binding procedure. Albumin, IgG, IgM, and IgA were measured in CSF and serum by kinetic nephelometry (Beckman Coulter IMMAGE). Age and sex-adjusted upper reference limits for total protein in CSF were used according to recent studies (17, 18). Blood-CSF barrier function was evaluated by CSF-serum albumin quotients (QAlbumin). The age-adjusted upper reference limit of QAlbumin was calculated using the formula QAlbumin = 4 + (age in years/15) which Reiber et al. suggested according to Faber et al. (19, 20). This formula is long established and evaluated for different neurological diseases (21–23). In addition, we also assessed the blood-CSF barrier function according to the more recent formula of age-adjusted upper reference limit: 8 + (age in years/25) which bases on a large cohort of control patients (17). Intrathecal synthesis of IgG, IgA, and IgM was calculated according to Reiber's revised hyperbolic function referring IgG, IgA, and IgM quotients to QAlbumin (14). CSF specific oligoclonal bands were determined

TABLE 1 | CSF cell distribution for CSF samples gained after artificial blood contamination incubated at room temperature and 4°C for 24 h.

CSF cell distribution after 24 h	Blood added to CSF *in vitro*					
	Incubated at room temperature ($n = 25$)			Incubated at 4°C ($n = 25$)		
	0.33 µl blood/1 ml CSF	0.5 µl blood/1 ml CSF	1 µl blood/1 ml CSF	0.33 µl blood/1 ml CSF	0.5 µl blood/1 ml CSF	1 µl blood/1 ml CSF
Erythrophages	0%	0%	0%	0%	0%	0%
Lytic cells	7%	9%	6%	8%	11%	6%
Lymphocytes	64%	57%	51%	48%	41%	41%
Monocytes	6%	8%	8%	7%	11%	5%
Neutrophils	23%	26%	35%	37%	37%	48%

by isoelectric focusing in polyacrylamide gels with consecutive silver staining (24).

Measlevirus, rubellavirus, and varicella zoster virus IgG antibody ELISAs in serum and CSF as well as subsequent calculation of the virus specific antibody index (AI) were performed at the MHH Institute of Virology on a EUROIMMUN Analyzer I using the respective Euroimmun ELISA kits. The AI was calculated according to the formula (CSF virus-IgG/serum virus-IgG)/(CSF IgG total/serum IgG total); AI values ≥ 1.5 were considered to indicate intrathecal production of virusspecific IgG (14).

Statistical Analysis

GraphPad Prism version 5.02 was used for statistical analysis. Fisher's exact and chi-square tests were used when analyzing categorical data. Whether data were normally distributed was analyzed with D'Agostino-Pearson test. ANOVA with Bonferroni *post-hoc* test was used for group comparison and paired *t*-test for comparison of two groups. The level of statistical significance was set to 5%. Data are described by means, standard deviation, medians and ranges.

RESULTS

Blood Added to CSF *in vitro* or Blood Contamination of CSF After Traumatic Lumbar Puncture Does Not Induce the Development of Erythrophages and Siderophages *in vitro*

Cytological examination results showed that the incubation of CSF samples with blood *in vitro* for 24 and 72 h did not induce the development of erythrophages or siderophages at two different storing conditions (room temperature, 4°C) (**Table 1** and **Supplemental Table 1**). In addition, evidence of erythrophages or siderophages could not be found in CSF which was blood contaminated by traumatic lumbar puncture after 24 and 72 h *in vitro* at room temperature and 4°C (**Table 2** and **Supplemental Table 2**). Xanthochromia did not occur after 24 and 72 h of incubation with artificial blood contamination.

In addition, we investigated the cell distribution in all samples after 24 and 72 h of incubation *in vitro*. The amount of lytic cells was between 6% (blood contaminated samples) and 15% (traumatic lumbar puncture at 4°C) of all cells after 24 h ($p = 0.0046$) and between 31% (blood contaminated samples) and 78% (traumatic lumbar puncture at 4°C) of all cells after 72 h (**Tables 1**, **2**; **Supplemental Tables 1**, **2**; $p < 0.0001$). Moreover, the frequency of lytic cells after 24 h was similar between both incubation conditions (room temperature, 4°C; $p = 0.8480$), while more cells were lytic after 72 h than after 24 h (**Table 1**, and **Supplemental Tables 1**, **2**; $p < 0.0001$). The amount of lymphocytes slightly decreased, while the amount of granulocytes slightly increased after addition of increasing amounts of blood in samples after 24 h incubation time (**Table 1**; lymphocytes: $p = 0.0887$; granulocytes: $p < 0.0001$). After 72 h, the amount of lytic cells and granulocytes was higher in samples which were incubated at 4°C than in samples incubated at room temperature (**Table 1** and **Supplemental Tables 1**, **2**; lytic cells: $p < 0.0001$; granulocytes: $p < 0.0001$).

The Effect of *in vitro* Blood Contamination on CSF Total Protein and Blood-CSF-Barrier Function (CSF/Serum Albumin Quotient) by Adding Blood

Five different blood volumes including erythrocyte amounts between 2,500 and 20,000 per µl CSF were investigated in order to imitate daily clinical experience. The addition of blood into the CSF continuously increased the mean level of total protein in CSF (445 ± 153 mg/l in untreated CSF and 606 ± 148 mg/l in CSF containing 20,000 erythrocytes/µl; $p < 0.0001$; **Figure 1A**; **Table 3**). Total protein was elevated in 3/25 patients (12.5%; $p = 0.2347$) before blood was added into CSF samples. The addition of blood resulted in a higher number of patients with pathological total protein results: 4/25 patients (16%; $p = 0.1099$) in CSF containing 2,500 erythrocytes/µl, 4/25 patients (16%; $p = 0.1099$) in CSF containing 5,000 erythrocytes/µl, 6/25 patients (24%; $p = 0.0223$) in CSF containing 7,500 erythrocytes/µl CSF, 8/25 patients (32%; $p = 0.0040$) in CSF containing 10,000 erythrocytes/µl CSF and 9/25 patients (36%; $p = 0.0016$) in CSF containing 20,000 erythrocytes/µl CSF.

The albumin quotient (QAlbumin) is generally accepted as the best indicator to describe a blood-CSF barrier dysfunction

TABLE 2 | CSF cell distribution for CSF samples gained after traumatic lumbar puncture incubated at room temperature and 4°C for 24 h.

CSF cell distribution after 24 h	Blood contamination of CSF by traumatic lumbar puncture	
	Incubated at room temperature ($n = 50$)	Incubated at 4°C ($n = 50$)
Erythrophages	0%	0%
Lytic cells	13%	15%
Lymphocytes	58%	51%
Monocytes	8%	7%
Neutrophils	21%	27%

for blood derived proteins. The mean serum albumin level was 40.1 g/l ± 5.2. Four patients had serum albumin levels between 32.6 and 34.8 g/l. Before blood was added into CSF samples, 4/25 patients (16%) showed an elevated QAlbumin indicating a blood-CSF barrier dysfunction ($p = 0.1099$; **Figure 1B**; **Table 3**). The addition of blood resulted in a higher number of patients with pathological QAlbumin values indicating a blood-CSF barrier dysfunction: 5/25 patients (20%) in CSF containing 2,500 erythrocytes/μl ($p = 0.05$), 7/25 patients (28%) in CSF containing 5,000 erythrocytes/μl ($p = 0.0096$), 7/25 patients (28%) in CSF containing 7,500 erythrocytes/μl ($p = 0.0096$), 7/25 patients (28%) in CSF containing 10,000 erythrocytes/μl ($p = 0.0096$), and 15/25 patients (60%) in CSF containing 20,000 erythrocytes/μl ($p < 0.0001$).

When using the formula 8 + (age in years/25), 3/25 patients (12%) had an elevated QAlbumin before blood was added. After addition of blood pathological QAlbumin was found in 3/25 patients (12%) in CSF containing 2,500 erythrocytes/μl ($p = 0.2347$), in 3/25 patients (12%) in CSF containing 5,000 erythrocytes/μl ($p = 0.2347$), in 3/25 patients (12%) in CSF containing 7,500 erythrocytes/μl ($p = 0.2347$), in 3/25 patients (12%) in CSF containing 10,000 erythrocytes/μl ($p = 0.2347$), and in 6/25 patients (24%) containing 20,000 erythrocytes/μl ($p = 0.0223$).

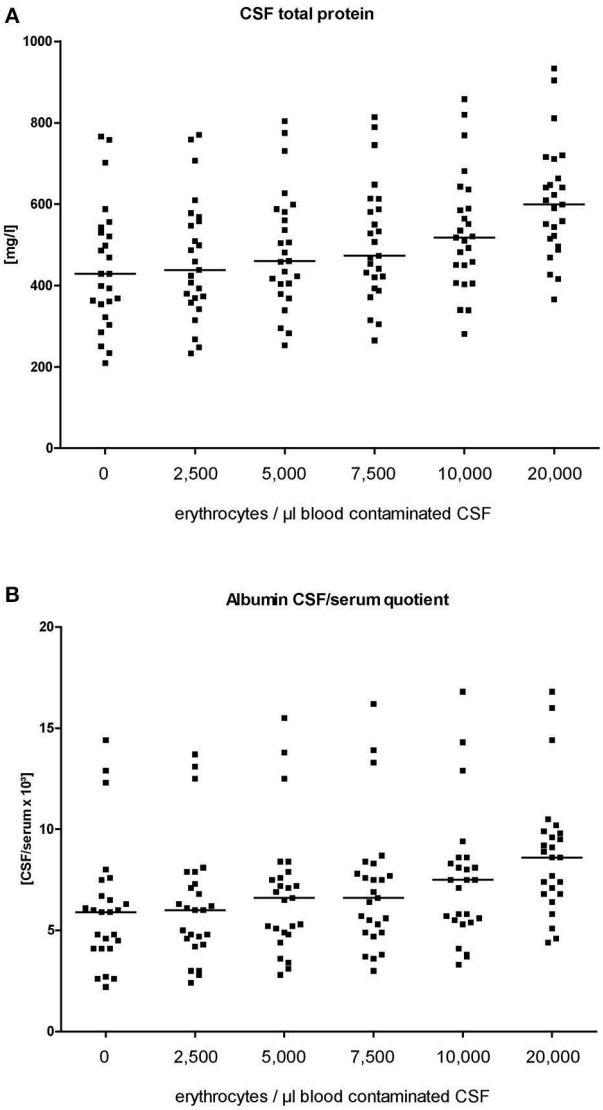

FIGURE 1 | Level of CSF total protein **(A)** and albumin quotients **(B)** at each titration step (erythrocytes/μl blood contaminated CSF). Horizontal bars indicate the medians.

The Effect of Blood Contamination on Intrathecal Synthesis of IgG, IgA, and IgM

In analogy to total protein and QAlbumin, the influence of five different blood volumes (the same samples as described above) on results indicating intrathecal production of immunoglobulins as referred to Reiber-graphs, was investigated. The mean serum IgG level was 11.0 g/l ± 3.7, the IgA level was 2.5 g/l ± 1.3, and the IgM level was 1.0 g/l ± 0.5. Serum IgG concentration <7 g/dl was found in one patient, serum IgA <0.7 g/dl in three patients, and serum IgM <0.4 g/l in two patients. Before blood was added to the CSF, none of the selected patients exhibited an intrathecal synthesis of either IgG, or IgA, or IgM (**Figure 2A**; **Table 3**).

The addition of blood containing 2,500 and 5,000 erythrocytes/ μl did not result in a false positive intrathecal synthesis of IgG. A false positive intrathecal synthesis of IgG was only detected with higher blood amounts: 1/25 patient (4%) in CSF containing 7,500 erythrocytes/μl ($p > 0.9999$), 3/25 patients (12.5%) in CSF containing 10,000 erythrocytes/μl ($p = 0.2347$) and 4/25 patients (16%) in CSF containing 20,000 erythrocytes/μl ($p = 0.1099$).

Similar to IgG, a false positive intrathecal synthesis of IgA was first detected in CSF contaminated with blood containing 7,500 erythrocytes/μl (1/25 patient; $p > 0.9999$). The addition of blood containing 10,000 erythrocytes/μl resulted in 3/25 patients (12.5%; $p = 0.2347$) with a false positive intrathecal synthesis of IgA, while blood containing 20,000 erythrocytes/μl showed a false intrathecal IgA synthesis in 9/25 patients (36%; $p = 0.0016$).

For intrathecal IgM synthesis, false positive results occurred already as consequence of blood addition containing 5,000 erythrocytes/μl (4/25 patients, 16%; $p = 0.1099$) and further increased with higher blood amounts. The addition of blood containing 7,500 erythrocytes/μl resulted in 9/25 patients (36%;

TABLE 3 | Overview of CSF protein results in response to different amounts of blood contamination.

CSF with artificial blood containing:	CSF parameters							
	Total protein	QAlbumin	Intrathecal synthesis			IgG antibody index		
			IgG	IgA	IgM	Measles	Rubella	VZV
2,500 erythrocytes/μl	↑	↑ (↑)*	–	–	–	–	–	–
5,000 erythrocytes/μl	↑	↑↑ (↑)*	–	–	↑↑	–	–	–
7,500 erythrocytes/μl	↑↑	↑↑ (↑)*	↑	↑	↑↑↑↑	–	–	–
10,000 erythrocytes/μl	↑↑	↑↑ (↑)*	↑	↑↑	↑↑↑↑↑↑	–	–	–
20,000 erythrocytes/μl	↑↑↑	↑↑↑↑↑ (↑↑)*	↑↑	↑↑↑↑	↑↑↑↑↑↑↑↑	–	–	–

*The arrows indicate false positive results as compared to naive CSF without blood contamination. ↑ = 1–10% of patients with false positive results; ↑↑ = 11–20% of patients with false positive results; ↑↑↑ = 21–30% of patients with false positive results; ↑↑↑↑ = 31–40% of patients with false positive results; ↑↑↑↑↑ = 41–50% of patients with false positive results; ↑↑↑↑↑↑ = 51–60% of patients with false positive results; ↑↑↑↑↑↑↑↑ = 71–80% of patients with false positive results. * ↑ in brackets indicate QAlbumin above the upper reference by using the formula 8 + (age in years/25).*

$p = 0.0016$) with a false positive intrathecal synthesis of IgM, while blood containing 10,000 and 20,000 erythrocytes showed a false intrathecal IgM synthesis in 14/25 patients (56%; $p < 0.0001$) and 20/25 patients (80%; $p < 0.0001$), respectively.

The Effect of Blood Contamination on Virus Specific Intrathecal IgG Antibody Synthesis

The influence of *in vitro* blood contamination on viral specific IgG synthesis against the viruses measles, rubella, and varicella zoster was investigated in analogy to the intrathecal synthesis of immunoglobulins as described above by using the same CSF samples. Before blood was added to the CSF, only one patient exhibited an elevated antibody index against the varicella zoster virus (**Figure 2B**; **Table 3**). The addition of blood containing 2,500, 5,000, 7,500, 10,000, and 20,000 erythrocytes/μl did not result in a false positive antibody index for any virus. The mean antibody index before blood was added was 0.8 ± 0.1 for measles virus, 0.7 ± 0.1 rubella virus and 1.2 ± 0.9 for varicella zoster virus. In the last titration step with 20,000 erythrocytes/μl the mean antibody index was 0.8 ± 0.1 for measles virus, 0.8 ± 0.1 rubella virus and 1.3 ± 1.5 for varicella zoster virus. The differences did not reach significant level.

DISCUSSION

In the present study we show that artificial blood contamination of the CSF did not induce the development of erythrophages and siderophages *in vitro* after 24 and 72 h of incubation. In contrast, the artificial contamination of CSF with blood resulted in false pathological CSF protein results and the proportion of false results continuously increased with increasing blood.

CT scan of the head is able to uncover a subarachnoid bleeding with a high sensitivity of approximately 86–93% 6 h after manifestation (1, 25). However, the sensitivity decreases to approximately 50% already after 1 week of bleeding occurrence (1, 25). Thus, in patients with suspected subarachnoid hemorrhage but normal CT of the head the diagnostic algorithm recommends a diagnostic lumbar puncture to detect subarachnoid hemorrhage 8–12 h after onset of the headache (9). However, diagnostic doubt arises when trauma induced by the needle causes artificial contamination of CSF with blood (2–4, 26). In such cases, the cytological examination represents a routine diagnostic approach to uncover erythrophages, which develop 12–18 h after contact with blood (9). After additional 36–48 h erythrophages produce haemosiderin deposits and are then called siderophages (1). Siderophages do not play a critical role in the first 3 days after bleeding, but the sensitivity and specificity increase during the progress and siderophages may even be detected several months after an insult (1, 10, 11). To date, it is controversially discussed if erythrophages and siderophages are exclusively generated in the CSF of patients with subarachnoid bleeding. It has been reported that blood contamination of the CSF as consequence of a previous lumbar puncture might induce the development of siderophages (27). Furthermore, it has been supposed that in some cases erythrophages might develop *in vitro* in the CSF in the presence of artificial blood contamination being not specific for a subarachnoid bleeding (12). Here we have shown that erythrophages and siderophages do not develop *in vitro* after blood contamination and an incubation time of 24 and 72 h. Our results are in line with the results of Dersch and colleagues who have recently reported that erythrophages do not develop *in vitro* after 7 h of incubation (13). The presence of xanthochromia in CSF is considered to be another indicator for subarachnoid bleeding in patients with negative CT scan (10, 28–30). In analogy to the absence of erythrophages and siderophages we did not detected visually xanthromic CSF in our experiments. We thus suggest that erythrophages and siderophages only develop *in vivo* in the CSF compartment as response to blood. The sensitivity of the human color vision is considered to be not sufficient to detect slight yellowish tint due to small amount of bilirubin (5, 31). In a previous study including patients with subarachnoid hemorrhage, visual inspection of CSF supernatant, revealed that only 47% of patients had xanthromic CSF while CSF of 53% patients was colorless (32). In a study using artificially lysed erythrocytes in distilled water the sensitivity of visual xanthromia was even 26.6% (5). The findings of these studies suggest that the lysis rate of erythrocytes *in vivo* might be higher. On the other side, the lack of xanthromia in our study might be explained by small amounts of erythrocytes, while

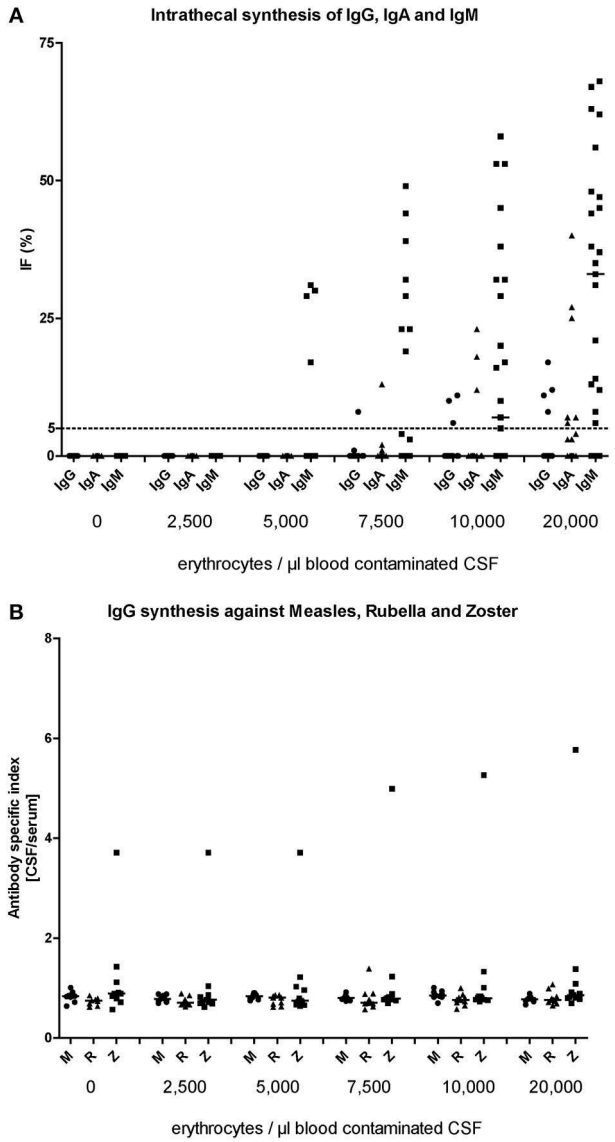

FIGURE 2 | Level of artificial immunoglobulin synthesis for IgM, IgA, and IgG **(A)** and specific IgG synthesis against the viruses **M**easles, **R**ubella, and Varicella **Z**oster Virus **(B)** at each titration step (erythrocytes/µl blood contaminated CSF).

true subarachnoid hemorrhages tend to have higher erythrocyte counts (26).

Artificial blood contamination of CSF samples presents not only a dilemma for the diagnosis of a subarachnoid bleeding but also influences CSF protein analysis leading to false pathological results (4). Our results show that already a low blood contamination (containing 2,500 erythrocytes/µl CSF) induced elevated levels above the cut-off of 500 mg/l of total protein and age related albumin quotients indicating a disturbed blood-CSF-barrier function. With increasing amounts of blood contamination (up to 20,000 erythrocytes/µl), the frequency of false pathological total protein and albumin quotient values increased up to every second patient of our study population.

Another diagnostic problem is the analysis of immunoglobulins (IgM, IgA, and IgG) in the CSF artificially contaminated with blood. The concentrations of immunoglobulins are magnificent higher in serum than in CSF and thus blood contamination may pretend to a false positive evidence of an intrathecal production of immunoglobulins indicating inflammatory processes in the CSF. Due to the different molecular weight of immunoglobulins (IgM>IgA>IgG) only very low concentrations of IgM are usually found in CSF. Blood contamination of the CSF will therefore affect the above proteins exactly in the same order with falsified IgM in the first place. The influence of blood contamination on CSF results should therefore be higher for IgM, followed by IgA and IgG. Our results are in line with these assumptions and conclusively show that false positive production of IgM was predominant in our experiments, followed by IgA, and IgG. A blood contamination of 5,000 erythrocytes/µl CSF resulted in a false positive intrathecal IgM production in nearly every fifth patient. In contrast, blood contamination with 5,000 erythrocytes/µl CSF did not induce a false positive intrathecal production of IgG and IgA. An artificial intrathecal synthesis of IgG and IgA occurred in every tenth patient after addition of blood containing 7,500 erythrocytes/µl CSF. We thus suggest a cut off of 5,000 erythrocytes/µl CSF for analyses of intrathecal IgG synthesis. In the case of a contaminated CSF with blood a falsely elevated intrathecal IgG synthesis will reliably be detected by the absence of oligoclonal bands in the CSF. This qualitative method to detect intrathecal IgG synthesis is less susceptible for blood contamination.

Interestingly, CSF contamination with even higher amounts of blood (up to 20,000 erythrocytes/µl CSF) did not lead to false positive IgG antibody index against viruses. The cut-off of blood contamination for analyses of the IgG antibody index against viruses might be therefore higher than 20,000 erythrocytes/µl CSF. However, since blood contamination increased the mean IgG antibody index against rubella and measles for 0.1, false positive results could be the consequence in cases of borderline IgG antibody indices.

In conclusion, erythrophages and siderophages did not develop *in vitro* and we thus suggest an extensive diagnostic work up for the source of blood when erythrophages and siderophages are found in the CSF. The contamination of CSF with blood resulted in false pathological CSF protein results in some patients. Contamination of the CSF with 5,000 erythrocytes/µl was the acceptable amount of blood which did not induce a false positive intrathecal synthesis of IgG.

DATA AVAILABILITY

Data supporting the findings can be found in the tables. Additional data extracted may be shared upon request.

ETHICS STATEMENT

The study was approved by the Ethic Committee of the Hannover Medical School, Carl-Neuberg-Str.1,

30625 Hannover (1 December 2012; 1322-2012). Only participants older than 18 years were included. All participants of this study gave written informed consent. The study is in accordance with the Declaration of Helsinki regarding ethical conduct of research involving human subjects.

AUTHOR CONTRIBUTIONS

PS: participated in the design of the study, collected and analyzed the data and drafted the manuscript. TJ: collected the data, analyzed the data and drafted the manuscript. UW was responsible for CSF analysis, analyzed the data and contributed in drafting the manuscript. FK: collected and analyzed the data. AN, JA, WP, LB and KS contributed in drafting the manuscript. MS and TG analyzed the data and contributed in drafting the manuscript. TS: conceived the study, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00584/full#supplementary-material

Supplemental Table 1 | CSF cell distribution for CSF samples gained after artificial blood contamination incubated at room temperature and 24°C for 72 h.

Supplemental Table 2 | CSF cell distribution for CSF samples gained after traumatic lumbar puncture incubated at room temperature and 4°C for 72 h.

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BRIEF RESEARCH REPORT
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Detection of Cell-Free Mitochondrial DNA in Cerebrospinal Fluid of Creutzfeldt-Jakob Patients

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Background: The current diagnosis method for Creutzfeldt-Jakob disease (CJD) is post-mortem examination, so early detection of CJD has been historically problematic. Auxiliary detection of CJD based on changes in levels of components of the cerebrospinal fluid (CSF) has become a focus of research. In other neurodegenerative diseases such as Alzheimer's disease (AD), cell-free mitochondrial DNA (mtDNA) in the CSF of patients may serve as a biomarker that could facilitate early diagnosis and studies of the mechanisms underlying the disease.

Methods: In this study, the cell-free mitochondrial DNA in the CSF of patients with sCJD and control patients was compared by digital droplet PCR.

Results: The cell-free mitochondrial DNA copy number in the CSF of sCJD patients was significantly increased in comparison with that of the control group, and this difference was pathologically related to CJD.

Conclusion: Therefore, we speculate that changes in cerebrospinal fluid mitochondrial DNA copy number play an important role in the study of CJD mechanism and diagnosis.

Keywords: mtDNA, Creutzfeldt-Jakob disease, cerebrospinal fluid, diagnosis, prion disease

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INTRODUCTION

Creutzfeldt-Jakob disease (CJD) is a neurodegenerative disease associated with an abnormal accumulation of proteinaceous infectious particles (prions) in neurons (1). CJD is the most frequent human transmissible spongiform encephalopathies (2–4). CJD can be confirmed by subjecting brain tissue from CJD patients to western blotting after digestion with proteases that is a demonstration of the protease resistance of the prion protein accumulated, or to use immunohistochemistry, abnormal PrP can be identified by its abundance, location and morphology

TABLE 1 | Cerebrospinal fluid sample background information.

N	Population distribution		Clinical test								Clinical diagnosis
	Gender	Age	1	2	3	4	5	6	Clinical performance	14-3-3 protein	
1	Male	56	+	−	+	+	−	−	1,3,4	+	sCJD
2	Female	67	+	−	−	−	−	−	1	+	sCJD
3	Male	40	+	+	−	−	+	+	1,2,5,6	+	sCJD
4	Female	58	−	−	−	+	−	+	4,6	+	sCJD
5	Female	79	+	+	+	+	+	+	1,2,3,4,5,6	−	sCJD
6	Female	57	+	−	−	−	−	−	1	−	sCJD
7	male	43	+	−	+	+	−	+	1,3,4,6	+	sCJD
8	Male	55	+	+	+	+	+	−	1,2,3,4,5	+	sCJD
9	Female	65	+	−	+	+	−	−	1,3,4	+	sCJD
10	Male	52	+	+	−	−	+	−	1,2,5	+	sCJD
11	Female	62	+	+	+	+	+	+	1,2,3,4,5	+	sCJD
12	Male	71	+	+	+	+	−	−	1,2,3,4	+	sCJD
13	Female	57	+	+	+	−	−	−	1,2,3	+	sCJD
14	Male	51	+	−	+	+	−	−	1,3,4	+	sCJD
15	Male	64	+	+	−	+	−	+	1,2,4,6	+	sCJD
16	Female	63	−	+	−	+	+	−	2,4,5	+	sCJD
17	Male	72	+	+	−	+	+	−	1,2,4,5	+	sCJD
18	Female	71	+	+	−	+	+	−	1,2,4,5	+	sCJD
19	Male	66	+	+	+	−	−	+	1,2,3,6	+	sCJD
20	Female	54	+	+	−	+	+	+	1,2,4,5,6	+	sCJD
21	Male	62	+	−	−	−	+	−	1,5	−	non-CJD
22	male	44	+	−	−	+	−	−	1,4	−	non-CJD
23	Female	50	−	−	+	+	−	+	3,4,6	−	non-CJD
24	Male	47	−	−	−	−	−	−	/	−	non-CJD
25	Male	65	+	−	−	−	−	−	1	−	non-CJD
26	Female	76	+	−	−	+	−	−	1,4	−	non-CJD
27	Female	62	+	+	−	−	−	−	1,2	−	non-CJD
28	Female	56	+	−	+	−	−	−	1,3	−	non–CJD
29	Male	45	−	−	−	−	−	+	6	−	non-CJD
30	Male	73	+	+	−	−	−	−	1,2	−	non-CJD
31	Female	52	−	−	−	−	−	+	6	−	non-CJD
32	Male	70	+	−	−	+	−	−	1,4	−	non-CJD
33	Female	55	−	−	−	+	−	−	4	−	non-CJD

"1" represent "Rapid progressive dementia," "2" represent "Myoclonus," "3" represent "Visual or cerebellar symptoms," "4" represent "Pyramids/Extrapyramidal dysfunction," "5" represent "akinetic mutism," "6" represent "other symptoms." "/" represent "inexistence," "Other symptoms" include paroxysmal convulsions, disturbance of consciousness, convulsions, slow progressive dementia, psychiatric symptoms, subacute progressive dementia, dual vision, cortical blindness, numbness of the right limb, dizziness, nausea, vomiting, memory loss, cerebellar symptoms. "+" represent "exist," "–" represent "inexistence."

(5). A diagnosis of pre-mortem CJD can be made based on autosomal dominant pathogenic mutations in the human prion protein gene (*PRNP)* (6) and using real-time quaking induced conversion (RT-QuIC) which detected the accumulation of misfolded PrP in the brain, CSF, or nasal brushings (7), combined with clinical manifestations, medical history, epidemiological reports, EEG, and MRI (8, 9). In recent years, non-invasive and less invasive detection methods have been used to diagnose CJD, and this idea can also be applied to the study of the mechanisms underlying CJD. CSF is in direct contact with the brain parenchyma and can indirectly reflect certain pathological indications of the central nervous system (10). Changes in the abundance of many proteins (14-3-3 protein, tau et al.) in CSF can be used in the diagnosis of CJD (11–14). RT-QuIC has proved to be a very valuable diagnostic CSF test for sCJD (7). The cell-free mitochondrial DNA content in CSF was found to be changed in a specific manner in patients with AD and PD, and such changes were distinct to particular neurodegenerative diseases (15–19). These findings suggest that particular changes in mitochondrial DNA content may be related to the mechanisms underlying specific diseases. In comparison

Abbreviations: AD, Alzheimer's disease; CJD, Creutzfeldt-Jakob disease; CSF, cerebrospinal fluid; ddPCR, digital droplet polymerase chain reaction; mtDNA, mitochondrial DNA; PD, Parkinson's Disease; sCJD, Sporadic Creutzfeldt-Jakob disease; TSEs, Transmissible spongiform encephalopathies.

with other neurodegenerative diseases, there have been few studies of changes in mitochondrial DNA in the CSF of patients with CJD (16).

In this study, we examined the mitochondrial DNA concentration in the CSF of sCJD patients, with the goal of determining the mechanisms underlying the development and progression of CJD and identifying if changes in cerebrospinal fluid mitochondrial DNA copy number could be suitable for diagnosing the disease.

MATERIALS AND METHODS

Subjects

CSF samples from 20 age-and sex ratio-matched sCJD and 13 age-and sex ratio-matched non-CJD controls were presented to the Chinese center for disease control and prevention. Both the control group and the CJD patient in the sample were Chinese. The average age of the sCJD and control patients was ∼55 years, and the ratio of males to females was nearly 1:1. The clinical symptoms of the subjects were: rapid progressive dementia, myoclonus, akinetic mutism, disturbance of consciousness, mental symptoms, etc. Detailed samples background information was shown in **Table 1**. The diagnosis of sCJD refers to the CJD diagnostic criteria standardized by the Chinese Center for Disease Control and Prevention. The CJD diagnostic criteria unified by the centers for disease control are as follows: ①Suspected cases are based on the exclusion of other diseases, and meet the following clinical manifestations 2 or more: rapid progressive dementia, passive silence, myoclonus, visual/cerebellar dysfunction, cone system/extrapyramidal dysfunction. ② The clinical cases are basically consistent with the suspected diagnosis cases, and meet any of the following items: EEG shows periodic three-phase waves during the course of the disease; laboratory tests for positive detection of 14-3-3 protein in cerebrospinal fluid (CSF); Early nuclear magnetic resonance imaging revealed abnormally high signals in the putamen/tail caudate nucleus, and diffusion-weighted images (DWI) showed a "ribbon" sign of symmetry/asymmetry cortex (or cortex). ③ The confirmed cases should meet one of the following items: spongiform lesions found in brain histopathology; immunohistochemical detection of brain tissue, presence of protease-resistant PrP^{sc} deposition; protein immunoblotting to detect brain tissue, protease-resistant PrP^{sc}.

Detection of CSF Mitochondrial DNA Copy Number in sCJD Patients

Digital Droplet Polymerase Chain Reaction (ddPCR)

The CSF mtDNA copy number of sCJD patients and non-CJD samples was directly determined by ddPCR. In this experiment, an amplification product containing 85 base pairs (mtDNA-85) was the only product generated. Three replicates were performed in 96-well plates. The sequences of the amplified mtDNA primer and probe were shown in **Supplementary Table 1-1** and Date **Supplementary Table 1-2**. The amplification reaction system and amplification reaction

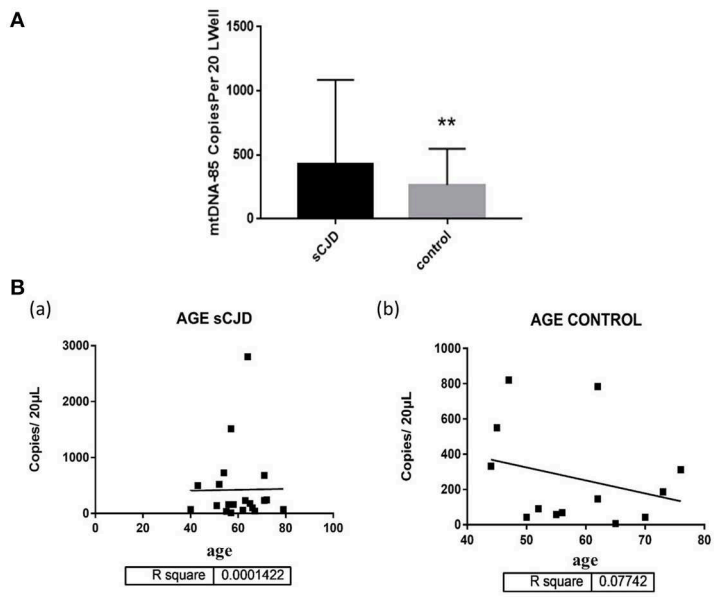

FIGURE 1 | (A) The mitochondrial DNA copy number of the CSF of the control group ($n = 13$) and patients with sCJD ($n = 21$). Subjects were divided into the following groups: possible sCJD patients (black bar) and non-CJD subjects (gray bar). The results are expressed as mean ± SEM. The mtDNA copy number of the CSF is expressed as mtDNA copies/20 μL. *$P < 0.05$; **$P < 0.01$, represents the sCJD group are significantly different from the control group, by unpaired *t*-test. **(B)** mitochondrial DNA copy numbers of the CSF of sCJD patients/non-patients of different ages. The correlation between age and the cell-free mitochondrial DNA copy number of the CSF was assessed for the experimental and control groups (a,b). There was no correlation between age and disease, By liner regression graph and pearson correlation analysis.

procedure were shown in Date **Supplementary Tables 1-3**, **1-4**. After the amplification reaction was complete, the QX200 Droplet Reader was used to read the signal, after which the data were analyzed.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism 7 software to compare the mitochondrial DNA copy number in CSF samples from the control group and sCJD patients. At the same time, the factors of gender, age, and symptoms were analyzed to assess their relationship with the CSF mitochondrial DNA copy number. The results are expressed as the mean mtDNA concentration in CSF (mtDNA copy/20 μL ± SEM). The statistical significance was set at $p < 0.05$.

RESULTS

Mitochondrial DNA Copy Number of the CSF of sCJD Patients (Calculated per 20 μL Copy Number)

The amplified mitochondrial DNA copy number of CSF in the experimental group and the control group results are shown in **Supplementary Table 2**. The mitochondrial DNA copy number of the CSF of sCJD patients (404.9 ± 142.2 copies/20 μL) was significantly increased in comparison with that of the control group (264.3 ± 78.62 copies/20 μL) (**Figure 1A**).

Correlation Between Changes in Mitochondrial DNA Copy Number and age in Cerebrospinal Fluid in Patients With Creutzfeldt-Jakob

We further evaluated the association between changes in mitochondrial DNA copy number and age in cerebrospinal fluid in CJD patients. The mitochondrial DNA copy number of the CSF was not associated with age in the sCJD or control group (**Figure 1B**).

Correlation Between Changes in Mitochondrial DNA Copy Number and Gender in Cerebrospinal Fluid in Patients With Creutzfeldt-Jakob

We also evaluated the association between changes in cerebrospinal fluid mitochondrial DNA copy number and gender in Creutzfeldt-Jakob patients. The mitochondrial DNA copy number of male sCJD patients (480.2 ± 263.1 copies/20 μL) was significantly higher than that of the control group (208.1 ± 103.9 copies/20 μL), and this difference was significant. The mitochondrial DNA copy number of female sCJD patients

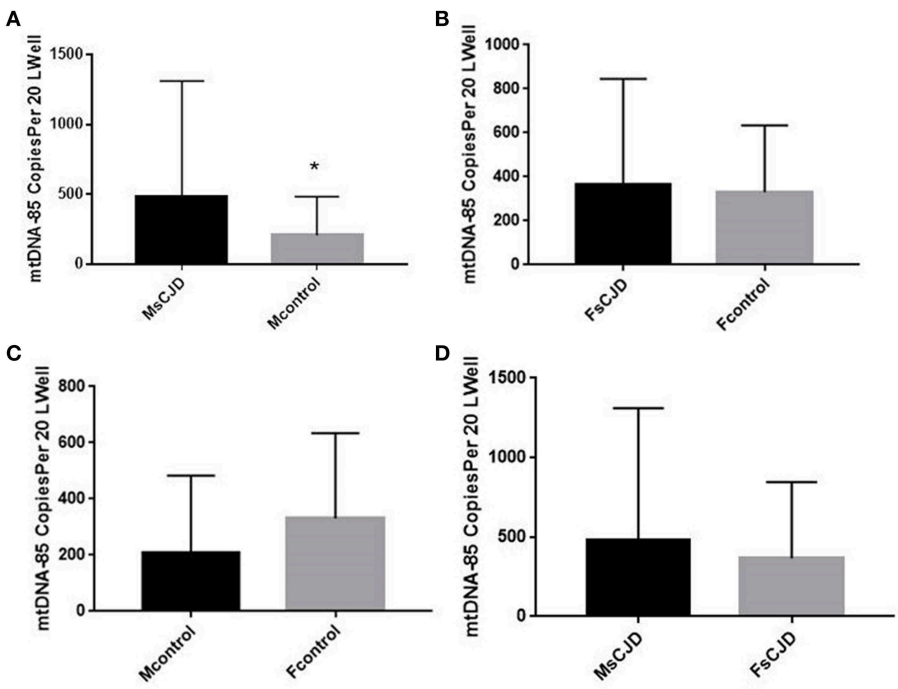

FIGURE 2 | Copies of mtDNA in CSF and correlation with gender. **(A)** The mitochondrial DNA copy number of the male sCJD patients was significantly higher than that of the control group. **(B)** The mitochondrial DNA copy number of the female sCJD patients ($n = 11$) was higher than that of the control group ($n = 10$), but this difference was not significant. **(C)** In the control group, the cell-free mitochondrial DNA copy number of female patients ($n = 6$) was higher than that of male patients ($n = 7$), but this difference was not significant. **(D)** Among sCJD patients, the mitochondrial DNA copy number of male patients was higher than that of female patients, but this difference was not significant. The results are expressed as mean ± SEM. The mtDNA copy number of the CSF is expressed as mtDNA copies/20 μL. $^{*}P < 0.05$; $^{**}P < 0.01$, represents the MsCJD group are significantly different from the control group, by unpaired *t*-test.

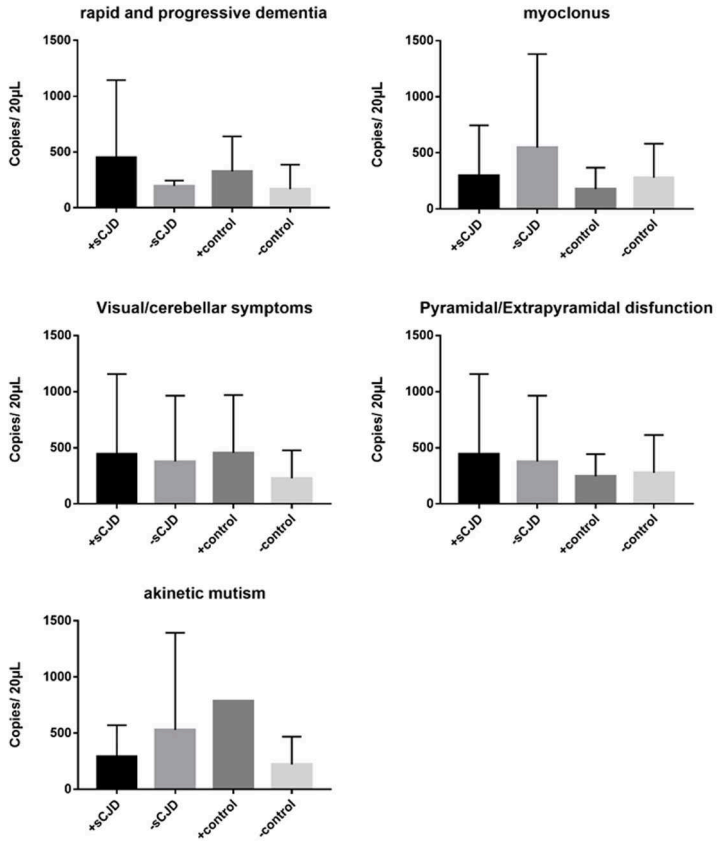

FIGURE 3 | The correlation between clinical symptoms and the cell-free mitochondrial DNA copy number of the CSF was analyzed for the experimental and control groups. The CSF mitochondrial DNA copy number was not found to be related to CJD symptoms. The results are expressed as mean ± SEM. The mtDNA copy number of the CSF is expressed as mtDNA copies/20 μL. *$P < 0.05$; **$P < 0.01$, represents the sCJD group are significantly different from the control group, by unpaired *t*-test.

(367.3 ± 151.1 copies/20 μL) was higher than that of the control group (330 ± 124.2 copies/20 μL), but this difference was not significant. In the control group, the cell-free mitochondrial DNA copy number of the CSF of female patients (330 ± 124.2 copies/20 μL) was higher than that of male patients (208.1 ± 103.9 copies/20 μL), but this difference was not significant. Among the sCJD patients, the mitochondrial DNA copy number of the male patients (480.2 ± 263.1 copies/20 μL) was higher than that of the female patients (367.3 ± 151.1 copies/20 μL), but this difference was not significant (**Figure 2**).

Correlation Between Changes in Mitochondrial DNA Copy Number and Clinical Symptoms in Cerebrospinal Fluid in Patients With Creutzfeldt-Jakob

We next evaluated the association between changes in cerebrospinal fluid mitochondrial DNA copy number and clinical symptoms in Creutzfeldt-Jakob patients. Clinical symptoms include rapid progressive dementia, myoclonus, akinetic mutism, visual/cerebellar symptoms, pyramidal/extrapyramidal dysfunction, mental symptoms. The mitochondrial DNA copy numbers of the CSF of the experimental and control groups were not significantly associated with differences in clinical symptoms (**Figure 3**).

Correlation Between Changes in Mitochondrial DNA Copy Number and 14-3-3 Protein in Cerebrospinal Fluid in Patients With Creutzfeldt-Jakob

Finally, we examined the association between the copy number of mitochondrial DNA and 14-3-3 protein in CSF. The mitochondrial DNA copy number of the 14-3-3 protein positive group (466.3 ± 161.9 copies/20 μL) was significantly higher than that of the 14-3-3 protein negative group (234.5 ± 70.81 copies/20 μL). Statistical analysis results were shown in **Supplementary Figure 1**.

DISCUSSION

Here, we investigated changes in the concentration of mtDNA in CSF from sCJD patients. In comparison with the control group with general neurological symptoms, the increase in the

mitochondrial DNA copy number of the CSF of patients with sCJD was significant. Therefore, we speculate that changes in cerebrospinal fluid mitochondrial DNA copy number play an important role in the study of CJD mechanism and diagnosis. Our data support previous work where, other than in AD, all the other non-AD type dementia, including sCJD have an increased of relative mtDNA copy numbers (16). They also found that the 14-3-3 protein was not associated with significant differences in mitochondrial DNA copy number in CSF. However, our experimental results showed that the mitochondrial DNA copy number was significantly increased in the 14-3-3 protein-positive group compared to the 14-3-3 protein-negative group, and in 20 cases of CJD cerebrospinal fluid samples, 18 samples of 14-3-3 protein were positive. In general, this result indicated that the change in mitochondrial DNA copy number has a certain correlation with 14-3-3 protein. We hypothesized that changes in mitochondrial DNA copy number are important for the diagnosis of CJD.

This study did not find a relationship between cell-free mitochondrial DNA content in CSF and the age/clinical neurological symptoms of CJD or non-CJD patients, which is similar to PD patients (18). However, Wei et al.'s research indicated that changes in mitochondrial DNA copy number in brain samples from Creutzfeldt-Jakob patients were strongly positively correlated with age (20). We speculate that because our sample size is small and the age span is small, our sample age is concentrated around 55 years old. The mitochondrial DNA copy number changes in this period of time are relatively small. Although they were different, they were not significant between each other, and the results obtained were one-sided.

The cell-free mitochondrial DNA copy number of the CSF of male patients was significantly increased compared with that of the control group, and it was significantly correlated with sCJD. However, there was no correlation between mitochondrial DNA copy number and sCJD in female patients. Therefore, changes in cerebrospinal fluid mitochondrial DNA copy number in male CJD patients play a better role than in female patients in studying CJD mechanisms and diagnosis. This phenomenon has also been observed in PD, AD, and Huntington's disease patients, in which mitochondrial DNA in men is more closely associated with the disease (18, 21–23).

We speculate that the extent of the increase in mitochondrial DNA in CSF directly reflects neuronal damage. When mitochondria are dysfunctional, an insufficient energy supply leads to neuronal degeneration, and an increased mitochondrial DNA copy number in CSF may be a response to neuronal mitochondrial dysfunction to maintain neuronal energy requirements. This hypothesis is consistent with the conclusion of Wei et al. (20) they observed a strongly positive correlation between age and mtDNA copy number in CJD, this could reflect compensatory mitochondrial biogenesis in older subjects. There are no effective prevention or treatment methods for most neurodegenerative diseases. Changes in the number of mitochondrial DNA copies are associated with several neurodegenerative diseases, including sCJD, PD, and AD, so we hypothesize that drugs that regulate the mtDNA copy number in neurons may have therapeutic effects in patients with certain neurodegenerative diseases.

CONCLUSIONS

The mtDNA-85 copy number in cerebrospinal fluid of patients with sCJD was significantly different from that of the control group, we speculate that changes in cerebrospinal fluid mitochondrial DNA copy number play an important role in the study of CJD mechanism and diagnosis. Due to the small number of samples we collected, so our results are indeed limited, we need a larger sample to further verify this result.

DATA AVAILABILITY

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

ETHICS STATEMENT

The study was ethically approved by the Institutional Ethics committee of the Chinese Center for Disease Control and Prevention. Written informed consent was obtained from each participant.

AUTHOR CONTRIBUTIONS

JL, YD, SS, and LY conceived the study. WW, XZ, ML, and QS collected the CSF samples and clinical data. JL, YD, ZG, HG, HZ, XW, and DY performed the experiments and data analyses. LY and DZ provided intellectual inputs. JL and YD wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00645/full#supplementary-material

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frontiers in Neurology

CASE REPORT
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Use of Cerebrospinal Fluid Biomarkers in Diagnosis and Monitoring of Rheumatoid Meningitis

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Rheumatoid meningitis is a rare extra-articular manifestation of rheumatoid arthritis, often with non-specific symptoms. In most cases brain MRI shows a patchy lepto- and pachymeningeal enhancement, but the diagnosis currently relies on examination of a meningeal biopsy with presence of plasma cells and rheumatoid noduli. Presence of IgM rheumatic factor (RF) has been found in several cases and recently four cases have shown high titer anti-cyclic citrullinated peptide (anti-CCP) in CSF, suggesting this as a potential marker for rheumatoid meningitis. We present a 62 year-old woman with sero-positive (IgM RF and anti-CCP) rheumatoid arthritis, presenting with headache and gait impairment. Brain MRI revealed the classical patchy meningeal enhancement and the diagnosis of rheumatoid meningitis was confirmed by neuropathological examination of a meningeal biopsy. Analysis of the CSF revealed positive IgM RF (92.7 IU/mL) and strongly positive anti-CCP (19,600 IU/mL) and CXCL-13 (>500 ng/L). After treatment with high-dose steroid and Rituximab the clinical symptoms resolved. A 6 month follow-up analysis of CSF showed a dramatic decrease in all these markers with negative IgM RF and a decrease in both anti-CCP (64 IU/mL) and CXCL-13 (<10 ng/L). Our case further underlines the potential use of CSF anti-CCP and IgM RF in the diagnosis of RM and the use of these markers and CXCL-13 in evaluation of treatment response. A case review of 48 cases of rheumatoid meningitis published since 2010, including, symptoms, serum, and CSF findings, treatment, and outcome is provided.

Keywords: rheumatoid meningitis, inflammation, anti-CCP, CXCL13, biomarker

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BACKGROUND

Rheumatoid meningitis (RM) is a rare but potentially aggressive extra-articular manifestation of rheumatoid arthritis (RA) involving both pachy- and leptomeninges (1, 2). It can occur at all disease stages, and manifestations are often non-specific, mimicking a variety of neurological disorders, malignancies, or infections (1–6). Brain MRI with patchy leptomeningeal contrast enhancement and cerebrospinal fluid (CSF) rheumatoid factor (RF) are useful to guide, but diagnosis still relies on

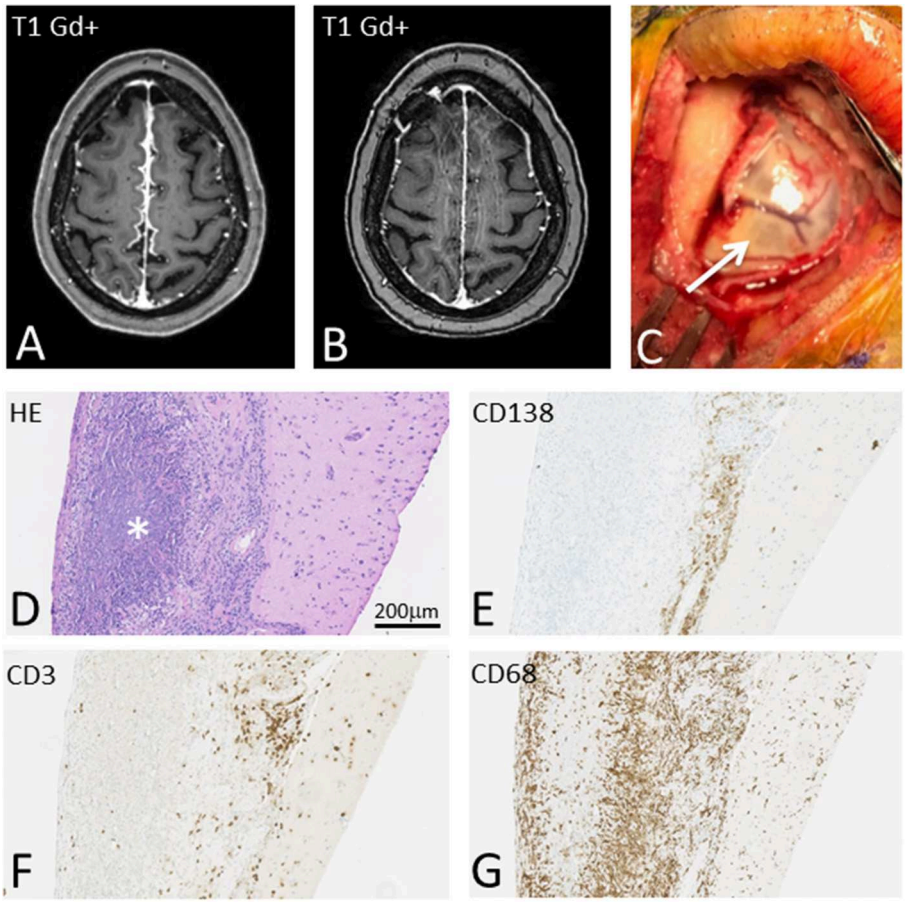

FIGURE 1 | T1-weighted brain MRI showing interhemispheric leptomeningeal Gd+ enhancement before **(A)** and after **(B)** treatment with high dose steroids, Methotrexate and Rituximab. On gross inspection meninges appear severely inflamed **(C)** and pathological examination reveals massive meningeal granulomatous inflammation **(D)** with pre-dominant CD138 positive plasma cells **(E)**, but also CD3 positive T cells **(F)**. Massive infiltration with CD68 positive histiocytes with rheumatic granuloma formation was also seen **(G)**.

pathological examination of a meningeal biopsy often showing unspecific inflammation, rheumatic noduli, and in some cases vasculitis (2, 7–11). Four recent cases have shown presence of CSF anti-cyclic citrullinated peptide (anti- CCP) in patients with RM (12–15). Here, we describe a patient with RM with strongly positive anti-CCP, IgM RF, and chemokine (C-X-C motif) ligand 13 (CXCL13) levels in CSF that normalized after treatment suggesting a potential use of these markers in both diagnosis and treatment management of RM. Furthermore, we review 48 cases of RM published in the English literature since 2010 focusing on symptoms, serum and CSF findings, treatment, and outcome.

CASE PRESENTATION

A 62 year-old woman was admitted after 4 months history of intermittent frontal headache, nausea, and gait and balance disturbances. She had a 3 year history of IgM-RF and anti-CCP positive RA, with a previously episode of pleuritis. Within the last year, she had been treated with Leflunomide, Infliximab, and was currently treated with Methotrexate and Salazopyrine entabs. Neurological examination was normal, except for a mild gait ataxia and her RA was well-controlled with no symptoms of active synovitis at time of admission.

Due to chronic headache a brain MRI was performed. This showed patchy interhemispheric pachy- and leptomenigeal enhancement adjacent to the parietal- and occipital lobes (**Figure 1A**). Blood tests revealed signs of inflammation with high levels of IgM RF (56 IU/mL), anti-CCP (>1,600 U/mL), Interleukin-2 receptor (ILR-2–1,065 kU/L) (**Table 1**), c-reactive protein (43 mg/L), and erythrocyte sedimentation rate (106 mm). Remaining systemic antibody examinations were negative (anti-DNA antibody, anti-nuclear antibody (ANA) IgG, anti-neutropil cytoplasmatic antibody (ANCA) IgG, Anti-Ro (SSA)/La (SSB), anti-cardiolipin antibody, phospholipid antibody, and lupus anticoagulant). Immunoglobulin A, G, and M levels were normal.

Cerebrospinal Fluid (CSF) analysis revealed a mononuclear pleocytosis (170 E6/L) and elevated protein level (1.16 g/L). Due to the pleocytosis, intravenous ceftriaxone, and aciclovir were administered, to cover for bacterial meningitis and Herpes

TABLE 1 | Serum and CSF markers before and after treatment.

Test/(range)	Pre-treatment	Post-treatment
Serum		
IgM RF (<15 IU/mL)	56	18
Anti-CCP (<25 U/mL)	>1,600	706
ILR-2 (158–623 kU/L)	1,065	N/A
CSF		
Leukocytes (<5 E6/L)	170	<5
Protein (0.40–0.70 g/L)	1.16	0.28
IgG index (<0,60)	1.45	0.45
Oligoclonal bands	Present	Absent
B lymfocytes (%)	7.80	–
Plasma cells (%)	1.80	–
RF IgM* (<15 IU/mL)	92.7	Negative
Anti-CCP* (<25 IU/mL)	19,600	64
CXCL-13 (<10 ng/L)	>500	<10

Range in serum; -, not performed.

Simplex Virus (HSV) encephalitis. Subsequent CSF cultures revealed no growth of bacteria, no Borrelia antibodies, and viral/bacterial PCR *(E. coli, hemophilus influenzae, Listeria monocytogenes, Neisseria meningitidis, hemolytic streptococcus, streptococcus pneumoniae*, *cytomegalovirus, enterovirus, herpes simplex virus, varicella zoster, Cryptococcus, and micromiome 16S/18S*), and flowcytometry, and cytological analysis for malignancy were negative. Therefore, antiviral- and antibiotic-treatment was terminated.

The following days the patient displayed sporadic confusion, delusions, and fever (38.5°C). Subsequent tests, including HIV, syphilis, and tuberculosis were negative. Re-examination of CSF showed continuous mononuclear pleocytosis (130 E6/L), high IgG index (1.45) and presence of oligoclonal bands, suggestive of inflammation. Repeated cultures for bacteria were negative and cytological analysis showed an inflammatory pattern with an elevated number of B-lymphocytes (7.8 %) and plasma cells (1.8%, **Table 1**).

To investigate possible systemic inflammation or malignancy whole-body FDG-PET CT was performed. This showed hypermetabolism of the cerebral cortex, adjacent to the meningeal enhancement found on MRI, and a right medial lobe infiltrate of the lung. CT of thorax and abdomen confirmed an infiltrate, slight pleural effusion, and pleural thickening. Endobronchial ultrasound with biopsy was performed revealing no malignancy or infection.

On suspicion of RM, we performed analysis on undiluted CSF showing moderately positive IgM RF (92.7 IU/mL) and strongly positive anti-CCP (19,600 IU/mL) and CXCL-13 (>500 ng/L, **Table 1**).

Subsequent, biopsy of meninges (**Figure 1C**) confirmed chronic inflammation dominated by CD138 positive plasma cells and a limited number of CD3 positive T-lymphocytes with limited infiltration into the underlying gray matter (**Figures 1E,F**). Additionally, granulomatous inflammation with dense infiltration of CD68+ histiocytes and the presence of rheumatoid nodules were found (**Figures 1D,G**). Microbial stains, PCR, and cultures of biopsy tissue for fungi, parasites, acid-fast bacilli, HSV 1, HSV 2, CMV, SV40, M. tuberculosis, and toxoplasmosis were negative.

Based on the (i) MRI findings with patchy meningeal enhancement, (ii) high titer of IgM-RF and anti-CCP in CSF and (iii) histopathological chronic inflammation of meninges with plasma cells and rheumatic nodules, the diagnosis RM was established. Concurrently, the patient displayed extra articular manifestations of RA in her lungs.

Intravenous high dose methylprednisolone (750 + 1,000 + 1,000 mg on three consecutive days) followed by oral tapering was administered in addition to current treatment with methotrexate. Within days symptoms improved, but did not completely resolve. The following weeks, the patient received Rituximab (1,000 mg intravenous, repeated after 14 days). CSF levels of IgM RF, anti-CCP, and CXCL-13 decreased accordingly to the patient reporting significant treatment response (**Table 1**). A 6 month follow-up MRI showed regression of meningeal enhancement (**Figure 1B**) and follow-up FDG-PET CT showed almost complete regression of pulmonary findings. Neurological examination at 6 month follow up confirmed resolution of clinical symptoms.

DISCUSSION

Meningitis in RA is a rare serious extra-articular complication (1, 2, 7, 16). Clinical neurological manifestations are often non-specific and duration and manifestations of RA is unreliable, as less than half of patients display active synovitis (2, 17). Sometimes CNS involvement even precedes the onset of arthritis (17–20). In cases published since 2010, 34% (13 of 38) had no history of RA before the diagnosis of RM (**Table A1**). CSF findings are variable but most often include a mild pleocytosis with elevated protein concentration and normal glucose (**Table A1**). Gadolinium enhanced MRI is often useful, showing asymmetrical pachy- or leptomeningeal enhancement (11, 18). Recently, a review of 29 cases of RM showed definite asymmetric meningeal involvement in 62% of patients, and most common neurological features were hemiparesis or hemisensory symptoms mimicking stroke or epilepsy related to localization of meningeal involvement (11). In comparison to this, we find that 70% (33 of 47) had transient or permanent weakness, sensory deficits, or speech disorders, whereas 36% (17 of 47) had seizures (**Table A1**) It is not uncommon that patients display other extra-articular manifestations of RA such as subcutaneous nodules or pulmonary manifestations, as seen in our case (2, 5, 21, 22).

Patients with RA are often treated with various immunosuppressants which increase the risk of aseptic meningitis or opportunistic infections. Therefore, it is important to rule out iatrogenic aseptic, septic, and fungal diseases before diagnosis of RM. Concurrently, autoimmune diseases, malignancies, other granulomatous diseases or IgG4-related disease can display a similar pattern of dural thickening, making them possible considerations in the differential diagnosis of RM (10, 11, 16).

Until now, there are no known RM biomarkers in CSF and meningeal biopsy is required for definite diagnosis. Biopsy shows thickening of meninges (**Figure 1C**) and histopathological features include pachy- and leptomeningeal inflammation with plasma cells and the presence of rheumatoid noduli, and in some cases vasculitis (1, 2, 17). Patients diagnosed at autopsy almost all display meningeal rheumatoid noduli, while patients diagnosed with meningeal biopsy most often show non-specific inflammation (2, 7). In some previous cases correlation between strongly elevated CSF RF and Il-6 and RM has been proposed (12, 13, 23, 24), however this still needs validation as a diagnostic tool.

No clear guideline for treatment of RM exists and cyclophosphamide, methotrexate, and azathioprine in combination with corticosteroids have all been described with improvement of symptoms (7, 17, 18, 25). In some cases, improvement on corticosteroid treatment alone has been described (5, 11, 12, 14, 20, 24, 26–31). In our case review 41% (18 of 44) were treated with corticosteroids alone, 2% (1 of 44) received no treatment, whereas the remaining received corticosteroids in combination with another therapy (**Table A1**). Seven patients (16 %) received rituximab. On these regimens only 1 case worsened (32), 8 (18%) had an incomplete improvement, whereas 80% improved (**Table A1**).

To our knowledge, anti-CCP in CSF has only been examined in four cases of RM and found to be elevated in three of these (12–15). Serum anti-CCP antibodies help distinguish RA from other types of arthritis, can help to identify patients with a higher risk of severe disease and are rarely found in other autoimmune conditions (33). They are often used in combination with IgM RF in the diagnosis of RA. In this case, anti-CCP level in CSF was strongly positive and a crucial element in both diagnosing RM and monitoring treatment response. With this case, we show a novel clear response of anti-CCP to the treatment of RM. Moreover, in addition to CSF anti-CCP and IgM RF, we also find the B cell chemoattractant CXCL-13 levels associated with treatment response, which to our knowledge has not previously been investigated.

We propose using anti-CCP, IgM RF, and CXCL-13 in CSF as potential biomarkers not only for diagnosis of RM, but also in evaluation of treatment response. Further studies are needed to clarify their potential use.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the Supplementary Files.

ETHICS STATEMENT

Clinical data in this case report was collected with the consent of the patient. A written informed consent was obtained from the patient for the publication of this case report.

AUTHOR CONTRIBUTIONS

MN and AN: design and draft of the manuscript and interpretation of data. JF and JM: draft of manuscript. MW and CB: acquisition of data and draft of manuscript. K-EB and TE: revised manuscript for intellectual content. MB: draft of manuscript, acquisition of data, and revised manuscript for intellectual content.

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APPENDIX

TABLE A1 | Summary of RM cases from 2010 to present.

References	Patient (Age, sex)	Years of RA	Treatment of RA	Symptoms of RM	Serum			CSF						MRI compatible with RM	Biopsi compatible with RM	Treatment	Outcome
					IgM RF (IU/mL)	Anti-CCP (IU/mL)	Other	Cells/μl	Protein (mg/L)	Glucose (mg/dl)	IgM RF (U/mL)	Anti-CCP (IU/mL)	Other				
Cianfoni et al. (32)	74, F	5	CS, MTX	Progressive left-side weakness and hypoesthesia	506	–	–	65	0.43	Normal	–	–	–	Yes	Yes	CS, IT MTX	Worsening
Matsushima et al. (24)	80, F	20	CS, sulfasalazine, bucillamine, etanercept	Transient weakness and numbness right-side	Normal	–	–	18	0.55	–	–	–	IL-6 = 4.6 pg/ml	Yes	Yes	CS	Improvement
Inan et al. (34)	70, F	0	None	Headache, nausea, vomiting, and confusion	108	–	ESR = 124 mm/h	140	1.13	34	98 (after treatment <20)	–	–	Normal	Not performed	CS, AZA	Improvement
Aguilar-Amat et al. (35)	71, F	15	NR	Seizures and PSP-like phenotype	27.9	–	–	Normal	Normal	Normal	–	–	–	Yes	Yes	CS, MTX	Improvement
Kim et al. (26)	66, M	0	None	Seizures (SE) and left-sided weakness	High levels	1,448	ANA high	11	Normal	Normal	–	–	–	Yes	Yes	CS	Improvement
Servioli et al. (36)	80, F	NR	CS, HCQ	Unsteady gait with falls. Progression to left-sided weakness	<20	–	ESR = 35 mm/h	2–7	0.75–0.77	60	–	–	–	Yes	Yes	Not reported	Not reported
Hasiloglu et al. (37)	62, F	4	CS, MTX	Headache, paresis, and paresthesia right UE	351	120	–	40	0.40	–	–	–	–	Yes	Not performed	CS, MTX	Improvement
Huys et al. (38)	58, F	9 month	MTX, Adalimumab	Headache and psychomotor retardation, seizures	–	–	–	30	0.55	–	–	–	–	Yes	Yes	CS, RTX, Leflunomide, MTX d/c, Adalimumab d/c	Improvement

(Continued)

TABLE A1 | Continued

References	Patient (Age, sex)	Years of RA	Treatment of RA	Symptoms of RM	Serum			CSF						MRI compatible with RM	Biopsi compatible with RM	Treatment	Outcome
					IgM RF (IU/mL)	Anti-CCP (IU/mL)	Other	Cells/μl	Protein (mg/L)	Glucose (mg/dl)	IgM RF (U/mL)	Anti-CCP (IU/mL)	Other				
Duray et al. (22)	73, M	1	CS, MTX	Disorientation, apathy, and astenia, walking difficulty	2,720	>340	–	83–91	1.3–2.22	42–58	–	–	–	First MRI normal, yes	Yes	CS, CYC	Improvement
Krysl et al. (5)	62, M	10	HCQ	Epilepsia partialis continua right side	1:320	760	–	0–32	0.245–0.345	–	–	–	OCBs in one CSF sample	Yes	Yes (2 year after initial symtoms)	CS	Improvement
Roques et al. (39)	60, M	NR	MTX	Transient right-sided paresis and hypoesthesia	–	–	–	Increased	Mild elevation	Normal	–	–	–	Yes	Yes	Not reported	Not reported
Hayashi et al. (4)	60, M	10	CS	Parkinsonism not responsive to levo-dopa	–	–	–	13	0.75	Normal	–	–	–	Yes	Yes	CS	Incomplete improvement
Bourgeois et al. (40)	70, M	NR	NR	Transient right hemiparesis, headache	Positive	–	–	68	0.47	2,9 mmol /L	–	–	–	Yes	Yes	CS, HCQ, sulfasalazine	Improvement
Rijkers et al. (6)	57, F	NR	NR	Tonic-clonic seizures	–	–	–	–	–	–	–	–	–	Yes	Yes	CS	Not reported
Yeaney et al. (9)	63, M	9	NR	Headache and paresis	–	–	–	–	–	–	–	–	–	Yes	Yes	Not reported	Not reported
Padjen et al. (20)	77, F	0	None	Seizures and right hemiparesis	171.7	405.3	–	Normal	Normal	Normal	–	–	–	Yes	Yes	CS	Improvement
Lu et al. (27)	60, F	23	CS, Auranofin	Headache, photophobia, insomnia, panic attacks, hallucinations	>1:160	Strongly positive	–	2	0.26	58	–	–	–	Yes	Yes	CS	Improvement

(Continued)

TABLE A1 | Continued

References	Patient (Age, sex)	Years of RA	Treatment of RA	Symptoms of RM	Serum			CSF						MRI compatible with RM	Biopsi compatible with RM	Treatment	Outcome
					IgM RF (IU/mL)	Anti-CCP (IU/mL)	Other	Cells/μl	Protein (mg/L)	Glucose (mg/dl)	IgM RF (U/mL)	Anti-CCP (IU/mL)	Other				
Roy et al. (3)	Late 50s, F	NR	MTX, sulfasalazine	Transient aphasia, confusion, headache right leg weakness, right facial drop	–	High	–	12	0.55	58	–	–	–	Yes	Yes	MTX, MMF, MTX d/c	Improvement
Magaki et al. (28)	37, M	0	None	Headache, facial weakness, speech disorder, right hand dysfunction	83	>250	–	10–16	0.35–0.50	50–89	–	–	–	Yes	Yes	CS	Improvement
Magaki et al. (28)	62, F	0	None	Confusion and transient loss of consiousness, seizures, and lower limb weakness	Negative	–	–	–	–	–	–	–	–	Not reported	Yes	CS	Incomplete improvement
Nihat et al. (41)	71, F	6	Adalimumab, MTX	Dysarthria, paresthesia left face and arm, difficulty walking, tremor, and headache	7,900 U/L	226	ESR = 76 mm/h; ANA 1:80	50–80	0.46–0.67	2.4 mmol/L	–	–	–	Yes	Yes	CS, CYC, MTX	Improvement
Saego et al. (29)	66, F	12	Infliximab	LE numbness, aphasia developing into headache, LE paralysis	–	–	–	213–216	4.4–8.59	41–44	RF elevated	–	–	Yes	Yes	CS	Improvement
Shibahara et al. (12)	63, M	0		Headache, vertigo, confusion	140	472	ESR = 18 mm/h	37	0.92	Normal	–	4.4–26.2	IL-6 = 482 pg/ml	Yes	Not performed	CS	Improvement

(Continued)

TABLE A1 | Continued

References	Patient (Age, sex)	Years of RA	Treatment of RA	Symptoms of RM	Serum			CSF						MRI compatible with RM	Biopsi compatible with RM	Treatment	Outcome
					IgM RF (IU/mL)	Anti-CCP (IU/mL)	Other	Cells/μl	Protein (mg/L)	Glucose (mg/dl)	IgM RF (U/mL)	Anti-CCP (IU/mL)	Other				
Matsuda et al. (21)	66, M	19	CS, MTX, iguratimod	Falls	160	310	ESR = 38 mm/h; ANA 1:5120; SSA and SSB positive	71	1.14	27		–	–	Yes	Not performed	CS, MTX d/c	improvement
Moeyersoons et al. (42)	49, F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Not performed	CS, RTX. adalimumab d/c, leflunomide d/c	Improvement
Tsuzaki et al. (43)	65, M	7 month	CS, MTX, Entanercept	Transient loss of consciousness, seizures, transient dysrthria, left leg weakness	12	275	sIL2R = 555 U/mL; ANA = 80; SSA 297 U/mL; SSB 18.6 U/mL	12	0.32	55		–	–	First normal, yes	Yes	CS, tocilizumab, etanercpt d/c	Improvement
Choi et al. (11)	65, F	3	CS, MTX, leflunomide	Headache, confusion, and recurrent left hemiparesis	69.3	48.8	–	20	1.134	43	RF 17.6	–	–	Yes	Yes	CS	Improvement
Degboé et al. (44)	59, M	6	MTX	Transient right-sided hypoesthesia and hemiparesis	–	–	–	30	0.75	3.2 mmol/L	–	–	–	Yes	Yes	CS, MTX, RTX	Improvement
Jessee and Keenan(45)	68, F	0	None	Confusion, right-sided weakness, and seizures	208	95.8	ANA 1:640	8	0.65	56	–	–		Not done (pacemaker)	Yes	CS, MTX	Incomplete improvement
Alexander et al. (46)	73, M	NR	Leflunomide	Transient speech disorder, behavoiral change and seizure	45	>340	–	18–100	0.69–1.03	2.5–3.1 mmol/L	–	–		Yes	Yes	CS, RTX	Incomplete improvement

(Continued)

TABLE A1 | Continued

References	Patient (Age, sex)	Years of RA	Treatment of RA	Symptoms of RM	Serum			CSF						MRI compatible with RM	Biopsi compatible with RM	Treatment	Outcome
					IgM RF (IU/mL)	Anti-CCP (IU/mL)	Other	Cells/μl	Protein (mg/L)	Glucose (mg/dl)	IgM RF (U/mL)	Anti-CCP (IU/mL)	Other				
Finkelshtein et al. (19)	66, F	0	None	Headache, transient paresthesia left leg	23–25	266	–	–	–	–	–	–	–	Yes	Yes	None	Improvement
Parsons et al. (47)	76, M	30	MTX	Transient left UE paresis, new onset seizures	Elevated	Elevated	ANA elevated	239	0.39	51	RF negative	–	–	Yes	Yes	CS, MTX	Improvement
Oono et al. (23)	36, F	13	CS, MTX	Headache and transient sensory disturbance right face and UE	–	–	ESR = 56 mm/h; anti-RNP = 15 U/mL	19	0.57	51		–	IL−6 = 843 pg/ml, OCBs	Yes	Not performed	CS, MTX d/c	Improvement
Akamatsu et al. (13)	55, F	6 month	MTX	Speech difficulty, left-sided hemiparesis, and spatial neglect	85 U/L	223.7		68	0.40	52		3.7	IL-6 = 271 pg/mL	Yes	Not performed	CS	Incomplete improvement
Gherghel et al. (10)	77, F	>9 year	Ethanercept, leflunomide	Recurrent speech disorder and left-sided paresthesia and hemparesis	86	119	ANA 1:160	5	0.49	–	–	–	–	Yes	Yes	CS, etanercept d/c, leflunomide d/c	Incomplete improvement
Schuster et al. (14)	48, M	0	None	Headache, recurrent left-sided weakness	298	>340	–	300	1.37	–	–	>340	–	Yes	Not performed	CS	Improvement
Schuster et al. (14)	62, F	Not stated	NR	Recurrent tingling and weakness	146	265	–	Normal		–	–	–	–	Yes	Not performed	CS, MTX	Improvement
Schuster et al. (14)	72, M	0	None	Recurrent sensory motor deficit left-side	133	154	–	51	Normal	–	–	–	–	Yes	Yes	CS	Improvement

(Continued)

TABLE A1 | Continued

References	Patient (Age, sex)	Years of RA	Treatment of RA	Symptoms of RM	Serum			CSF						MRI compatible with RM	Biopsi compatible with RM	Treatment	Outcome
					IgM RF (IU/mL)	Anti-CCP (IU/mL)	Other	Cells/μl	Protein (mg/L)	Glucose (mg/dl)	IgM RF (U/mL)	Anti-CCP (IU/mL)	Other				
Schuster et al. (14)	62, M	11	NR	Alexia, agraphia, acalculia, headache, seizures	22.3	329	–	Normal	Normal	–	–	–	–	Yes	Not performed	CS, tocilizumab	Improvement
Schuster et al. (14)	65, F	11	NR	Recurrent sensory motor deficit left-side, speech disorder	313	26	–	8	0.653	–	–	–	–	Yes	Yes	CS, tocilizumab, leflunomide d/c; MTX d/c	Improvement
Schuster et al. (14)	45, M	30	NR	Recurrent left-side hypoesthesia, headache, ataxia	113	7	–	37	4.6	–	–	–	–	Yes	Yes	CS, CYC, MTX, leflunomide d/c; HCQ d/c	Improvement
Ching et al. (31)	72, F	0	None	Left-sided weakness, psychiatric symptoms, seizures	Negative	197.5	ESR = 39 mm/h	12	0.25	58	–	–	–	Yes	Yes	CS	Improvement
Harrison et al. (48)	53, M	NR	CS, leflunomide, tofacitinib citrate	Headache, seizures, right LE paresis	293	250	ESR 46 mm/h	7	0.64	48	–	–	–	Yes	Yes	CS, RTX	Improvement
McKenna et al. (30)	59, M	0	None	Headache and left-sided weakness, focal onset seizures	88.2	>340	ACE = 70 U/L	Pleocytosis	0.672	3.4 mmol/L	–	–	–	Yes	Yes	CS	Improvement
Pellerin et al. (1)	74, M	3–4	CS, HCQ, MTX	Expressive aphasia, imbalance, potural tremor, parkinsonism, seizures	High	High	ACE 66 U/L, beta 2 mikroglobulin 4,6 mg/L	6	0.86	Normal	–	–	–	Yes	Yes	CS, CYC, MTX d/c	Incomplete improvement

(Continued)

TABLE A1 | Continued

References	Patient (Age, sex)	Years of RA	Treatment of RA	Symptoms of RM	Serum			CSF						MRI compatible with RM	Biopsi compatible with RM	Treatment	Outcome
					IgM RF (IU/mL)	Anti-CCP (IU/mL)	Other	Cells/μl	Protein (mg/L)	Glucose (mg/dl)	IgM RF (U/mL)	Anti-CCP (IU/mL)	Other				
Grose et al. (49)	87, F	NR	None	UE weakness, confusion, hallucinations	143	>200	ANA 1:640	104	1.55	Normal	–	–	–	Yes	Not performed	CS	Incomplete improvement
Scheitel et al. (50)	75, F	9	CS, leflunomide	UE paresthesia, weakness, headache, facial jerks, Rytmic jerks	High	High	ESR = 92 mm/h	14	0.69	–	–	–	–	Yes	Not performed	CS, RTX	Improvement
Lubomski et al. (15)	49, M	0	None	Headache, deterioration in mental state, delusions	8	>600	–	1	0.39	3.4 mmol/l		Strongly positive		Yes	Yes	CS, RTX	Improvement

ACE, angiotensin converting enzyme; ANA, antinuclear antibodies; AZA, azathioprin; CS, corticosteroids; CYC, cyclophosphamide; d/c, discontinued; ESR, erythrocyte sedimentation rate; F, female; HCQ, Hydroxychloroquine; IL-6, interleukin-6; IT, intrathecal; LE, lower extremity; M, male; MTX, methotrexate; MMF, Mycophenolate mofetil; N/A, not avaliable; NR, not reported; RA, rheumatoid arthritis; RF, rheumatic factor; RM, rheumatoid meningitis; RNP, ribonucleoprotein; RTX, Rituximab; sIL2R, soluble interleukin-2 receptor; SSA, Anti-Sjögren's-syndrome-related antigen A; SSB, Sjögren's-syndrome-related antigen B; UE, upper extremity.

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BRIEF RESEARCH REPORT
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Immunity in Gilles de la Tourette-Syndrome: Results From a Cerebrospinal Fluid Study

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Background: Several lines of evidence support the hypothesis of an autoimmune origin of Gilles de la Tourette-Syndrome (GTS). Accordingly, in a recent study we detected positive oligoclonal bands (OCB) in cerebrospinal fluid (CSF) in >30% of adult patients indicating an intrathecal antibody synthesis. However, until today no corresponding antibodies could be identified. The aims of this study were to replicate our findings of positive OCB in an independent sample and to detect CSF autoantibodies.

Methods: In this prospective study, 20 adult patients with GTS (male: female = 18:2, median age 36.1 years ± 14.34 SD) were included. All patients were thoroughly clinically characterized. Magnetic Resonance Imaging (MRI) and CSF standard measurements were performed. Isoelectric focusing on polyacrylamide gels with silver staining was used to detect OCB. To examine specific and unspecified autoantibodies, we used transfected Human Embryonic Kidney (HEK) cells expressing different surface antigens (NMDA-, CASPR2-, LGI1-, AMPA-, or GABAB1/B), indirect immunofluorescence on different brain tissue sections, and enzyme-linked visualization. Additionally, we differentiated Glioma stem cells SY5Y (human neuroblastoma) using retinoic acid and astrocytes (rat).

Results: CSF analyses showed positive OCB (type 2) in 4/20 patients (20%). Using transfected HEK cells we did not find specific surface-autoantibodies. Immunohistochemistry on tissue-sections, SY5Y Glioma stem-cells, and astrocytes showed no specific binding patterns either.

Conclusions: Our results corroborate previous findings and demonstrate positive OCB in a substantial number of patients with GTS (prevalence in healthy controls: 5%). Although this is the largest study investigating CSF autoantibodies in GTS using several techniques, we failed to detect any specific or unspecified autoantibodies.

Keywords: Tourette-syndrome, autoimmunity, cerebrospinal fluid, oligoclonal bands, antibodies, tics, immunology

INTRODUCTION

Gilles de la Tourette-Syndrome (GTS) is a neuropsychiatric disorder characterized by childhood onset motor and vocal tics (DSM-5) that fluctuate spontaneously over time (1). It is thought that GTS is caused by alterations in cortico-striato-thalamo-cortical circuits. Several lines of evidence suggest that both genetic and non-genetic influences contribute to the etiology of GTS (2, 3). It has been demonstrated that multiple common genetic variants of small effect play a role, but in recent genome-wide association studies (GWASs) no single-nucleotide polymorphisms (SNPs) met criteria for genome-wide significance (4, 5). While in a first genome-wide *epigenetic* analysis, no methylation site reached significance (6), altered methylation levels of different dopaminergic genes (dopamine D2 receptor, DRD2, dopamine transporter, DAT) could be detected, when measuring peripheral DNA methylation (7). Finally, several interacting environmental factors seem to be involved in the pathogenesis of GTS such as psychosocial stress (8), perinatal risk factors (9), and immunological changes (10). Accordingly, several different abnormalities in the peripheral immune system have been described including increased serum levels of Tumor Necrosis Factor-Alpha (TNF-α), Interleukin 12 (IL-12) (11) and several other interleukins (IL) such as IL-6, IL-8, IL-1β, and IL-17 as well as interferon-gamma induced protein 10 k (IP-10), an indicator for activation of cellular immunity (12). Furthermore, increased levels of antinuclear antibodies (ANA) (13), C-reactive protein (CRP), and neopterin, increased numbers of monocytes (14), increased concentrations of CD4-, CD95-, CD8-, CD69-, B-, and T-cells, and an overexpression of natural killer (NK)-cells (15) in patients' sera suggest increased inflammatory activity in patients with GTS. Accordingly, in an animal model for GTS, striatal dysfunction could be provoked by intrastriatal microinfusion of sera from patients with GTS (16) suggesting abnormalities in the immune response in the central nervous system. However, there is only one study examining inflammatory changes in cerebrospinal fluid (CSF) ((17), see below) whereas most other studies examined patients sera, and therefore assessed changes in the peripheral immune system.

In line with these findings, the PANDAS (=Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections) concept has been suggested, based on the hypothesis that diseases associated with tics and/or obsessive compulsive disorder (OCD) might be caused by group A streptococcal (GAS) infections (18). The fact that many patients with a clinically similar syndrome to PANDAS have no evidence of streptococcal infection, resulted in the generation of the term PANS (=Pediatric Acute-onset Neuropsychiatric Syndrome) describing a syndrome with abrupt onset of obsessive-compulsive symptoms, anxiety, and sensory symptoms in previously healthy children (19). However, results from the recently completed European-wide EMTICS study (20) failed to demonstrate evidence for a causal role of streptococcal and non-streptococcal bacteria in the onset or exacerbation of tics, but confirmed recent data for an abnormal immune responsiveness in patients with GTS with lower levels of pro-inflammatory cytokines IL-6 and TNF-α and soluble TNF-receptor as well as higher immunoglobulin levels soluble monocytes activation marker CD14 (21).

In order to further explore immunological changes in GTS, in a recent study, we analyzed autoantibodies in sera of 51 patients, but failed to detect any abnormalities for N-methyl-D-aspartic acid- (NMDA), contactin-associated protein related 2- (CASPR2), Leucin-rich glioma inactivated protein (LGI1), or gamma-aminobutyric acid (GABAB1/B2) (22). In contrast, Dale et al. (23) reported elevated levels of antibodies against dopamine-2 receptors in 4/44 of patients with GTS.

Unfortunately, the vast majority of studies investigating antibodies in GTS is limited by the fact that blood sera have been used, but not cerebrospinal fluid (CSF). This is of paramount importance, since in neuropsychiatric disorders results from CSF are more meaningful, not only because the blood-brain barrier segregates blood from CSF limiting antibodies and other determinants to pass, but also the fact that CSF contains less proteins than blood, which may influence the results. Even though in a recent case-control study in five children with GTS (age range, 6–12 years) no CSF specific oligoclonal bands (OCB) could be detected (17), in a recently performed larger study in a mixed population (median age 29 ± 12 SD, range, 9–51 years), we found positive CSF OCB in 8/21 (38%) of patients (24). This finding further corroborates an autoimmune hypothesis in GTS, since OCB are IgG antibodies produced by plasma cells that are found in only 5% of healthy people (25) and therefore document—in nearly all cases—a pathological antibody synthesis within the central nervous system.

The aim of this study was to perform extensive CSF analyses in a group of adult patients with GTS. We expected to replicate recent findings of positive OCB (24) and to detect CSF autoantibodies particularly in those patients with positive OCB.

METHODS

Patients

In this prospective study, we included 20 adult patients (male: female = 18:2) with GTS according to DSM-5. In all cases, the diagnosis was confirmed by one of the authors (KMV). Patients were recruited from our Tourette outpatient clinic at the Hannover Medical School and internet calls between 10/2015 and 3/2016. All examinations were conducted in accordance with the declaration of Helsinki and after approval of the Local Ethics Committee (no. 6987) of the Hannover Medical School. All patients gave written informed consent before entering the study. The inclusion criteria were: (i) age >18 years, (ii) confirmed diagnosis of GTS, (iii) no other severe psychiatric or neurological diseases such as schizophrenia, alcoholism, epilepsy, and mental retardation as well as no (autoimmune) diseases that are known to cause CSF changes including OCB. Additionally, all patients were screened for contraindications of a lumbar puncture such as disturbances of blood coagulation (including measurement of International Normalized Ratio (INR), Partial Thromboplastin Time (PTT), and thrombocyte count), increased intracranial pressure, and spinal tumors.

Magnetic Resonance Imaging (MRI)

Before lumbar puncture, in all patients cerebral Magnetic Resonance Imaging (MRI) was performed to exclude (i) brain pathologies associated with increased intracranial pressure, and (ii) immunological diseases known to cause positive OCB such as multiple sclerosis (MS). All images were acquired on a 3T Siemens Skyra MRI scanner equipped with 32 channel head coils. For structural images, T1 weighted images were acquired by means of a MPRAGE sequence (slices = 192, FoV = 256 mm, voxel size = 1 × 1 × 1 mm, TR = 2.5 s, TE = 4.37 ms, flip angle = 7°, distance factor = 50%).

Clinical Assessments

All patients were carefully clinically assessed using the following standardized assessments: (i) Yale Global Tic Severity Scale—otal Tic Score (YGTSS-TTS) (26) to measure tic severity, (ii) Premonitory Urge for Tics Scale (PUTS) (27) to assess tic-related urges, and (iii) Gilles de la Tourette-Syndrome Quality of Life Scale (GTS-QOL) (28) to measure patients health-related quality of life. To assess common comorbidities, we used (i) Beck Depression Inventory-II (BDI-II) (29), (ii) Conner's Adult Attention deficit/hyperactivity disorder (ADHD) Rating Scale (CAARS) (30), (iii) DSM-IV symptom list for ADHD (31), (iv) Wender Utah Rating Scale short version (WURS-K) (32), (v) Beck Anxiety Inventory (BAI) (33), (vi) Yale Brown Obsessive Compulsive Scale (Y-BOCS) (34), and (vii) Brief Symptom Inventory (BSI) for psychological distress and psychiatric disorders (35).

CSF Analysis

After lumbar puncture, CSF samples were immediately frozen at −80°C for up to 4 months prior to analyses.

Routine Parameters

CSF was analyzed by standard methods assessing routine CSF parameters including (i) CSF cell count using a Fuchs-Rosenthal counting chamber, (ii) manual assessment of cytology and (iii) blood-CSF barrier function by CSF-serum albumin quotients [QAlb (36, 37)]. The age-adjusted upper reference limit of QAlb was calculated using the following formula: QAlb = 4 + (age in years/15) [formula of Reiber-Felgenhauer (38)], and (iv) OCB by isoelectric focusing on macro polyacrylamide gels with consecutive silver staining simultaneously in CSF and blood sera (39).

Detection of Specific CSF Autoantibodies

For the assessment of well-known specific CSF autoantibodies, we used transfected Human Embryonic Kidney Cells (HEK293, Autoimmune-Encephalitis-Mosaik 1 FA 112D 1003-1, Euroimmun, Lübeck, Germany), which express either NMDA-, CASPR2-, LGI1-, AMPA- or GABAB1/B2. The HEK-cells were incubated with 30 µl undiluted CSF and washed afterwards to detect antibodies in CSF. Bound autoantibodies in CSF were labeled with secondary fluorescein-conjugated goat anti-human antibodies (Mosaik 1 FA 112D 1003-1, Euroimmun, Lübeck, Germany). As a positive control we used the NMDAR antibody provided in the kit (Mosaik 1 FA 112D 1003-1, Euroimmun, Lübeck, Germany).

Detection of Unspecified CSF Autoantibodies

To detect antibodies targeting unknown antigens, we used indirect immunofluorescence and incubated (30 min) tissue sections from monkey cerebellum (all tissues: Glutamate-Receptor-Mosaik 3 FA 111m 1003-3, Euroimmun, Lübeck, Germany), hippocampus (rat), basal ganglia (rat), and Eu90-cells (as negative control) with undiluted patient CSF. In order to visualize bound antibodies, we used fluorescein isothiocyanate (FITC) marked anti-human IgG (Mosaik 3 FA 111m 1003-3, Euroimmun, Lübeck, Germany) provided by the kit as secondary antibody. To control for immunofluorescence staining artifacts, we used enzyme-linked visualization by incubating tissue sections from cerebellum (monkey) with 1:1 diluted patient CSF and anti-human IgG antibodies, adding 3 3′-diaminobenzidine (DAB) as dye (Vector Laboratories, Burlingame, USA). 1:100 diluted Anti-Yo and anti-Hu positive sera (of patients with immunoblot-confirmed antibodies) were used as positive controls. Antinuclear antibodies were described if a typical nuclear binding to all cell nuclei was present in tissue sections.

Glioma Stem Cell Line

To measure IgG binding to cell surface neuronal antigens, we differentiated a human Glioma cell line (neuroblastoma, SY5Y). Therefore, SY5Y cells were incubated for 3 days in nutritional medium with 10 µl retinoic acid (DMEM/F-12, ThermoFisher Scientific). After plating on poly-l-lysine-coated wells (20.000 cells/well), we incubated the cells with 200 µl undiluted patient CSF. Antibody binding was visualized by fluorescing goat-anti human IgG (Alexa Fluor 488, Invitrogen, 1:500) as secondary antibody. Cells were double labeled using rabbit-anti Microtubule-Associated Protein 2 (map-2, Millipore, 1:500) as second primary antibody and fluorescing goat-anti rabbit (Alexa Fluor 555, Invitrogen, 1:500) as second secondary antibody. Cells incubated with Hu- and Yo-receptor positive sera (patient sera with immunoblot-confirmed antibodies), diluted 1:10 using phosphate buffered saline and triton 0.1%, served as positive controls.

Glia Cells: Astrocytes

Since astrocytes closely interact with neurons and regulate synaptic transmission and plasticity, we additionally assessed antibody binding on this cell type. On that account, we prepared astrocytes from newborn rat brains. Preparations were done in accordance with the international guidelines for the use of laboratory animals. Therefore, 50,000 astrocytes/well were plated and incubated with nutritional medium (DMEM + 1% Pen/Strep + 10% FCS) for 3 days. Assessed by Glial Fibrillary Acid Protein, the purity of astrocyte cultures was over 95%. For detection of antibody binding, the astrocytes were incubated with undiluted patient CSF and fluorescing goat anti-human IgG (Alexa Fluor 488, Invitrogen, 1:500) as secondary antibody. Anti-Glial Fibrillary Acid Protein antibodies (GFAP, Millipore, 1:200) as a second primary antibody and fluorescing goat

anti-rabbit (Invitrogen, Alexa Fluor 555, 1:500) as a second secondary antibody were used to label astrocytes. As positive control we used aquaporin 4 (AP4) positive serum (patient sera with confirmed AP4-antibodies, 1:200). Additionally, CSF of all patients were examined for AP4- and MOG-antibodies using a commercially available AP4-antibody kit (Aquaporin4 FA1128-1005-1, Euroimmun, Lübeck, Germany).

Blood Sera

Referring to the PANDAS hypothesis of streptococcal infections in GTS, in addition, we measured serum anti-streptolysin titers. Based on recent findings of positive dopamine-2 receptor antibodies (23), we incubated SY5Y cells with 1:10 diluted patient's sera and compared binding patterns with a D2-positive control (primary antibody mouse anti-human D2 IgG, 1:500; secondary antibody goat anti-mouse IgG, 488, 1:500).

Statistical Analyzes

All statistics were calculated using SPSS. For statistical analysis a p-value < 0.05 was considered significant. All statistical analyses were performed using two-tailed testing. As all dependent variables were normally distributed (tested using Kolmogorow-Smirnow test), parametric tests were used throughout. Due to the small sample sizes, we assumed variance homogeneity for all tests.

RESULTS

Clinical Assessments

We included 20 patients with GTS (median age 36.1 ± 14.34 SD, range, 19–64 years) with a mean age at tic onset of 7.7 years (±2.8 SD, range, 3–13 years). Mean tic severity according to YGTSS-TTS was 23.2 (±9.1 SD, range, 10–39). All other clinical details are summarized in **Table 1**.

MRI

T1-weighted cerebral MRI did not reveal significant abnormalities suggesting increased intracranial pressure or an immunological or inflammatory disease in any of the patients.

CSF Analysis

Routine Parameter

In none of the patients an elevated CSF cell count (≥5 cells/μl) was found. Using QAlb, CSF analyses showed a slightly dysfunctional blood-CSF-barrier in 4/20 patients (4/4 male patients, in none OCB type 2). In ten patients (50%), no OCB (type 1) could be detected. In four patients (20%, 2/4 male, 2/4 female), we found positive OCB in CSF only (type 2) indicating intrathecal IgG synthesis. One of these patients also showed an activated lympho-monocytic cell profile. None of the patients showed OCB type 3 (=combination of both identical OCB in CSF and serum and additionally OCB in CSF only). Identical OCB in serum and CSF (type 4) were observed in six patients (30%).

Detection of Specific CSF Autoantibodies

Evaluation of the transfected HEK cells revealed no specific binding patterns to any of the expressed surface antigens as depicted in **Figures 1A,B**.

Detection of Unspecified CSF Autoantibodies

In two patients (10%), we identified positive ANA in sera and CSF (no OCB type 2), but failed to detect any other antibody binding pattern in any of the tissue sections (**Figures 2A–D**). By using enzyme linked immunohistochemistry on tissue-sections of monkey cerebellum, we were able to confirm results as seen in immunofluorescence (**Figures 2E,F**).

Glioma Stem Cell Line

To evaluate immune reactivity against different cells in the central nervous system, we used a human Glioma stem cell line (SY5Y). We detected positive ANA in the same two patients (10%), where we also identified positive ANA using immunoreactivity on tissue sections (see above). No other antibody binding was found as depicted in **Figures 1C,D**.

Glia Cells: Astrocytes

When comparing patients' CSF-incubated and dyed astrocytes to aquaporin 4 positive controls, we were unable to detect antibodies (**Figures 1E,F**). Presence of aquaporin 4 antibodies was additionally checked using transfected HEK cells providing the same negative results.

Blood Sera

Measuring anti-streptolysin titers, five patients (25%) demonstrated elevated values (>200 IU/ml). Noteworthy, two of these patients (10%) also showed positive OCB type 2 in CSF and another two patients mirrored OCB in CSF and serum (pattern type 4). Only two patients (no. 9 and 13) reported a history of streptococcal infections in childhood. However, none of these showed elevated anti-streptolysin titers. None of the patient's sera demonstrated positive dopamine-2 antibodies.

Relation of Clinical Data and CSF Findings

There were no statistically significant differences in mean age at tic onset between patients with positive OCB type 2 (age at tic onset: 6 years ± 0.81) and without OCB (age at tic onset: 8.1 years ±3 SD, $p = 0.93$). We also did not find any other significant differences between patients with and without positive OCB referring to tic severity (according to YGTSS-TTS), pre-monitory urges (PUTS), and comorbidities including OCD (Y-BOCS), ADHD (CAARS, WURS-K, DSM-IV symptom list), depression (BDI-II), anxiety (BAI) and psychological distress and psychiatric disorders (BSI) (for further details see **Table 2**).

DISCUSSION

The main result of this study was the finding of positive OCB type 2 in 4/20 of patients (20%) corroborating recent findings from our group in an independent sample of patients with GTS [positive OCB type 2 in 8/21 of patients (38%)] (24). However, the percentage of patients with positive OCB type 2 in this study was lower compared to our previous findings (20 vs. 38%). This could be explained either by the relatively small sample sizes or differences in clinical characteristics. For example, 16/21 patients in the study of Wenzel et al. (24)—but only 3/20 in this study—were medicated (e.g., antipsychotics, benzodiazepines, or

TABLE 1 | Clinical characteristics of patients.

Type of OCB	Age-range	No.	Albumin quotient Q(Alb) CSF/Ser	Streptolysine titer (IU/ml)**	ANA	Medication	Age at onset of tics	YGTSS-TTS (range 0–50)	Y-BOCS (range 0–40)	PUTS (range 10–40)	GTS-QoL (range 0–100)	CAARS *t*-score (range 25–90)	WURS-k (range 0–84)	BDI-II (range 0–63)	BAI (range 0–63)	BSI, t-score (range 21–80)	DSM-IV, inattention (range 0–9)	DSM-IV, hyperactivity (range 0–9)
1	21–25	3	2.43	108	–	–	10	37	**17**	22	33.3	**72**	5	**13**	9	**71**	2	0
1	21–25	5	3.19	184	–	–	3	10	8	29	32.4	60	22	**15**	**21**	**68**	**6**	2
1	21–25	8	4.66	<50.3	–	–	11	35	7	36	28.7	52	**32**	**13**	**10**	62	3	2
1	21–25	17	4.64	<50.3	+	–	9	21	**17**	29	28.7	63	25	**15**	**23**	**65**	4	4
1	26–30	20	4.18	<50.3	–	–	8	19	0	24	19.4	47	24	**12**	6	57	4	2
1	36–40	19	**7.36**	73.4	–	–	5	15	0	31	13	42	**31**	**17**	1	50	2	1
1	41–45	10	5.69	**201**	–	–	12	11	**12**	29	49	**75**	14	**29**	**12**	**80**	2	2
1	46–50	13	5.41	104	–	–	4	24	1	13	25	61	**40**	**16**	**31**	**80**	4	**8**
1	51–55	6	**8.57**	<50.3	–	–	10	28	12	24	13.9	46	**40**	9	**11**	60	**7**	5
1	51–55	16	**8.37**	74	–	–	7	18	11	26	13	n.a.*	**60**	**11**	**20**	**71**	**8**	**6**
2	21–25	2	4.43	**547**	–	Aripiprazole	6	15	1	20	31.5	n.a.*	7	**16**	0	49	2	1
2	26–30	7	2.50	**224**	–	–	7	18	0	34	19.4	**71**	**37**	8	**19**	61	2	**6**
2	41–45	14	6.07	66.6	–	–	5	19	**14**	20	10.3	28	**36**	4	4	57	**6**	4
2	61–65	12	2.76	71.6	–	–	6	18	1	22	28.7	58	14	**13**	**12**	**67**	0	0
4	<21	15	3.98	67	+	–	13	16	10	30	24.1	n.a.*	19	**27**	**17**	**80**	2	1
4	21–25	4	2.94	**455**	–	Dronabinol	5	25	9	29	22.2	61	18	0	3	56	5	2
4	36–40	9	5.13	141	–	Nabiximols	9	38	13	29	14.8	45	22	2	4	52	3	3
4	36–40	11	**6.65**	**387**	–	–	10	33	8	21	3.7	33	0	1	0	41	0	0
4	46–50	1	3.92	<50.3	–	–	5	26	5	19	6.5	57	14	0	8	56	4	5
4	56–60	18	4.74	<50.3	–	–	9	39	**15**	29	38	61	7	**15**	8	**66**	1	1

*M, male; f, female; OCB, oligoclonal bands; type 1, no OCB; type 2, positive OCB in CSF only; type 3, identical OCB in CSF and serum plus OCB in CSF only; type 4, identical OCB in CSF and serum. ** cut-off value: >200 IU/ml. Values in bold = exceeding cut-off values, ANA, antinuclear antibodies; YGTSS-TTS, Yale Global Tic Severity Scale—Total Tic Score; Y-BOCS, Yale Brown Obsessive Compulsive Scale; PUTS, Premonitory Urge for Tics Score; GTS-QoL, Gilles de la Tourette-Syndrome Quality of Life Scale (normalized); CAARS, Conner's Adult Attention deficit/hyperactivity disorder Rating Scale; n.a., not available. * data not available due to high inconsistency index, WURS-k, Wender Utah Rating Scale short version; BDI-II, Beck Depression Inventory II; BAI, Beck Angst Inventory; BSI, Brief Symptom Inventory.*

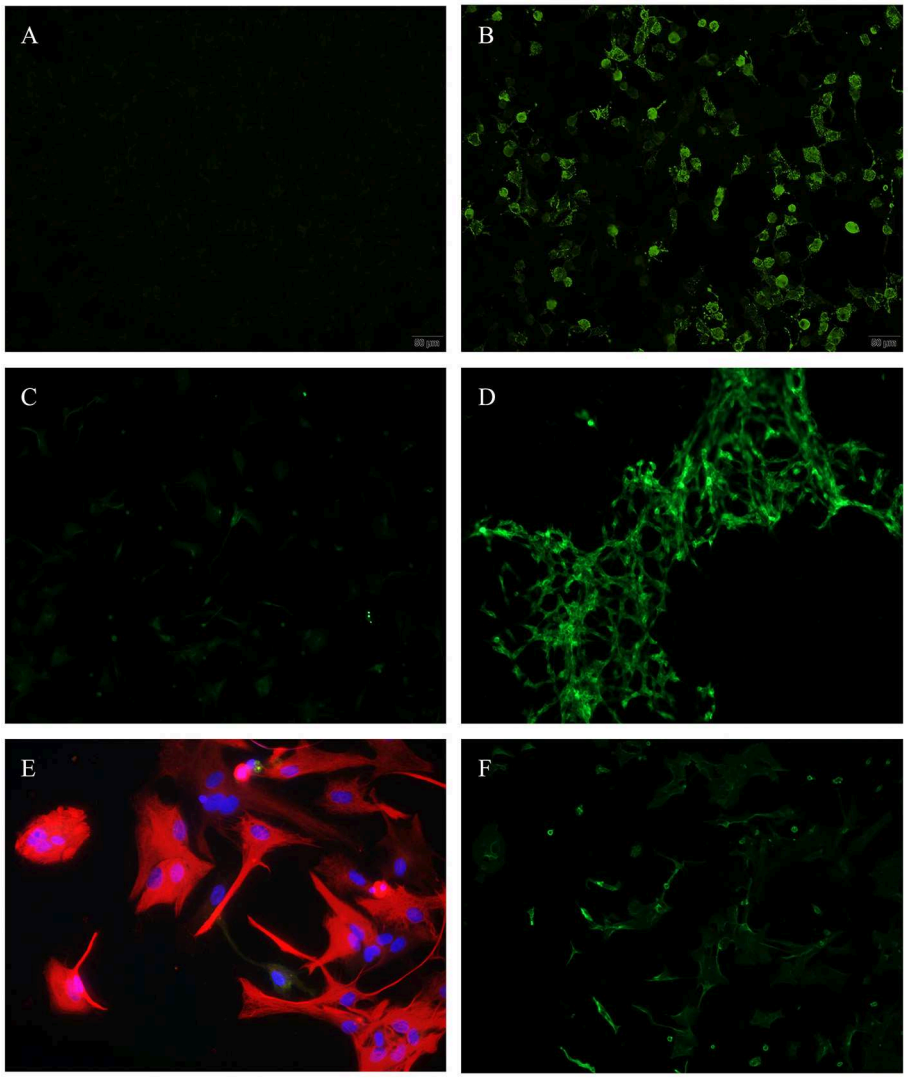

FIGURE 1 | (A,B) Transfected HEK-cells, N-methyl-D-aspartate (NMDA), immunofluorescence, **(A)** incubated with patients' CSF—showing no antibody binding, **(B)** positive control. **(C,D)** Differentiated Glioma stem cells SY5Y (human), immunofluorescence, **(C)** incubated with patients' CSF—showing no antibody binding, **(D)** positive control. **(E,F)** Astrocytes (rat), **(E)** incubated with patients' CSF (red = anti glial fibrillary acidic protein (GFAP)—showing no antibody binding, **(F)** positive control (green = anti aquaporin 4).

serotonin reuptake inhibitors). Accordingly, one might speculate that patients included in this study suffered from less severe GTS. In addition, in this study, no children (age < 18) were included, while Wenzel et al.'s sample comprised four children (9–17 years), of whom two showed positive OCB type 2. In contrast, in a recent study in five children with GTS none had positive OCB (17).

When combining results from our recent (24) and current studies (since samples were completely independent, but analyses were performed in the same lab), CSF OCB were positive in 29% (12/41 patients) of patients. Since in the general population positive OCB type 2 are found in only 5% of healthy people (25) and can be demonstrated only rarely in patients with non-inflammatory diseases (40, 41), our results clearly suggest a pathological immune process in terms of an intrathecal production of IgG antibodies. While in other diseases such as multiple sclerosis, an association between OCB and several inflammatory mediators linked to B-lymphocyte activity and other pro-inflammatory molecules has been demonstrated (42), in GTS such investigations have not been done yet. Since we detected OCB only in a subset of patients, from our results it is suggested that GTS represents a heterogeneous and multi-factorial disease. However, the clinical relevance of OCB in GTS needs further investigation as we were unable to find any differences between patients with and without positive OCB type 2 with respect to age, medication, age at tic onset, tic severity and number, kind and severity of comorbidities. Even a more detailed consideration of patients' history and clinical

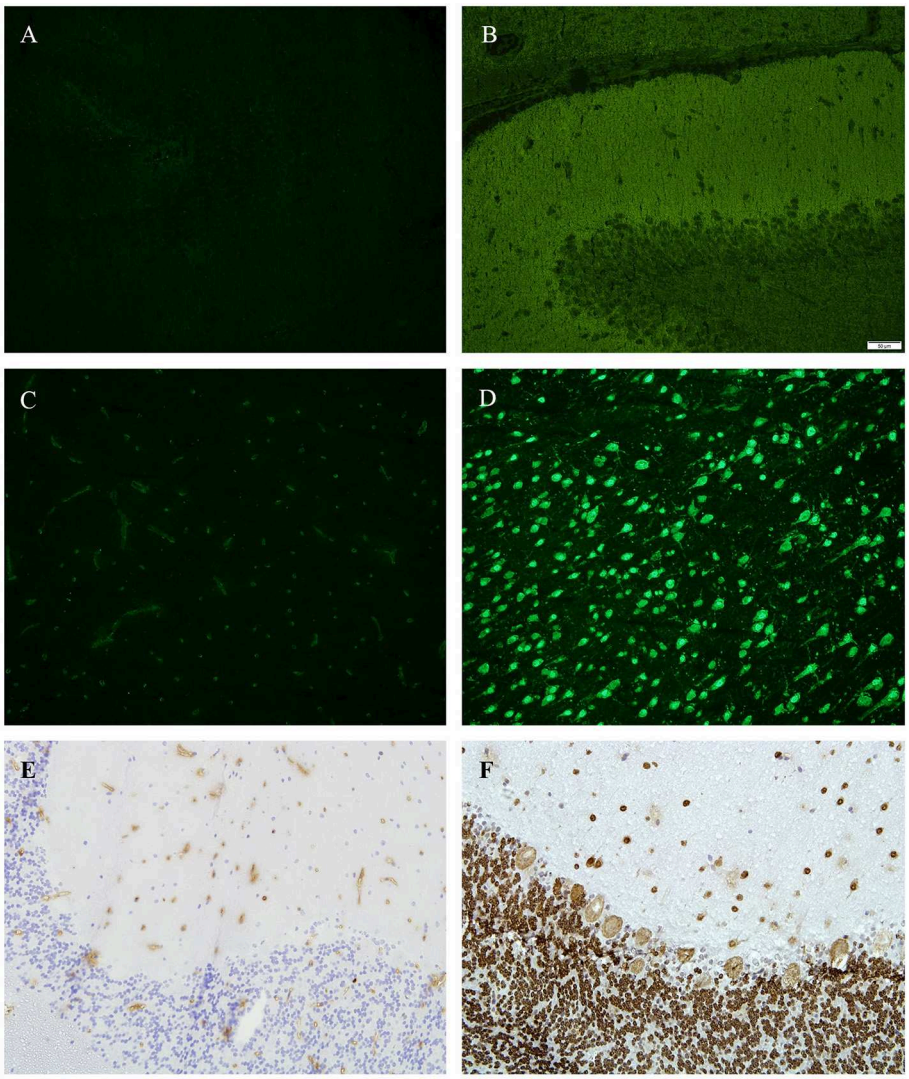

FIGURE 2 | (A,B) Tissue sections from hippocampus (rat), immunofluorescence, **(A)** incubated with patients' CSF—showing no antibody binding, **(B)** positive control. **(C,D)** tissue sections from basal ganglia (rat), immunofluorescence, **(C)** incubated with patients' CSF—showing no antibody binding, **(D)** positive control (green = anti D2). **(E,F)** Tissue sections from cerebellum (monkey), 4′,6-diamidin-2-phenylindol (DAPI), **(A)** incubated with patients CSF—showing no antibody binding, **(E)** positive control (anti-Hu).

presentation did not reveal any conclusive clinical differences between these patients' groups. However, it should be kept in mind that in psychiatric diseases several different processes may trigger a B-cell response leading to the presence of CSF OCB (43).

Our findings are completely in line with results from a recent study based on the Swedish National Patient Register, where the authors describe a general increased risk (36%) for autoimmune diseases in patients with chronic tic disorders (44) and the data from the EMTICS study demonstrating altered levels of IL-6, TNF-α, TNF-receptor, and CD14 (21). Similar conclusions have been drawn in a review of 74 studies suggesting a connection between some autoimmune diseases and OCD/tic disorders (45). More specifically, previous data provided substantial evidence that in GTS a dysfunctional immune response is at least partially T-cell mediated [e.g., elevated TNF-α, IL-12 secretion (11)], while data supporting a B-cell driven, antibody mediated process is controversial, and a specific target antigen has not yet been identified (10). However, in all available studies autoantibody production has been assessed in patients' blood sera, but not CSF. Even though we were unable to detect a specific antibody in CSF, the high percentage of patients with CSF OCB suggests an involvement of a B-cell mediated immune response.

In two patients (10%) we detected positive ANA. Since the prevalence rate of positive ANA is about 27% in the general population (46) and because none of those patients exhibiting positive ANA also showed positive OCB or increased anti-streptolysin titers, this result can be considered as not pathological.

TABLE 2 | Relation between CSF findings and clinical data.

Assessment	All patients ($N = 20$)	Patients without OCB (type 1 or 4) $N = 16$	Patients with positive OCB (type 2), $N = 4$	Mean difference	*p*-value (patients with vs. patients without positive OCB)
Age at lumbar puncture	36.1	35.4	38.7	+3.3	n.s.
Age at onset	7.7	8.1	6	−2.1	n.s.
YGTSS-TTS	23.2	24.7	17.5	−7.2	n.s.
Y-BOCS	8.0	9.1	4.0	−5.1	n.s.
PUTS	25.8	26.2	24.0	−2.2	n.s.
GTS-QOL	22.8	22.9	22.5	−0.4	n.s.
BDI-II	11.8	12.0	10.2	−1.8	n.s.
BAI	10.9	11.5	8.7	−2.7	n.s.
CAARS, t-score	54.8*	55.3*	52.3*	−3.0	n.s.
WURS-K	23.3	23.3	23.5	+0.2	n.s.
DSM-IV symptom list					
i. Inattention	3.3	3.6	2.5	−1.1	n.s.
ii. Hyperactivity	2.7	2.7	2.7	0.0	n.s.
BSI, t-score	62.5	63.3	58.5	−4.8	n.s

**Results obtained from three patients could not be analyzed due to inconsistencies, n.s., not significant; YGTSS-TTS, Yale Global Tic Severity Scale—Total Tic Score; Y-BOCS, Yale Brown Obsessive Compulsive Scale; PUTS, Premonitory Urge for Tics Score; GTS-QoL, Gilles de la Tourette-Syndrome Quality of Life Scale (normalized); CAARS, Conner's Adult Attention deficit/hyperactivity disorder Rating Scale; WURS-k, Wender Utah Rating Scale short version; BDI-II, Beck Depression Inventory II; BAI, Beck Angst Inventory; BSI, Brief Symptom Inventory.*

The second main result is the lack of autoantibodies detectable with the methods as described above. This is the first study investigating CSF samples for antibodies targeting both known and unknown antigens in a large number of adult patients with GTS. Although we used a wide spectrum of different methods including HEK cells expressing specific antigens, tissue sections of rat cerebellum, basal ganglia, and hippocampus as well as differentiated human Glioma stem cells and rat astrocytes, we failed to detect any CNS specific autoantibodies. In particular, we used not only tissue sections, but also living cells preventing possible loss of immunological reactivity to isolation or fixation processes. We primarily focused on IgG antibodies targeting cell surface antigens on live cells due to their functional impact on living cells.

This negative result can be interpreted in different ways: (i) a true absence of an autoantibody-related mechanism, at least directed to a single antigen; (ii) a failure to detect the target antigen on the cells and tissues used in this study, and (iii) the use of insufficient methods to detect autoantibodies.

Based on the autoimmune hypothesis for GTS, immunomodulatory interventions commonly used in autoimmune diseases have also been suggested for the treatment of GTS including plasmapheresis (47–49), the non-steroidal anti-inflammatory drug celecoxib (50), and intravenous immunoglobulin (IVIG) (47, 49, 51–54). Available studies resulted in conflicting findings with positive (47–50, 52–54) and negative results (51). However, all these studies are limited by the fact that no pre-selection of patients was performed depending on a marker indicating increased immune activation. It can be hypothesized that immunomodulatory treatments might be effective only in a subgroup of patients with underlying (auto)immunity (55, 56). We suggest that positive OCB may serve as such a marker to identify patients with an immune activation who might benefit from immunomodulatory treatments.

The following limitations of our study have to be addressed: (i) the sample size was quite small. However, relatively high effort and the invasive procedure related to lumbar puncture have to be taken into account as well as the fact that in clinical routine CSF analysis is not recommended in patients with tic disorders. When combining data from our two independent samples in GTS, our sample consists of 41 patients, of whom nearly one third exhibits positive OCB; (ii) although patients received a compensation fee for participating in the study, we do not believe that this has caused any bias in patient selection; and (iii) antibody binding was not tested on human brain sections. Yet this is due to the limited availability of these sections. A possible approach to margin this limitation is the use of CSF on protein arrays containing large numbers of CNS proteins. Furthermore, the prior fixation of tissue might have biased the results by possible loss of immunoreactivity. Data presented are based on immunochemistry results. Antibody detection via fluorescent cell sorting is possible (23) yet the clinical significance of low-level antibodies possibly detected by this is unclear. Therefore, for routine diagnostic of CNS autoantibodies mainly immunofluorescence or blot techniques are used.

In conclusion, although we failed to detect any specific autoantibodies, our finding of positive CSF OCB in a subset of patients points a possible humoral immune contribution to GTS and therefore supports the assumption of autoimmune processes being involved.

DATA AVAILABILITY

All datasets for this study are included in the manuscript and the supplementary files.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Local Ethics Committee (no. 6987) of the Hannover Medical School with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Local Ethics Committee of the Hannover Medical School.

AUTHOR CONTRIBUTIONS

KM-V and K-WS contributed conception and design of the study. CB, KM-V, K-WS, JK, PS, TS, MS, and CS contributed acquisition of data and organized the database. CB wrote the first draft of the manuscript. All authors contributed analysis and interpretation of data and contributed to manuscript revision, read, and approved the submitted version.

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frontiers in Neurology

BRIEF RESEARCH REPORT
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Routine Cerebrospinal Fluid Cytology Reveals Unique Inclusions in Macrophages During Treatment With Nusinersen

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Background: Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder characterized by degeneration of spinal motor neurons leading to muscular weakness. The antisense oligonucleotide nusinersen was approved for the treatment of patients with 5q-associated SMA. Treatment must be repeatedly administered intrathecally by lumbar puncture. So far, data regarding cerebrospinal fluid (CSF) parameters are sparse and examinations of CSF cytology during nusinersen treatment are completely missing.

Methods: 87 CSF samples from 19 adult SMA patients who underwent repeated lumbar punctures for intrathecal injections of nusinersen were investigated. CSF specimens were quantitatively assessed regarding leukocyte subpopulations by routine cytology after Pappenheim staining. A control group with 38 CSF samples from 10 patients with repeated lumbar punctures due to other diseases was used.

Results: Treatment with nusinersen did not result in persistent inflammatory cellular changes or a relevant shift of leukocyte subpopulations in the CSF. During nusinersen therapy unique macrophages with numerous sharply defined purple and granular inclusions were detected in all patients. These macrophages were not found in CSF of patients with other diseases who underwent repeated lumbar punctures.

Discussion: Routine CSF cytology performed by experienced personnel represents an important and feasible tool for safety monitoring during treatment with intrathecally administered therapeutics. Analysis of leukocyte subpopulations did not raise safety concerns during nusinersen therapy. The potential significance of the unique phagocytic cells for disease course and treatment response needs to be further elucidated in the future.

Keywords: nusinersen, spinal muscular atrophy (SMA), cerebrospinal fluid (CSF), cytology, macrophage, monocyte, lymphocyte

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder characterized by degeneration of lower motor neurons in the spinal cord with subsequent progressive muscular atrophy and weakness (1). The disease is caused by disruption of the survival motor neuron 1 (*SMN1*) gene on chromosome 5q13 (2). The antisense oligonucleotide nusinersen modifies the pre-mRNA splicing of the survival motor neuron gene *SMN2* which leads to increased production of full-length SMN protein, thereby compensating for the genetic defect in the *SMN1* gene and improving motor function in patients with different SMA phenotypes (3–5). Nusinersen (Spinraza®) was approved by the FDA in the USA in 2016 and by the EMA in the European Union in 2017 for the treatment of patients with 5q-associated SMA. Since nusinersen cannot pass the blood–brain barrier it has to be repeatedly administered by intrathecal injection via lumbar puncture on days 0, 14, 28, and 63 and subsequently every 4 months (6). During phase 1 and 2 clinical studies prior to approval of nusinersen, cerebrospinal fluid (CSF) was analyzed for safety assessments regarding total cell count, protein, glucose, inflammatory cytokines, and anti-nusinersen antibodies which revealed no safety concerns (7, 8). Apart from a recent letter reporting a decrease of elevated tau and neurofilament light chain levels in an infant with SMA (9) and the description of the macroscopic condition of the CSF after lumbar puncture in adolescent and adult SMA patients (6), data regarding CSF parameters during nusinersen therapy are sparse and investigations of CSF cytology during nusinersen therapy are completely missing. Therefore, we examined the impact of repeated intrathecal administrations of nusinersen in adult SMA patients on CSF cytology.

PATIENTS AND METHODS

Patients

Routine CSF cytology specimens of adult patients with genetically proven 5q-SMA who were treated with nusinersen at the Department of Neurology at Hannover Medical School between November 2017 and January 2019 were analyzed. In total, we collected 87 CSF samples from 19 adult patients with SMA (8 patients with SMA type 2, 11 patients with SMA type 3) who underwent repeated lumbar punctures (ranging from three to seven) for intrathecal injections of nusinersen on days 0, 14, 28, 63, and at month 6, 10, and 14. Median age was 35 years, ranging from 19 to 64 years. Seven female and 12 male patients

A

				Phagocytes					Granulocytes		
LP	LY (%)	PC (%)	MO (%)	MA (%)	NP (%)	SI (%)	ES (%)	E (%)	NG (%)	EG (%)	BG (%)
d0	70.09 ± 3.56	0	21.36 ± 2.31	0	0	0	0	0	8.99 ± 3.9	0.07 ± 0.06	0.26 ± 0.026
d14	65.75 ± 4.73	0	22.27 ± 3.06	0.66 ± 0.21	0.51 ± 0.16	0	0.03 ± 0.03	0	10.2 ± 3.96	0.54 ± 0.28	0.05 ± 0.053
d28	66.38 ± 4.5	0	19.12 ± 2.45	0.62 ± 0.22	1.32 ± 0.3	0.01 ± 0.01	0	0.12 ± 0.12	11.78 ± 4.26	0.42 ± 0.16	0.19 ± 0.14
d63	66.85 ± 4.16	0	17.57 ± 2.37	0.29 ± 0.15	1.37 ± 0.43	0.19 ± 0.13	0.04 ± 0.04	0	13 ± 4.32	0.64 ± 0.4	0
m6	66.59 ± 7.86	0	20.31 ± 2.51	0.44 ± 0.16	2.51 ± 0.77	0	0	0.14 ± 0.14	9.29 ± 7.23	0.87 ± 0.87	0
m10	67.8 ± 10.84	0	12.7 ± 0.41	0.22 ± 0.22	0.58 ± 0.36	0.16 ± 0.16	0	0	17.84 ± 10.03	0.6 ± 0.6	0.16 ± 0.16
m14	82.5	0	14.7	0	2.9	0	0	0	0	0	0

B

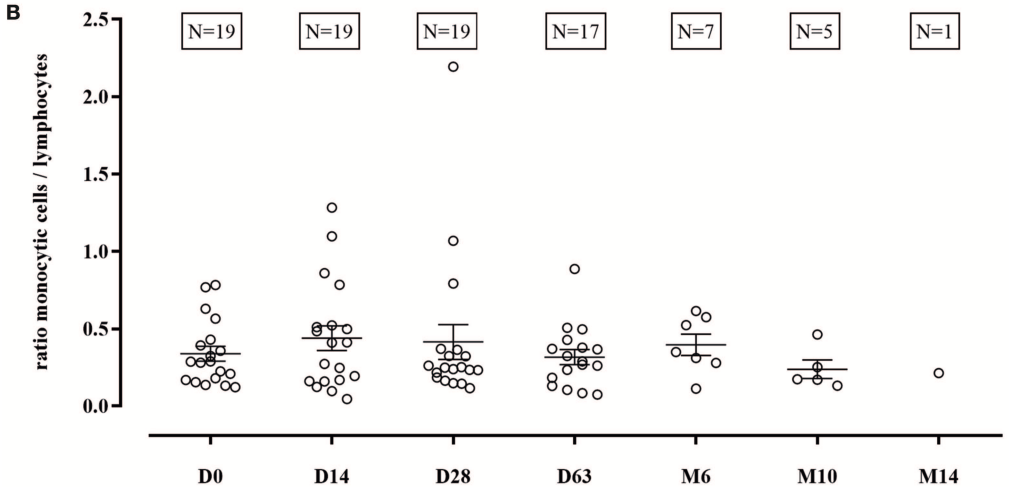

FIGURE 1 | Cell distribution of leukocyte subpopulations during treatment with nusinersen. Respective cell populations are given as proportion of total leukocytes in percentage with mean ± standard error of the mean (SEM) **(A)**. Ratio between share of monocytic cells (including monocytes and different forms of macrophages termed as "phagocytes") and lymphocytes is depicted at different time points of nusinersen therapy **(B)**. Dot plots show mean ± SEM. LP, lumbar puncture; LY, lymphocytes; PC, plasma cells; MO, monocytes; MA, macrophages; NP, nusinophages; SI, siderophages; ES, erythrosiderophages; E, erythrophages; NG, neutrophil granulocytes; EG, eosinophil granulocytes; BG, basophil granulocytes.

were included into the analysis. 38 CSF cytology samples from mostly age-matched patients who underwent repeated lumbar punctures at least twice within 6 months between 2016 and 2019 with diagnosis of idiopathic intracranial hypertension (IIH) ($n = 8$), meningitis ($n = 1$) or chronic inflammatory CNS disorder ($n = 1$) served as control group. Median age was 35 years, ranging from 28 to 61 years. This investigation was approved by the Ethics Committee of Hannover Medical School (No. 3142-2016).

Preparation of CSF Samples

CSF was drained prior to administration of nusinersen and was subjected to standard diagnostic procedures within 1 h after lumbar puncture in the neurochemistry laboratory at the Department of Neurology as described previously (10). Briefly, cells in the CSF were manually counted using a Fuchs-Rosenthal counting chamber and a CSF cell count of > 4 cells/µl was considered as elevated. Fifteen minutes pre-centrifugation with 145 g of 2–5 ml CSF was performed to enrich the cells. Cell sediment was resuspended in 0.2 ml of cell culture medium and cytospins were prepared in a Shandon Cytospin 3 (Thermo Shandon Limited, Cheshire, UK) at 90 g for 10 min (11). After air drying CSF specimen were stained using a panoptic Pappenheim stain by performing May-Grünwald (Merck, Darmstadt, Germany) followed by Giemsa staining (Sigma-Aldrich, St. Louis, USA). To distinguish macrophages with blue or purple inclusions from siderophages (**Figure 2C**),

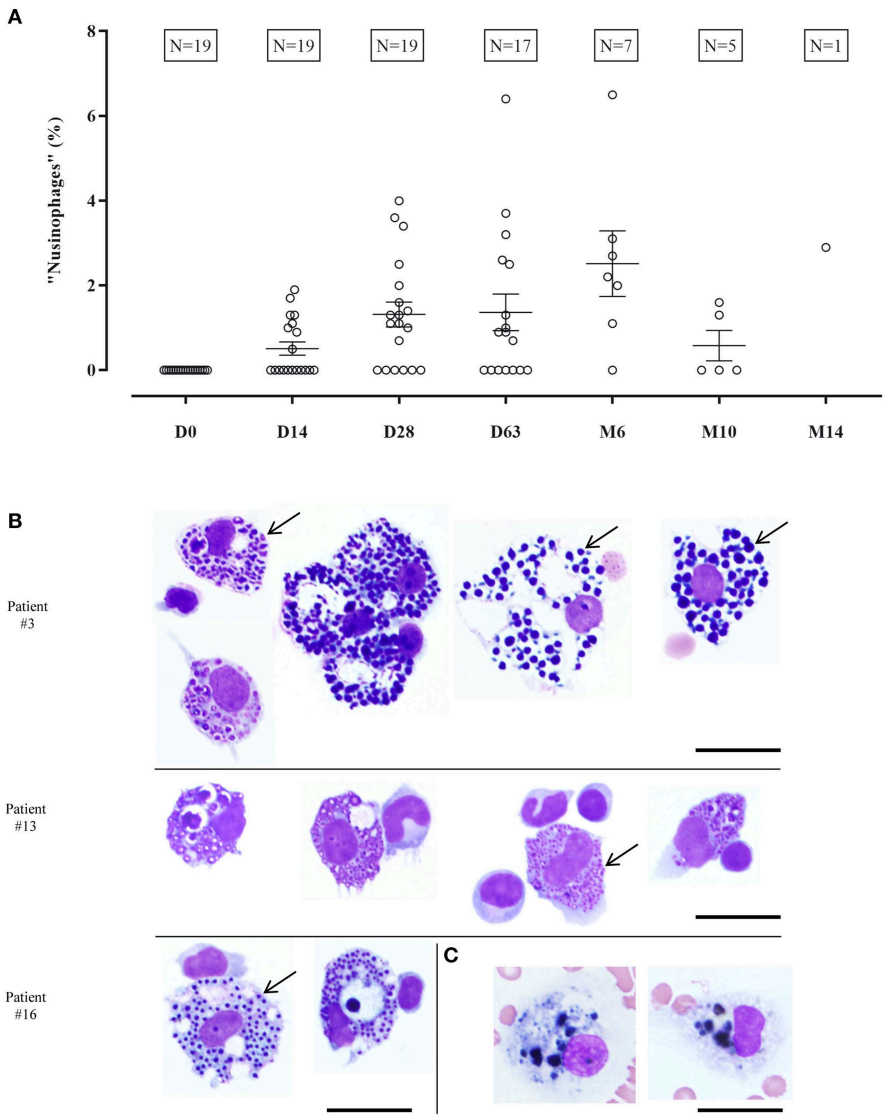

FIGURE 2 | Incidence of characteristic macrophages containing numerous sharply defined blue or purple granules ("nusinophages"). The proportion of "nusinophages" at the time of the respective lumbar puncture is stated as percentage of all leukocytes **(A)**. Dot plots show mean ± SEM. Macrophages with characteristic sharply demarcated inclusions classified as "nusinophages" are exemplarily shown in CSF specimen of patients who had no relevant admixture of erythrocytes in the prior lumbar punctures **(B)**. Arrows display characteristic inclusions in different "nusinophages." Phagocytes graded as siderophages from a CSF specimen of a patient under Nusinersen treatment after a prior lumbar puncture with >1,000 erythrocytes/µl **(C)**. Scale bar = 25 µm.

prussian blue staining for iron was performed regularly. Dried cells were immersed in a solution of 2% potassium hexacyanoferrate and 1% hydrochloric acid (Merck, Darmstadt, Germany) followed by a counterstaining with nuclear fast red aluminum sulfate solution (Carl Roth, Karlsruhe, Germany) (12).

Analysis of CSF Cytology Specimens

Specimens were independently examined by two raters experienced in CSF cytology who were blinded to condition and time point. In samples from SMA patients, the following leukocyte populations were assessed quantitatively: lymphocytes, plasma cells, monocytes, macrophages, erythrophages, erythrosiderophages, siderophages, neutrophils, eosinophils, and basophils. Cartilage and bone marrow cells were present in some specimens but were not analyzed. In cases where raters either disagreed on the presence or absence of any cell type or the percentage of lymphocytes and/or monocytes differed by more than 10% between the two raters' assessments, the specimen was reviewed and discussed by both raters together. After consensus was reached and data were corrected accordingly, mean values of both raters' assessments were calculated for each specimen and cell population. Specimens from repeatedly punctured patients with other diseases were assessed as a control. Prussian blue stainings were analyzed regarding iron containing siderophages. Specimens were examined using an x40 objective (Axiostar, Carl Zeiss, Jena, Germany).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego). Data are given as arithmetic means ± standard error of the mean (SEM).

RESULTS

CSF Cytology During Nusinersen Treatment

At the time of first lumbar puncture before treatment with nusinersen, all patients showed a normal CSF leukocyte cell count (<4 cells/μl). Evaluation of all 87 CSF samples revealed mild pleocytosis (ranging from 5 to 21 cells/μl) in 4 specimens which did not persist at the subsequent lumbar punctures (**Supplemental Table 1**). The proportions of the different leukocyte subpopulations showed considerable variation between the individual punctures which was predominantly attributable to admixture of peripheral blood cells to the CSF samples (**Supplemental Table 1**). Apart from presumably peripherally derived granulocytes, other phagocyte populations were not detected at the first lumbar puncture but were evident during repeated punctures at a small percentage (**Figure 1A**). In addition, there were no relevant changes in the ratio of the CSF-resident cell populations of monocytic cells (comprising monocytes and macrophages termed as "phagocytes") and lymphocytes (**Figure 1B**). In addition, plasma cells were not detectable in any of the 87 CSF specimens. Taken together, quantitative analysis of leukocyte subpopulations did not provide evidence for a sustained inflammatory cellular reaction in the CSF or a relevant shift of the cellular composition of the CSF under treatment with nusinersen.

Emergence of Unique Macrophages ("Nusinophages")

Beginning with the second lumbar puncture, macrophages with sharply defined purple and blue granular inclusions were detected whereas these cells were not present in any specimen of the first lumbar puncture before treatment with nusinersen (**Figure 2A**). These macrophages were found in CSF samples of every patient at least at one time point during nusinersen therapy and accounted for 0.5–6.5% of all leukocytes (**Supplemental Table 1**). At the time of the second lumbar puncture, no CSF specimen contained more than 2% of these macrophages and a higher percentage of up to 6.5% was found in samples from later time points. However there was no clear increase in the percentage of macrophages with inclusions over time and the two outliers with >6% of these cells at d63 and m6 as well as the small number of samples at later time points should be noted (**Figure 2A**).

These cells, which we termed "nusinophages" for convenience, were characterized by numerous sharply demarcated inclusions varying in size and in color between purple and blue (**Figure 2B**). Prussian blue staining for iron did not show siderophages in specimens in which only "nusinophages" but not siderophages had previously been detected by Pappenheim staining. In addition, 38 CSF samples of 10 patients who had undergone repeated lumbar punctures due to other diseases were investigated for the presence of "nusinophages." No phagocytic cells resembling these macrophages with characteristic sharply defined granules seen in patients under treatment with nusinersen ("nusinophages") were observed in these patients (**Supplemental Table 2**).

DISCUSSION

The antisense oligonucleotide nusinersen (Spinraza®) must be repeatedly administered intrathecally for the treatment of patients with 5q-associated SMA. Monitoring of potential alterations of CSF parameters and especially changes of the cellular composition of the CSF is crucial.

Here, we systematically analyzed leukocyte subpopulations in adult SMA patients treated with nusinersen. Since no evidence for sustained inflammatory cellular reactions or a relevant shift of the cellular composition in the CSF was found, cytological analysis did not reveal safety issues of nusinersen treatment in adult patients with SMA. These findings complement the extensive clinical data demonstrating safety and feasibility of nusinersen therapy (6, 13–15).

Interestingly, during the treatment with nusinersen unique macrophages with numerous sharply delineated blue and purple inclusions were detected in all patients. These cells were not found in patients who had undergone repeated lumbar punctures due to other diseases and we are not aware of the existence of similar CSF macrophages in other conditions. However, different intervals between the lumbar punctures of

SMA patients and control group patients represent a limitation of this comparison. Since stainings for oligonucleotides in an infant treated with nusinersen indicated the presence of nusinersen in neuronal and non-neuronal cells in brain and peripheral tissue (8) we speculate, that the inclusions found in the macrophages (colloquially termed "nusinophages") might also contain nusinersen. However, the origin and significance of these remarkable phagocytes remains unclear.

In summary, cells with unique inclusions were detected in the CSF of patients after repeated administration of nusinersen. Further investigations are needed to clarify whether these inclusions might contain nusinersen and if these cells may influence the response to therapy. We suggest that analysis of CSF parameters should supplement clinical measures of safety and therapy response during treatment with intrathecally delivered drugs.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the institutional ethics committee of Hannover Medical School with written informed consent from all subjects (No. 3142-2016). All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the institutional ethics committee of Hannover Medical School.

AUTHOR CONTRIBUTIONS

SG, MH, SA, LB, and TS evaluated the specimens. SG, MH, SA, KJ, PS, K-WS, LM, AO, OS-K, MB, MS, SP, and TS analyzed the data. SG, MH, SA, KJ, MS, SP, and TS drafted and wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Cerebrospinal Fluid Findings in Patients With Autoimmune Encephalitis—A Systematic Analysis

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Autoimmune encephalitides (AIE) comprise a group of inflammatory diseases of the central nervous system (CNS), which can be further characterized by the presence of different antineuronal antibodies. Recently, a clinical approach for diagnostic criteria for the suspected diagnosis of AIE as well as definitive AIE were proposed. These are intended to guide physicians when to order the antineuronal antibody testing and/or facilitate early diagnosis even prior to the availability of the specific disease-confirming test results to facilitate prompt treatment. These diagnostic criteria also include the results of basic cerebrospinal fluid (CSF) analysis. However, the different antibody-defined AIE subtypes might be highly distinct with regard to their immune pathophysiology, e.g., the pre-dominance of specific IgG subclasses, IgG1, or IgG4, or frequency of paraneoplastic compared to idiopathic origin. Thus, it is conceivable that the results of basic CSF analysis might also be very different. However, this has not been explored systematically. Here, we systematically reviewed the literature about the 10 most important AIE subtypes, AIE with antibodies against NMDA, AMPA, glycine, $GABA_A$, and $GABA_B$ receptors as well as DPPX, CASPR2, LGI1, IgLON5, or glutamate decarboxylase (GAD), with respect to the reported basic CSF findings comprising CSF leukocyte count, total protein, and the presence of oligoclonal bands (OCB) restricted to the CSF as a sensitive measure for intrathecal IgG synthesis. Our results indicate that these basic CSF findings are profoundly different among the 10 different AIE subtypes. Whereas, AIEs with antibodies against NMDA, $GABA_B$, and AMPA receptors as well as DPPX show rather frequent inflammatory CSF changes, in AIEs with either CASPR2, LGI1, $GABA_A$, or glycine receptor antibodies CSF findings were mostly normal. Two subtypes, AIEs defined by either GAD, or IgLON5 antibodies, did not fit into this general pattern. In AIE with GAD antibodies, positive OCBs in the absence of other changes were typical, while the CSF in IgLON5 antibody-positive AIE was characterized by elevated protein.

Keywords: autoimmune encephalitis, antineuronal antibodies, cerebrospinal fluid, pleocytosis, oligoclonal bands, NMDAR antibodies, LGI1 antibodies, GAD antibodies

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INTRODUCTION

Autoimmune encephalitides (AIE) are inflammatory diseases of the central nervous system (CNS) (1). As a differential diagnosis for infectious encephalitides, epilepsy of other causes, or cognitive deterioration of non-inflammatory origin, the diagnosis of AIE is often established by the detection of subtype-specific antibodies against different neuronal surface antigens in the cerebrospinal fluid (CSF), the blood or both. It has been suggested that rapid immunosuppressive treatment improves the outcome of patients with AIE (2). However, the specific antibody testing usually takes several days. Thus, CSF findings like CSF pleocytosis, increased protein, and the presence of oligoclonal bands (OCB) restricted to the CSF might prove an inflammatory origin of neurological disturbances compatible with an AIE prior to the specific test results, thereby supporting the diagnosis and triggering early treatment.

Recently, diagnostic criteria for AIEs were proposed, which also incorporate CSF findings (3). Of note, only CSF pleocytosis was chosen as supporting findings for the diagnostic categories of possible AIE or definitive limbic encephalitis of autoimmune origin. In contrast, for the diagnostic category of possible NMDAR encephalitis, both positive OCB and pleocytosis were considered as supportive CSF findings. Of note, it has been reported that inflammatory CSF changes, although common in patients with NMDAR encephalitis (4), might be rare in other AIE subtypes, e.g., AIE associated with LGI1 antibodies (5, 6). Thus, the likelihood that inflammatory CSF findings support the suspected AIE diagnosis might strongly depend on the underlying disease subtype in an individual patient. However, this relationship has not been studied systematically.

In this analysis, we systematically reviewed the literature regarding 10 AIE subtypes with well-defined antibodies. We extracted the reported CSF findings both on the basis of group findings as well as of data reported for individual patients whenever possible. In these different data sets, we analyzed the cumulative reported frequencies and levels of CSF pleocytosis, elevated total protein as well as the frequency of positive OCB with respect to the antibody-defined specific AIE subtype. In addition, we analyzed the typical combination of the three basic values when reported for individual patients to characterize the typical CSF result pattern in the 10 AIE subtypes.

METHODS

We reviewed the published literature using the PubMed data base (https://www.ncbi.nlm.nih.gov/pubmed) of the National Center for Biotechnology Information for publications published until December 31st, 2018 with regard to AIE with AMPA receptor (AMPAR), CASPR2, DPPX, glutamate decarboxylase (GAD), glycine receptor (GlyR), IgLON5, $GABA_B$ receptor ($GABA_BR$), $GABA_A$ receptor ($GABA_AR$), LGI1, and NMDA receptor (NMDAR) antibodies using the following search terms: "NMDA," "AMPA," "CASPR2," "DPPX," "GAD," "GlyR," "IgLON5," "$GABA_BR$," "$GABA_AR$," or "LGI1" in combination with "encephalitis," "IgLON5" combined with "case report," "CASPR2" in combination with "seizure." The hits were critically reviewed and all publications that reported CSF findings for two or more patients for one of the 10 specific antibodies were selected for further analysis. However, in AIE subtypes for which this strategy identified very few patients (<15), namely AIEs with CASPR2, DPPX, $GABA_BR$, and $GABA_AR$ antibodies, for individual CSF data comprising all three parameters, we also incorporated data from reports of two or one patients. It was carefully checked whether some patients might be reported in more than one publication. These patients were only included once. As characterizing the CSF abnormalities in children was beyond the scope of this paper, patients younger than 13 years were excluded.

Basically, two types of data were extracted from the selected publications, group data and individual data. Most often, only group data were reported, e.g., percentage of patients with pleocytosis among all with information about CSF cell count available. For female gender, CSF pleocytosis, increased protein as well as the presence of OCB, the percentage of patients positive for one of these findings was calculated as follows: For each specific AIE subtype, the total number of patients positive for one of these findings was obtained by adding up all these patients reported in all selected publications. These were compared to the total number of patients for whom information regarding this parameter could be extracted from these publications. If one CSF parameter was reported as either normal or abnormal for some patients but no information was given for other patients, we assumed that the results of this analysis were not available for the latter. Thus, these patients were not counted for the total number of patients for this parameter. If only pathological values were specifically reported, it was assumed that the data were available but normal in all other patients unless it was specifically mentioned that these values were not available. Of note, the normal values for CSF cell count varied from up to 4 to 5 cells/μl among the publications. In addition, the upper normal limit for total protein ranged from 350 to 500 mg/l. When reported, we extracted the exact results for the CSF cell count as cells/μl and CSF total protein as mg/l and regarded cell counts up to 4 cells/μl and protein levels up to 450 mg/l used for majority of publications applied these cut-offs. Median, minimum, maximum, and interquartile range were calculated using the Graph Prism Software.

RESULTS

For all antibody-defined AIE subgroups combined, we could identify 116 publications that matched our search criteria and contained relevant information for the intended analyses. Ten were identified for AIE with AMPAR antibodies (7–16), 15 for CASPR2 (16–30), eight for DPPX (31–38), four for $GABA_AR$ (15, 39–41) with patients reported by the Pettingil et al. being excluded as these were not tested for $GABA_AR$ antibodies in CSF and represented a different phenotype (42), 15 for $GABA_BR$ antibodies (12, 15, 16, 43–54), 15 for GAD antibodies (19, 22, 55–67), five for GlyR antibodies (68–72), 14 for IgLON5 antibodies (73–86), 25 for LGI1 antibodies (5, 6, 15, 16, 19–22, 30, 52, 77, 87–100), and finally 27 for NMDAR antibodies (4, 15, 16, 19, 22,

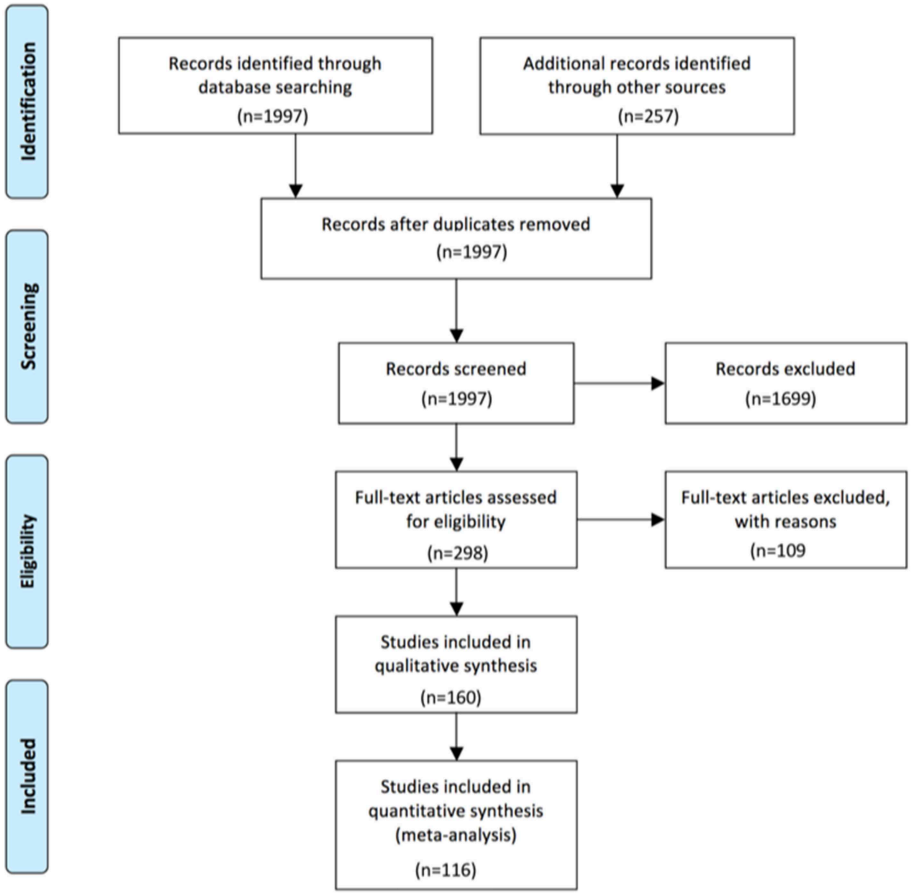

FIGURE 1 | Study profile.

52, 62, 88, 89, 101–118). The history of the data search and the results are depicted in **Figure 1**. With regard to the group data, information regarding the presence or absence of CSF pleocytosis was available for 1,305 patients total, while less information was available for increased CSF protein and presence of OCB with a total of 1,001 and 610 patients, respectively (**Table 1**). The data set for AIE with DPPX antibodies was the smallest with 29, 16, and 19 patients for percentages of either pleocytosis, increased protein or presence OCB, respectively, while the number of reported CSF findings was highest for AIE patients with NMDAR antibodies. Here, the frequency of pleocytosis was reported in 532 patients, the occurrence of increased protein in 433, and the presence of OCB in 196 patients. However, this kind of group data did neither allow to analyze the typical age of onset, CSF cell count or CSF protein levels nor the frequency of co-occurrence of pleocytosis, increased protein and presence of OCB in individual patients. Thus, whenever reported individually, we also extracted the age, gender, CSF cell count in cells per μl, total protein as mg/l and presence or absence of OCB for each patient. However, the size of the groups of patients with individual data available was substantially lower compared to the cohorts for which group data were combined (**Table 1**, **Supplementary Table 1**). The group size for each antibody-defined AIE was smallest for individual patients with information regarding all three basic CSF parameters whether normal of pathological available, ranging from 5 to 34 for DPPX- and GAD-antibody associated AIE, respectively (**Table 1**). In general, the proportion of patients with individual values compared to group data for gender, cells, CSF protein, and OCB was lowest for patients with NMDAR, GAD, $GABA_AR$, and again NMDAR antibodies with 12, 8, 9, and 18%, respectively (**Supplementary Table 1**). In contrast, for gender and AMPAR and $GABA_AR$ antibodies and OCB and $GABA_AR$ antibodies all individuals identified had individual data. When the frequencies of CSF pleocytosis, elevated CSF protein, or positive OCB in the cohorts with individual exact values was compared to the those generated by adding up group data, we found that for 6 of the 10 well-defined antibodies the percentage of either pathological CSF cell count and elevated protein values was significantly higher in patients with individual exact values given compared to the group data. This indicates a strong bias for over-reporting of pathological in comparison to normal values (**Supplementary Table 2**).

When comparing the basic demographic variables, age, and gender, among the different cohorts with antibody-defined AIE

TABLE 1 | Number of patients with AIE with disease-specific antibodies identified by the literature search either as grouped data, with individual exact data or all three major parameters available.

Anti-body target	Group data					Individual exact data					Cells + TP + OCB
	Age	Gender	Cells	TP	OCB	Age	Gender	Cells	TP	OCB	
AMPAR	51	50	51	43	19	50	50	34	18	16	15
CASPR2	103	100	91	59	38	29	22	17	8	8	6
DPPX	39	39	29	16	19	19	19	15	5	18	5
$GABA_AR$	24	24	24	22	24	24	24	10	2	24	8
$GABA_BR$	130	130	112	72	19	125	124	79	46	18	8
GAD	192	192	76	76	163	65	65	6	12	65	34
GlyR	77	77	62	62	62	32	32	11	11	18	18
IgLON5	52	52	37	36	29	20	20	4	9	16	14
LGI1	351	348	291	182	41	112	109	30	25	33	25
NMDAR	486	504	532	433	196	58	60	65	42	35	21
Sum	1,505	1,516	1,305	1,001	610	534	525	270	178	251	154

TP, total protein; OCB, oligoclonal IgG restricted to the CSF; Cells+TP+OCB, Data for CSF cells, total protein as well as OCB.

subtypes prominent differences became apparent. Whereas, the median age was 60 years or higher for patients with AIE associated with $GABA_BR$, IgLON5, LGI1, CASPR2, and AMPAR antibodies, patients with $GABA_AR$, DPPX, GAD, and GlyR antibody-associated AIE were considerably younger (**Figure 2A**). Patients with $GABA_AR$ antibodies showed a bimodal age distribution with an additional group of patients with a very young age. Patients with NMDAR antibody-associated AIE were the youngest with a median age of 27 years (**Figure 2A**). In addition to age, also the gender distributions were very different among the different AIE subtypes. While females were exceedingly rare among patients with CASPR2 antibodies (14%) and males among patients with GAD antibodies (19%), there was a moderate male pre-dominance in AIE with DPPX, LGI1, and $GABA_BR$ antibodies and a moderate female pre-dominance in AMPAR, $GABA_AR$, and NMDAR antibody-positive patients (**Figure 2B**).

The cumulative reported frequencies of CSF pleocytosis were also highly divergent among the 10 different antibody-defined AIE subtypes: while present in 50% or more of the patients with NMDAR, AMPAR, $GABA_BR$, and DPPX antibodies, CSF pleocytosis was rare in patients with GAD, LGI1, and IgLON5 antibodies with frequencies of 9, 16, and 24%, respectively (**Figure 3A**). Patients with the remaining antibodies, anti-GlyR, -$GABA_AR$, and -CASPR2, had reported frequencies of CSF pleocytosis ranging from 29 to 36%. Analyzing individual exact CSF cell counts if pathological only, reduced the number of data points considerably. Due to the scarcity of data points, GAD antibodies–only reportedly elevated in two patients with 7 and 56 cells/μl–were omitted from graphical depiction of this analysis (**Figure 3B**). Median values for CSF pleocytosis of 20 cells/μl and higher were found in AIE associated with AMPAR and NMDAR antibodies as well as for $GABA_AR$ antibody-associated AIE. The latter findings are surprising, as pleocytosis in these subtypes was found to be rather infrequent (**Figure 3A**). However, the number of data points were limited with three and eight for GlyR and $GABA_AR$ antibodies, respectively. A relevant pleocytosis of 20 cells/μl or more was reported for >60% of patients with $GABA_BR$, AMPAR, and NMDAR antibodies, corresponding to those with highest percentage of reported pleocytosis, whereas CSF cell counts in this range were found in 40% of patients with DPPX antibodies and finally in 25% or less in patients with AIE associated with IgLON5, LGI1 as well as CASPR2 antibodies, the three subtypes where CSF pleocytosis was least common. Pleocytosis of >100 cells/μl was found in 2 of 58 patients (3%) with $GABA_BR$ antibodies and in 2 of 30 patients (7%) with AMPAR antibodies, 2 of 15 patients with DPPX antibodies (13%) and 18 of 52 patients (35%) with NMDAR antibodies but not in the other AIE subtypes. Maximal cell counts well above 500 cell/μl were reported for patients with $GABA_BR$ (950 cell/μl) and NMDAR antibodies (730 cells/μl) only. However, for $GABA_BR$ antibody-positive AIE this cell counts can be judged as exceptionally high as the next highest cell count was considerably lower (159 cells/μl).

An elevated CSF protein reportedly occurred in <25% in AIE patients with GAD, $GABA_AR$ as well as GlyR (**Figure 4A**). In contrast, in AIE patients with antibodies against AMPAR and $GABA_BR$ elevated CSF protein levels was reported with a frequency of 43 and 47%, respectively. With reportedly elevated CSF protein in 53% of patients, AIE with IgLON5 antibodies showed in highest prevalence of this finding. Again, the frequencies of elevated individual CSF protein values (>450 mg/l) among cases with individual exact values reported were substantially higher when these were compared to group data (**Supplementary Table 2**). As for the CSF cell count, we thus omitted all individual patients with normal exact CSF protein levels to avoid that this reporting bias distorts our analysis. For AIE with $GABA_AR$ antibodies, only two CSF protein values (520 or 600 mg/l) were available. We thus omitted their graphical depiction as these two values were judged as

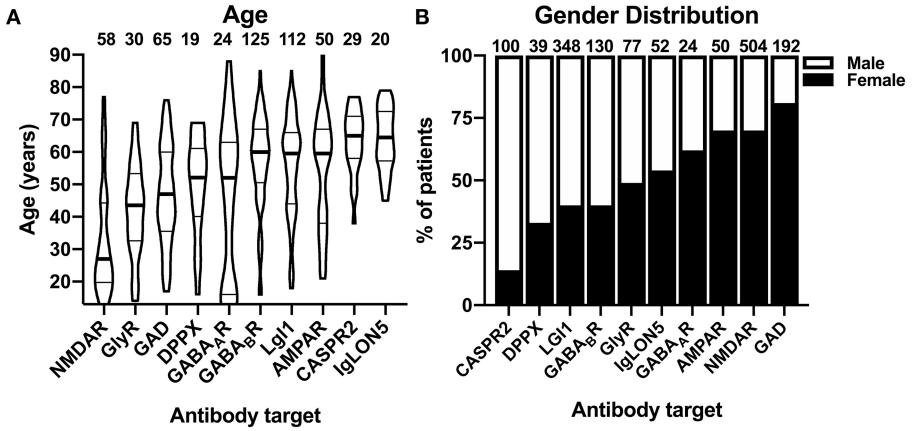

FIGURE 2 | Demographic characteristics of patients with autoimmune encephalitis subtypes defined by 10 different antibodies. **(A)** Distribution of individual reported ages in patients with autoimmune encephalitides (AIE) of the antibody-defined subtypes. The distribution is depicted as violin plots. Patients younger than 12 years were excluded. The median age is indicated as bold line, the interquartile range is indicated by fine lines. Note the bimodal age distribution of the patients with $GABA_AR$ antibodies. **(B)** Gender distribution of the combined groups of patients with group wise data about the gender distribution available. The percentage of females is depicted as the black part of the bar.

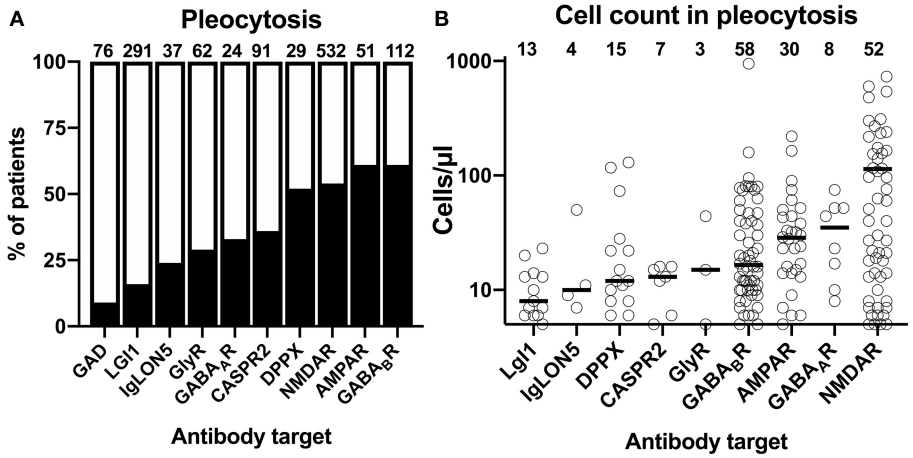

FIGURE 3 | Differential frequency of CSF pleocytosis and CSF cell count in patients with different antibody-defined autoimmune encephalitis subtypes. **(A)** The cumulative percentage of the patient groups identified in the literature with CSF pleocytosis. The percentage of patients with reported CSF pleocytosis is indicted as black. The total number of patients is indicated above each bar. **(B)** Distribution of reported CSF cell counts in individual patients with antibody-defined autoimmune encephalitis (AIE) subtypes and pleocytosis. The median cell count identified as a bold line. The origin of the y-axis is set to 5 cells/µl, the lowest pathological cell count.

not representative (**Figure 4B**). When ranking the combined individual pathological protein levels according to their median, two of the AIE subtypes with the highest percentage of reportedly increased in protein, AIE associated $GABA_BR$ and AMPAR antibodies, were among the four subtypes with the highest median pathological CSF protein levels (**Figure 4B**). In addition, protein levels reported for patient with LGI1 and NMDAR antibodies, although increased protein was reported much less often, also showed relatively high median pathological protein levels. When analyzed for the frequency of pathological protein levels >1,000 mg/l, these were detected in four of nine patients (44%) with IgLON5, 1 or 4 patients (25%) CASPR2, 4 of 18 patients (22%) with NMDAR, and 4 of 25 patients (16%) with $GABA_BR$ antibodies.

The third CSF finding we extracted from the published data was the reported presence or absence of isolated OCB in the CSF. In more than 50% of patients with GAD, $GABA_BR$, and NMDAR antibodies, positive OCB in the CSF were reported (**Figure 4C**). With 37%, the frequency of positive OCB was considerably lower in patients with AMPAR antibodies. Between 23 and 32% of patients with antibodies against GlyR, $GABA_AR$, CASPR2, and DPPX antibodies were OCB-positive, while positive OCBs were exceedingly rare in the groups of patients with LGI1 and IgLON5 antibodies with a percentage of only 5 and 7%, respectively.

In summary, the reported percentages of pathological values for the three basic CSF analyses are highly different among the 10 antibody-defined subtypes of AIE examined. However,

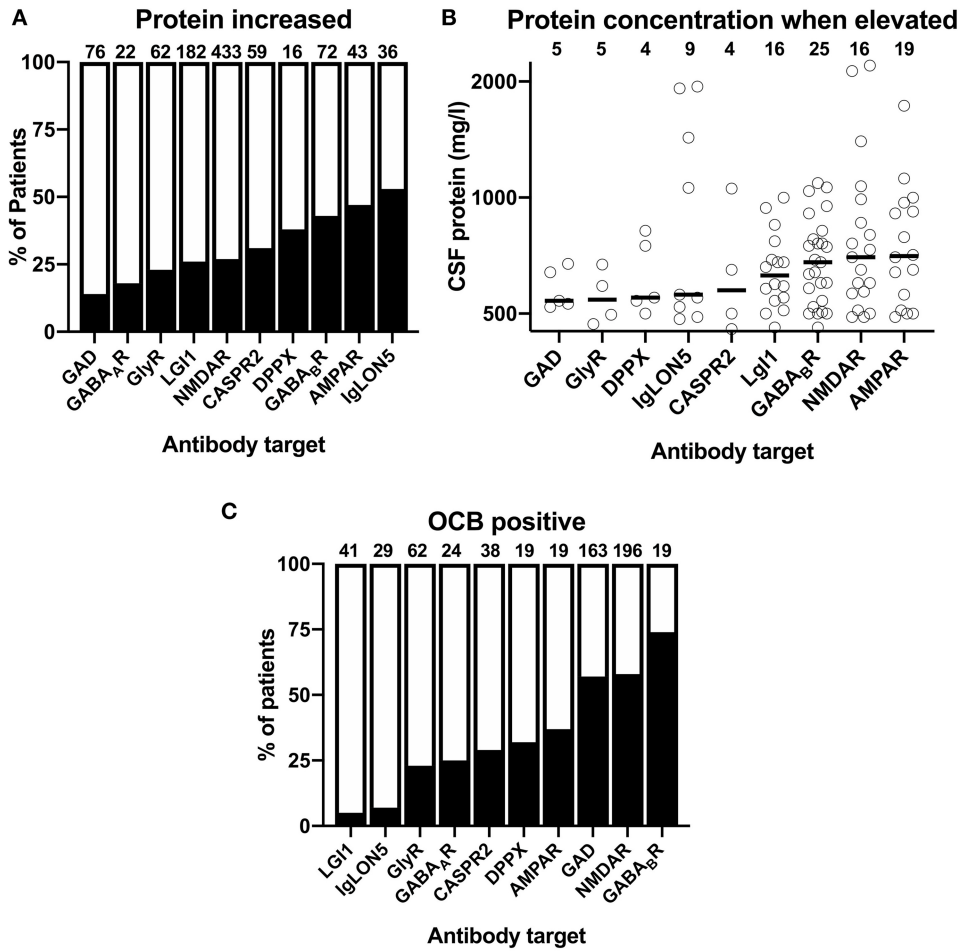

FIGURE 4 | Reported frequencies of increased protein and oligoclonal IgG in the CSF of patients with different antibody-defined autoimmune encephalitis subtypes. **(A)** The cumulative percentage of the antibody-defined AIE patient groups identified in the literature with increased CSF protein. The percentage of patients with reported CSF protein is indicated as black. The total number of patients is indicated above each bar. **(B)** Distribution of reported pathological CSF protein values in individual patients with antibody-defined autoimmune encephalitis (AIE) subtypes. The median cell count identified by a bold line. The origin of the y-axis is set to 450 mg/l as upper normal limit for CSF protein. **(C)** The cumulative percentage of the antibody-defined AIE patient groups identified in the literature with isolated oligoclonal bands (OCB) in the CSF. The percentage of patients with positive OCB is indicated as black. The total number of patients is indicated above each bar.

when the percentage of reported pleocytosis was plotted against the percentage of reportedly positive OCB, it became apparent that subtypes with frequent pleocytosis are in general also characterized by frequently positive OCB (**Figure 5A**). There was one exception however, as GAD antibody-associated CNS diseases only rarely show CSF pleocytosis while this subtype ranked among high with respect to OCB positivity. When the frequency of reportedly elevated CSF protein was plotted against the frequency of pleocytosis, a similar relationship became apparent. However, here the frequency of elevated CSF protein seemed to be disproportionately high in patients with IgLON5 antibodies (**Figure 5B**). Similar observations were made when the frequency of elevated CSF protein was plotted against relative OCB positivity (**Figure 5C**). It seems that AIE subtypes with antibodies against either NMDAR, $GABA_BR$, AMPAR, or DPPX are characterized by rather frequent pathological changes in all three analyses, while these CSF abnormalities seem to be rather infrequent in those subtypes with either CASPR2, LGI1, GlyR, or $GABA_AR$ antibodies. GAD and IgLON5 antibody-positive AIEs deviate from this general pattern with either disproportionally frequent positive OCB or elevated protein levels, respectively.

Having thus established that the reported data about single CSF findings seem to share certain patterns, we next investigated the relative co-occurrences of pleocytosis, elevated CSF protein, and positive OCB in individual patients suffering from 1 of the 10 antibody-defined AIE subtypes. For that purpose, we expanded our literature search to publications with <3 patients for AIE associated with AMPAR, CASPR2, DPPX, $GABA_BR$, $GABA_AAR$, and IgLON5 antibodies as for these <15 patients were identified with the full data set. Thereby, we increased the number of patients by 10 for AIE with IgLON5 antibodies (74, 75, 79–86), three for AIE with AMPAR antibodies (8, 10, 11), by two

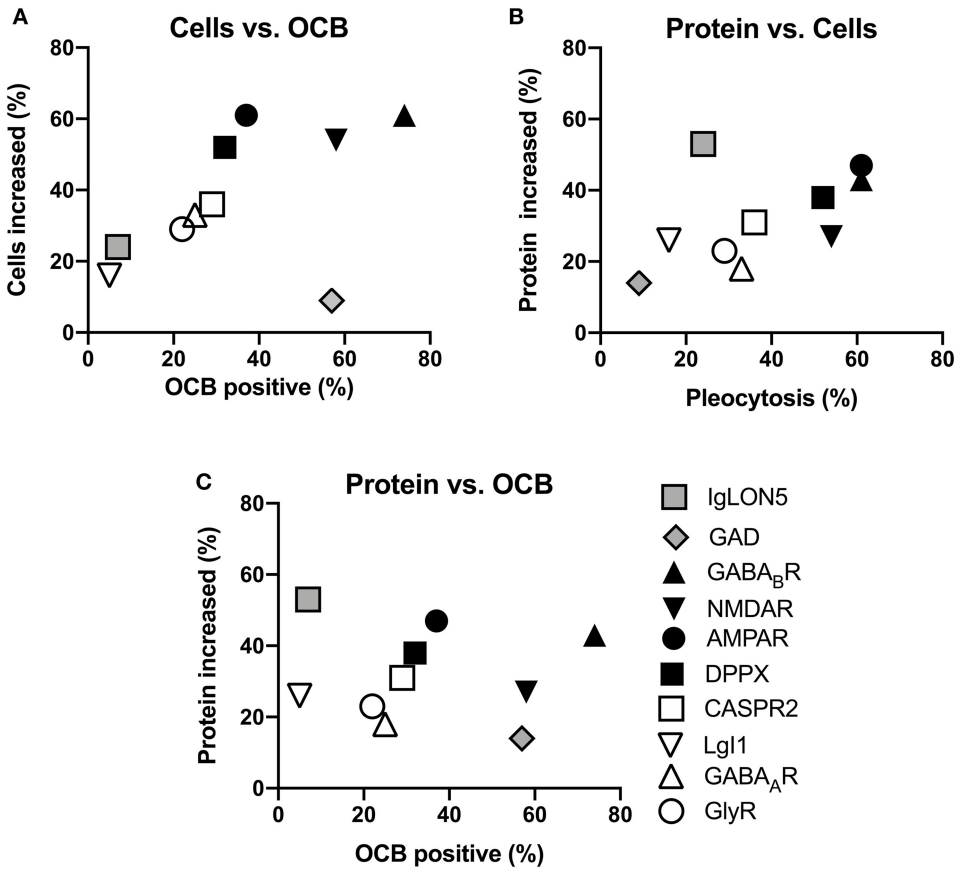

FIGURE 5 | Relationships of the reported frequencies of pleocytosis, elevated protein, and presence of oligoclonal IgG in the CSF of patients with different antibody-defined autoimmune encephalitis subtypes. Graphs depict the cumulative frequencies of reported increased CSF cell counts plotted against the frequencies of reportedly positive oligoclonal IgG [OCB positive, **(A)**], increased CSF protein against pleocytosis **(B)** as well as the frequency of increased protein plotted against OCB positivity **(C)**. The antibody-defined autoimmune encephalitis (AIE) subtypes were grouped as those with infrequent pathological changes (empty symbol: LGI1, $GABA_AR$, GlyR, CASPR2), frequent pathological changes (black symbols: AMPAR, DPPX, $GABA_BR$, NMDAR) as well as those rather distinct patterns of CSF pathologies (gray symbols: IgLON5, GAD). Compared to the rare occurrence of CSF pleocytosis and positive OCB, CSF of patients with IgLON5 antibodies were frequently reported to exhibit elevated CSF protein. CSF findings in patients with GAD antibodies are characterized by a high frequency of positive OCB while pleocytosis and elevated CSF protein rarely occur.

for AIE with DPPX antibodies (35, 38), by one for AIE with CASPR2 (21) and $GABA_BR$ antibodies each (47). However, the number of individual patients with information of all three CSF parameters remained <10 for AIE with DPPX, CASPR2, $GABA_BR$, and $GABA_AR$ antibodies (**Supplementary Table 3**). Although the scarcity of data prohibited a more detailed analysis for these four antibodies, cases with CASPR2 antibodies had mostly normal CSF while inflammatory changes were observed in the majority of the other three subtypes. The analysis of the remaining AIE subtypes showed that basic CSF results were normal or unspecifically pathological with elevated CSF protein only in more than 2/3 of patients with LGI1, IgLON5, and GlyR antibodies, while only ∼1/3 were normal or unspecifically pathological in AIE with GAD and AMPAR antibodies. In contrast, all patients with NMDAR antibodies had definitively inflammatory CSF findings (**Figure 6**). In AIE with AMPAR and NMDAR antibodies, the vast majority of patients with inflammatory CSF had pleocytosis with or without positive OCB. However, there was a substantial proportion of patients with GAD antibodies with positive OCB in the absence of pleocytosis (56%), a combination also present in 4, 14, and 17% of patients with LGI1, IgLON5, or GlyR antibodies, respectively.

DISCUSSION

In clinical neurology, the differential diagnosis of AIE is often considered in patients presenting with new-onset epilepsy, psychiatric diseases, especially in younger patients, and dementia or delirium in the elderly. The rationale is that early identification of AIE might lead to a favorable response to immunosuppressive therapy (2) and missing the diagnosis might lead to life-long cognitive deficits. It is thus not surprising that proving the inflammatory origin of neurological sequelae by CSF findings plays a role in the diagnostic criteria for AIE recently proposed by multiple experts in this field (3). However, it is known that CSF in AIE sometimes lacks inflammatory changes (90, 119).

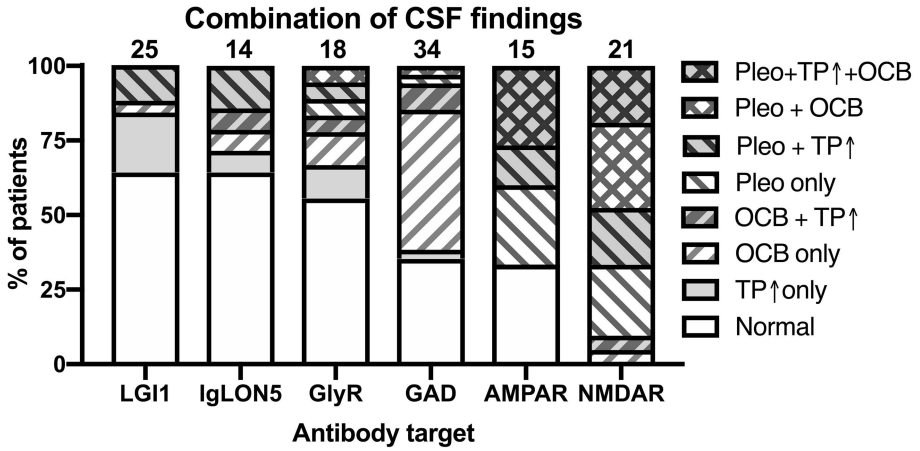

FIGURE 6 | Combination of pathological CSF findings in patients with antibody-defined autoimmune encephalitis. Patients with individual data with regard to all three basic CSF analyses–presence of pleocytosis, elevated CSF protein and positive OCBs–were analyzed for the frequencies of the eight possible combinations of all the tree pathologies. The order of the different antibodies was determined by the percentage of patients with normal or not definitely inflammatory CSF findings (increased protein only). Pleo, pleocytosis; TP ↑, total protein increased; OCB, positive isolated oligoclonal bands in CSF. Diagonal stripes upwards from left to right: positive OCB, downwards: pleocytosis.

Within the last years, it became apparent that the different antibody-defined subtypes of AIEs actually represent different diseases with typical clinical presentations, subtype-specific typical ages of onset and gender prevalences as well as imaging results (1). In addition, genome-wide linkage studies demonstrated that fundamentally different genetic risk factors (120). For the two most frequent AIE subtypes, those associated with LGI1 or NMDAR antibodies (6, 121), it is already well-acknowledged that AIE with LGI1 antibodies is rarely associated with inflammatory changes (5, 6, 90) while pleocytosis and/or positive OCB occur in most cases of AIE with NMDAR antibodies (4, 103).

Thus, it is conceivable that each antibody-defined AIE subtype has characteristic CSF findings that reflect its immune pathophysiology. To generate data that support this hypothesis, we performed a systematic evaluation of the CSF findings in published cases with 10 different types of AIE associated with well-defined antineuronal antibodies. In total, we combined the results of 1,305 patients for the presence of pleocytosis, while information about the CSF protein and especially OCB was less often available (**Table 1**). In addition, only in a minority of patients with individual results, data for all the three CSF parameters were reported. The fact that the exact CSF cell count or CSF protein level were more likely to be reported when abnormal introduced prominent bias and led us to analyze pathological CSF cell count and protein values only to prevent a distortion of our analysis due to differential reporting of normal values among the 10 AIE subtypes. Moreover, the normal values for cell count and CSF protein, as probably the techniques for the determination, slightly differed. In addition, in none of the reports CSF erythrocyte count, which may artificially increase CSF cell count, was reported. Finally, information about the time point of the reported CSF analysis with regard to disease onset or potential immunosuppressive medication administered beforehand was not available in the vast majority of patients. Thus, our results have to interpreted with caution.

Our group analysis indicates that in addition to AIE with NMDAR antibodies also the much rarer AIE subtypes with $GABA_BR$ and AMPAR and maybe DPPX antibodies not only frequently show CSF pleocytosis but also positive OCB. In contrast, diseases associated with LGI1, IgLON5, CASPR2, and GlyR antibodies rarely show positive OCB as well as pleocytosis. Of note, in these AIE subtypes cell counts when pleocytosis is present, with exception of GlyR, where only a limited number of patients was published, are relatively low compared to AIEs with NMDAR, AMPAR, and $GABA_BR$ antibodies. These findings were corroborated by our analysis of the typical patterns of individual patients with all three parameters. Thus, it can be expected that AIEs with NMDAR, AMPAR, $GABA_BR$, and DPPX antibodies in most cases will show inflammatory CSF changes supporting the diagnosis of an AIE before the results of a specific antibody testing are available, but this will not be the case in most patients with LGI1, IgLON5, CASPR2, and GlyR antibodies. Of note, of the two basal CSF findings to unequivocally prove an inflammatory process, CSF pleocytosis and isolated OCB in the CSF, currently only pleocytosis is included as a criterium for the diagnostic category of possible AIE and definitive limbic encephalitis of autoimmune origin, while for category of possible NMDAR encephalitides both pleocytosis and OCB are considered (3). Correspondingly, with few exceptions in patients with NMDAR-antibody associated AIE with OCB only, pleocytosis was always detected when CSF was OCB-positive in AIE with AMPAR and NMDAR antibodies. However, isolated OCB without pleocytosis were present in patients with LGI1, IgLON5, GlyR, and most prominently with GAD antibodies. Thus, we are not convinced that OCB positivity should be weighted differently than pleocytosis in the diagnostic work-up of suspected AIE. Although, it was estimated that positive OCB

occur with a frequency <5% in the healthy individuals (122) and thus their specificity for an active and symptomatic inflammatory process is <100%, the same can be assumed for mild CSF pleocytosis as slightly increased CSF cell counts nowadays might be a consequence of an automated cell count, which frequently overestimates low CSF cell counts (123), although pleocytosis is exceptionally rare in patients with neurodegenerative disease when CSF cell are counted manually (124). In addition, both parameters cross-validate each other when positive.

In general, in AIE subtypes with frequent definitively inflammatory CSF changes, pleocytosis, and/or OCB, increased CSF protein levels reportedly also occurred more frequently and vice versa. Of note, there are two notable exceptions of this rule. Firstly, GAD antibody-associated diseases prominently show a disproportionately high frequency of positive OCB, while CSF pleocytosis or elevated protein are exceptionally rare. Secondly, patients with IgLON5 antibodies might be characterized by rather frequent and high elevations of CSF protein in the absence of pleocytosis and positive OCB. Of note, IgLON5 antibody-associated encephalopathy differs from both AIE subtypes with rare and frequent inflammatory CSF changes by its poor response to immunosuppression (73).

In our analysis, CASPR2 antibody-associated AIE showed an intermediate to low frequency of pleocytosis and OCB. Of note, for this AIE, different subtypes have been described: limbic encephalitis associated with high CASPR2 antibodies in CSF, Morvan's syndrome (MoS) with low anti-Caspr2 antibodies in serum only, and finally cerebellar ataxia (23, 27). However, CSF findings of MoS are not included in our CASPR2 antibody-positive cohort, as these were without exception reported grouped with either CSF findings of LGI1 antibody-positive patients (125) or patients with peripheral hyperexcitability only (23).

$GABA_AR$ antibodies have been reported in a variety of neurological diseases, even in patients finally diagnosed to suffer from a hereditary disease (39, 42). However, a specific AIE subtype presenting as a rather acute and severe encephalopathic syndrome with multifocal T2 hyperintensities upon MRI as well as severe epilepsy is characterized by detection of anti-$GABA_AR$ antibodies in both serum and CSF (40). We focused our analysis on patients with positive CSF antibodies. Although, the number of patients was limited, our analysis indicates that although pleocytosis is only observed in a minority of patients, cell counts frequently exceed of >20 cells/μl when elevated. Further studies have to investigate the clinical relevance of this finding.

Autoimmune encephalitides (AIE) associated with NMDAR antibodies is the most frequent AIE subtype (121), preferentially occurs at younger age (4) and, in our analysis, is almost always associated with inflammatory CSF changes, while AIE associated with LGI1 antibodies, which might be the second most common form with an annual incidence of more that 1:1 million (6) and typically occurs at older age and in males (5), rarely shows inflammatory CSF changes. Taken together, this strongly supports the hypothesis that inflammatory CSF changes might have a much higher discriminatory power to tell AIE from schizophrenia within the second or third decade of life, especially in females, than delirium or rapid progressive dementia from AIE in late life, especially in males. Of note, our findings underscore a recent report of antibody-associated neurological syndromes without signs of inflammation in the elderly (119).

The assumption that different immunological processes underlie the AIE subtypes is corroborated by the pre-dominant IgG subclasses reportedly involved (1). While the most prevalent antigen-specific IgG subclass is IgG4 in AIEs associated with IgLON5 (73), CASPR2 (24), LGI1 (126) an DPPX antibodies (1), in AIEs with $GABA_BR$, NDMAR, and AMPAR antibodies these were classified as pre-dominantly IgG1 (1). However, in line with IgG1-dependent complement fixation in AIE with LGI1 antibodies (127, 128), this subclass might be additionally important in this AIE subtype (126). AIEs with AMPAR and NMDAR antibodies, in our analysis with highly similar CSF findings, also share the pathogenic mechanism, receptor internalization rather than complement fixation (129–131).

AIE subtypes reportedly characterized by antigen-specific IgG1 reportedly are more likely to be paraneoplastic than those where antigen-specific IgG4 prevails (1). Combining these categorizations with the results of our analysis allows the hypothesis that a pathophysiology more likely to be paraneoplastic and driven by antigen-specific antibodies of the IgG1 subclass might be associated with robust and frequent inflammatory CSF findings, while non-paraneoplastic AIE subtypes with IgG4 as pre-dominant antigen-specific antibody rarely show an inflammatory CSF. GAD antibody-associated AIEs do not fit into this scheme. However, GAD is an intracellular antigen and thus cytotoxic T cells might play a prominent role in GAD antibody-associated AIE (127), as demonstrated for diabetes type 1 associated with GAD antibodies (132), a related and often co-existing disease. GAD antibody-associated CNS diseases are characterized by a much more chronic course compared to the other AIE subtypes (57). Correspondingly, we show that the typical pattern of CSF changes in GAD antibody-associated CNS disease is very different from all other AIE subtypes due to the pre-dominance of positive OCB while pleocytosis and increased CSF protein are rare.

In summary, our findings suggest that different antibody-defined AIE subtypes are associated with characteristic CSF findings. Rather non-paraneoplastic and IgG4 pre-dominant disease subtypes tend to have less CSF inflammatory activity compared to diseases with IgG1 pre-dominance, which more frequently are paraneoplastic. AIE with NMDAR antibodies is the most frequent AIE subtype at younger age and almost always associated with inflammatory CSF findings while anti-LGI1 AIE, the most frequent AIE subtype in the elderly, in the majority of patients CSF is normal. We thus conclude that in suspected AIE in the elderly, normal basic CSF findings should not lead to the decision against testing for antineuronal antibodies. As this assumption is based on a retrospective review of the literature, they have to be confirmed prospectively diagnosed patients.

DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/pubmed/.

AUTHOR CONTRIBUTIONS

TB performed the literature research, extracted the data and did the analysis, and critically revised the manuscript. JL envisioned the concept of the analysis, supervised data acquisition, and wrote the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00804/full#supplementary-material

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frontiers in Neurology

CASE REPORT
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Severe Anti-*N*-Methyl-D-Aspartate Receptor Encephalitis Under Immunosuppression After Liver Transplantation

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Anti-NMDA receptor encephalitis is a rare and often therapy-responsive autoimmune disease that usually affects young adults and causes neuropsychiatric symptoms. Here, we describe a 69-year-old patient who developed anti-NMDA receptor encephalitis while being under adequate immunosuppressive therapy following liver transplantation. Although a broad spectrum of different immunotherapies was applied and anti-NMDA receptor antibody titers gradually decreased, the clinical course could not be affected positively. Autoimmune encephalitis after transplantation is only described in a few cases and not well-recognized. Our case adds further evidence for anti-NMDA receptor encephalitis as the cause of neuropsychiatric symptoms even under immunosuppressive therapy in a post-transplant setting.

Keywords: Epstein-Barr virus, anti-NMDA receptor encephalitis, immunosuppression, liver-transplantation, autoimmune encephalitis

BACKGROUND

The anti-*N*-methyl-D-aspartate (NMDA) receptor encephalitis was first described in female patients under 50 years who developed neuropsychiatric symptoms after a non-obligate prodromal phase with flu-like symptoms (1–3). The core symptoms of the disease are cognitive deficiency, behavioral changes, dyskinesia, and seizures (2). Some patients may develop autonomic dysfunctions and reduced consciousness leading to the need of intensive care (2). This spectrum of encephalitis is associated with antibodies that target extracellular epitopes of cell-surface or synaptic proteins such as the GluN1 subunit of the NMDA receptor (2). Associated neurological syndromes often respond to immunotherapy, achieving substantial, or complete recovery in >75% of the patients (2).

Since a strict immunosuppressive treatment after solid organ transplantation is mandatory, it could be assumed that those patients have only marginal risk in developing autoimmune encephalitis. However, anti-NMDA receptor encephalitis was described in five post-transplant cases (4–8). Here, we report the first case of anti-NMDA receptor encephalitis after liver transplantation during sufficient anti-rejection immunosuppressive therapy.

CASE PRESENTATION

A 69-year-old woman presented with a 10-day history of progressive mental impairment to the emergency department of a community hospital. Acquaintances of her had observed that she withdraw from social life, barely spoke, and appeared mentally absent. The clinical examination showed that she was aphasic and not oriented. The remaining neurological examination was unremarkable.

A decade before onset of the neurological symptoms, she was diagnosed with liver cirrhosis due to chronic hepatitis c virus infection of which she suffered for 13 years and received an allogenic transplant. The cause of hepatitis c virus infection remained unclear. From then on, she was permanently on immunosuppressive therapy with tacrolimus and mycophenolate mofetil. At the time of onset of neurological symptoms, tacrolimus was administered with 1.5 mg/day and mycophenolate mofetil with 1,000 mg/day. Blood dosage of tacrolimus was 2.2 μg/L, and blood dosage of mycophenolate mofetil was not examined. White blood cell count and distribution revealed normal values for leukocytes (8,300/μl) and lymphocytes (1,300/μl).

After first admission, contrast enhanced magnetic resonance imaging (MRI) of the brain demonstrated leukoencephalopathy but no signs of a brain tumor or encephalitis. Basic cerebrospinal fluid (CSF) diagnostic revealed pleocytosis (58 cells/μl) and an elevated protein level (695 mg/L), while the lactate concentration was within the normal range (2.1 mmol/L). A viral encephalitis was assumed and the patient was treated intravenously with acyclovir. In the course of disease, she suffered from generalized epileptic seizures and an anticonvulsive therapy with levetiracetam 2 × 500 mg/day was initiated. The patient was then transferred to our university hospital. Follow-up MRI showed no change (**Figure 1**, *A1*) and electroencephalogram (EEG) was unremarkable. Due to a rapid progressive disturbance of consciousness within a week that led to a vegetative state, she had to be treated on the intensive care unit. The immunosuppressive therapy was changed from tacrolimus and mycophenolate mofetil to intravenous hydrocortisone. One month after immunosuppression with intravenous hydrocortisone, blood dosage of tacrolimus was 2.5 μg/L and that of mycophenolate mofetil was 3.2 mg/L, while the according white blood cell count and distribution revealed values within the reference range for leukocytes (6,600/μl) and lymphocytes (1,200/μl). Since C-reactive protein (CRP) and leucocytes were increasing, the anti-infectious therapy was changed to ganciclovir and piperacillin/tazobactam was added. CSF analysis was repeated 1 day after admission in our clinic and revealed pleocytosis (25 cells/μl, thereof 80% lymphocytes, 15% monocytes, 4% granulocytes, and 1% plasma cells), a disturbed blood–CSF barrier function (Qalbumin 12.3; protein 791 mg/L), and a lactate concentration of 2.6 mmol/L. Oligoclonal bands (OCB) restricted to CSF were found. Laboratory tests for autoimmune causes such as connective tissue diseases (antinuclear antibodies, anti-DNA antibodies, and antiphospholipid antibodies) were unremarkable. A broad diagnostic screening for infectious agents was performed. Analysis for bacterial (conventional cultural growth, mycobacterial cultures, *Treponema pallidum*, and *Borrelia burgdorferi* antibody tests), viral [antibody-specific index (AI) for herpes-simplex virus, varicella zoster virus, Epstein-Barr virus (EBV), measles virus, and rubella virus; polymerase-chain-reaction (PCR) for DNA of herpes-simplex virus, varicella zoster virus, Epstein–Barr virus, entero-virus, parecho-virus, adeno-virus, JC-virus, and human herpesvirus-6], and fungal (cultural growth and antigen test to Aspergillus and *Cryptococcus neoformans*) pathogens at different time points revealed only signs of CNS infection with Epstein–Barr virus. Polymerase-chain-reaction (PCR) analysis detected Epstein–Barr virus DNA in CSF (<3,200 copies/ml) and an elevated antibody-specific index for Epstein–Barr virus (45.3) suggesting intrathecal synthesis of EBV-specific IgG. In addition, anti-NMDAR-IgG antibodies in serum (titer 1:200) and CSF (titer 1:100) were found (**Figure 1**, *B1*) by using the commercially available cell-based assay of Euroimmune, confirming the diagnosis of anti-NMDAR encephalitis.

Additionally, flow cytometry of the CSF was performed to exclude post-transplant lymphoproliferative disorders (9, 10).

Corticoid treatment with 1 g of intravenous methylprednisolone was administered for 5 days followed by five courses of immunoadsorption therapy.

The patient's symptoms did not improve and thus a therapy with two cycles of intravenous immunoglobulins (60 g in total) was performed followed by a second course of methylprednisolone (1 g daily for 5 days) and two applications of rituximab (2× 1,000 mg within 14 days). Because of the devastating disease course without any improvement, an additional immunosuppressive therapy with cyclophosphamide (750 mg/m^2) was performed. White blood cell population count after extended immunosuppressive therapy revealed a decrease of leukocytes (2,400/μl) and lymphocytes (700/μl). As the patient experienced further epileptic seizures, the anticonvulsive treatment was expanded with valproate and lacosamid. Due to persisting epileptic seizures, lacosamid was changed to phenytoin.

Meanwhile, the gynecologic diagnostic including ovarian ultrasound remained unremarkable. Whole-body PET-CT screening showed no signs of a paraneoplastic etiology of the autoimmune encephalitis. Nevertheless, the patient underwent oophorectomy of both sides, as a rescue option that can be considered in imaging-negative anti-NMDA receptor encephalitis patients without obvious ovarian teratoma (11). Histological examination of the ovarian tissue did not detect a teratoma.

In the course of the disease, the patient slightly regained consciousness. Follow-up CSF diagnostic 8 weeks after first symptoms and 5 weeks after the first dose of steroids showed decreasing pleocytosis (10 cells/μl, thereof 90% lymphocytes and 10% monocytes) and reduced anti-NMDAR-IgG antibodies titers (1:100 in serum, 1:50 in CSF, **Figure 1**, *B2*). The

Abbreviations: Anti-NMDA receptor, anti-*N*-methyl-D-aspartate receptor; MRI, magnetic resonance imaging; CSF, cerebrospinal fluid; EEG, electroencephalogram; PCR, polymerase chain reaction; EBV, Epstein–Barr virus; CRP, C-reactive protein; OCB, CSF-specific oligoclonal bands; Qalbumin, CSF-serum albumin quotients; PET, positron emission tomography; CT, computed tomography.

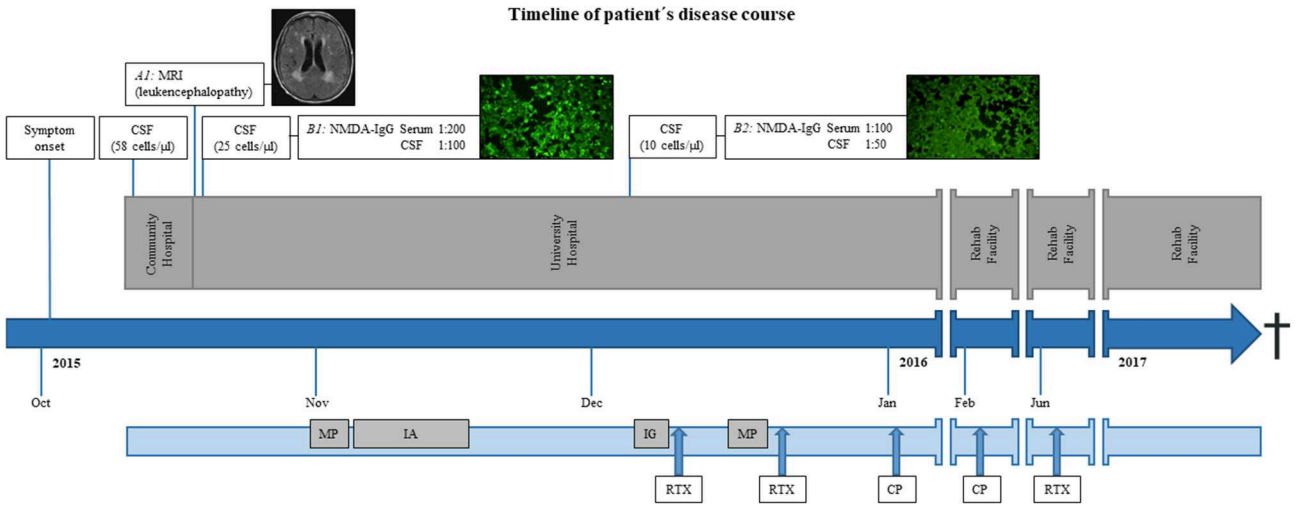

FIGURE 1 | Timeline of patient's disease course. *(A1)* Exemplary axial fluid-attenuated inversion recovery (FLAIR) magnetic resonance imaging (MRI) of the brain demonstrated leukoencephalopathy but no signs of inflammation. Immune-fluorescence microscopy of anti-NMDA receptor staining with high (*B1*) and low titers (*B2*) in serum (depicted) and CSF. Bright green cells represent an antibody-antigen-interaction (*B1*) while dim cells do not reveal such interaction (*B2*). CSF, cerebrospinal fluid; MRI, magnetic resonance imaging; MP, methylprednisolone; IA, immunoadsorption-therapy; IG, intravenous immunoglobulins; RTX, rituximab; CP, cyclophosphamide; Anti-NMDA receptor, Anti-N-methyl-D-aspartate receptor.

immunosuppressive therapy was switched back to oral treatment with tacrolimus and mycophenolate mofetil and the patient was transferred to a rehab facility. The patient regained consciousness and orientation but showed a reduced general condition with cachexia and was not able to walk.

After 6 weeks, she was readmitted to our hospital for another course of cyclophosphamide and after 6 months for rituximab treatment. In the course, repeated tumor screening including cerebral, abdominal, and thoracic imaging showed no evidence of concomitant malignant diseases. However, the patient did not fully recover and died 2 years after disease onset due to septicemia (see timeline figure for overview).

DISCUSSION

Here, we present the first case of anti-NMDA receptor encephalitis developing despite immunosuppressive therapy after liver transplantation. Mycophenolate mofetil and tacrolimus are both highly effective drugs and were developed to prevent autoimmunity in patients after transplantation of solid organs (12–14). Mycophenolate mofetil has inhibitory effects on B- and T-cells, while tacrolimus reduces activation of T-cells (14, 15). Since the pathomechanisms of anti-NMDA receptor encephalitis are considered to be driven by complement-independent antibody effects, it could be assumed that this autoimmune disease should not occur under adequate immunosuppressive therapy with mycophenolate mofetil and tacrolimus (7). However, similar cases have been described in three patients after kidney transplantation (4, 5, 8), in one patient after repeated stem-cell transplantations in childhood and kidney transplantation in the course (7), and in one patient after heart transplantation (6). In all five published cases, immunosuppressive therapy at the time of encephalitis onset consisted of mycophenolate mofetil in addition to either tacrolimus or prednisolone (4–8). Furthermore, several reports showed that patients after allogeneic or autologous stem cell transplantation developed autoimmune diseases such as polymyositis, myasthenia gravis, Guillain–Barré syndrome, and anti-LGI1 and anti-GABAAR encephalitis, concluding that the inhibitory effect of mycophenolate mofetil on B- and T-cells and tacrolimus on T-cells might not be sufficient to prevent additional neuroimmunological diseases (16–18).

In all reported cases of anti-NMDAR encephalitis, symptoms began at least 6 years after transplantation (4–8). The symptom onset in our patient occurred even 10 years after liver transplantation. Dysfunctional immune tolerance and autoimmune phenomena are described as long-term effects of immunosuppressive therapy (16–18). It can be hypothesized that after several years of immunosuppression therapy, immune cell populations might be imbalanced, causing the breakdown of immune tolerance, in particular on the side of B-cells since chronic immunosuppression is rather T-cell targeting (12, 14).

Another interesting observation in our case is the concomitant presentation of Epstein–Barr virus DNA in CSF. In four of the five published cases, Epstein–Barr virus DNA was also found in the CSF of patients with autoimmune encephalitis (4–8). The authors suggested either reactivation of latent virus infection or first infection under immunosuppressive therapy (5, 7). An involvement of Epstein–Barr virus in autoimmune diseases such as multiple sclerosis is the subject of ongoing discussion. Casiraghi et al. propose that Epstein–Barr virus infection of brain endothelial cells could cause an upregulation of inflammatory mediators, which then induces a local breach in the brain–blood barrier and attraction of

autoreactive lymphocytes into the brain (19). In analogy to this hypothesis, a similar pathomechanism with diffusion of peripheral autoreactive lymphocytes across a dysfunctional blood–brain barrier and subsequent intrathecal production of anti-NMDAR-IgG antibodies could be assumed (19).

On the other side, in another infectious disease, the herpes-simplex virus encephalitis, the herpes-simplex virus is regarded to trigger processes of autoimmunity in the CNS directly, which was demonstrated in a study involving patients with herpes-simplex encephalitis who subsequently developed autoimmune encephalitis (20, 21).

There are several other hypotheses about a possible relationship between virus infection and autoimmune encephalitis. As it is known, that EBV acquires its definite envelope by budding through the plasma membrane of infected host cells, the virus might incorporate specific components of their membrane like expressed receptors in its envelope (22). Possible targets for infection with Epstein–Barr virus are B- and T-lymphocytes, NK-cells, and epithelial cells of the nasopharyngeal and gastrointestinal area (23). If the infected host cells express the specific NMDA receptor on their surfaces as it is shown for different neuronal and extra-neuronal cells, for example, of the gastrointestinal tract, the envelope of the Epstein–Barr virus might include this receptor (24). The specific immune response against Epstein–Barr virus might also lead to an immune reaction against the virus envelope-associated NMDA receptor.

Furthermore, it might be possible that the Epstein–Barr virus infection of already primed B-lymphocytes against the NMDA receptor promotes the proliferation of this specific B-cell type and thus increases the synthetization of anti-NMDA receptor antibodies (25).

Another remarkable aspect of our case is the clinical poor response to the broad spectrum of immunosuppressive therapeutics. Although clinical trials are not yet available, clinical experience in the therapy of anti-NMDA receptor encephalitis suggests intravenous methylprednisolone followed by plasma exchange or immunoadsorption therapy and/or intravenous immunoglobulins and an escalation therapy with rituximab and/or cyclophosphamide (26–28). In our case, all the recommended therapies were performed but did not improve the patient's symptoms. White blood cell count showed laboratory efficacy of the immunosuppressive therapy and even anti-NMDAR antibody titers in serum and CSF decreased. However, a correlating positive clinical effect could not be achieved. Even oophorectomy of both sides did not lead to clinical improvement as described in some cases (11, 28).

Early treatment in autoimmune encephalitis patients without previous immunosuppressive therapy has been shown to have a better prognosis (2). Our patient was diagnosed already 3 weeks after the onset of symptoms and had a fatal outcome. In contrast, the other published cases of post-transplant patients with autoimmune encephalitis and full recovery were diagnosed even later (3 and 5 months) after clinical manifestation (5, 7). Thus, the duration of symptoms and onset of a sufficient therapy was rather not the reason for the fatal outcome in our patient.

CONCLUSION

Anti-NMDA receptor encephalitis can develop fatally despite previous immunosuppressive therapy. The etiology of anti-NMDA receptor encephalitis is still not fully understood but seems to involve autoimmune mechanisms that are not sufficiently inhibited by mycophenolate mofetil and tacrolimus. In addition, an EBV infection of the cells forming the blood–brain barrier might play a role in the pathogenesis of anti-NMDA receptor encephalitis. An autoimmune encephalitis should be considered in post-transplant patients with neuropsychiatric symptoms.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FK participated in the design of the study, collected and analyzed the data, and drafted the manuscript. PS collected the data, analyzed the data, and drafted the manuscript. KJ, MH, FW, and K-WS contributed in drafting the manuscript. MS analyzed the data and contributed in drafting the manuscript. TS conceived the study, analyzed the data, and drafted the manuscript. All authors read and approved the final manuscript.

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frontiers in Neurology

HYPOTHESIS AND THEORY
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Albumin Exchange in Alzheimer's Disease: Might CSF Be an Alternative Route to Plasma?

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Amyloid β (Aβ) in brain parenchyma is thought to play a central role in the pathogenesis of Alzheimer's disease (AD). Aβ is transported from the brain to the plasma via complex transport mechanisms at the blood-brain barrier (BBB). About 90–95% of plasma Aβ may be bound to albumin. Replacement of serum albumin in plasma has been proposed as a promising therapy for AD. However, the efficacy of this approach may be compromised by altered BBB Aβ receptors in AD, as well as multiple pools of Aβ from other organs in exchange with plasma Aβ, competing for albumin binding sites. The flow of interstitial fluid (ISF) into cerebrospinal fluid (CSF) is another major route of Aβ clearance. Though the concentration of albumin in CSF is much lower than in plasma, the mixing of CSF with ISF is not impeded by a highly selective barrier and, hence, Aβ in the two pools is in more direct exchange. Furthermore, unlike in plasma, Aβ in CSF is not in direct exchange with multiple organ sources of Aβ. Here we consider albumin replacement in CSF as an alternative method for therapeutic brain Aβ removal and describe the possible advantages and rationale supporting this hypothesis.

Keywords: Alzheimer's disease, amyloid-beta-protein, CSF (cerebrospinal fluid), BBB (blood–brain barrier), therapy

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INTRODUCTION

Aggregation of amyloid-β (Aβ) in the brain parenchyma and arterial walls and the formation of neurofibrillary tangles in neurons due to phosphorylated tau protein accumulation are the main histologic hallmarks of Alzheimer's disease (AD). While familial AD is associated with an increased production of $A\beta_{1-42}$, the amyloid form most prone to aggregate, sporadic AD may be related to an imbalance between the production and the clearance of different forms of the Aβ peptide (1). Interstitial monomeric Aβ is in equilibrium with Aβ in oligomers and large insoluble fibrils (plaques) and cleared from the central nervous system (CNS) via at least three pathways: metabolic degradation, efflux across the blood–brain barrier (BBB) and efflux via perivascular and cerebrospinal fluid (CSF) routes. The relative importance of each of these pathways is under investigation (1, 2).

To cross the BBB, soluble Aβ in the interstitial fluid (ISF) of the brain's extracellular spaces must first diffuse to the capillary basement membrane, past pericytes and astrocytic endfeet, to reach the endothelium (3). Blockage of Aβ transcytosis across the endothelium accelerates the abnormal deposition of Aβ and is closely associated with neuronal degeneration (4–6). In addition to drainage via ISF to capillaries, Aβ reaches the CSF via perivascular spaces, where it can be cleared

via CSF drainage routes (2). While the details and dynamics of these pathways are currently under investigation and the subject of much debate, the concentration of Aβ in CSF has been shown to be significantly altered in AD (7, 8).

Aβ is produced throughout the body and has a concentration in plasma and CSF of ∼0.1–0.5 nM (9, 10). Its clearance relies on carrier proteins such as albumin, beta-2-macroglobulin, apolipoprotein E, apolipoprotein J (clusterin), low-density lipoprotein receptor-related protein-1 (LRP1), and transthyretin (11–15). A very small quantity of plasma proteins diffuse into CSF from plasma (e.g., albumin). Unlike plasma (∼7 g protein/100 ml) or milky lymph (∼2 g protein/100 ml), the CSF has only ∼0.025 g protein/100 ml—mainly albumin. All albumin is synthesized in the liver and initially released into blood. Albumin is the most abundant protein in both plasma (∼640 μM) and CSF (∼3 μM) (16) and has been reported to be the primary carrier of Aβ in blood (17). However, there is also evidence that a soluble cleavage product of LRP1 (sLRP) may be the major plasma carrier of Aβ (15). In addition to Aβ, albumin also transports a variety of other endogenous and exogenous molecules and is involved in regulating oncotic pressure.

Some kinetic studies have supported a 1:1 stoichiometry for the binding of monomeric Aβ to albumin with a dissociation constant (K_d) of ∼5–10 μM (18–20), while others have shown that albumin preferentially binds oligomeric Aβ at 3 independent binding sites with much lower K_d's, in the range of 1–100 nM (21). A recent study has also supported a lower K_d for monomeric Aβ than previously reported (e.g., 180 nM for monomeric $Aβ_{1-42}$) (22). Regardless of the study, these K_d values would explain the high percentage of binding of Aβ to albumin in blood (∼90%) (17). In CSF, based on an assumed K_d of 5 μM, it has been estimated that ∼40% of Aβ is bound to albumin (20). However, if the lower Kd's reported for Aβ binding to albumin are more accurate, then a much higher fraction of Aβ could be bound to CSF albumin. For example, if the effective CSF albumin-Aβ K_d is as low as 180 nM, as measured by Litus et al. for $Aβ_{1-42}$, and assuming 1:1 monomeric binding without interference from other ligands, the percentage of albumin-bound Aβ in CSF could be as high as 94%.

Whether by direct binding of monomeric forms of Aβ or indirectly via oligomer binding and inhibition of further monomer addition, albumin also attenuates the growth of Aβ fibrils (20, 21, 23–25). The binding of cholesterol and fatty acids to albumin have been shown to weaken the binding of Aβ (22, 26), possibly contributing to the association of high dietary levels of these molecules with AD (26).

Changes in albumin are independently associated with aging and neurodegeneration. Low serum albumin is associated with increased odds of cognitive impairment in the elderly (27), and both plasma and CSF albumin oxidation are higher in AD patients compared to healthy controls (28). In a study on sheep, an increase in CSF albumin concentration in older sheep was attributed to a reduced production of CSF by the choroid plexus, even though the latter was observed to increase in size with age (29).

There is preliminary evidence from both animal and human studies that replacing plasma albumin using interventions such as hemodialysis and plasmapheresis may be effective in treating AD (30–33). In an ongoing clinical trial (34), the safety and efficacy of using plasma exchange to remove albumin-bound Aβ for treating AD is being explored (35–37). The concept behind this approach is that a reduction of the albumin-bound Aβ pool in plasma will, in turn, reduce Aβ levels in the parenchyma of the brain, known as the "peripheral sink" hypothesis (38–40). However, equilibrium between the brain and blood pools of Aβ is complex, involving distinct one-way receptor-mediated transport mechanisms on either side of the BBB, which have been shown to be altered in AD, thus reducing the net efflux of Aβ from the brain (2, 41). Moreover, Aβ bound to plasma albumin comes from several organ sources, not solely from the brain. These factors could potentially limit the additional efflux of Aβ from the brain after plasma albumin replacement.

While large insoluble Aβ fibrils forming the plaques of AD are the most conspicuous makers of the disease, a large and growing body of evidence supports that smaller soluble oligomers may be the most cytotoxic forms of Aβ. Soluble Aβ oligomers are capable of forming transmembrane ion channels (42, 43), disrupting homeostasis, and have been shown to have a number of cell surface and intracellular targets implicated in AD pathology, including components of synaptogenesis and neurofibrillary tangle formation (25, 44–46). Hence, trapping toxic oligomeric forms of Aβ is likely to be a major underlying mechanism for the therapeutic effect of albumin replacement on AD (47, 48).

Other possible therapeutic roles for albumin include its role as a transporter of the labile pool of Cu^{2+} ions in blood plasma (49). Dysregulation of Cu^{2+}, leading to increases in oxidative stress and cellular damage, has been implicated in Aβ aggregation and fibril formation (50–52). Plasma exchange therapy may also serve to replace aged and glycated albumin. Both CSF and plasma proteins are involved in a vicious cycle of glycation during aging, as aging reduces CSF turnover and increases the exposure time of CSF proteins to glucose, resulting in more glycation of CSF proteins (53, 54). Protein properties are altered after glycation, resulting in a decreased degradation rate and a longer time to be eliminated (55). Glycated albumin, which has less positive charge than non-glycated albumin, more readily crosses brain barriers from the blood to the CSF (56). The increasing glycation of CSF proteins during aging may stimulate the formation and the consequent deposition of AGEs as well as oxidative stress in the brain (53).

In addition to plasma exchange, adding synthetic albumin to CSF has been explored as a more direct route to augmenting the capture and elimination of toxic forms of interstitial brain Aβ. In the 3xTg mouse model of AD, Ezra et al. showed that intraventricular infusion of synthetic serum albumin for 28 days, using an osmotic pump, led to a decrease of both Aβ monomers and oligomers in brain homogenates and amyloid plaques in histological samples (48). Additionally, performance in memory and fear conditioning tasks improved in treated animals and hyperphosphorylated tau was reduced and tubulin increased in brain homogenates, suggesting increased microtubule stability.

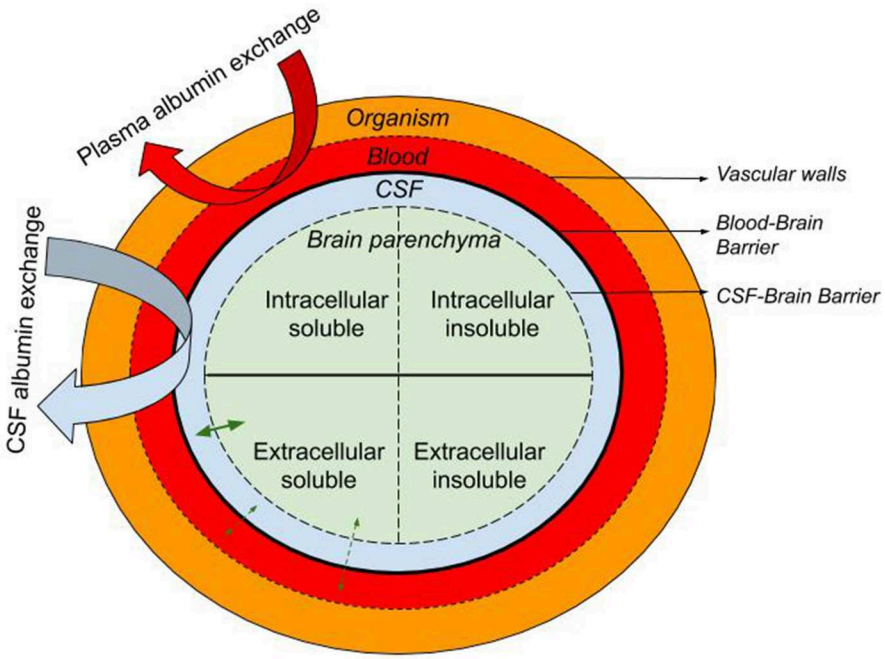

FIGURE 1 | Schematic representation of the the blood, CSF, and brain intra- and extra-cellular pools of albumin-containing fluids with the organism. The equilibrium of soluble molecules (i.e., soluble Aβ) between the extracellular ISF, CSF, and plasma is represented with straight green arrows while curved arrows illustrate the CSF and plasma albumin exchange.

Markers of BBB and myelin integrity were also assayed and demonstrated improvement in treated mice.

The positive findings of Ezra et al. on direct infusion of albumin into CSF along with the potential limitations of plasma exchange motivate us to propose an approach that combines aspects of both: CSF exchange with albumin replacement.

THE THERAPEUTIC HYPOTHESIS

Here we describe the rationale supporting the therapeutic approach of exchanging endogenous albumin in CSF for synthetic albumin for the treatment of AD. Like plasma exchange, CSF exchange is a "peripheral sink" approach to affecting brain levels of Aβ, whereby Aβ in a pool that is in equilibrium with the brain pool of Aβ is replaced with Aβ-free fluid (**Figure 1**). This process is either continuous, as in dialysis, or repeated at intervals, as in plasma exchange. During or after the process, the Aβ-free pool re-establishes an equilibrium with the brain pool, drawing out extracellular brain Aβ as it does.

Though both plasma and CSF may act as "sinks" for interstitial Aβ, the drainage routes for either differ substantially. An early study on the relationship between plasma and CSF levels of Aβ in a transgenic mouse model of AD observed a significant and strong correlation between the levels in mice without detectable Aβ plaques but no significant correlation in mice after Aβ plaque formation (7). Subsequent studies have revealed that distinct and complex brain efflux and influx transport mechanisms are involved in the transfer of Aβ across the BBB (57, 58). Though the relationship between plasma and ISF levels is still not fully understood, in AD the Aβ transporter LRP1 on the abluminal side of the capillary endothelium has been reported to be reduced in number (41, 59) while the Aβ transporter receptor for advanced glycation end products (RAGE) on the luminal side of microvascular endothelium is increased (59). These alterations have been suggested to contribute to the net accumulation of interstitial Aβ in AD, favoring the formation of insoluble Aβ fibrils.

Given the possible impairment of the BBB Aβ transport mechanisms in AD, CSF exchange may create an Aβ sink with less impediments to drainage than plasma exchange, since there is no tight cellular barrier between the CSF and ISF compartments. A substantial fraction of the brain's metabolic waste and cellular/pathogen degradation debris is cleared by CSF/ISF drainage routes to lymphatic and venous outlets. Estimates for the elimination of Aβ via CSF have been as high as 25% (60) [reviewed by (61)]. The CSF that flows along perivascular spaces originates in the subarachnoid space and, by mechanisms still being explored, it may mix with ISF deep within the parenchyma, promoting the passage of cellular waste back to the CSF. Though the dynamics of these pathways, including that of the so-called "glymphatic" system (62), are still being debated, several studies have demonstrated the involvement of CSF drainage routes in normal Aβ clearance [reviewed by (61)].

The specific approach to CSF exchange we propose is a variation on liquorpheresis (cerebrospinal fluid filtration). Liquorpheresis is the process of filtering CSF in order to clear "toxic" molecules from the CSF (63). Though currently not in

common use, it has been used to treat a number of neurological diseases and recent developmental efforts may foster its future use. Specifically, we propose to use liquorpheresis with albumin replacement to create a "CSF sink" of low Aβ within the brain. We hypothesize that this sink will pull the equilibrium between free and CSF albumin-bound Aβ in the brain more toward albumin-bound Aβ, as the latter is cleared from the brain either via the natural routes of CSF drainage or via the ongoing liquorpheresis filtering process itself.

DISCUSSION

Could CSF exchange be more efficacious than plasma exchange for the replacement of albumin to treat AD? We summarize three points from the discussion above that support the hypothesis that it could be:

1) The origin of CSF Aβ is mostly cerebral, whereas the origin of plasma Aβ is from the entire body, with just a small proportion coming from the brain. Hence, plasma exchange is non-specific for brain-derived Aβ and alters the homeostasis of Aβ across multiple organs. Indeed, plasma exchange has been shown to be followed by an initial overshoot of plasma Aβ (a "sawtooth" concentration pattern) (37), presumably as the multiple tissue-plasma equilibria are re-established. In contrast, CSF albumin replacement is a much more targeted therapeutic approach. Though the concentration of albumin is a factor of 20 less in CSF, it only binds Aβ that exists in the brain, even if some fraction of this Aβ derived from plasma via the BBB or choroid plexus.
2) If sLRP is the principal carrier of brain-derived Aβ as some studies suggest (15), then plasma albumin replacement may have only a minor effect on brain-derived Aβ, since the sLRP carrier is released from the luminal side of the BBB after LRP1-Aβ transcytosis and is hypothetically already tightly bound to Aβ. As acknowledged by Boada et al. in a preliminary report on clinical plasma exchange, its beneficial effects on AD may be due to factors not related to albumin-Aβ binding (37).
3) Even though the BBB may be the major route of interstitial Aβ clearance in healthy subjects, alterations in BBB Aβ transporters with age, leading to lower net Aβ efflux, may exacerbate or be a cause of AD. Adding to this potential limitation of plasma exchange is that Aβ efflux across the BBB is a saturable process (64, 65). Under these circumstances, the barriers to CSF-ISF exchange may present less of an impediment for Aβ clearance than the highly selective and transporter-dependent BBB barrier and CSF-ISF drainage routes might act as compensatory routes for clearing brain Aβ in AD (4).

There are several unknown factors that will impact the capacity of CSF exchange to enhance the clearance of brain Aβ. Most of these factors would be very challenging to model, given our still evolving knowledge of the normal routes and dynamics of CSF/ISF drainage, let alone the uncertainties regarding the *in vivo* binding kinetics of Aβ to albumin. Nonetheless, Ezra et al. (48) have shown that a relatively small addition of synthetic, Aβ-free albumin to intraventricular CSF, delivered via an osmotic pump, had measurable effects on Aβ plaque formation as well as several other markers of disease in a mouse model of AD. These results suggest similar studies on CSF albumin replacement via liquorpheresis to explore the possibility of achieving comparable effects with a relatively straightforward clinical tool that has been shown to be efficacious in treating other neurological diseases. Experimentation in animal models of AD will also be useful to compare the relative efficacies of liquorpheresis and plasma exchange to reduce brain Aβ levels.

Clinically, liquorpheresis may offer advantages in terms of safety over either CSF infusion by osmotic pump or plasma exchange. Infusing albumin either intrathecally or intraventricularly in humans poses the risk of increased intracranial pressure due to the osmotic properties of albumin, while equimolar albumin replacement during CSF exchange circumvents this possibility. Plasma exchange needs to be performed during long sessions in a hospital setting, with potential side effects associated with osmotic changes and the removal of other circulating factors, such as metabolites, cytokines, clotting factors, and hormones. Liquorpheresis, on the other hand, does not entail the removal of such factors and requires shorter periods of time since the CSF volume is much

TABLE 1 | Comparison between plasma albumin exchange and CSF albumin exchange.

Plasma albumin exchange	CSF albumin exchange
The blood-sink mechanism of action relies on transport of Aβ through the BBB, which is a saturable process and is damaged in AD.	The CSF-sink mechanism of action relies on transport of Aβ through the CSF-brain barrier, circumventing BBB transport which is compromised in AD.
Many endogenous and pharmaceutical molecules can bind plasmatic albumin. Therefore, removing plasmatic albumin might interfere in many physiological functions and treatments.	The number of endogenous and pharmaceutical molecules binding CSF albumin is much lower than the number binding albumin in plasma. Therefore, removing CSF albumin would interfere less with normal physiologic function or other treatments.
Levels of albumin in plasma are much higher than in CSF. Therefore, the amount of albumin that needs to be removed in order to achieve a "sink effect" is also higher.	The amount of albumin in CSF is much lower than in plasma; therefore, the amount of albumin that needs to be removed in order to achieve a "sink effect" is relatively much lower.
Potential systemic side effects affecting multiple organs, including the CNS.	Potential side effects limited to the CNS.
Nonspecific for cerebral Aβ.	Specific for cerebral Aβ.
Plasma albumin exchange is a well-developed technique. • Infusion of albumin in plasma is a common clinical practice. • Devices for plasmapheresis are available in most hospitals. • Requires venous puncture.	• CSF albumin exchange would be a novel use of liquorpheresis. • Intrathecal infusion of albumin has never been attempted in humans. • Requires a lumbar puncture.

Positive points are in green and negative points in red.

smaller than the blood volume. However, an advantage of plasma exchange is that it can be performed through a venous access while liquorpheresis requires a lumbar puncture. A comparison of the potential advantages and disadvantages plasma vs CSF exchange is given in **Table 1**.

CONCLUSIONS

The accumulation of toxic oligomers of Aβ in the brain due to inadequate clearance from ISF may be a cause of AD. Reduction of Aβ in fluids peripheral to brain ISF may enhance Aβ clearance within the brain. Plasma exchange has been shown to be one promising approach to accomplish this in humans, as has intraventricular infusion of synthetic albumin in a mouse model of AD. Intrathecal CSF exchange (liquorpheresis) with albumin replacement may be an alternative approach that circumvents the potential disadvantages and clinical risks of either plasma exchange or CSF infusion. CSF exchange targets the pool of Aβ directly in exchange with brain ISF, without being impeded, as with Aβ transport via the BBB, by a highly selective, saturable, and possibly dysfunctional barrier. In terms of efficacy and safety, CSF exchange is a clinically tested therapeutic approach for the treatment of other neurological diseases and, unlike CSF infusion, does not risk disruption of osmotic homeostasis. Experimentation with animal models is needed to establish that CSF exchange performs as well as CSF infusion in reducing the hallmarks of AD, as well as to optimize its application. Additionally, such experiments could shed more light on Aβ dynamics in health and disease.

AUTHOR CONTRIBUTIONS

MM-G is the author of the therapeutic hypothesis. MM-G and CG wrote the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Psychiatric Presentation of Anti-NMDA Receptor Encephalitis

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Background: Anti-N-methyl D-aspartate (NMDA) receptor encephalitis is an autoimmune condition characterized by neuropsychiatric symptoms, including epileptic seizures, movement disorders, autonomic instability, disturbances of consciousness, paranoia, delusions, and catatonia. Ovarian teratomas and viral infections, typically Herpes simplex viruses, have previously been demonstrated to precipitate anti-NMDA receptor encephalitis, but in many cases, the trigger remains unclear. The detection of anti-NMDA receptor antibodies in cerebrospinal fluid (CSF), in combination with other CSF, electroencephalography (EEG), or magnetic resonance imaging (MRI) abnormalities, typically leads to diagnostic clarification.

Case Presentation: We present the case of a 22-year-old female patient who developed an acute polymorphic psychotic episode 3 days after receiving a booster vaccination against tetanus, diphtheria, pertussis, and polio (Tdap-IPV). Her psychiatric symptoms were initially diagnosed as a primary psychiatric disorder. Her MRI, EEG, and CSF results were non-specific. Anti-NMDA receptor IgG antibodies against the GluN1 subunit were detected in her serum (with a maximum titer of 1:320), but not in her CSF. [18F]fluorodeoxyglucose positron emission tomography (FDG-PET) showed pronounced relative hypermetabolism of her association cortices and a relative hypometabolism of the primary cortices, on the basis of which an anti-NMDA receptor encephalitis diagnosis was made, and treatment with a steroid pulse was initiated. The treatment led to fast and convincing clinical improvement with normalization of neuropsychological findings, considerable improvement of FDG-PET findings, and decreasing antibody titers.

Conclusion: The patient's psychiatric symptoms were most likely caused by anti-NMDA receptor encephalitis. Her polymorphic psychotic symptoms first occurred after she had received a Tdap-IPV booster vaccination. Although the vaccination cannot have caused the initial antibody formation since IgG serum antibodies were detected only 3 days after administration of the vaccine, the vaccine may have exerted immunomodulatory effects. MRI, EEG, and CSF findings were non-specific; however, FDG-PET identified brain involvement consistent with anti-NMDA receptor encephalitis. This case shows the importance of implementing a multimodal diagnostic work-up in similar situations. The negative CSF antibody finding furthermore fits to the hypothesis that the brain may act as an immunoprecipitator for anti-NMDA receptor antibodies.

Keywords: anti-NMDA receptor encephalitis, encephalopathy, autoimmune psychosis, antibodies, steroids, vaccination

BACKGROUND

Anti-N-methyl D-aspartate (NMDA) receptor encephalitis was first identified as a paraneoplastic disorder associated with ovarian teratoma (1). Since then, large case series with hundreds of cases have allowed researchers to describe its typical clinical course (2). After a prodromal phase with flu-like symptoms, behavioral abnormalities and acute polymorphic symptoms, often accompanied by catatonic symptoms, are typically observed. In addition, movement disorders, such as dyskinesia or rigidity, autonomous instability, or central hypoventilation, typically occur as the disease progresses, as do disturbances of consciousness. Epileptic seizures may also occur at any time (3, 4). Thus, the typical clinical syndrome is neuropsychiatric in nature and thereby points to an organic cause, but there are occasional cases that present like primary idiopathic psychiatric disorders, typically with catatonia (5, 6). Young women are most frequently affected (2).

Patients with anti-NMDA receptor encephalitis usually have IgG antibodies against the GluN1 subunit of NMDA receptors in their cerebrospinal fluid (CSF) (7–10). Electroencephalography (EEG) results are usually abnormal (3), and in some patients a specific EEG phenomenon, the so-called "extreme delta brush," has been observed (11). It is well-known that ovarian teratomas and viral infections can trigger autoimmune reactions. In paraneoplastic cases associated with ovarian teratomas, the tumor itself contains NMDA receptors, which are typically found in brain tissue, and this seems to trigger the encephalitic autoimmune response (3). In addition, most authors suggest that virus-induced neuroinflammation can trigger antibody formation. While Herpes simplex is most widely blamed in this context, other viruses or bacteria (e.g., varicella zoster, mycoplasma) may also potentially trigger the encephalitic autoimmune response (3). However, often no specific causes for antibody production can be identified.

Vaccinations may also play a role as unspecific triggers (12, 13). A PubMed search for "vaccination and NMDA" (on 24 April 2019) identified 27 published articles addressing this issue, including two case reports. The first case was reported to have developed anti-NMDA receptor encephalitis 5 weeks after a booster vaccination against tetanus, diphtheria, pertussis, and polio (Tdap-IPV) (14). In the second, a patient was reported as having developed a postural tachycardia syndrome with anti-NMDA receptor antibodies after receiving a human papillomavirus vaccination (15). An additional vaccine-related case was also mentioned in a publication by Wang (12), who reported that an individual developed anti-NMDA receptor encephalitis after receiving a vaccination against Japanese encephalitis. A further search for "vaccination and autoimmune encephalitis" resulted in only 12 results and no case reports.

CASE PRESENTATION

Here, we present the case of a 22-year-old female German patient who experienced an acute polymorph psychotic episode after receiving a Tdap-IPV booster vaccination (Repevax®). The patient received the vaccination at the beginning of January 2019 and reported an immediate "strange" feeling. She rapidly developed flu-like symptoms (headache, jittering), and 3 days later, she was admitted to a hospital emergency department with psychosis-like symptoms: She was hearing her thoughts and developed paranoia (e.g., she felt that she was being used as a test subject by the clinic staff). Her neurological and general medical examinations were normal, and her CSF samples, magnetic resonance imaging (MRI) scans of her brain, and the EEG showed no relevant abnormalities. Anti-NMDA receptor IgG antibodies against the GluN1 subunit (titer 1:80 3 days after symptom-onset; titer 1:320 after 6 days, both times using biochip-assays) were detected in her serum, but the CSF antibody testing was negative (3 days after symptom-onset, not performed after 6 days; using cell-based assay). After 6 days, the patient was discharged from the hospital and began outpatient psychotherapy with the assumption that her problems were caused by being separated from her parental home. In the meantime, the patient stopped her vocational training and moved house back to her parents. The mother reported that her daughter behaved like a toddler and that she was helpless, unbalanced, and dependent. She was readmitted to the hospital 47 days after vaccination due to her persistent symptomatology with strong

anxiety and ambivalence. Treatment with lorazepam (2 mg) led to a reduction in her anxiety.

Fifty-three days after the vaccination, the patient presented herself to our hospital. At this time, she reported that she had pronounced anxiety, an inability to make decisions, strong restlessness, and sensory overload, and that she was emotionally unstable (i.e., she could suddenly burst into tears) and had cognitive deficits (i.e., was not able to keep conversations for a longer period of time in a concentrated manner). She also reported difficulties in falling asleep and sleeping through the night, reduced appetite, and a weight loss of 4–5 kg over 2 months. In addition, she reported experiencing burning sensations on her chest and head and intermittent tachycardia.

Diagnostic Findings

A high-resolution 3 Tesla structural MRI of her brain was normal except for a few slight, non-specific bifrontal white matter lesions (**Figure 1**). An EEG depicted a β-rhythm (likely related to lorazepam intake), with rare intermittent slow wave activity of 6–7/s. Independent Component Analysis (ICA) showed a frontocentral topography compatible with tiredness (**Figure 2**). Her CSF basic analysis results were inconspicuous (normal white blood cell count/albumin quotient/protein concentration, no CSF specific oligoclonal bands). In her serum, anti-NMDA receptor GluN1-IgG antibodies were still positive (titer 1:160, using cell-based-assays), while IgA and IgM antibodies were negative. CSF antibody testing was again negative, including biochip assays, cell-based assays, and tissue testing. An [18F]fluorodeoxyglucose positron emission tomography (FDG-PET) examination showed a pronounced relative hypermetabolism of parts of the association cortices and a relative hypometabolism of the primary cortices, well compatible with encephalitis (**Figure 3**). A whole-body FDG-PET/CT detected no lesions suspicious of malignancy, including no ovarian teratoma. A gynecologic ultrasound and an MRI of the abdomen were unremarkable. A long-term electrocardiogram (ECG) showed recurrent sinus tachycardia that stopped at night (**Figure 4**). Neuropsychological testing showed significant deficits in working memory (**Figure 5**). All diagnostic findings are summarized in **Table 1**.

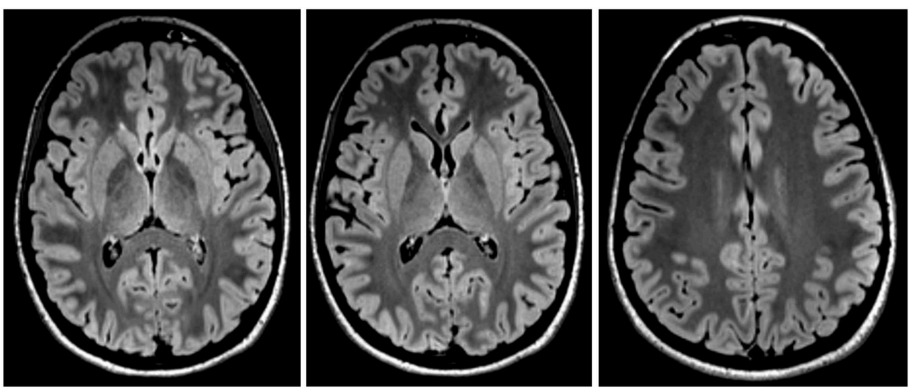

FIGURE 1 | Magnetic resonance imaging showed slight, non-specific bifrontal medullary lesions, but was otherwise inconspicuous.

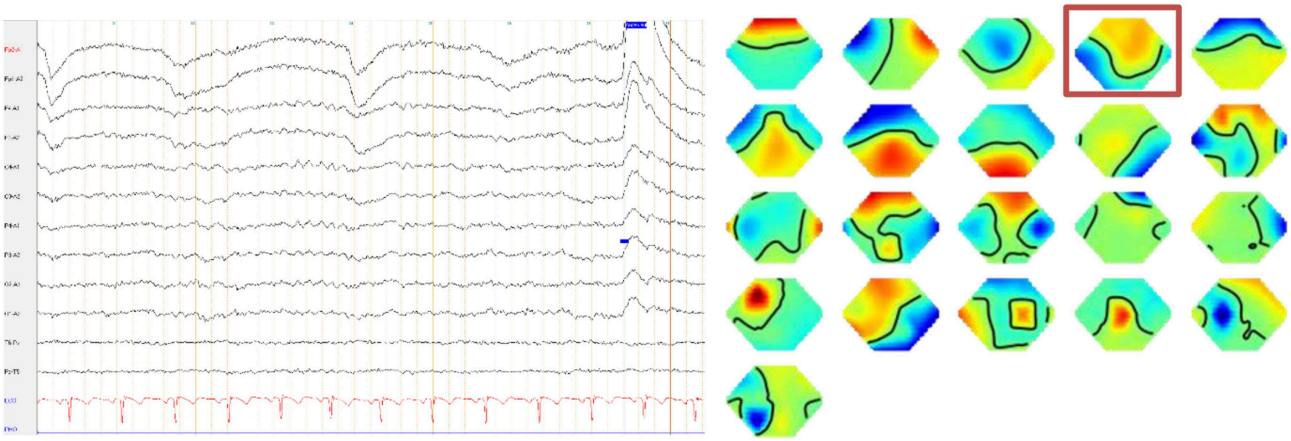

FIGURE 2 | The electroencephalography depicted a ß-rhythm, with an intermittently slow wave activity of 6–7/s. The independent component analyses showed a frontocentral topography (component 4, framed) compatible with tiredness.

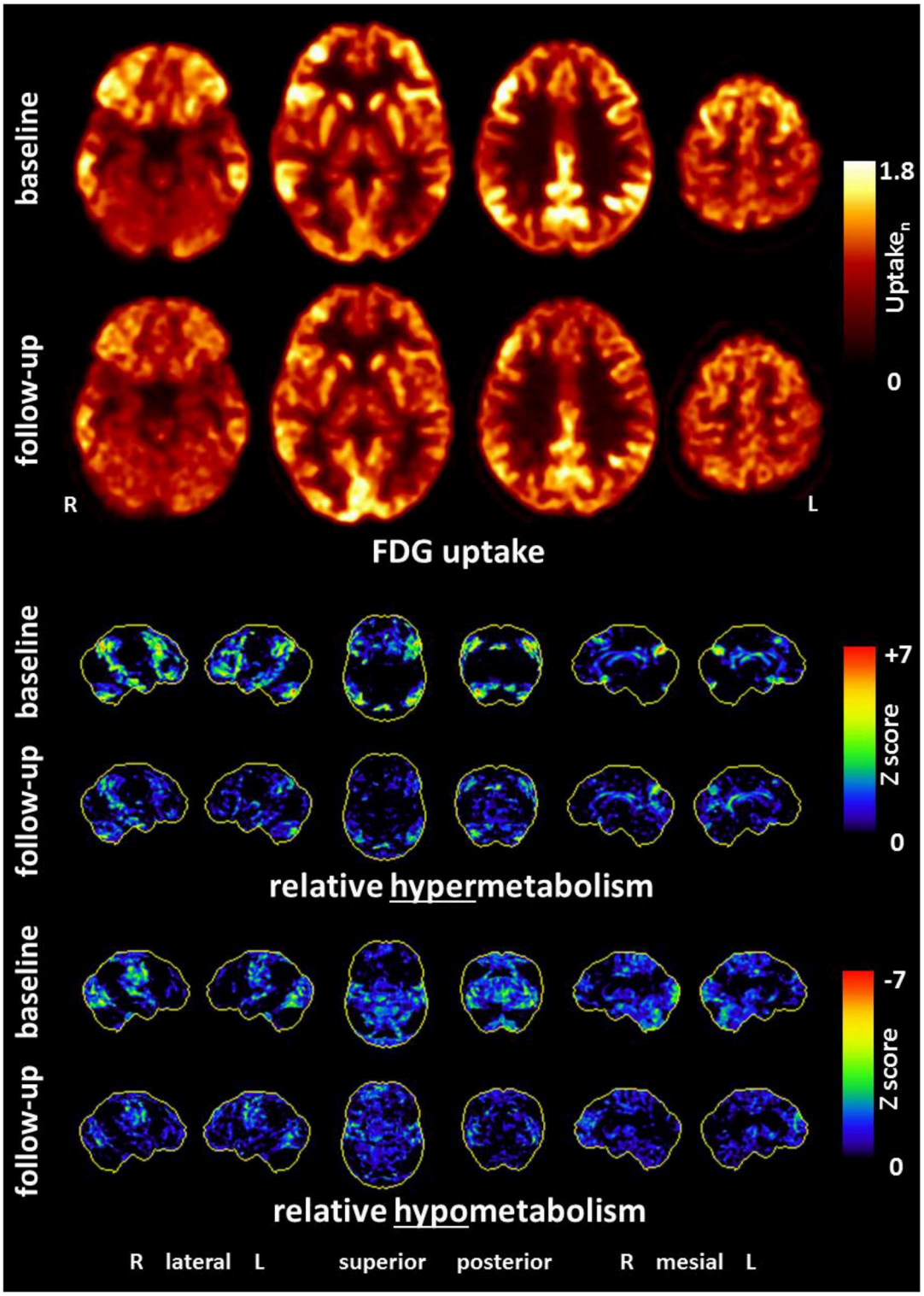

FIGURE 3 | An [18F]fluorodeoxyglucose positron emission tomography (FDG-PET) examination at baseline showed a pronounced relative hypermetabolism of parts of the association cortices and mild relative hypometabolism of the primary cortices, consistent with encephalitis. These metabolic findings markedly improved at follow-up. **(Upper panel)** Transaxial FDG-PET images at baseline and follow-up. **(Middle and lower panel)** Metabolic deviations from healthy controls, given as three-dimensional stereotactic surface projections (3D-SSP), color-coded as Z score (middle, relative metabolic increases; lower, relative metabolic decreases). All analyses were performed with Neurostat/3D-SSP [LIT] (16).

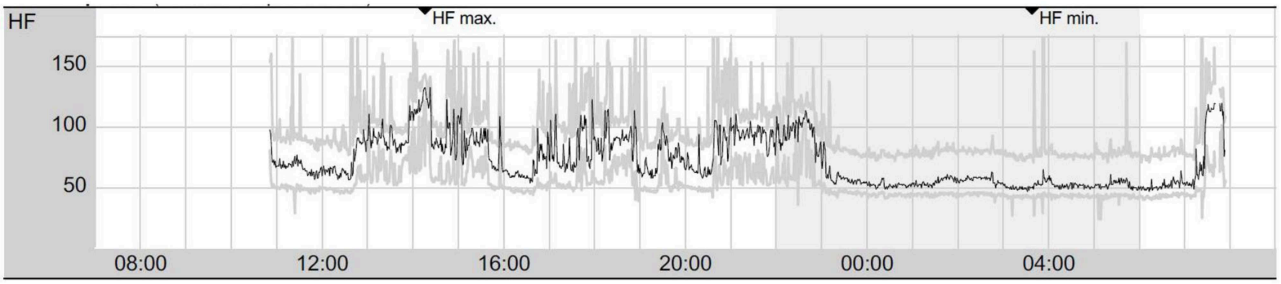

FIGURE 4 | Twenty-four hours of electrocardiogram showing signs of vegetative dysbalance with intermittent sinus tachycardia during daytime and sinus rhythm with stable frequencies during the night (presented are mean heart rates per minute). HF, heart rate; HFmax, maximum heart rate; HFmin, minimum heart rate.

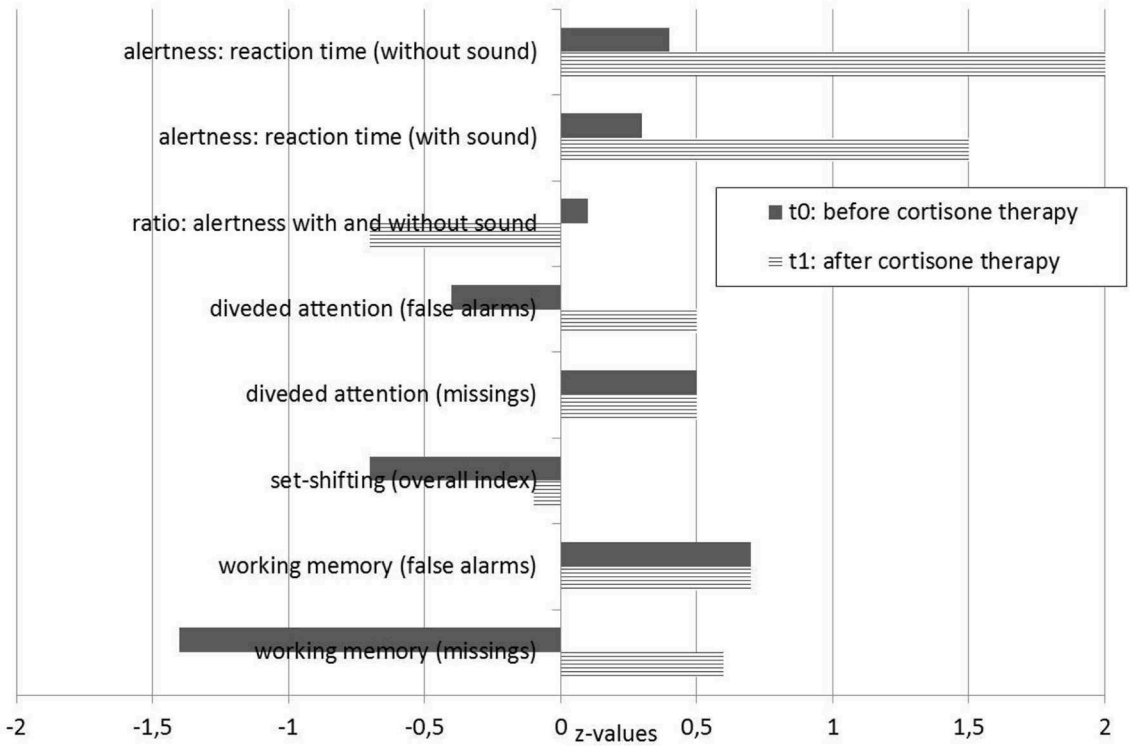

FIGURE 5 | Neuropsychological test results. t0 testing was performed before treatment, and t1 testing was performed 14 days after the steroid pulse treatment.

Illness, Somatic, and Family Histories

The patient's history was negative for *in-utero* or birth complications, febrile convulsions, craniocerebral traumata, or inflammatory brain diseases. During her first two decades of life, she had no evidence of a developmental disorder such as autism spectrum syndrome, attention deficit hyperactivity disorder, or a tic syndrome. The patient described herself as a rather anxious and insecure person; however, she clearly did not fulfill the criteria for any personality, affective, or anxiety disorder. She had no history of cancer or of autoimmune, neurological, or other somatic disorders. Apart from her paternal grandfather having suffered from Alzheimer's disease she had no family history of psychiatric disorders (including her siblings, parents, and grandparents). Her maternal grandmother had died of a pancreatic carcinoma, her maternal grandfather had died of an unspecified tumor at the age of 38.

Treatment and Outcome

Lorazepam led to a slight improvement in the patient's anxiety symptoms. After pausing the medication at the patient's request, anxiety symptoms became more severe. We then treated her with 7.5 mg diazepam. A steroid pulse (5 × 500 mg methylprednisolone, with oral tapering from 40 mg and halving every week over 4 weeks; further reductions took place in 2 mg steps) was performed for immunological treatment. Directly after the steroid pulse, we were able to discontinue diazepam without any worsening of the patient's anxiety symptoms. On the 5th day of the steroid pulse, the patient reported that she felt well

TABLE 1 | Diagnostic findings (∼8 weeks after onset of symptoms).

Physical examination	• Inconspicuous neurological and general medical examinations.
Basic blood/urine analyses	• Blood cell count, electrolytes, liver/kidney/pancreas values, vitamin B12, and selenium were normal. Vitamin D was normal, but not optimal (28.9 ng/ml; optimal: >30 ng/ml), folic acid was reduced (4.1 ng/ml; reference ≥4.8 ng/ml). • Thyroid-stimulating hormone, triiodothyronine, and thyroxine levels were in normal ranges. • The urine analyses showed normal findings, screening on illegal drugs was negative.
Immunological blood testing	• No antibodies against the intracellular onconeural antigens Yo, Hu, CV2/CRMP5, Ri, Ma1/2, SOX1, or the intracellular synaptic antigens GAD65/amphiphysin were found (using Ravo line assay). • Antibodies against different neuronal cell surface antigens (*AMPA-R, GABA-B-R, VGKC-complex [LGI1, Caspr2]*) were negative (using biochip-assays). IgG anti-NMDA-R antibodies were positive (titer 1:160 using cell-based assay [reference <1:20] and 1:32 [++, reference <1:10] using biochip-assay), anti IgA and IgM antibodies were negative (using biochip-assays). Negative tissue testing for known and unknown antineuronal antibodies. • Aquaporin 4 and MOG antibodies were negative. • Autoantibodies against thyroglobulin, TSH receptor, and thyroid peroxidase were not increased. • Screening for antinuclear antibodies (ANA) in IIF was normal. Anti-neutrophil cytoplasmic antibodies, antiphospholipid antibodies, rheumatoid factor, and anti-mitochondrial antibodies were negative. CH50 was slightly increased (119, reference: 65–115%), no other changes in the complement system (C3, C4, CH50, C3d) were observed. • Normal serum IgA, IgM, and IgG immunoglobulin concentrations; immunofixation showed no monoclonal antibody production. • B-cell panel showed no relevant pathologies beside of slightly reduced percentage of lymphocytes (24.1%, reference: 27–34%). All other cell counts were normal.
Infectiological blood testing	• Serologies for Lyme borreliosis, syphilis, and HIV were negative. • The vaccination titers showed the following values: Anti-tetanus toxoid IgG: >5 IU/ml (positive from >0.1 IU/ml), diphtheria -antitoxin IgG: >2 IU/ml (positive from >0.1 IU/ml), anti-pertussis IgG: 154.67 IU/ml (positive from >50 IU/ml), anti-polio 1: >1:512 (sufficient protection), and anti-polio 3: >1:512 (sufficient protection).
Cerebrospinal fluid analyses	• Normal white blood cell count (1/μL; reference <5/μL). • Normal protein concentration (207 mg/L; reference <450 mg/L), and normal age-corrected albumin quotient: 2.4; age-dependent reference $<6.5 \times 10^{-3}$). • No CSF specific oligoclonal bands; IgG index not increased (0.49; reference ≤ 0.7). • CSF lactate not increased (1.39 mmol/l; reference 1, 5–2, 1 mmol/L). • Antibodies against neuronal cell surface antigens (*NMDAR, AMPA-R, GABA-B-R, VGKC-complex [LGI1, Caspr2]*) were negative (Biochip assay). The live-cell assay and the tissue antibody test was also negative.
Cerebral magnetic resonance imaging	• Inconspicuous, except for a few slight, non-specific bifrontal medullary lesions.
Electroencephalography	• β-rhythm (appropriate due to lorazepam intake), with an intermittently slow wave activity of 6–7/s. The independent component analyses showed a frontocentral topography (component 4, framed) compatible with tiredness.
[^{18}F]fluorodeoxyglucose positron emission tomography	• Pronounced relative hypermetabolism of parts of the association cortices and a relative hypometabolism of the primary cortices. • No lesion suspicious of malignancy on whole-body PET/CT.
Ophthalmological examinations	• Ophthalmological examinations were inconspicuous. • Macular scans performed using SPECTRALIS® optical coherence tomography device (spectral-domain OCT) showed normal retinal volume and normal full retina thickness in all subfields with reference to normative data given by Nieves-Moreno et al. (17).
Heart examinations	• Inconspicuous resting electrocardiography (ECG). The long-term ECG measurement showed a sinus rhythm with intermittent sinus tachycardic pulse; no relevant pauses or severe cardiac arrhythmia.

again. Over the following 2 weeks, her fears and ambivalence were greatly reduced, her emotions stabilized, and neither inner restlessness nor stimulus overload occurred. A slight form of sleeping disorder persisted; however, her appetite improved. The burning sensations in the chest and head no longer occurred. Follow-up cognitive testing results were within the normal range (**Figure 5**), and a follow-up FDG-PET (20 days after starting the steroid pulse) showed considerable improvements of the metabolic findings (**Figure 3**). Her anti-NMDA IgG antibody titers decreased (1:40 ∼1 week after steroid pulse and 1:80 ∼2 weeks after steroid pulse treatment, using cell-based assays in both analyses). Approximately half a year after the steroid pulse treatment, the patient was completely free of symptoms.

DISCUSSION

Our case report describes a female patient with anti-NMDA receptor encephalitis with isolated psychiatric manifestation presenting with acute polymorphic psychotic symptoms that

developed directly after the patient had received a Tdap-IPV booster vaccination, most probably in the context of preexisting anti-NMDA receptor IgG antibodies.

Diagnostic and Pathophysiological Considerations

Basic diagnostic procedures using EEG, MRI, and CSF examinations only showed non-specific findings. While the patient initially displayed acute polymorphic psychotic symptoms, she did not present the typical neurological symptoms of encephalitis or encephalopathy, such as movement disorders or epileptic seizures, and her psychiatric symptoms were initially misdiagnosed as a primary mental disorder. However, her vegetative symptom (sinus tachycardia) was typical for anti-NMDA receptor encephalitis, possibly caused by catecholaminergic hyperstimulation, similar to the well-known ketamine effects (18). However, since the sinus tachycardia only occurred during the day, it was more likely due to the patient's anxiety than to a primarily antibody-induced genesis. There were no indications of cancer, particularly ovarian teratoma, neither from the whole-body FDG-PET/CT nor from the gynecological examination. Also, there was no observed viral association nor any preexisting immunological predisposition.

The combination of GluN1-IgG antibodies in the patient's serum and the FDG-PET finding of relative hypermetabolism in the patient's association cortex and a relative hypometabolism of the primary cortices led us to the probable diagnosis of anti-NMDA receptor encephalitis (19). Primary vaccination-induced encephalitis seemed less likely due to the clinical course and the FDG-PET findings, the latter of which were consistent with anti-NMDA receptor encephalitis (20–22), including an increased frontal to occipital metabolic ratio that markedly improved upon treatment (23). Indeed, the present patient showed strong frontal hypermetabolism and occipital hypometabolism at baseline, which resolved almost entirely after treatment.

However, due to the timing of symptom onset, there was a possible association with the Tdap-IPV booster vaccination. The presence of isolated anti-NMDA receptor IgG antibodies in the acute phase (IgG antibodies are typically not detectable in the first week after antigen contact) and the rapid onset of symptoms after vaccination clearly indicate the presence of preexisting antibodies or latent, non-clinically manifest encephalitis. Therefore, the vaccination cannot be the initial reason for anti-NMDA receptor antibody production. However, vaccinations physiologically lead to an immunological response with T-cell and B-cell activation. Preexisting specific T- and B-lymphocyte clones might have been re-stimulated, causing them to proliferate and leading to excessive antibody synthesis. Indeed, recent work suggested that antibody-secreting cells releasing functional anti-NMDA-R antibodies are part of the human naïve B cell repertoire and might thus be much more common than previously assumed (24). It is possible that the vaccine's effect of boosting antibody production led to a short-term increase in the patient's anti-NMDA receptor antibody levels, which could in turn have caused her clinical symptoms. One could also speculate that a temporary dysfunction of the blood-brain-barrier function might have led to increased antibody levels in the central nervous system (CNS). In summary, it can be hypothesized that too many anti-NMDA-R antibodies were present in the CNS for a short time and that this has led to a reversible internalization of the anti-NMDA receptors, thereby altering glutamatergic signaling and, consequently, changing neuronal glucose metabolism, with the described FDG-PET alterations and psychiatric symptoms [c.f. (6, 8, 25)]. The FDG-PET finding probably does not reflect classic neuroinflammation, so it may be better to speak of encephalopathy rather than encephalitis in such cases [cf. (8, 26)].

Normally, antibody detection in CSF characterizes anti-NMDA receptor encephalitis (7–9). The negative CSF result in the patient presented here is nonetheless valid because neither the biochip nor cell-based assays nor the tissue tests were conspicuous. The inconspicuous CSF finding could be due to the relatively low antibody levels compared to typical anti-NMDA receptor encephalitis with broader neurological symptoms (i.e., epileptic seizures). In the present constellation, it is plausible that all antibodies reaching the CNS compartment were bound to brain tissue. Laboratory experiments have shown that the brain can act as an immunoprecipitator for anti-NMDA receptor antibodies (27). Such cases with negative CSF findings could be associated with milder psychiatric manifestations. A similar situation has been found in neuromyelitis optica spectrum disorders: Aquaporin-4 (AQP4) and myelin oligodendrocyte glycoprotein (MOG) antibodies develop in the periphery, but if AQP4 or MOG antibodies reach the CNS, they can have pathological effects. They also typically cannot be detected in the CSF, and even oligoclonal bands are usually negative in those affected (28).

To date, only one comparable report of an association between anti-NMDA receptor antibodies and Tdap-IPV booster vaccination has been published, in which a 15-year-old boy developed anti-NMDA receptor encephalitis 5 weeks after receiving the vaccination. This previous patient presented with a severe neuropsychiatric syndrome with dyskinesia and choreatiform movements, and antibodies were also detected in his CSF; however, detailed information about antibody type was not reported (14).

Clinical Implications

A study of the Tdap vaccination without the additional inclusion of IPV found that there were neurological complications in 41 out of 2,090 vaccinated persons, including three who suffered from encephalitis/encephalopathy. The same study also reported demyelinating disorders ($N = 4$), seizures ($N = 7$), Bell's palsy ($N = 7$), and Guillain-Barré syndrome ($N = 10$) (29). Future research should investigate whether patients who report neurological side effects after receiving the Tdap-IPV booster vaccination are positive for anti-NMDA receptor antibodies. Antibody status could then be used to warn patients against vaccination side effects.

In addition, our case shows that an inconspicuous CSF anti-NMDA receptor antibody status does not rule out brain involvement. Despite non-specific MRI, EEG, and CSF findings, profound metabolic changes suggestive of encephalitis may be detected using FDG-PET. This case therefore demonstrates the importance of using multiple modes of examination to clarify and confirm or reject diagnosis in patients who are

seropositive for anti-NMDA receptor antibodies and who have acute polymorphic psychotic episodes. The broad spectrum of additional examinations (EEG, MRI, CSF, FDG-PET) were of great relevance for our patient, since a steroid pulse treatment resulted in rapid clinical improvement. We discussed with the patient the possibility of additional temporary maintenance therapy with azathioprine or rituximab, but the patient chose not to receive this treatment due to her clinical improvement. Further research should investigate how patients with similar diagnostic criteria and symptoms can best be treated over longer periods of time.

Limitations

Although the pathophysiological considerations discussed here are plausible, they cannot be definitively proven by this case report since the possible disease-causing effects of anti-NMDA receptor antibodies could only be shown indirectly via FDG-PET changes.

CONCLUSIONS

This case report shows a possible association between vaccination and the immediate development of anti-NMDA receptor encephalitis in a young female patient with likely preexisting anti-NMDA receptor antibodies. Since IgG antibodies were already initially detectable, the vaccination cannot have led to the initial antibody formation, but it may have exerted immunomodulatory effects. The further particularities of our case are the patient's mild psychiatric manifestation that mimicked an idiopathic psychiatric syndrome, her non-specific basic diagnostics (MRI, EEG, and CSF), and ultimate diagnostic clarification by FDG-PET. Detection of an immunological cause was essential, since it suggested immunosuppressive treatment, which led to rapid clinical improvement.

DATA AVAILABILITY STATEMENT

All necessary data can be found in the paper.

ETHICS STATEMENT

Written informed consent was obtained for the publication of any images or data included in this article.

CONSENT FOR PUBLICATION

The patient has given her signed written informed consent for this case report, including the presented images, to be published.

AUTHOR CONTRIBUTIONS

DE performed the data research, and wrote the paper. DE and LT treated the patient. SR performed the neurological interpretation. HP supported the neurological interpretation and performed the tissue antibody tests. BF and LT performed the EEG analyses. WK performed the infectiological interpretation. TH interpreted the cardiological findings. PM was responsible for the nuclear medicine investigations and interpretation. KE and SM performed and interpreted the MRIs. NV performed the rheumatological measurements and interpreted the results. KR, PS, KN, KD, and LT supported the clinical interpretation. All authors were critically involved in the theoretical discussion and composition of the manuscript. All authors read and approved the final version of the manuscript.

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ORIGINAL RESEARCH
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A Score Based on NfL and Glial Markers May Differentiate Between Relapsing–Remitting and Progressive MS Course

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Background: The diagnostic use of biomarkers in body fluids of multiple sclerosis (MS) patients allows the monitoring of different pathophysiological aspects of the disease. We previously reported elevated cerebrospinal fluid (CSF) and serum levels of glial fibrillary acidic protein (GFAP) but not neurofilament light chain (NfL) in progressive (PMS) compared to relapsing–remitting MS (RRMS) patients.

Objectives: We analyzed the glial marker chitinase-3-like protein 1 (CHI3L1) in the CSF and serum of PMS and RRMS patients. To capture the extent of glial processes in relation to axonal damage in each individual patient, we established a score based on CHI3L1, GFAP, and NfL and compared this score between RRMS and PMS patients and its association with the extended disability status scale (EDSS).

Methods: For this retrospective study, we included 86 MS patients (47 RRMS and 39 PMS) and 20 patients with other non-inflammatory neurological diseases (OND) as controls. NfL and GFAP levels were determined by the single-molecule array (Simoa). CHI3L1 levels were measured with classical enzyme-linked immunosorbent assay. A score was calculated based on glial to axonal markers (CHI3L1*GFAP/NfL, referred to as "Glia score").

Results: CHI3L1 showed higher CSF levels in PMS vs. RRMS and controls ($p < 0.001$ and $p < 0.0001$, respectively), RMS vs. controls ($p < 0.01$), and higher serum levels for PMS vs. RRMS ($p < 0.05$). The Glia score was higher in the CSF of PMS compared to RRMS patients ($p < 0.0001$) and in the serum of PMS patients compared to RRMS ($p < 0.01$). Furthermore, the Glia score and CHI3L1 in serum but not in CSF correlated with the disability as determined by EDSS in the PMS group but not in the RRMS group (Spearman $\rho = 0.46$ and 0.45, $p = 0.003$ and 0.004, respectively).

Discussion: Our data indicate the involvement of glial mechanisms during the pathogenesis of PMS. Moreover, a calculated score may help to differentiate between PMS and RMS in the CSF and monitor disease progression in the serum of PMS patients.

Keywords: multiple sclerosis, CSF, serum, glial markers, neurofilament light chain, progressive MS

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INTRODUCTION

The pathophysiology of multiple sclerosis (MS) is a complex interplay of B and T lymphocytes, demyelination, and axonal demise (1). Whereas, for relapsing–remitting MS (RRMS), the driving mechanism for disability in patients is supposed to be demyelination and the acute axonal damage (2). In progressive MS patients (PMS), the glial activation seems to be one of the major contributors to disability progression (3–5). Various glial processes are involved in MS, regardless of clinical subtype RRMS or PMS, like astrogliosis, microglial activation, scar formation, secretion of proinflammatory secretion of cytokines, and alteration of the metabolism of the neuroaxonal structures (6, 7).

The most extensively investigated marker for axonal damage is neurofilament light chain (NfL), which was shown to be elevated in the cerebrospinal fluid (CSF) of MS patients, correlating with MRI parameters of patients and being a potential prognostic biomarker (8–11). On the other hand, the concentration of glial fibrillary acidic protein (GFAP) is shown to be elevated in the CSF of PMS patients compared to RRMS patients (12) and correlate with the extent of contrast enhancement in RRMS patients (13). A further marker for glial activation, especially microglial activation, chitinase-3-like protein 1 (CHI3L1), was shown to be elevated in the CSF of MS patients compared to controls and a putative prognostic biomarker in patients with a clinically isolated syndrome (CIS) (14–18).

Highly sensitive detection methods as the single-molecule array allow the detection of brain-derived proteins in serum at low concentrations (19). Here, the determination of serum NfL is already well-established as a promising marker for prognosis and therapy efficacy (20–25) and as a possible additional endpoint for clinical trials (26). We could recently show that serum GFAP might be a more suitable marker for disease progression than serum NfL, as serum levels were higher in PMS patients compared to RRMS patients and correlated with extended disability status scale (EDSS) (27), also in a multicenter cohort (28). These findings were already independently confirmed by others (29).

Based on this first impression that glial processes might be an important driver of the disability in PMS patients, we analyzed CHI3L1 as an additional glial activation marker in the CSF and serum of PMS and RRMS patients. Together with the previously reported NfL and GFAP levels (27), we calculated a score based on those three markers for CSF and serum and compared its levels between RRMS and PMS patients and the correlation with EDSS.

MATERIALS AND METHODS

Patient Selection

CSF and serum samples from 86 MS patients were collected at the Department of Neurology of the University Hospital Ulm between 2012 and 2017. Patients were characterized according to the revised McDonald criteria 2017 (30). Patients' disability status was determined by the EDSS. Relapses were defined as a focal neurological disturbance within the last 3 months lasting more than 24 h, without an alternate explanation.

Controls were selected from patients visiting the Department of Neurology of the University Hospital Ulm for a neurological examination but not showing abnormal MRI or CSF analysis (elevated cell count, total protein or albumin quotient, and no intrathecal immunoglobulin production). Diagnoses at the time of lumbar puncture were as follows in descending order: migraine or tension headache, functional disorders (e.g., non-organic hypoesthesia), and dissociative disorder. There were no statistical differences between the control group and the PMS or RRMS patient group concerning age and sex.

CSF and Serum Sampling

CSF and serum samples were taken on the same day and processed according to the consensus protocol for CSF and serum collection and biobanking (31).

CHI3L1 Measurements

CHI3L1 levels were determined using the Human Chitinase 3-like 1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). The assay was performed according to the manufacturer's instruction, and CSF was diluted 1:100 and serum 1:50. All samples were measured in duplicates, and intra-assay coefficients of variation (CVs) were <5% and interassay CV was <10%.

Calculation of Glia Score

A biomarker score to represent glial processes compared to axonal damage for CSF and serum was calculated based on GFAP, CHI3L1, and NfL. GFAP and NfL values were published separately before (27) and used for this calculation. For the illustration of the extent of glial processes in relation to axonal damage, CHI3L1 and GFAP were placed in the numerator and NfL in the denominator of the equation. Thereby, the score is higher if glial processes are dominant and lower if axonal damage predominates.

The score was calculated for CSF and serum values as follows:

$$\frac{\text{GFAP}*\text{CHI3L1}}{\text{NfL}}$$

Statistical Methods

All statistical tests were performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). The Shapiro–Wilk test was used to examine the distribution of the data. Mann–Whitney *U*-test and Kruskal–Wallis with Dunn's multiple comparison test was used to compare medians in skewed distributed datasets. Spearman's rho test was used to measure correlation. Receiver operating characteristics (ROC) analyses were performed to assess the discriminability of parameters between two groups. A $p \leq 0.05$ was considered as statistically significant.

RESULTS

Patient Characteristics

Paired serum and cerebrospinal fluid (CSF) samples of 86 MS patients (39 PMS patients−13 secondary PMS and

26 primary PMS; 47 RRMS patients) were analyzed as well as 20 other non-inflammatory neurological diseases {OND; median age, 44 years [interquartile range (IQR), 27–52]}. Seven RRMS patients received disease-modifying therapy (DMT); two received interferon-1-β (INF-β), three received Natalizumab, one Teriflunomide, and another one was on Alemtuzumab.

Three secondary PMS patients were on DMT (Natalizumab, INF-β, and Fingolimod). All clinical characteristics of the patients are summarized in **Table 1**.

CHI3L1 and Glia Score in the CSF and Serum of PMS and RRMS Patients

CHI3L1 levels were higher in PMS patients compared to RRMS in the CSF (median, 181 vs. 124 ng/ml, $p < 0.0001$) and in serum (median, 36 vs. 26 ng/ml, $p = 0.0096$) (see **Figure 1**).

The calculated Glia score showed significantly higher levels in PMS patients compared to RRMS patients in the CSF (median, 1,369 vs. 519, $p < 0.0001$) and in serum (median, 239 vs. 163, $p = 0.0032$) (see **Figures 2**, **3**). We could not observe a correlation between age and CHI3L1 or the Glia score in CSF and serum of PMS patients (Spearman $\rho < 0.3$) and only for age and the Glia score in RRMS patients (Spearman $\rho = 0.53$ for CSF and 0.38 for serum, $p = 0.0002$ and 0.008, respectively).

ROC Analyses

To compare the utility of the analyzed parameters regarding discrimination between PMS and RRMS patients, ROC analyses for CSF and serum were performed (see **Figures 4**, **5**). Here, the Glia score showed the highest area under the curve (AUC) of 0.81, followed by CHI3L1 with 0.76, GFAP with 0.70, and NfL with 0.53 (**Figure 4**). Similarly, the Glia score in serum showed the highest AUC of 0.68 again, followed by CHI3L1 with 0.66, GFAP with 0.65, and NfL with 0.61.

Correlation With EDSS

Correlation of CHI3L1 and Glia score in CSF and serum with the EDSS was calculated by Spearman correlation analysis, and Spearman rho and *p*-values are given in **Table 2**. Here, CHI3L1 and Glia score in serum showed a moderate correlation with the EDSS ($\rho = 0.45$ and 0.46, respectively) in PMS but not in RRMS patients.

TABLE 1 | Patients' characteristics.

	n = 86 MS patients		
Diagnosis	**RRMS**	**PMS**	**Controls (OND)**
Number (*n*)	47 (44%)	39 (37%)	20 (100%)
Female (*n*)	29 (62%)	21 (54%)	13 (65%)
Age (years)	34 (27–47)	53 (47–59)	44 (27–51)
Recent relapse*	26 (55 %)	0	n/a
EDSS	2.0 (1.5–4.0)	6.0 (4.0–7.0)	n/a
DMT at LP	7	3	n/a
GFAP (pg/ml) (27)			
CSF	6836 (4,695–10,654)	11,131 (7,459–14,740)	6158 (2,425–8,064)
Serum	107 (74–141)	131 (98.6–224)	92.3 (57.2–140)
NfL (pg/ml) (27)			
CSF	1612 (871–3,205)	1,450 (1,045–2,340)	585 (358–835)
Serum	14.6 (9.2–26.8)	19.9 (13.9–28.4)	9.2 (6.0–12.3)
CHI3L1 (ng/ml)			
CSF	124 (88.3–162)	181.0 (151–254)	74.4 (47.5–96.1)
Serum	26.4 (21.4–35.4)	35.6 (23.8–96.3)	25.8 (21.7–43.2)

Numbers are given as median and IQR or percentages in brackets.
**A recent relapse was defined as focal neurological disturbance lasting more than 24 h, without an alternate explanation.*
RRMS, relapsing–remitting MS; PMS, progressive MS; PPMS, primary progressive MS; OND, other neurological diseases; EDSS, expanded disability status scale; DMT, disease-modifying treatment; LP, lumbar puncture.

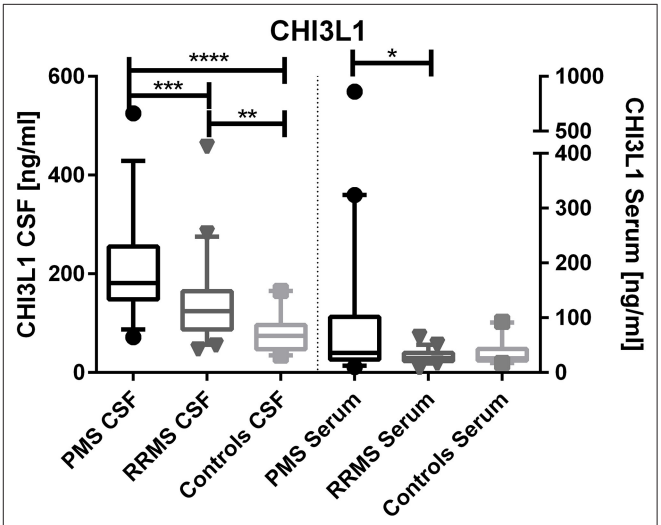

FIGURE 1 | Chitinase-3-like protein 1 (CHI3L1) levels in cerebrospinal fluid (CSF) and serum of progressive multiple sclerosis (PMS) and relapsing–remitting MS (RRMS) patients. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

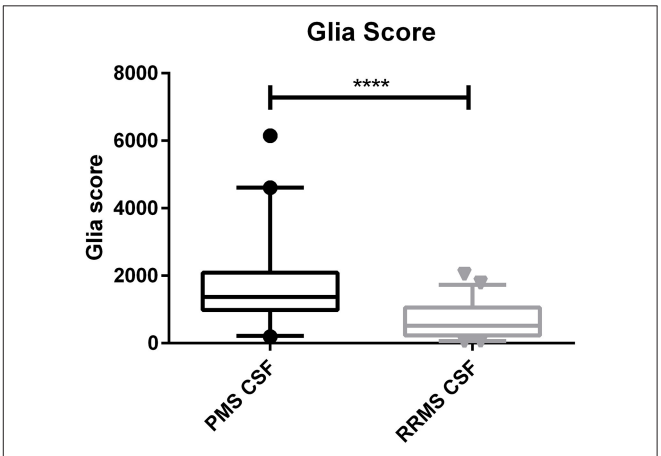

FIGURE 2 | Glia score in the cerebrospinal fluid (CSF) of progressive multiple sclerosis (PMS) and relapsing–remitting MS (RRMS) patients. ****$p < 0.0001$.

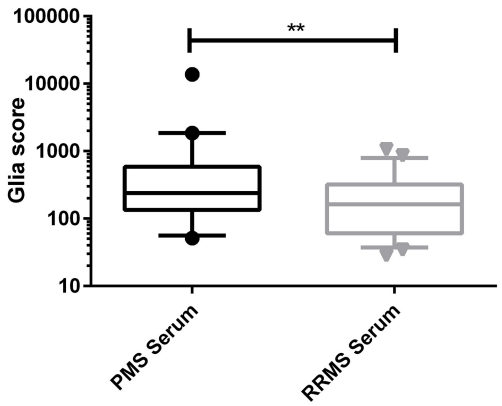

FIGURE 3 | Glia score in the serum of progressive multiple sclerosis (PMS) and relapsing–remitting MS (RRMS) patients. $^{**}p < 0.01$.

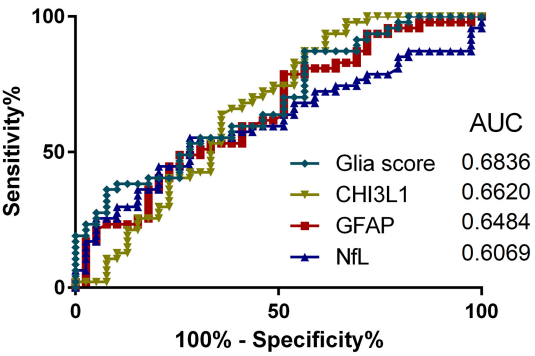

FIGURE 5 | Receiver operating characteristics (ROC) analyses of Glia score (diamonds), CHI3L1 (triangle down), glial fibrillary acidic protein (GFAP) (square), and neurofilament light chain (NfL) (triangle up) in the serum and for the comparison of progressive multiple sclerosis (PMS) vs. relapsing MS (RMS) patients. AUC, area under the curve.

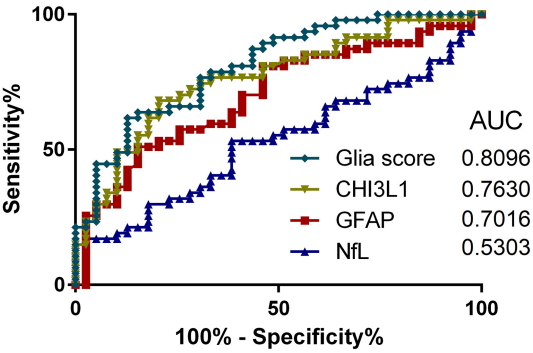

FIGURE 4 | Receiver operating characteristics (ROC) analyses of Glia score (diamonds), CHI3L1 (triangle down), glial fibrillary acidic protein (GFAP) (square), and neurofilament light chain (NfL) (triangle up) in the cerebrospinal fluid (CSF) and for the comparison of progressive multiple sclerosis (PMS) vs. relapsing MS (RMS) patients. AUC, area under the curve.

TABLE 2 | Spearman correlation of CHI3L1 and Glia score in CSF and serum of progressive MS (PMS) and relapsing–remitting MS Patients (RRMS).

Spearman ρ	EDSS	
	PMS	RRMS
CHI3L1 CSF	−0.05	0.14
CHI3L1 serum	0.45**	0.22
Glia score CSF	0.17	0.13
Glia score serum	0.46**	0.09

$^{**}p < 0.01$.

DISCUSSION

Various factors contribute to the disability progression in multiple sclerosis, e.g., infiltration with different immune cells, glial cell activation, iron accumulation, and mitochondrial dysfunction. Those different pathophysiological aspects can occur in the same patient leading ultimately to demyelination and neuroaxonal demise. Nevertheless, some might overweight the others depending on the disease stage. The acute inflammatory reaction and the resulting demyelination and active axonal damage through the immune cells in the acute active plaques are apparent in RRMS and decrease over time (2, 32). On the other hand, the glial activation is a prominent driver of the disability in PMS through various mechanisms, including axonal dysfunction, and is not necessarily accompanied by remarkable acute neuroaxonal damage, as seen in the chronic inactive MS lesions (4, 5, 33–35). Based on this assumption, GFAP and CHI3L1 as markers of astrocytic and microglial activation showed higher levels in the CSF and serum of PMS patients compared to RRMS patients (11, 12, 18, 27, 29, 36–38). Moreover, GFAP, but not NfL, in serum correlated with the disease severity in PMS patients (27, 28).

In this work, we report similar findings using a marker of microglia activation in CSF and serum. In some scenarios, it might be challenging to determine the disease course solely based on the clinical presentation, like in patients with the first manifestation at older age or patients in the transitional phase between RRMS and SPMS. In our study, the proposed Glia score in the CSF might be a helpful tool to differentiate between RRMS and PMS patients, as it showed the highest area under the curve of all parameters.

There was a correlation between CHI3L1 and Glia score in serum and the EDSS, however only in PMS but not in RRMS patients. The same was shown for GFAP previously (27–29). Why these markers especially in serum but not in CSF show a correlation with EDSS remains a subject to be investigated. However, we hypothesize that glial processes that happen at the

branches of astrocytes and astrocytic endfeet, which constitute a part of the blood–brain barrier and are in direct contact with blood vessels (39, 40), are well-reflected in the serum of those patients. Additionally, apoptosis and necrosis of astrocytes might release glial proteins as GFAP and CHI3L1 that are then drained via the glymphatic system into the blood (41). One example, therefore, is the very high levels of GFAP in the serum of neuromyelitis optica patients, where especially aquaporin-4 positive astrocytes are damaged (42, 43).

As this is an explorative study, these findings need to be confirmed in prospective, independent, and multicentric approaches with a higher number of patients including a comparison of active and inactive MS patients (44) and detailed MRI data. The suggested score is a first quite simplified approach to detect individual processes of neurodegeneration and glial activation in each patient. However, it needs further revision by a statistical weighting of single markers or the addition of further disease markers of MS.

Nevertheless, our data suggest that the glial markers GFAP and CHI3L1 might be a more suitable readout for disease progression and therapy response in PMS patients than NfL. Furthermore, we need to gain a deeper understanding of which of the various glial processes might be the driving mechanism of disease pathology in PMS, as neither GFAP nor CHI3L1 reflects specific glial processes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee University of Ulm, Ulm, Germany (approval number 20/10). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MO, MS, and HT: study concept. AH and AA: data acquisition, data analysis, and interpretation. AH, AA, and HT: drafting of the manuscript. MO, HT, and AL: study supervision and critical revision. All authors: critically reviewed and approved the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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