ΚΛΑΣΣΙΚΕΣ ΚΑΙ ΝΕΟΤΕΡΕΣ ΑΝΟΣΟΛΟΓΙΚΕΣ ΔΙΑΓΝΩΣΤΙΚΕΣ ΠΡΟΣΕΓΓΙΣΕΙΣ ΓΙΑ ΤΗ ΜΥΑΣΘΕΝΕΙΑ ΚΑΙ ΤΙΣ ΔΙΑΤΑΡΑΧΕΣ ΤΟΥ ΦΑΣΜΑΤΟΣ ΤΗΣ ΟΠΤΙΚΗΣ ΝΕΥΡΟΜΥΕΛΙΤΙΔΑΣ

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Περίληψη

Η μυασθένεια Gravis (MG), ή μυασθένεια, και οι διαταραχές του φάσματος της Οπτικής Νευρομυελίτιδας (NMOSD) είναι αυτοάνοσα νευρολογικά νοσήματα που μεσολαβούνται από αυτοαντισώματα, των οποίων η διάγνωση βασίζεται στον συνδυασμό κλινικών, απεικονιστικών και εργαστηριακών ευρημάτων, με έμφαση στην ανίχνευση συγκεκριμένων αυτοαντισωμάτων. Οι περισσότεροι ασθενείς με μυασθένεια φέρουν αντισώματα κατά του υποδοχέα της ακετυλοχολίνης (AChR) και λιγότεροι κατά της ειδικής μυϊκής κινάσης (MuSK) και της σχετιζόμενης με τον υποδοχέα λιποπρωτεΐνης πρωτεΐνη-4 (LRP4). Αντίστοιχα, από τους ασθενείς με φαινότυπο NMOSD οι περισσότεροι ασθενείς φέρουν αντισώματα κατά της ακουαπορίνης-4 (AQP4) ενώ λιγότεροι φέρουν αντισώματα κατά της γλυκοπρωτεΐνης της μυελίνης των ολιγοδενδροκυττάρων (MOG). Ωστόσο, υπάρχουν ορισμένοι «οροαρνητικοί» ασθενείς, οι οποίοι δημιουργούν σημαντικές διαγνωστικές και θεραπευτικές προκλήσεις, αναδεικνύοντας τη σημασία της ταυτοποίησης νέων βιοδεικτών και την καθιέρωση προηγμένων διαγνωστικών δοκιμασιών. Η παρούσα ανασκόπηση εστιάζει στην παρουσίαση των κλασσικών μεθόδων ανίχνευσης των σχετικών αυτοαντισωμάτων κάθε ασθένειας (όπως ανοσοκαθίζηση και ELISA) καθώς και στον σημαντικό ρόλο και τις αρχές νεότερων τεχνικών (όπως ο κυτταρικός ανοσοφθορισμός) για την επίτευξη της βέλτιστης διάγνωσης των ασθενών. Επιπλέον, περιγράφονται τα τρέχοντα δεδομένα για τις αναδυόμενες ανοσολογικές διαγνωστικές προσεγγίσεις σχετικά με πιθανούς βιοδείκτες ιστικής βλάβης και ενεργοποίησης του συμπληρώματος, εμπλουτίζοντας τον συμβατικό ορολογικό έλεγχο με διευρυμένα πάνελ αυτοαντισωμάτων και βιοδείκτες. Αυτές οι εξελίξεις θα επιτρέψουν στους κλινικούς ιατρούς να επιτυγχάνουν αξιόπιστη διάγνωση σε προηγουμένως χαρακτηρισμένους «οροαρνητικούς» ασθενείς και καλύτερη παρακολούθηση των ασθενών για την επίτευξη εξατομικευμένων θεραπευτικών παρεμβάσεων.

Λέξεις-κλειδιά: Μυασθένεια, αυτοαντισώματα, NMOSD, ανοσολογικές διαγνωστικές δοκιμασίες, βιοδείκτες

ESTABLISHED AND EMERGING IMMUNOLOGICAL DIAGNOSTIC APPROACHES FOR MYASTHENIA GRAVIS AND NEUROMYELITIS OPTICA SPECTRUM DISORDER

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Abstract

Myasthenia Gravis (MG) and Neuromyelitis Optica spectrum disorder (NMOSD) are both antibody-mediated autoimmune neurological disorders whose diagnosis is based on clinical, imaging, and laboratory findings, and particularly on the detection of specific antibodies. Regarding their serological antibody status, most MG patients express antibodies against acetylcholine receptor (AChR) and fewer express antibodies against muscle specific kinase (MuSK) and the lipoprotein receptor-related protein-4 (LRP4). Similarly, most patients with NMOSD phenotype express antibodies against aquaporin-4 (AQP4), while fewer have antibodies against myelin oligodendrocyte glycoprotein (MOG). However, some "seronegative" patients pose diagnostic and therapeutic challenges, highlighting the importance of novel biomarkers and the parallel establishment

of advanced diagnostic assays. In this review, we focus on presenting the established methodologies used to detect the related autoantibodies in each disease (such as immunoprecipitation and ELISA), as well as the role and principles of some of the latest techniques (such as cell-based immunofluorescence assays) in achieving an optimal patient diagnosis. Furthermore, we describe the current data on emerging immunological diagnostic approaches regarding potential biomarkers of tissue damage and complement activation, enriching the conventional serological testing with expanded autoantibody panels and markers. These advancements will enable clinicians to achieve a reliable diagnosis in previously "seronegative" patients and to effectively monitor patients to implement truly personalised therapeutic strategies.

Keywords: Myasthenia Gravis, autoantibodies, NMOSD, immunological diagnostic assays, biomarkers

INTRODUCTION

Myasthenia Gravis (MG) disease pathophysiology

MG is an autoimmune disorder caused by antibodies (Abs) targeting neuromuscular junction (NMJ) proteins, impairing synaptic transmission and muscle contraction.[1-3] With a prevalence of 150-200 cases per million and an annual incidence of 4-30 per million, MG shows bimodal age distribution: women are more frequently affected in their 20s-40s, while male incidence rises after the age of 50.[4] Diagnosis relies on point-of-care tests,[5] electrophysiological (e.g., repetitive nerve stimulation, single-fibre electromyography),[6,7] and serological testing. Accurate diagnosis has enabled effective immunotherapies to improve quality of life and reduce disease mortality.[3,8-10]

The NMJ ensures precise conversion of action potentials into muscle contraction. More specifically, presynaptically released acetylcholine binds to clustered Acetylcholine receptors (AChRs), a ligand-gated ion channel, triggering depolarisation of postsynaptic membrane. This highly specialised synapse depends on molecular regulation: nerve-derived agrin binds to low-density lipoprotein receptor-related protein 4 (LRP4), activating muscle specific kinase (MuSK) autophosphorylation and downstream signalling, that drives rapsyn-mediated AChR clustering. Higher AchR cluster density directly enhances synaptic transmission efficiency.[11,12]

Approximately 85% of patients harbour anti-AChR antibodies, of whom about 10% have thymoma.[13] Another 5-8% exhibit anti-MuSK antibodies, whereas anti-LRP4 antibodies are rarer (~2%).[14-17] Recent evidence suggests that anti-agrin antibodies may also be pathogenic, often co-occurring with anti-LRP4, anti-AChR, or anti-MuSK Abs.[18-21] Some patients lack detectable autoantibodies and are designated as seronegative (SNMG). In several cases, antibodies to intracellular antigens are also found, such as titin,[22] ryanodine receptor (RyR),[23] voltage-gated K+ channel (Kv1.4),[24] rapsyn,[25] cortactin,[26] and collagen Q (ColQ) and XIII,[27,28] which may serve as biomarkers, although their pathogenic role remains uncertain.[16]

The pathophysiological role of MG Abs in disease mechanism and diagnosis

In AChR MG, autoantibodies of the IgG1 and IgG3 subtype bind mainly to the extracellular domain of the AChR α-subunit, where they either promote receptor endocytosis or activate the complement system, ultimately leading to receptor degradation or block the receptor activation site.[29] AChR MG patients usually present with ocular muscle weakness, in most cases followed by bulbar or/and limb muscle weakness. Respiratory muscle involvement is rare but, when present, can be clinically severe and associated with high mortality.[3]

Differing from AChR antibodies, MuSK antibodies are predominantly of the IgG4 subclass, a subclass with unique characteristics, including the inability for complement or immune effector cell response or promotion of receptor endocytosis and the capacity to mediate Fab-arm exchange (FAE).[30] These antibodies typically target MuSK's extracellular domain, disrupting the agrin/LRP4/MuSK interaction, ultimately leading to the inhibition of AChR cluster formation and impairment of the synaptic transmission.[31] Clinically, MuSK antibody titres correlate with disease severity in both individual patients and population-wide analyses,[32] and patients typically manifest a more severe clinical phenotype than those with AChR-MG, presenting prominent bulbar weakness and, sometimes, respiratory failure.[1]

LRP4 antibodies are mainly of the IgG1 and IgG2 subclasses, which disrupt the interaction between agrin and LRP4.[17,33-35] Unlike anti-AChR, anti-LRP4 antibodies appear less dependent on complement activation. Recent studies demonstrate that their primary pathogenic effects are mediated through antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) mechanisms.[36] Clinically, the symptoms tend to be milder compared to other MG subgroups.[1]

Strational antibodies target skeletal and cardiac muscle proteins, with anti-titin and anti-RyR antibodies serving as biomarkers for MG-associated thymoma. Titin maintains muscle elasticity and in-

tegrity,[37] while RyR regulates calcium release for excitation-contraction coupling.[38] These antibodies are associated with AChR-seropositive early-onset MG (EOMG) with thymoma, but also with late-onset MG (LOMG) independently of the presence of thymoma. They serve as biomarkers for MG thymoma and for disease severity.[16,22,23,39]. Interestingly, RyR antibodies are predominantly detected in thymoma-associated MG patients and correlate with more severe disease manifestations, including potential cardiac complications due to RyR expression in cardiac muscle, highlighting their clinical utility as markers for identifying high-risk patients who may benefit from intensified monitoring or therapy.[39,40] Although anti-titin antibodies are known to be present mainly in AChR-seropositive patients (routinely detected by a commercial ELISA), in a multicentre European study, anti-titin antibodies, detected by a sensitive radioimmunoassay, were also detected in 13% of triple-SNMG patients (AChR/MuSK/LRP4-negative; tSNMG), while demonstrating 100% specificity as no antibodies were detected in healthy or neurological disease controls,[41] offering an additional diagnostic tool for MG. However, their intracellular localisation likely precludes direct pathogenicity.[16]

MG patients may develop antibodies against additional NMJ components, with distinct clinical profiles, although more studies are needed in most cases to verify/confirm the extent of their frequency and role. Anti-agrin antibodies (0.6-15% prevalence) are enriched in SNMG, but may co-exist with AChR, MuSK, or LRP4 antibodies,[19,21,42-44] showing diagnostic value due to their MG-specificity and variable clinical severity correlation.[16,43] Anti-Kv1.4 antibodies found in 17-18% of MG patients were associated with severe symptoms (bulbar involvement, myocarditis, QT prolongation) in the Japanese population and with milder ocular forms in Caucasians.[24,45,46] Anti-rapsyn antibodies (~15%) may be associated with thymoma [25] but lack disease severity correlation or MG-specificity.[47,48] In contrast, anti-cortactin antibodies were detected in 20-24% of double-SNMG (dSNMG; lacking both AChR/MuSK antibodies) and 9.5% of AChR-MG, and were linked to milder phenotypes without bulbar involvement, albeit with limited specificity.[26,49,50] Anti-AChE antibodies may occur in some ocular MG patients but show cross-reactivity with other disorders and no correlation with demographics or thymoma.[51-53] Rare antibodies against ColQ (3%) may co-occur with AChR/MuSK antibodies, while anti-collagen XIII antibodies showed higher prevalence in AChR-negative versus AChR-positive MG (16% vs 7%); both with unclear clinical significance.[27,28,54-56]

Neuromyelitis Optica Spectrum Disorder (NMOSD) pathophysiology

NMOSD is an inflammatory demyelinating central nervous system (CNS) spectrum of disorders with relapsing course that was once considered a variant of multiple sclerosis (MS). NMOSD is characterised by severe demyelination and axonal damage that is targeted mainly to the optic nerves and the spinal cord but can also affect the area postrema, the brainstem, diencephalon, or cerebrum as described in the 2015 NMOSD criteria.[57] A recent systematic review concluded that the pooled prevalence of NMOSD is 2.16 per 100.000 people,[58] although it may vary depending on the genetic background and a few environmental factors across the populations analysed. It is known that it predominantly affects females,[59,60] its prevalence peaks in middle-aged adults,[61-63] and is more common in Asian and African-American populations.[64,65]

The diagnosis of NMOSD requires a comprehensive approach, integrating clinical manifestations with neuroimaging techniques and specific laboratory findings.[57,66] NMOSD encompasses a range of diseases that include AQP4-IgG positive NMOSD and some closely related diseases without aquaporin-4 immunoglobulin G (AQP4-IgG) Abs. AQP4-IgG serves as a critical biomarker for differentiating NMOSD from other inflammatory CNS disorders[67] such as MS, which is pivotal to the patients' course, as most treatments destined for MS can be detrimental in NMOSD.[68] Moreover, recent treatments for NMOSD have shown effectiveness in patients seropositive for AQP4 antibodies.[69,70]

MOG antibodies may be detected in patients with NMOSD phenotype, seronegative for AQP4-IgG. Patients with MOG-Abs may either belong in the NMOSD group or, more frequently, to the MOG antibody-associated disease (MOGAD). MOGAD is typically associated with acute disseminated encephalomyelitis, optic neuritis, or transverse myelitis, and is less commonly associated with cerebral cortical encephalitis, brainstem presentations, or cerebellar presentations.[71]

The pathophysiological role of Abs in NMOSD disease mechanism and diagnosis

NMOSD's pathophysiology is mediated by the humoral immune system. AQP4 autoantibodies are disease-specific, and more than 80% of patients with NMOSD express antibodies against AQP4,[72] a water channel protein that transports water across cell membranes. It is the most widely expressed water channel in the brain, spinal cord, and optic nerves; mainly expressed at the terminals of astrocytes and the glia limitans.[73] AQP4 is a tetrameric channel com-

posed of monomers of 2 different mRNA isoforms, M1 and M23.[74] Its main role is the transport of water from the blood into the brain and between the brain parenchyma and the cerebrospinal fluid (CSF),[73,75] thus ensuring CNS homeostasis,[76-78] while also contributing to the elimination of toxic byproducts of cellular metabolism, cell migration, the management of cerebral oedema, and cellular homeostasis.[77]

AQP4-Abs are predominantly of the IgG1 isotype. The antibodies bind to AQP4 channels on astrocytes, leading to complement-dependent cytotoxicity and chemotaxis of T and B lymphocytes, macrophages, neutrophils, and eosinophils.[79-81] Ultimately, the induced inflammation causes significant injury of the astrocytes, with loss of aquaporin-4 channels and glutamate transporters that disrupt cellular water and glutamate homeostasis,[82] leading to oligodendrocyte damage and severe demyelination.[81,83]

Antibodies against AQP4 play an important role in the pathogenesis of the disease and are a specific biomarker for NMOSD.[84] As a result, new effective therapies targeting specific molecular pathways of AQP4-NMOSD, such as complement (C5) and interleukin-6, have been approved.[85] Although anti-CD20 therapies can be used in these diseases, there are now more specific therapeutic approaches depending on the underlying pathophysiological mechanisms of each disease,[85] i.e., inebilizumab for AQP4-positive NMOSD patients targeting anti-CD19 positive B-cells.[69,70]

MAIN IMMUNOLOGICAL ASSAYS FOR THE DIAGNOSIS AND DISEASE MONITORING OF MG AND NMOSD

Overview of laboratory methods for antibody detection

As previously mentioned, antibody detection is crucial for MG and NMOSD diagnosis, as identifying the disease subtype helps determine the most appropriate therapeutic approach.[86] Ensuring accurate antibody detection requires method selection tailored to each antibody's traits. The following section outlines the general principles of the main methods.

Radioimmunoprecipitation assay (RIPA) remains particularly valuable for quantifying antibodies, including very low-titre antibodies.[29,87] This method uses radiolabeled ligands to tag target antigens, or directly radiolabeled antigens, which are then immunoprecipitated by patients' sera and quantified by a γ-counter.[88] While highly specific, RIPA requires radioactive facilities, poses safety risks, is costly, and may alter epitopes during antigen solubilisation.[89,90] It is mostly used for the detection and quantitation of anti-AChR and anti-MuSK antibodies in MG.

Cell-based assays (CBA) have revolutionised neuroimmunological diagnostics because they allow the utility of properly folded, membrane-anchored antigens in transfected cells, i.e., allowing binding of only the potential pathogenic antibodies.[91] Live CBAs preserve the native antigenic structure, enabling the highly sensitive detection of conformation-specific and even low-affinity antibodies via fluorescent labelling,[92-95] albeit requiring cell culture expertise and immediate analysis.[96] In contrast, fixed-cell CBAs utilise chemical permeabilisation to stabilise the antigens, mostly for easier commercialisation, although it often reduces both sensitivity and specificity for most targets.[97-100] While live CBAs remain optimal for detecting antibodies against structurally sensitive membrane proteins, fixed CBAs are more easily available to routine diagnosis laboratories.[97,98]

Cell-based-flow cytometry enables high-sensitivity antibody detection by quantifying the binding to antigen-expressing cells, combining high throughput, rapid analysis, and small sample volumes, while preserving native antigen conformation.[101] It supports multiplex autoantibody detection and is particularly effective for low-affinity antibodies, though it requires specialised instrumentation, expertise, and is costly.[101,102] While not a universal replacement for conventional methods, its flexibility is valuable for complex antibody profiling.

Enzyme-linked immunosorbent assay (ELISA) is a widely used standardised method for high-throughput antibody detection.[103] While reliable for linear epitopes,[104] its sensitivity and specificity decrease for conformational targets.[105] Although convenient and suitable for routine use, ELISA has limited sensitivity and specificity compared to RIPA or CBA.[106]

The most important strengths and weaknesses of the methods analysed here are presented in summary in **Table 1**.

MG autoantibodies

AChR Abs

Detection of AChR antibodies, the most prevalent in MG, is the primary diagnostic approach, with RIPA (using AChR-125I-α-bungarotoxin) serving as the gold standard for nearly 50 years (~99% specificity, ~82% sensitivity).[88,90,107-109] By using larger serum volumes and reducing background radioactivity, the detection threshold can be improved fivefold (from 0.5nM to 0.1nM), significantly enhancing the sensitivity.[110] However, RIPA's cost and safety concerns have driven the adoption of non-isotopic alternatives.[16,90] While ELISA offers simplicity, its lower specific-

ity (~96%) and sensitivity (~70%) than RIPA limit its diagnostic utility.[16,104,109,111,112] Similarly, FIPA may have comparable sensitivity and specificity to RIPA but requires specialised equipment and expertise[113] (**Table 1**). Luciferase IP system (LIPS) with luciferase-labelled recombinant AChR α1-subunits shows limited sensitivity due to its restricted epitope targeting, reducing diagnostic reliability.[114] CBAs are capable of detecting antibodies in some AChR-RIPA negative sera, likely because they can detect low-affinity antibodies and also antibodies to epitopes that have been damaged during AChR detergent-solubilisation necessary for the RIPA.[86,91,109,115] Overall, CBAs are thought to detect AChR antibodies in about 20% of RIPA-negative MG cases.[91,92,110,116-120] However, fixed CBA is not very efficient in detecting AChR antibodies in sera with low RIPA titres (46% sensitivity for 1.0-3.0 nM RIPA-titre sera.[16,100] For CBA sensitivity enhancement, the use of both adult and foetal AChR isoforms is recommended.[121]

MuSK Abs

MuSK antibody detection is typically considered when patients with MG symptoms test negative for AChR antibodies. Although ELISA was the first assay for MuSK antibody detection[14] and commercial kits are available, its lower sensitivity makes it less frequently used.[30,122] RIPA is considered the gold standard for the detection of MuSK Abs. Lately, CBAs for MuSK antibodies have also emerged, successfully identifying MuSK antibodies in previously SNMG patients from different nationalities[91,113,123,124] (**Table 1**). Despite their high sensitivity and specificity, IgG-Fcγ-specific secondary antibodies should be used instead of anti-IgG (H+L)[125], and live rather than fixed CBAs should be used, whenever available, to further improve the sensitivity of anti-MuSK antibodies.[98]

LRP4 Abs

While LRP4 antibody assays remain less standardised, several studies have investigated assays for reliable antibody detection. ELISA is a more accessible method that can detect LRP4 antibodies in 9 to 15% of dSNMG patients; however, no validated commercial kit is available.[19,33,107] LIPS was employed in a study and LRP4 antibodies were detected in 3% of dSNMG and 1% of MuSK-MG patients.[17] CBA is the most used assay for LRP4 autoantibody detection, though studies report varying prevalence rates (2-50%) in dSNMG.[126-129] This variation likely results from differences in methods, antigen sources (e.g., rat[33] and solubilised mouse LRP4[17]), as well as patients' demographics, ethnic diversity, and variability in diagnostic criteria. Such inconsistencies highlight the need for standardisation in LRP4 antibody testing. Serum LRP4 antibodies have also been detected in a significant fraction of patients with amyotrophic lateral sclerosis.[130]

Titin Abs

Due to the rarity of anti-titin antibodies, research and the development of specific assays for their detection are limited. Nevertheless, ELISA and immunoblotting have been used for anti-titin,[131,132] while commercial ELISA kits are also available. An advanced method that combines CBA with flow cytometry can enhance the sensitivity for anti-titin antibody detection[24] (**Table 1**). Interestingly, since 2016, a highly sensitive RIPA for detecting anti-titin antibodies has been established.[41] The authors developed the assay and screened 300 serum samples from tSN-MG patients across various European clinics, 114 samples from healthy individuals and 30 samples from ''disease-controls'' and showed that approximately 13% of the seronegative MG patients had detectable anti-titin antibodies, while none of the healthy or neuropathy control samples were positive.[41] These data suggest that the RIPA assay could be considered as a valuable serological tool for diagnosing tSN-MG, as anti-titin Abs may serve as a useful prognostic marker of disease outcome[128] or monitoring and indication of the severity of MG associated with thymoma.[41,107,133]

MG Monitoring

Antibody titre monitoring in MG offers valuable insights into disease progression and treatment response. For AChR antibodies, titre fluctuations may reflect clinical improvement, though their correlation with disease severity remains inconsistent.[134,135] Notably, AChR antibody titer increases up to two years before symptom onset[136] and could predict the risk for neonatal MG,[137] underscoring the importance of serial monitoring.[16] In contrast, MuSK antibody titres, especially of the IgG4 subclass, strongly correlate with disease severity, guiding therapeutic strategies.[32,122,138] RIPA remains the gold standard for titre quantification.[16] Regarding the other MG-related autoantibodies, titre monitoring has not been widely adopted.

NMOSD autoantibodies

AQP4 Abs

Various methodologies have been developed to identify AQP4-IgG, each with its strengths and limitations, influencing the sensitivity and specificity of NMOSD diagnosis. The gold standard for the detection of anti-AQP4 Abs is the live CBA. Live CBAs are consistently proven to be superior to fixed CBAs for

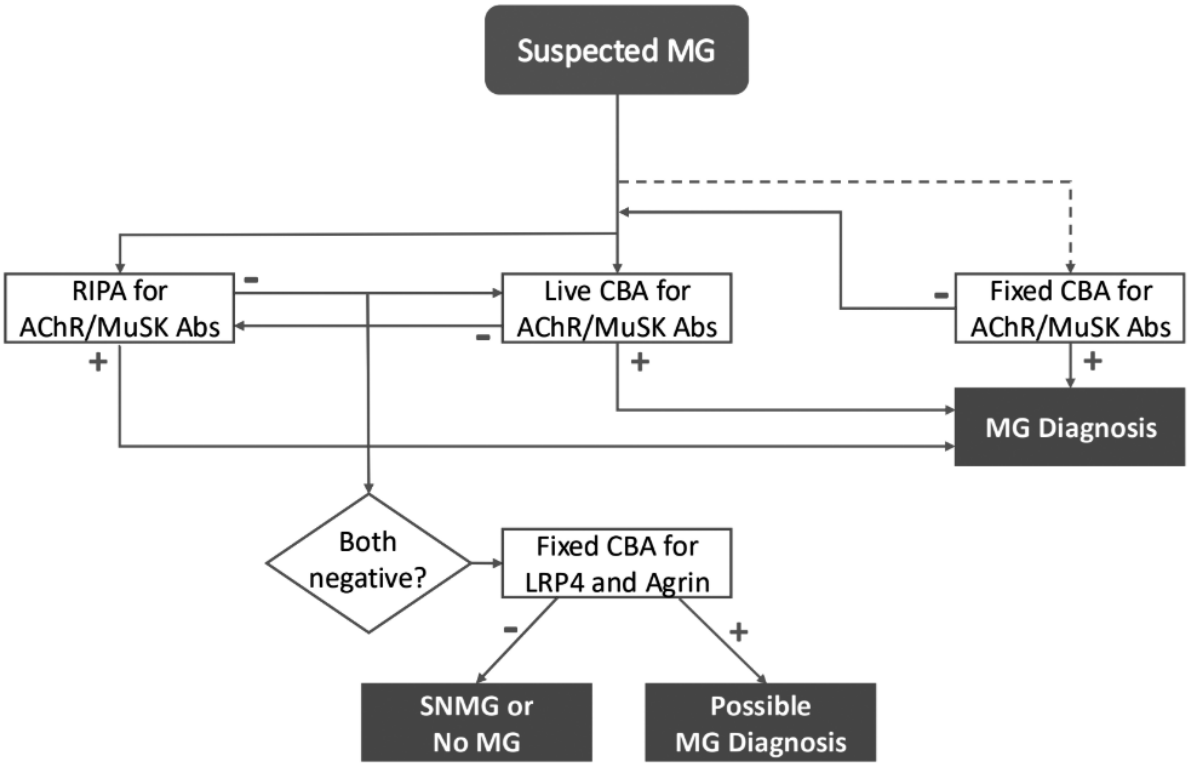

Figure 1. Diagnostic algorithm for MG.
Ideally, RIPA and live CBA should be available. Testing may start with either of them, but if negative, it should then be tested by the other assay. If neither RIPA nor live CBA is available, then fixed CBA, with lower sensitivity (or ELISA, not shown, with lower both sensitivity and specificity) may be used, but confirmation with at least one of the first two assays may be needed. It's important to note that binding to LRP4 or agrin is not exclusive to MG. **MG: Myasthenia** Gravis; RIPA: Radioimmunoprecipitation assay; AChR: acetylcholine receptor; MuSK: muscle specific kinase; CBA: Cell-based assay; LRP4: lipoprotein receptor-related protein-4, SNMG: seronegative Myasthenia Gravis

the detection of AQP4-Abs.[139,140] The advantages of live CBAs for AQP4-Abs are their high specificity and sensitivity.[95,140] However, in some cases, live CBAs may not be available due to technical issues,[141] in which case fixed CBAs are used because they are more easily available. While the specificity of fixed CBAs remains high, the sensitivity decreases compared to live CBAs[95,140] (**Table 1**).

Flow cytometry is considered equally reliable as CBAs in detecting AQP4-Abs.[72] Protein-based assays such as ELISAs, FIPA, western blot, or RIPA that use radiolabelled AQP4 and detect antibodies via precipitation should be used with caution, as they are no longer considered for routine testing due to their lower sensitivity and possible false-positive results[57,72,139,142,143] (**Table 1**). Tzartos NeuroDiagnostics has developed a highly sensitive 2-step RIPA by the pre-enrichment of the serum in AQP4 antibodies by affinity semi-purification, followed by RIPA with I125-labelled AQP4. It detects antibodies in all CBA-positive sera and allows quantitative measurement of the AQP4 antibodies, which may be useful for disease monitoring (unpublished results). Yet, because RIPA may also detect non-pathogenic antibodies to the cytoplasmic side of AQP4, the assay is used only for antibody quantification and disease monitoring of CBA-positive sera.

Differences between assays' sensitivity can be attributed to multiple factors including the isoform of AQP4 that is used, with M23 isoform of AQP4 showing in some studies higher sensitivity than M1 isoform without sacrificing the assay's specificity,[105], type of fixation for cells, florescent tags and volume of serum used for each assay.[139]

MOG Abs

Testing for anti-MOG Abs is primarily performed with CBAs[141] while FACS has also shown similar results.[144] It has been shown that live MOG-CBAs are more sensitive than fixed CBAs, apparently because of the preservation of the native spatial conformation of the protein and, thus, are preferred.[102,140] In a recent study, MOG-IgG FACS with live cells exhibited 95.1% (95% CI: 88%–99%) sensitivity and specificity, whereas MOG-IgG fixed CBA had much lower sensitivity and specificity[140] (**Table 1**). Additionally,

Method	Pros	Cons	Myasthenia Gravis		NMOSD	
			Range of Sensitivity	Target of autoAbs	Range of Sensitivity	Target of autoAbs
RIPA	- High specificity & sensitivity - Quantitative - Commercially available	- Radioactive - Does not differentiate between Abs to extracellular and cytoplasmic sites - Limited sensitivity for low-affinity & strictly conformational Abs	High	**AChR, MuSK**	High (limited use, for quantitation of CBA-positive Abs)	AQP4
Live CBA	- High sensitivity and specificity - Detection of only potentially pathogenic Abs - Detection of low-affinity Abs - Native antigen structure	- Labor-intensive - Qualitative - Expertise Requirement - Live Cell Requirement	High	**AChR, MuSK**	High	**AQP4, MOG**
Fixed CBA	- Easier than live CBA - No need for Cell Culture - Commercially available for some Abs	- Lower sensitivity and specificity than live CBA - Qualitative - Altered antigen conformation	Moderate to high	AChR, MuSK **LRP4**	Moderate to high	**AQP4**, MOG
ELISA	- Quantitative - Simple - Non-radioactive - Commercially available for some Abs	- Lower sensitivity - False positives & negatives	Moderate	AChR, LRP4 MuSK	Moderate	AQP4
FACS-CBA	- Advantages of live CBA - Quantitative	- Technical Complexity - Expensive Equipment	High	AChR, MuSK	High	**AQP4, MOG**

Table 1. Advantages and disadvantages of the most used antibody-detection methods for MG and NMOSD autoantibodies.
Each method has different strengths and weaknesses, which should be taken into consideration when they are utilised. Here is also presented the sensitivity range of these methods for each autoantibody. It is noted for which target autoantibody each method could be used, and in bold lettering, the antibody for which each method is preferred.
RIPA: Radioimmunoprecipitation assay; CBA: Cell-based assay; ELISA: Enzyme-linked immunosorbent assay; FACS: Fluorescence-Activated Cell Sorting; NMOSD: Neuromyelitis Optica spectrum disorder; autoAbs: autoantibodies; AChR: acetylcholine receptor; MuSK: muscle specific kinase; LRP4: lipoprotein receptor-related protein-4; AQP4: aquaporin-4; MOG: myelin oligodendrocyte glycoprotein; AQP1: aquaporin-1.

among the different antibody subclasses, it has been shown that IgG1-specific CBAs may be more sensitive and possibly more specific than MOG-IgG CBAs while also providing a correlation between MOG titre and disease state.[99,144,145] This is both because MOG Abs are predominantly of the IgG1 subclass and because MOG IgG1 CBAs can exclude reactivity from other less specific antibody isotypes such as IgM while IgG CBAs utilise IgG heavy and light chains as secondary antibodies that have non-specific binding therefore increasing the likelihood of false-positive results.[71,99,144,146]

NMOSD Monitoring

It is now known that AQP4-Ab titres do not directly correlate with disease activity, [147-149] however, it has been shown that the antibodies' titre in some cases increases in clinical relapses and decreases following B-cell directed or anti-IL-6-R therapies.[147,150] Therefore, for interpreting a given result, it is essential to consider the status of the disease and any treatments given at the time of sample collection. Ultimately, even though AQP4-Abs might decrease with immunosuppressive therapy, they may not be considered very suitable biomarkers for monitoring NMOSD.[151]

Regarding MOG Abs, it has been shown that patients with MOG Abs exhibit a highly heterogenous disease course and studies suggest a correlation between Ab titre and disease severity[71,152,153]; as antibody persistence has been linked to a relapsing disease phenotype whereas low titre or conversion of patients to seronegative was highly indicative of milder or monophasic disease course.[154] However, this is not universal, and seronegative conversion does not guarantee permanent remission; MOG Abs may still reappear, and relapses may occur.[152] Patients, therefore, may be advised to be tested every 6 to 12 months[71] as antibody monitoring may help with early recognition of monophasic course or not and subsequently aid in determining the need for ongoing immunosuppression and treatment strategies; additional evidence is needed to establish more definitive monitoring and treatment guidelines.

Emerging diagnostic assays and future approaches for MG and NMOSD in clinical practice

The role of Ig classes/subclasses in MuSK-MG

As mentioned before, MuSK-Abs are predominantly of the IgG4 subclass,[15,155] however, some MuSK-MG patients also produce anti-MuSK IgG1,[91] IgG2, or IgG3[31,122] antibodies. The presence of various Ig isotypes in MuSK-MG may represent promising biomarkers of clinical outcomes and disease states; thus, it has to be investigated.

Recently, Spagni et al. collected 43 samples from 20 MuSK-MG patients (45% treated with rituximab) at different time points during their disease course and screened them for the estimation of IgG levels, affinity binding, and the detection of the predominant MuSK-IgG subclasses.[122] In all samples, MuSK-IgG4 was the most frequent isotype present, representing on average 90.95% of MuSK antibodies, followed by IgG3 (3.29%), IgG2 (3.27%), and IgG1 (2.47%) subclasses.[122] In all patients' samples, during the acute phase, MuSK-IgG4 was the predominant subclass and remained the dominant isotype along the disease course. The authors observed an interesting event in one patient who switched from IgG4 to IgG1 during remission, and simultaneously, the total MuSK-IgG and MuSK-IgG4 levels remained stable, perhaps representing an event of immune remodelling. They observed a significant reduction of both IgG4 and IgG2 titres in samples characterised by a better clinical status based on the MGFA criteria. However, no significant changes in MuSK-IgG2 and IgG4 levels were reported in patients who did not improve after treatment.[122] The authors concluded that total MuSK-IgG and MuSK-IgG4 levels and the combination of reduced IgG4 and MuSK-IgG2 antibodies may represent a valuable biomarker of disease severity and clinical outcomes, data that clinicians could interpret and guide more effective and personalised treatment choices.

Recently, in Tzartos NeuroDiagnostics, we screened sera from MuSK-MG RIPA-positive patients, and from other neurological diseases and healthy control sera by live CBA and identified all MuSK-IgG isotypes (IgG1-4), but also antibodies of the IgA type. We found that although some MuSK-MG sera had only IgG4 autoantibodies, the majority had additionally other IgG subtype autoantibodies, while some had IgA anti-MuSK antibodies with or even without IgG anti-MuSK antibodies, as recently presented at the 15th MGFA International Conference on Myasthenia and Related Disorders by our group (Gkotzamani et al., May 2025). **F**uture studies should determine whether MuSK-IgA autoantibodies activate the complement system and correlate with clinical status**.**

Anti-MOG IgA and IgG3 Ab

Emerging evidence suggests that anti-MOG IgG3 and IgA antibodies may have clinical relevance in MOGAD.[156,157] While IgG1 remains the dominant and best-characterised antibody in MOGAD, IgG3 has been detected in some patients, coexisting with IgG1 (sometimes even as the predominant subclass) or present alone without detectable IgG1. Separately, anti-MOG IgA antibodies have been identified in a subset of patients seronegative for MOG-IgG/IgM and AQP4-IgG and may be associated with distinct clinical phenotypes, possibly influencing disease presentation through regional neuroinflammation or mucosal immune involvement.[157] Notably, a patient with exclusive IgG3 and IgA positivity, without IgG1, has been reported.[156] Their presence raises important questions about their role in disease mechanisms, particularly in atypical cases or mucosal involvement. While IgG1 remains the primary diagnostic marker, anti-MOG IgG3 and IgA detection could provide additional prognostic value for patient stratification, therapy prediction, and explaining some seronegative cases.[156,157] However, further large-scale studies are needed to validate their clinical utility, standardise testing methods, and clarify whether these antibodies directly contribute to pathogenesis or serve as secondary biomarkers of immune dysregulation.

Complement-based diagnostic assays in MG and perspectives for adaptive treatment strategy

As previously mentioned, the IgG1 and IgG3 pathogenic antibodies in AChR-MG activate the complement system.[158-161] The complement cascade gets activated when the AChR-IgGs bind to AChRs on the postsynaptic membrane of muscle cells, and this antigen-antibody complex activates the classical pathway. C1 complex binds to the Fc region of the antibodies, initiating a proteolytic cascade that activates C4 and C2 to form the C3 convertase (C4b2a). This enzyme cleaves C3 into C3a and C3b, leading to the formation of the C5 convertase (C4b2a3b), which cleaves C5 into C5a and C5b. Then, C5b initiates assembly of the membrane attack complex (MAC: C5b-9), which forms pores in the muscle membrane, leading to cell damage and loss of AChRs, impairing the neuromuscular transmission.[158-161] The activation of the complement system is the result of a serial cascade of events, in which many potential target molecules could serve as candidates for therapeutic tailoring and disease monitoring.

Iacomino et al. performed comprehensive complement profiling in AChR-MG, MuSK-MG, and healthy

controls using multiplex immunoassays and ELISAS.[162] They identified significant changes in AChR-MG, namely decreased C2/C5 and elevated C3/C3b/C5a levels compared to controls, while this pattern was absent in MuSK-MG.[162] These findings align with prior studies[163] and suggest that these proteins could serve as complement-related biomarkers for MG, promoting the early identification of patients who may benefit from anti-complement therapies.[162]

Hoffman et al. conducted the first histopathological analysis in tSN-MG patients, performing a cross-sectional study in treatment-refractory SNMG patients who had undergone muscle biopsy [164]. They reported C1q-positive signal, indicating classical complement pathway activation, and significant colocalisation of C5b-9 with IgG1 at the motor endplates in all SNMG and AChR-Abs positive patients.[164] Non-disease controls, with no muscle or serological abnormalities, stained negative for C5b-9 and IgG1,[164] consistent with findings from another group.[163] The authors propose muscle biopsy as a novel diagnostic approach to identify SNMG patients who may benefit from complement-targeted therapies based on histopathological findings in external intercostal muscles.[164]

The clinicoserological screening for the detection of either complement blood or/and histopathological biomarkers as an upcoming diagnostic approach for MG could be combined with a novel cell-based assay developed by Obaid et al., which measures AChR autoantibody-mediated complement membrane attack complex (MAC) formation via flow cytometry.[165] In this study, HEK293T cells were utilised and modified using CRISPR/Cas9 genome editing tools to disrupt the gene expression of the complement regulators CD46, CD55, and CD59. The authors screened serum samples from 96 clinically confirmed AChR MG patients and 32 healthy controls. AChR autoantibodies were detected in 139 of the 155 (89.7%) MG samples through a cell-based assay, and in 83 /139 AChR-positive samples, autoantibody-mediated MAC formation was detected, whereas MAC formation was undetectable in the healthy controls.[165] Moreover, in most patient samples, the MAC formation complex was positively associated with autoantibody binding.[165] Additionally, the authors did not observe significant differences in autoantibody-mediated complement activation between early onset and late onset MG clinical subgroups, nor in MG patients who underwent thymectomy.[165]

These findings warrant the necessity for the establishment of new biomarkers for the diagnosis and monitoring of both MG and SNMG patients whose condition is solely supported by heterogeneous clinical symptoms, since the routine clinicoserological diagnosis alone is challenging and carries the risk of underdiagnosis.

Rapid, Easy, and Low-Cost Assays for Non-Specialised Laboratories and for the point-of-care

Despite advances in immunodiagnostic tests for MG, often simpler, more rapid assays may be required for use in non-specialised laboratories where they can provide immediate results, thereby improving diagnostic efficiency and patient comfort. Trakas and Tzartos invented a method for AChR antibody detection using a modified ELISA onto a solid support stick (Immunostick), which dramatically reduces incubation times without increasing nonspecific background, offering a simple, and quick diagnostic solution with high sensitivity (91%) and specificity (99%) compared to regular RIPA.[166] Similarly, Bikoliya et al. proposed the nonradioactive dot blot assay for a rapid, simple, and low-cost detection of AChR antibodies with similar efficiency as ELISA.[167] Though potentially beneficial for MG diagnosis, these strategies need more thorough evaluation before clinical integration.

Similarly, even though the gold standard for identifying AQP4 Abs in NMOSD patients is CBA, their reliance on specialised laboratories limits access in clinical settings, and a more time- and cost-effective method could be highly valuable. To address that, Fu et al. developed a rapid enzyme immunodot assay in which patient serum is applied to a nitrocellulose filter strip (immunodot), dramatically shortening assay time and requiring only standard laboratory equipment.[168] In their study, this assay showed concordance with the CBA, exhibiting high sensitivity and specificity[168]; however, more studies are needed to confirm these results and to further validate the assay's sensitivity. Given its speed, ease of use, and low cost, this immunodot approach could enable broader AQP4-IgG screening, though real-world implementation studies are needed before it can be recommended for routine clinical practice.

AQP1-Abs in "seronegative" NMOSD

Although AQP4- and MOG-Abs are valuable biomarkers for NMOSD patients, about 10-20% of NMOSD patients are seronegative,[72] and the diagnosis of seronegative NMOSD remains challenging. AQP1 is expressed in various tissues and organs; regarding the CNS, it is highly expressed in areas affected in NMOSD patients, the spinal cord, and the optic nerves,[169] therefore AQP1 could also be the target of pathogenic antibodies in NMOSD. Tzartos et. al, identified anti-AQP1 autoantibodies in a subset of patients with chronic CNS demyelination, showing clinical similarities to anti-AQP4-seronegative NMOSD.[170] This finding suggests a new potential

biomarker for CNS demyelinating disorders, especially considering their potentially pathogenic role that has been presented in various studies,[171,172] however, further validation is needed.

Emerging biomarkers for monitoring NMOSD

Some emerging blood-based biomarkers that might be able to transform monitoring for NMOSD by measuring astrocytic and neuroaxonal damage, include neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP).

NfL is a biomarker of neuro-axonal damage and may be used in NMOSD as a marker of disease activity, prognosis, and treatment response when interpreted within the clinical and therapeutic context. NfL is an axonal cytoskeletal protein released into the interstitial fluid upon neuronal damage. Because its half-life in neurons spans a few months, elevated NfL reflects ongoing or recent neuro-axonal injury rather than a single acute event. Standard ELISA assays are used to measure NfL in CSF, while, after the recent advancements in detection technologies, the single-molecule arrays (SIMOA) and chemiluminescence (by Lumipulse) assays are preferred for blood measurements.[173]

Serial serum NfL monitoring may be able to detect subclinical disease activity and gauge remission stability, particularly when samples are timed before immunotherapy or during relapse.[174] Elevated NfL during acute NMOSD attacks provides an objective measure of neuro-axonal damage, correlating with MRI lesion burden,[175] and with Expanded Disability Status Scale (EDSS) worsening and aiding risk stratification for future disability[176,177]; however, serum GFAP may be superior to serum NfL in predicting future NMOSD attacks.[177] Moreover, NfL measured at relapse predicts post-attack disability worsening better than many other biomarkers, and higher baseline NfL associates with greater risk of spinal cord atrophy and long-term motor decline.[177] Reductions in NfL and GFAP levels over time have been observed in AQP4-IgG+ NMOSD patients receiving targeted therapies (e.g., ravulizumab, eculizumab, inebilizumab), indicating that NfL can serve as a pharmacodynamic biomarker of treatment efficacy.[177,178] Additionally, another study showed that NfL changes correlate with MOG Abs titre changes, thus making NfL a promising biomarker for patients with MOG Abs as well.[179]

Despite its utility, Nfl is not a disease-specific biomarker, and its levels are affected by prior therapies such as corticosteroids or plasma exchange before sampling can lower NfL levels, risking false-negative interpretations. Therefore, documentation of disease phase and therapies at collection is essential.[180]

GFAP is an intermediate-filament protein mainly expressed by astrocytes. Upon astrocyte injury or activation, GFAP is released into the interstitial fluid and subsequently into CSF and blood, which can be quantified by ultrasensitive immunoassays [181]. GFAP levels correlate with astrocytic injury, disease severity, MRI lesion burden, and subsequent disability, and when combined with NfL, provide complementary information on astrocyte versus axonal damage.[173]

GFAP is an emerging biomarker of astrocytic injury in NMOSD in addition to MS. Studies show that NMOSD patients exhibit significantly higher serum GFAP levels compared to healthy controls, MS, and MOGAD patients.[173,180,182] GFAP concentrations in serum may be associated with disease activity and severity in NMOSD patients.[183,184] Peak GFAP levels occur during acute relapses, with median remission levels remaining above control ranges days to weeks post-NMOSD attack —evidence of ongoing subclinical astrocyte injury.[174,185] Elevated baseline GFAP predicts a greater risk of early relapse and higher Expanded Disability Status Scale (EDSS) scores at follow-up,[174] while reductions in GFAP after initiation of targeted therapies (e.g., inebilizumab, eculizumab, ravulizumab) could mirror clinical improvement and decreased MRI activity.[178,186] SIMOA is believed to be the most accurate method for the detection of GFAP.[187,188]

While GFAP is more specific for astrocytic injury and may better predict relapse risk, NfL uniquely reflects axonal damage severity and disability progression. Therefore, combining NfL with GFAP measurement could enhance diagnostic accuracy and yield complementary insights into both astrocyte and neuron pathology in NMOSD.[173,180,182] Although not entirely disease-specific, GFAP may be useful in the differential diagnosis of AQP4-IgG+ NMOSD from MS and MOGAD.[189]

Overall, serum NfL and serum GFAP have a potential role as biomarkers for disease severity and future disease activity in AQP4-IgG+ NMOSD patients.[174] The GFAP/NfL quotient at relapse has also been suggested as the most specific serum biomarker for monitoring NMOSD. Lastly, NfL and GFAP have a potential role as biomarkers for disease severity and future disease activity in AQP4-IgG+ NMOSD patients,[174] and the GFAP/NfL quotient at relapse is a potential diagnostic marker for NMOSD.[180,190]

CONCLUSIONS

The field of diagnostics for antibody-mediated neurological disorders, including MG and NMOSD, is undergoing significant advancement. While traditional methods such as ELISA and immunoprecipitation con-

tinue to serve as a diagnostic foundation, the adoption of newer technologies, particularly cell-based immunofluorescence assays, has greatly improved both the sensitivity and specificity of antibody detection. Already, it has become a usual approach for AChR and MuSK RIPA-negative sera to be subsequently tested by CBAs for clustered AChR, MuSK, and LRP4 antibodies. It is also important to note that sera that test negative for AChR, LRP4, and MuSK antibodies in fixed CBAs, especially in cases of high clinical suspicion, should be examined further by live CBA, which offers higher sensitivity, thus reducing the possibility of false-negative results. However, antibody detection assays may require target-specific optimisation to maintain antigens in their physiological state, including structure, modifications, and oligomerisation, for epitope integrity. This assay optimisation may assist in the possible distribution of patients across different clinical sub-entities, the association with disease severity and stage, as well as the prognostic value of the findings for patient monitoring and the selection of the most appropriate treatments. Additionally, the identification of emerging biomarkers linked to tissue injury and complement system activation (i.e., decreased C2/C5 and elevated C3/C3b/C5a levels) offers new opportunities, particularly in addressing the diagnostic difficulties associated not only with seronegative patients, but also with MuSK MG patients bearing IgG4 Abs along with other Ig subclasses/classes. Treatment needs to be adapted to each patient's profile based on their MG subgroup, which could be identified based on these biomarkers and clinical features.

For NMOSD, early and accurate diagnosis is paramount in preventing further relapses and facilitating the prompt initiation of appropriate treatment strategies, which are crucial for mitigating long-term optic nerve damage and neurological disability.[139] Furthermore, the role of serum GFAP and NfL as prognostic biomarkers in patients with AQP4-IgG+ NMOSD remains to be defined in prospective cohorts. All in all, the aforementioned innovations and strategies enhance the precision of diagnosis, enabling more individualised therapeutic approaches, ultimately contributing to better patient management and clinical outcomes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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