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Glial Fibrillary Acidic Protein in Blood as a Disease Biomarker of Neuromyelitis Optica Spectrum Disorders

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Glial fibrillary acidic protein (GFAP) is a type III intermediate filament protein found in astrocytes in the brain. Damaged astrocytes release GFAP into cerebrospinal fluid and blood. Thus, GFAP levels in these body fluids may reflect the disease state of neuromyelitis optica spectrum disorder (NMOSD), which includes astrocytopathy, characterized by pathogenic antibodies against aquaporin 4 located on astrocytes. Recently, single-molecule array technology that can detect these synaptic proteins in blood, even in the subfemtomolar range, has been developed. Emerging evidence suggests that GFAP protein is a strong biomarker candidate for NMOSD. This mini-review provides basic information about GFAP protein and innovative clinical data that show the potential clinical value of blood GFAP levels as a biomarker for NMOSD.

Keywords: glial fibrillary acidic protein, neuromyelitis optica spectrum disorder, blood, biomarker, anti-aquaporin-4 antibodies, GFAP, NMOSD

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INTRODUCTION

Neuromyelitis optica spectrum disorder (NMOSD) is a chronic inflammatory disease of the central nervous system (CNS) (1, 2). The main pathogenesis of NMOSD is autoimmune channelopathy/astrocytopathy that targets the water channel aquaporin-4 (AQP4) on perivascular astrocytic endfeet, and antibodies against AQP4 (AQP4-Ab) have been established as a diagnostic biomarker (3–5). Because NMOSD is a lifelong disease characterized by unpredictable attacks, subsequent severe neurological disability, and variable responses to treatments, blood biomarkers for monitoring and predicting the course of the disease would be useful (6–8). Serum AQP4-Ab titers may serve as such a disease biomarker; however, they have failed to show consistent results regarding their correlations with disease activity, severity, outcome, or responses to therapy (9–14). Currently, no blood biomarkers for monitoring are available in clinical practice.

Glial fibrillary acidic protein (GFAP) is the specific intermediate filament protein that constitutes the cytoskeleton of astrocytes (15). Damaged astrocytes release GFAP into interstitial fluid, cerebrospinal fluid (CSF), and finally the blood. Because NMOSD is an astrocytopathy, GFAP blood levels may be a useful biomarker for NMOSD. The recent development of ultrasensitive single-molecule array (Simoa) technology has expedited the realization of the potential of GFAP as a biomarker for NMOSD (16, 17).

In this review article, we will first briefly provide basic information about GFAP protein and its function in the brain. Then, we will review detection methods for GFAP protein in the blood and the recent evidence for the potential of GFAP as a blood biomarker for NMOSD. Finally, we will discuss several considerations in using GFAP as a disease biomarker and future directions.

GFAP

GFAP, a type III intermediate filament protein that was discovered by Dr. Eng in 1969 (18, 19), is responsible for the main cytoskeletal structure of astrocytes (19, 20). Apart from being present in the CNS, GFAP is also present in non-myelinated Schwann cells in the peripheral nervous system (PNS) and in enteric glia cells, which constitute the enteric nervous system (21, 22).

The human GFAP gene consists of nine exons and is located on chromosome 17 (17q21), spanning 10 kb (23). Alternative splicing occurs, and several GFAP isoforms have been identified (**Figure 1A**) (24). The three major domains of GFAP protein are the head, rod, and tail domains. The head domain is followed by the rod domain, which is composed of four α-helical coils. The N-terminal head domain is crucial for filament assembly, the rod domain plays a role in filament formation by coiling between polypeptides, and the C-terminal tail domain is important for stabilizing intermediate filaments (25). GFAP-α is the most abundant isoform in the brain and spinal cord but is also present in the PNS (26). This is the most commonly detected and analyzed isoform in the literature (20). GFAP-β is primarily expressed in non-myelinated Schwann cells in the PNS and has an alternate N-terminal (27). GFAP-γ also has an alternative N-terminal and is mainly located in the corpus callosum (28). GFAP-δ/ε is specifically expressed in neurogenic niches, such as the subventricular zone, and has an alternate C-terminal known to interact with presenilin (29–31). In addition, GFAP-δ/ε plays a role in modulating intermediate filament network dynamics (24, 32). GFAP-κ and GFAP-ζ also have distinct alternative C-terminals, which can modulate the properties of intermediate filaments (31, 33). Furthermore, an additional four isoforms of GFAP are collectively called GFAP+1, indicating isoform formation by a single nucleotide frameshift. GFAP+1 is found in a limited number of astrocytes in patients with Alzheimer's disease, Down syndrome, and chronic epilepsy; however, its implications remain to be elucidated (34–36). Although the precise functions of the different isoforms are not well-known, these isoforms seem to play a role in modulating intermediate filament networks during physiological and pathological states (37).

GFAP serves numerous pivotal functions in the CNS. GFAP is important for maintaining the mechanical strength of astrocytes and supporting neighboring neurons (38). In addition, GFAP participates in astrocytic motility and mitosis (39–41), maintains the integrity of the blood-brain barrier (BBB) and myelination (42, 43), protects neurons against neurotransmitter excess (44, 45) and injury (46, 47), regulates vesicle trafficking and autophagy (48, 49), and promotes synaptic plasticity (50, 51). Because GFAP is a major structural scaffold of astrocytes, damaged astrocytes release GFAP into their environment, e.g., interstitial fluid and CSF. Such released GFAP finally reaches the blood through an impaired BBB and/or glymphatic efflux (**Figure 1B**) (52–56). As such, blood GFAP exhibits much potential as a biomarker reflecting the state of NMOSD.

ULTRASENSITIVE DETECTION OF GFAP: SINGLE-MOLECULE ARRAY

GFAP concentrations can be detected with immunoassays such as enzyme-linked immunosorbent assay (ELISA) (57, 58). Conventional ELISA typically measures proteins at concentrations above 10^{-12} M (16). However, its sensitivity may be insufficient to reliably measure GFAP in the blood, of which concentrations in most patients with neurological disorders range from 10^{-14} to 10^{-10} M (0.5–5,000 pg/mL) (59–64). In patients with demyelinating diseases, the median CSF GFAP level is 8,601 pg/mL and the median serum GFAP level is 167 pg/mL from NMOSD and MS patient (59). The limit of quantification of commercial ELISA varies from 62.5 pg/mL (Eagle Biosciences, NH, USA) to 1,500 pg/mL (MilliporeSigma, MA, USA). Accordingly, although conventional ELISA measured CSF GFAP levels that showed promise as a potential biomarker for NMOSD (65–67), the blood GFAP levels demonstrated inconsistent results, indicating little clinical value for NMOSD (68, 69).

Recently, an ultrasensitive digital ELISA technology, Simoa, has been developed (16). The technique detects fluorescent signals from each single protein molecule by using femtoliter-volume chambers that isolate a single bead holding an immuno-complex with an enzymatic reporter generating fluorescence. High sensitivity to enzyme labeling and low background signals due to digitizing the detection of proteins has enabled the technology to detect blood proteins at subfemtomolar concentrations ($<10^{-15}$ M) (16). There are also other quantifying methods for GFAP such as electrochemiluminescence-based immunoassays and mass spectrometry (70). However, Simoa not only requires the smallest amount (only femtoliters) of blood for testing, but also shows the best analytical sensitivity with the limit of quantification of serum GFAP of 0.467 pg/mL (71). The reliability of Simoa for detecting blood neuronal and glial proteins is also high, as shown by the strong correlations between CSF and serum levels measured by Simoa technology (59, 72).

GFAP IN BLOOD AS A BIOMARKER FOR NMOSD

Recently, several studies have demonstrated that blood GFAP levels measured by Simoa have potential as a useful NMOSD biomarker for (1) differentiating NMOSD from other demyelinating diseases, (2) identifying and predicting clinical attacks, (3) monitoring disease disability and progression, and (4) evaluating treatment effects (59, 71, 73–78) (**Table 1**).

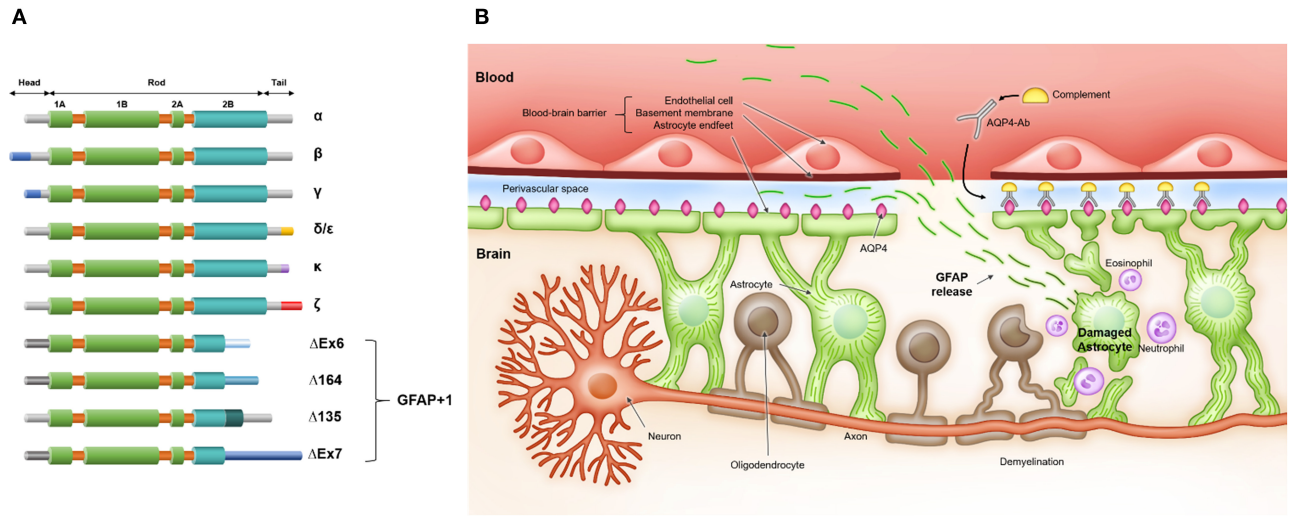

FIGURE 1 | (A) Glial fibrillary acid protein (GFAP) isoforms and **(B)** release of GFAP after astrocyte injury in neuromyelitis optica spectrum disorder. **(A)** GFAP protein consists of three domains: N-terminal head, central rod, and C-terminal tail. The head domain is crucial for filament assembly, the rod domain has a role in filament formation by coiling between polypeptides, and the tail domain is important in stabilizing the intermediate filament. **(B)** Serum anti-aquaporin-4 antibodies (AQP4-Ab) penetrate the blood-brain barrier and bind to aquaporin-4 (AQP4) on astrocyte endfeet. Antibody- and complement-dependent cellular cytotoxicity results in inflammatory cell recruitment, astrocyte damage, demyelination, and neuronal loss. After astrocyte damage, GFAP, an astrocytic scaffold protein, is released into interstitial and cerebrospinal fluid and finally reaches the blood through an impaired blood-brain barrier and/or glymphatic efflux.

TABLE 1 | GFAP in blood as a biomarker for NMOSD.

Author	Comparison of levels*			Attack vs. remission*	Correlation with		Prediction for future attack	Treatment effect
	vs. HC	vs. MS	vs. MOGAD		Age	EDSS	(Elevated vs. non-elevated)	
Watanabe et al. (59)	↑ (207.7 vs. 97.2)	↑ (207.7 vs. 121.1)	N/A	↑ (540.9 vs. 152.9)	NS	+	N/A	N/A
Kim et al. (73)	N/A	N/A	↑ (123.1 vs. 90.2)	↑ (253.8 vs. 104.4)	NS	+	N/A	N/A
Aktas et al. (75)	↑ (128.3 vs. 71.3)	↑ (128.3 vs. 97.5)	N/A	↑ (2,160 vs. 168.4)	+	N/A	Hazard ratio 3.09	Inebilizumab†
Schindler et al. (76)	NS (109.2 vs. 67.7)	N/A	NS (109.2 vs. 81.1)	N/A	+	+	Hazard ratio 11.6	N/A
Kim et al. (71)	↑ (154.1 vs. 98.9)	N/A	N/A	↑ (275.5 vs. 153.7)	NS	N/A	N/A	Rituximab¶
Chang et al. (77)	↑ (274.1 vs. 61.4)	↑ (274.1 vs. 66.5)	NS (274.1 vs. 136.7)	↑ (284.4 vs. 147.1)	NS	+	N/A	N/A
Zhang et al. (78)	↑ (149.7 vs. 68.7)	N/A	N/A	↑ (2,691 vs. 114.0)	NS	+	N/A	Tocilizumab, rituximab§

EDSS, expanded disability status scale; GFAP, glial fibrillary acidic protein; HC, healthy control; MOGAD, myelin oligodendrocyte glycoprotein antibody-associated disease; MS, multiple sclerosis; N/A, not available; NMOSD, neuromyelitis optica spectrum disorder; NS, not significant.

**The unit for GFAP levels is pg/mL. The figures in parentheses are median level of blood GFAP of each group.*

†Inebilizumab attenuated the attack-related increase in serum GFAP levels [inebilizumab, median fold change (FC): 1.1 vs. placebo, median FC: 20.2], and decreased serum GFAP levels in patients who did not experience attacks (inebilizumab, −12.9% vs. placebo, +2.9% at week 16).

¶Rituximab-treated patients manifested stable serum GFAP levels over time, but other immunosuppressant-treated patients, treated with corticosteroids and/or immunosuppressants (azathioprine, mycophenolate mofetil, or methotrexate), showed significantly increased serum GFAP levels over time (rituximab: baseline 145.6 pg/mL → follow-up 168.1 pg/mL, $p = 0.433$; immunosuppressant: baseline 128.6 pg/mL → follow-up 153.0 pg/mL, $p < 0.001$).

§Tocilizumab and rituximab decreased plasma GFAP levels by 36 and 23%, respectively, compared to the change between baseline and follow up of the prednisone-treated group.

Differentiating NMOSD From Other Demyelinating Diseases

It is important in clinical practice to differentiate NMOSD from other demyelinating diseases, including multiple sclerosis (MS) and myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD), because treatments for these diseases differ considerably. Inappropriate treatments may result in poor outcomes. For example, treating NMOSD patients with therapies for MS could worsen the disease (79–81). Although the testing of AQP4-Ab is essential for the diagnosis of NMOSD, differentiation of the diseases remains crucial. Contrary to the high specificity of the AQP4-Ab assay (96.6–99.8%), the sensitivity of the AQP4-Ab assay (48.7–76.7%) varies according to the assay methodology, indicating a high risk of false-negative results (82). In addition, a patient's treatment and clinical status can affect the result of an antibody assay (83, 84).

Serum GFAP levels could be used as a diagnostic marker for NMOSD, as they are significantly higher in NMOSD patients compared to those in healthy controls (59, 71, 75, 77, 78) and patients with other demyelinating diseases (MS or MOGAD) (59, 73, 75, 77). These findings are in line with immunopathological studies which showed that GFAP-positive astrocytes are highly destroyed only in active lesions of NMOSD but not in those of MS (85–90). Neurofilament light chain (NfL), a scaffolding protein of the neuronal cytoskeleton that is released upon axonal damage, may represent another diagnostic biomarker because it is also elevated in the blood of NMOSD patients, compared to healthy controls (59, 71, 75, 77, 78). However, serum NfL levels do not differ between NMOSD patients and MS or MOGAD patients (59, 73, 77), suggesting that NfL lacks specificity as a biomarker for NMOSD. A recent study proposed that the serum GFAP/NfL quotient at attack state could be a useful biomarker that differentiates NMOSD from MS with a sensitivity of 73.0% and a specificity of 75.8% (59). The serum GFAP/NfL quotient also distinguished AQP4-Ab-seropositive NMOSD from MOGAD and MS (77).

Identifying and Predicting Clinical Attacks

Identifying and predicting clinical attacks in NMOSD patients would be useful. Attack or relapse is defined as new or worsening neurological symptoms with an objective sign on neurological examination correlating with new or aggravating magnetic resonance image (MRI) lesions (91). However, pseudo-attacks or pseudo-relapses, i.e., clinical exacerbations with similar symptoms and signs but without true lesions, also occur in NMOSD patients, and clinically distinguishing between the two conditions can be difficult (91). Furthermore, although currently no method can predict future clinical attacks, a recent report revealed that clinically silent MRI lesions may represent a high risk of relapse (92). However, clinically silent brain or spinal cord lesions are rare in NMOSD patients, and thus performing regular MRI to predict future relapses would be inefficient.

Serum GFAP levels may help identify and predict clinical attacks in NMOSD patients, as they are higher in the attack state than in the remission state, and their elevation is associated with recent relapses (59, 71, 73–75, 77, 78). In a longitudinal NMOSD cohort (median follow up: 17 months), serum GFAP levels alone successfully discriminated clinical attacks from remission with a sensitivity of 94.7% and a specificity of 74.6% (area under the receiver characteristic curve = 0.876). Remarkably, this performance was better than that of other blood biomarkers, such as NfL and the GFAP/NfL quotient (71). In line with this, another study on a longitudinal NMOSD cohort (median follow up: 12 months) showed that plasma GFAP levels were the most powerful contributor in a random forest model to differentiate relapses from remissions, compared to other biomarkers (NfL, GFAP, and GFAP/NfL) and clinical variables [age, annual relapse rates, expanded disability status scale (EDSS) score, disease duration, and treatment status] (78). After relapse, serum GFAP levels decrease over time, and most patients show reduced serum GFAP levels below the predefined cut-off value ($\geq$3 standard deviations of mean levels in age-/sex-matched healthy controls) within 3 months (71, 74).

Notably, increased serum GFAP levels may indicate forthcoming clinical relapses. In a substudy of the N-MOmentum study, significantly increased serum GFAP levels were already observed 1 week before a clinical attack (93), and serum GFAP levels were linearly correlated with the risk of an upcoming attack (75). Additionally, patients with elevated serum GFAP levels at baseline ($\geq$2 standard deviations of the mean level of healthy controls) showed a 3-fold higher risk of having future NMOSD attacks than patients without elevated serum GFAP levels at baseline (75). Similar results were shown by another study on a prospective longitudinal cohort. NMOSD patients with high serum GFAP levels (>90 pg/mL, the cut-off value was derived from the 75th percentile of serum GFAP levels in healthy controls) at baseline had a shorter time to a future attack than those without [adjusted hazard ratio (95% confidence interval):11.6 (1.3–105.6)] (76). Conversely, in the same NMOSD cohort, baseline serum NfL levels were not significantly associated with a risk of future attack (76).

Monitoring Disease Disability and Progression

Monitoring disease disability is necessary to determine the severity and track the progression of the disease, and to assess treatment effectiveness (94). The most popular and widely used instrument is the EDSS. However, considering that the inter-rater variability of EDSS is as high as 30%, establishing an objective and easily measurable biomarker would be preferable (95). Many studies have demonstrated that blood GFAP concentration is independently associated with EDSS score in NMOSD patients (59, 73, 76–78, 96). Serum GFAP levels are also correlated with other clinical disability parameters, including the MS functional composite score, 9-Hole Peg Test, and paced auditory serial addition test (76). Blood NfL levels also tend to increase with EDSS score in NMOSD patients. However, the degree of association is not as strong as that of blood GFAP levels; positive correlations were significant in some studies (59, 77, 78, 96) but not in others (73, 76).

Serum GFAP levels may also be useful to monitor disease progression. NMOSD is considered to lack subclinical disease activity, and all disabilities are related to attacks (97, 98). Conversely, MS exhibits subclinical progression (99, 100). However, recent optical coherent tomography and visual evoked potential studies suggested subclinical neurodegeneration in NMOSD patients (101, 102). More recently, silent progression of brain atrophy was documented in NMOSD patients, even in clinically inactive patients (103). Additional studies on blood GFAP levels further support the concept of ongoing subclinical neurodegeneration in NMOSD. First, median blood GFAP levels during remission periods are significantly higher in NMOSD patients than those in healthy controls (59, 71, 75, 77, 78). Second, blood GFAP levels gradually increase over time even in patients with no clinical relapse, and the rate of increase of GFAP levels is faster than that related to normal aging (71). Third, monoclonal antibody treatments such as inebilizumab, tocilizumab, and rituximab decrease serum GFAP levels more than treatments with placebo or prednisolone (75, 78). This

indicates that gradual increases in GFAP levels may reflect ongoing pathological processes and may be alleviated by active treatment. However, it should be noted that most of these findings have been derived from small studies conducted at single centers or from substudies of clinical trials that may be different from real clinical situations. Future larger studies are warranted to confirm these findings.

Evaluating Treatment Effects

It would be useful to have blood markers as objective endpoints in determining therapeutic effects, as shown in a recent clinical trial (104), or as index markers for selecting optimal personalized treatments (105). Recent data suggest that blood GFAP levels may represent such markers. In a longitudinal follow-up study, rituximab (anti-CD20 monoclonal antibody)-treated NMOSD patients exhibited stable serum GFAP levels over time, in contrast to patients with other immunosuppressant treatments who showed significantly increased serum GFAP levels during the same period (71). Inebilizumab, an anti-CD19 monoclonal antibody, also prevented increases in serum GFAP levels. It attenuated the attack-related increase in serum GFAP levels (75) and significantly decreased serum GFAP levels in patients who did not experience an attack, as compared to placebo treatment (75). Tocilizumab, an anti-IL6 monoclonal antibody, also significantly reduced plasma GFAP levels in NMOSD patients, as compared to prednisolone (78). These findings are remarkable because they indicate that blood GFAP levels can reflect treatment responses during silent periods without clinical relapses.

SPECIAL CONSIDERATIONS

Age

Physiological aging gradually affects the brain (106), and serum GFAP levels increase with aging in healthy controls (59, 71, 75, 77). However, this positive association has not been consistently demonstrated in NMOSD patients (59, 71, 73, 75–78). One explanation for such inconsistent GFAP–age correlations could be that NMOSD patients tend to have high serum GFAP levels even at a young age. Furthermore, aging-related processes, such as increased astrogliosis, also appear to affect the clinical implication of GFAP in elderly patients. In a study that analyzed the effect of age on serum biomarkers in NMOSD patients, positive GFAP–EDSS correlations were distinctively stronger in the youngest ($\leq$45 years) compared to the oldest ($\geq$55 years) group (96). The association between GFAP levels and disease severity may have been compromised in elderly patients due to increased astrogliosis following neurodegeneration (96). Therefore, age should be considered when interpreting blood levels of neuronal and glial proteins in NMOSD patients.

Temporal Trajectories

The temporal dynamics of GFAP and date of blood sampling are also important. After brain injury, the serum GFAP levels peak at 20 h and decline over 72 h, indicating estimated half-life as 24–48 h (107, 108). It should also be noted that GFAP levels increases from 1 week before the advent of clinical symptoms (75). Even detected during the remission state, GFAP levels in NMOSD patients are still higher than healthy controls (59, 77, 78). In reflecting acute NMOSD attacks, GFAP may represent the event most appropriately within 7 days after attack, since 92% samples drawn within 1–7 days following attacks showed elevated level of blood GFAP ($\geq$2 standard deviations of mean level of healthy controls) (75).

Specificity

Blood GFAP levels increase not only in NMOSD but in various neurological diseases (109), thus the specificity of GFAP as an NMOSD biomarker should be discussed. Blood GFAP levels in patients with NMOSD, which often increase more than 1,000 pg/mL during relapses, tend to be higher than in patients with other diseases such as relapsing remitting MS (59, 60, 77), progressive MS (59–61), and even ischemic stroke (62). This is because patients with NMOSD are accompanied by direct damage of astrocytes. However, blood GFAP levels can also increase very high in glioblastoma, traumatic brain injury, and hemorrhagic stroke, as the level of NMOSD during relapses (63, 64, 110, 111). Therefore, it is difficult to regard that blood GFAP levels alone are a pathognomonic biomarker for NMOSD. Another parameter like GFAP/NfL ratio may enhance the specificity in terms of discriminating NMOSD from other diseases (59). However, it should also be emphasized that GFAP alone reflects well the longitudinal disease course of NMOSD and may be the most appropriate marker to monitor the disease changes within the NMOSD cohort (71).

NfL

As a representative biomarker of neuronal damage, serum NfL has also demonstrated disease association with NMOSD as well as MS (59, 71, 73, 76–78, 112, 113). However, serum NfL was not useful to distinguish NMOSD from other demyelinating diseases, and less sensitive and specific than serum GFAP in identifying and predicting NMOSD relapses (59, 71, 76–78). The value of NfL may be more pronounced elsewhere. Given that NfL is a neuronal structural component, serum NfL might be a better biomarker for monitoring the degree of neurodegeneration of NMOSD (101–103) and associated cognitive impairment (113) than serum GFAP. This possibility should be elucidated in future studies.

OUTLOOK

For GFAP to be used as a biomarker in clinical practice, several limitations that hinder the applicability of blood GFAP in clinical settings should be addressed. First, standard protocols and quality control criteria should be established across different laboratories (113). In addition, age-specific and sex-specific reference should also be developed. The dynamics of GFAP after releases upon NMOSD attacks should be explored to determine accurate blood GFAP half-life. This work should be paralleled with unraveling mechanisms and pathways of GFAP released from brain into the blood. Finally, the intervals for testing blood GFAP levels and guidelines for biomarker-based decision making should also be established.

Clinically, management strategies could be available by stratifying the risk of future attack based on both age-adjusted cut-off values and intraindividual changes in blood GFAP levels. Based on an individual's different strata of attack risk, clinicians could decide treatment initiation, continuation, and escalation/de-escalation of NMOSD patients. For example, it could be possible to set a serum GFAP range for the treatment response of patients and classify patients into treatment-responsive and treatment-resistant groups. This classification would enable precision treatment strategies that quickly change from one option to another suitable before it is too late (e.g., the advent of clinical relapses).

CONCLUSIONS

Although more than 50 years have passed since GFAP was first discovered, only recently has GFAP been suggested as a reliable blood biomarker in clinical practice. The role of GFAP as a biomarker for NMOSD shows promise because GFAP not only has pathophysiological specificity that can reflect astrocytopathy as much as AQP4-Ab, but it also has the advantage of being quantifiable with much more sensitivity than AQP4-Ab. After several clinical and technical issues are resolved, blood GFAP levels may expedite the process of personalized care of NMOSD patients.

AUTHOR CONTRIBUTIONS

HK and E-JL contributed to conception and design of the review. HK wrote the manuscript. E-JL acquired funding. Y-ML, K-KK, and E-JL supervised the study and revised the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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