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CONTENTS: PROCEEDINGS OF THE 14TH INTERNATIONAL CONFERENCE ON MYASTHENIA GRAVIS AND MYASTHENIC DISORDERS

Proceedings of the 14th International Conference on Myasthenia Gravis and Myasthenic Disorders

Message from the Founding Facilitator

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I am delighted that the Proceedings of the 14th MGFA International Conference of Myasthenia Gravis and Myasthenic Disorders is being published in the RRNMF Neuromuscular Journal. This conference has a long, impressive, and impactful history My understanding is that the conference began in 1954. For decades the proceedings were published in the Annals of the New York Academy of Science and these issues became a great source of information and authority for several generations of scientists and physicians involved in the field of myasthenia gravis (MG) and other disorders of the neuromuscular junction. Some of the biggest breakthroughs in the field were communicated at these meetings and in the published proceedings. As I type this I am in my library and looking at the proceedings from 1976 and the fifth annual conference. This was volume 274 in the NY Academy of Science series, and in that issue there were many papers on experimental autoimmune myasthenia gravis by both the J. Lindstrom laboratory in southern California and E. Lambert and V. Lennon laboratory at Mayo Clinic. This conference was held just three years after the Lindstrom lab first produced EAMG and proved the immune basis of myasthenia gravis. This the fifth annual conference I see as a pivotal year in our understanding of the disease. In addition to papers by the scientists noted above, there were reports by M Seybold, A Engel, S Ringel, D Drachman, D Grob, E Stalberg, A Pestronk, K Toyka, S Appel, J Griffith, D Sanders, A Penn, R Lovelace, J Daube, WK Engel, TR Johns, HJHG Oosterhuis, M McAQuillen, and many others that began the field of modern myastheniology.

We have come such a long way both in understanding MG and in treating patients with the disorder. Now we have a new generation of myasthenia experts who gathered in 2022, emerging from the Covid-19 pandemic. It is a privilege that the conference has chosen to publish the proceedings in this relatively new open access, on-line neuromuscular journal. When Drs. Carolina Barnett-Tapia and Kevin O'Connor approached me about this opportunity we immediately made the journal available for their use to publish the conference proceedings. I hope that the scientific communications published in this issue will be as impactful as those from the 5th conference in 1976.

As always, we abide by our mission to publish open access papers that the authors own, at no charge to the author or the reader.

- Rick

Proceedings Editors' introduction

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Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction (NMJ) that specifically affects neuromuscular transmission. The clinical hallmark of MG is skeletal muscle weakness, worsened by physical activity. Early in their disease, most patients present with extraocular muscle weakness which can generalize to involve limb, bulbar, and respiratory muscles. The disability can be severe and muscle atrophy may occur over time. The immunopathology of autoimmune MG is directly attributed to circulating autoantibodies specifically targeting postsynaptic membrane proteins at the NMJ. In contrast to many autoimmune diseases, autoantibodies in MG are unmistakably pathogenic. Consequently, MG serves as an archetype for human autoantibody-mediated autoimmune diseases. Due to several recent approval of therapeutics, MG patients can now benefit from a wider spectrum of treatment options including biologics that target specific underlying immune mechanisms.

The MGFA International Conference on Myasthenia Gravis and Related Disorders is considered the *major* meeting focused on MG pathology, treatment, and epidemiology. The meeting brings together clinicians and scientists, covering different aspects of MG: from basic science to new treatments, to the personal and societal impacts of the disease. Thus, this meeting is driven by the shared goal to improve the care and lives of people living with MG and related disorders. The meeting had been held every five years, providing a unique opportunity to discuss advances in the field, while also serving as a venue for idea exchange, establishing collaborations, and the opportunity to refocus the field while moving forward. In addition, this international conference aims to engage the next generation of clinicians and investigators, nurturing their MG-specific investigative programs and clinical practices.

The 14th MGFA International Conference on Myasthenia Gravis and Related Disorders was held from May 10th - 12th, 2022 in Miami, Florida. The meeting was sponsored by the Myasthenia Gravis Foundation of America (MGFA). National presenting partners included argenx, Alexion, UCB, Takeda and national sponsors included Regeneron, Catalyst, Immunovant, Sanofi, CSI Pharmacy, Janssen, and Horizon. The importance of the meeting is demonstrated by its high attendance, with upwards of 360 scientists and clinicians from around the world. The plenary sessions included 51 oral presentations and 102 posters were presented; there were 16 exhibit booths.

The Keynote address was given by Professor Angela Vincent, who has had a long-standing interest in understanding the pathophysiology of MG. She is credited with several major discoveries that have deepened our understanding of the disease. She also has trained and mentored many clinicians and scientists that have, in turn, made important contributions to the field. In her talk she shared her experience while attending the 5th International Conference on MG in New York City in 1975. She then provided her first-hand perspective of the last 50 years of MG-focused research, highlighting seminal discoveries made by Drs. John Newsom-Davis, Jon Lindstrom, Daniel Drachman, Vanda Lennon, Ricardo Miledi, her own lab and other key investigators.

Additional highlights from the sessions included new data on the role of autoantibody-mediated complement activity, and human monoclonal autoantibodies that revealed pathomechanisms underlying both AChR and MuSK MG. Topics that were also covered included ocular MG, experimental MG, biomarkers, fetal AChR autoantibodies, immune checkpoint inhibitor induced MG, sero-negative MG, cytokines and immune cells, and the biology of the NMJ.

The meeting also included an outstanding series of talks focusing on congenital myasthenia syndromes (CMS), which comprise a heterogeneous group of rare genetic disorders. Mutations underpinning CMS are found in genes encoding proteins with expression largely restricted to the neuromuscular synapse. Newly identified mutations were presented along with successful demonstrations of therapeutic intervention targeting the mutated proteins.

During the five years since the last conference, remarkable progress has been made in MG research. Perhaps most importantly is the approval of several biologic therapeutics that are highly effective in treating MG, and which are targeted, as opposed to traditional treatments. Therefore, we are entering a new era on how we treat people living with MG. This transition comes with new gaps in our scientific knowledge and healthcare systems, which will drive our research efforts in the next years. Highlighting these new therapeutics were presentations on complement inhibitors that interrupt autoantibody-mediated complement activity, neonatal FcRn inhibitors that decrease circulating autoantibodies, and cytotoxic therapeutics that target and deplete B cells by leveraging engineered T cells. Additional presentations focused on preclinical studies that investigated the induction of immune tolerance in experimental models of MG, shedding light on novel candidate treatments that represent a worthy focus of future research.

Given the growing interesting in MG research and the accelerated pace of new treatments coming into clinical

practice, the MGFA has decided that the international conference will be held every three years. Additionally, to be able to reach a larger number of international clinicians, scientists and trainees, the next meeting will be outside of the United States, with the 15th International Conference scheduled to be held in Europe.

Finally, the co-chairs of the organizing committee would like to thank Dr. Richard J. Barohn, the editor of the RRNMF Neuromuscular Journal, for supporting the publication of these proceedings. Marianne Reed, Eric Bader and Jiji Oufattole at the journal were gracious and extremely helpful through all phases of developing these proceedings. We also thank Dova Levin and Samantha Masterson of the MGFA for managing the meeting logistics, and the steering committee members, Drs. Anna Punga, Rosen Le Panse, Chip Howard, Amanda Guidon and Linda Kusner. Others who played integral roles include Dr. Lawrence Phillips, Dr. Meg Mendoza, Calli Dreveni, Annabel Wallace, Dr. Gianvito Masi, and all the authors who contributed papers and provided peer review.

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and Kevin C. O'Connor, PhD

Co-chairs, of the 14th International Conference on
Myasthenia Gravis and Related Disorders

Myasthenia research over the last 50 years – a personal perspective

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ABSTRACT

Myasthenia gravis (MG) research has, in many respects, been a trail blazer for the growing number of autoantibody-mediated disorders that affect the nervous system. The breakthroughs in MG understanding were made in the 1970s and even 50 years later, MG still remains a topic which scientists, clinicians and, most recently Pharma, return to as the most common and well-studied disorder. Here, some of the main discoveries will be reviewed very briefly focusing on how the knowledge of the disease evolved during the first decades after the discovery of acetylcholine receptor antibodies. It should be noted that this is a personal perspective and not a systematic or fully referenced review.

Keywords: History of myasthenia gravis, acetylcholine receptor, muscle specific kinase, autoantibodies, thymus

Earliest Times

MG was a topic of interest to neuroscientists and neurologists for three centuries before the discoveries of the 1970s. Table 1 lists the most important contributors to the history of MG research starting with Thomas Willis[1] in the 17th century. The clinical and physiological characterization began to move forward with Erb[2] and Goldflam[3] who described the fluctuating fatigue, and Jolly[4] helped explain fatigue by demonstrated the decreasing muscle contraction during repetitive nerve stimulations. By 1901, Campbell and Bramwell[5] had published a detailed description of myasthenia gravis. Meanwhile, Weigert[6] noticed collections of lymphocytes in MG patient muscle and later Buzzard[7] hypothesized that there might be an "autotoxic" agent. The description in 1934 by Walker of how, as in curare poisoning, the symptoms of MG were rapidly reversed by the cholinesterase inhibitor, physostigmine, led to the first systematic treatment for the disease.[8] All these observations helped demonstrate that MG was a disease of the neuromuscular junction and was likely due to some sort of inhibitory circulating substance. The history of MG research is covered briefly in a 2002 review,[9] and in a more detailed and beautifully illustrated book by Keesey.[10]

By 1960, several groups, including neurologists Straus and Nastuk, examined the role of the immune system on muscle fibers, finding cytotoxic damage caused by MG sera, and immunoglobulins and complement bound; importantly, however, these signs of autoimmunity were not at the neuromuscular junction itself but very evident on the muscle fiber striations.[11,12] In retrospect, the patients whose sera were positive in these experiments almost invariably had thymomas; these antibodies later became known as anti-striated muscle antibodies, strongly associated initially with the tumors. At this time, tissue specific antibodies were beginning to be recognized more widely, particularly those involved in thyroid disease.[13] In 1960, Simpson published a hypothesis,[14] reviewing the clinical associations of MG, including the often-enlarged hyperplastic thymus, the fluctuating disease course, the associations with a number of other autoimmune conditions (including thyroid disorders), and the transfer of disease to neonates. He proposed, with some prescience, that MG was a condition caused by an antibody to an "endplate" protein.

In 1952, Fatt and Katz[15] had identified miniature endplate potentials as the postsynaptic depolarization resulting from the release of single packets or quanta of ACh. Elmqvist and colleagues in Sweden[16] found that the miniature endplate potentials were reduced in amplitude in MG muscle. They concluded from their studies, somewhat tentatively, that the defect lay in the release of acetylcholine rather than in the response of the postsynaptic muscle.

Until that point, there was no way of identifying the postsynaptic "receptor" for ACh. It took the work of Taiwanese scientists, Chang and Lee,[17] whose main interest was snake toxin envenomation, to identify a component of venom from Bungarus multicinctus, the banded krait, that paralyzed rodent neuromuscular preparations. Conveniently, the toxin, α-bungarotoxin, was a polypeptide and could be easily radio-iodinated. They found that ^{125}I-α-bungarotoxin bound essentially irreversibly to the postsynaptic muscle membrane, exclusively at the NMJ, suggesting that it was binding to the elusive "receptor" for ACh.

The question was how to purify this large membrane protein. First, there was a much easier source than mammalian tissue. It had been known for years that the electric organs of electric eel or Torpedo were innervated somewhat similarly to muscle and responded strongly to acetylcholine (reviewed in detail by Keesey[18]). Second, in 1968, a group at the Weizmann Institute led by Cuatrecasas[19] had shown that it was possible to purify a protein to high specificity if you could immobilize its ligand on an insoluble matrix, apply the protein soup, wash and then "elute" the specific protein by introducing a ligand that competed with the matrix-attached ligand. This seminal discovery eventually led to the use of cobratoxin-columns to purify the toxin-binding protein from the electric organs of electric eel or torpedo (and subsequently human muscle).[20,21] By eluting with high concentrations of carbachol or d-tubocurarine, a number of groups achieved relatively pure ACh receptor (AChR) proteins and began to study its subunit structure.

Quinquennial meeting, New York 1975

All these findings came together in the early 1970s, and the results were presented at the MGFA conference on MG in 1975. I was lucky enough to be there, having been asked to write a conference report for Nature News and Views,[22] an opportunity that, although approached with considerable timidity at the time, turned out to be a wonderful stepping stone for my future career.

Firstly, Fambrough, Drachmann and Satyamurti had answered the pre- or post-synaptic question – to a large extent – by showing that there were less ^{125}I-α-bungarotoxin binding sites at the MG NMJ compared with control NMJs.[23] In the same year, Patrick and Lindstrom found that rabbits immunized against the purified AChRs from electric eel developed an MG-like syndrome, reversible by cholinesterase inhibitor, that could be transferred to healthy rabbits by the serum that contained antibodies to the immunizing AChR.[24] Lindstrom had devised a radio-immunoprecipitation method for measuring these antibodies that relied on incubating the serum with ^{125}I-α-bungarotoxin bound to solubilized electric eel AChR, and then immunoprecipitating with an antibody specific for rabbit IgG. The precipitate formed with the rabbit serum IgG contained the ^{125}I-α-bungarotoxin-AChR. [24] This led Almon and others to demonstrate that MG patients also had antibodies that interfered with binding of a-bungarotoxin to the AChR.[25] Meanwhile, Lindstrom together with Seybold, Lennon and others, used solubilized human muscle in the radio-immunoprecipitation assay, with precipitation by antibodies specific for human IgG, and found that 85% of patients were positive for AChR antibodies compared with a variety of controls. This test has formed the basis for an assay which, despite the radioactivity (which in fact is minimal), is still used widely.[26] Control sera are very rarely positive and the levels in patients vary but are often orders of magnitude higher than the controls.

Role of the antibodies

The question then became were these antibodies the cause of MG or could they be an epiphenomenon with no pathogenic role? Toyka, Drachmann and colleagues reported at the 1975 meeting that when MG IgG antibodies were injected into mice daily, the mice developed weakness and their endplate had very small miniature endplate potentials – reproducing well the neurophysiological hallmark of the disease.[27]

This was strong evidence that the serum IgG was causative; the reverse was to remove or reduce the AChR antibodies from patients and see if they improved. It was reasonable to suspect that antibodies were being made in the thymus or lymph nodes draining the thoracic cavity. Already before the antibodies were discovered, Matell and others[28] had found improvement in patients treated with adrenocorticotropic hormone and begun to use azathioprine as an immunosuppressive treatment. Impressively, they also found that thoracic duct drainage achieved clinical improvement, and that injection of the drainage fluid back into one patient caused deterioration – the perfect human experiment.

In the UK, plasma exchange was beginning to be used regularly for Goodpasture's disease (autoimmune glomerulonephritis) and the procedure was tried in MG by Pinching et al.[29] They found dramatic clinical improvement within days and, on further investigation, AChR antibody levels showed a striking inverse relationship with strength during the five day procedure and in the following weeks as the AChR antibody levels recovered and the patient's symptoms returned.[30] It should be noted that to get these results, each MG serum had to be titrated to find the optimal serum concentration for measuring that individual's antibodies over time, and this concentration varied considerably between different patients; this is seldom done nowadays and routine AChR antibody titers are seldom helpful in assessing treatment responses.

Since those seminal findings (reviewed in 1980[31]), MG research has expanded in many directions. Figure 1 uses a heatmap to illustrate the main topics and how interest in them has waxed and waned over time. The following sections will cover the topics asterisked.

Levels and characteristics of antibodies to the AChR in MG

The antibodies were found to be polyclonal IgG, predominantly IgG1 with some IgG3, and they appeared to react differently with AChR from normal muscle, denervated muscle and extraocular muscles.[32] They were very high affinity for the native AChR – as identified by binding to AChR in the solubilised muscle extracts – and did not bind well to denatured protein on western blots. However, monoclonal antibodies (mAbs), raised against purified eel AChR could bind to human AChR and one in particularly bound to a well-defined epitope on the surface of each of the two alpha subunits.[33] Since this monoclonal antibody (mAb 35) inhibited a variable but often large proportion of MG patients' antibodies, the two binding sites were termed the main immunogenic regions or MIR.[34] Similar results were obtained with mAbs raised against the human AChR, one of which, mAb M3D6, competed with mAB 35 and showed similar ability to compete with patient AChR antibodies.[35] In addition, other AChR mAbs bound to the beta or delta subunits, and four bound only to the fetal isoform in which the gamma subunit replaces the adult epsilon subunit[36] (see Figure 2). In fact, studies on the human antibodies binding to human AChRs (mostly identified by competition with subunit defined mAbs) showed considerable heterogeneity both in the levels and in their specificities, raising questions regarding which antibodies might be most pathogenic, and whether some are non-pathogenic and potentially protective; these questions have still not been clearly addressed.

Figure 1. A heat-map displaying some of the main topics of interest from International Conferences on Myasthenia Gravis over the last 50 years. Note that publications until 2008 included short papers from submitted abstracts as well as the contributions from invited speakers. For 2022, in order to include here some of the newer topics, all invited and submitted abstracts were searched.

Fig 1.

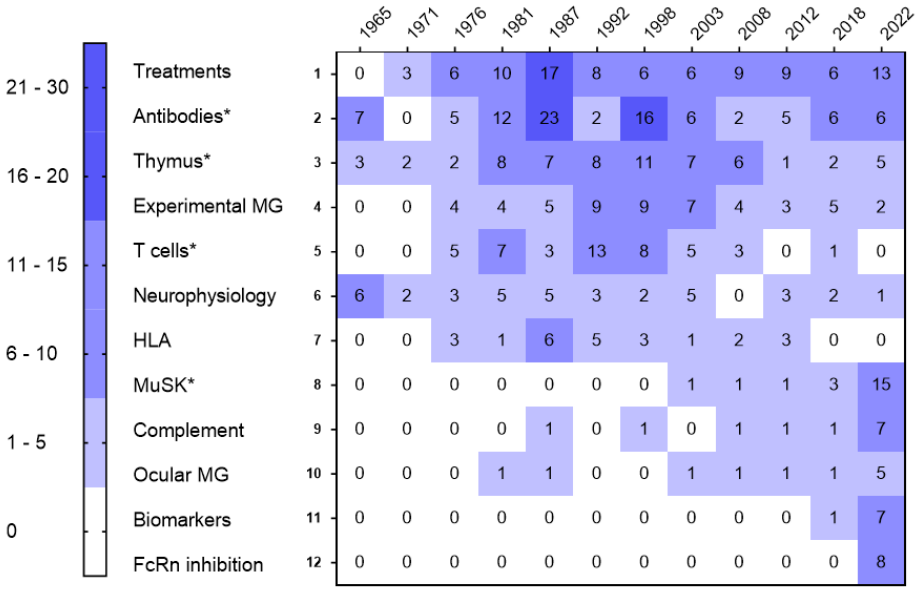
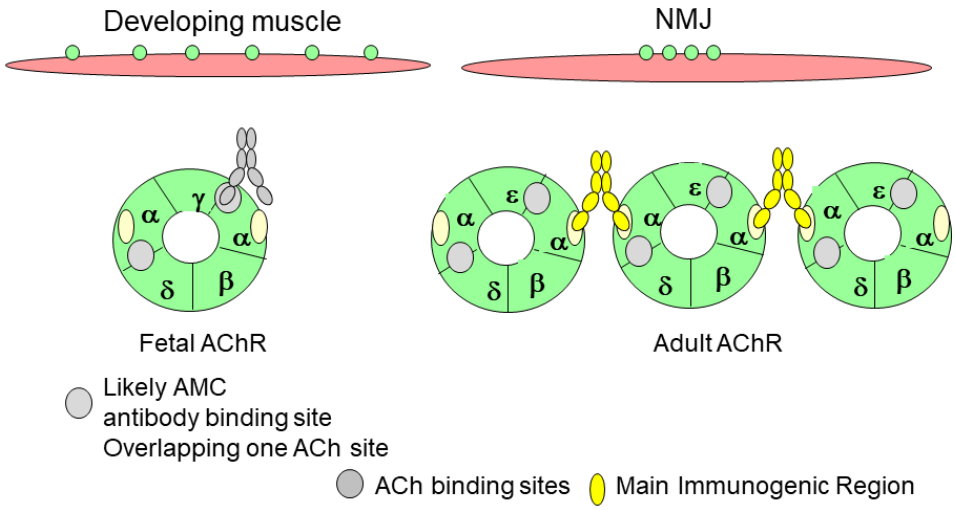


Figure 2. Simple diagrams of the adult and fetal AChRs and the most important binding sites for antibodies.
A. In humans, the fetal AChR can still be detected up to 31 weeks gestation[96] and it is likely that adult AChRs are present for some time before that. In mothers whose children develop AMC (arthrogryposis multiplex congenital), the antibodies block the AChR ion channel function and are assumed to bind to a fetal-specific site overlapping the ACh binding site. This is less clear in maternal antibodies of children with the recently described FARAD (fetal acetylcholine receptor antibody-associated disorder). Note also that because the fetal AChR shares the other three subunits with the adult form, antibodies to any of these subunits will bind both forms. Nevertheless, many of the FARAD mothers' antibodies are highly selective for binding to fetal AChRs (on the gamma subunit); but these may not necessarily inhibit fetal AChR function.

B. The adult AChR and how MIR antibodies can easily cross-link the receptors. Note that additional antibodies can help build up complexes that stimulate complement activity.[46]

Fig 2.



Mechanisms of action

The pathogenic mechanisms of the antibodies were identified in the late 1970s and 1980s. Engel's electron-microscopic studies demonstrated clearly that the NMJs were damaged with reduced numbers and depths of the secondary folds and widened synaptic gap.[37] Within the synapse, he and his colleagues found IgG bound and complement factors including C3 and the membrane attack complex.[38] The reduced binding of peroxidase labelled α-bungarotoxin confirmed relationships between IgG bound complement activation and AChRs lost. Curiously, despite the evident involvement of complement-mediated damage in MG, it is only over the last few years that attention has begun to focus on complement-mediated activity in MG. In the first of several trials, anti-complement therapy was effective in refractory MG[39] and a recent publication describes a method for assessing the complement-activating ability of individual patients' AChR antibodies that should help stratify patients who will respond to this type of therapy.[40]

Another mechanism discovered early was that of internalisation of the AChR.[41] This is particularly likely to occur with antibodies binding the MIR because, as illustrated in Figure 2, they can easily cross-link AChRs. It should be noted that the creation of complexes of this kind will also increase the likelihood of complement activation; however, using human-derived monoclonal antibodies bound to epitopes on different AChR subunits, complement activation was much more effective using combinations of the antibodies rather than antibodies to single subunits;[42] this suggests a role for the heterogeneous antibodies to other subunits that are found in MG.

It was disappointing that the antibodies did not often show direct inhibitory effects on the AChRs. This would likely need antibodies that bind to at least one of the two ACh binding sites, which are distinct from the MIR and at the interfaces with the two adjacent subunits (Figure 2). Those antibodies appear to be rare, and the mechanisms are more likely dominated by complement-mediated damage and internalisation. One exception, however, is fetal specific antibodies as described below.

Maternal MG and fetal AChRs

In the 1990s, a small number of women, mostly with MG, had babies who had stopped moving in utero and were born with severe, often fatal, arthrogryposis multiple congenita (AMC) rather than the well-known transient neonatal myasthenia. AMC is due to lack of fetal movement of any cause, including many genetic disorders, but the presence of AChR antibodies in the mothers, and the fact that consecutive pregnancies were affected, strongly implicated a maternal cause. IgG antibodies from two of the mothers, unusually, rapidly blocked fetal AChR currents while having no effect on adult AChR currents.[43] This suggested that they bound to the fetal gamma subunit in such a way as to block the binding of ACh to the adjacent alpha subunit (Figure 2); moreover, passive transfer of the mothers' antibodies to pregnant mice resulted in pups born with deformities and respiratory failure.[44] The numbers of reported cases with this condition is small, but it is now recognized that some children have milder symptoms in utero and survive, but have long-term consequences, a syndrome initially termed fetal acetylcholine receptor inactivating syndrome (FARIS).[45] The antibodies often bind preferentially to the fetal AChR but since the functional studies have not yet been performed, fetal acetylcholine receptor antibody associated disorder (FARAD) is more appropriate. The features in 40 children, all of whose mothers had AChR antibodies, include polyhydramnios and mild contractures in utero as well as hypotonia, feeding and respiratory difficulties at birth and dysmorphism, feeding difficulties, and speech impairment long term; only 50% of the mothers had diagnosed MG raising the possibility that FARAD could be a, previously undiagnosed, cause of neuromuscular developmental disorder in some neonates.[46]

Subgroups of MG

There were early hints in the 1970s of interesting associations between MG, gender, age of onset, and specific HLA (human leucocyte antigen) polymorphisms. Over the next decade many groups enlarged on these findings.[47] As the number of MG patients increased (partly the result of having diagnostic antibody tests available), three different subgroups of MG began to emerge: early onset (before 40 years), late onset (after 40 years) and those with thymoma.[48] Only when separated into these three groups was it clear that there were different gender ratios and HLA polymorphisms. Although the genetic analysis has since become much more complex, these distinctions remain; moreover, as the population ages the number of patients developing MG after the age of 50 years, predominantly males, now far exceeds those, mainly female, who develop MG as children or younger adults. However, there is still little understanding of how these genetic polymorphisms, and the more recent GWAS studies contribute to the aetiology of MG.

The role of the thymus.

Involvement of the thymus in the pathology of MG was seen in autopsies from earlier times but possibility of thymectomy for MG was serendipitous. Removal of the thymus by Sauerbruch[48] when performing thyroidectomy for a woman with thyroid disease led to marked improvement in her MG, and Blalock noted improvement in a woman when he removed her thymomatous gland.[50]

Since then, thymectomy, mainly for early onset MG, has been the source of much research material. Surprisingly, lymphocytes derived from the thymus could be shown to make AChR antibody spontaneously in culture.[51] In fact, the thymus contains B and T cells, some of which have been shown to be specific to AChR, which are surrounded by muscle-like cells that express AChRs on their surface.[52]

It is not surprising, therefore, that the levels of AChR antibody often decreases after removal of the thymus.[53,54] In most cases, the clinical response to thymectomy is slow, and given the success and quicker effect of immunotherapies, particularly steroids, it was questioned whether thymectomy was necessary. As Gronseth and Barohn reported in their retrospective review of controlled, non-randomized studies,[55] thymectomy conferred only moderate benefits. This was the basis for the multicentre international trial of thymectomy, first established in 2003 by John Newsom-Davis, which was eventually reported in 2016 led by Wolfe and colleagues;[56] this showed that thymectomy plus steroids conferred significant clinical improvement with less requirement for steroids, compared to steroids alone.

Thymic tumours are found in about 10% of MG patients, usually between the ages of 30 and 60, and they are mainly lymphoepithelial.[57] Thymoma patients seldom improve after removal of the tumour (unlike Blalock's patient) and may even get worse. They are always AChR-Ab positive but also often have antibodies to striated muscle proteins, specifically titin and ryanodine receptor.[58,59] These bind to intracellular proteins and are unlikely to be causative, but their presence in MG patients can be helpful as a biomarker for thymoma, especially in younger individuals. Antibodies to cytokines IFNa and IL12 can also help predict thymoma recurrence[60] but are seldom measured. The thymoma itself does not express native AChRs, but the epithelial cells express individual subunits of the AChR[61] which are thought to sensitise T cells which then migrate to the periphery.[62] Finally, in late-onset MG, the thymus is usually atrophic (ie. normal for age), yet these patients, whose numbers are growing owing to the increasing life expectancy of the general population, often have antibodies that are specific for titin and ryanodine receptor, despite no evident thymoma.

T cells in MG

As soon as it became clear that MG was a high affinity IgG antibody mediated disorder, it was assumed that the B cell antibody response was dependent on AChR-specific T cells, and that the epitopes recognized by the T cells would likely be more restricted than the B cells that produced the heterogeneous antibodies. The hope was that, if a specific T cell receptor response could be identified, the responding T cells could be selectively deleted. From the 1980s, the individual subunits of the AChR from Torpedo electroplax and then human muscle, were sequenced and cloned for expression studies.[63,64] Several groups produced recombinant AChR subunits by E. coli expression, and looked for proliferative T cell responses to the purified subunits, then epitope mapping the responses with overlapping synthetic peptides sequences, either in peripheral blood mononuclear cells or thymic lymphocytes. Hohlfeld and colleagues first found peripheral-blood lymphocytes responding to purified *Torpedo* AChR[65] and, when the human AChR subunits were sequenced, he and others went on to clone T cells specific for responding to human AChR.[66-68]

Disappointingly, there was diversity of responses to AChR peptides between MG patients, and sometimes control cells also responded. T cell responses could be restricted by the appropriate MG-associated HLA but often they were restricted by a less MG associated HLA.[66] Pools of overlapping peptide sequences frequently stimulated T cell responses, but it was not clear whether these cells would have responded to the native AChR as presented to B cells in vivo. When recombinant proteins were used as antigen, some of the responses were shown to be to E. coli contaminants rather than the AChR itself.[69] More encouraging, a small number of patient T cells responded, surprisingly, to the AChR epsilon subunit (adult receptor), and the response could be mapped to one specific epitope.[70] It was possible to cause apoptosis in responding T cells cloned from one patient by means of a tetrameric class II peptide complex in vitro[71] but, unfortunately, the hope of a specific T cell epitope that could be the target for such a therapy in a high proportion of patients has not yet been realised.

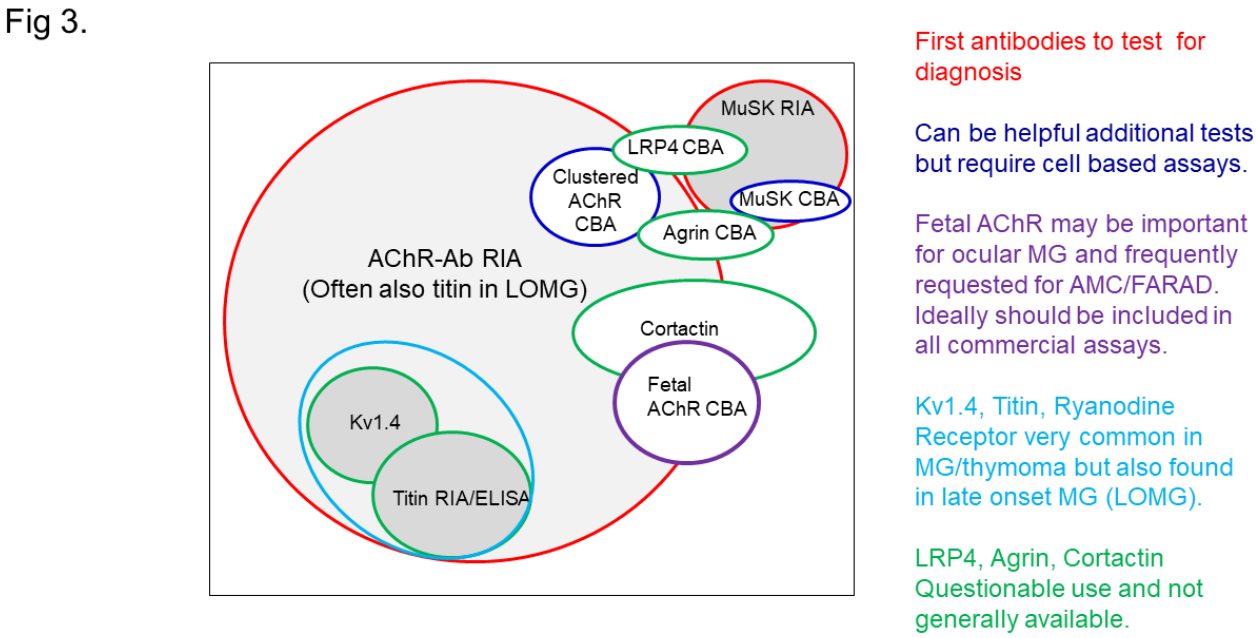
Origin of the immune response

Could the autoimmunity in MG be secondary to an infection? In the 1980s, there was considerable interest in the work of Jerne[72] who described antibody idiotypes and how their networks could control immune responses. A few publications appeared to show that AChR specific antibodies arose as a result of dysregulation of an "idiotypic" network, perhaps initiated by a microbial antigen[73] or by cross-reaction with epitopes shared on microbial antigens,[74,75] although the ELISA techniques used were questioned.[76] Moreover, the absence of an infectious history in most patients, the very high affinity of the AChR antibodies, and their clear preference for binding to the native protein rather than isolated subunits or synthetic peptides, strongly implied that the B cells are stimulated by the native human antigen. It is still possible, however, that low affinity antibodies to the AChR, possibly induced by cross-reaction with a microbial antigen, precedes the production of high-affinity pathogenic antibodies. Nevertheless, two attempts to demonstrate the presence of viruses in myasthenia gravis patients, including in the thymus itself, were unsuccessful.[77,78] A review in 1998 discussed these issues in more detail.[79]

Seronegative MG

In 1976, when reporting the AChR antibody assay results, Lindstrom[26] drew attention to the presence of some patients who appeared completely negative, and this "seronegative" MG group has been a focus of interest ever since. Importantly, these patients usually responded very well to plasma exchange, confirming that they probably did have an antibody-mediated condition, and passive transfer of their IgG to mice resulted in some changes in NMJ function, but not as clear-cut as transfer of those with AChR antibody positive IgG;[80] moreover, clinically the patients

Figure 3. Antibodies in myasthenia gravis patients. Note that a number of antibodies have been reported in MG, but not all of them are tested widely, and there are still around 5% of patients with generalised MG who have no detectable MG-related antibody and a higher proportion of those with ocular MG.



were somewhat different, often with more bulbar features.[81] One improvement was the much later introduction of the cell-based "clustered AChR' antibody test which detected antibodies in a proportion of those who were otherwise seronegative.[82] More exciting, was the discovery in 1994 by DeChiara et al. of a new potential antigen at the NMJ, muscle specific kinase (MuSK),[83] and the subsequent identification of MuSK's interaction partner low density lipoprotein-related protein 4 (LRP4).[84] Antibodies to MuSK[85,86] and LRP4 are now detected routinely in many labs, by radio-immunoprecipitation or cell-based assays. These patients can be severely affected with weakness and long-term muscle atrophy often predominant in the facial, bulbar and respiratory muscles,[87] and they have been difficult to treat effectively. The thymus is seldom hyperplastic, and thymectomy is not usually undertaken.[88] Intriguingly, however, they respond well to rituximab, and indeed better than the patients with AChR antibodies.[89] Nevertheless, some patients relapse which has provided an opportunity to explore the characteristics of the emerging B cells ($CD27^{high}CD38^{high}$ plasmablasts) and to identify the affinity-matured MuSK antibodies they produce.[90]

MuSK antibodies are different from AChR antibodies since they are mainly IgG4, not IgG1, they are monovalent, and they inhibit the interaction between LRP4 and MuSK that initiates MuSK phosphorylation and AChR clustering during development, and maintains AChR clusters in mature muscle.[91,92] In MuSK-MG, monovalent cloned human IgG4 antibodies had more pathogenic potential than the same antibodies when made divalent.[93] On the other hand, IgG1,2 and 3 MuSK antibodies exist in most patients and they also reduce AChR clusters in vitro.[92] However, instead of inhibiting MuSK phosphorylation as IgG4 antibodies do, they either have no effect (Cao et al. in preparation) or enhance MuSK phosphorylation.[94] IgG4 antibodies are proving to be of particular interest in a number of antibody-mediated diseases, including several that affect the central nervous system[95], but in most conditions co-existing divalent IgG1-3 antibodies exist and the mechanisms need to be explored comprehensively.

Since the discovery of MuSK antibodies, LRP4, agrin and other neuromuscular junction proteins have been tested for antibody binding (see Figure 3). Although antibodies to these proteins can be found in a minority of patients, they are not widely tested in routine laboratories, and despite many attempts by a number of research centres, there remain some patients (perhaps 5%), usually with relatively mild symptoms, who are persistently negative.

Final comments

There is a long history of research into the neuromuscular junction and the diseases that affect it; myasthenia gravis remains one of the best studied neurological diseases, and has provided a model, although with some obvious limitations, for understanding and treatment of the now well-defined antibody-mediated disorders of the central nervous system.

There are new approaches to study of myasthenia gravis that have flourished over the last 20 years, particularly in genetics, human derived monoclonal antibodies, biomarkers such as miRNAs, and trials of better targeted immunotherapies. Nevertheless, there are still many aspects that are

unexplained and deserve further research, some are now being investigated more intensively as was clear in the 2022 meeting (Figure 1), particularly ocular MG, novel biomarkers and the roles of complement and fetal FCR.

Conflicts of interest

I received a proportion of royalties for MuSK antibody assays until 2021. No other disclosures.

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Table. Important developments in the early research into myasthenia gravis

Year	Author	Observations
1672	Willis (1)	A woman with long-standing paralysis that affected her limbs and her tongue. "She speaks freely and readily enough for a while, but after a long period of speech ... she is not able to speak a word and is as mute as a fish. Her voice does not return for one or two hours". Hypothesis: a failure of some circulating substance to reach the muscles.
1895	Jolly (4)	Repetitive stimulation of the nerve that innervates a muscle produces a decreasing muscle contraction in MG patients, which explains their weakness and fatigue.
1901	Weigert (6)	Collections of lymphocytes ('lymphorrhages') in muscle and other tissues (but not brain) from MG patients.
1905	Buzzard (7)	Hypothesis: a circulating toxin, possibly an 'autotoxic' agent, was the cause of the disorder.
1934	Walker (8)	Mary Walker, recognizing the similarities between MG and curare poisoning, tried the curare antidote, physostigmine, with success in an MG patient.
1952	Fatt and Katz (14)	First demonstration of miniature end-plate potentials using fine glass electrodes inserted into muscle fibres. Acetylcholine is released in small quanta that cause small depolarisations of the muscle membrane.
1960	Nastuk (10)	Cytolytic effect of MG sera on frog muscle fibres in vitro and MG sera contain a complement-activating substance.
1960	Strauss (11)	Complement-fixing antibodies specific for muscle fibres in MG. IgG and complement are involved in MG.
1960	Simpson (13)	The female bias, fluctuating course, other autoimmune disorders, thymic abnormalities, and transfer of myasthenia to neonates indicated a circulating immunoglobulin was responsible for MG. Hypothesis: MG caused by an antibody to an "endplate (NMJ)" protein
1962	Chang and Lee (16)	Demonstrated that bungarotoxin from Bungarus multicinctus bound to postsynaptic membrane blocked neuromuscular transmission. Hyp: it binds to the muscle acetylcholine receptor.
1964	Elmqvist et al. (15)	First description of reduced miniature end-plate potentials at NMJs of MG patient. Could be pre- or post-synaptic; but they concluded that a reduction in acetylcholine release was more likely than a reduction in the postsynaptic response.

1968	Cuatrecasas (18)	Showed how a ligand bound to an insoluble substance (such as bead polymers) could be used to purify the receptor for that ligand.
1970 – 1972	Changeux and Miledi (17,18)	Cuatrecasas method employed cobra-toxins to purify AChRs from torpedo and eel electric organs. The AChR is a membrane, detergent-soluble protein that retains bungarotoxin binding in solution.
1973	Patrick and Lindstrom (20)	Rabbits immunized against purified electric eel AChR developed weakness, that responded to anti-cholinesterase. Hyp: an experimental model of MG.
1973	Fambrough, Drachmann and Satyamurti (21)	Used radioactive bungarotoxin to measure AChRs and found reduced AChRs in MG muscle.
1974	Almon et al. (22)	MG sera inhibit binding of ^{125}I-α-bungarotoxin binding to rat denervated muscle AChR. First demonstration of effect of MG antibodies on AChR.
1976	Lindstrom et al. (23)	Radio-immunoprecipitation by patient IgG antibodies of ^{125}I-α-bungarotoxin human AChR demonstrated in 85% of patients.
1975, 1977	Toyka et al. (24)	Injection of immunoglobulin G from MG patients into mice produced weakness and a reduction in the number of AChRs at the NMJ.
1977 1978	Pinching et al. (26) Newsom-Davis et al. (27)	Plasma exchange, which removes circulating antibodies and other soluble factors, produced a marked clinical improvement. For an individual MG patient, the clinical benefit correlated inversely with the level of AChR specific antibody.
1980	Engel et al. (33)	Both IgG and complement present at the NMJs of MG patients and co-localize with the remaining AChRs

These landmarks are focused on early observations and the most relevant work of the 1970s.
Hypothesis = hypothesis-generating.

Pharmacological treatment of Lambert-Eaton Myasthenic Syndrome

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ABSTRACT

Lambert-Eaton myasthenic syndrome (LEMS) is a very rare antibody-mediated autoimmune disease of the neuromuscular junction. Therapy can be divided in symptomatic treatment and immunosuppressive treatment. Symptomatic treatment with amifampridine is the only therapy currently authorized for use in LEMS patients. In the Netherlands the first-choice drug is amifampridine base in an extended-release formulation instead of the currently authorized immediate release amifampridine phosphate. The extended-release formulation has lower costs and is possibly safer due to lower peak concentrations. Other therapy used in LEMS patients is prescribed off-label and is based on experience in patients with myasthenia gravis. In many cases pyridostigmine is added as symptomatic treatment. In almost half of patients immunosuppressive therapy is started, mostly corticosteroids with or without azathioprine. Intravenous immunoglobulins and plasma exchange are used as emergency treatment.

Currently no randomized clinical trials with new therapies are ongoing or announced in patients with LEMS, although multiple new therapies for myasthenia gravis are being investigated. These future therapies can be differentiated in symptomatic and immunomodulating drugs. The immunomodulating drugs can be further differentiated in early-stage drugs which target the B-cell, later stage drugs which target the circulating autoantibodies and targeted therapy which have a disease-specific target. Some early and later stage immunomodulating drugs show promising results in myasthenia gravis although high cost and uncertain long-term safety may be limiting for incorporating these drugs in LEMS treatment guidelines.

Clinical trials in LEMS patients are lacking due to the rarity of the disease and we suggest the following requirements for future trials of potential new treatments: Sufficient power by performing multicenter or N-of-1 trials when appropriate, a cross-over design to reduce the number of patients and using a LEMS-specific quantitative primary outcome measure like the Triple Timed-Up-and-Go (3TUG) score.

Key words: Lambert-Eaton Myasthenic Syndrome, amifampridine

Introduction

Lambert-Eaton myasthenic syndrome (LEMS) is an autoantibody-mediated immune disease of the neuromuscular junction. LEMS is a very rare disease with a point prevalence between 2.3 and 3.5 per million (1-3). Autoantibodies to P/Q-type voltage-gated calcium channels (VGCC) can be detected in 90% of patients (4, 5). Autoantibodies against presynaptic VGCCs inhibit the release of the neurotransmitter acetylcholine in the neuromuscular junction (6) causing muscle weakness and autonomic dysfunction (3). In approximately 60% of patients, LEMS is associated with a malignancy, in most cases small cell lung cancer (SCLC) (3). It is believed that autoantibodies directed against VGCCs expressed on the tumor surface cross-react with the VGCCs expressed on the presynaptic nerve terminal at the neuromuscular junction (7). LEMS is often compared to myasthenia gravis (MG), since they are both associated with muscle weakness due to pathology in the neuromuscular junction, however autoantibodies in MG are directed at the postsynaptic membrane and the symptoms differ. Ocular and bulbar muscle weakness causing ptosis, diplopia, difficulties in swallowing and talking is usually rather mild compared to MG patients, and mostly not present as presenting symptoms (3). In contrast, proximal leg weakness is almost invariably present in the early phase of LEMS and relatively rare in MG. Furthermore, patients with LEMS are less likely to be hospitalized due to disease specific symptoms than patients with MG (8), probably because respiratory muscles are less likely to be affected.

Therapy for LEMS can be divided into symptomatic treatment and immune-directed treatment (9). Amifampridine has been the symptomatic drug of choice since 1983 (10) and is the only drug currently authorized at the FDA and EMA for the treatment of LEMS. Since its approval by the FDA, multiple review articles have been published to highlight amifampridine as the first drug of choice in the symptomatic treatment of LEMS (11-14). Other therapies used in the treatment of LEMS are prescribed off-label. Due to the low prevalence of LEMS, clinical trials needed for the regulatory approval of new therapies are difficult to carry out and have not been done. In addition, older clinical trials in LEMS patients often used outcome parameters developed for MG, making it difficult to assess the efficacy of the investigated therapies. The Triple

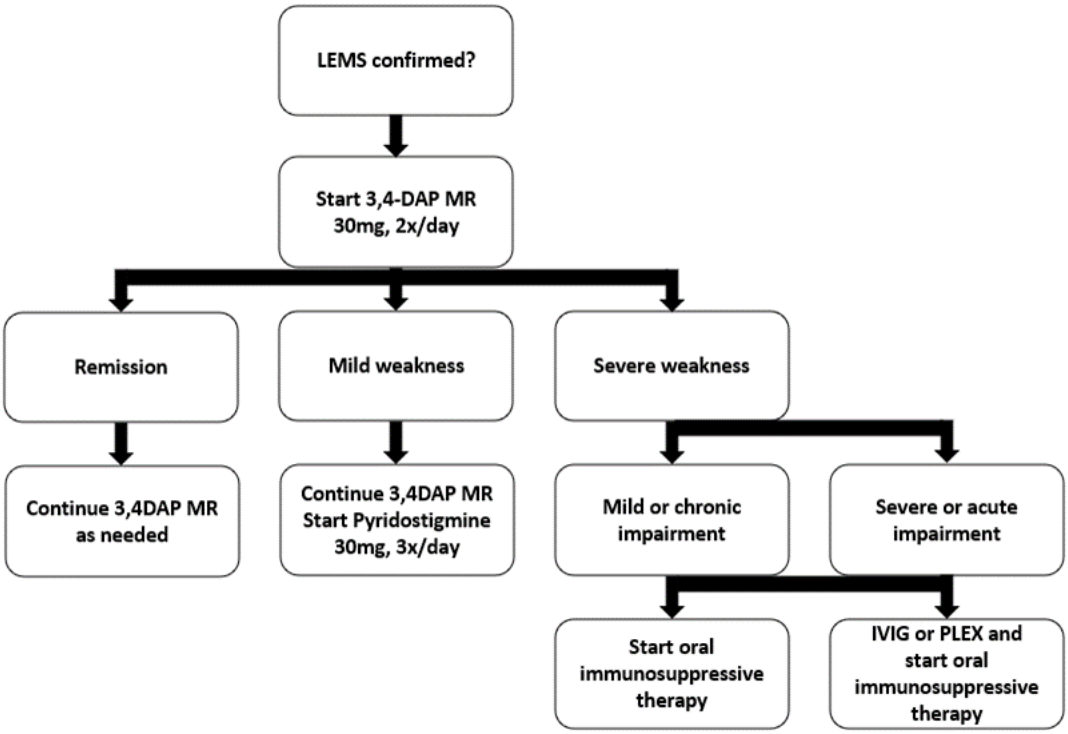

Figure 1: Treatment scheme for LEMS used in the Netherlands. 3,4-DAP MR = 3,4-diaminopyridine base modified release tablets. Illustration of a decision tree for the therapeutic options for patients with confirmed LEMS. This decision tree is based on data collected between 1998 and 2015 in the Netherlands and Belgium (4). Ninety-five percent of patients used amifampridine and 68% used pyridostigmine; 40% used immunosuppressive treatment of whom 29% used the combination azathioprine and prednisolone and 14% used prednisolone alone; intravenous immunoglobulins and plasma exchange were used as emergency treatment and were used in 26% of patients. Based on the Dutch registry for disorders of the neuromuscular junction, the use of immunosuppressive treatment in patients with LEMS is lower than in patients with MG, 49% and 69% respectively (8).

Timed-Up-and-Go (3TUG) score, a more disease-specific measure with a better representation of the functional disability of LEMS has been validated and introduced in most recent clinical trials in LEMS patients (15-17). As MG and LEMS show some similarities in pathogenesis and pathology, most therapeutic decisions in LEMS are based on experience with these treatments in MG patients. Several emerging treatments in MG may be useful in LEMS patients as well. In this article, the most applied therapeutic options for LEMS are reviewed. Treatment directed at the primary tumor is outside the scope of this review. Finally, potential future therapies will be discussed.

Existing therapies

Amifampridine

Most patients with confirmed LEMS start with amifampridine. Amifampridine is the International Nonproprietary Name (INN) of 3,4-diaminopyridine (3,4-DAP). Use of the name amifampridine may refer to 3,4-DAP phosphate (Firdapse) or 3,4-DAP base. Amifampridine blocks the efflux of potassium ions in the presynaptic nerve by blocking the presynaptic voltage gated potassium channel. This prolongs the duration of depolarization in the presynaptic nerve which then increases the calcium influx, thereby improving the efflux of acetylcholine in the synaptic cleft (2).

The formulation of amifampridine currently approved at EMA and FDA for LEMS is 3,4-DAP phosphate in an immediate release formulation. The approval of amifampridine by the EMA has been based on two pivotal studies performed with another formulation, 3,4-DAP base, which confirmed a positive risk-benefit balance (18, 19). The market authorization holder assessed the bioequivalence in a relative bioavailability trial of 3,4-DAP phosphate and 3,4-DAP base to include these studies in the application for marketing authorization. For the approval of amifampridine (as phosphate and as base) by the FDA, more recent randomized clinical trials (RCTs) have been performed using a withdrawal design (15, 16, 20). In a withdrawal trial, patients who already use a stable dose of amifampridine are included in the trial and, after randomization, either receive a tapered withdrawal using a placebo or receive their usual dose of amifampridine. Combining these RCTs a total of 168 patients were included of whom 93 patients received amifampridine. A summary of the main trial findings is shown in Table 1.

Table 1: Summary of main trial results of RCTs with amifampridine.

Study	Study drug	Trial type	Number of Patients	Outcome	Main trial findings	Serious drug reactions
McEvoy 1989(19)	Amifampridine base capsules	Double blind placebo-controlled crossover	12	NDS Isometric muscle strength Autonomic function CMAP amplitude	Significant improvement in all outcome measures	1 patient had a seizure when 3,4DAP was increasing from 90-100mg and pyridostigmine from 120mg-240mg
Sanders 1993(21)	Amifampridine base capsules	Double blind placebo-controlled crossover trial	18 (10 with LEMS)	QMG	significant lower QMG scores	2 patients had seizures who took 100mg 3,4DAP per day, 1 had toxic levels of theophylline, no seizures recurred after theophylline was discontinued, 1 had no seizures after dose reduction to 40mg per day
Sanders 2000(18)	Amifampridine base capsules	Double blind placebo- controlled parallel	26 (12 3,4-DAP)	QMG score change	Significant lower QMG scores	No serious drug reactions
Oh 2009(22)	Amifampridine tablets	Double blind placebo-controlled crossover	7	SS score LEMS classification MRC QMG CMAP amplitude	Significant improvement in all outcome measures	1 patient withdrew due to chills, weakness, shortness of breath, wooziness in the stomach and difficulty sleeping
Wirtz 2009(23)	Amifampridine base IV, pyridostigmine IV, placebo or combination	Double blind placebo-controlled crossover	9	Isometric muscle strength CMAP amplitude	Significant improvement in both outcome measures in amifampridine or combination treatment, no improvement in pyridostimine or placebo, no additive effect of combination therapy	2 patients withdrew due to pain in upper arm into which medication was administered
Oh 2016(20)	amifampridine phosphate tablets (Firdapse)	Double blind placebo-controlled parallel withdrawal trial	38 (16 3,4-DAPP)	Primary endpoints: QMG and SGI	Significant improvement in both primary endpoints	No serious drug reactions
Sanders 2018(16)	Amifampridine base tablets	Double blind placebo-controlled parallel withdrawal trial	32 (14 3,4-DAP)	Primary endpoint: 3TUG score	Significant change in 3TUG scores	No serious drug reactions
Shieh 2019(15)	Amifampridine phosphate tablets (Firdapse)	Double blind placebo-controlled parallel withdrawal trial	26 (13 3,4-DAPP)	Primary endpoints: SGI and QMG	Significant improvement in both primary endpoints	No serious drug reactions

NDS: Neurologic Disability Score, QMG: Quantitative Myasthenia Gravis score, SS score: Subjective Symptoms score, MRC: Medical Research Council score, SGI: Subject Global Impression of Improvement, 3,4-DAP: 3,4-diaminopyridine, 3,4-DAPP: 3,4-diaminopyridine phosphate.

In the Netherlands, 3,4-DAP base is available in a modified release tablet. The available strength of 3,4-DAP base is 30mg and patients usually start with 1 to 2 tablets a day. Based on the clinical response and side effects, the dosage can be increased to up to 3 tablets a day. Amifampridine is metabolized into the inactive metabolite 3-N-acetylated amifampridine by the enzyme N-acetyltransferase (NAT). Amifampridine and its metabolite are almost completely eliminated through the urine, resulting in an elimination half-life of approximately 2 hours (24). Patients with slow NAT phenotypes have a higher exposure to amifampridine than patients with a fast NAT phenotype (25). Pharmacogenetic testing is not recommended, because dosage is based on clinical response and amifampridine shows an immediate effect on clinical improvement of LEMS symptoms and side effects. The main side effects of amifampridine described in clinical trials are oral and digital paresthesia. Less frequently headache and gastrointestinal symptoms may occur (12). The most frequent serious side effect are seizures, which appear to be dose dependent. The occurrence of seizures is mainly described in patients with daily dosages of 100mg or more (19, 21). In addition, side effects are associated with high serum peak concentration of amifampridine (26). Of 93 LEMS patients who received amifampridine in RCTs, three patients had a seizure, of whom all received daily doses of 100mg amifampridine or more.

The modified release formulation will reduce the peak concentration of amifampridine, making it a safer option. Moreover, due to less frequent dosing it is more patient friendly. The market approval of amifampridine as the phosphate salt in Europe was based on efficacy data of the base and therefore the efficacy of amifampridine phosphate and base are comparable. Combined with the much lower price of the base and the possibly safer toxicity profile, the National Health Care Institute of the Netherlands concluded that 3,4-DAP modified release remains the first drug of choice in LEMS patients (27). A reason for using the market approved amifampridine mentioned in literature was that the base was not as stable as the phosphate salt, with a supposed maximum shelf life of 12 months (28). However, amifampridine base as a raw material as well as in the modified release formulation was found to have a shelf life of at least 36 months (personal observation by GMP licensed quality control laboratory).

Pyridostigmine

If the symptoms of LEMS are not adequately treated with amifampridine alone, pyridostigmine might be added, although there is limited evidence (19, 29). Pyridostigmine is an acetylcholine esterase inhibitor and increases the amount of acetylcholine by inhibiting the breakdown of acetylcholine in the synaptic cleft. Since amifampridine and pyridostigmine increase the amount of acetylcholine at the neuromuscular junction, but at a different site of action, they may have a synergistic effect. The only RCT to address the question whether the combination of amifampridine and pyridostigmine provides additional effect compared to amifampridine or pyridostigmine monotherapy, showed that the addition of pyridostigmine did not yield a significant benefit on isometric muscle strength and CMAP amplitude (23). In this randomized crossover trial, nine patients were treated with a single intravenous dose of amifampridine, pyridostigmine and the combination of these drugs. Nevertheless, in some cases pyridostigmine is still being used and in one study, 67% of patients noticed a subjective improvement due to pyridostigmine (4). The starting dose of pyridostigmine is usually 30mg 3 times a day and can be increased up to 6 times 60mg daily. The main side effects of pyridostigmine can be attributed to its cholinergic effects and include flatulence, urinary urgency, muscle cramps, blurred vision, hyperhidrosis, diarrhea, abdominal cramps, increased salivation, and light-headedness. Diarrhea has been reported to be the most frequent cause for treatment discontinuation or lowering the dose (30).

Immunosuppressive therapy

If symptoms are not adequately controlled with amifampridine and/or pyridostigmine, the introduction of immunosuppressive therapy can be considered, to inhibit the production of VGCC autoantibodies. There is little evidence, in terms of clinical trials, of its effect on the clinical severity of LEMS. The first-choice oral immunosuppressive treatment is a corticosteroid such as prednisolone, either with or without azathioprine. The use of the combination of these drugs is based on RCTs in patients with MG (31, 32). In one study of six patients with non-tumor related LEMS treated with the combination of prednisolone and azathioprine, three had sustained remission, while the other three improved. However two of the latter three were azathioprine intolerant (33). The corticoid sparing effect is another reason to add an immunosuppressive to prednisolone, in an attempt to avoid the serious side effects of prednisolone if high doses are needed for longer periods of time (34). Indeed, weight gain was less pronounced in patients using the combination of prednisolone and azathioprine compared to prednisolone alone and the overall dose of prednisolone was lower when combined with azathioprine (31).

The usual starting dose of prednisolone is 60mg after which the dose is tapered to a low maintenance dose. The standard daily dose of azathioprine is 2-3mg/

kg. Prednisolone can have major side effects including hyperglycemia, weight gain, opportunistic infections, hypertension, depression, and osteoporosis (34). Side effects of azathioprine include hepatotoxicity and myelosuppression. Because bone marrow toxicity is associated with the activity of thiopurine methyltransferase (TPMT), pharmacogenetic testing is recommended in patients in whom azathioprine is initiated (35). Another gene associated with azathioprine related toxicity is NUDT15. Patients who are homozygous for the inactive NUDT15-variant also need a dose reduction of azathioprine (36). Other corticosteroid sparing immunosuppressives can also be used, including tacrolimus, mycophenolate mofetil, cyclophosphamide and ciclosporin. Again, there is little evidence from RCTs, but the limited evidence in generalized MG does not show a clear difference in efficacy between these drugs, although the dose of the corticosteroid may be less when combined with other immunosuppressive drugs (37).

Intravenous Immunoglobulins (IVIG) or plasma exchange (PLEX) are used as a third line treatment when the disease is inadequately controlled by symptomatic treatment and immunosuppressive drugs. PLEX results in a rapid decrease in circulating antibodies (38). IVIG also leads to a reduced concentration of pathogenic autoantibodies, although the underlying mechanism is not fully understood. Possible explanations include neutralization by anti-idiotypic antibodies, downregulation of antibody production and accelerated autoantibody degradation by competing with the neonatal Fc receptor (39). One RCT in LEMS patients showed that IVIG therapy had a significant improvement on limb strength compared with placebo (40). Improvement in strength peaked at 2-4 weeks and declined after 8 weeks. Serum titers of VGCC autoantibodies declined significantly. Research in MG patients showed that IVIG and PLEX are comparable in effectiveness (41-43).

The usual dose of IVIG therapy is a total of 2 g/kg, divided over five daily doses of 0.4g/kg/day. Common side effects of IVIG therapy include headache, fever, chills, and nausea. However, side effects of IVIG therapy are subjectively less severe than PLEX (44). Reported side effects of PLEX are arterial bleeding, bleeding disorders, septicemia, and venous thrombosis. A typical PLEX schedule is performed by removing 1 plasma volume every other day in 5 sessions (45). The choice between PLEX and IVIG therapy depends on different factors. PLEX is considered when a rapid response is needed, but cannot be used in patients with sepsis, whereas IVIG treatment cannot be used in patients with renal failure (46).

Cost Of Therapy

The daily costs for a daily dose of 60mg of the licensed product with amifampridine phosphate are €130,80 in the Netherlands. This corresponds with annual costs of €47.742. In contrast, the daily costs of amifampridine base are €13,28, corresponding with annual costs of €4.847 (47). In the Netherlands, the total population of LEMS patients is estimated to be approximately 65 (4). If 95% of these use amifampridine, the estimated annual cost saving of using amifampridine base instead of amifampridine phosphate would be €42.895 per patient per year or €2.659.490 for the total estimated users of amifampridine. In particular in the United States, where amifampridine phosphate is priced in excess of $400.000 per patient per year, the annual savings achieved with a more affordable alternative would be immense. Licensing a medicinal product will increase its costs due to extra requirements, like post marketing pharmacovigilance. However, as the efforts undertaken by the pharmaceutical company that obtained marketing authorization at the time appear to be very limited, this enormous difference in drug pricing seems disproportionate (48).

The costs of pyridostigmine are €0,05 for the 10mg tablet and €0,20 for the 60mg tablet. With dose ranges between 3 times 30mg and 6 times 60mg the respective daily costs vary between €0,45 and €1,20 which corresponds with €164,25 to €438 per patient per year (49).

Prednisolone tablets are also relatively cheap with an estimated cost of €0,10 to €0,30 per patient per day and a respective yearly cost between €36,50 and €109,50 (50). However, the costs of prednisolone tablets do not provide an accurate representation of the total annual costs considering that these patients require monitoring and regular lab testing, bone density measurements and osteoporosis prophylaxis. In addition, the costs accrued through the occurrence of side effects of corticosteroids, including a 2.5-fold increased risk of cardiovascular events, are likely to be far higher.

The estimated annual costs per patient of other oral immunosuppressive therapies are varying between €365 and €1.825 depending on the dose and choice of drug (51-53). The cost of PLEX and IVIG therapy are not directly available and depend on multiple variables including, but not limited to costs of personnel, costs of a hospital visit, insertion of a central line if needed, departmental and equipment costs. A cost-minimalization analysis has been performed in a neurological center in the UK comparing PLEX and IVIG, showing an estimated total cost-per course- of £4.432 for PLEX and £8.890 for IVIG (54), which is approximately €5.000 and €10.000 per course respectively.

Future Therapies

As mentioned before, the only therapy currently approved for the treatment of LEMS is amifampridine. New treatment modalities for LEMS are not yet in the clinical phase. As LEMS has a low prevalence, and thus low commercial value, it remains to be seen whether clinical trials will be eventually performed. Other off-label prescribed drugs used in the treatment of LEMS are mostly based on experiences with these drugs in MG. Therefore, it will be interesting to see which new treatment modalities are or will become available for MG and which of these drugs may be of added value in the treatment of LEMS. An overview of these new drug modalities tested in clinical trials is shown in Table 2.

Table 2: An overview of drugs being tested in clinical trials in myasthenia gravis (source clinicaltrials.gov and clinicaltrialsregister.eu).

Drug classes	Drug	Drugtarget
Symptomatic drugs	Tirasemtiv	troponin activator
	Salbutamol	beta 2 receptor agonist
	Ephedrine	beta 1 receptor agonist
Immunomodulating drugs		
target B cell / early stage	Inebilizumab	CD-19
	Rituximab	CD-20
	Mezagitamab	CD-38
	Iscalimab	CD-40
	Satralizumab	IL-6
	Tocilizumab	IL-6
	Descarted-08	BCMA (CAR-T)
	Telitacicept	BAFF and APRIL
	Tofacitinib	JAK inhibitor
	Tolebrutinib	BTK inhibitor
	Abatacept	CTLA-4 inhibitor
	Bortezomib	Proteasome inhibitor
target circulating autoantibodies/ later stage	Batoclimab	FcRn blocking
	Efgartigimod	FcRn blocking
	Nipocalimab	FcRn blocking
	Orilanolimab	FcRn blocking
	Rozanolixizumab	FcRn blocking
	Vemircopan	Complement pathway (factor D)
	Zilucoplan	Complement pathway (C5)
	Eculizumab	Complement pathway (C5)
	Gefurulimab	Complement pathway (C5)
	Pozelimab	Complement pathway (C5)
	Ravulizumab	Complement pathway (C5)
Targeted therapy	MuSK-CAART	Muscle specific tyrosine kinase chimeric autoantibody receptor T-cells
	CAR-T	RNA-engineered chimeric antigen receptor T-cell therapy targeting B-Cell Maturation Antigen (BCMA)

BCMA = B-Cell Maturation Antigen, BAFF = B-Cell Activation Factor, APRIL = Proliferation-Inducing Ligand, JAK = Janus Kinase, BTK = Bruton Tyrosine Kinase, FcRn = neonatal Fc Receptors.

In terms of symptomatic treatment, two types of drugs have been tested in randomized clinical trials in MG patients in the past decade. Tirasemtiv is a fast skeletal troponin activator, which has been tested in patients with acetylcholine receptor MG. This drug showed potential but not significant efficacy and had an acceptable safety profile (55). However, in the past decade, no new randomized clinical trials have been started or announced and the use of tirasemtiv in LEMS is not expected soon. Beta receptor agonists like salbutamol (beta 2) and ephedrine (beta 1) have shown some efficacy in MG and especially in congenital myasthenic syndrome (56, 57). In 2019 an RCT was started to study the effect of salbutamol as adjuvant therapy in MG, but no results are currently available. The mechanism of action is not clear, but researchers have hypothesized that beta agonists provide a compensatory mechanism to stabilize motor endplate structures. This is especially the case in patients treated with pyridostigmine, which has been suggested to have a destabilizing effect on the neuromuscular junction (56). A large effect of beta agonist in the symptomatic treatment of LEMS seems doubtful. However, one case report on the use of ephedrine in one patient with LEMS showed clinical improvement. The improvement was most marked with a combination of amifampridine and ephedrine, although potential cardiovascular side effects could limit its use (58).

Most new treatment modalities studied in MG have an immune modulating effect (59, 60). These new drugs are not specifically designed for MG but have their origin in other autoimmune diseases such as multiple sclerosis, ulcerative colitis, or systemic lupus erythematosus. Some of these new drugs exert their effect early in the immune response at the B-cell level and act by inhibiting the production of autoantibodies. Other drugs have their effect at a later stage in the immune response and act by diminishing the autoantibody levels. Of all immunomodulating drugs being tested in RCTs in MG, only rituximab has been mentioned in patients with LEMS in case reports. Three patients were treated with rituximab, of whom all three experienced improvements, but did not achieve remission (61, 62). Presumably, other new immunomodulating drugs have potential benefit in LEMS patients as well, although uncertainty on their long term safety, high cost and low level of evidence are barriers for incorporating these drugs in treatment guidelines of LEMS (63).

A drug specifically developed for MG is MuSK-CAART. This drug targets B cells that produce autoantibodies against muscle-specific kinase (MuSK) (64). By design this therapy is only effective in MuSK positive MG, but effectiveness of this therapy can accelerate the development of a comparable drug targeting VGCC autoantibody producing B-cells to treat LEMS. Another targeted therapy, CAR-T therapy, investigated in the Descartes-08 trial comprises of patients' own T-cells that have been modified ex-vivo with RNA to target B-cell maturation antigen (BCMA) (65). This therapy shows promising results in severe MG, however serious adverse reactions might prove a limitation of implementing CAR-T therapy in mild to moderate disease (42).

Towards Novel Treatment Options For Lems

Implementation of novel treatments for LEMS has been hampered by the rarity of the disease and relative paucity of data on valid outcome measures. Previous trials have sometimes used MG-specific outcome measures, which are not ideal for LEMS as they tend to be heavily tilted towards ocular and bulbar weakness, which is rarely the main limitation in LEMS patients.

We suggest the following requirements for a future trial on a potential novel treatment: 1) sufficient power (due to the rarity of the disease) by performing a multicenter trial or using an alternative trial design. 2) a cross-over design to reduce the number of patients required. 3) LEMS-specific but relevant and quantitative primary outcome measure. As a primary outcome measure, we would suggest the 3TUG (three Times Up and Go) test which has been used in the most recent RCTs (15, 16) in LEMS and which has been shown to have a high reliability (17). Potential secondary outcome measures could include neurophysiological outcome measures, the 15-item revised version of the Myasthenia Gravis Quality of Life (MG-QOL15r) questionnaire and muscle force dynamometry, which provides objective, reproducible measures of muscle force in arm and leg muscles. In addition to requirement 1, an alternative trial design can be an N-of-1 trial, in which the patient functions as its own control and can be entered in multiple treatment cycles. Evidence of these treatment cycles can be aggregated to produce population treatment effect estimates. An N-of-1 trial requires fewer patients to assess a meaningful treatment effect than a traditional RCT (66, 67). This trial design is suitable in LEMS because LEMS is a chronic or slowly progressive disease and symptoms are relatively stable and quantifiable. However, the use of N-of-1 trials is limited to treatments with a rapid response and few lasting carryover effects, so disease modifying therapy such as the new immunomodulating therapies tested in MG are not ideal candidates for an N-of-1 trial (66, 68).

Disclosures Of Conflicts Of Interest

LRN reports no disclosures relevant to the manuscript. WRB, KJMS and TvG are employed by the Department of Clinical Pharmacy and Toxicology which produces and

supplies the 3,4-diaminopyridine base modified release tablets to 40-50 users in the Netherlands. In the last 3 years TvG has received lecture fees and consulting fees from Roche Diagnostics, Thermo Fisher, Vitaeris, CSL Behring, Astellas and Aurinia Pharma. In all cases money has been transferred to hospital accounts, and none has been paid to his personal bank accounts. JJGMV has been involved in MG research sponsored by the Princes Beatrix Fonds, Health Holland and consultancies for Argenx, Alexion, and NMD Pharma. Reimbursements were received by the LUMC. He is coinventor on patent applications based on MuSK-related research. The LUMC receives royalties for MuSK antibody assays. He is a member of the Target-to-B! consortium. MRT reports trial support from Argenx and Alexion, consultancies for Argenx and UCB Pharma and research funding from NMD Pharma, with all reimbursements received by Leiden University Medical Center. LRN, JJGMV and MRT are members of the European Reference Network for Rare Neuromuscular Diseases (EURO-NMD).

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Challenges Managing Myasthenia Gravis: An International Perspective

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ABSTRACT

There have been increasing breakthroughs in the diagnosis and treatment of myasthenia gravis over the past decades. However, most published research in myasthenia is conducted in developed regions, such as the US, Canada and Europe. The challenges faced in these regions may be different from other areas of the world, often under-resourced, such as having fewer neurologists, limited or no access to specialised testing for myasthenia, and poor access to some therapeutic interventions. During the 14th International Conference for Myasthenia Gravis and Myasthenic Disorders, we organized a panel of neurologists and researchers who work with people living with myasthenia in different world regions. The goal was to stimulate discussion around common challenges as well as those that are specific for given areas. Ultimately, we aimed to develop networks of clinicians caring for people living with myasthenia gravis around the world, to improve patient care. We present a summary of challenges using a case format by region, and a discussion around common threads and potential next steps.

Key words: Myasthenia gravis, low-resource settings, global health

Introduction

Over the past century, the prognosis of myasthenia gravis (MG) has dramatically changed. From a described mortality close to 90% in the early 1900s to ≤ 10% in the 2000s, MG is a treatable disease where approximately 90% of patients improve with available treatment[1]. We have also seen an increase in the incidence of MG over the last decades, likely a combination of greater awareness and improved diagnostic abilities, as well as a probable increase in incidence with recognition of late and very-late onset of disease.[2–4] Causative antibodies can be found in up to approximately 90% of people with autoimmune MG, and genetic testing for congenital myasthenic syndromes is more readily available in developed countries. However, we know that there is still a large number of patients who, despite available treatments, live with high disease burden.[5, 6] Newly approved treatments for MG, such as eculizumab and efgartigimod, have the potential to further improve patient care due to rapid onset of benefits and good safety profiles;[7, 8] however, there are marked limitations to their implementation in practice, in large part due to their very high costs.[9]

These challenges have been recognized in well-resourced, developed countries, heavily biased towards the US, Canada and Europe; however, the perspectives from other populations are not usually incorporated. To understand gaps in MG care around the world, we assembled a panel of neurologists caring for people with MG in Argentina, United Arab Emirates, South Africa and South Korea, as well as a social scientist researching MG patient experiences in China. During the meeting, each panelist presented cases representing specific challenges they face in their countries. Other panelists commented on how that situation would present in their respective countries, and the impact of resource limitations. Additionally, we had rich audience interactions from participants from different countries. We will present a summary of challenges by country, as presented by each panelist, followed by a discussion of common and divergent issues discussed during the meeting.

Perspectives from South Africa

Treatment-refractory ophthalmoplegia in MG is increasingly recognised as an indication for urgent attention.[10, 11] We have reported several cases with AChR-antibody positive (AChR-pos) moderate to severe generalized MG, who showed excellent responses to MG immune regimens except for their extraocular muscles (EOMs), which remain paretic and treatment refractory. Observational data collected in our clinic previously showed that patients with MG-associated ophthalmoparesis who are treated with higher doses of prednisone within 12 months of symptom onset, compared to those who start prednisone >12 months, have a significantly higher chance of resolution of the weak EOMs.[10]

We discussed a case with AChR-pos MG who started immune treatment for MGFA class IVB within 6 months; the patient received steroids, azathioprine, underwent a thymectomy and subsequent cyclophosphamide pulses, but remained with refractory ophthalmoplegia/ptosis for which he was referred for further management 3.5 years later. Thyroid function was normal. MRI of the orbits showed normal EOM STIR sequence intensities. As the referral occurred during the COVID-19 pandemic, he received a vaccination (uncomplicated) three months in advance of a planned single rituximab infusion. Despite residual partial ophthalmoplegia on examination, the patient's EOMs showed substantial functional improvement which remained stable for seven months on continued maintenance azathioprine alone.[12] However, within a week of receiving a COVID-19 vaccination booster, the extraocular muscles/ptosis decompensated. The case raised discussion around treatment-resistant MG-related ophthalmoplegia, which appears to be more common among Chinese and African children and adolescents with AChR-pos MG,[13] although we have reported cases in older patients and those with and without MuSK-antibody positive MG.[14] Longitudinal observations of MG patients with severe EOM involvement, likely in the setting of genetic susceptibility, have shown that earlier immune treatment to prevent prolonged loss of muscle contractility as a result of antibody-mediated 'functional denervation', will impact the activation of atrophy pathways in EOMs and thereby clinical reversibility.[11] MG patients with refractory ophthalmoplegia may benefit from the use of crutch glasses and prisms. Moreover, in selected cases, surgical correction may be considered, although the success of treatment-refractory ptosis surgery is far better than EOM re-alignment surgery.

The other point of discussion was related to the "MG decompensation" noted within days of an mRNA COVID-19 vaccine. A prospective clinic cohort of 91 stable MG patients, of whom 79% were on a single immunosuppressant, were followed after receiving an mRNA COVID-19 vaccination; at 7 days, 58% developed transient non-specific vaccine-related symptoms, but only 2 experienced mild emergence of MG symptoms.[15] Surveillance groups/networks in the United Kingdom identified seven new MG cases developing symptoms within 2-14 days of a COVID-19 vaccination, although two cases only developed their MG symptoms after a 3rd vaccine dose.[16] Most of these patients developed generalised MG with significant bulbar symptoms. The authors reviewed seven other published cases from elsewhere in whom five developed MG symptoms after the second vaccine dose.[16] Therefore, although MG symptoms emerged in rare cases within days of a COVID-19 vaccine, most known MG patients on treatment tolerated the vaccine well.[15]

An adolescent with double seronegative (AChR- and MuSK-antibody negative by radioimmunoassay (RIA)) and moderately-severe refractory MG (MGFA class 3B) was discussed. She remained with moderately-severe and fatigable leg weakness which was refractory to >2 years each of azathioprine and cyclosporine, in addition to dependence on 30mg daily prednisone. A single infusion of 600mg rituximab resulted in >50% reduction in prednisone and pyridostigmine dosing and minimal leg fatigability (MGFA class IIA) after 3 months, which was sustained for > 12 months similar to previous cases.[12]. This case highlights the cost-effectiveness of a single dose of rituximab in resource-limited settings, and is in keeping with a recent trial of low-dose rituximab in AChR-pos MG.[17]

Accessibility to diagnostic assays other than RIA vary from country to country and can make a difference in the therapeutic management of "seronegative" by RIA MG patients.

Perspectives from South Korea

This is a fictional case of refractory AChR-pos MG. A 52-year-old male with Masaoka stage IVa WHO type B2 thymoma. Due to severe bulbar palsy, he often developed aspiration pneumonia, which led to myasthenic crisis. The dose of corticosteroids could not be lowered to less than 15 mg/day. Immunosuppressant agents including azathioprine, cyclosporine, and tacrolimus were not effective. IVIG and rituximab showed only partial effect. Due to prolonged use of corticosteroids, CMV retinitis, osteoporosis, iatrogenic Cushing's syndrome occurred.

For refractory MG patients, newly developed therapeutic agents such as complement inhibitors can be a good treatment option.[7] In South Korea, National Health Insurance (NHI) is mandatory and covers almost all of the population. The reimbursement and price of drugs is strictly regulated by the government.[18] After approval of new drugs by Ministry of Food and Drug Safety (MFDS), major factors that hinder access to the new drugs are delays in drug pricing negotiations between the National Health Insurance Service (NHIS) and the relevant drug manufacturer and in process of determining whether to reimburse the drugs or not.[19] Eculizumab was approved by MFDS in 2019; however, reimbursement and eculizumab pricing negotiations have been stalled for a long time. The NHIS is concerned about the financial risk from introducing the expensive new drug that costs more than $400,000 a year per a patient, whereas the manufacturer wants to maintain its drug price internationally. In the treatment of MG, eculizumab is available but not accessible in South Korea. Because policymakers may refer to a drug price information from other countries in their own negotiation on the drug price, low price of a drug in one country can lead to price cuts in other countries.[20] Therefore, a country's low drug pricing policy may force some manufacturers to abandon the market of the country. This situation seriously hinders refractory MG patients in the country from accessing new treatment options. In order to improve accessibility to new

treatment, patient-oriented approaches with reasonable policies and drug prices are needed.

A second case was a 49 year-old AChR-pos MG patient with Masaoka stage I WHO type AB thymoma in stable condition with MG-ADL 1 or 2. However, about two weeks after COVID-19 infection, MG exacerbation occurred. He was not vaccinated for COVID-19. He was treated with plasma exchange in the intensive care unit.

Most of previous studies about effects of COVID-19 infection on MG were performed in the early stages of the COVID-19 pandemic, when COVID-19 vaccination was not available.[21, 22] The situation in South Korea in early 2022 was different from those in other countries at the time. Most COVID-19 infections have occurred since February 2022. Almost of all COVID-19 infections are caused by SARS-CoV-2 Omicron variants, of which severity is milder than the other previous variants.[23] As of May 2022, full vaccination rate was about 86% of the population and booster was given to more than 63% of the population.[24] Therefore, a substantial number of MG patients had been vaccinated against COVID-19. In an analysis of 40 Korean MG patients infected with COVID-19, 28 patients were vaccinated before COVID-19 infection and 12 patients were not. The comparison between the vaccinated and unvaccinated MG patients are summarized in Table 1. The vaccinated MG patients had lower frequency of hospitalization for COVID-19 and MG worsening or exacerbation after COVID-19 infection than the unvaccinated MG patients. This is in keeping with previous studies showing that severe COVID-19 outcomes are less frequent in vaccinated than unvaccinated individuals.[25,26] Because the severity of infection can influence the disease activity of MG, vaccination against COVID-19 may have preventive effect of MG worsening or exacerbation through protection against severe COVID-19 infection. Although there have been studies showing the safety of vaccination against COVID-19 in MG patients,[27–30] no studies have evaluated the effect of the vaccination on MG deterioration after COVID-19 infection. Further large-scale studies are necessary to investigate the preventive effect of COVID-19 vaccination on MG worsening or exacerbation triggered by COVID-19 infection.

Perspectives from the United Arab Emirates

A 42-year-old woman who has been diagnosed with generalized seronegative MG (negative AChR, MuSK and LRP4 antibodies) for almost 10 years. Her disease started with ocular and bulbar manifestations followed by limb weakness. Her diagnosis was supported by the significant decrement response (> 60%) with 3Hz repetitive nerve

Table 1. Comparison between vaccinated and unvaccinated against COVID-19 MG patients who infected with COVID-19 in Korea

	Vaccinated (n = 28)	Unvaccinated (n = 12)	P-value
Age at COVID-19 infection	49.50 [38.25 – 61.5]	46 [41.25 – 56.5]	0.873
Age at MG onset	35 [24.25 – 48.5]	39 [32 – 47]	0.192
Sex			1.000
Male / Female			
Body mass index	24 [22.5 – 27]	23 [18 – 26]	0.118
Antibody status			0.833
AChR-Ab	22	9	
MuSK-Ab	1	1	
No detectable Abs	5	2	
Generalized Disease	23	11	0.648
MGFA at nadir			0.827
I	5	1	
II	8	4	
III	7	4	
IV	2	1	
V	6	2	
MG-ADL score at last visit before COVID-19 infection	2 [0.75 – 5]	3 [0.5 – 5]	0.425
Hospitalization for COVID-19 infection			0.001
Non-hospitalized	27	6	
Hospitalized	1	6	
Change in MG status			0.021
Worse or Exacerbation	5	7	
Improved or Unchanged	23	5	
Recovery after COVID-19 infection			1.000
Completely recovered	22	9	
Partially recovered	6	3	

stimulation. Over the years, she has been on different immunosuppressive medications with either poor response, or significant adverse events. IVIG was not effective; she developed significant psychiatric side effects, elevated liver enzymes and intolerance to steroids, methotrexate and azathioprine respectively. The patient was eventually started on rituximab, which resulted in subjective 30% improvement in her strength and respiratory function, and over 18 months she received 3 cycles. During this period, her MG-ADL score ranged between 9-12 points, and her MG-QoL15 score between 19-21 points, without significant objective benefit after rituximab. Patient declined to try other medications such as tacrolimus, eculizumab or efgartigimod, despite severe limitations to her daily life activities, preferring to stick with the medication that is "keeping me out of trouble" (patient's words).

Discordance between physician and patient perception of disease control and symptom severity has been a subject in research, especially in prevalent chronic conditions such as asthma[31] and rheumatoid arthritis.[32] Presence of patient-physician discordance contribute to poor symptom control while concordance leads to better clinical and patient reported outcomes.[33] Several factors had been implicated in patient-physician discordance, these include health literacy, race/ethnic minority, poor communication and use of anti-depressant medications (Hirsh & Kenney-Riley).[34,35]

MG is a chronic and potentially disability condition. Studying patient-physician discordance (or concordance) in disease control is an important step in improving the care for MG patients, especially in the current era of emerging new therapies.

Perspectives from China

Our presentation focuses on preliminary findings from a patient journey study on myasthenia gravis patients in China. Ethical approval of this study was obtained from the Survey and Behavioral Research Ethics Committee of the Chinese University of Hong Kong (approval no: SBRE-21-0260). The purpose of the study is to identify the factors contributing to MG relapse in China and to provide insight on how to improve care for MG patients. The findings were based on semi-structured in-depth interviews conducted between January 2022 and May 2022, with 28 MG patients or their main caregivers, 3 neurologists, 2 thoracic surgeons, and 2 Traditional Chinese Medicine practitioners in China.

According to a recent study, after adjusting age and sex, the incidence of MG in China is 0.68 per one-hundred thousand. The disease can occur at all ages but occurs most frequently between the ages of 30 to 50. There is a slightly higher incidence rate among females (0.76 per 100,000) than males (0.60 per 100,000).[36] The in-hospital mortality rate is 14.69%, with the main causes of death being respiratory failure and pulmonary infection. More than 64% of the MG patients with thymomas had thymectomy. Consistent with previous research, the majority of our MG patient participants were female aged between 18 and 65. All our participants had generalized MG, and 70% of them had undergone thymectomy. Eight of them self-identified as refractory cases.

Our study revealed that, although most of the patient participants were AChR-pos, 26 out of 28 patients experienced relapses, or even recurrence of crises, with varying reasons. Patient compliance was identified as the most common cause of relapse among MG patients in China. Many patients took medication not prescribed by their doctor, or made changes to the dosage of their medication at their own discretion. This was often due to ineffective or inefficient communication with their doctors, deteriorated doctor-patient relationships, or a lack of regular follow-ups. This is a significant problem as it can lead to the worsening of the patient's condition and even to a crisis.

Another factor contributing to the relapses of MG patients in China was the lack of a regular doctor or medication plan for migrant workers who had to work inter-provincially. These patients often had to seek medical help from different hospitals and doctors, making it difficult to establish a consistent treatment plan. This highlights the need for more coordinated care and better understanding of the unique challenges faced by migrant workers in the management of MG.

Overwork and emotional impact were also identified as significant factors that led to MG relapses. Patients who continued to work after the onset of their symptoms, or who were overworked due to household chores, childcare, and other factors were more likely to experience relapse. Emotional stressors, such as death of relatives, problems with family or spousal relationships, economic and psychological pressures, were also identified as potential causes of relapse. This highlights the need for a more holistic approach to the management of MG, which should not just focus on the physical symptoms but also on the emotional and psychological well-being of the patient.

Other factors contributing to MG relapse in China included seasonal flu, or, for female patients, menstrual periods, pregnancy and childbirth. Some patients did not allow family members to participate in disease management due to their strong personalities, which might further contribute to MG relapse. These findings emphasize the need for multidisciplinary teams for managing pregnancy and childbirth, stronger social support in disease management, as well as the importance of patient education to increase awareness of the disease.

There was one patient whose patient journey could mostly illustrate many of the factors we discussed above. The patient was a 37-year-old female who experienced multiple relapses while trying to reduce the dosage of steroids, as per her doctor's advice. As the quote indicates, the patient, after the several relapses, lost trust in doctors and frequently changed her attending doctors, often

increasing or decreasing her medication according to her own assessment of her condition. She would only go to her hometown hospital to receive IVIG when her symptoms worsened, as the doctors there did not know much about the disease. The patient had to dictate the dose and infusion method for the IVIG treatment to the doctors.

In conclusion, the study highlights the various factors that contribute to MG relapse in China. It emphasizes the need for effective communication between patients and their doctors, especially in terms of medication compliance, regular follow-up, and multidisciplinary teams for managing pregnancy and childbirth. The study also underlines the importance of social support, as well as patient education to increase awareness of the disease.

Perspectives from Argentina

Case 1 is about a 46-year-old patient who at 33 years old was diagnosed with MG, AchR-pos, associated with thymoma, MGFA class IIA at onset. He underwent a video-assisted thymectomy (VATS), with pathology consistent of thymoma WHO type AB, Masaoka-Koga Stage I. Afterwards, he was diagnosed with Morvan Syndrome, with positive leucine-rich glioma inactivated 1 (LGI1) and contactin-associated protein like-2 (CASPR2), and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) antibodies. In two opportunities, he presented a crisis/exacerbation of both conditions simultaneously. He remained with invasive mechanical ventilation dependence despite IVIG, steroids and azathioprine. Successively and/or concurrently, different organisms were isolated in the sputum. The impossibility to eradicate the respiratory infection led to a reduction of the dose of azathioprine, with worsening of MG. A chest MRI showed images suspicious of pulmonary neoplasia in the right inferior lobe, previously interpreted as pneumonia. The biopsy showed a recurrence of the thymoma.

Thymomatous patients can have a more severe presentation and a higher risk of death. In one series, ~35% of the deaths were attributable to thymoma recurrence and dissemination.[37] Of these recurrences, 48% coincided with an MG flare-up or crisis. In our case, the red flags for tumor recurrence were antibodies positivity, symptom worsening when azathioprine was reduced, and antecedent of VATS (not the gold-standard procedure for thymoma).

This case highlights many issues, including managing the coexistence of two autoimmune neurological disorders, and the difficulties managing adequate immunosuppressive treatment when there are chronic infections that contribute to MG exacerbations. Finally, this case highlights the difficulties in managing patients with refractory MG.

The second case is about a 38-year-old woman diagnosed with AChR-pos MG at 16 years-old. The initial MGFA class was IIB. Since her diagnosis, she receives pyridostigmine and prednisone. She had several therapeutic failures, and was considered as refractory MG. She suspended azathioprine because of the elevation of liver enzymes and presented three myasthenic crises. She received cyclophosphamide IV, and 18 months later, she developed her fourth crisis. In this opportunity, treatment was initiated in three steps with IVIG followed by rituximab. She then received tacrolimus for a long time. She miscarried her first pregnancy, then she had her only daughter, who suffered from neonatal MG. Later, there was an important reduction in the steroid dose and QMG score. During the puerperium and the following years, she presented frequent exacerbations. She suffered a fifth and a sixth myasthenic crisis, in the context of a renal abscess, and discontinued tacrolimus. Later, and after numerous difficulties with the health system, and many years after her initial diagnosis, she started treatment with eculizumab.[7] Her MGFA-Post-intervention status was improved.

In patients with refractory MG, the therapeutic decision is conditioned by the availability and access to medications and interventions in the health system. Difficulty in monitoring, controlling, and acquiring the drug can perpetuate the refractory or pseudo-refractory status in these patients.

Discussion

A common thread across different presentations was the difficulty accessing new therapeutics for MG, especially in low-resource settings. This is especially relevant for treating patients with MG who are refractory to first-line treatments that are more commonly available. We discussed the use of rituximab that, as an older drug, is less costly than newer medications and maybe more accessible. During the panel discussion it also became evident that access to diagnostic testing for MG varies by region, such as variable access to antibodies, including RIA and cell based-assay for AChR, as well as for MuSK, and variable access to specialised electrophysiology testing such as single fiber EMG. There are also major differences in access to genetic panels for patients with suspicion of congenital myasthenia syndromes, for example in refractory seronegative patients.

Another common thread among presenters was the management of chronic infections and the relationship between infections and MG exacerbations, especially as people with MG have a higher risk of infections—especially respiratory.[38] This has become more relevant with the COVID-19 pandemic, where clinicians had to make therapeutic decisions early in the pandemic before evidence specific to MG became available.

The factors associated with MG relapse in China are also present in other countries, and during discussion the importance of communication between patients and physicians was emphasized, although it was also noted that there can be discrepancies in the assessment of disease status. The lack of detectable autoantibodies may raise diagnostic uncertainties, which may further compromise patients' trust in the physician. Of note, patients with seronegative

MG represent a small— but not negligible— proportion of MG cases. The importance of multidisciplinary teams for managing pregnancy and childbirth was highlighted in the presentation from China, but was also reflected in cases from other panelists.

In summary, our international panel identified many aspects of MG care that are hindered in different countries. In some cases it is due to lower resources overall, but sometimes it has to do with health policies around access to expensive medications, access to high risk perinatal care and overall robust multidisciplinary health teams. The importance of studying infections in MG and developing related guidelines, can help prepare for future epidemics. Developing networks of clinicians who care for people living with MG in different regions will be important to help overcome some of these limitations and improve patient care.

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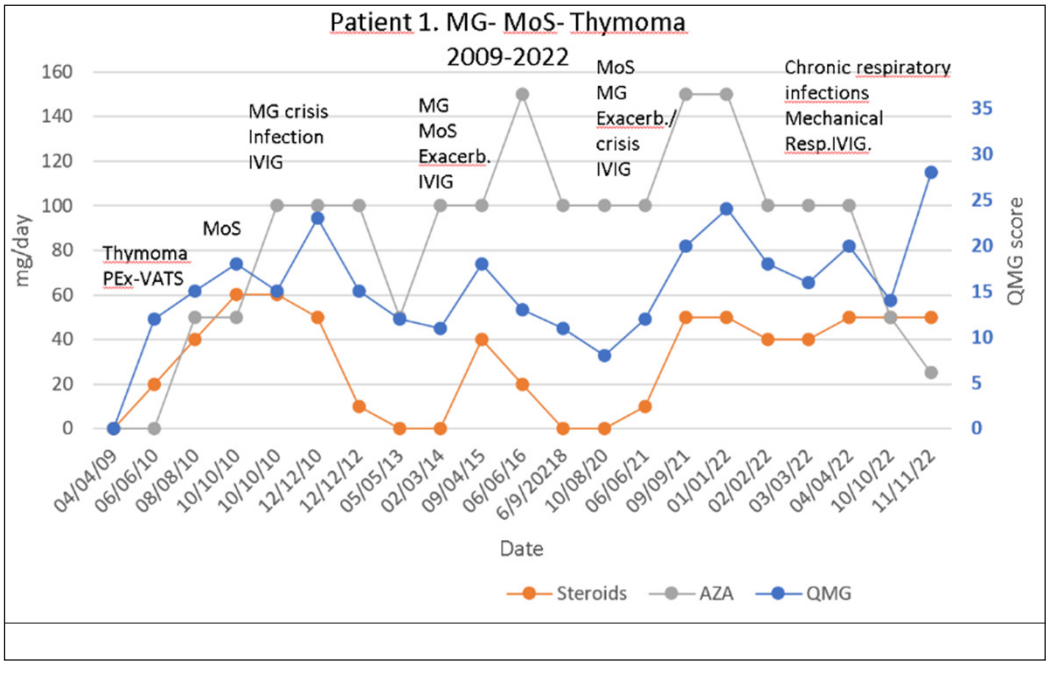
Figure 1. Clinical course of patient with MG and Morvan Syndrome



Congenital myasthenic syndromes: β-adrenergic receptor agonist treatment

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ABSTRACT

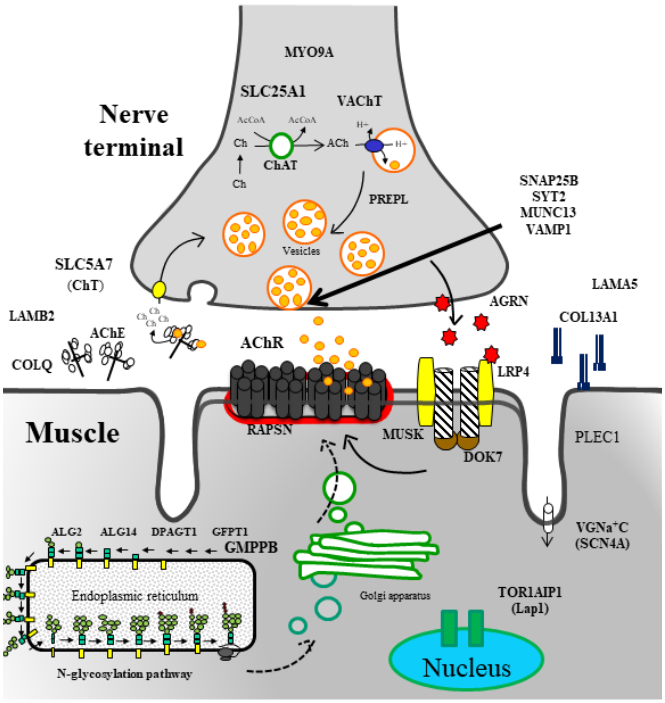
Acetylcholinesterase inhibitors, such as pyridostigmine, are the standard symptomatic treatment for myasthenia gravis (MG), and so have naturally been applied to the genetic forms of myasthenia, termed congenital myasthenic syndromes (CMS). Although effective for many CMS in others there is no clear response, and in some it is actually harmful. Now, with greater understanding of the mutations and molecular mechanisms underlying CMS, treatments can be tailored for the specific syndrome, and, depending on disease severity and patient response, this can include utilizing different combinations of drugs. In CMS, over the last 15-20 years β2-adrenergic receptor agonists have moved from occasional use to a mainstream medication. Many patients have life-transforming improvement both when the β2-adrenergic receptor agonists are used alone or in combination. Here we feature how the identification of DOK7-CMS first highlighted the consistent benefit of β2-adrenergic receptor agonists as medication and how its application to many different CMS subtypes evolved. The molecular pathogenic mechanisms for many CMS subtypes are now established, and this report will also discuss a hypothetical rationale for which forms of CMS are likely to benefit from the β2-adrenergic receptor agonists.

Key words: *Congenital myasthenic syndrome, β2-adrenergic receptor, ephedrine, salbutamol, albuterol, DOK7, COLQ, CHRNE*

Introduction

More than 30 genes have been identified in which mutations can underlie defective neuromuscular transmission (Figure1)[1,2]. The mutations can have their effect through a variety of molecular mechanisms, and even mutations within the same gene can lead to different phenotypes and very different clinical pictures. The congenital myasthenic syndromes (CMS) are hereditary disorders, and therefore there is no role for immunomodulatory agents. However, there are a number of drugs that can be used to provide symptomatic treatment

Figure 1. Diagrammatic representation of a motor endplate illustrating the potential location of the many genes/proteins in which mutations that underlie a congenital myasthenic syndrome are identified.



for the various different underlying molecular pathologies. The present repertoire includes acetylcholinesterase inhibitors (mainly pyridostigmine), 3,4-diaminopyridine (3,4-DAP), acetylcholine receptor (AChR) open-channel blockers (fluoxetine, quinidine), the β2-adrenergic receptor agonists ephedrine and salbutamol/albuterol, or different combinations of these agents [1,3]. It is important to recognize that drugs that benefit one form of CMS may be harmful in another, even when the mutations lie in the same gene.

Reversible, competitive acetylcholinesterase inhibitors, such as pyridostigmine have been the mainstay of treatment for myasthenia gravis (MG) for many years. By blocking the action of acetylcholinesterase, the presence of ACh within the synapse is prolonged, thus giving a greater probability of reaching the depolarization threshold for generation of a muscle action potential. Although effective for many CMS, in others there is no clear response, and in some it is harmful. Pyridostigmine is quite clearly contraindicated for endplate AChE deficiency due to mutations in COLQ, as there is already a deficit of acetylcholinesterase function [4]. Similarly, in the dominantly inherited slow channel syndrome, increasing the level and duration of ACh within the synaptic cleft is only likely to exacerbate this excitotoxic disorder [5]. The use of AChR open channel blockers, fluoxetine or quinidine, can be remarkably effective for

some slow channel mutations [6], but the response is less marked for others. Ephedrine, a β2-adrenergic receptor agonist that was originally derived from the *ephedra* plant family in China, was reported to produce some benefit for patients suffering from MG in the 1930s [7,8], but it was largely replaced once acetylcholinesterase inhibitors were found to give consistent and effective symptomatic treatment for the disease [9]. In CMS, clearly an alternative to cholinesterase inhibitors was required for endplate AChE deficiencies, and in these patients a beneficial response to ephedrine was reported [10]. In addition, anecdotally, in other CMS, patients would sometimes report having benefit from ephedrine or other adrenergic agonists. However, the identification of DOK7 mutations as a major cause of CMS [11] and their slow but remarkable improvement with β2-agonist medication [12] provided the impetus for their more widespread adoption and thus re-emerging as a mainstream option in treatment.

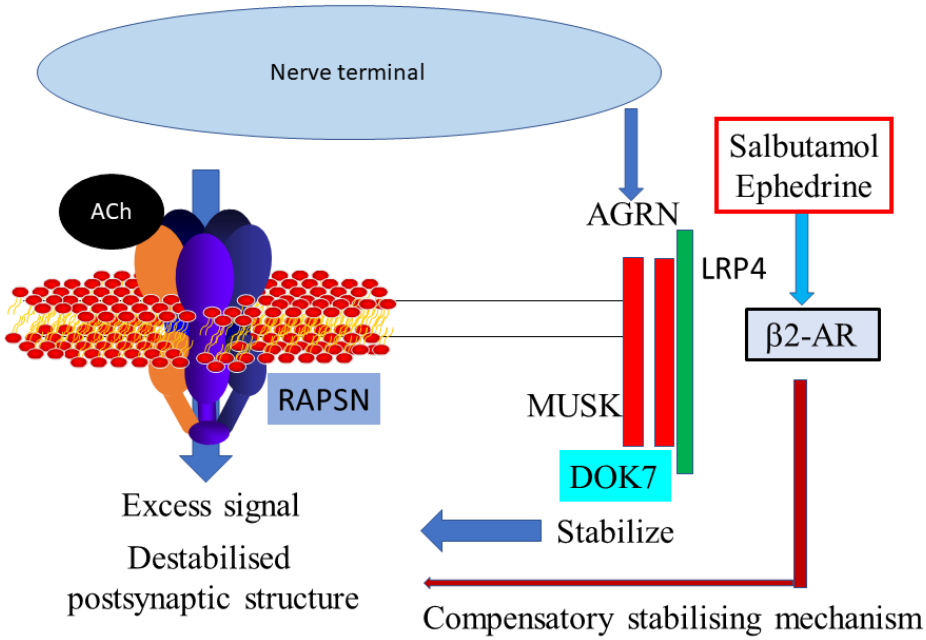
β2-adrenergic receptor agonists in the treatment of DOK7-CMS

After detecting mutations in DOK7 in a cohort of CMS patients with unknown genetics [13] it quickly became apparent from the clinical notes that there was a lack of response to cholinesterase inhibitors, but many patients insisted they felt better when taking β2-agonist medication, either ephedrine or salbutamol/albuterol [14]. Following this observation, a prospective study was set up to record the long-term response to ephedrine of patients with newly identified DOK7 mutations who were not previously on β2-agonist medication [12]. Ephedrine given at doses between 15 and 90 mg/ day improved muscle strength as measured by the quantitative myasthenia gravis (QMG) severity score and mobility scores [15]. Unlike treatments such as pyridostigmine or 3,4-DAP which in other forms of CMS take effect quickly, ephedrine was found to lead to delayed and progressive improvement in muscle strength taking place over months. Indeed, patients would often be found to be continuing to improve over a year from first starting treatment. The QMG score, designed for MG, is not an ideal method for severity scores in DOK7-CMS, and those scores that reflected the pattern of proximal muscle weakness seen in DOK7-CMS (such as times of arm raise, leg raise, or neck raise) were those that showed the most improvement. Moreover, the patients themselves reported profound benefit in their everyday living activities. What is also of note is that, while patients take a long time to improve, if they stop taking their medication they weaken rapidly back to baseline, usually within three or four days. Although ephedrine was used in this initial study, in a number of countries ephedrine is not easily available, in which case salbutamol/albuterol has been found to be an equally good alternative medication [16]. Salbutamol/albuterol is well tolerated in children. Many child neurologists have experience with using it in other neuromuscular disorders, and so it is frequently the drug of choice. Our initial observation and results of the prospective study are supported by numerous reports of the beneficial use of β2-agonists for DOK7-CMS where it is seen to be effective from early childhood through old age [17-21]

Treatment of CMS due to mutations in the AGRN-LRP4-MuSK-DOK7 pathway governing neuromuscular junction formation and stability

MuSK, plays a critical role in the formation of neuromuscular synapses and in maintenance of the synaptic structure [22]. MuSK is activated following the interaction of nerve-derived AGRN with LRP4, which in turn interacts with MuSK through its third β-propeller domain, leading to MuSK dimerization and phosphorylation [23]. Neuronal AGRN contains specific short RNA splicing inserts (of 4, 8, and 11 amino acids) that give it effective AChR clustering activity and that are not present in other AGRN forms, such as muscle-derived AGRN. Neuronal AGRN is secreted from the motor nerve terminal to perform its function within the synaptic cleft. DOK7, an intracellular protein, interacts with MuSK at the juxtamembrane phosphotyrosine binding site to amplify phosphorylation of both MuSK and DOK7[24]. This initiates what is still a poorly understood signalling pathway; it is thought to include the recruitment of Crk and Crk-L by DOK7 [25] that is crucial both for efficient clustering of the AChR on the postsynaptic membrane and development and maintenance of the synaptic structure. Mutations in DOK7 impair AChR cluster formation and cluster complexity in myotube cell cultures [26]. In muscle biopsies from patients with DOK7 mutations the neuromuscular junctions are found to be smaller than normal, and there is evidence for unstable or reforming synaptic structures [13,24]. It would appear that β2-agonists are able to partially compensate for the impaired DOK7 function, presumably through affecting the pathway responsible for maintaining synaptic structure somewhere downstream of DOK7. Therefore, it was not surprising to find that patients with mutations in MuSK, in the β-propeller domain of LRP4, or in AGRN also have a marked beneficial response to β2-adrenergic receptor agonists. However, the precise molecular mechanism has yet to be elucidated. It is likely to be through the increase in intracellular cAMP and activation of various protein kinases in the vicinity of the motor endplate. Although many protein kinases have been shown to activate or enhance AChR cluster formation in cell culture models, a definitive understanding of their

Figure 2. Representation of the destabilizing effect of neurotransmission which can lead to dispersal of AChR clusters and deconstruction of synaptic structure with the balancing signal from the AGRN-LRP4-MuSK-DOK7 signaling pathway that stabilizes endplate structure. It is hypothesized the β2-adrenergic receptor activation can provide additional input into this pathway downstream from DOK7.



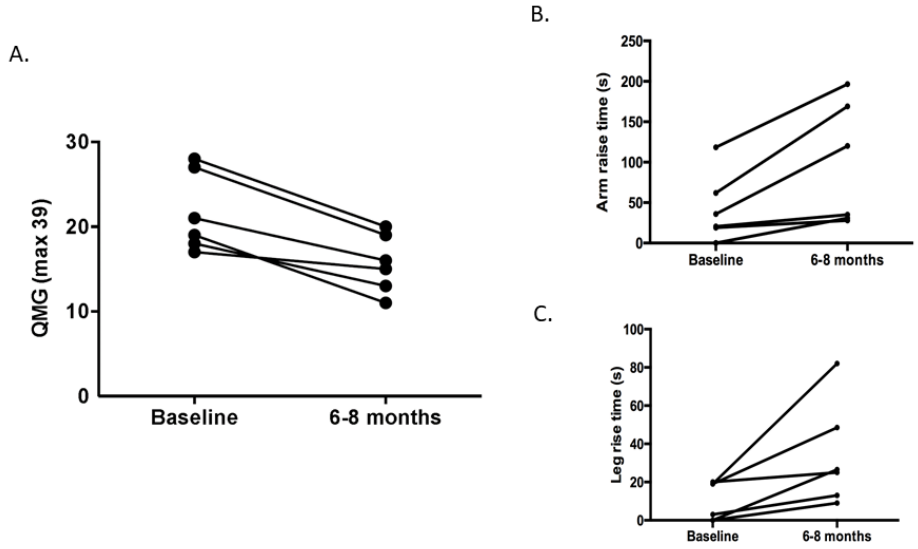
role at the neuromuscular junction *in vivo* is lacking. The slow and gradual response to β2-adrenergic receptor agonists treatment would argue against a direct effect on components of the AChR clustering pathway but rather for enhancement of the environment favoring stabilization of the synaptic structures [27]. Patients with MuSK or LRP4 mutations tend to respond equally as well as DOK7-CMS patients, but with AGRN mutations the response tends to be far less marked. This may be because AGRN is also synthesized by muscle (though not the neuronal RNA-spliced isoforms required for interaction with LRP4), and thus patients with mutations that also affect muscle AGRN often have a myopathic component to their weakness as well as impaired neuromuscular junction function. Though the β2-adrenergic receptor agonists may improve neuromuscular junction function they do not have a similar effect on the myopathic damage.

Patients with endplate acetylcholinesterase deficiency due to mutations in COLQ were identified well before DOK7-CMS was characterized and were reported to have a beneficial response to ephedrine [28], and this response has been confirmed in many subsequent reports [29,30]. The prolonged presence of acetylcholine in the synaptic cleft resulting from impaired breakdown of acetylcholine is thought to lead to excess calcium entry through the AChR and results in an endplate myopathy [31]. β2-adrenergic receptor agonists may help in repair of the disrupted neuromuscular junctions. Alternatively, there is some evidence suggesting that COLQ can interact with MuSK and contribute to the MuSK signalling pathway [32]. In which case the medication would be exacting a similar effect as seen in other cases with mutations in the AGRN-LRP4-MuSK-DOK7 pathway.

β2-adrenergic receptor agonists in treatment of severe AChR deficiency syndrome

Our current understanding of the maintenance of neuromuscular junction synaptic structure is largely based on a series of experiments in mice in which elements of the neuromuscular synaptic apparatus were 'knocked out' [22]. In the model derived from these experiments it has been proposed that the neurotransmitter ACh itself acts to destabilize both the neuromuscular junction structure and the aggregation of AChR on the postsynaptic membrane, but that the AGRN-LRP4-MuSK-DOK7 pathway works to counter this (Figure 2) [33,34]. COLQ mutations or anticholinesterases, by increasing the effective concentration and duration of ACh in the synaptic cleft, are likely to exacerbate destabilization of synaptic structures. Some evidence for the effect of anticholinesterases on the neuromuscular junction was obtained in the early 1970s, where in long-term usage they were found to affect the neuromuscular junction fine structure [35]. If β2-adrenergic receptor agonists can somehow enhance the AGRN-LRP4-MuSK-DOK7 pathway, they should be able to nullify this detrimental destabilizing effect of the cholinesterase

Figure 3. The response of patients with severe AChR deficiency syndromes on optimized pyridostigmine to the introduction of salbutamol/ephedrine to their medication. **A**. Reduction of the QMG severity score at 6-8 months. **B, C.** Response of arm raise and leg raise times after 6-8 months, illustrating the marked and consistent improvement seen for two quantitative components of the QMG scoring system.



inhibitors. Using this model as a basis, a rational hypothesis can be put forward that many other forms of CMS that are treated with anticholinesterase medication might find additional benefit from β2-adrenergic receptor agonists.

Cholinesterase inhibitors such as pyridostigmine are the first line treatment for patients with a deficiency of endplate AChR due to mutations in the AChR ε-subunit (CHRNE). These patients respond well to cholinesterase inhibitors but also often have some structural changes in their endplates with the loss of postsynaptic folds and the area of the endplates that stain with α-bungarotoxin elongated along the muscle fiber [36]. A number of AChR deficiency patients seen in clinic were found to initially respond very well to pyridostigmine, but over time the response diminished. This cohort became severely affected despite many attempts at optimizing their treatment. It was therefore hypothesized that these patients might benefit from the addition of β2-agonists to their medication that would counter the long-term detrimental effect of the cholinesterase inhibitors on the synaptic structures in these patients. A prospective study was set up to quantify any potential improvement. Medication was given on an outpatient basis with incremental dosage dependent on body weight and tolerability; the final dose ranged between 0.5 and 1 mg/kg/d for ephedrine and 0.05 and 0.2 mg/kg/d for salbutamol. In all patients, baseline therapy with pyridostigmine and 3,4-DAP or pyridostigmine alone remained unchanged for at least a year before adding salbutamol or ephedrine and during the follow-up period. Blood pressure, heart rate, and ECG were performed before treatment and at each dosage increment. All patients showed unequivocal improvement in functional ability as measured using the QMG severity score (Figure 3). Four patients who had been non-ambulant for many years acquired the ability to walk independently. Whereas patients with DOK7-CMS tend to see the benefit from their medication with β2-agonists as a gradual improvement over a period of months, the CHRNE AChR deficiency patients were found to respond more quickly with the majority of the improvement felt within the first month after initiation. Follow up of the patients showed that in most cases the improvement was sustained for years [37]. In our experience less severe cases of AChR deficiency due to CHRNE mutations frequently also benefit from the addition of β2-agonists but the improvement may not be so dramatic due to starting from a less severe baseline score.

β2-adrenergic receptor agonists in in mouse models of CMS

While it is clear that CMS patients benefit from β2-adrenergic receptor agonists, it is important to establish that this is truly due to a function effect at the neuromuscular junction.

Since CMS are rare, the easiest way to investigate is through mouse models. One mouse model that accurately reflects the respective human condition is the model for AChR deficiency syndrome [38]. In humans the fetal form of the AChR that contains the γ-subunit is expressed at low levels in adult muscle throughout life, whereas in mice, expression of the γ-subunit is turned off by three weeks

after birth. To reflect the human condition, the human γ-subunit was introduced into the mice under the muscle actin promoter to induce continuous low-level expression of the γ-subunit along the muscle fiber. The mice generated are myasthenic with fatigable muscle weakness, reduced endplate receptor number, and electrophysiological evidence of impaired neuromuscular junction function [38]. These mice were subjected to different treatment regimens to mirror what might occur in clinic, and in particular two cohorts were compared where one was given pyridostigmine alone and a second had salbutamol/albuterol added six weeks after pyridostigmine was initiated. The results of the study showed that addition of salbutamol reduced fatigable muscle weakness, reduced amplitude decrement of the compound muscle action potential on repetitive stimulation, and increased postsynaptic area labelled by α-bungarotoxin. Whereas pyridostigmine treatment reduced postsynaptic folds, the addition of salbutamol restored postsynaptic folding [37]. Thus, there is direct confirmation of the beneficial effect of salbutamol on neuromuscular junction structure and function. Similar results have been seen in a mouse model of acetylcholinesterase deficiency [39] and DOK7-CMS [40], although the DOK7-CMS mouse model is so severely affected that it is difficult to make direct comparison with the human situation. However, salbutamol did increase survival and the number of detectable endplates in the DOK7 CMS mouse model, again demonstrating its effect at the neuromuscular junction.

Concluding remarks

Treatment of CMS is often challenging. The current repertoire of drugs is not specifically licensed for CMS largely due to rarity and consequent lack of randomized controlled trial evidence of efficacy. Nevertheless, the CMS are a group of genetic disorders that mostly respond well to the current symptomatic treatments, which are often life-transforming. As stated earlier, an agent that provides benefit in one CMS subtype can be harmful in another. Thus, it is important to obtain an early genetic diagnosis, and it may also be crucial to establish molecular pathology for a particular mutation to guide treatment. It should be noted that some syndromes such as DOK7-CMS or MuSK-CMS may give the impression of a good response to cholinesterase inhibitors at first dosing but may subsequently suffer severe deterioration in their condition, emphasizing the imperative of a molecular diagnosis. Moreover, because each patient's response may be different or vary over time, it is important to optimize treatment and treatment combinations, and to provide follow up.

Over the last 10–15 years, β2-agonists have re-emerged as a mainstream option in treatment. Clearly an alternative to cholinesterase inhibitors was required for endplate AChE deficiencies, and in these patients a beneficial response to ephedrine was reported [28]. However, it was following the identification of DOK7 mutations as a major cause of CMS and their slow but remarkable improvement with β2-agonist medication that provided the impetus for its more widespread adoption. The idea that acetylcholinesterase inhibitors can be detrimental to neuromuscular junction structure suggests that the β2-agonists are potentially beneficial as a counteracting agent wherever cholinesterase inhibitors are appropriately used. In simple terms, this is whenever increased synaptic duration and density of acetylcholine can enhance signal transmission at the neuromuscular junction, then β2-agonists could be used to alleviate long term detrimental effects. Ephedrine and salbutamol can be used interchangeably, although we currently use salbutamol more frequently, because there is more safety data for its use in children and it is easier to prescribe. However, ephedrine is a good alternative for those in whom salbutamol causes side effects. Higher doses appear to give a greater response, but this needs to always be weighed against the side effect profile. In general, we would recommend increasing salbutamol progressively up to 4 mg twice a day over the course of 6 months when side effects are not apparent. Medication can be increased further up to 8 mg twice a day in older children/adults if required. In a few patients we, and others, have found that the beneficial response can diminish over time, which in most of the cases we have observed is associated with an adolescent growth spurt. Some have tried a 'drug holiday' to restore efficacy, but in our experience patients suffer an often serious and rapid decline with the withdrawal of medication which can then take many weeks or months to regain the functional levels seen prior to drug withdrawal. The precise function of β2-adrenergic receptors at the neuromuscular junction is not known, but there are reports that they are present at high density and that neuromuscular junctions may receive direct sympathetic innervation [41,42]. It is also known that β2-adrenergic receptor blockers are detrimental for myasthenia gravis or CMS patients, which further suggests a direct role at the neuromuscular junction. With time the role of β2-adrenergic receptors at the neuromuscular junction will be elucidated, but until then it is useful to view treatment for the many phenotypically different CMS as a balancing act between functional enhancement of signal transmission by cholinesterase inhibitors, that long-term are detrimental to synaptic structure, with the counterbalancing enhancement of structure by β2-agonists.

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Exercise training for autoimmune myasthenia gravis: A review of safety and effectiveness based on existing literature

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Introduction

Whilst autoimmune myasthenia gravis (MG) is a rare disease, it is the most common disease of the neuromuscular junction. Despite the significant advances in diagnosis and treatment, there is currently no cure for MG. Management consists of diverse pharmaceutic strategies to relieve symptoms and reduce the disease process with the ultimate aim of inducing disease remission.[1] Individuals not only suffer from the primary symptoms of MG but may also have secondary deconditioning as well as experience negative effects of medications such as corticotherapy. In recent times, the prevalence of MG has increased and whilst mortality has decreased over this century,[2] morbidity remains high, with symptoms and MG treatment creating huge burden for those living with this chronic disease. Health-related quality of life (HRQoL) is reduced, and MG has a negative impact on psychological, social, and economic well-being.[3,4]

Whilst a plethora of medications exist, with different therapeutic targets as well as varied management strategies,[5] the role of non-pharmacological management in MG is underdeveloped and underexploited.[6] Non-pharmacological treatments include allied health care such as physiotherapy, speech therapy, occupational therapy, psychological therapy but also music therapy, art therapy and exercise training.

Exercise is especially relevant to individuals with MG as exercise could have an effect on both the primary symptoms of the disease as well as the secondary consequences of MG. Exercise has demonstrated benefits in the general population as well as in various chronic neurological and non-neurological diseases.[7,8] Benefits include a reduction in pain,[9] fatigue,[10] anxiety,[11] depression[12] and morbimortality as well as improvements in strength and functional capacity. As MG is becoming more prevalent in older age, individuals have multiple comorbidities as well as age-related functional decline, which could be improved or managed with exercise. Exercise could also counter possible corticotherapy-induced myopathy and osteoporosis from long-term corticosteroid use. Further, exercise could play an immunomodulatory role in MG.[13] In addition, unlike many pharmacological agents, exercise has minimal, if any, side effects when adapted to the individual.

Observational studies evaluating daily physical activity (PA) demonstrate that individuals with MG may be less active and more sedentary than the general population.[14-16] Sedentary behaviour and reduced activity increase the risk for cardiovascular disease, type 2 diabetes, cancers and overall morbimortality.[17-19] Further, deconditioning creates a vicious cycle, increasing fatigue and weakness and consequently further limiting participation in activities of daily living (ADLs).[20] In addition to the health benefits that exercise can provide, individuals with MG express the desire to exercise. In a recent survey including 455 participants, 56% report exercising and of those that do not currently exercise, 77% express the desire to (NCT05408702, in writing).

In the past, exercise for individuals with MG was discouraged, even contraindicated as it was thought to worsen symptoms as well as the disease, causing exacerbations and even possible crises. This was presumably because individuals with MG typically experience *fatigability* with effort or repetitive movements. Similar to other neurological and neuromuscular diseases, this dogma was never supported by any scientific evidence of harmful effects and has been reconsidered recently in light of the emerging evidence demonstrating the safety of exercise in stable disease. Simultaneously, the dangers of disuse atrophy and sedentary behaviour have become omnipresent and it appears that fatigability in MG is likely exacerbated by weakness.[21]

There are currently no published guidelines to inform or guide patients nor healthcare practitioners working with individuals with MG. Several narrative reviews concerning exercise and MG have been published;[22-25] however, the most recent studies were not included.[26-28] Thus, the aim of this review is to present the current research evaluating the safety aspects as well as the effectiveness of exercise as an intervention for adults with autoimmune MG.

Method

To conduct this narrative review, Pubmed, Cochrane Central Register of controlled trials, the Physiotherapy Evidence Database and the clinical trials registry were searched using the terms autoimmune myasthenia and exercise with no limit on publication date. The last search was completed in December 2022. Reference lists of identified publications and previous reviews were also searched to identify additional studies. Due to the limited body of existing literature, all interventional trials (regardless of methodology) and without specific outcome measure requirement (i.e. all outcome measures were accepted) were included if published and available in English or French. Interventional studies involving exercise interventions regardless of duration, type, frequency, or delivery were included. Only studies of adults with MG were considered. Although exercise is a subcomponent of the broad term PA which is "any bodily movement produced by contraction of skeletal muscle that results in a substantial increase in energy expenditure,"[29] this review specifically focuses on exercise interventions. PA can include transport, leisure, occupational and household activities whereas exercise is defined as a "planned, structured, and repetitive form of PA with the intention or goal of maintaining or improving one's fitness and/or health."[29] Although important, studies involving exercise for electromyography-related evaluations and studies evaluating rehabilitation or self-management or specific respiratory training were not included nor were observational studies on PA in MG or case reports of exercise or sport in MG.

Exercise interventions are often classified into either strength/progressive resistance training (RT), aerobic (endurance) training (AT), or a combination of both. RT generally consists of repetitive lifting of weights or moving against high load resistance with the main aim of increasing strength by inducing muscular and neural adaptations. AT induces physiologic adaptations that differ from strength training. AT usually involves large muscle groups for longer durations, lower loads, with the aim of inducing adaptations in the heart, peripheral circulation, and skeletal muscle systems.[8,30]

Results

This review included nine interventional studies (one with abstract only) which evaluated the effects of exercise in adults with MG (details in supplementary data Table 1). An additional study evaluating a physical and psychological education programme to manage fatigue in MG was identified.[31] Whilst the programme incorporated some light physical exercises, the main focus was on education and empowerment so it was excluded from this review. The earliest study was published in 1993 and the remaining eight were published in the last decade. A total of 189 participants were enrolled and 174 were included in post-intervention analyses. Of those analysed and based on available data, the majority had generalised MG which was mild (MGFA II) for 49.7%, moderately severe (MGFA III) for 46.1%, severe (MGFA IV) for 0.6% and 3.6% had ocular MG (MGFA I). The mean age of participants ranged from 45-65 years and the average disease duration ranged from 8 to 19 years. Based on available data from eight studies, both sexes were represented however, there was a large female majority (91%[32] and 93%[26]) in two studies. Five studies did not report antibodies; of the other four studies, the majority included participants with acetylcholine receptor antibodies (73-100% of participants), two studies included participants with muscle-specific kinase antibodies and three studies included participants without known MG antibodies. Four studies explicitly stated that participants required stable disease to be eligible.

Exercise training interventions

Exercise interventions varied in terms of exercise type, session duration, session frequency, programme duration, exercise intensity, presence of supervision and setting (Table 1). Exercise type included aerobic training (AT),[26,33] resistance training (RT),[32,33] mixed AT/RT,[28,34-36] walking training[27] and balance training.[37] Where specified, session duration ranged from 30 to 90 minutes, frequency ranged from once per day to once per week and programme duration ranged from 8 to 24 weeks. The overall exercise intervention duration ranged from 8.5 hours to 36 hours depending on the study. AT intensity was defined by % maximum heart rate (HR) in three studies,[26,33,34] RT intensity was defined by repetition-maximum in three studies,[33-35] exercise intensity was otherwise undefined in five studies.[27,28,32,36,37] Exercise intensity was maintained or progressed by adjusting the resistance level, increasing weights, time, speed and/or number of repetitions or adjusting target HR for AT. The majority of studies included individually tailored training that was supervised in all but three studies.[26-28] Where specified, settings included hospital,[34] university,[33] physiotherapy gymnasiums[35,37] and home[26,28] or community-based.[27]

Study withdrawal and adherence to exercise training

Of a total of 9.5% reported dropouts, 10.9% were those participating in exercise and 7.5% were from control groups (only 2 studies with control groups). Of the 13 dropouts that were participating in exercise only one was possibly related to exercise due to worsening bulbar symptoms with RT[33] (Table 2). Other reasons for study withdrawal

were either not reported (1)[28] or due to lack of time (3),[33,34] work-related health problems (1),[34] spontaneous lumbar vertebral compression fracture (1),[35] spinal stenosis (1),[35] prescheduled thymectomy (1),[35] work-related injury (1),[33] work commitments (1),[37] or illness and cardiac arrhythmia (1).[37] One study did not provide information regarding dropouts.[36]

Adherence to the exercise programme was not reported in two studies.[36,37] One participant randomised to exercise refused exercise training.[26] Otherwise, whilst exact details are missing from most studies, based on available data, mean adherence to exercise was high ranging from 70-97%.[26,27,33-35] Reasons for missing sessions were only reported in one study: work commitments for most missed sessions and flu, weekend away, and menstrual pain/tiredness for missing occasional sessions.[26] One study reported difficulties in following the number of repetitions and training load.[32]

Exercise tolerance

Safety/tolerance of exercise training is summarised in Table 2. Of all nine studies, there was only one myasthenic crisis reported and this was in the control (rest) group.[27] No myasthenic crisis was reported in relation to exercise in any of the studies. Six MG exacerbations (3.2%) were reported with two necessitating hospitalisation. Five of these (2.7%) were in the control (usual care) group, thus unrelated to exercise[5] and one (0.5%) was a participant in the RT group.[33] However, it is possible that bulbar symptoms worsened prior to beginning RT as the Quantitative Myasthenia Score (QMGS) increased (speech and facial muscle strength items) during the run-in phase of the study prior to beginning exercise.[33] Five studies did not report adverse events (AEs).[28,32,34,36,37] One study reported bulbar symptoms in two participants (one temporary, the other withdrew as described previously).[33] The same study reported increased fatigue in three participants that was mild and temporary. For the 62 AEs reported over nine months in one study, there was no difference between the control and exercise arm.[26] Two other studies reported two AEs each which were unrelated to exercise.[27,35] Concerning changes in medication, six studies did not evaluate or did not report changes.[28,32,33,35,36] One single-arm study reported a decrease in acetylcholinesterase inhibitors (AchEi) following exercise in three (21%) participants.[34] Out of two controlled studies, one observed a decrease in both AchEi and corticosteroids (CS) in the exercise compared to the control (rest) arm[27] whilst the other study found no significant difference in dosage change of AchEi and CS between the two groups.[26]

Effectiveness of exercise

The benefits of exercise training are summarised in Table 3. HRQoL using the MG-specific patient-reported MGQOL-15 was evaluated in three studies but no improvement was found in favour of the exercise intervention.[26,27,33] Within-group analyses demonstrated worsening of HRQoL in the AT group in the Danish study.[33] Of the six studies evaluating knee extension strength, four studies demonstrated improvements with exercise (with RT but not AT in the study with 2 exercise arms),[26,32-34] whilst two studies did not show any change in knee extension strength with exercise.[27,35] Upper limb strength (elbow flexion,[26,32,35] elbow extension,[32] thumb abduction and finger extension[35]), was evaluated in three studies but no improvements were observed with exercise. Only one of five studies evaluating handgrip strength demonstrated an improvement with exercise.[28] With respect to function, walking capacity increased with exercise in three studies[26,35,36] whilst there was no change in five studies.[27,28,33,34,37] Timed-Up-and-Go performance improved in two[36,37] out of three studies,[34] 30-second sit-stand improved in all three studies that used this outcome.[33-35] Improvements were also observed in the stair climb test (RT not AT),[33] static standing balance[37] and box and blocks test (RT).[33]

Of three studies that used the MG-ADL as an outcome measure, only one showed an improvement following exercise.[26] Seven studies used various MG clinical scores including the Myasthenia Gravis Composite scale (MGC), the QMGS and the Myasthenia Muscle Score (MMS). Of these, three non-controlled studies showed improvements in post-exercise analyses on the QMGS[28,37] and MGC[34] and one controlled study showed improvements in the MMS in favour of exercise.[27] Two studies evaluated lower limb fatigability, one demonstrated a slight increase in resistance to fatigue with RT compared to AT[33] and the other study could not conclude due to the large inter-subject variability.[32] Two studies evaluated self-reported fatigue but did not demonstrate improvements with exercise.[33,34] One study demonstrated an improvement in exercise self-efficacy with exercise.[35] Finally, one uncontrolled study demonstrated improvements in immune markers with exercise[35] whilst another randomised controlled trial (RCT) found no between-group differences[26].

All studies evaluated the effects of exercise *immediately* post-intervention. Only two studies also included a no intervention follow-up period. Gains made immediately following the exercise intervention were unsustained at the 3-month follow-up in the MGEX study.[26] On the contrary, in the study by Wong et al., gains made in the QMGS and standing balance were sustained at the 4-week follow-

up whereas improvements in the TUG-cognitive were not maintained at follow-up.[37] Exercise dose-response, evaluated in two studies demonstrated that those that performed more exercise had greater benefits in leg strength and walking speed.[26,28]

Study design and methodological quality

The smallest sample size included 7 participants and the largest, 45 participants. Study designs varied between RCTs[26,27,33] and quasi-experimental single-group pre-post-test studies.[28,32,34-37] Only one study performed intention to treat analyses,[26] with the remaining studies performing per-protocol between group analyses, per-protocol within group analyses, or both. Only two studies included blinded assessors.[26,33] Concealed allocation was reported in only one of the three RCTs.[26] Only three studies calculated the sample size prospectively.[26-28] One study is only available as an abstract thus details are lacking.[36] Due to the nature of the intervention, no participants in any of the studies could be blinded. Participant retention was 80% or below in three studies[33-35] and unreported in one study.[36]

Discussion

The aim of this review was to summarise the current literature with respect to safety aspects and effectiveness of exercise interventions in adults with MG. Nine studies (one abstract only) were included. Evaluating exercise as an intervention presents certain challenges. Firstly, exercise is a complex intervention, consisting of multiple elements; exercise type, duration, frequency, intensity, individualised or generic, delivery (supervision and motivation) as well as setting. Secondly, exercise requires active participation which presents the challenge of adherence, particularly if the programme is ongoing, sessions are long and frequent. Not only can exercise be time consuming but it also has to fit into one's current lifestyle. Considering the age of the participants in this review, they are still likely to be working and may have children to care for. As with all therapies, the effects of exercise cannot be observed if adherence is not maintained.

Although few studies explicitly focused on safety and not all studies reported AEs, an important finding from this review, from precedent reviews and published case reports,[13,38-40] is that there is no data to support exercise as a harmful intervention in MG. Only four studies explicitly stated that participants had stable disease. There is no study to date demonstrating evidence of an exercise-related myasthenic crisis. One incidence of MG worsening was reported however as stated by the authors this may have occurred prior to exercise participation and, symptoms are known to fluctuate in MG so it is possible that this was the natural course of the disease, reinforcing the necessity for a non-exercise control group in future studies. The MGEX study demonstrates the possibility of MG exacerbation unrelated to exercise. The MGEX study actually supports the hypothesis of a protective effect of exercise as all five exacerbations were in the control group.[26] A similar finding has been reported in multiple sclerosis[41] and warrants further investigation in MG. Several studies from this review observed symptom improvement and medication reduction. There were several dropouts but adherence to exercise was otherwise reasonably high in most studies.

In terms of effectiveness, compared to a non-exercise control group, improvements were observed in walking capacity,[26] MG-ADL score,[26] knee extension strength[26] and MMS[27] in favour of exercise. In the single-group studies or within-group analyses, improvements were observed in knee extension,[32,34] handgrip strength,[28] walking capacity,[35,36] 30s sit-stand,[33-35] hand dexterity[33] and clinical scores (QMGS or MGC).[28,34,37] When comparing two exercise modes there was an improvement in the stair climb test and a reduction in knee extension fatigability in favour of RT compared to AT.[33] The minimal detectable change (MDC) and minimal clinically important difference (MCID) were rarely considered; the small observed gains were often below the MDCs or MCIDs (where known).[42] Improvements were not sustained in the 3-month follow-up in the MGEX trial, which reinforces the notion that the exercise programme was responsible for observed gains with benefits being lost with cessation of the programme.[26] In the study by Wong et al., two of the three improvements were sustained which may be explained by the fact that the four-week follow-up was shorter than the 3-month follow-up in the MGEX study.[37]

Two important outcomes directly reported by participants, HRQoL and self-perceived fatigue did not improve with exercise. Whilst it is preferable to use outcomes that are meaningful to participants, in a pragmatic trial, it can be challenging to identify sensible, reliable and meaningful outcomes. For example, in the MGEX study, the largest RCT to date and the only multicentre trial, HRQoL, did not demonstrate any change with exercise. In MG, HRQoL is most commonly evaluated using the MGQOL-15, an MG-specific standardised self-reported questionnaire. However, patient-reported outcomes can be impacted by expectations (positive or negative) and/or a response-shift phenomenon.[43] Response shift phenomenon has been defined as a change in the meaning of one's self-evaluation of a target construct i.e. HRQoL or fatigue which could be explained by various mechanisms such as a change in one's internal standard of measurement (recalibration), change in the importance (repriorisation) of component

domains, or a redefinition of the target construct (reconceptualization).[44] Response shift may attenuate treatment effects as individuals adapt to treatment side effects over time. Further, the fatigue scales used were not MG-specific and their responsiveness has not been evaluated in MG, which may be an explanation for their lack of change or improvement.

The scope of current evidence of exercise intervention in MG is small with only eight studies published and one abstract. The existing studies are of mixed quality with small sample sizes, keeping in mind that MG is a rare disease. Uncontrolled studies makes it difficult to interpret findings. Multiple different outcomes were used. There is an effort to improve standardization of existing outcome measures (MGNet, Benatar)[50]; however, more thought may be required as to which outcomes are most appropriate for exercise studies in MG, taking into account what is most important to the individual. Based on current evidence, it is impossible to compare safety and/or effectiveness of one type of exercise to another type (e.g AT vs RT), keeping in mind that intensity, duration, frequency and delivery varied amongst studies. We are also not able to conclude as to which type of exercise is best, how much should be done nor how often or at what intensity. Reporting of exercise interventions, adherence to exercise and AEs was lacking and/or insufficient in several studies. However, this is not unique to these specific studies.[45]

Other unanswered questions include when is best to begin or continue exercise in the MG disease course and whether a relationship exists between exercise and pharmacological therapies (e.g. exercise has an enhancing action on pharmacological therapies). With the plethora of new treatments being studied and becoming available in MG, it will be vital to understand the role and complementarity of exercise. Further studies are necessary to understand possible disease-modifying autoimmune response effects of exercise in MG. A future area of research could be whether exercise plays a role in preventing secondary generalisation in ocular MG.

Future studies should also consider wearables. These could be used as a monitoring tool, to stratify groups taking into consideration pre-intervention PA levels and to evaluate and encourage behaviour change[46] to further understand long-term and dosage-effects of exercise. A control group is important to truly understand the effects of exercise and whilst it is not possible to blind participants, assessors should systematically be blinded. Further, it is crucial to consider transferability. It is not a given that being enrolled in an exercise study and undergoing supervised or structured exercise over a period of time will transfer into incorporating exercise into daily life. One study demonstrated that the beneficial effects of exercise had worn off in the follow-up non-exercise period of the study.[26] Thus for sustained effects, it is necessary to continue exercise over a long-term period, making it important to find an activity that is feasible and enjoyable. Engaging in exercise without the structured environment of a trial, for those out of practice or having never undergone exercise is challenging. Multiple barriers exist including those related to and those unrelated to MG (NCT05408702, in writing).

Although no specific recommendations exist, we propose that general recommendations regarding moderate-intensity exercise can be applied safely to well-regulated individuals with mild-moderate MG.[47] Individuals may need to be reassured that mild-moderate intensity exercise will not worsen their disease. Healthcare providers should endorse and promote the safety and possible benefits of exercise and lifestyle PA.[48] Neurologists and treating physicians could play an essential role in promoting exercise by regularly enquiring about PA and exercise habits. Prescribing exercise and/or referral to a physiotherapist and/or exercise physiologist and/or coach is highly recommended to assist individuals in starting and progressing their exercises as well as educating and empowering individuals.[49] An individual exercise plan is useful not only from a physical/physiological perspective but also from a psychological and behavioural standpoint to assist individuals in finding an activity they enjoy which is fundamental for long-term adherence. This should incorporate the needs and priorities of the individual with the aim of achieving or maintaining the individual's highest or optimal function within their capacities. Smartphone and smartwatch applications are widely developing and can be useful for motivating as well as monitoring exercise levels with regular data being fed back to the individual and/or the prescriber.

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Table 1: Details of exercise interventions in the included studies

Study	Study design	Type exercise Aerobic (AT) Resistance (RT)	Intensity	Programme duration	Session duration	Frequency	Total planned training	Setting/Supervision
Birnbaum 9 months	Multicentre RCT, ITT analyses	Aerobic	Target HR = 70% maxHR	3 months	40 minutes	3/week	24 hours (1440 mins)	Home/unsupervised (1st 2-3 training sessions supervised)
Misra 3 months	RCT Per-protocol b/w grp & w/i grp analyses (proportions)	Walking		12 weeks	In 1-2 sessions 10min: week 1 20min: week 2 30min: week 3	Daily	8.5 hours (510mins)	Home/community Unsupervised
Chang 24 wks	Single-grp uncontrolled	Mixed AT/RT		24 weeks	30 minutes	At discretion of participant	Minimum 12 hours: 720 mins (1/week)	Home/unsupervised 1 supervised session per month
Westerberg 18 12 wks	Single-grp uncontrolled	Mixed AT/RT	AT: aim 80% maxHR RT: 10-RM	12 weeks	90 minutes	2/week	36 hours (2160 mins)	Hospital/supervised
Westerberg 17 12 wks	Single-grp uncontrolled	Mixed AT/RT	"moderate" AT: high load RT: 10-RM	12 weeks	70 minutes	2/week	28 hours (1680 mins)	PT setting/supervised
Rahbek 8 wks	RCT: 2 exercise arms Per-protocol b/w grp & w/i grp analyses	AT OR RT	AT: 70-85% maxHR RT: 15-RM to 8-RM	8 weeks 20 sessions	~ 40mins*	5/2 weeks	13.3 hours (800mins)	Sport Science University/ supervised
Hafer-Macko 3 months	Single-grp uncontrolled	Mixed AT/RT		3 months	60mins	3/week	36 hours (2160 mins)	Supervised
Wong 4wk pre, 16wks post + 4wk F/U: up to 24wks	Single-grp uncontrolled	Functional/ balance		16 sessions	~60mins* (based on Nitz & Choy)	1-2/week	16 hours (960mins)	PT setting/supervised
Lohi 10 wks	Single-grp, opposite untrained limb used as control	Resistance		10 weeks 27-30 sessions	~40mins*	2-3/week	20 hrs (1200mins) to 24.7 hrs (1480mins)	Supervised (< 20% unsupervised)

*specific data not provided, time is assumed. Grey cells: unspecified. AT: aerobic training, RT: resistance training, RM: repetition maximum

Table 2: Summary of safety/tolerance of included studies

Safety/tolerance								
Study	**Dropouts**	**Drop-outs possibly related to exercise**	**MG crisis**	**MG exacerbation**	**Other adverse events**	**Change in dose AChEI/CS or both**	**Electrophysiology**	**Worsening of MG possibly due to exercise**
Birnbaum EG vs CG (usual care) 9mo (3mo F/U)	2 (CG)^ 95% (41/43) completed + 2 prior to randomisation	0	0	CG: 5 (2 hospitalised)	62 31 EG & 31 CG	NS b/w grp difference in change AChEI or CS		0
Misra EG vs CG (rest) 3mo	2 (1 CG, 1 EG) **95%** (38/40) completed	0	1 CG (rest)	NR	EG: 1 FSGS	↓ dose AChEI & CS in EG compared to CG	ND	0
Chang Single-grp, 24 wks	1 **97%** (34/35) completed 24wks.	0	NR	NR	NR	NE/NR		0
Westerberg 18 Single-grp, 12 wks	3 **79%** (11/14) completed 12wks.	0	0	0	NR	↓ dose AChEI, n=3	↑ CMAP amp: RF ND CMAP: BB RNS: No deterioration~	0
Westerberg 17 Single-grp, 12 wks	3 **77%** (10/13) completed 12wks.	0	0	0	1: spontaneous lumbar compression fracture 1: spinal stenosis	NE/NR	↑ CMAP amp: BB & RF. ND CMAP: APB & EDB ND RNS post	0
Rahbek EG (RT) vs EG (AT) 8 wks	3 **80%** (12/15) completed 8wks	1 bulbar symptoms (RT)	0	1	2 : bulbar symptoms 3 : ↑ fatigue	NE/NR		1 (may have preceded exercise)
Hafer-Macko Single-grp, 3mo	NR	NR	NR	NR	NR	NR		NR
Wong Single-grp, 24wks	2 83% (6/7) post, 71% (5/7) F/U	0	NR	NR	NR	NE/NR		0
Lohi Single-grp, 10 wks	0	0	0	0	NR	NE/NR		0
TOTAL	**18 (9.5%)**	**1**	**1**	**6 (3.2%)**	**70**			**1**
EG (9 studies)	**13 (10.9%)**			**1 (0.5%)**	**39 (20.9%)**			**1**
CG (2 studies)	**3 (7.5%)**		**1**	**5 (2.7%)**	**31 (16.6%)**			
Before randomisation	**2**							

AChEi: Acetylcholinesterase inhibitors, APB: abductor pollicis brevis, AT: aerobic training, BB: biceps brachii, CG: control group, CS: corticosteroids, EDB: extensor digitorum brevis, EG: exercise group, F/U: follow-up, grp: group, ND: no difference, NE: not evaluated, NR: none reported, NS: not significant FSGS: focal segmental

glomerulosclerosis, mo: months, post: post-intervention, RF: rectus femoris, RNS: repetitive nerve stimulation, RT: resistance training, ~1 decrement post compared with 4 pre, ^ post-randomisation

Table 3: Summary of effectiveness of exercise on various outcomes used in the included studies

		Effectiveness of exercise on																	
			Strength			Function						Clinical MG score							
Study	Adherence to exercise	HRQoL (MGQOL)	KE	UL	Hand grip	Walking 6MWD	TUG	30STS	SCT	B&B dom	MG-ADL	QMGS	MGC	MMS	ESES	Fatigue	Fatigability	Depression/ Anxiety	Immune markers	Follow-up
Birnbaum	**96%** (22/23) participated in ET 70% adherence (of n=23). Mean 24 (range 0-38) 40min sessions.	ND b/w EG & CG	+ (CACE)	ND	ND	+					+			ND				ND	ND IL-6, TNF α	Not sustained
Misra	97% adherence of 19/20 (1 drop-out EG)	ND b/w grps	ND		ND	ND b/w grps					ND			+						
Chang	Median 56.3min/wk of 97%	ND			+	ND						+								
Westerberg 18	Mean 88±7% sessions of 79% (n=11/14, remaining participants)	ND	+		ND	ND*	ND	+				ND	+		ND	ND FSS				
Westerberg 17	2 = 71%, 8=95% of 79% of 10/13 remaining participants		ND	ND	ND	+		+					ND		+				+ miR-150-5p, miR-21-5p, IL-6	
Rahbek	Of 80% remaining participants, n=12/15: Mean 95%±8. AT: 91.7±9.8% RT: 98.3±4.1%	↓ AT (w/i grp) compared to RT (sig b/w grp)	+ RT w/i grp			ND		+ w/i grp both	+ RT b/w grp	+ RT w/i grp						ND MFIS	+ KE: RT			
Hafer-Macko	No information	ND				+	+				ND	ND								
Wong	NR					ND	+# & FoamEC					+								QMGS, FoamEC maintained
Lohi	Not all could complete repetitions or training load as planned.		+	ND													inconclusive			

Grey cells – outcome measure not used or no follow-up period, Electrophysiological measures not included. AT: aerobic training, CG: control group, EG: exercise group, ESES: Exercise self-efficacy, ET: exercise training, FoamEC: FoamEC: standing balance on foam with eyes closed, FSS: Fatigue Severity Score, KE: knee extension,

MFIS: Modified Fatigue Impact Scale, MGC: Myasthenia Gravis Composite Score, MMS: Myasthenia Muscle Score, ND: no difference, QMGS: quantitative myasthenia gravis score RT: resistance training, SCT: Stair Climb Test, TUG: Timed Up and Go test, UL: upper limb, 6MWD: Six-minute walking distance, 30STS: 30-Second Chair Stand Test, *12MWD, #TUGcognitive.

Supplementary data Table 1 presents all included interventional studies (most recent first)

Study, design, location	Design/method	Participants	Exercise Group (EG)/Control group (CG)	Adherence	Outcome measures (OM)	Adverse events	Dropouts	Results
Birnbaum, 2021 [3, 2] Multicentre RCT Study duration 9mo for each participant (3mo run-in, 3mo ex, 3mo F/U) Paris, France	**Single-blind parallel grp multicentre** **Randomised** 1:1 - computer generated, permuted blocks of randomly varying sizes, stratified by centre, **concealed allocation**	Eligibility Mild-mod gMG: MGFA II-III 18-70yrs, **Stable** for ≥ 6mo MGQOL score ≥ 15 No CI to exercise N= 45 included N=43 randomised Female: 40 (93%) Mean age: 45.5±10 yrs AChRab+ve: 35 (81%) MuSK+ve: 3 (7%) Seronegative: 5 (12%) MGFA II: 23 (53%) MGFA III: 20 (47%) Mean DD: 14.3±11 yrs Juvenile: 7 (16%) EOMG: 30 (70%) LOMG (> 50yrs): 6 (14%) Mean BMI: 28.4 (5.5) Obese (BMI ≥ 30): 13 (32%) Mean MGQOL: 22.1±9 Mean MMS: 86.6±11 Mean MG-ADL: 2.6±2.4 Mean 6MWD: 498±83m Mean FVC%: 84.6±13.1	**EG:** N = 23 **40min sessions, 3/week, 12 wks** 2 – 3 supervised sessions, then **unsupervised** at home with HR monitor **Individualized** target HR (70% of their HRmax, using 220-age as their HRmax) **AT:** Rowing machine Each 40 min moderate-intensity rowing session consisted of: 10min warm-up to reach individual target HR, followed by 20min plateau of constant aerobic activity at **70%HRmax**, followed by 5min power interval phase (5 sets of 10 consecutive pulls at maximum effort each minute, followed by regular intensity strokes for the remainder of each minute), 5min active cool-down. **CG:** N = 20 Usual care, nothing added	Training sessions (distance, time, Watts, date) recorded by the rowing machine N = 1 refused exercise. Adherence defined as having completed ≥ 20 (frequency) 30min (duration) sessions. Including n=23, mean 24 sessions & 70% adherence Non-adherence mainly due to work commitments. Reasons for missing occasional sessions: the flu, weekend away, menstrual pain/tiredness.	Primary: MGQOL-15 Secondary: MG-ADL score MMS score Strength (isometric MVC) KE + EF (Biodex) Handgrip (MyoGrip) 6MWD FVC/FEV1 MIP & MEP Dose AChEi Dose prednisone WHO-QoL BREF BDI (depression) STAI (anxiety) SEI (self-esteem) Serum IL-6 & TNF α	62 AEs reported, no difference b/w grps. CG: 5 MG exacerbations (2 hospitalised) EG: zero exacerbation, zero hospitalization.	2 dropouts CG 95.3% completed Lost to F/U < 5%	Analyses ITT, n=43 No b/w grp difference in MGQoL EG: ↓ MG-ADL & ↑ 6MWD, not maintained at 3mo F/U EG CACE analyses (based on compliance): ↑ KE strength, not maintained at 3mo F/U
Misra, 2021 [7] RCT Lucknow, India	**Randomisation** computer generated random numbers (no concealed allocation) No blinding	Eligibility Mild-mod gMG: MGFA II-III 15-70 years, MGQOL ≤ 45 No CI to exercise n = 40 included n = 38 analysed Median DD : 4.5 (1.2-24) yrs Median age: 45 (16-70) yrs	**12 weeks** **EG:** N= 20 Self-walking in 1 or 2 sessions: Week 1 10min **daily** Week 2: 20min daily Week 3 onwards: 30min daily Steps & distance recorded using "Step Tracker" (smartphone), verified fortnightly by telephone & at F/U	Monitored fortnightly by telephone. Subject & caregivers instructed to maintain a diary of Step Tracker including # steps & distance. Walking details	Primary: > 50% ↑ MGQOL-15 Secondary: > 50% improvement MG-ADL 6MWD (15m corridor) # steps (6MWT) MMS score Handgrip strength Dose AChEhI	EG: 1 – FSGC leading to renal failure at 2 months CG: 1 - MG crisis at 1 month	1 in each arm (cf AEs) 94.7% completed Lost to F/U 5.3%	N =38 analysed (per protocol) In favour of EG 1°: More subjects in EG had > 50% improvement in MGQOL & 6MWD than CG. However, comparing MGQOL score between the 2

	Analyses: Per-protocol baseline-3mo (compared proportions)	Female: 16 (42%) MGFA II: 8 (20%) MGFA III: 30 (80%) EG/CG Median MGQOL: 19/18 Median MMS: 68/60 Median 6MWD: 132/108 MG-ADL, Antibodies: no data	**Intensity** undefined **CG (Rest) :** N=20 Rest (sitting or lying) 30mins daily in 1 or 2 sessions (each ≥ 6-8h apart)	verified at F/U visits. Non-compliance of >30% on 2 consecutive sessions would lead to study exclusion. EG: 97% adherence 89% completed walking in 1 session. CG: 98%, all completed rest in 2 sessions.	Dose CS Decrement trapezius EMG (RNS 3Hz)			groups there was no difference b/w grps (supp data). Pre-post = improvement in both grps in MGQOL, MMS but no improvement in 6MWD ↓ dose AChEI + CS in EG compared to CG
Chang, 2021 [4] New Taipei City, Taiwan	Pre-post (baseline, 24-wks) No blinding	Eligibility Mild-mod gMG: MGFA II-III **No change meds** ≥ 6mo No IVIG/PLEX within 6mo prior to enrolment N = 35 included Female: 22 (63%) Mean age: 56.1± 8.6 yrs AChRab+ve: 100% MGFA II: 21 (60%) MGFA III: 14 (40%) Mean DD: 12.3±10.6 yrs Obese: 40% Sarcopenia: 8 (22.9%) MGQOL: 14.9±11.3 QMGS: 10.5±4.8 6MWD: 396±90m FVC%: 72.6±18.5 N=34 analysed (21 female)	**30-min sessions, 24-wks** **Individually** tailored **Aerobic resistance training** **Supervision by a researcher once per month** at hospital PT setting **Home, unsupervised**, sessions at the discretion of subject Session: 5min warm-up, 7 x 3min cycling intervals, 5min cool-down + squats, sit-stand, arms-out stretch, squat jumps, sprint on the spot, own body weight exercises. If easy, intensity ↑ gradually by ↑ reps + speed. Stretching. **Intensity** undefined Participants were free to decide how many exercise sessions per week they would perform and regularly reported their weekly exercise time. No CG	Median 56.3min/wk Median 2.9 sessions/wk	No 1° OM defined QMG score Handgrip strength FVC MG-QOL Gait speed - mean of 2 6MWT Body composition (DXA)	No negative effects reported – no info provided	1 dropout reported – no details provided Lost to F/U < 5% (2.9%)	Pre-post analyses Feasible, well-tolerated ↑ QMG 9 to 10.47±4.78 ↑ handgrip strength ↑Android/gynoid fat ratio High ex grp (>56.3min/wk) compared to low ex grp (<56.3min/wk): greater deterioration in arm muscle mass (high grp), greater ↑ FVC, ↑ gait speed, improvement QOL & QMGS low grp
Westerberg, 2018 [10]	Pre-post No blinding	Eligibility age ≥18 years, living nearby no concomitant condition no severe CVD, other disabling disease, pregnancy.	**90-min sessions, 2/week, 12-wk, Supervised –** Hospital setting Intensity & weights - **individually tailored** Each session: **AT, RT & balance**	11 completed the 12-wk program 75% to 96% (88±7%), max 24 sessions.	CMAP RF, BB. RNS 10 stimuli, decrement recorded b/w 1st & 4th (4 abnormal decrement)	None of them showed any signs of clinical deterioration	3 dropouts unrelated 2 – lack of time 1 work-related	↑ CMAP amplitude in RF (no correlation with change in RNS decrement). ND CMAP BB

Uppsala, Sweden Safety & efficacy, effects on functional muscle parameters		N= 14 included N= 11 analysed Mean age: 60±18 yrs Female: 6 (55%) Mean BMI: 26.3 Obese: 2/11 (18%) Mean DD: 16.4±11.6 yrs AChRab +ve: 8 (73%) MuSKab +ve: 1 (9%) Seronegative: 2 (18%) EOMG: 5 (45%) LOMG: 6 (55%) MGFA I: 2 MGFA IIa: 1, MGFA IIb: 2 MGFA IIIa: 3, MGFA IIIb: 2 MGFA IVa: 1 Mean MGC: 3.8 [0-9] Mean QMGS: 2.5 [0-6] 6MWD: 486±91m Accelerometer: median 8801 steps, SB 18.8h/24, 10h (waking hrs) Self-reported: strenuous exercise 0 to>120min/wk (median: <30min/wk). PA not regarded as exercise <30 min/wk to >300min/wk (median: 150–300min/wk).	**AT:** stationary bicycle interval training 5min warm-up, 7 intervals of 2min cycling against high load & 1min cycling against minimum load, 5min cool down. Level of resistance was set, continuously adjusted, according to HR **aiming for 80% of maxHR** during the 2min high load periods. **RT:** 7 resistance exercises (weightlifting, resistant band exercises, or exercises using own body weight) biceps curl, latissimus dorsi pulldown, triceps pushdown, leg curl, cable rowing, sit-ups, & leg press were carried out, each with 2 sets of **10 RM**. Increasing adjustments of RT weights were done individually. The active training program was followed by a set of 2 balance & 6 stretching exercises which were not changed over time. No CG	72–100% exceeded 70% of HRmax during the 2-minute high load periods. Ten (91%) increased weights ≥ 4 of the 7 strength exercises. All ↑'d resistance weights for leg press. Eight (73%) ↑'d bicycle resistance in the second half of the training.	Isometric muscle strength HHD (Lafayette): BB, KE Handgrip strength (Jamar) U/S muscle thickness: BB, RF, VI MGC score QMGS PEF% TUG 12MWT 30STS MGQOL FSS ESES Blood samples Body composition: DXA–BIA	(MGC/QMGS) or described other uneasiness regarding the training. No deterioration (RNS).	health problems 78.6% completed Lost to F/U 21.4%	↑ Isometric quadriceps force ↑ U/S muscle thickness (RF + VI) ↑ 30STS (median +2) ↑ median MGC (3 to 2)* DXA: ↓ fat (%),↑ muscle (%) RNS : only 1 subject had abnormal decrement compared with 4 prior to training Majority (72-100%) exceeded 70% of HRmax each session during the 2min high load. ↑ level of resistance in multiple exercises
Westerberg, 2017 [9] Uppsala, Sweden	Pre-post No blinding	Eligibility >18yrs, Well-regulated MG with ongoing treatment &/or mild fatigue: MGFA class I-II N=13 included N= 10 analysed MGFA I: 4 (40%) MGFA IIa: 3 (30%) MGFA IIb: 3 (30%) Female: 5 (50%) Mean age: 65±14 Mean DD: 19±13 [4-40] Mean BMI: 27.5±4.5	**70min sessions, 2/week, 12-wk** **AT (bicycle interval training)** & **RT** **Supervised by a PT,** PT setting **Individually tailored** Every session: **AT, RT & balance** **AT:** Stationary bicycle 30min: 5min warm-up, 7 intervals of 2min cycling against high load/resistance (**max tolerated**), 1min "recovery cycling" minimum load/resistance, ending with 5-min cool-down. **RT:** 40min, 8 resistance exercises - each with 2 sets of **10 repetition max.**	2 = 71% 8=95%	MGC score PEF CMAP, RNS 10 @ 3Hz decrement b/w 1st & 4th - APB, BB, RF, EDB Right-side isometric strength HHD (Lafayette): APB, BB, RF, EDB Handgrip strength (Jamar) Performance-based measures: TUG	Physical exercise was well tolerated & MGC score was unchanged. No change RNS	3 dropouts 1 – spontaneous lumbar vertebral compression fracture 1 – spinal stenosis 1 – prescheduled	↑ 6MWD ↑ 30STS ↑ CMAP amplitudes (mV): BB & RF ↑ ESES (↑ confidence) ↓ disease-specific micro-RNAs miR-150-5p & miR-21-5p. DXA-BIA - ↑% muscle ↓%fat Pulse (% of max; [220-age]) was

		AChRab +ve: 8 (80%) AChRab -ve: 2 (20%) Median MGC: 4.5(2.8) Mean 6MWD: 486±91 Mean 30SCS: 13.6±5.6 Mean TUG: 8.5±1.5 Baseline PA level (accelerometer): median 7872 steps/day N = 1 abnormal decrement (RNS)	Biceps curl, triceps pushdown, seated leg curl, cable pull-down, leg extension, cable rowing, sit-ups, leg press. **Balance**: 1-leg standing for 1min on each leg on variable surfaces. **Progression:** Increasing adjustments of bicycle resistance load & RT weights were done over the 12 wks as participants improved. **Intensity** "moderate intensity" No CG		6MWT 30STS Romberg test Toe-rise Endurance Test Serum levels IL-6, muscle enzymes, Disease-specific micro-RNAs (miR-150-5p & miR-21-5p) Body composition: DXA–BIA ESES		thymectomy 76.9% completed Lost to F/U 23.1%	consistent among subjects over the training period, whereas the resistance (Watt) gradually increased over the period, indicating a positive AT effect. Muscle resistance weights ↑ UL & LL
Rahbek, 2017 [8] 4wk run-in & 8wks exercise Arhus, Denmark	2 arms - type of exercise **randomised** - stratified by gender & QMG score 4 week run-in period Within grp (pre/post) & between grp analyses **Assessor-blinded**	Eligibility gMG: MGFA II-IV, 18-80 yrs Living nearby, No cardiorespiratory, orthopaedic or metabolic comorbidities, no dementia or pregnancy N=15 included MGFA IIa: 10 (66.7%) MGFA IIb: 4 (26.7%) MGFA IIIa: 1 (6.7%) Mean age: 55.6±17.2 Median QMGS: 5.5 (0-17) Mean BMI: 25.8±3.8 Female: 8 (53%) Mean DD: 7.6±6.6 PRT grp = 7 AT grp= 8 Antibodies: not reported N=12 analysed MGFA II: 11 (91.7%) MGFA III: 1 (8.3%)	**Both arms intervention:** **8 weeks, 20 training sessions** Schedule: 5 sessions per 2wks. **Moderate-high intensity PRT & AT** At the Sport Science training facilities, Aarhus University. All sessions were **supervised** by the same exercise physiologist. All sessions of both grps were preceded by a 5-min low-intensity aerobic warm-up. Most sessions were conducted on an individual basis, but some sessions overlapped, resulting in 2 or more subjects exercising concurrently. **AT protocol:** 3 sets of 10–12min cycling on a bicycle ergometer with 3min rest periods. Intensity progressed from **70 to 85% of maxHR** during the 8wk intervention. **PRT protocol:** Full-body including; weighted step-up, smith bench-press, leg-press, pull-down, hip flexion & lateral raises. All exercises progressed from 3 sets of 12 repetitions performed at **15-RM** in wk 1, to 3 sets of 8 repetitions	Adherence defined as % of sessions attended (of the 20 scheduled). **Only subjects who completed the intervention were included in adherence calculation.** AT: n = 6 completed PRT: n = 6 completed Mean adherence: 95%±8. AT: 91.7±9.8% PRT: 98.3±4.1%	Isokinetic dynamometer - isometric strength (MVC): KE, shoulder abd, EF, HE, HF Max neural drive iEMG – VL (during isometric test). Concentric isokinetic KE 100-0° at 90°/s Fatigability: 25-repetition isokinetic test of KE. Functional: 6MWT STS B&B SCT Aerobic Power: Incremental cycle test to exhaustion within 8–12 min (individual dependant). The highest recorded 30s average O_2 uptake rate attained during the test considered the peak rate of oxygen consumption (VO_2peak). MG-QoL15	Transient training-induced muscle soreness not regarded as an AE. Both grps reported AEs: bulbar symptoms (n = 1 PRT → withdrew, n = 1 AT temporary & did not affect participation) and mild, temporary ↑ fatigue both grps. No change in QMGS in either grp.	3 (20%) dropouts 1 PRT potentially related to PRT (bulbar symptoms requiring CS 4wks into the PRT) 2 AT grp unrelated to AT 1 = work related injury 1 = lack of time 80% completed Lost to F/U 20%	AT and PRT were feasible for most patients with mild MG. B/w grp analyses: MGQOL deteriorated in AT grp SCT improved PRT grp (AT worse) Within grp analyses: PRT ↑ KE strength (10%) PRT ↑ B&B^{dom} performance ↑ STS both grps ↓ fatigability end of test in PRT group.

			performed at **8-RM** in wk 8. Sets were interspaced by a 90- to 120-s rest period. No non-exercise CG		MDI MFIS			
Hafer-Macko, 2016 (abstract) [5] **3months**	Single grp	Eligibility: no data N = 9 Mean age: 63 **Stable** Mild-mod MG	**3 months** **1h 3/week** **AT(walking), RT (therabands) & breathing exercises** **Intensity** undefined	No information provided	MG-ADL MGQOL-15 QMGS TUG 1-RM leg press 6MWT Self-selected walking speed VC	None reported (abstract)	None reported	Improvement TUG, 1-RM leg press, peak walking speed, peak ventilator exchange
Wong 2014 [11] Brisbane, Australia 16wks & 4wk F/U Effects of a BST program on balance, strength & fitness	Single grp Repeated measures (pre/post & 4-week follow-up) No blinding	Eligibility Required confirmation from treating Dr that MG was controlled, symptoms were **stable**, & medication would not be changed during the study. Excluded: Cognitive deficits & any additional neurological or musculoskeletal condition that affected mobility. N = 7 included MGFA II: 5 (71%), MGFA III: 2 (29%) Female: 4 (57%) Mean age: 53.9 yrs [range 24–75] Mean DD: 7.9 yrs [range 5–20] N = 6 completed post-intervention assessment + analysed MGFA II: 5 (83%), MGFA III: 1 (17%) Female: 3 (50%) Mean age: 59±12 yrs [range 43–75] Mean DD: 10±5 yrs [range 5–20]	**1-2/week** depending on work commitments. **BST: 16-session workstation intervention within an exercise grp** **BST, strengthening, endurance training** **Exercises tailored individually** to physical ability as determined by initial assessment. **PT students** delivered the intervention **under PT supervision**. Examples: heel–toe walking, sit to stand, ball catching & throwing. Progressive increases in challenge were introduced if subject was able to cope. This was done by increasing the number of repetitions, altering the speed, introducing dual tasks, or changing the base of support or support surfaces. **Intensity** undefined No CG	1 dropout during the intervention period. 2 subjects participated once a week, 4 subjects twice a week. Compliance was otherwise not reported.	Improvement defined as ≥ 15% improvement b/w pre & post (& F/U 4wks post-intervention.) 6MWT TUG TUGmanual TUGcognitive Standing stability (foamEC) When subjects were taking AChEIs, assessments were undertaken approx. 3hrs after ingestion.	No subject reported or showed any AEs.	2 dropouts: 1 during intervention due to work commitments. 1 post-intervention due to illness and cardiac arrhythmia 71.4% completed Lost to F/U 28.6%	Improvement in QMGS (median 29%), TUGcognitive, FoamEC (change of 29% representing a ↓in COP sway velocity). Only improvement in QMGS (41%) & FoamEC (45%) indicating greater postural stability) maintained at F/U.

		Antibodies: not reported						
Lohi, 1993 [6] Gothenburg, Sweden	Within subject control – contralateral limb Randomised training to right or left UL & LL, comparator = contralateral UL & LL No blinding	<u>Eligibility</u> <50 years old Mild-mod MG Living nearby Excluded – other severe or disabling disease N=11 analysed Female: 10 (91%) 25-50yrs UL/LL Mild: 6 (55%) UL/LL Mod : 2 (18%) Oculo/bulbar: 3 (27%) → mod for calculations Antibodies: not reported	**2-3/per week, 10weeks** (unilateral UL & LL), **27-30 supervised sessions** + **≤ 5 unsupervised sessions** **Session time unspecified** **Weights based on individual MVC** EF, KE trained sitting, EE trained supine – upper arm vertical, forearm horizontal **Intensity** undefined	EE: Only 1 (9%) could perform as planned, 9 (82%) could not manage number of repetitions in each training set & 8 (73%) were unable to ↑ training load as planned. EF: 6 (55%) managed well whereas 4 (36%) had problems with number repetitions & 3 (27%) with ↑ing workload. KE: only 1 (9%) unable to use initially predicted training weight but managed later as did all others.	MVC EF, EE, KE - fixed dynamometer Fatigability test (EF, EE, KE): max contractions over 3mins – 3s on/2s off – peak value of each & mean decline calculated using linear regression analysis	AEs noted at each training session. None reported. No one complained of muscular pain or discomfort during the training period but not all completed	No dropouts Lost to F/U 0%	All reported that they gained better strength and resistance to fatigue during the training period. Two subjects improved their daily level of functioning, reporting that their walking distance had increased (not an outcome measure). Slight ↑ KE strength compared to untrained side Fatigability results inconclusive No change fatigue or max force EF/EE

AChEIs: Acetylcholinesterase inhibitors, AT: Aerobic training, BST: Balance strategy training, BDI: Beck Depression Inventory, BB: biceps brachii, B&B: Box and Block Test, CACE: compliers average causal effect, CG: Control group, CI: contraindication, CS: corticosteroids, CVD: cardiovascular disease, D: Duration, DD: disease duration, EE: elbow extension, EF: elbow flexion, EG: Exercise group, EMG RNS: electromyography repetitive nerve stimulation, ESES: Exercise Self-Efficacy Scale, FSS: Fatigue Severity Score, FoamEC: standing balance on foam with eyes closed, FSGC: focal segmental glomerulosclerosis F/U: Follw-up, F: frequency, HHD : hand-held dynamometer, HRQoL: Health-related quality of life, HR: heart rate, ITT: Intention-to-treat, I: Intensity, KE: knee extension, LL: lower limb, MD: missing data, MDI: Major Depression Inventory, MFIS: Modified Fatigue Impact Scale, MGC: Myasthenia Gravis Composite Score, MGQOL-15: Myasthenia Gravis health-related quality of life scale, MG-QoL15r: Myasthenia Gravis Quality of Life 15 revised, MG-ADL: impact of MG on activities of daily living scale, MMS: Myasthenia Muscle Score, PA: Physical activity, QMGS: quantitative myasthenia gravis score, SCT: Stair Climb Test, SEI: Self-esteem Inventory scale, STAI: State Trait Anxiety Inventory, STS: 30s Sit-to-stand test, 6MWT: Six-minute walking test, 6MWD: Six-minute walking distance, RCT: randomised control trial, RF: rectus femoris, RA: research assistant, RT: resistance training, 30STS: 30-Second Chair Stand Test, TUG: Timed Up and Go test, TUGmanual: TUG with dual task, TUGcognitive: TUG with dual task, 12MWT: Twelve-Minute Walk Test, UL: upper limb, VI: vastus intermedius, VAFS: visual analogue fatigue scale, WHOQoL BREF: World Health Organisation QoL scale, 1-RM: 1-repetition maximum, VC: vital capacity *Minimal important difference for improvement: QMGS 2 or 3 points, MGC 3 points [1]. NB: Where outcomes are listed, if there is no change they are not necessarily mentioned in the results column, Mean ± SD (range), median (range), [] min, max

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MiR-146a in myasthenia gravis thymus: from uncontrolled innate immunity to B-cell-mediated autoimmunity

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ABSTRACT

The thymus is the main trigger site of autoimmunity in myasthenia gravis (MG) associated with anti-acetylcholine receptor (AChR) autoantibodies, a prototypic autoimmune disease affecting the neuromuscular junction. The majority of patients with early-onset MG have follicular hyperplastic changes of the thymus that are critically implicated in the initiation and perpetuation of the autoimmune response against the AChR. Uncontrolled activation of Toll-like receptor (TLR)-mediated innate immune responses, chronic inflammation, and ectopic germinal center (GC) formation are key pathological features of the hyperplastic thymus in MG, indicating that a close link between innate immunity and B-cell-mediated autoimmunity underlies the intra-thymic pathogenesis of MG.

MiR-146a is an "immune-miR" that acts as a key modulator of both innate and adaptive immunity and is a potent inhibitor of TLR signaling pathways. It is able to prevent and avoid overstimulation of the inflammatory response by targeting the NF-κB signaling transducers IRAK1 and TRAF6. At the same time, miR-146a modulates the expression of c-REL, ICOS, and ICOSL, which are crucial regulators of B-cell function and GC response. Dysregulation of miR-146a expression is a common molecular event in several autoimmune disorders. Recent findings have found defective expression of miR-146a in follicular hyperplastic MG thymuses, associated with over-expression of its TLR- and B-cell-related target genes, which suggests that loss of regulatory functions of this miRNA may contribute to the immunopathological steps leading to MG. Of note, corticosteroids have been found to increase miR-146a expression thus suggesting that miR-146a can mediate the effects of these drugs in inducing immunosuppression and control of autoimmunity.

In this review, we discuss the role of miR-146a as a molecular bridge between innate and adaptive immunity and summarize the current knowledge on the miRNA contribution to the intra-thymic pathogenesis of MG associated with follicular hyperplastic thymus. We also highlight the role of miR-146a as a potential biomarker for therapeutic monitoring and as a target of future advanced RNA-based therapies to modulate the immune system and counteract the autoimmune response in AChR-MG.

Key Words: *autoimmunity, innate immunity, miR-146a, myasthenia gravis, thymus*

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Introduction

Myasthenia gravis (MG) is a chronic autoimmune disease characterized by fluctuating muscle weakness and fatigability of ocular, bulbar, and skeletal muscles caused by autoantibodies to neuromuscular junction (NMJ) components. In about 80% of patients the autoimmune response is directed against the acetylcholine receptor (AChR); less frequently, autoantibodies target the muscle-specific tyrosine kinase (MuSK) or the lipoprotein-related protein 4 (LRP4). Patients in which specific autoantibodies cannot be detected are currently classified as seronegative (1,2).

A consensus-based stepwise approach is recommended for treatment of MG, including symptomatic therapy with cholinesterase inhibitors, immunosuppressive (IS) therapy with corticosteroids, alone or combined with other IS agents, thymectomy in selected patients, and plasmapheresis/immunoglobulins for acute exacerbations (3). The prognosis of MG has greatly improved over the past half century. Nevertheless, up to 80% of patients fail to achieve complete stable remission and need lifelong IS treatment. Moreover, about 10% of patients are treatment refractory or intolerant to IS drugs (4,5), highlighting the importance of gaining a better understanding of the disease-specific molecular events in order to design more effective therapeutic strategies.

The thymus is the main site of autoimmunity development in MG associated with anti-AChR antibodies. AChR-MG patients frequently present morphological and functional changes of the thymus including follicular hyperplasia and thymoma (6,7). Follicular hyperplasia is the most common alteration in early-onset (< 50 years) MG patients. It is characterized by an expanded thymic medulla containing germinal centers (GCs) forming follicles, as observed in secondary lymphoid organs (6). Thymectomy improves the clinical outcome in a considerable proportion of patients with hyperplastic thymus (8), thus supporting a role for this organ in sustaining the autoimmune reaction against the AChR.

The hyperplastic MG thymus may be considered a

prototypic autoimmune organ, since it encompasses a number of immunological alterations commonly observed in target organs of autoimmune disorders, including chronic inflammation, abnormal T- and B-cell activation, B-cell dysfunction, and GC formation (6,9). Experimental data over the past two decades have pointed to a critical role for uncontrolled Toll-like receptor (TLR)-mediated innate immune responses to pathogenic infections in driving and perpetuating the inflammatory autoimmune process in this organ (10-15). However, factors that cause persistence of innate immunity and inflammation, and ultimately chronicity of the autoimmune response in MG thymus still remain to be determined.

The innate immune system consists of a variety of factors that control and participate in all aspects of inflammation and immunity. The innate immune system is the body's first line of defense from invading pathogens, but its improper activation may lead to autoimmunity (16). In normal conditions, innate immune pathways are kept under control by fine-tuning mechanisms to avoid hyper-activation of immune cells and autoimmune phenomena (16). Thus, identification of the molecular events underlying the loss of regulation of innate immunity is an important field of research in MG and other autoimmune diseases in which a dangerous link between innate and adaptive autoimmunity has been demonstrated. A deeper understanding of these molecular events could promote the design of new targeted therapies.

MicroRNAs (miRNAs) modulate many biological processes, including innate and adaptive immune responses (17). MiR-146a-5p (hereinafter called miR-146a) is one of the most important miRNAs known to orchestrate TLR-mediated innate immune signaling, as well as T- and B-cell function, including GC response (18-20). This regulatory property makes this miRNA a good candidate to play a role in the intra-thymic pathogenesis of MG associated with thymic hyperplastic changes and a target for innovative therapeutic interventions to treat long-term inflammation and autoimmunity.

We review the key role of miR-146a in modulating innate and adaptive immune responses and discuss its contribution to AChR-MG by highlighting its biomarker and therapeutic potential.

Innate autoimmune mechanisms in follicular hyperplastic MG thymus

The hyperplastic MG thymus provides a complex microenvironment where the anti-AChR autoimmune reaction can develop and perpetuate. The presence of thymic epithelial cells (TECs) and myoid cells expressing the autoantigen, along with antigen-presenting cells, favors specific antigen presentation/cross-presentation, leading to intra-thymic T- and B-cell autosensitization (9). AChR-specific T- and B-cells and autoantibody-producing plasma cells are present in hyperplastic thymuses of MG patients (21,22). Moreover, abnormal neoangiogenic processes, consisting of high endothelial venule development and over-expression of chemokines (e.g. CXCL13 and CCL21) promoting peripheral cell recruitment into the thymus have been described (6,9), indicating that autoimmunity can be triggered and then perpetuated.

Chronic inflammation, with over-expression of pro-inflammatory cytokines, including interleukin-6 (IL-6), IL-17, and type I Interferons (IFN-I), and up-regulation of TLRs (i.e. TLR3, TLR4, TLR7, TLR9), is likely to play a role in inducing thymic hyperplastic changes and intra-thymic anti-AChR sensitization in MG patients (6-15). Cufi and colleagues demonstrated that TLR3 signaling selectively increased the expression of the AChR-α subunit in TECs via IFN-β (14). Moreover, stimulation of both TLR3 and TLR4, via a combination of Poli(I:C) and lipopolysaccharide (LPS) induced thymic hyperplasia, anti-AChR antibody production, and MG symptoms in mice without immunization, suggesting that lymphoid neogenesis and anti-AChR autoreactivity could result from dysregulated TLR signaling in the thymus (15). These events can be mediated by TLR-induced production of the antiviral mediator IFN-β. Indeed, IFN-β can increase AChR-α expression and apoptosis in TECs, thereby favoring protein uptake by dendritic cells (DCs) and antigen presentation, at the same time increasing CXCL13, CCL21, and BAFF expression, that result in peripheral immune system cell recruitment and enhanced survival of B-cells, including autoreactive cells (23-25).

Viral infections are likely the main trigger for abnormal TLR activation and IFN-I production in hyperplastic MG thymuses, although a role for endogenous molecules, such as nucleic acids (25), is also plausible. Poliovirus persistence was demonstrated in TLR4-positive macrophages in the thymus of some MG patients, suggesting a viral contribution to persistent TLR4 activation and inflammation (26). However, since TLR4 over-expression, but not poliovirus, was common in MG thymuses, it is plausible that in some cases autoimmunity might become clinically apparent when the triggering pathogen has already been cleared by the thymus ("hit-and-run" hypothesis), or viruses other than poliovirus can trigger dangerous TLR4 hyper-activation. Epstein-Barr virus (EBV), a highly B-cell-tropic virus, has been associated with several autoimmune disorders. EBV persistence and reactivation was found to be a common pathological feature of hyperplastic MG thymuses, suggesting a contribution of the virus to abnormal TLR and B-cell activation in the inflamed MG thymic milieu (10,12). EBV nucleic acids can stimulate TLR3, TLR7, and TLR9, with the last two being over-expressed in intra-thymic MG B-cells positive for EBV proteins (12). Since TLR7 and TLR9 can act as co-stimulatory signals for proliferation and survival of B-cells, including autoreactive B-cells, their EBV-driven signals could well participate in perpetuation of autoimmunity in MG thymuses (12,13). Dysregulated

TLR pathways can also affect the balance between effector (Teff) and regulatory T-cells (Treg), in favor of Teffs, as demonstrated for TLR4 pathways (11), thus supporting a TLR contribution to T-cell dysfunction and autoreactive T-cell responses in MG thymuses (11).

The overall data in the literature strongly indicate that a dangerous link between innate immunity and autoimmunity underlies intra-thymic MG pathogenesis. Nevertheless, the reasons why TLR-mediated responses are not properly regulated and turned off in hyperplastic MG thymuses to avoid sustained activation and chronicity of the inflammatory cascade, ultimately leading to autoimmunity, remain to be elucidated.

MiR-146a role in modulation of innate and adaptive immune response

MiR-146a is one of the most important "immune-miRs" capable of regulating TLR signaling and the inflammatory response, and its dysregulated expression has been associated with several autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis, and multiple sclerosis (MS) (27-30). MiR-146a acts as a dominant, potent inhibitor of MyD88-dependent TLR pathways via suppression of two recognized target genes, the tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) and the interleukin 1 receptor associated kinase 1 (IRAK1), which are key components of the TLR pathways functioning as NF-κB signaling transducers (18).

The gene encoding miR-146a is located within the *MIR3142HG* host gene on chromosome 5 (5q33.3) and has a promoter locus with binding sites for NF-κB, IRF3/7, and c-myc transcription factors (18,31-33). Regulation of TLR signaling via the miRNA occurs through a negative feedback loop: miR-146a is induced by NF-κB in response to TLR stimulation, and it then targets TRAF6 and IRAK1, thus inhibiting TLR signaling to dampen the magnitude of the immune response and guarantee maintenance of immunological tolerance (32,33). Indeed, mice lacking the miR-146a gene have several immune defects and spontaneously develop autoimmunity, pointing once again to miR-146a function as an effective control on autoimmune processes (33).

Normally, suppression of TRAF6 and IRAK1 via miR-146a leads to reduced expression of NF-κB target genes, such as IL-6, IL-8, IL-1β, TNF-alpha, IFN-I, and inflammatory chemokines (18,32), the excessive production of which may favor an autoimmune response in a susceptible background. In SLE, reduced miR-146a levels correlate with higher levels of inflammatory molecules and IFN-I, and with worse clinical manifestations; contrariwise, introduction of the miR-146a into patients' PBMCs alleviates the activation of the IFN-I pathway (34). Along with TRAF6 and IRAK1, miR-146a has been shown to target the signal transducer and activator transcription 1 (STAT-1) and interferon regulatory factor 5 (IRF-5) to control the antiviral IFN-I response (18). Since STAT-1 is a transcription factor required for Teff differentiation, its repression via miR-146a is also important for the suppressive function of Tregs. Indeed, miR-146a is highly expressed in Tregs, and its knock-out expression in these cells leads to a fatal tolerance breakdown in mice which results in CD4+ T helper lymphocyte-mediated immunopathology (35). MiR-146a has also been demonstrated to block the autocrine IL-6- and IL-21-induced Th17 differentiation pathways in autoreactive CD4+ T-cells. In this regard, miR-146a-deficient mice developed a more severe experimental autoimmune encephalomyelitis (EAE), an animal model of MS, associated with increased differentiation of T-cells into Th17 cells (36). There is considerable evidence of miR-146a involvement in the control of adaptive immunity by modulating not only T- but also B-cell functions, particularly the GC response (36). Indeed, miR-146a deficiency promotes activation of c-Rel, an NF-κB subunit implicated in B-cell proliferation and differentiation (36). Moreover, miR-146a limits the accumulation of follicular T helper (Tfh) cells and GC B-cells by targeting the inducible T-cell costimulator (ICOS) and its ligand (ICOSL), as demonstrated in mice by Pratama and colleagues (19). Additionally, increased miR-146a expression was associated with down-regulation of Fas cell surface death receptor (FAS) in naïve B-cells, which disrupts lymphocyte homeostasis and leads to hyper-lymphoproliferation and GC formation (20).

Taken together, the aforementioned studies strongly point to an extensive role for miR-146a as a critical negative regulator of innate and adaptive immune reactions (Table 1), highlighting its deficiency as harmful, and its normalization as a potential therapeutic approach for treating inflammatory autoimmune disorders. However, determination of the optimal miR-146a dosage, as well as identification of the optimal target cells, would be of outmost importance for its use as a therapeutic agent, since superabundant miR-146a expression can lead to imbalanced immune homeostasis and side effects (e.g. spleen and lymph node enlargement) (20).

MiR-146a in MG associated with follicular hyperplastic thymus

Despite the critical involvement of miR-146a in modulation of the innate and adaptive immune system, its possible contribution to intra-thymic MG pathogenesis has only recently been investigated. Defective miR-146a expression was found to be a key alteration in hyperplastic thymuses from early-onset (< 50 years) MG patients, with a profound impact on the expression of genes involved in TLR signaling, as well as genes controlling B-cell function and GC formation (37).

Table 1. Main target genes of miR-146a involved in innate and adaptive immune response

	Symbol	Function	MiR-146a effect	References
Innate immunity	TRAF6, IRAK1	Key mediators of MyD88-dependent TLR signaling pathways	***Down-regulation:*** Inhibition of MyD88-dependent TLR signaling pathways and suppression of the inflammatory response	18, 32, 33
	TLR4	TLR family member for recognition of LPS, and other bacterial and viral components whose signaling leads to NF-kB activation and pro-inflammatory gene expression	***Down-regulation:*** Inhibition of TLR4 signaling pathways and suppression of inflammatory response	38
Adaptive immunity	STAT-1	Transcription factor required for Teff differentiation	***Down-regulation:*** Reduced Teff differentiation and increased Treg function	35
	IRF-5	Transcription factor for IFN-I pathway activation	***Down-regulation:*** Inhibition of IFN-inducible gene expression and IFN-I-mediated antiviral response	18
	c-REL	NF-kB subunit implicated in B-cell proliferation and differentiation	***Down-regulation:*** Negative regulation of B-cell proliferation and differentiation	36
	ICOS	Inducible T-cell costimulator acting as a T-cell response activator and positive regulator of Tfh cell differentiation	***Down-regulation:*** Inhibition of Tfh cell accumulation and GC formation	19
	ICOSL	Cell surface antigen acting as ICOS ligand to activate T-cell response and positively regulate Tfh cell differentiation	***Down-regulation:*** Inhibition of Tfh cell accumulation and GC formation	19
	FAS	Cell death receptor leading to apoptosis pathway by Fas ligand	***Down-regulation:*** Interference with Fas-mediated apoptosis; increase of B-cell survival, activation and GC response	20, 42

Abbreviations: TRAF6: tumor necrosis factor receptor associated factor 6; IRAK1: interleukin 1 receptor associated kinase 1; MyD88: Myeloid differentiation primary response 88; TLR: Toll-like receptor; TLR4: Toll-like receptor 4; LPS: lipopolysaccharide; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B-cells; STAT-1: signal transducer and activator transcription 1; Teff: effector T-cells; Treg: regulatory T-cells; IRF-5: interferon regulatory factor 5; IFN-I: type I interferon; c-REL: proto-oncogene c-REL; ICOS: inducible T-cell costimulator; Tfh: follicular T helper; GC: germinal center; ICOSL: inducible T-cell costimulator ligand; FAS: Fas cell surface death receptor

Defective control of innate immune response

The expression of miR-146a and its TLR-related target genes was recently assessed in follicular hyperplastic thymuses from early-onset AChR-MG patients and normal control thymuses from patients without autoimmune diseases (37). MiR-146a levels were significantly lower in hyperplastic MG compared to control thymuses, whereas the expression levels of the miRNA targets TRAF6 and IRAK1 were increased (37). No significant difference in intrathymic miRNA levels was found between male and female patients. In view of the crucial miR-146a inhibitory role discussed above, this finding pointed out the lack of efficient control of innate immune responses and inflammation in hyperplastic MG thymuses (37).

MiR-146a is a key regulator of MyD88-dependent TLR signaling pathways, including those of TLR4, known to be over-expressed in hyperplastic MG thymuses (11). A close relationship between defective miR-146a expression and TLR4 up-regulation in MG thymic tissues can be postulated. Indeed, an interaction between TLR4 and miR-146a has been demonstrated via a consensus bioinformatics approach, and decreased expression of the miRNA was found to be concomitant with TLR4 up-regulation in macrophages. Conversely, TLR4 down-regulation was accompanied by over-expression of miR-146a (38). In line with these observations, double immunofluorescence analyses disclosed increased expression of IRAK1 in macrophages and myeloid DCs (mDCs), known to over-express TLR4 (11), in hyperplastic MG compared to control thymuses (37). This links miR-146a deficiency with increased TLR activation and pro-inflammatory cytokine production via these cells, that in turn may contribute to chronic inflammation. TLR7 and TLR9 were also found to be up-regulated in hyperplastic MG thymuses, likely due to active EBV infection (10,12). Of note, macrophages and mDCs were found to over-express TLR7, the expression levels of which were correlated with those of IFN-β (12), suggesting a relationship among low miR-146a levels, TLR7 over-activation, and IFN-β over-expression in the above-mentioned cells. Of note, EBV proteins are able to modulate miR-146a expression and function: EBV nuclear antigen 2 (EBNA2), expressed in newly infected naïve B-cells, down-regulates miR-146a, thus increasing IRAK1 and antiviral IFN-I expression (39). On the contrary, latent membrane protein 1 (LMP1), expressed in latently infected cells, induces miR-146a expression to decrease the intensity or duration of IFN-I response in a negative feedback loop for latency maintenance (40). Thus, defective expression of miR-146a in chronically inflamed hyperplastic MG thymus, characterized by active EBV infection, might be a critical factor contributing to the loss of regulation of IFN-I pathways, that in turn promote anti-AChR autosensitization (23,24).

Impact on B-cell function and GC response

The expression of B-cell-related miR-146a target genes was assessed in hyperplastic MG thymuses characterized by reduced levels of the miRNA (37). Transcriptional levels of c-Rel, an NF-κB subunit implicated in proliferation and differentiation of B-cells and GC formation (36), were significantly increased in MG pathological tissues compared to controls, suggesting that miR-146a deficiency may favor intra-thymic B-cell dysregulation via c-REL in MG patients. Indeed, the miRNA and target mRNA levels were negatively correlated, supporting a functional relationship with each other (36). At the protein level, c-REL was markedly expressed in both GCs and infiltrating B-cells of the MG thymic medulla (37). Similarly, the expression of ICOS, another recognized miR-146a target implicated in GC formation (19), was significantly increased in hyperplastic MG versus control thymuses, further supporting a link between low miRNA levels and GC development in the thymus of MG patients (37). This idea is based on considerable data that show miR-146a ability to repress ICOS, which is expressed in Tfh cells, and ICOSL, which is expressed in GC cells (19). Interestingly, Cho and colleagues demonstrated that specific miR-146a deletion in T-cells can increase Tfh cell number, strongly enhancing GC reactions (41). Thus, it is reasonable that the miRNA decrease observed in MG thymuses (37) can promote accumulation of Tfh and GC B-cells. The relationship between miR-146a deficiency and the presence of GCs was explored by laser-capture microdissection experiments, showing that the miRNA was expressed in GCs, whereas its levels were defective in the thymic medulla surrounding the GCs in MG thymic tissues (37). Of note, FAS mRNA levels were reduced in miR-146a-positive GCs compared to the surrounding medulla, in line with data in the literature that indicate miR-146a ability to induce GC formation via inhibition of FAS (20). The importance of FAS in GC formation was supported by data showing that B-cell-specific FAS-deficient mice develop fatal lymphoproliferation due to B-cell activation, and ablation of FAS specifically in GC B-cells may reproduce lymphoproliferation (42).

In summary, a critical role for miR-146a in B-cell dysfunction and GC response in MG thymuses can be postulated: on the one hand its defective expression in Tfh can increase the Tfh cell number, hence enhancing GC formation via the ICOS/ICOSL axis; on the other, the miRNA is expressed in B-cells and can promote GC response by targeting FAS (37).

MiR-146a in MG animal models

MiR-146a involvement in MG immune responses has been investigated in experimental autoimmune MG (EAMG) models. Zhang and colleagues proved that miR-146a is up-regulated in activated B-cells in response to

the AChRα-subunit R97-116 peptide in EAMG mice, and this up-regulation was significantly attenuated by the antagomiR-146a (43). Silencing of the miRNA in B-cells led to decreased total IgG levels *in vitro* and to significant improvement of symptoms in mice with ongoing disease (43). In a subsequent study, miR-146a expression was found to be significantly different between EAMG and control rats in immune organs, including the thymus, lymph nodes, and spleen (44). MiR-146a levels were decreased in the EAMG thymus and drainage lymph nodes compared with those in the same organs of the control animals in line with data obtained in thymuses from MG patients (37); contrariwise, in splenic tissue, higher levels of miR-146a were observed in EAMG compared to control animals (44). Since the thymus and drainage lymph nodes are enriched by T-cells, while the spleen is composed mainly of B-cells, differential expression of miR-146a in these tissues could be related to the cell content. Indeed, miRNA levels were down-regulated in Th17 and Treg cells and up-regulated in B-cells, of EAMG compared to control rats (44). Decreased miR-146a levels in T-cells from EAMG animals (44) was in line with the contribution of defective miR-146a expression to pathogenic T-cell function (35).

To assess the therapeutic effects of miR-146a in EAMG, Yin and colleagues (45) produced exosomes from miR-146a overexpressing DCs and observed that they suppressed ongoing disease in mice, altering the Th cell profiles from Th1/Th17 to Th2/Tregs both in serum and spleen. These therapeutic effects were antigen-specific and partly dose dependent (45).

MiR-146a as mediator of corticosteroid effects, treatment monitoring biomarker, and new therapeutic target for MG

Palagani and colleagues (46) demonstrated that glucocorticoids can regulate the expression of multiple genes involved in cell cycle control, cell organization, cell death, and immune response, as well as a number of miRNAs, termed glucocorticoid-inducible miRNAs, including miR-146a. In line with these observations, defective expression of miR-146a was found in hyperplastic thymuses from corticosteroid-naïve but not corticosteroid-treated MG patients, suggesting that IS treatment before thymectomy could have normalized/restored miRNA levels (37). MiRNA normalization in the thymus of treated patients was accompanied by down-regulation of TRAF6, IRAK1, c-REL, and ICOS genes, thus supporting a link between anti-inflammatory and IS effects of corticosteroids and miR-146a induction (37). *In vitro* studies strengthened this idea, since treatment with prednisone enhanced miRNA expression in peripheral blood cells (37). Considering the key role of the miR-146a/target gene axis in the regulation of GC formation, restoration of miR-146a levels by corticosteroids could partially explain the previously demonstrated ability of these drugs to reduce thymic GCs in MG patients (47). According to data obtained in the thymus, significant down-regulation of miR-146a was also observed in serum of corticosteroid-naïve AChR-MG patients compared to controls, whereas in corticosteroid-treated patients, serum miR-146a levels were normal (37), supporting a role of the miRNA as a therapeutic monitoring biomarker in AChR-MG patients. Based on overall findings, we suggest that miR-146a may mediate the effects of corticosteroids and that its levels in individual patients can affect, or be related to, the therapeutic responses to these drugs. Indeed, sensitivity and specificity performances of serum miR-146a discriminated AChR-MG patients from healthy controls (AUC: 0.78, P=0.027) (37). The potential role of the miRNA as a biomarker to predict or monitor AChR-MG patients' response to IS drugs deserves further study.

The ability of miR-146a to control both innate and adaptive immune response strongly highlights its modulation as a prospective molecular option to counteract autoimmunity in MG and potentially other autoimmune diseases. However, due to the multifaceted functions of miR-146a in different immune system cells, its therapeutic manipulation could result in beneficial or detrimental effects in a cell-dependent manner. Silencing of miR-146a in B-cells improves MG symptoms in the EAMG animal model (43), as described above. Metformin improves EAMG by reversing the expression of miR-146a in AChR specific B- and Th17 cells, partially inhibiting the pathogenic functions of these cells: beneficial effects were associated with decreased expression of miR-146a in B-cells and its increase in Th17 cells (44). Over-expression MiR-146a in DCs inhibits their maturation and leads to generation of exosomes able to reduce T-cell proliferation and polarize them toward an anti-inflammatory phenotype in EAMG animals and suppressing the ongoing disease (45).

The overall data indicate that miR-146a may serve as a potential therapeutic target for MG, but the challenge will be to design miRNA-modulating cell-specific therapies based on advanced delivery vehicles for administration of RNA therapy.

Conclusions

MiR-146a is a regulator of innate and adaptive immune responses implicated in the pathogenesis of several autoimmune conditions, including intra-thymic MG pathogenesis. A model of miR-146a as a molecular bridge linking innate and adaptive autoimmunity in hyperplastic MG thymus is shown in Figure 1. Based on literature data, miR-146a offers an important resource for innovative strategies to modulate immune system cells in the context of MG, and restore immune regulation. Thus, a deeper understanding of the miRNA mimicking/inhibition impact on specific cell types (e.g. dendritic cells, T- and B-lymphocytes) could prospectively pave the way to development of advanced molecular strategies to disrupt the link between innate immune activation and adaptive autoimmune response in MG.

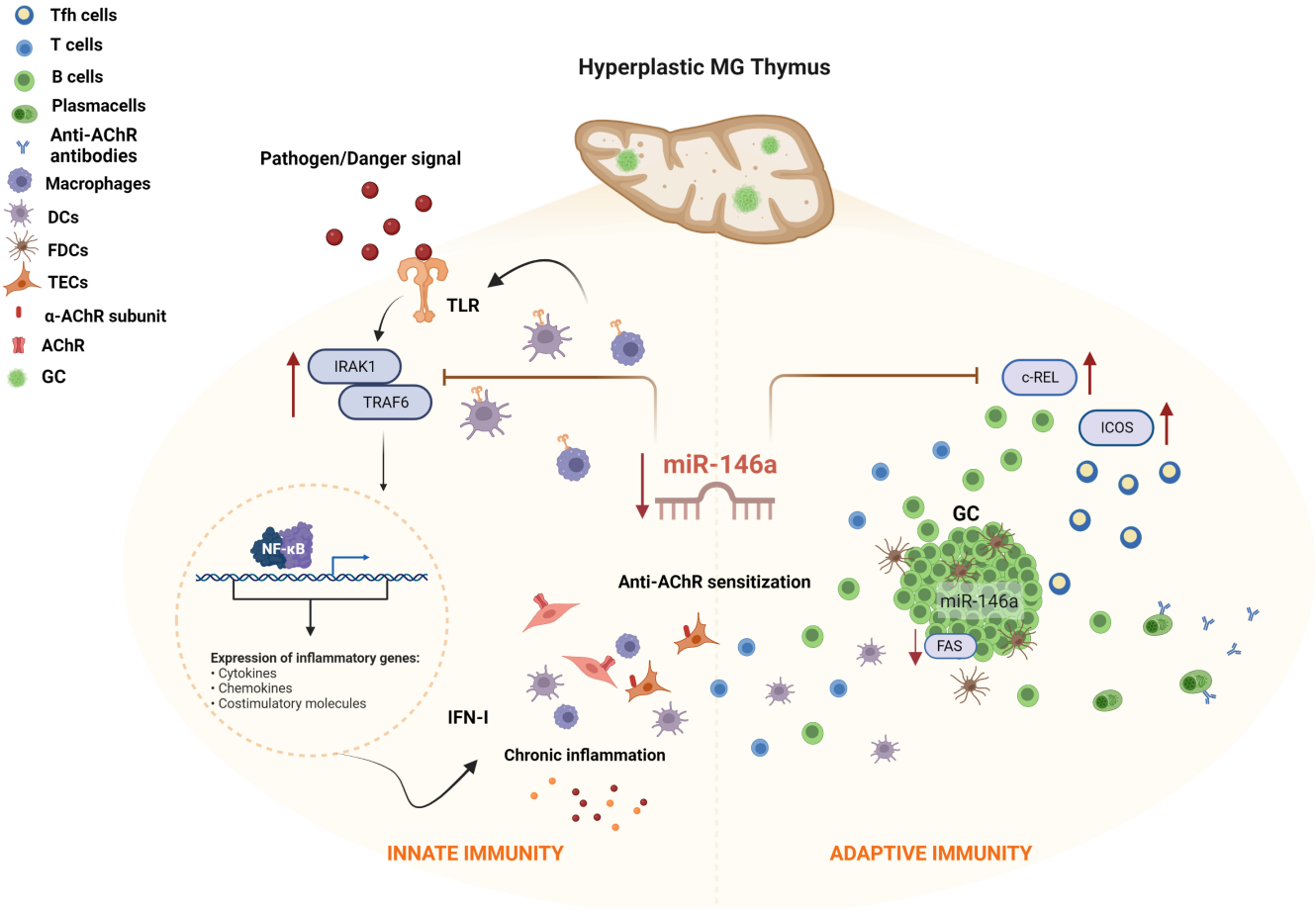

Figure 1. Model of miR-146a involvement in intra-thymic pathogenesis of MG associated with follicular hyperplastic thymus. Defective expression of miR-146a in innate immune system cells (e.g. macrophages, dendritic cells) of the thymus contributes to uncontrolled activation of pathogen-stimulated MyD88-dependent Toll-like receptor (TLR) signaling pathways due to loss of the miRNA inhibitory/regulatory effects on IRAK1 and TRAF6 expression. IRAK1 and TRAF6 increases cause sustained NF-kB activation and hence over-expression of pro-inflammatory cytokines, chemokines, and type I interferons (IFN-I), in turn promoting intra-thymic chronic inflammation. MiR-146a deficiency also contributes to over-expression of c-REL and ICOS, favoring B-cell proliferation and differentiation, and accumulation of follicular T-helper (Tfh) cells that, along with follicular dendritic cells (FDCs), promote germinal center (GC) formation. Decreased expression of Fas via miR-146a allows GC maintenance. IFN-I production in the inflamed thymic milieu, favorable to B-cell activation and survival, ultimately leads to autosensitization to the locally expressed acetylcholine receptor (AChR), and perpetuation of autoimmunity in the context of genetic backgrounds prone to MG. Figure created with BioRender.com.

Acknowledgments

This article is dedicated to the memory of our friend and colleague Pia Bernasconi, a passionate scientist whose findings significantly contributed to the understanding of an involvement of Toll-like receptor (TLR)-mediated innate immune response in the intra-thymic pathogenesis of myasthenia gravis (MG). Her ideas inspired and continue to inspire us to carry on our research on MG with passion and motivation.

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Biomarker Development, Methodological Challenges

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ABSTRACT

Biomarker development is a common endeavor in medical research. The purpose is to find indicators of disease occurrence or prognostic markers for response. The process of development of biomarkers often starts with showing mean differences between responders and non-responders or those with a disease or condition versus those without. However, these statistically significant mean differences, while necessary are not sufficient to validate a biomarker. Sensitivity, specificity, positive and negative predictive value are at least as important and the relative increase in performance using the biomarker over the usual clinical variables should be demonstrated. This paper discusses the various assessments in the context of use for the biomarker, the need for characteristics in addition to mean differences and the importance of independent validation of putative biomarkers. Lastly, it is hoped that the process and thoroughness necessary be considered with recognition that the task is at best difficult.

Key Words: Biomarkers, Surrogate Outcomes, Prentice Criteria, Prognostic Biomarkers, Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value, Validity

Introduction

The search for biomarkers is not new. Fever has long been used as a sentinel biomarker for illness in the body. This common tool for lay and professionals alike is, of course, a consequence of disease rather than a predictor of disease, although it may be a harbinger of a consequence indicative of the need for treatment or the impending consequences of disease. Often in the search of biomarkers we use a similar fallacy called the *post hoc ergo propter hoc* fallacy, whereby one assumes that one event must have caused a later event simply because it happened after the other. One might argue that this happens with acetylcholine receptor (AChR) antibodies in myasthenia gravis. The fact that these are defining the disease does not mean that the severity or course of disease is predicted or identified by the levels seen. Prior occurrence is not sufficient to define a predictive biomarker. Biomarkers may indicate what will happen or they can be useful to avert something happening.

"Biomarkers are biological substances, characteristics, or images that provide an indication of the biological state of an organism." (group 2001) (Medicine 2009). The FDA defines 5 categories that need to be considered when developing or evaluating a biomarker:

- Context of use (purpose, population, and nature of disease)
- Analytical validity
- Clinical validity
- Clinical utility
- Gold standard validation

The above categories are somewhat self-evident. The context of use (FDA) or COU in FDA nomenclature, defines two steps in the development of a biomarker. First is the category of use into one of 7 categories: Diagnostic; Monitoring; Predictive; Prognostic; Pharmacodynamic/Response; Safety; Susceptibility/Risk. Then within each category, there is the determination of how the biomarker will be used. For example, the diagnostic use might be for subject selection in a trial: an AChR antibody test might be the cardinal biomarker of myasthenia gravis (MG) and the level might be used to quantify the selection criteria for qualification for a trial as was done in the Thymectomy Trial in Non-Thymomatous Myasthenia Gravis Patients Receiving Prednisone Therapy (MGTX) trial (Wolfe GI 2016) .

Often biomarkers are classified in other related ways, such as a *surrogate* endpoint which is assessed pre- and post-treatment as an early measure of clinical outcome; a *pharmaco-dynamic* biomarker which is assessed pre- and post-treatment as a measure of the effect of treatment on disease; a *prognostic* biomarker, to identify which patients need treatment; and a *predictive* biomarker to determine which patients are likely to benefit or respond from a specific treatment.

Biomarkers aimed at treatment should be able to improve on the prediction of responders over the clinical variables available. That is, having the biomarker results in hand should lead to better prediction of the likelihood of response. Thus, biomarkers may improve treatment decisions by identifying responders in general or identifying treatments that work better in subgroups or vice versa. One example might be the muscle-specific receptor tyrosine kinase (**MuSK**) which identifies patients who are less likely to respond to conventional MG treatments. There are a number of ways statistically that this can be done: Show that the area under the receiver operator characteristic (ROC) curve is increased (Pencina MJ 2010); achieve improvements in the net reclassification index (Hlatky MA 2009); use the integrated discrimination index (IDI) (Pencina MJ 2008). Each of these measures are calculations that return a number that is used to assess if the classifications have been improved by the addition of the biomarker to the prediction equation. The increase in the area under the ROC curve is commonly used, indicating improved sensitivity and/or specificity of the

biomarker under consideration, but is only an indicator of improvement and does not always imply the improvement is clinically meaningful. Thus, a combination of statistical tools is needed to assess the added value of the biomarker.

Surrogate Biomarkers

Validating a biomarker as a surrogate for a clinical outcome is extremely difficult. Usually this requires a series of randomized trials with both the biomarker and clinical outcome measured demonstrating correlated differences in the outcome and/or mediation of the treatment effect by the biomarker. While there are criteria for defining when this has occurred, it is rare that such surrogates can be found. Even the concept of surrogate is dubious because often a large treatment effect on the surrogate corresponds to only a small treatment effect on the true clinical outcome. Think of blood pressure treatment for hypertension and the outcome of cardiovascular disease. Blood pressure treatments often lower blood pressure by 15% to 20%, but the impact on mortality may be less than 5%. However, on a population level this impact is large and clinically meaningful indicating again the context of use is important.

Prentice (RL 1989) created what may be considered the most stringent criteria or the goal of a surrogate outcome. Within a randomized clinical trial (RCT):

- The treatment must have an effect on the surrogate.
- The treatment must have an effect on the clinical outcome.
- The surrogate and the clinical outcome must be correlated.
- The treatment effect on the true clinical outcome must disappear after adjusting for the surrogate.

The last of these criteria is, for the most part, unachievable. It is this last criterion that is often relaxed to significantly mediate the outcome and link the concept of a surrogate to a mediating variable. Thus, a surrogate endpoint (Biomarker) is said to be an intermediate (instrumental) variable that can be used to indicate the true clinical endpoint. If the full effect of treatment on the responder status is mediated through the biomarker, then we have a surrogate as defined by the Prentice criteria.

Prognostic Biomarkers

Most prognostic factors are not used, because they are not therapeutically relevant. For example, age is strong predictor of poor outcomes in many situations, yet it is not something we can intervene on therapeutically. We want prognostic biomarkers in the concept of surrogates, which are subject to manipulation and therapeutic intervention. However, to develop such markers requires carefully designed studies even though many are identified via retrospective analyses of existing datasets. That said, most prognostic factor studies are poorly designed. They are not focused on a clear therapeutic decision context and often use a convenience sample of patients for whom material or information is available. Generally, the patients are too heterogeneous to support therapeutically relevant conclusions, and, commonly, they address statistical significance, rather than predictive accuracy, relative to standard prognostic factors.

Two examples might help clarify these issues. Low density lipoprotein receptor-related protein 4 (LRP4-Ab) has recently been considered as a potential biomarker in seronegative MG patients (Chung HY 2023). These authors attempt to develop a cell-based assay (CBA) for the detection, however, they report that "there is no gold-standard test for LRP4-Ab that can be used to compare the performance of the present CBA. The possibility of false-positive results cannot be ruled out. Further studies using different methods for detecting LRP4-Ab are necessary." This lack of a gold standard for validation is equally important with clinical outcomes, which often use a specified amount of change, such as 2 or 3 points on the MG-ADL scale as indicative of being a responder. This often ignores the recruitment requirement to have scores above some cut point, such that responders are mixed with individuals measured in error at baseline with values higher than they actually are. This leads to regression toward the mean and in a randomized trial is expected to be the same in both treatment groups, but in biomarker discovery is confounded with response. Another example is the use of statistically significant differences to infer biomarker status. In the paper by Cavalcante et al. (2019), a microRNA signature was associated with being a biomarker of responsiveness to treatment in MG, and while significant differences are seen, the sensitivity is only around 50%.

Predictive Classifiers

Many treatments benefit only a minority of patients to whom they are administered. This is particularly true for molecularly targeted drugs. Predictive classifiers seek to be able to predict which patients are likely to benefit and which patients can be saved from unnecessary toxicity. Thus, predictive classifiers are focused on the benefit/risk equation of treatment and enhance the patient's chance of receiving a drug that helps them or does not hurt them. If we knew that a person/patient with a specific HLA type when given a certain drug has a higher likelihood of drug-induced liver injury, we might avoid the use of this treatment in favor of some other. Similarly, if we know that a specific HLA type responds better, we would use the treatment associated with the better response. These biomarkers can help control medical costs while improving the success rate of treatment and even clinical drug development.

Validity

Validity implies correctness, but it requires more than simply opinion or face validity. It should demonstrate that the biomarker is predictive *a priori* rather than *a posteriori*. Even though identification and performance characteristics

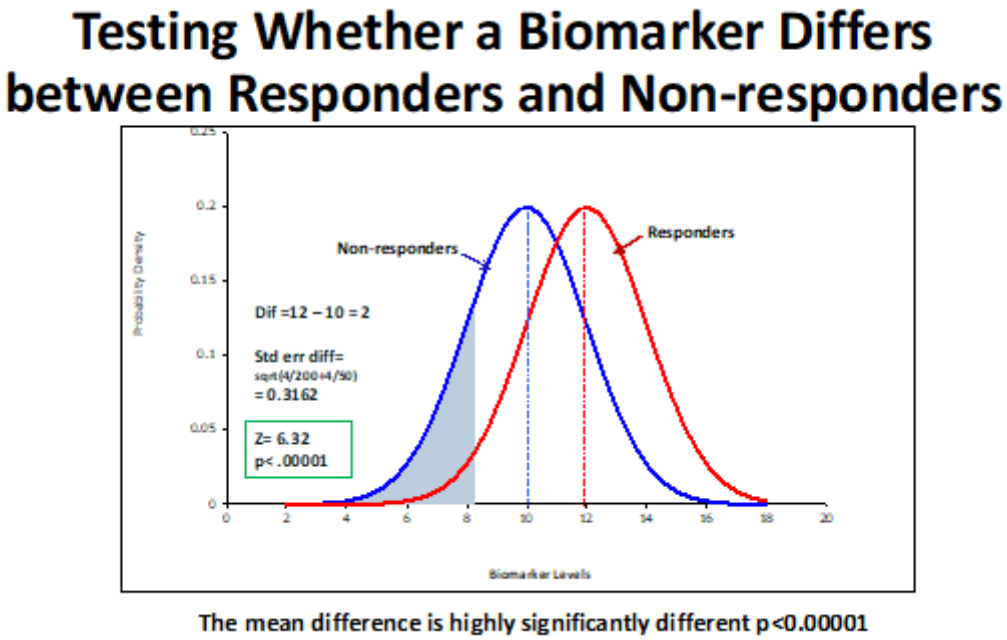

Figure 1: Relative Frequency of Biomarker Levels in Responders (red) and Non-Responders (blue)

are often evaluated by comparing cases to controls, the true test is from prospectively applying the putative biomarker in studies or trials that demonstrate the predictive value. Consider a biomarker for disease diagnosis. Was there an independent, blind comparison with a reference standard of diagnosis? Was the test evaluated in an appropriate spectrum of patients (like those actually seen in clinical practice, where there is diagnostic uncertainty)? Was the reference standard applied regardless of the diagnostic test result? When tests are invasive or expensive, we often only perform these after a higher suspicion of disease is present, this leads to verification bias. For example, because of cost, yield and small risk, routine CTs as the gold standard for detecting thymomas are given to patients only when symptoms are present. Thus, a study of a biomarker for thymoma might underestimate false negatives because patients with symptoms under the threshold were not offered a CT. Additionally, for establishing a biomarker, it is important to ask whether the test is validated in a second group of patients. The last of these questions is essential to provide independent confirmation of the value of the biomarker.

As noted above, too often developers of biomarkers use statistical significance of differences between those with the disease compared to those without the disease as evidence for a putative biomarker. Let's look at an example. Suppose we want to assess whether a biomarker differs between responders and non-responders.

Suppose amongst **non-responders** to CMP (Cutter's Magic Potion) the mean interleukin-17 (IL-17) was found to be 10 with a standard deviation of 2 (sample size of n_1=200). In **responders** it was found, on average, to be 12 with a standard deviation of 2 (sample size of n_2=50). Is IL-17 a biomarker of response? Figure 1 shows the hypothetical distribution of IL-17 for responders (red frequency distribution) and non-responders (blue frequency distribution). The blue curve to the left shows the distribution of the non-responders and the red one to the right are the responders. Approximately 10% of the responders had levels of IL=17 lower than a little over 8 (shown as the shaded area on the blue non-responders curve).

As is often done by researchers when they are attempting to identify a biomarker, they will test the mean differences between responders and non-responders or cases versus controls to convince the reader that the biomarker is indeed a predictor of response. Here we see a mean difference of 2 units (mean of 12 for responders and 10 for non-responders). The t-test for the difference uses the standard error of the mean difference between responders and non-responders to decide if this difference is larger than that expected by change, and this takes into account the standard deviation of the responders and non-responders and the respective sample sizes.

Thus, standard error of mean difference is:

= square root of (variance in non-responders/n_1 + variance in responders/n_2)

= sqrt (4/200+4/50) = 0.3162

And the t-test for the difference between the two groups:

= 2/0.3162 = 6.33 yielding a p-value of 0.00001

This tells us that the means are significantly different, but is this sufficient to establish IL-17 as a biomarker of response? Many investigators think this is so, but while this result is necessary, it is not sufficient. There are other summarizations that are important and meaningful. Four of them are: Sensitivity, which is the probability of a positive test among patients with disease; Specificity, which is the probability of a negative test among patients without disease; Positive Predictive Value (PPV) and Negative Predictive Value, (NPV). PPV means of those that have a positive test, the probability that the individual has the disease or condition (or doesn't have the disease or condition – NPV). The former two, sensitivity and specificity, are what developers of biomarkers generally focus on; however, PPV and NPV are the most important to the patient. Why? While sensitivity, specificity, and false positives and negatives help a discipline, the clinician or patient decide whether to advocate for a biomarker being useful or perform a test with a biomarker because it is useful; patients (and their clinicians) are not directly interested in false positives and false negatives, once they have the result. They want to know what the test means for them! "I have a positive test – what does that mean for me?" For example, if the sensitivity of mammography for detecting breast cancer is 75% and specificity is 98%, this may help policy makers and clinicians recommend a mammogram. However, because so many more women do not have cancer, the false positives greatly outnumber the true positives with this screening test (biomarker). Thus, a clinician can be a calming force for a woman with a positive mammogram informing her that of those with a positive mammogram only about 10% actually have breast cancer. The clinician is using the positive predictive value to assuage the panic of the positive mammogram.

Let's look a bit closer at sensitivity and specificity in our IL-17 example from Figure 1. Recall from Figure 1, that the mean IL-17 in responders was 12 and in non-responders it was 10. If we use 10 as our critical value for determining sensitivity and specificity for those above and below the mean of the non-responders, we would ask in assessing if IL-17 is a biomarker for response, what is the probability of being a responder if their IL-17 is above 10? Similarly, what is the probability of being a non-responder if their IL-17 is below 10. In Figure 1, we see that for responders 10 is 1 standard deviation below the mean (recall the standard deviation is 2 and thus 1 standard deviation below the mean of 12). This translates into 64% of the responders being above 10 (this results from assuming a normal distribution of the IL-17, where 1 standard deviation below the mean separates the population into 64% above the -1 standard deviation and below -1 standard deviation). Similarly, among non-responders, the mean was 10 and thus 50% of the non-responders are below 10 (in a normal distribution 50% are below the mean). If one used IL-17 as a biomarker with the value set at 10, it would not be a good biomarker because so many participants would be misclassified: 50% of the non-responders would be above 10 and thus false positives! In the responder predicted category, 36% of the responders would be below 10 and thus false negatives.

What were the PPV and NPV from Figure 1? There were 200 non-responders and 50% of them are expected to be above 10 or 100 individuals. Of the 50 responders, 64% or 32 were above 10. Thus the PPV = 32/(100+32) = 0.242. Stated another way, if your IL-17 was above 10, you had a 24.2% chance of being a responder. If you just had historical data and no putative biomarker, you would guess that 50/(200+50) = 20% would be responders. This naïve estimate (not taking into account the biomarker) is only slightly below the information that is coming from having the biomarker, that is 24.2% compared to 20%. Thus, while it is an increase in the estimated chance of response, it probably is insufficient to convince users that it is a relevant biomarker.

It is also important to remember that positive and negative predictive value depend on the prevalence of the disease or the outcome. Myasthenia Gravis is estimated at a prevalence of 20 per 100,000 population. Suppose we develop a questionnaire that we think can identify MG. In the clinic we show it has 95% sensitivity in correctly identifying the MG patients, but only 90% specificity, what is the positive predictive value? Consider 100,000 individuals evaluated in a population survey. We expect 20 cases with a sensitivity of 95% and thus, 19 of the 20 cases would be positive on our questionnaire. However, because the specificity is only 90% of the 99,980 individuals without MG, 10% or approximately 10,000 would be flagged as potential cases. Our PPV would then be 19/10,000 or 0.19% and virtually an NPV of 1.

Another common approach to establishing a biomarker is to compare the extremes of the distribution of the biomarker. Investigators often compare the lowest decile or quartile to the highest decile or quartile to show their biomarker works. This too is necessary for a biomarker's performance, but it is not sufficient to establish a biomarker. Consider the lower quartile compared to the upper quartile. Increased response in one quartile compared to the other still leave 50% (quartiles 2 and 3) out of the quantification. This can lead to substantial misclassification and poor performance by the biomarker. The value as a biomarker actually then relies on what happens in the middle rather than at the extremes. There is an especially prevalent use of these extreme comparisons in epidemiological studies and specifically diet studies. Part of the rationale for this prevalent use is that diet is poorly measured and thus the misclassification is not from the performance of the biomarker, but rather the error in assessment of the underlying diet. This may be true, but one needs to exercise caution when interpreting a biomarker determined solely on the basis of comparison of the extremes. In the search for biomarkers, statistically significant differences between

these groups are necessary BUT NOT SUFFICIENT. Achieving high levels of sensitivity and specificity require low variability within a population and high variability between populations and good biomarkers or classifiers require high sensitivity, specificity, PPV and NPV.

The Sequential Organ Failure Assessment or SOFA score is a widely used biomarker of disease prognosis. It has been shown to predict mortality in a variety of settings from the intensive care unit to results of COVID-19 infection. AChR and Aquaporin4 are often thought of as biomarkers, but since they are often used in the definition of the disease and do not clearly associate the levels found with prognosis, they fail to meet these requirements. CD4 counts in HIV and/or hemoglobin A1C in diabetes have been successfully used to characterize these as biomarkers. Although they fail to meet the Prentice criteria cited above, they have proven to be very important biomarkers of response.

Quite common in the development of biomarkers, is the question: how many or much more do I need? This question often comes to biostatisticians brought in to help "bless" a biomarker being considered. While this is a reasonable question, especially in this era of adaptive designs, where incrementally evaluating data is used to arrive at a more firm conclusion, it is also a problematic question. This is because the biostatistician doesn't know what has been done to get to this point in the research. Were outliers tossed, samples rerun, was the development of the data done under a defined or strict protocol or has this evolved and the researcher gained interest in the putative biomarker with further experiments and analyses? While it is important and natural to conduct exploratory data analyses to develop a biomarker, the process is not a continuous one. At some point in the development, a more formal evaluation should occur. This often is done by adding the formal evaluation to a clinical trial providing objective and rigorous evaluation of the putative biomarker. Irrespective of whether this is done within a trial, a formal evaluation under a defined protocol is essential. Adaptive designs require carefully crafted protocols to ensure adequate control of type I errors and *a priori* decision-making.

We are in the era of digital and remote monitoring which will lead to more and more putative biomarkers. The digital biomarker development process has been categorized (Bent B 2020) into: State the goal; define the sensor data to be used; specify other data needed; define the preprocessing necessary; perform exploratory data analyses to evaluate relationships; identify feature engineering and feature selection. What seems missing from this development process is the utility of the biomarker or biosensor. Defining the context of use and the utility in that context are often ignored as the rush to apply or market the device occurs. The utility is often assumed or implied, but not formally evaluated. This last step is critically important lest the information derived from the device is of limited value clinically.

Some digital biomarkers have been shown to improve care. Digital glucose monitors which free the patients from finger sticks and provide real time monitoring of blood glucose continue the known benefits of tight control in diabetes. The plethora of step counters, however, have not been shown to provide improved health despite their widespread use other than in small studies and anecdotal experiences. This latter example exemplifies several issues. First is the rapid escalation in the availability of digital monitoring and the benefits may take much longer to assess. Studies of the control of mild hypertension and tight control of diabetes evaluated mortality over a 5-year period and of course took several years longer in real time to get answers due to funding, initiation, recruitment, etc. In addition, there are the concepts of efficacy and effectiveness. Can the digital monitor work as proposed, that is, is it fit for purpose. These are issues with home step counters and home pulse oximeters. Then, assuming they achieve the technical details of measuring what they purport to measure, do they, in ideal settings, change the clinical outcome (efficacy)? Finally, if they work and possess efficacy, do people use them? The use in practice results in effectiveness and incorporates both accuracy and precision of the device with efficacy and individual compliance.

On the other hand, even small increments in some biomarkers can be important. If we can develop behavioral threat assessments for mass shootings as biomarkers and they lead to actions and/or interventions that prevent mass gun violence, then the biomarker doesn't have to have great sensitivity to be valuable. As long as there are few negative consequences for the false positives, even a poorly performing biomarker might be helpful. The benefit is great, and risk is low or non-existent. Thus, the question being addressed is central to the interpretation of the purported biomarker.

A final word of caution. Developing biomarkers is harder than most investigators think. Without validation, and independent validation, they are just another outcome measure. Investigators need to remember the difference between a correlate and a surrogate. Further, while the excitement of finding mean differences on a putative biomarker are encouraging, mean differences are necessary but not sufficient to establish a biomarker.

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Diagnostic challenges in myasthenia gravis: a clinical approach

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ABSTRACT

The development of antibody tests and neurophysiological techniques have aided in confirming the diagnosis of myasthenia gravis (MG) over the years. However, there still remains an unmet diagnostic need in the subgroup of MG patients with weakness restricted to ocular muscles (OMG) as routine diagnostic tests are less sensitive in this group: around 50% of these patients have no positive antibody test and around 71% have no significant decrement with repetitive stimulation EMG. Moreover, virtually all disorders that can cause a pupil-sparing ptosis or diplopia have been reported to be confused with OMG. Among the most mentioned mimics for OMG are Graves ophthalmopathy, cranial nerve palsies, ocular tendinomuscular deficits (such as levator dehiscence), myopathy, demyelinating disease and stroke. Diagnostic delay and confusion of OMG with mimicking disorders might lead to a worse prognosis due to a possible increased risk of generalization of disease and the need of emergency treatments. A careful clinical follow-up of patients with suspected OMG by systematically assessing changes in ocular weakness patterns between visits can aid in confirming the diagnosis. In addition, the ice pack test can be a diagnostic aid in cases of both evident ptosis and ophthalmoparesis. In the foreseeable future, cell-based assays (CBA) for antibodies to clustered acetylcholine receptor might aid in the diagnostic confirmation of OMG. There is a need of studies that investigate the yield of new and not-routinely used diagnostic tests in suspected OMG with negative antibody and inconclusive EMG and SF-EMG, such as the repetitive ocular vestibular evoked myogenic potentials (RoVEMP) test and CBA. Lastly, the effect of early immunosuppressive treatment should be further investigated in OMG.

Key Words: myasthenia gravis, ocular myasthenia gravis, diagnosis, differential diagnosis, diagnostic tests

Introduction

Myasthenia gravis (MG) is a heterogenous autoimmune disease characterized by fatigable muscle weakness with clinical patterns ranging from purely ocular to different combinations of limb/bulbar and axial weakness. In the second half of the 19th century, the disorder was known as Erb's or Erb–Goldflam disease.[1,2] Jolly observed that MG could be distinguished from 'true' paralyses and coined the term 'myasthenia gravis pseudoparalytica' (myo, muscle; asthenia, weakness; gravis, severe).[3] The broad phenomenological rather than etiological/pathophysiological name for this disease is in concordance with various clinical presentations of MG and the absence of a single laboratory of neurophysiological test that can confirm or exclude the diagnosis.

In 1976, Lindstom showed the presence of antibodies directed towards the acetylcholine receptor (AChR) in 85% of MG patient cohort.[4] This both confirmed the pathophysiological hypothesis of MG being an autoimmune disorder and boosted MG research towards identifying additional antibody targets in the remaining 15% 'seronegative' MG patients. Even though new antibody targets have been identified and neurophysiological tests were developed to support the diagnosis, there remain cases in which the diagnostic tools are not satisfactory. The aim of this review is to discuss diagnostic challenges and to offer a clinical approach for hard-to-diagnose MG patients.

Routine diagnostic procedure

When there is a clinical suspicion of MG due to a typical history of fluctuating fatigable muscle weakness without neurological deficits in other domains, the first line of testing is antibodies, starting with AChR and MuSK antibodies. Testing for striated antibodies (such as for ryanodine receptor and titin) have less of a diagnostic value and are mostly used for prognostic purposes.[5] When antibody tests are negative, electrophysiological tests can be employed to confirm the diagnosis of MG. Firstly, electromyography (EMG) repetitive stimulation is performed and, in the case of no significant decrement, single-fibre EMG (SF-EMG) can be used to find jitter blocking. SF-EMG is not widely available as it requires a certain level of expertise. If all above mentioned tests result negative, the acetylcholinesterase (AChE) inhibitor test can be used. For this test, there must be a clear form of weakness that can be objectively improved during the test, such as a severe ptosis. Lastly, the ice pack test can be used to confirm the diagnosis of MG in patients with evident ptosis (or severe objectifiable ophthalmoparesis).[6-8] Arguably when applicable, this test should be done at the start of the diagnostic procedure. This bedside test, however, does not widely have a specific place

in the diagnostic sequence and is not routinely used in all MG expertise centers.

New and experimental diagnostic tests

In "double-seronegative" MG, when AChR and MuSK antibodies have not been found (~ 5% of all MG patients), antibodies against low-density lipoprotein receptor-related protein 4 (LRP4) and agrin can be tested.[9-11] In addition, cell-based assays (CBA) can be used to increase the sensitivity of antibody detection: Rodríguez et al. showed that 38.1% of radioimmunoassay-negative cases showed positive results on CBA for antibodies to clustered acetylcholine receptor.[12] Regarding new electrophysiological tests, repetitive ocular vestibular-evoked myogenic potentials (RoVEMP) test is used in an experimental setting and is not yet part of the standard diagnostic procedure. In the studies performed until now, RoVEMP test had a sensitivity of 71-89% and a specificity of 64-86%.[13,14] RoVEMP differentiated between MG patients and patients with other neuromuscular disorders, and a significant correlation was found between the magnitude of decrement and the time since the last intake of pyridostigmine.[14] With regards to imaging, quantitative MRI of extra-ocular muscles has been investigated and shown to reveal EOM atrophy and fatty replacement, but until now has not shown to be a potential addition in the diagnostic process.[15,16]

Hard-to-diagnose MG patients

Patients that are particularly hard to diagnose are isolated ocular MG (OMG) patients. Around 50% of these patients have no positive antibody test and around 71% have no significant decrement with repetitive stimulation EMG; see figure 1.[17] SF-EMG has a relatively high sensitivity in OMG of 86%, as high as 94% in a single-center study, but has the problem of not being widely available as discussed earlier and has a relatively low specificity (73-79%) even in specialized centers.[17,18] Particularly in other neuromuscular disorders, SF-EMG results can be abnormal. The AChE inhibitor test is not widely used, because of the risk of serious side-effects and the necessity of an evident and objectifiable form of ocular muscle weakness at the time of testing, such as severe ptosis. Alternatively, a beneficial response to treatment with acetylcholinesterase inhibitors can be used to support the diagnosis of MG. Another problem with suspected OMG is that with it comes a more expansive differential diagnosis as compared to generalized MG.

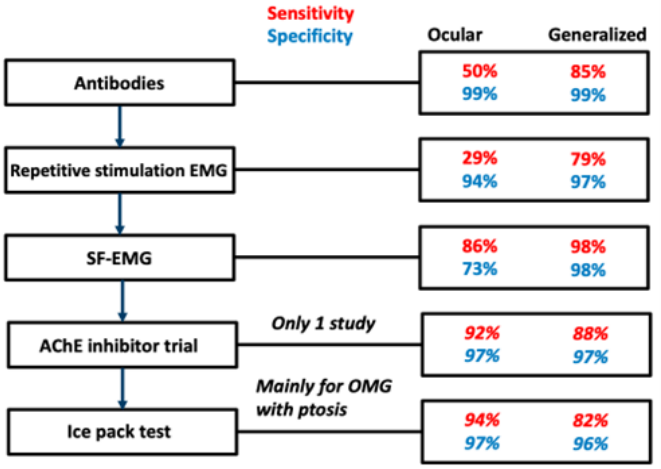

Figure 1. A summary of the sensitivity (red) and specificity (blue) of routine diagnostic tests in ocular and generalized myasthenia gravis derived from Benatar's systematic review.[17] The bottom two tests have a note and italicized numbers because of the lesser generalizability of the study findings.
Abbreviations: EMG = electromyography; SF-EMG=single-fiber-EMG; AChE = acetylcholinesterase.

Comparable disorders and risks of late diagnosis

Virtually all disorders that can cause a pupil-sparing ptosis or diplopia have been reported to be confused with OMG.[19] The most commonly mentioned disorders are Graves ophthalmopathy (GO), cranial nerve palsies, ocular tendinomuscular deficits (such as levator dehiscence), myopathy, demyelinating disease and stroke.[19,20] Especially GO is often reported to be confused with OMG.[20-25] It is controversial whether early treatment with corticosteroids might prevent the progression of ocular MG to a generalized form of MG as the only randomized controlled trial on this topic (Efficacy of prednisone for the treatment of ocular myasthenia (EPITOME) study) had a too small sample size and short follow-up to give a conclusive answer.[26] However, this trial provided support in favor of starting with a therapy of low-dose prednisone in OMG and several experts hold that early corticosteroid treatment in OMG might result in a better prognosis.[27,28] Therefore, early confirmation of the diagnosis of ocular MG is of great importance. Cases of OMG mimicking as GO have necessitated emergency treatments possibly because of diagnostic delay and the late start of adequate immunosuppressive therapy.[21,24]

Diagnostic tools in seronegative OMG

In the case of suspected OMG with negative antibody tests, negative repetitive stimulation EMG test and negative SF-EMG, the first test to consider – if not already performed – is the ice pack test. Several recent reports have again

confirmed the high yield of the test.[7,18] Marinos et al. showed that the ice test is superior to comparable tests (the rest test and the heat test).[8] If this bedside test does not confirm the diagnosis, the next step would be CBA. Studies have shown a relatively high sensitivity of CBA for antibodies to clustered acetylcholine receptor in OMG, probably because of relatively low circulating antibody levels in OMG compared to generalized MG.[12] In the future, RoVEMP might play a role in these hard-to-diagnose patients.[29] In one study, the RoVEMP test was positive in 6 of 7 seronegative OMG patients with a negative repetitive stimulation EMG test.[14] It has to be noted that there is no specific data on the yield of the above tests in the specific group of suspected OMG patients.

Clinical recommendations in diagnostic uncertainty

Besides the role of the above mentioned tests, a careful clinical follow-up of patients with suspected OMG is of great aid to make the diagnosis.[30-33] Detailed testing of extra-ocular muscle (EOM) weakness by assessing diplopia in all eight gaze directions for at least 30 seconds and carefully reporting of the extent and side of ptosis, might reveal changes in the specific ocular muscles that are involved. Such changes are typical of MG, and can help in excluding other causes of ocular muscle weakness.[20] In one study, at the second visit the side most affected by ptosis changed in 10% of MG patients. Over the whole follow-up, 50% of seronegative MG patients had a change in form of ptosis. In that cohort, patients with diplopia had double vision with both a vertical and horizontal component in 95%. In these patients, 83% manifested double vision in other gaze directions at the second visit. Of patients with ptosis, 42% manifested after 30 seconds of looking upwards. In the case of EOM weakness, diplopia manifested after 30 seconds only in 13% of gaze directions tested. So, in cases of suspected OMG it might pay off to invest time to test the upward gaze direction for 60 seconds (for ptosis and diplopia) and the other seven gaze directions for at least 30 seconds (for diplopia solely; even though sometimes ptosis might become more evident when a patient looks in a lateral direction).[30] Furthermore, specific clinical tests can be of aid to reveal ocular weakness. The Cogan's lid twitch is an overshoot of the eyelid on an upward gaze after a period of rest. Also, a "quiver" movement can be observed with saccadic examination in the case of severe ophthalmoplegia.[31]

Conclusions and future directions

Confirmation of suspected MG has improved over the years by the development of antibody tests and neurophysiological techniques. However, in the subgroup of MG patients with weakness restricted to ocular muscles, there still remains an unmet diagnostic need as these tests are less sensitive in this group. Moreover, the absence of generalized weakness makes it harder to clinically distinguish MG from other disorders that cause ptosis or diplopia. Early confirmation of the diagnosis of ocular MG is of great importance as a timely start of adequate immunosuppressive therapy might prevent generalization of disease and the need of emergency treatments due to a myasthenic crisis. A careful clinical follow-up of patients with suspected OMG, by systematically testing ptosis for 60 seconds and diplopia in eight gaze directions for 30 seconds each, might reveal changes in ocular weakness pattern between visits typical for OMG. In addition, specific clinical signs such as the Cogan's lid twitch and the ease-to-perform ice pack test (both for ptosis and evident ophthalmoparesis) can aid in making the diagnosis. Regarding diagnostic tools, CBA is most likely to aid in diagnostic confirmation of OMG in the foreseeable future. Other tests that are being used in an experimental setting, such as the RoVEMP test, might get a future role in the diagnostic process of hard-to-diagnose patients. There is a need of studies that investigate the yield of new diagnostic tests in suspected OMG with negative antibody tests and inconclusive routine electrophysiological tests. Lastly, the effect of early immunosuppressive treatment should be further investigated in randomized controlled trials including OMG patients.

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The mechanisms of immunopathology underlying B cell depletion therapy-mediated remission and relapse in patients with MuSK MG.

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ABSTRACT

In a series of studies, we applied reverse translational medicine, which affords understanding of immune pathogenesis via therapeutic intervention, to the MuSK subtype of myasthenia gravis (MG). Treatment with CD20-specific B cell depletion therapy (BCDT) demonstrated that MuSK MG patients respond remarkably well; the majority invariably reached remission accompanied by a remarkable drop in autoantibody levels. Circulating antibodies are primarily produced by bone marrow resident plasma cells, which do not express CD20. So, how does BCDT diminish MuSK autoantibodies and induce rapid remission? We developed a mechanistic model, which hypothesized that plasmablasts, which are short-lived antibody secreting B cell populations, produce MuSK-specific autoantibodies. Anti-CD20-mediated BCDT is expected to deplete CD20-expressing plasmablasts or CD20 expressing memory cells that supply the plasmablast population. To test this hypothesis, we performed a series of investigations, which were reported over the last seven years and are summarized in this review. First, we isolated plasmablasts from patients and generated human recombinant monoclonal autoantibodies (mAb) which bound MuSK and had pathogenic capacity, demonstrating that MuSK autoantibodies can be produced by this specific cell population. The characterization of the mAbs showed that MuSK autoantibodies can include unique properties including unusually high antigen binding affinity, and an elevated frequency of *N*-linked glycosylation in their binding domains. Further characterization suggested that MuSK autoantibody-producing cells may form in the early stages of B cell development due to defective tolerance mechanisms. Finally, we sought to determine how these pathogenic B cell clones behave over time. High throughput B cell receptor sequencing was applied to investigate longitudinally collected samples from patients treated with anti-CD20-mediated BCDT. MuSK-specific clonal variants were detected at multiple timepoints spanning more than five years and reemerged after BCDT-induced remission, predating disease relapse by several months. These collective investigations provide a more detailed mechanistic understanding of MuSK MG, the key features of which include the production of autoantibodies by circulating plasmablasts that can be diminished by CD20-specific BCDT, but a subset of which persist which then seed a reemergence of pathogenic clones prior to manifestation of clinical relapse.

Key Words: Myasthenia gravis, muscle-specific tyrosine kinase (MuSK), B cells, autoantibodies, B cell depletion therapy, rituximab, tolerance, reverse translational medicine, remission, relapse, longitudinal specimen collection, immunomechanisms, mechanistic model

Introduction

Autoimmune myasthenia gravis is an archetypal autoantibody-mediated disease (1, 2). The autoantibodies target molecules at the neuromuscular junction (NMJ), which leads to increased fatigability and muscle weakness in patients (1, 2). Disease subtypes can be defined by autoantibody specificity. The most frequently observed MG subtype is characterized by autoantibodies against the nicotinic acetylcholine receptor (AChR), comprising approximately 85% of patients (1). The remaining patients can harbor autoantibodies targeting muscle-specific kinase (MuSK) (3) or lipoprotein receptor-related protein 4 (LRP4) (4, 5), while a small fraction do not have detectable circulating autoantibodies to known targets. Accordingly, this group is collectively categorized as seronegative. The pathogenic capacity of autoantibodies targeting AChR and MuSK have been clearly demonstrated with both *in vitro* (6-11) and *in vivo* approaches (12).

The immunopathology of the subtypes can differ substantially, which is well highlighted by the AChR and MuSK subtypes. The immunopathology of AChR MG is mediated by IgG1 and IgG3 subclass autoantibodies, which effect disruption of AChR signaling through complement activation and subsequent tissue damage, initiating receptor internalization, and interfering with ACh binding. Conversely, MuSK MG is largely governed by IgG4 autoantibodies. These autoantibodies are ineffective in activating complement and mediate pathology by physically blocking NMJ protein-protein interactions. Specifically, MuSK Abs inhibit the interaction between MuSK and LRP4, which is essential for MuSK phosphorylation and subsequent effective AChR clustering and signaling (13). Moreover, the pathogenic capacity of MuSK autoantibodies is partly dependent upon fragment antigen-binding (Fab)-arm exchange, which generates functionally monovalent IgG4 antibodies (14).

While much of the underlying immunopathology of MuSK MG is understood, further details are needed. Over the last decade, we established a potential mechanism describing how pathogenic autoantibodies develop in MuSK MG through applying reverse translational medicine. That is, by using knowledge observed in clinical studies in combination with basic immunological research (15, 16). Spe-

cifically, we leveraged the positive effect of anti-CD20-mediated B cell depletion therapy (BCDT) in treating MuSK MG patients, to build a model in which CD20-expressing plasmablasts are the key disease-relevant cells that produce MuSK autoantibodies (17). We pursued testing of this model and further investigated the immunopathology of relapse that can occur following anti-CD20-mediated BCDT-induced remission in MuSK MG patients (17). This mini-review will focus on different aspects of the immunopathology of MuSK MG and will provide insights into the immunopathology of relapse after CD20-mediated BCDT.

What we learned from anti-CD20-mediated B cell depletion in MuSK MG – the basis of our mechanistic model.

B cells express different surface markers at different stages of B cell development and these markers can be used to identify and target specific B cell subsets (18). The cluster of differentiation molecule 20 (CD20) is not expressed on B cells at early stages of development or when they have differentiated to plasma cells (18). Targeting CD20 with the monoclonal antibody, rituximab (RTX), was first successfully used for the treatment of B cell malignancies (19-21). Rituximab was then shown to be effective in autoimmune diseases including antibody-mediated chronic inflammatory demyelinating polyneuropathy (CIDP), pemphigus vulgaris, multiple sclerosis, rheumatoid arthritis (22-25), and MuSK MG, first in 2008 by the research group of Isabel Illa (26), then shortly afterward in a number of corroborative studies (17, 27, 28), including several by our group at Yale (29, 30).

The B cell subsets that secrete autoantibodies (31) are short-lived plasmablasts and plasma cells. Some plasmablasts may express low levels of CD20, while plasma cells do not express CD20 (18, 32, 33). The response to RTX observed in MuSK MG patients often includes a rapid and near-complete reduction of autoantibody titer and subsequent disease remission. The Illa group elegantly demonstrated that, in contrast to the MuSK autoantibody titer, both total circulating IgG and tetanus vaccine specific IgG titers did not significantly diminish after BCDT (17). A sensible hypothesis explaining these findings is that the observed effect was based on the depletion of MuSK autoantibody-expressing, CD20-positive, short-lived plasmablasts and/or CD20-positive memory B cells that supply this plasmablast population (16). To test this mechanistic hypothesis, we isolated plasmablasts from MuSK MG patients with the intent of determining whether they produced MuSK specific autoantibodies (34). We took considerable care in the flow cytometry-based isolation, as these cells are challenging to identify because they are rare within the circulation and share surface markers with other B cell subsets. The additional step of examining the isolated cells via morphology was performed, as plasmablasts are distinctly bigger than naive or memory B cells due to an enlarged cytoplasm. These isolated plasmablasts were cultured in a manner that allowed for antibody secretion into culture media, which was then tested for binding specificity towards MuSK using a live cell-based assay (34). We found that the secreted antibodies bound to MuSK demonstrating that plasmablasts are a source of autoantibodies in MuSK MG (34).

To perform a more rigorous experimental demonstration, we next produced recombinant human MuSK monoclonal autoantibodies (mAbs) from these plasmablasts (33-35). We also included experienced (memory) B cells in our cell isolation approach; the result of which was that most of our MuSK mAbs originated from plasmablasts, while the rest were derived from memory B cells (33-35). Recombinant production of human mAbs allowed for an unlimited source of human autoantibodies for study, given that those secreted in the culture media by stimulated B cells are limited in quantity. Additionally, experiments could be performed with individual autoantibody clones rather than a heterogeneous mixture found in the bulk cell culture media or serum. In addition to validating binding properties, we leveraged these mAbs to further investigate the development of pathogenic B cells in MuSK MG and the pathogenic effect of autoantibodies at the NMJ.

Development of autoantibodies in MuSK MG

Human serum contains a multitude of distinct antibodies with different variable regions, which is vital for the broad reactivity to a vast array of potential pathogens (36). Although broad reactivity is important for protection against foreign antigens, self-reactivity is a possible by-product of the process that generates a diverse B cell and serum antibody repertoire. This is because random combinations of antibody variable region genes are assembled to produce a repertoire with many different antigen specificities during B cell development. However, that initially generated repertoire can include reactivity to self (37). Both central and peripheral tolerance checkpoints prevent these self-reactive B cells from further development (38, 39). The fidelity of these checkpoints is compromised in several autoimmune disorders. The result of which is increased frequencies of self-reactive B cells within the naïve B cell repertoire (40). We found that the central and peripheral tolerance checkpoints are defective in MuSK MG (41). Therefore, it is reasonable to propose that the development and origin of MuSK autoantibodies is partly due to unsuccessful counter-selection of self-reactive B cells due to tolerance defects.

The MuSK mAbs that we (33-35) and others (42) generated contain multiple mutations in the sequences of their variable region, which is the characteristic hallmark of the affinity maturation process. The reversion of these sequences to their corresponding germline-encoded form, which would be found in the naïve B cell precursors, is a common approach that is used to investigate the development or origin of autoantibodies (43, 44). Given that some small

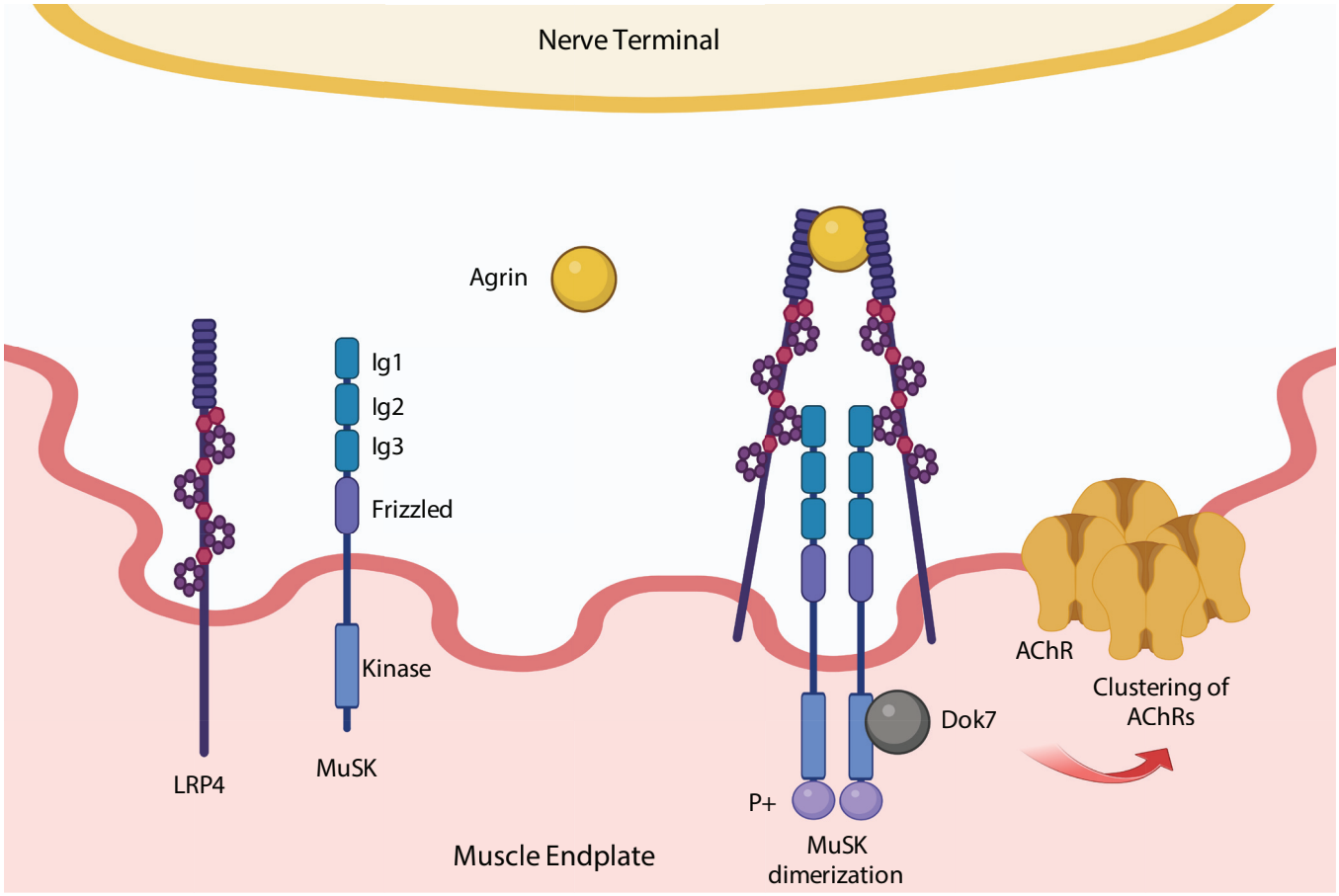

Figure 1. Schematic of the interaction of MuSK, LRP4, agrin and Dok7 at the neuromuscular junction.
The MuSK/LRP4 pathway is involved in the clustering of AChRs at the neuromuscular junction. MuSK has three immunoglobin-like domains 1-3 (Ig1-3) and a cysteine-rich domain (frizzled domain) on the ectodomain and an intracellular tyrosine kinase domain (65, 66). LRP4 is the (membrane-bound) ligand of MuSK and binds to the Ig-like domain 1 (54). The interaction of MuSK and LRP4 is enhanced when agrin binds to LRP4 which changes its conformation (54). Downstream of kinase-7 (Dok7) is an intracellular activator and substrate of MuSK, which binds to the kinase domain (65). Dok7 facilitates the autophosphorylation of MuSK (65, 67). The activation of the MuSK/LRP4 pathway results in the dimerization and autophosphorylation of MuSK, which is important for the activation of downstream pathways that lead to the clustering of AChRs at the NMJ. LRP4 = Low Density Lipoprotein Receptor-Related Protein 4; MuSK = muscle-specific tyrosine kinase; AChR = nicotinic acetylcholine receptor. P+ = phosphorylation. This figure was created with Biorender.com.

sequence areas of the antibody variable region (namely parts of the complementary determining region 3 (CDR3)) are not encoded by gene segments, the best approximation of the naïve, unmutated sequence is commonly called the unmutated common ancestor (UCA). Testing the binding properties of UCAs to the antigen recognized by the mature form can lead to at least two potential outcomes. The first is that UCA antibodies recognize the antigen, suggesting that the parental naïve B cell bound the antigen and that the same self-antigen is driving the affinity maturation process. The second outcome is that UCA antibodies do not recognize the antigen, suggesting that the mature B cell may gain antigen specificity during the affinity maturation process. UCAs in several autoimmune diseases have been investigated; there is no clear conclusion whether autoantigens predominantly drive the development of autoantibodies or whether antigen reactivity develops during affinity maturation. UCA autoantibodies in neuromyelitis optica spectrum disorder (NMOSD), pemphigus vulgaris (PV) and systemic lupus erythematosus (SLE) do not recognize the associated self-antigen (43, 45-47), whereas UCAs in rheumatoid arthritis and other mAbs in PV can exhibit specific reactivity to the disease-associated self-antigen (48, 49). We found that UCAs of MuSK mAbs recognize MuSK (33, 44) and that these UCAs have strikingly high affinities (nanomolar) for MuSK (44). Thus, we speculate that MuSK might be both the initiating and affinity maturation-driving self-antigen of MuSK specific B cells, and that they escaped elimination as a consequence of defective tolerance mechanisms.

Pathogenic and functional properties of MuSK autoantibodies

Understanding the role of MuSK is an essential prerequisite for investigating how pathogenic MuSK autoantibodies interfere with neuromuscular signaling at the NMJ **(Figure 1)**. MuSK is associated with the development and preservation of the NMJ (50-53) and it forms a functional unit with low density lipoprotein receptor-related protein

4 (LRP4) (54). The activation of the MuSK/LRP4 pathway results in the dimerization and autophosphorylation of MuSK, which is important for the activation of downstream pathways that lead to the clustering of AChRs at the NMJ **(Figure 1)** (51). Most serum-derived MuSK autoantibodies recognize the Ig-like domain 1 of MuSK, which interacts directly with LRP4 **(Figure 1)** (54, 55). It has been demonstrated, with both *in vitro* and *in vivo* approaches, that MuSK autoantibodies prevent the interaction of MuSK and LRP4, which leads to diminished clustering of AChRs and subsequent impaired neuromuscular signaling (14, 33, 42, 56-58). Some of the MuSK mAbs that we generated (33-35), specifically recognized the Ig-like domain 1 (33) while several others recognized the Ig-like domain 2 (35). Irrespective of their domain specificity, these mAbs reduced AChR clustering when tested with an *in vitro* approach (33, 35).

IgG4 subclass antibodies have a unique property in that they can exchange half-molecules with other IgG4 subclass antibodies during a process termed Fab-arm exchange (FAE), which produces bispecific IgG4 that bind to their target antigen in a monovalent manner (59, 60). MuSK MG autoantibodies are mainly of the IgG4 subclass (61-63) and functional monovalency potentiates their pathogenic effect at the NMJ (14, 42, 44, 64). In work we performed collaboratively with Angela Vincent and Michelangelo Cao (35), we found that recombinant divalent MuSK mAbs phosphorylate MuSK and reduce AChR clusters in comparison to non-disease relevant, control antibodies. In contrast, monovalent variants of these same antibodies are much more pathogenically potent because they robustly diminish AChR clustering (44). Given these observations, we proposed that divalent antibodies can crosslink and activate MuSK **(Figure 1)**. Monovalent antibodies, in contrast, block the interaction of MuSK with LRP4 without any artificial crosslinking of MuSK. Thus, our work, along with key findings from the Leiden University group led by Maartje Huijbers and Jan Verschuuren (14, 42), demonstrate that monovalency - generated by IgG4 FAE - is important for the pathogenic effect of MuSK autoantibodies at the NMJ.

In addition to valency, we found that affinity is important for the pathogenic capacity of MuSK autoantibodies (44). We found that only monovalent Fabs of mature, mutated autoantibodies prevented agrin-induced clustering of AChRs, while UCA Fabs did not show any pathogenic capacity despite having high affinities (nanomolar range) for MuSK (44). Thus, we hypothesized that binding kinetics (association and dissociation) may play a key role in the different pathogenic capacities. To investigate this further, we turned to affinity measurements. Our autoantibodies recognize MuSK over a wide range of concentrations when using live cell-based assays (CBAs) (35, 44). However, the static nature of these assays does not provide any information on the kinetics of antibody association and dissociation. Consequently, CBAs are not ideal for properly measuring affinity. Accordingly, we used bio-layer interferometry and monovalent Fabs to measure the affinity of our antibodies to MuSK, rather than divalent mAbs, which would have provided avidity values. We found that mature MuSK autoantibodies had exceptionally high affinities (sub-nanomolar) and that the high Ka was driven by fast association and slow dissociation whereas their UCA counterparts associated slower and dissociated faster (44). Thus, high affinity, characterized by rapid association and delayed dissociation, together with monovalency appear to be key properties for the pathogenic development of MuSK mAbs and are necessary for potent monovalent pathogenic capacity at the NMJ (44).

Unique features of the circulating B cell repertoire in MuSK MG

We next turned our attention to studying the B cells in MuSK patients. We started by examining the BCR repertoire using adaptive immune receptor repertoire (AIRR) sequencing. Although conspicuous changes in the overall repertoire of MuSK MG patients relative to healthy controls were not observed, we observed some unique abnormalities (68). These changes in the B cell repertoire in MuSK MG are subtle but seem to be specific as the repertoire of AChR MG showed different abnormalities (68). The B cell repertoire of MuSK MG shows differences in preferential usage of variable region gene segments and indicates impaired mechanisms of central tolerance during B cell development (68). The most conspicuous observation provided by the BCR repertoire analysis concerned the frequency of *N*-linked glycosylation site motifs (N-X-S/T, X cannot be proline) in the antibody variable region (IgG-$V^{N\text{-}Glyc}$). The frequency of IgG-$V^{N\text{-}Glyc}$ is elevated in AChR and MuSK MG in comparison to healthy individuals (42, 69). These glycosylation sites were either acquired through affinity maturation or present due to a preferential usage of the select gene segments containing glycosylation sites in their germline configuration (69). Several of our MuSK mAbs included IgG-$V^{N\text{-}Glyc}$ motifs affording us the opportunity to test whether they were involved in binding, given their conspicuous occupation of the variable region. The removal of these glycosylation sites, however, did not alter the binding capacities of these mAbs (42, 69). Thus, the functional purpose of *N*-linked glycosylation sites in the variable region of autoantibodies in MG is currently not understood but might be connected to altered B cell activation (70).

Immunomechanisms underlying relapse after anti-CD20-mediated B cell depletion

While most MuSK patients reach clinical remission following anti-CD20-mediated B cell depletion, patients can experience relapse years later (17, 71). Therefore, we wanted to study the immunomechanisms underlying

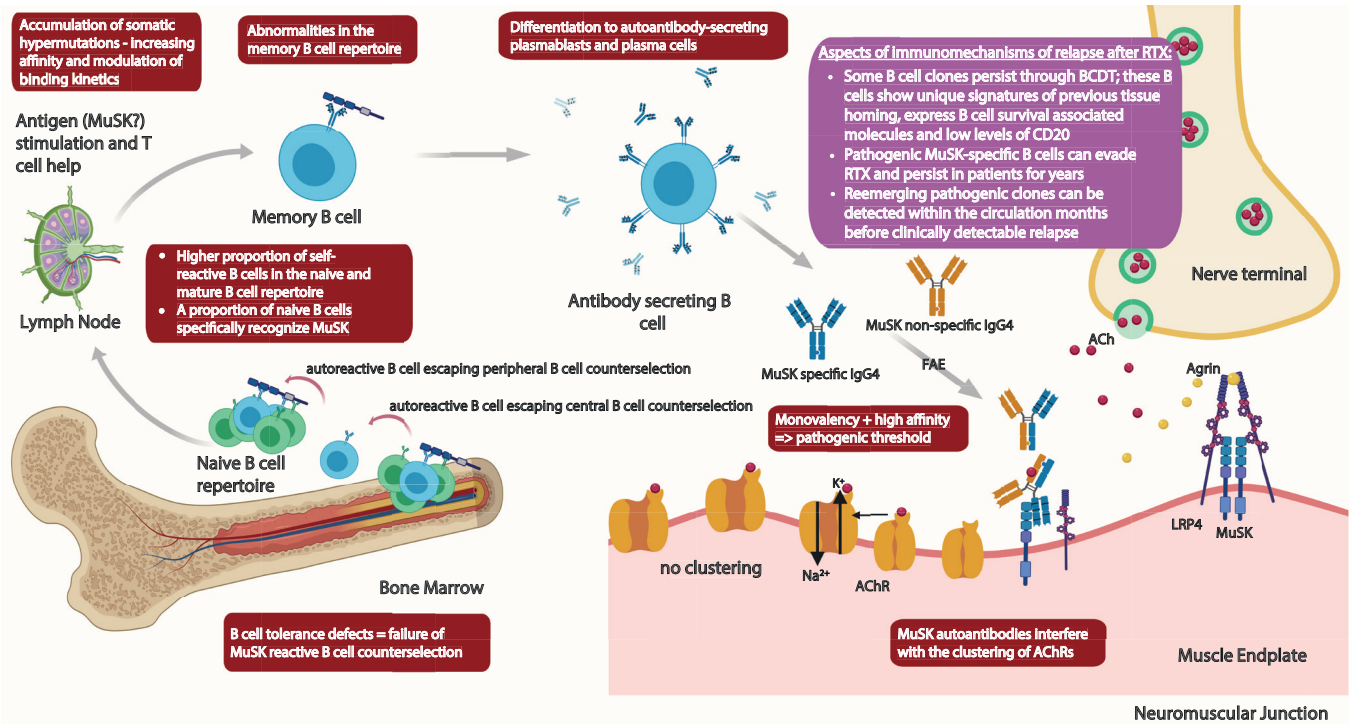

Figure 2. Schematic diagram showing the development of the pathogenic B cell repertoire and features of autoantibodies that mediate disease in MuSK MG. This figure was created with Biorender.com.

these relapses. We specifically focused on whether relapse is the consequence of reemerging historic clones or the development of newly generated pathogenic clones. To that end, we leveraged our MuSK mAbs, longitudinally collected samples, and AIRR sequencing. Specifically, with the BCR sequence of validated MuSK mAbs in-hand, we used AIRR sequencing to search for related clones present in longitudinal samples collected over several years prior to the mAb isolation. These longitudinal samples were collected during periods of both BCDT-induced remission and relapse. We found one pathogenic mAb and its corresponding clonal variants in a patient who had received several cycles of anti-CD20-mediated BCDT over almost 79 months (33). These clonal variants acquired changes in the antibody variable region sequence indicative of continuous affinity maturation in germinal centers; these changes did not alter the binding and pathogenic properties of the identified MuSK clone (33). The clonal variants reemerged before clinically-detectable relapse, concurrent with increasing MuSK autoantibody titer (33).

These persistent B cells express low levels of CD20 and show expression signatures associated with previous tissue homing and B cell survival (32). Likewise, plasmablast populations examined at the time of relapse expressed molecular signatures associated with B cell survival, B cell proliferation, and tissue homing (32, 33). Anti-CD20-mediated BCDT, however, is effective in eliminating antigen specific B cells in the lymph nodes in NMOSD (72), and decreases the levels of B cells in both the circulation and bone marrow in RA (73). Thus, it is not clear whether tissue homing is protective or indicative of recent repopulation and proliferation in germinal centers.

Summary

Over the last decade, we developed a model to describe the development of pathogenic B cells in MuSK MG **(Figure 2)**: The proportion of self-reactive B cells is elevated in the naïve B cell repertoire due to defects in the central and peripheral tolerance checkpoints (41). Among these self-reactive naïve B cells are clones that show strong and specific binding to MuSK indicating that the MuSK antigen might be initiating B cell activation and may also drive affinity maturation of these B cells in germinal centers (44), followed by differentiation into antibody-secreting plasmablasts (34). The secreted antibodies are mostly of the IgG4 subclass (61-63) and become functionally monovalent through the process of Fab-arm exchange (64). Binding of the monovalent pathogenic mAbs to MuSK impedes the clustering of AChRs which impairs the signaling from the nerves to the muscles (14, 33, 42, 44). Thus, affinity maturation and monovalency are necessary for the pathogenic development of MuSK autoantibodies and their pathogenic capacity at the NMJ (14, 33, 42, 44, 64). Characteristic abnormalities in the B cell repertoire of MuSK MG patients include the elevated frequency of *N*-linked glycosylation motifs within the variable region (68, 69); the functional relevance of these observations is the object of future investigations. Lower expression of CD20 on persistent B cells, together with molecular signatures associated with B cell survival and tissue homing

(32), may contribute to survival of persistent clones during BCDT as well as continuous antigenic stimulation. Among these persistent clones are pathogenic B cell clones that can be traced longitudinally over several years and through continuous BCDT treatments (33). These pathogenic clones can reemerge months before noticeable clinical relapse together with increasing autoantibody levels (33). Overall, this body of research provides both a mechanistic understanding of MuSK MG immunopathology and how disease relapse develops during a commonly used treatment strategy.

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Competing interests
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Refractory myasthenia gravis: the more we learn, the less we know.

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ABSTRACT

Refractory myasthenia gravis (MG) identifies the group of patients who have inadequate symptom control and persistent muscle weakness and fatigability despite the use of multiple immune modulatory therapies. This manuscript highlights what is currently known about refractory MG and underlines major knowledge gaps, drawing attention to the unmet needs in our understanding of this disease subset. This review raises questions about our current understanding of refractory disease and how emerging data as well as therapies may alter our thinking and patients' disease course.

Key words: *refractory myasthenia gravis; quality of life; acetylcholine receptor; muscle specific kinase; thymectomy; eculizumab; rituximab; tacrolimus; cyclophosphamide*

Introduction

Myasthenia gravis (MG) is the prototype immune-mediated neuromuscular disorder with autoimmunity against components of the neuromuscular junction causing disruption of neuromuscular transmission and subsequent characteristic fatigable muscle weakness (1). As an autoimmune disorder, MG is categorized in several different ways including clinical phenotype (ocular versus generalized), early versus late onset (initial symptoms before or after age 50 years), association with thymoma, and serological subtypes (antibodies against acetylcholine receptor [AChR], muscle specific kinase [MuSK] or lipoprotein-related protein 4[LRP4])(2). MuSK+ MG, which accounts for <10% of all myasthenia, is unique from AChR+ antibody disease based on several differences including IgG subclass (IgG4 versus IgG1 and 3 subclass), target protein, clinical phenotype, association with thymoma, response to cholinesterase inhibitors, disease course, and immune modulatory treatment response. MuSK+ MG tends to have worse clinical nadir and faster progression than AChR+ disease. Given its propensity to affect bulbar muscles, there is greater risk of myasthenic crisis. A greater proportion of MuSK+ MG patients have refractory disease compared to AChR+ patients (3–5), though it is important to keep in mind that AChR+ disease is proportionally greater among most refractory MG cohorts. Thymoma-associated MG is similarly more difficult to treat than non-thymomatous MG. Across different populations, younger age of disease onset and women have been identified as patient-specific risk factors for poorer response to therapy.

Treatment response has been included in the conceptual framework of MG for as long as disease-modifying treatments have been a part of disease management strategy (6–8). Most studies estimate the prevalence of refractory MG to be between 10-20% of generalized MG (3,4,9). Refractory disease poses a significant challenge for clinicians and patients, as it is associated with impoverished quality of life, lifestyle challenges, health care resource utilization, and increased morbidity. There is a need to better understand the underlying mechanisms of refractory MG, identify biomarkers to guide therapy, and develop more effective treatments.

This review aims to provide an overview of refractory MG, including diagnostic criteria, disease burden and current treatment options. The manuscript will also discuss emerging therapies, including biologics and immunomodulatory agents, as well as the challenges and opportunities in managing refractory MG. By advancing understanding of refractory MG, the hope is to improve outcomes and quality of life for patients with this challenging condition.

Defining Refractory Myasthenia Gravis

Several publications (Table 1) have operationalized the term "refractory MG" for describing an MG cohort that in some way experiences suboptimal response to immune modulatory treatment, be it lack of response in terms of symptom relief, occurrence of disease exacerbations, clinician impression of treatment response, need for adjunct therapy, frequency of disease exacerbations, or undesired or intolerable side effects (3,5,10–12).

These definitions have variable degrees of subjectivity associated with them, both on the part of patients and providers. More importantly, while there may be considerable overlap between these definitions, the separation of refractory and non-refractory disease states differs significantly. The University of Toronto group applied these various criteria to a cohort of 237 patients within their group practice at two time points (at the time of the original cohort inception [2014-16] and at the last clinical visit [August 2019]) and found a high degree of

Table 1: Definitions of refractory myasthenia gravis arranged by date of publication, adapted from Tran C, *et al* (13).

PUBLICATION	DEFINITION
Drachman et al. 2008 (10)	1. Failure to respond to otherwise adequate doses and durations of conventional immunosuppressive treatments. 2. Have unacceptable adverse side effects of the treatments. 3. Require an excessive amount of potentially harmful agents. 4. Have comorbidities that preclude the use of conventional therapy. 5. Require repeated rescue with short-term intravenous immunoglobulin or plasma exchange treatments.
Suh et al. 2013 (3)	1. Unable to lower immunotherapy without clinical relapse. 2. Not clinically controlled on immunotherapy regimen. 3. Severe side effects from immunosuppressive therapy.
Sanders et al. International Consensus Guidance, 2016 (11)	Myasthenia Gravis Foundation of America (MGFA) Task Force post-intervention status (PIS) is unchanged or worse after corticosteroids and at least 2 other IS agents used in adequate doses for an adequate duration WITH (a) persistent symptoms OR (b) side effects that limit functioning, as defined by patient and physician.
Howard et al. REGAIN Study, 2017 (12)	1. Treatment with two or more immunosuppressive therapies for 12 months without symptom control, OR 2. At least one immunosuppressive therapy with intravenous immunoglobulin or plasma exchange given at least four times per year.
Mantegazza et al. 2018 (5)	1. Failure to respond adequately to conventional therapies: insufficient response to maximal safe doses of steroids and at least one immunosuppressive drug at an adequate dose and duration. 2. Inability to reduce immunosuppressive therapy without clinical relapse or a need for ongoing rescue therapy such as intravenous immunoglobulin (IVIg) or plasma exchange (PLEX). 3. Severe or intolerable adverse effects from immunosuppressive therapy ("treatment intolerant"). 4. Comorbid conditions that restrict the use of conventional therapies (also "treatment intolerant"). 5. Frequent myasthenic crises even while on therapy.

variability between the criteria (13). While the Drachman, Suh, and Mantegazza criteria identified about 40% of patients as refractory, this number significantly dropped to 10% and 3% when applying the Sanders/International Consensus Guidance and Howard/REGAIN Study criteria. Furthermore, there was significant difference in classification even between the Sanders and Howard criteria. Conversely, the Myasthenia Gravis Impairment Index (MGII), Neuro-QoL-Fatigue, and Myasthenia Gravis Quality of Life 15 (MG-QOL15) scores all showed worse patient-reported symptom states in patients classifiable as refractory using the Sanders and Howard criteria versus the other 3 criteria. Thus, comparing results from studies is challenging based on the differences amongst criteria.

These criteria may exclude certain disease subtypes within MG. For example, studies from a South Africa cohort of patients showed that Blacks were more likely than Whites to develop treatment-resistant oculoparesis and ptosis, termed the ophthalmoplegic variant of MG (14). Escalation of therapy may be considered an exercise in futility and higher risk than benefit for such patients by their providers. Based on this, patients would not fulfill

criteria for "refractory" yet would experience persistent and debilitating symptoms.

All the above criteria do not account for thymectomy as a potential therapy, for either thymoma-associated or non-thymomatous MG. Thymoma-associated MG is well known to pose greater therapeutic challenges than non-thymomatous disease. Conversely, the benefit of thymectomy in acetylcholine receptor antibody-positive, generalized, non-thymomatous MG now is indisputable on the basis of the MGTX study (15).

The term "refractory" also carries a sense of futility for a disease, and yet this is hardly the case. This point is emphasized by the pivotal phase 3 REGAIN study of eculizumab which required "refractory" status for inclusion into the trial. Despite this disease categorization, eculizumab therapy resulted in clear and rapid improvement in patient-reported and provider-assessed measures (12). Several retrospective studies have suggested efficacy of rituximab and cyclophosphamide in refractory MG (10,16–18). In their study, Tran *et al* found that some patients who fulfilled criteria for "refractory" status at the initial study period (2014-2016) subsequently moved to "non-refractory" status at the later study timepoint (2019), again supporting the notion that this designation is not exactly a "point of no return".

Burden Of Refractory Disease

That refractory disease associates with persistent MG symptoms is self-evident. Analyses of the MGFA Patient Registry showed that MG-QOL15, Myasthenia Gravis Activities of Daily Living (MG-ADL), and NeuroQoL Fatigue scores were higher in the refractory compared with the non-refractory cohort (19).

Another analysis of enrollment data from the MGFA Registry showed that, compared to patients with non-refractory disease, those with refractory disease were significantly more likely to have experienced at least one MG exacerbation, ER visit, hospitalization, ICU admission at any time for reasons associated with MG, or previously required a feeding tube (27). Data analysis from two administrative health plan databases showed that refractory patients had 4 times higher odds of experiencing a myasthenic crisis and 4.7 times higher odds of experiencing MG exacerbation compared with non-refractory patients (28). A Spanish MG Registry study showed that drug-refractory patients (defined per Sanders/ICT criteria) needed IVIg (86.9% vs 23.7%, $P<0.0001$) and PLEX (19% vs 4.4%, $P<0.0001$) more frequently compared with non-drug refractory patients (4). Whether or not patients with refractory MG are at higher risk of mortality compared to non-refractory patients is not certain though one Korean study reported higher hazard ratio (2.49) for the former group (29).

Danish and Japanese studies have shown that MG negatively impacts employment productivity among patients with MG (30,31). Patients with refractory disease fare worse: the MGFA Registry enrollment survey showed that non-refractory patients had higher odds of previous (2.643) and current (2.777) employment compared with refractory patients (32).

In recent years, there has been increasing interest in studying the impact of MG on symptoms and experiences other than those related to muscle weakness. There is increasing evidence that patients with MG have higher burdens of anxiety, depression, and poor sleep (20). While no studies have specifically compared the presence of these issues between refractory and non-refractory disease, findings of recent studies suggest a higher burden with more severe disease (21,22).

The generalized feeling of fatigue reported by many patients, distinct from muscle fatigability with continuous or repeated use, has been a particularly challenging issue in MG care. Providers often struggle with this symptom as it is difficult to understand from the pathophysiologic standpoint and difficult to correlate with disease activity. Thus, the tendency is to limit intervention on the basis of observable muscle weakness and muscle fatigability and not the perceived experience of patients. Yet, multiple studies point to fatigue being an important symptom of the disease even in patients with mild disease (23–25). At least one prospective study, the REGAIN phase 3 trial of eculizumab, reported improved fatigue that mirrored improvements in other MG scales (26). This is not to say that patient-reported fatigue should become a part of the conversation around refractory disease nor that it should lead to consideration of complement inhibitor therapy. Yet, there is increasing awareness of it as a contributor to disease burden, and its impact would presumably be greater in sub-optimally controlled disease.

Treatment Options

The initial International Consensus Guidance manuscript suggested the use of chronic IVIg or PLEX, cyclophosphamide, and rituximab in addition to other conventional immunosuppressive therapies (IST; azathioprine, cyclosporine, mycophenolate mofetil, methotrexate, and tacrolimus) for treatment of refractory MG (11). This work was completed prior to the publication of the pivotal phase 3 REGAIN study of eculizumab in refractory MG. A subsequent update included the use of eculizumab for severe refractory AChR+ generalized MG (33). Several studies have reported on the use of these agents

in mixed MG cohorts (AChR+, MuSK+, seronegative), whereas few studies have specifically studied refractory MG patients. To date, no clinical trials have assessed the efficacy of IVIg or PLEX in refractory MG.

The data on rituximab effectiveness in generalized MG were primarily based on observational studies and systematic reviews until recent years (34,35). These studies have shown improvement in both AChR+ and MuSK+ MG patients with both refractory and non-refractory disease, though response may occur more frequently in MuSK+ MG. Improvements were noted in clinical state (MGFA PIS, MG specific scores), clinical relapse, and need for immunosuppressive therapy. Rituximab was largely well-tolerated in all studies. Two recent randomized trials in AChR+ gMG are noteworthy. The phase 2 BEAT MG study randomized patients to two cycles of rituximab (four weekly infusions of 375 mg/m^2) six months apart versus placebo (36). The primary endpoint was a greater than 75% reduction of mean prednisone dose in the four weeks prior to week 52 compared to the four-week period prior to baseline with either clinical improvement or no worsening (≤2 point increase) in MGC scores and with rituximab treatment accounting for at least 30% of the observed difference between the two groups in a futility design; this primary outcome was not observed. Similarly, no significant differences were noted in several secondary outcomes. Patients treated with rituximab had a numerically lower relapse rate and need for rescue therapy compared to placebo. More recently, a multi-center, prospective, double-blind, placebo-controlled trial of low dose rituximab (single 500 mg infusion) in early gMG had more favorable results (37). The primary endpoint of achieving a QMG score ≤4 and prednisone dose ≤10 mg/day at week 16 with no rescue needed between weeks 9-16 was achieved by 71% of rituximab treated patients compared to 29% in the placebo group (p=0.007). Need for rescue therapy was also significantly lower in the rituximab group. Currently, rituximab treatment is well-recognized as being effective for, and is an early consideration in, MuSK+ MG. The Rinomax study suggests the same might be true in early management of AChR+ disease.

Several studies have shown potential therapeutic benefit of tacrolimus in MG, including a randomized, placebo-controlled study (38,39). One study looked at its use in "refractory" patients, though this was defined loosely as those patients who did not respond well to conventional treatment or were unable to withstand side effects (40). Wu *et al* treated 24 refractory MG patients with 3 mg/day oral tacrolimus. QMG, manual muscle testing (MMT), MG-ADL, and MG-QOL15 scores were significantly lower at 2, 6, and 12 months compared to baseline (40). Mean prednisone dose was reduced by about 60%, and therapy was generally well-tolerated with mild side effects. Tacrolimus use is recommended as next in line to prednisone in Japan (41).

A few small studies have shown benefit of cyclophosphamide in gMG. A small randomized trial showed statistically significant reduction in prednisone doses in both cyclophosphamide- and placebo-treated patients at 6 and 12 months and a significant difference between the two treatment groups at those time points (42). Drachman and colleagues treated 12 refractory MG patients with their "rebooting the immune system" protocol of high dose cyclophosphamide (50 mg/kg/day for 4 days) (10). Eleven patients had "clinically obvious beneficial effects", 6 had "very good to excellent responses" for at least a year, and 2 remained in complete remission for multiple years. Another retrospective study showed improvement by at least 1 point on the Osserman scale in six out of eight refractory MG patients treated with monthly cyclophosphamide at 30-50 mg/kg for at least 6 months (18). Response was maintained for a mean duration of nine months.

Eculizumab, a selective inhibitor of C5 activation, is the only agent exclusively tested in the refractory MG cohort in a large, randomized, double-blind phase 3 study (12). Based on worst-rank ANCOVA analysis, the study did not meet its primary efficacy endpoint of change in MG-ADL in treated versus placebo groups. However, QMG and MG-QOL15 scores did achieve significance on the worst-rank analyses, and all measures (MG-ADL, QMG, MG composite [MGC], and MG-QOL15) showed significant improvement compared with placebo on prespecified repeated-measures sensitivity analyses.

Several neonatal Fc receptor (FcRn) antagonists are currently in late stage development with efgartigimod being the first-in-class approved agent after the positive pivotal ADAPT study (12). Clinical trials with these agents have included, though not exclusively, some patients who would fulfill various criteria for refractory disease. It stands to reason that targeting this mechanism of action will be considered in patients with both refractory as well as non-refractory disease.

Discussion

"*When language is ambiguous, thought is imprecise and vice versa*" (43).

What exactly does "refractory MG" denote and how is this designation helpful with regard to management of MG? If this really identifies a group of patients who have difficult to treat disease with higher disease burden and worse outcomes, then ideally there should be ways to identify them beforehand. This in turn would better guide treatment approaches and create the ability to forecast their disease

course. However, we currently have no such ability, and we know precious little about what separates refractory from non-refractory disease. All current definitions determine refractory disease on a retrospective basis and in a somewhat arbitrary fashion.

Younger age, female gender, thymoma-associated MG, and MuSK+ disease confer greater risk of refractory MG. Yet, treatment choices are made moreso based on side effect profile rather than age and gender for the first two factors. For example, weight gain and teratogenic potential are important considerations, rather than potentially higher risk of refractory disease, when deciding on steroid and non-steroidal immunosuppressant use, respectively in young women. Similarly, the decision to perform thymectomy is based on the treatment of the thymoma itself, not to alter MG disease course. Treatment decisions are certainly influenced by the known worse disease course for MuSK+ disease; hence earlier consideration of rituximab in these patients, similar to other IgG4 mediated neurological and non-neurological disorders. However, a greater number of refractory MG patients are AChR+ rather than MuSK+ and, as discussed above, the data for rituximab in AChR+ are not as encouraging. Based on the seminal MGTX study, we know that early thymectomy in AChR+ non-thymomatous gMG confers significant advantages over prednisone alone in terms of clinical improvement, long-term steroid exposure, relative risk of exacerbations and crises, and need for adjunct non-steroidal immunotherapy (15,44). *Does this also confer relative risk reduction for refractory disease?*

All criteria for refractory MG require adequate dose and time on specific therapies. For steroids, the dose and duration are not specifically defined in any of the criteria. There is greater consensus among experts on the dose and duration for non-steroidal therapies like azathioprine, mycophenolate, methotrexate, and others. Even with the most lenient criteria, any individual patient would have to spend at least a year on steroids and non-steroidal immunotherapy while demonstrating a suboptimal response before being considered "refractory". Does a longer duration of sub-optimally treated disease adversely affect potential for improvement? This may hold true for at least a subset of patients, such as those with the "ophthalmoplegic" variant of MG (45). Conversely, though, the mean disease duration was nearly 10 years in the REGAIN study cohort, and yet these patients showed rapid and clinically meaningful improvements with eculizumab therapy (12). *Is the propensity for poor recovery uniform across the disease, or are there subsets within the disease that have better or poorer odds of recovery?*

Multiple other recent clinical trials of complement and FcRn inhibition have shown rapid, clinically meaningful, and statistically significant treatment responses compared to placebo, within days to weeks. *How will these newer therapies impact our current definitions of refractory MG? More importantly, would earlier use of these newer therapies "buy" more time and alter the odds of becoming refractory?*

The REGAIN trial and other studies also highlight the point that patients with "refractory" disease may still improve (4,12). So, defining a patient as having refractory MG does not signify a disease nadir from which there is no hope of improvement. It may simply mean that the correct treatments have not been tried. One study found that certain single nucleotide polymorphisms (SNPs) in the glucocorticoid gene influence steroid response in patients with MG (46). Similarly, another study identified polymorphisms in cytochrome P450 3A5 (CYP3A5) and heat shock protein 90AA1 (HSP90AA1) associated with refractory versus non-refractory MG (47). Rose *et al* demonstrated that AChR antibodies have varied specificity for epitopes on the acetylcholine receptor. While antibodies with a single specificity bind AChR, they alone do not activate complement. However, antibodies with different epitope specificities act synergistically, strongly activate complement, and damage the neuromuscular junction (48). Obaid *et al* showed that complement activation varied significantly between sera from different AChR+ MG patients, with only 60% sera activating complement and resulting in detectable membrane attack complex (MAC) formation (49). All of this points to the possibility that patient specific factors play a significant role in determining response to specific therapies and explain why one size does not fit all. Assays measuring levels of complement activation through patient sera are experimental and are not currently available for clinical use.

Conclusion

Refractory MG, in its current definition, describes a clinical response-based cohort of patients with suboptimal improvement and/or tolerability to current treatment options. While this group constitutes a smaller proportion of MG patients, they have a considerably higher burden of disease and impact on daily life, reduction in productivity, and increased health care resource utilization. At present, the designation of refractory MG does not provide any significant clinical utility and should certainly not imply therapeutic futility.

Current clinical tools do not afford the luxury of identifying these patients beforehand.

Determination of the underlying pathophysiology that modulates treatment response to specific therapies as well as factors unique to patients, such as genetic determinants, immune system function and interaction, and antibody

function and pathogenicity would form better substrates for classifying patients into treatment response therapies. Recent studies have provided important clues to potential mechanisms, but a lot of work remains before the field can transition from hind sight and reactive decision-making to proactive care and improved outcomes.

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Complement inhibition in Myasthenia – from basics to RCT data

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ABSTRACT

Myasthenia gravis (MG) is the prototypic autoimmune neurological disorder causing fatiguable muscle weakness either limited to the ocular muscles or becoming generalised involving the limb and bulbar muscles. Nine out of ten generalised MG patients have IgG1 or IgG3 antibodies against the acetylcholine receptor (AChR). AChR antibodies cause neuromuscular weakness by internalisation of AChR, receptor blockade and activation of the complement pathway. Complement activation causes formation of the membrane attack complex (MAC), leading to degradation of the neuromuscular junction (NMJ). Several animal models have confirmed the role of complement in the pathogenesis of MG, with the experimental autoimmune MG models (EAMG) often needing complement inhibitory therapies to prevent or reverse the disease. Various molecules that target the complement system have now been developed to treat myasthenia gravis. The vast majority of the currently studied molecules target the C5 protein, thereby preventing the formation of MAC and subsequent NMJ destruction. The currently studied anti-complement therapies for MG include Eculizumab, Zilucoplan, Ravulizumab, Pozelimab, Cemdisiran, Gefurilimab, Danicopan and a few others in the pipeline. Eculizumab has been shown in clinical trials to be effective in the treatment of refractory MG, but further sub-group analysis and real-life experience have shown that this drug can be beneficial in various patients including those receiving regular intravenous immunoglobulin (IVIG), plasma exchange or Rituximab. It was approved for use by the FDA in October of 2017. Ravulizumab is a long-acting monoclonal antibody which has similar mechanism of action to Eculizumab and was approved for use in MG by the FDA in April 2022. Zilucoplan is a macrocyclic peptide which can be given subcutaneously and binds to C5 and C5b, thus preventing terminal complement activation (FDA new drug application accepted in Nov 2022). Many of these have also been shown to have long-term benefit in different sub-groups of patients with MG. Patients would need to be vaccinated against Neisseria meningitidis because of the risk of Gram-negative septicaemia, although no major safety signatures have been noted in the studies so far. Future studies may be able to identify specific biomarkers which might aid in selecting the most appropriate patients who might respond to these therapies.

Keywords: Myasthenia Gravis, Complement, Eculizumab, Ravulizumab, Zilucoplan

Introduction

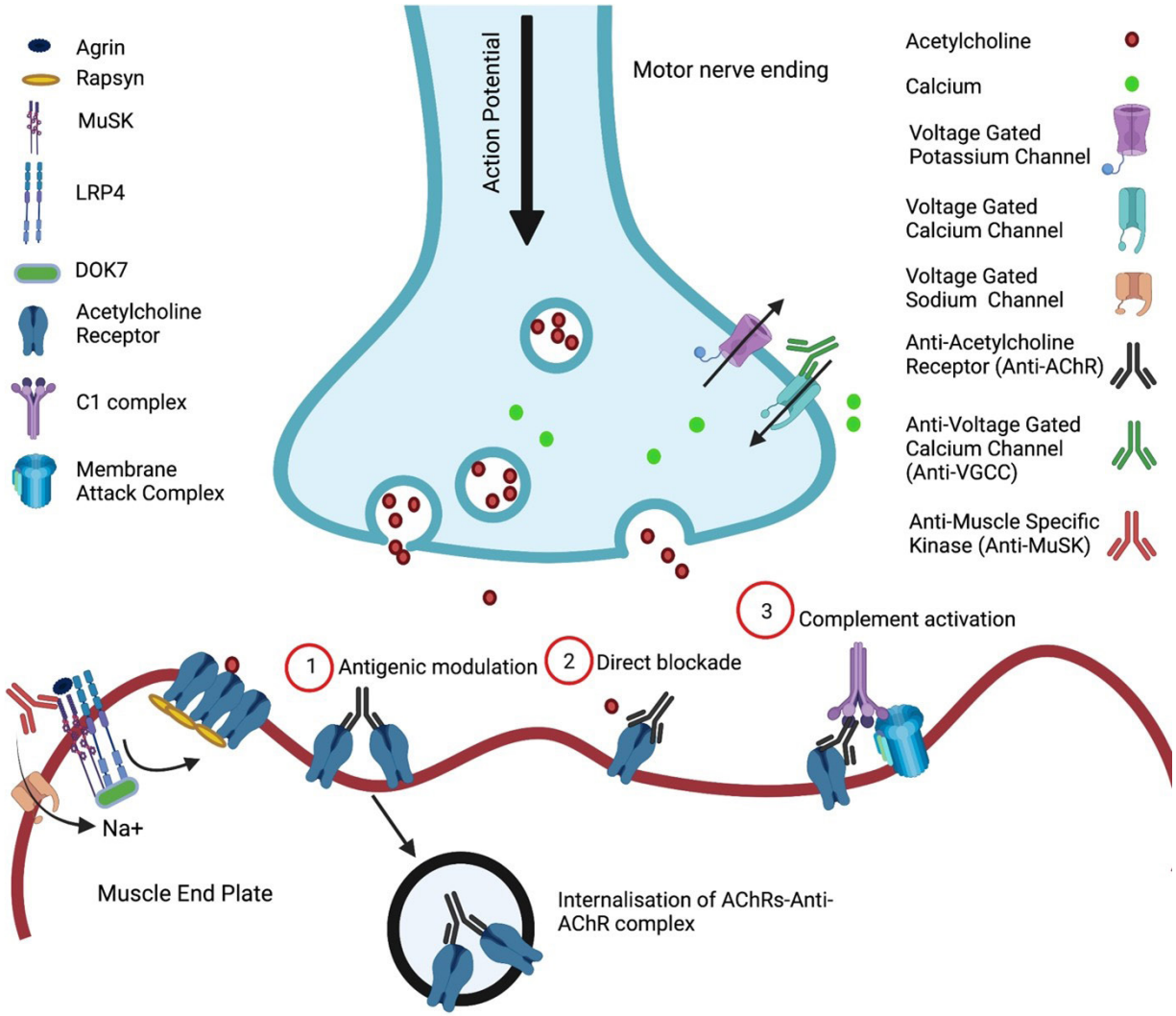
Myasthenia gravis (MG) is the most well recognised autoimmune nervous system disease characterised by fatiguable muscle weakness.[1] Patients can have symptoms localised to the eye muscles causing ptosis and double vision (ocular MG) or progress to develop weakness in the limbs or bulbar muscles causing dysphagia, dysarthria and breathing difficulties (generalised MG, gMG). Generalised MG is caused by antibodies against the nicotinic acetylcholine receptor (AChR) in over 85% of patients.[2] Other main antibodies involved in myasthenic syndromes include those against the muscle-specific tyrosine kinase (MuSK), which is seen in 5-8% of generalised MG, and the pre-synaptic voltage gated calcium channels (VGCC), causing the related Lambert Eaton Myasthenic syndrome (LEMS). Low density lipoprotein receptor related protein 4 (LRP4) antibodies are seen in up to 2% of generalised MG patients. [1] Antibodies against several other molecules have been described including acetylcholinesterase, agrin, ColQ, titin, ryanodine, Kv1.4 and cortactin, but their exact pathophysiological role is unknown. [3, 4] The main molecules involved in neuromuscular transmission and the pathogenetic mechanisms in MG are schematically represented in **Figure 1.**

The action potential arriving at the pre-synaptic terminal opens voltage gated calcium channels (VGCC) triggering release of Agrin and Acetylcholine receptor (AChR) to the synaptic cleft. The binding of ACh to its receptor (AChR) opens voltage gated sodium channels leading to muscle contraction. The clustering of AChRs at the neuromuscular junction (NMJ) is promoted by Agrin binding to the MuSK-LRP4 complex. There are three main mechanisms by which AChR antibody causes neuromuscular damage: antigenic modulation where Anti-AChR cross links AChRs, increasing the internalisation of AChRs (1), direct blockade when Anti-AChR blocks the ligand binding site of Acetylcholine to AChR (2) and complement activation (3). Anti-AChR-AChR complex activates the complement system, leading to the destruction of muscle end plate by Membrane Attack Complex.

Other NMJ syndromes include Anti-MuSK MG (Anti-MuSK binds MuSK-Lipoprotein Receptor Related Protein 4 (MuSK-LRP4), interferes with interaction of MuSK with other NMJ molecules and reduces AChR clustering) and LEMS (anti-VGCC binds VGCC at motor nerve terminal, blocking the calcium influx and calcium driven AChR vesicle release into NMJ).

There are three possible ways by which the AChR antibodies are likely to impair neuromuscular transmission.[5] These include:

Figure 1
Neuromuscular transmission and immunopathogenesis of neuromuscular junction disorders



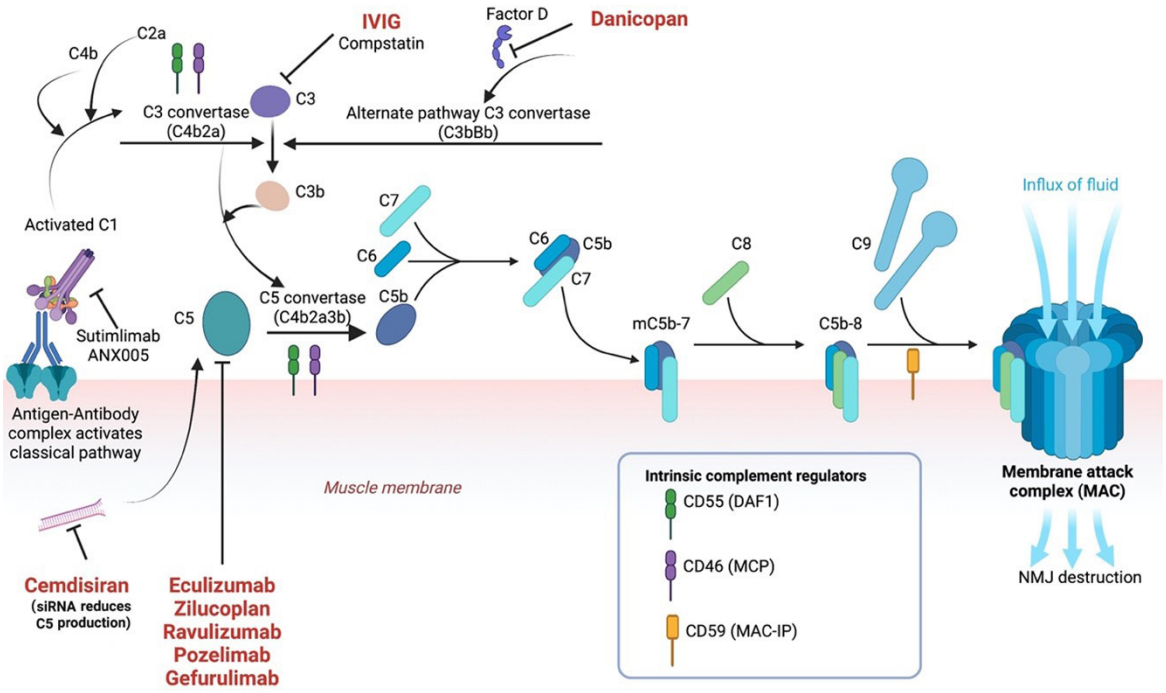
1. Antigenic modulation –antibodies cross-link the receptors, accelerating internalisation and degradation of AChR
2. Direct blockade – antibodies prevent the acetylcholine from binding to the AChR
3. Reduction of AChR density –activation of the complement cascade causes lysis of the post-synaptic membrane and simplification of the neuromuscular junctional folds

It is currently not easy to demonstrate in vivo which of the three mechanisms might be the predominant component in an individual MG patient, although complement activation is thought to play the major role in the pathogenesis, at least in AChR-MG patients.[5-7] Antibodies against AChR, LRP4 and VGCC are predominantly of the IgG1 sub-class and are more likely to fix complement as opposed to MuSK antibodies, which are usually IgG4. The vast majority of complement inhibition studies in MG have been done on AChR antibody positive patients and hence this review primarily focuses on this sub-group of MG.

Complement pathway

The complement system is an integral part of innate immunity and is composed of over fifty proteins primarily responsible for defending the host from infections by eliminating pathogenic organisms. It also serves as a link between innate and adaptive immunity by interacting with the T and B-cell receptors or by dendritic cell modulation.[8] The complement system is also involved in the clearance of immune complexes and dead cells.[9] This delicate balance can occasionally be disrupted, leading to autoimmune neurological disorders and may contribute to some neurodegenerative conditions (e.g.: Alzheimer's disease, amyotrophic lateral sclerosis and Huntington's disease).

Figure 2
The classical complement pathway and molecules used to inhibit this pathway in Myasthenia Gravis



This has now led to a growing interest in complement modulatory therapies in various neurological diseases, involving the peripheral (e.g.: Guillain-Barre syndrome, chronic demyelinating neuropathies, dermatomyositis) and central nervous system (e.g.: neuromyelitis optica, autoimmune encephalitis, multiple sclerosis). [6]

A detailed review of the complement pathway is beyond the scope of this article. The main aim of the complement pathway is the formation of the membrane attack complex (MAC), which leads to destruction of microorganisms or tissue damage when triggered by autoimmunity. The activation of the pathway involves three different initiation loops [6], although we will concentrate on the one responsible for myasthenia pathogenesis in more detail:

1. **Classical pathway** – C1 activation after binding of antigen-antibody complexes, which leads to a cascade of reactions explained below.
2. **Mannose Binding Lectin (MBL) pathway** – Lectin binds mannose or other carbohydrates (e.g.: ficolins or collectins) on the bacterial surface, activating the Mannan-binding lectin serine proteases (MASP1 and MASP2) and leading to the formation of C3 convertase, with subsequent steps common with the classical pathway.
3. **Alternate pathway** – Spontaneous activation of C3 leads to a low rate, "tick-over" pathway which is an integral part of innate immunity. Unlike the other two pathways, C1, C2 and C4 are not needed with Factor B and properdin Factor D helping to produce the alternate C3 convertase (C3bBb). This, when combined with high concentrations of C3b, leads to the production of alternate C5 convertase (C3bBbC3b).

Classical pathway and terminal complement complex

The activation of C1 complex by the multi-valent C1q binding to the Fc portion of the AChR-bound antibody (usually IgG1 or 3, less commonly IgG2), generates enzymatically active C1r and C1s. C1s cleaves C4 to C4a and the larger C4b, and the combination of C1r, C1s and C4b converts C2 to C2a and C2b. The C4b2b complex is called C3 convertase because it cleaves C3 to C3a and C3b, the latter combining with the C3 convertase to form C5 convertase. C5 convertase (C4b2a3b) initiates the terminal complement pathway by cleaving C5 into C5a and C5b. C5a is a chemoattractant protein and is involved in anaphylactic reactions along with the C3a released earlier. The C5b component sequentially accepts C6 and C7, and then translocates to the outer lipid bilayer of cell membrane, exposing its lipophilic structure due to the transmembrane location. C8 and several (up to 17) molecules of C9 are added, widening the pore size and subsequent formation of the osmolytic membrane attack complex (MAC, C5b-9)[6] **(Figure 2)**. MAC formation at the post-synaptic membrane leads to lysis and disruption of the NMJ folds.

Complement regulators

To avoid spontaneous activation of the complement pathway that leads to cell injury, there are several inhibitory molecules in the plasma (C4 binding protein and factor H) and cell surface (CD55 - decay-accelerating factor (DAF1), including CD46 – membrane co-factor protein (MCP) – and CD59 – membrane attack complex inhibitory protein (MAC-IP)). CD55 and CD46 are concentrated at the NMJ and inactivate C3 and C5 convertases, whereas CD59 inhibits C9 polymerisation and hence the formation of the MAC complex. From a clinical point of view, the complement regulators are expressed less abundantly at the extraocular muscle NMJs, possibly suggesting the predilection of these muscles in myasthenia.[10]

The binding of AChR to ACh activates C1 leading to the formation of C3 convertase (C1C4b2Ca) which cleaves C3 to form C3b. The C3b binds to the C4b2a complex forming the C5 convertase (C1C4bC2aC3b), which cleaves C5 to C5a and C5b. C5b initiates the lytic pathway leading to the formation of membrane attack complex (MAC). The intrinsic complement regulators which prevent spontaneous activation of the pathway and the targets of some of the main anti-complement therapies in MG are shown (IVIG – Intravenous immunoglobulin). Factor D is a serine protease which cleaves Factor B to Bb and helps in the formation of the alternate pathway C3 convertase, which is a potential signal amplification pathway of the complement pathway.

Evidence for the role of complement in Myasthenia

Experimental autoimmune myasthenia gravis (EAMG) models have been established in rodents to investigate the pathogenesis of MG. The animal models mirror human MG in that the rodents develop fatigable muscle weakness and show decremental response on repetitive nerve stimulation. EAMG models can be made either by immunising with purified AChR or its sub-unit (active) or by transferring antibodies from patients with MG (passive). In addition to a clinical response similar to human MG, the EAMG models show deposition of immunoglobulins and complement components (C3 and MAC) at the neuromuscular junction of affected animals, with destruction of the end-plate and also reduction in the miniature endplate potential (MEPP) amplitude.[11, 12]

EAMG induction can be inhibited either by depleting the complement by giving cobra venom factor or by knocking out complement components C3, C5 or C6.[13-16] Animals show serum AChR antibodies and also deposition of IgG but not the corresponding complement components at the end-plates. Similarly, animals lacking the complement regulators (e.g.: DAF1 and CD59) are known to be susceptible for EAMG, with severe end-plate damage, loss of AChR and significant complement deposition seen in the double knock-out models.[17-19]

The role of complement in human myasthenia has been established since the 1970s by the demonstration of C3 and MAC deposition at the NMJ, causing degenerated junctional folds.[11, 20, 21] Patients are shown to have depleted serum complement components and the neurophysiology often correlates with the serum complement-fixing capacity demonstrated in-vitro.[19, 22] More recently, by measuring the serum levels of complement proteins and regulators, it has been shown that the inflammatory pathogenesis in MG is associated with activation of the complement pathway, especially in AChR antibody positive MG patients [23, 24]. Techniques are being developed to identify complement activity in individual patients using modified cell lines [25] or CH50 hemolysis assays [26], so that appropriate patients can be selected for complement therapies.

Complement inhibition as therapy for MG

Experimental models

Initial experiments confirmed that the administration of anti-complement therapies reduces clinical weakness in EAMG models and minimises complement deposition at the neuromuscular endplates. This can be achieved either by using inhibitors of the classical pathway (anti-C1q) or the terminal lytic pathway (anti-C6, anti-C5) or by using siRNA which causes prolonged suppression of the liver C5 expression.[27-30]

Clinical trials

Eculizumab

The first anti-complement therapy studied in MG is an IgG2/4 monoclonal antibody directed against the C5 protein. Binding of Eculizumab to C5 prevents its breakdown to C5a and C5b, thereby reducing chemotaxis by inflammatory cells and formation of MAC, respectively. Eculizumab has already been in clinical use for other complement-mediated conditions like paroxysmal nocturnal haemoglobinuria (PNH) and atypical haemolytic uremic syndrome (aHUS). The initial phase II study in MG (NCT00727194) was done using 14 patients for 16 weeks followed by a cross-over, with significant improvement in the Quantitative Myasthenia Gravis (QMG) scores in the Eculizumab patients, which was rapid and clinically meaningful. [31]

The encouraging Phase II results led to the phase III study in a multi-centre, randomised double-blind, placebo-controlled fashion (REGAIN, Safety and Efficacy of Eculizumab in AChR positive Refractory Generalised Myasthenia Gravis; NCT01997229) followed by an open label extension (OLE).[32, 33] REGAIN enrolled 125 AChR antibody positive refractory MG patients to either Eculizumab or placebo for 26 weeks. The induction dose of Eculizumab was 900 mg on day 1, weeks 1, 2 and 3 and 1200 mg in week 4, and thereafter maintenance dose of 1200 mg every 2 weeks. The primary endpoint assigned was the change in Myasthenia Gravis Activity of Daily Living (MG-ADL) score from baseline to week 26 using worst-rank ANCOVA and the secondary endpoints assessed were the change from baseline in the total scores of QMG,

Myasthenia Gravis Composite (MGC) and Myasthenia Gravis Quality of Life 15 (MG-QOL-15), and the proportion of responders.[32]

REGAIN failed to attain significance for the primary endpoint (mean rank of 56.6 vs 68.3, p=0.0698). However, the intervention group showed significantly better secondary outcomes including changes in QMG (p=0.0129) and MGQoL-15 (p=0.0281) scores but without significant change in MGC. In the pre-specified sensitivity analysis, significant difference in all scores was noted between the two groups in favour of Eculizumab starting as early as week 1 and sustained through week 26. A major drawback detected in the trial design and possibly the reason for the negative result in primary endpoint was the use of the worst rank analysis. This relegated all patients who discontinued therapy to the lowest rank irrespective of the reason for such discontinuation. This was notable in the eculizumab group where 3 patients who had a good therapeutic response discontinued due to side effects other than myasthenic worsening, namely prostatic carcinoma, Moraxella bacteremia and bowel perforation. The side effects were mild to moderate, with headache, upper respiratory infection and nasopharyngitis being the most common and reported equally in both the groups. No patients developed Meningococcal infection. Fewer patients in the eculizumab group needed rescue therapy for MG exacerbations.[32]

117 patients from the double-blind phase of REGAIN (56 in Eculizumab/ Eculizumab group and 61 in the placebo/ Eculizumab group) entered the OLE phase for up to 4 years. After a blinded induction phase (active drug provided as 1200 mg every 2 weeks for Eculizumab group and 900 mg on day 1 and weekly for 3 weeks for the previous placebo group), all patients were continued on 1200 mg once in 2 weeks. The primary endpoint was the change in mean MG-ADL score over time. Interim analysis showed a reduction of 75% in the episodes of myasthenic worsening compared to the baseline. Infections of specific interest occurred in less than one-fifth of the study group and none had meningococcal meningitis. Improvements in myasthenia scores and quality of life scores were sustained with rapid improvements in the patients who switched over from placebo to Eculizumab after the double-blind phase (called the placebo/ Eculizumab group above).[33]

Various post hoc analysis of the REGAIN trial and OLE have underlined the efficacy and broad-spectrum responses with Eculizumab. In the REGAIN trial, Eculizumab-treated patients were two times more likely to have achieved minimal manifestation post intervention status compared to placebo at week 26In the OLE at 130 weeks, a substantial majority (88%) patients had attained improved status and 57.3% had reached minimal manifestation status. [34] Minimal symptom expression defined as MG-ADL score of 0-1 or MG-QOL-15 score of 0-3 was attained by a significantly higher proportion of Eculizumab-treated patients at week 26 of REGAIN.[35]

By week 12 of the randomised control trial (RCT), 67.3% and 56.1% Eculizumab-treated patients were classified as responders based on clinically meaningful improvements in MG-ADL (≥3 points) or QMG scores (≥5 points), respectively. While the majority were early responders (i.e. response within 12 weeks), new responders continued to emerge with longer term therapy. At the end of the OLE, the corresponding numbers were 84.7% and 71.4%, showing sustained response to treatment.[36]

Eculizumab was shown to be beneficial in subgroups of subjects in REGAIN and OLE who presumably had the worst spectrum of refractory MG as defined by failed use of chronic IVIg therapy and Rituximab. Eculizumab was administered in both these subsets after a sufficient washout period. The 17 patients on chronic IVIg who completed OLE (8 in Eculizumab/Eculizumab and 9 in placebo/Eculizumab groups respectively) had a higher exacerbation rate in the year preceding randomization compared to the total REGAIN cohort. Eculizumab in the REGAIN and OLE produced rapid and sustained improvement in the majority and reduced the exacerbation rate by more than two-thirds between pre-treatment years and during treatment (i.e. reduced from 150 to 47 exacerbation per 100 patient-years).[37] In addition, 14 patients who were previously exposed to Rituximab did not show any difference from the unexposed group in terms of efficacy or safety of Eculizumab.[38] There are also reports of successful transitioning from thrice-weekly plasmapheresis (PLEX) to Eculizumab.[39]In one study, three ventilator-dependent AChR-MG patients who were previously resistant to other immunotherapies, IVIg and PLEX were given Eculizumab. While two achieved minimal manifestations status in 4 to 6 weeks of therapy, the third had partial amelioration of symptoms allowing transition to non-invasive ventilation.[40] Eculizumab has also been found to be useful in refractory myasthenic crisis.[41]

More recent real-world evidence has shown improvement in MG-ADL scores (4.4 vs 6.33) and reduction in exacerbations (7 vs 42) at 12 months (vs baseline) in 15 treatment-refractory AChR-MG patients. The average exacerbations per patient/year reduced from 2.8 to 0.46, with a mean reduction of Prednisolone dose of 23.33 mg/ day. In addition, the mean single breath count improved from 28.13 to 50.26 seconds with IVIG being discontinued in all 6 patients receiving them and 9/15 patients could also come off the Pyridostigmine.[42]

In a retrospective 24-month observational study, 57 MG patients treated with Rituximab and 20 with Eculizumab were compared. The primary end point of change in QMG scores as well as more frequent minimal manifestation state were achieved by the Eculizumab cohort, although the risk of myasthenic crisis remained the same in both groups.[43]

The role of Eculizumab as rescue therapy in refractory MG has been firmly established via the RCT and OLE, various subgroup analysis and case reports, but its role as

a first-line agent and duration of therapy are still unclear. It is currently licensed to be used in generalised AChR-MG (USA, FDA approval – Oct 2017), refractory AChR-MG (EU) and AChR-MG unresponsive to IVIG/PLEX (Japan). Even though all the current approvals are for AChR antibody positive patients, Eculizumab has also been successfully used in some seronegative patients.[44] Paediatric and thymoma-associated MG patients may need to be studied further although early anecdotal reports are promising.[45, 46] The annual cost of therapy, which exceeds half a million US dollars, has been a major deterrent to the wider use of this drug around the world.[47, 48]

Ravulizumab

Ravulizumab, a recombinant human monoclonal antibody, is a long-acting C5 complement inhibitor with a similar mechanism of action to Eculizumab. The long half-life of this molecule necessitates fewer intravenous infusions for maintenance (once every 8 weeks, as opposed to every 2 weeks for Eculizumab). This drug was previously approved for treatment of PNH and is under investigation for atypical HUS and IgA nephropathy.[49]

175 adults with symptomatic AChR antibody positive gMG were recruited to receive Ravulizumab infusion versus placebo (1:1) in the phase 3 randomized placebo-controlled CHAMPION-MG study (NCT03920293). The dosage of Ravulizumab was weight-based given as 2400 – 3000 mg single loading dose on day one followed by maintenance doses of 3000 – 3600 mg every 8 weeks starting from day 15. The primary efficacy endpoint of significant improvement in MG-ADL and the secondary outcomes were achieved in the treatment group at 26 weeks. No marked difference in adverse effects was noted between the two groups.[50] The open label extension phase of the study is ongoing. Ravulizumab is currently approved for use in MG by the FDA (Apr 2022) and potentially can be used for a wider range of patients.

Zilucoplan

Zilucoplan prevents the terminal activation of the complement cascade by two mechanisms. It binds to the C5 complement component to prevents its cleavage and binds to the existing C5b to prevent its attachment to C6. It is a small macrocyclic peptide given as a subcutaneous (SC) injection. The advantages of this molecule are its ability to be self-administered, good NMJ penetration because of its small size, and the ability to concomitantly administer IVIg therapy or neonatal Fc receptor (FcRN) inhibitors as this is not an antibody, unlike Eculizumab and Ravulizumab.[51]

In the phase 2 clinical study over 12 weeks in symptomatic adult AChR-MG patients, 44 patients were randomized and received one of the three interventions: once daily SC injection of Zilucoplan at 0.3 mg/kg, once daily Zilucoplan at 0.1 mg/kg or placebo. The main efficacy endpoints were changes in MG-ADL and QMG scores and the high dose Zilucoplan group showed a rapid and statistically significant improvement in the scores compared to placebo (MG-ADL 3.4 vs 1.1; QMG 6.0 vs 3.2). They also had reduced need for rescue therapies. No serious treatment emergent adverse reactions were reported with Zilucoplan.[52] The phase 3 study to study the efficacy and tolerability of 0.3 mg/kg Zilucoplan (n=86) versus placebo (n=88) (RAISE; NCT04115293) has been completed, with significant benefits shown in the primary outcome (MG-ADL, $p<0.001$) and also the secondary outcomes (QMG, $p<0.001$; MGC, $p=0.0023$; MG-QoL15r, $p=0.0128$). Clinically meaningful improvement in the MG-ADL score (≥3 points) was achieved in 73.1% of Zilucoplan patients versus 46.1% of those receiving placebo. The corresponding QMG improvement (≥5 points) was seen in 58% patients receiving the active drug (vs 33%). [53]

Pozelimab

Pozelimab is a fully humanized IgG4 monoclonal antibody which blocks C5 and can be used alone or in combination with Cemdisiran, a small siRNA which interfere with the hepatic production of C5. Cemdisiran reduces the circulating C5 protein levels and Pozelimab blocks any remaining C5, thus preventing the MAC deposition at NMJ. Loading dose of Pozelimab at 15 mg/kg IV followed by four repeat doses of Pozelimab at 400 mg SC administered once weekly was found to inhibit complement activation in healthy volunteers.[54] In animal studies, combination of Pozelimab with Cemdisiran allowed lower doses and decreased dosing frequency compared to use of the individual agents separately.[55] The phase 3 randomized controlled trial of the combination (intravenous Pozelimab loading followed by 4 weekly SC injections along with Cemdisiran subcutaneous 4 weekly) versus placebo in gMG is ongoing (NCT05070858).

Other anti-complement therapies

The main complement therapies in MG are summarised in **Table 1.** Of the existing immunomodulatory therapies for MG, IVIG has multiple actions along the complement cascade. These include binding of C1q, neutralisation of C3a and C5a leading to uptake, inhibition of C3b and C4b and prevention of MAC deposition.[56]

The newer therapies which are under various stages of clinical trials (although not necessarily in MG) include Tesidolumab, Crovalimab, Zimura, Gefurulimab and Nomacopan (all anti-C5), SKY59 (anti-C5 and also inhibits FcRn), Compstatin (family of cyclic peptides which inhibits C3), ANX005 (anti-C1q), Cinryze, Berinert and Ruconest (all anti-C1r/s), and Sutimlimab (anti-C1s). Danicopan (anti-Factor D) and Avacopan (anti-C5aR1), are orally administered complement blockers.[57, 58]

Table 1
Complement therapies currently used or being studied in Myasthenia Gravis

Molecule	Mechanism of action	Target group	Route and dose of administration	Current evidence
Eculizumab	Recombinant humanised IgG2/4 monoclonal antibody against C5 complement	AChR+ MG	IV induction of 900 mg weekly for 4 weeks followed by 1200 mg maintenance every 2 weeks	QMG: Eculizumab Vs Placebo = 54.7 Vs 70.7 (P=0.0129); MG-QoL15: Eculizumab Vs Placebo = 55.5 Vs 69.7 (P=0.0281) **(REGAIN)** Approved for treatment of adults with AChR+ gMG
Ravulizumab	Long-acting recombinant humanised monoclonal antibody against C5 complement	AChR+ MG	IV weight-based dose. Single loading dose of 2400 – 3000 mg and maintenance doses of 3000 – 3600 mg every 8 weeks	QMG total scores improved by 5 points or more – 30% in treated group Vs 11.3% in placebo group **(CHAMPION MG)** Approved for treatment of adults with AChR+ gMG
Zilucoplan	Macrocyclic peptide binding C5 and C5b complement components	AChR+ MG	SC, once daily dose of 0.3 mg/kg	Phase 3 study showed positive results **(NCT04115293, RAISE)** Primary outcome (MG-ADL, p<0.001) Secondary outcomes (QMG, p<0.001; MGC, p=0.0023; MG-QoL15r, p=0.0128)
Pozelimab	Fully humanised IgG4 monoclonal antibody inhibiting C5 complement	AChR+ or LRP4+ MG	SC alone or in combination with Cemdisiran	Phase 3 study is ongoing **(NCT05070858)**
Cemdisiran	siRNA suppressing hepatic C5 synthesis	AChR+ or LRP4+ MG	SC alone or in combination with Pozelimab	Phase 3 study is ongoing **(NCT05070858)**
Gefurulimab (ALXN1720)	Anti-C5 humanised bi-specific VHH antibody (nanobody)	AChR+ MG	SC weight-based dose once weekly	Phase 3 study is underway (**NCT05556096**)
Danicopan (ALXN2050)	Small molecule complement pathway factor D inhibitor	AChR + MG	Oral, multiple dosages in trial	Phase 2 study ongoing (**NCT05218096**)

(Abbreviations: AChR – Acetylcholine receptor, MG – Myasthenia Gravis, MG-ADL – Myasthenia Gravis Activities of Daily Living score, MGC – Myasthenia Gravis Composite score, MG-QoL15 – Myasthenia Gravis Quality of Life Score, gMG – generalised Myasthenia Gravis, LRP-4 – Low-density Lipoprotein receptor related protein 4)

Safety

Anti-C5 complement therapies have been in use for over a decade for PNH and more than five years in MG. No major safety markers have been identified, even in patients receiving other immunosuppressive therapies like Rituximab. The main risk is the development of Gram-negative infections, especially meningococcal sepsis since MAC formation is the primary defence against these organisms. Subsequently, meningococcal vaccinations are mandatory prior to initiation of complement therapies, and many countries stipulate the use of prophylactic antibiotics to prevent any serotypes which may not be covered by the vaccine. No safety concerns have been raised in pregnancy and lactation.[59] When using antibiotics, fluoroquinolones and macrolides are best avoided to minimise MG exacerbations. If complement therapy is used in children in the future, additional vaccinations (e.g.: against Streptococcus pneumoniae and Haemophilus Infleunzae type B) may be required. So far, clinically significant neutralising antibodies have not been identified.

Biomarkers for complement therapy

Currently, there is a dearth of biomarkers which will predict the sub-group of patients who may respond better to complement inhibitory therapies. Serological studies assessing circulating C3 levels, C5 functional activity and total complement activity estimated by CH50, or a combination of these assays (e.g.: C3:CH50 ratio) are currently being studied. A CRISPR/Cas9 genome modified HEK293T cell line with reduced complement regulator expression has been used to develop a novel assay that may be helpful to assess complement activity in AChR-antibody positive patients, thereby helping to identify patients who may benefit from anti-complement therapies. [25] Rare missense C5 heterozygous variants (c.2654 G →A; c.2653 C →T) have been shown to replace Arginine with Histidine or Cysteine on C5, preventing its ability to bind Eculizumab making the drug ineffective. Similarly, complement related gene panels may help identify the ideal "complotype" which will help develop personalised medicine.[60] A new bioassay is currently being developed enabling functional characterisation and complement-mediated neuromuscular synaptic damage.[61] It has to be noted that the complement activity as measured using the available assays do not correlate well with disease severity or AChR antibody levels [26], even though older papers suggested a link between C3 levels and disease severity. [62]

Summary

The existing model for treatment in myasthenia revolves around three main actions – inhibiting ACh breakdown by cholinesterase inhibitors, suppressing the immune system by steroids and immunomodulatory therapies and thymectomy to modify specific autoimmune activity, especially in AChR antibody-positive patients. Current steroid-sparing immunotherapies in MG are limited by their slow onset of action (often taking several months to be effective) and rescue therapies like plasma exchange/ IVIG are unlikely to be useful for long term management. The newer complement-mediated therapies are useful for selective blocking of one of the main mechanisms of antibody-mediated myasthenic syndromes. These have had extensive experimental and pre-clinical evidence and more recently have had consistently positive results in Phase II and Phase III studies. Even though there is a theoretical risk of infections with Gram-negative organisms like Neisseria, this has not been shown to be a major concern in studies so far. Future studies may be able to identify biomarkers predicting which patients might be better suited for these targeted therapies.

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Congenital Myasthenic Syndromes: A paradigm shift.

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ABSTRACT

Very few areas of medical genetics have been so profoundly impacted by the advent of next- generation sequencing (NGS) as the field of congenital myasthenic syndromes (CMS). This is due to the formidable genetic heterogeneity of CMS, a dearth of diagnostic clinical clues of CMS types, and the imperative need to establish an accurate molecular diagnosis of CMS type before any medication is started. A molecular diagnosis of CMS is fundamental not only to provide an appropriate therapy, but more importantly, to avoid potential deleterious treatments. Thus, NGS has transformed the tedious and expensive task of searching for causative mutations in an ever-expanding list of genes linked to CMS into an effective, and relatively inexpensive process that can rapidly identify the variant of CMS in question. One of the consequences of this transformation is a paradigm shift in the clinical practice of CMS that no longer requires, with rare exceptions, the use of special muscle biopsies that enable the analysis of the function and ultrastructure of the neuromuscular junction to determine the type of CMS. Another technological advance of recent years is CRISPR/Cas9, which allows genome editing at the zygotic stage, thus greatly simplifying the generation of mouse models carrying the same human CMS mutations in orthologous mouse genes. This permits an in-depth analysis of the pathogenesis and treatments of CMS caused by specific gene mutations. In terms of therapy, in addition to the classical pharmacologic treatments of CMS, including pyridostigmine sulfate, albuterol and 3,4 diaminopyridine, AAV-based gene therapies are now at the preclinical stage for several types of CMS. In this brief review, CMS are classified in six major groups: (1). presynaptic CMS, (2) synaptic CMS, (3) postsynaptic CMS; 4. CMS affecting the agrin-signal transduction pathway, (5) CMS linked to disorders of glycosylation, and (6) CMS associated with abnormalities of the cytoskeleton.

Keywords: Congenital myasthenic syndrome, neuromuscular junction, presynaptic, synaptic, postsynaptic

Introduction

Congenital myasthenic syndromes (CMS) continue being a topic of broad interest for clinicians and scientists alike because CMS are treatable disorders and because the understanding of these conditions provides fundamental knowledge about the function of the neuromuscular junction (NMJ).

Heterogeneity of CMS and patterns of genetic transmission:

The mechanisms of failure of neuromuscular transmission in CMS are quite heterogeneous, and all stem from defects of genes encoding proteins that participate directly or indirectly in neuromuscular transmission. Often, more than one mechanism contributes to the pathogenesis of a single disorder.

Mutations causing CMS usually involve single genes, except for large DNA deletions that affect more than one gene. The most common inheritance of CMS is Mendelian autosomal recessive, however mutations in several genes, including those encoding the adult subunits of the acetylcholine receptor (AChR), Synaptotagmin 2 *(SYT2)*, and SNAP25 can also be dominantly inherited.[1-4] *De novo* mutations, which are often seen in dominant forms of CMS, are the only type of mutations that have so far been described in CMS caused by defects of SNAP25.[5] The X-linked pattern has not yet been associated with the pathogenesis of CMS.

CMS linked to proteins that are exclusive vs non-exclusive of the NMJ:

The first described variants of CMS were those caused by mutated proteins participating directly in the process of neuromuscular transmission and present only at the NMJ. Examples of these variants are CMS caused by mutations in the subunits of the adult AChR and rapsyn. Pathogenic mutations in these genes result only in CMS. By contrast, mutations of genes encoding proteins that participate indirectly in neuromuscular transmission and that are not present exclusively at the NMJ result in less consistent and more complex phenotypes in which CMS is only part of broader syndromes. An example of this is mutations in *DPAGT1* that can result in a limb-girdle congenital myasthenic phenotype along with other features of glycosylation type Ij disease, including developmental delay, microcephalia and seizures. Another example is mutations in *LAMB2* that can result in CMS along with other features of Pierson syndrome, including microcoria and congenital nephrotic syndrome.

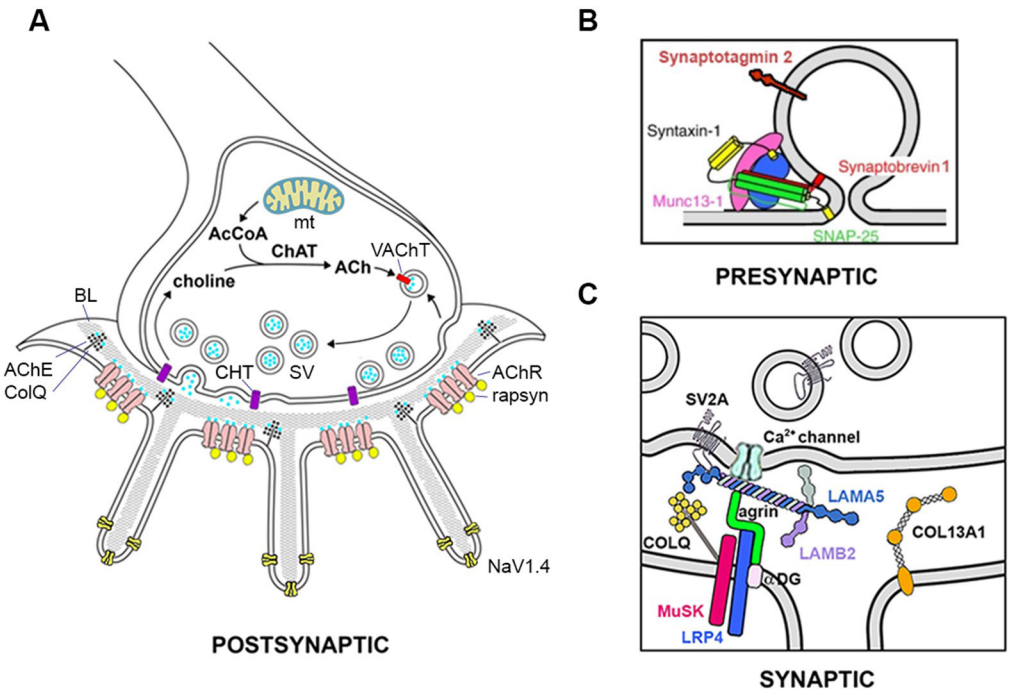

Figure 1: Diagram showing the most important proteins linked to the pathogenesis of CMS in the postsynaptic (A), presynaptic (B) and synaptic (C) compartments. Abbreviations: AcCoA: acetyl coenzyme A, AChE: acetylcholinesterase catalytic subunits, BL: basal lamina, CHT: high-affinity choline transporter, ColQ: collagen-like tail subunit, mt: mitochondria, NaV1.4: sodium channel protein type 4 subunit alpha *(SCN4A),* VAChT: vesicular acetylcholine transporter, SV2A: synaptic vesicle protein 2A.

Classification of CMS:

CMS are traditionally classified based on the location of the protein encoded by the gene causing the disease in three major groups: presynaptic, synaptic, and postsynaptic types (Figure 1). This classification is helpful to arrange CMS according to the primary site of pathology. However, in many types of CMS, such as those resulting from deficient proteins of the agrin signaling pathway and glycosylation disorders, there are both pre- and postsynaptic defects. Table 1 presents a proposed classification of CMS based on the primary site of the defect, while Table 2 lists the most important allelic variants of genes linked to CMS Another approach to classify CMS is by sequential numbers in the order that they were discovered, and this is the way CMS variants are listed in the NCBI OMIM web site https://www.ncbi.nlm.nih.gov/omim

Table 1. Classification of CMS

Presynaptic

a. Defects of the cholinergic pathway:
ChAT deficiency *(CHAT)†**
High-affinity presynaptic choline transporter deficiency *(SLC5A7)*
Vesicular ACh transporter deficiency *(SLC18A3)*

b. Defects of mitochondrial function with presumptive effect on the cholinergic pathway:
PREPL deficiency *(PREPL)*
Mitochondrial citrate carrier *(SCL25A1)*

c. Defects of SNAREs
SNAP25 deficiency *(SNAP25B).* ***DOMINANT***‡
VAMP1 deficiency *(VAMP1)*

d. Defects of Ca^{2+} sensors, active zone linkers, and kinetic proteins:
Synaptotagmin2 defect *(SYT2).* ***DOMINANT***

Synaptotagmin2 recessive deficiency *(SYT2)*
Munc13-1 deficiency *(UNC13A)*
Rabphilin3a *(RPH3A)*
Myosin9a deficiency *(MYO9A)*

Synaptic

a. Defects of collagen proteins:
ColQ deficiency *(COLQ)*
COL13A1 deficiency *(COL13A1)*
b. Defects of laminins:
Laminin beta2 deficiency *(LAMB2)*
Laminin alpha5 deficiency *(LAMA5)*

Postsynaptic

a. Defects of the ACh receptor:
Without major kinetic changes:
Receptor deficiency (CHRNA1/B1/D/E)
With major kinetic changes:
Slow-channel syndrome (CHRNA1/B1/D/E) **DOMINANT**
Fast-channel syndrome (CHRNA1/B1/D/E)
b. Prenatal myasthenia (Escobar Syndrome) (CHRNG)
c. Defects of rapsyn (RAPSN)
Generalized
With facial deformities
d. Defect of the sodium channel
Sodium channel myasthenic syndrome (SCN4A)

Defects of signaling pathways

Agrin deficiency *(AGRN)*
Proximal
Distal with presynaptic deficit
MuSK deficiency *(MUSK)*
LRP4 deficiency *(LRP4)*
DOK7 deficiency (*DOK7*)

Defects of glycosylation

GFPT1 deficiency (*GFPT1*)
DPAGT1 deficiency *(DPAGT1)*
ALG2 deficiency *(ALG2)*
ALG14 deficiency *(ALG14)*
GMPPB deficiency *(GMPPB)*

Defects of the cytoskeleton

Plectin deficiency *(PLEC1)*

*The most frequent forms of each group are bolded.
† Linked gene is shown in parenthesis.
‡Indicates dominant forms.

Table 2. Most important phenotypic and allelic variants of genes linked to CMS

Presynaptic

SLC5A7 (choline transporter) hereditary motor neuropathy (dominant)

VAMP1 spastic ataxia (dominant)

SNAP25 epileptic encephalopathy, ataxia, and intellectual disability

SYT2 hereditary motor neuropathy (dominant)

Synaptic

LAMB2 microcoria, congenital nephrotic syndrome (Pierson syndrome)

LAMA5 congenital nephrotic syndrome, bent bone dysplasia, myopathy

Postsynaptic

CHRNA1, CHRNB1, CHRND receptor deficiency and slow channel syndrome

CHRNE receptor deficiency, slow channel syndrome and fast channel syndrome

RAPSN proximal, focal with facial malformations in Jewish people from Iran and Iraq (E-box mutations)

SCN4A paramyotonia congenita, periodic paralysis (dominant)

Defects of signaling pathways

AGRN proximal variant and distal variant with LEMS-like features

LRP4 Cenani-Lenz syndactyly syndrome

Defects of glycosylation

DPAGT1 congenital disorder of glycosylation (developmental delay, seizures)

ALG2 congenital disorder of glycosylation

ALG14 Myopathy, seizures, and progressive cerebral atrophy

GMPPB Muscular dystrophy, intractable seizures

Defects of the cytoskeleton

PLEC1 myopathy, epidermolysis bullosa, pyloric atresia

Defects linked to mitochondrial metabolism

PREPL hypotonia-cystinuria syndrome

SCL25A1 combined D-2- and L-2-hydroxyglutaric aciduria, agenesis of corpus callosum, developmental delay, seizures.

PRESYNAPTIC DEFECTS

CMS caused by presynaptic defects are rare, and with the exception of deficiency of choline acetyltransferase (ChAT) most are represented by single case reports or only by a few families.

Defects of the cholinergic pathway:

ChAT deficiency *(CHAT)*: The disorder was initially referred to as familial infantile myasthenia and later changed to CMS associated with episodic apnea.[6,7] However, since not all cases of ChAT deficiency present with episodic apnea and not all the CMS associated with episodic apnea are due to *CHAT* mutations, it is preferrable to refer this condition simply as ChAT-CMS. The severity of this disease is extraordinary variable: it can range from mild forms that tend to improve after puberty to extremely severe forms resulting in wheelchair-bound status, continuous ventilatory support and gastric tube.[7-9] This variant of CMS has several distinctive features including: (1) association with apneas, (2) fast-developing muscle fatigue (within minutes), (3) paradoxical impairment with cold temperatures such as weakness triggered by cold water of a swimming pool,[10] (4) in mild cases no decrement to repetitive nerve stimulation (RNS), but decrement only after 5 minutes of nerve stimulation at 10 Hz.[10,11] and (5) ptosis without ophthalmoparesis and unsatisfactory long-term response to pharmacologic treatments. Severe cases of ChAT-CMS present with psychomotor delay,[9,12] but autonomic dysfunction is surprisingly absent. Mutations in *CHAT* has been described in other species, including dogs,[13] zebrafish,[14] *C elegans* [15] and *Drosophila*.[16] Several molecular defects have been associated with ChAT-CMS, including missense, nonsense, frameshift, and microdeletions.[7,8,9,17] Large deletions are peculiar because they also involve the VAChT gene located in the first intron of *CHAT*.[18] This condition has been reported world-wide in North America,[7,19,17] South America,[9] Europe,[19] the Middle East,[20] Malaysia,[21] and China.[8]

High-affinity choline transporter *(SLC5A7)*: Patients with mutations in this gene present many of

the symptoms described above for ChAT-CMS, thus representing an example of locus heterogeneity.[22] However, the choline transporter CMS can present with antenatal forms resulting in arthrogryposis or stillbirths, and CNS involvement is more frequent than in ChAT-CMS.

Vesicular ACh transporter deficiency *(SLC18A3)*: This is a rare condition that shares many clinical features with ChAT-CMS, including muscle fatigability, apneas and paradoxical worsening with low temperatures (swimming pool sign).[23]

PREPL deficiency *(PREPL)*: This condition results from recessive deletions, involving the *PREPL* gene and other contiguous genes on chromosome 2p21.[24] When the *SLC3A1* gene is included in the deletion there is also cystinuria. The clinical manifestations include severe neonatal hypotonia, fluctuating ptosis, facial paresis, dysarthria, feeding difficulties and growth hormone deficiency. An anconeus biopsy in one patient showed severe reduction of MEPP amplitudes with normal AChR density strongly suggestive of an underlying abnormality of ACh synthesis. Beneficial response to pyridostigmine and albuterol is variable and often transient.

Deficiency of mitochondrial citrate carrier *(SCL25A1)*: Biallelic mutations in this gene can result in mild proximal weakness and variable ocular and bulbar involvement.[25] Patients often show developmental delay and dysmorphic features. The mutation p.(Arg247Gln) is a recurrent mutation present in individuals of different ethnic groups.[26] As in the previous group an anconeus biopsy performed in a single patient showed normal MEPP amplitudes with normal AChR density, which points to a defect of ACh synthesis. Reported patients showed no consistent beneficial response to either anticholinesterase medication or albuterol.

Defects of SNAREs:

SNAP25: This severe and dominant form of CMS is associated with arthrogryposis, cortical excitability, ataxia, and developmental delay.[4,5]

VAMP1 (synaptobrevin 1): VAMP-CMS is a recessive CMS characterized by hypotonia, impaired external ocular muscle function, developmental delay, joint contractures, and Lambert-Eaton myasthenic syndrome (LEMS)-like features on EMG testing.[27]

Defects of Ca^{2+} sensors, proteins of the active zone, and kinetic proteins:

Synaptotagmin 2 defect *(SYT2)* (dominant): This is a relatively mild form of CMS with motor axonal neuropathy as an allelic variant. All mutations so far described are missense mutations altering calcium binding sites in the CB2 domain. There is frequent multigenerational involvement and LEMS-like features on electrophysiologic testing. The condition usually responds to treatment with 3,4 diaminopyridine (DAP).[28]

Synaptotagmin 2 defect *(SYT2)* (recessive): This is a severe form of CMS with onset at birth or prenatally. Most of the reported cases involved consanguinity and nonsense or frameshift mutations resulting in protein truncation.[29-31] There is modest ocular involvement, but severe bulbar and generalized weakness with muscle atrophy. The EMG shows denervation and LEMS-like features in response to RNS. Patients show modest response to albuterol, pyridostigmine and 3,4 DAP.

Munc13-1 deficiency *(UNC13A)*: This is a severe form of CMS, which has been so far only described in a single patient. Munc13-1 has a C2A and C2B domains that interacts with SNARES and participates in calcium homeostasis. The reported patient had a homozygous nonsense mutation predicting a large truncation of the protein. The patient had microcephaly, developmental delay, cortical EEG irritability, joint contractures, and LEMS-like features on electrophysiologic testing. A muscle biopsy showed normal NMJ ultrastructure and LEMS-like electrophysiology.[32]

Rabphilin 3a deficiency *(RPH3A)*: Pathogenic mutations in the *RPH3A* gene have been found in two independent families of patients with a mild presynaptic CMS associated with hand incoordination and tremors.[33,34] The muscle biopsies showed double membrane sacs encircling synaptic vesicles. The pathogenic mechanism of this condition is unclear, but rabphilin 3a, as Synaptotagmin 2 and Munc13-1, encompasses a C2A and C2B Ca^{2+}/phospholipid binding domains that when altered may affect synaptic vesicle homeostasis.

Myosin 9a deficiency *(MYO9A)*: Two non-related patients affected with ptosis, ophthalmoparesis, global weakness, bulbar involvement, and respiratory crises were found to have deleterious mutations in *MYO9A*,[35] which encodes the unconventional myosin 9a. CNS symptoms, including learning difficulties and vertical nystagmus were also reported. Muscle biopsies were not available. Patients responded to pyridostigmine. The underlying pathogenic mechanism is unclear, but expression studies in cell lines and zebrafish indicated that myosin 9a is fundamental for neurite extension and axonal transport.[36]

SYNAPTIC DEFECTS

Except for ColQ deficiency synaptic CMS are rare forms of CMS.

Defects of collagen proteins:

ColQ deficiency *(COLQ)*: Deficiency of ColQ, is

a relatively common variant of CMS and is the first one that was completely characterized by microelectrode recordings and electron microscopy of the NMJ.[37] The condition results from mutations in *COLQ*, the gene that encodes the triple-helix strands that assemble with three homotetramers of the AChE catalytic subunit and holds the enzyme at the endplate.[38] The ultrastructure of the NMJ in ColQ-CMS shows a characteristic triad consisting of: (1) reduced size of nerve terminals, (2) encasement of nerve terminals by the Schwann cell, and (3) focal degeneration of the postsynaptic folds.[37] In some cases, numerous endocytic vesicles in the subsynaptic region can be seen, a feature in common with slow-channel CMS (SCCMS). Because ACh cannot be hydrolyzed, once it is released from the nerve terminal it accumulates at the synaptic cleft re-exciting the AChR ion channel. This in turn results in endplate potentials (EPPs) of prolonged duration that remain above threshold level longer than the refractory period of the muscle fiber enabling them to trigger multiple muscle action potentials. This feature of ColQ-CMS is also shared with the SCCMS and can be clinically observed by EMG recordings showing repetitive compound muscle action potentials (CMAPs) in response to a single nerve stimulation. Failure of neuromuscular transmission in ColQ-CMS occurs as a result of multiple mechanisms, including presynaptic deficit, staircase summation of EPPs leading to depolarization of the endplate and AChR desensitization. Treatment is limited to sympathomimetic drugs, such as albuterol.

COL13A1 deficiency *(COL13A1)*: This is a rare recessive CMS characterized by early onset in life and predominant involvement of bulbar and axial musculature without significant impairment of external ocular muscle function.[39,40] The mechanism of failure of neuromuscular transmission is unknown, but studies in *Col13a1* $^{-/-}$ mice indicate both pre- and post-synaptic involvement.[39] Affected patients show a moderate response to albuterol and 3,4 DAP.[40]

Defects of laminin proteins:

Laminin beta2 deficiency *(LAMB2)*: This is a very rare form of CMS occurring in survivors of Pierson syndrome after a successful renal transplant. Only two cases reported in the literature, both showing ultrastructural changes of the NMJ reminiscent of ColQ-CMS.[41,42] In one case there was a favorable response to 3,4 DAP, but pyridostigmine resulted in an adverse effect.

Laminin alpha5 deficiency *(LAMA5)*: A rare recessive form of CMS with only one case formally reported.[43] The described case showed LEMS-like features. The clinical manifestations of biallelic LAMA5 mutations are protean and include congenital nephrotic syndrome,[44] bent bone dysplasia and myopathy.[45] The reported case responded to 3,4 DAP, albuterol and pyridostigmine.

POSTSYNAPTIC DEFECTS

More than half of CMS are caused by mutations in the genes encoding the adult subunits of the AChR or rapsyn.

Deficiency of AChR expression *(CHRNA1, CHRNB1, CHRND, CHRNE)*: This is the most common variant of CMS and can result from mutations in any of the genes encoding the adult subunits of the AChR. There is an overwhelming majority of mutations in the gene encoding the epsilon subunit.[46] The reason for this is unclear, but a possible explanation is that since the adult epsilon subunit can be compensated by re-expression of the fetal gamma subunit (encoded by *CHRNG*), these patients tend to have milder forms of CMS. Thus, they are less vulnerable to natural selection pressure enabling them to pass their mutated genes to their offspring. Examples of this include *CHRNE 1267delG* in Roma people and *CHRNE 1293insG* in Eastern Europeans.[47,48]

Biallelic mutations in *CHRNA1*, which encodes the ACh binding alpha-subunit usually result in severe and potentially fatal CMS. By contrast mutations in *CHRNG* result in prenatal CMS and represent one of the multiple causes of the Escobar syndrome, which is characterized by arthrogryposis multiplex, joint contractures, pterygia, and respiratory distress.[49]

Ocular involvement is usually prominent in patients with deficiency of AChRs. Patients respond well to pyridostigmine and surprisingly also to albuterol and 3,4 DAP, likely because the sizes of nerve terminals in these patients are normal allowing increased ACh output without depletion.

Slow-channel CMS *(CHRNA1, CHRNB1, CHRND, CHRNE)*: SCCMS is the most common dominant form of CMS, and it can result from mutations affecting the AChR transmembrane domains M1 and M2, the M2–M3 linker, and the N-terminal.[50] The most severe forms are those involving the M2 domain, while those affecting the N-terminal are milder.[51] The SCS shares a number of similarities with ColQ deficiency even though they result from very different pathogenic mechanisms. The similarities include repetitive CMAPs to a single nerve stimulus, depolarization block from staircase summations of EPPs, subsynaptic degenerative changes and poor or adverse response to anticholinesterase medications. Treatment involves medications that shorten the channel open time, such as quinidine, quinine, and fluoxetine.[52]

Fast-channel CMS *(CHRNA1, CHRNB1, CHRND, CHRNE)*: Mutations in all the adult subunits of the AChR

can cause low agonist affinity with shortened AChR ion channel kinetics and result in the fast-channel syndrome. However, as in the case of receptor deficiency, these mutations are most common in the epsilon subunit. The εP12L mutation is indeed the most common fast-channel mutations, and it results in a serious disease with a potentially fatal outcome.[53,54] The treatment of this condition is similar to that of AChR deficiency.

Rapsyn deficiency *(RAPSN)*: Mutations in the gene encoding rapsyn is another relative common cause of CMS. Rapsyn is a 43-kD postsynaptic protein intimately associated with the receptor and essential for clustering of AChRs.[55,56] The severity of this disease is extraordinary variable, it can range from severe and potentially fatal neonatal forms to very mild forms with onset during childhood or adulthood. Often patients are born with arthrogryposis multiple indicating prenatal disease.[57] Patients with severe forms suffer recurrent respiratory crises, which at variance with patients with ChAT mutations, do not occur spontaneously, but are usually triggered by intercurrent infections. A predominant bulbar involvement with facial malformations has been described in Jewish people from Iran and Iraq, who were found to possess pathogenic E-box mutations.[58]

The mutation N88K, which derives from an old Indo-European founder is often found at least in one of the alleles of patients with Rapsyn-CMS.[59,60] In contrast with patients with AChR ε subunit mutations, patients with RAPSN mutations seldom show involvement of extraocular muscles. Treatment is similar to that for patients with AChR deficiency.

Defect of the skeletal muscle sodium channel *(SCN4A)*: This is a unique type of CMS characterized by recurrent episodes of generalized and bulbar weakness reminiscent of periodic paralysis. However, the clinical presentation also includes muscle fatigue, ptosis and ophthalmoparesis more consistent with CMS. [61,62] Decrement of CMAP amplitudes in response to repetitive nerve stimulation at 2 Hz is modest but becomes obvious with nerve stimulations at higher rates. The management of this condition is based on a dual therapy with pyridostigmine and acetazolamide.

DEFECTS OF SIGNALING PATHWAYS (AGRN, MUSK, LRP4, DOK7)

This is an important group of CMS involving a signal transduction pathway that is fundamental for the development and maintenance of the NMJ.[63-66] The clinical presentations of these disorders are very heterogeneous, but all share predominant proximal limb weakness, variable bulbar and ocular involvement and poor or adverse response to pyridostigmine. Stridor is also common, particularly in the DOK7-CMS.[67] The disease can start anytime in life and weakness of neck muscles, sometimes presenting as a drop-head syndrome, is a distinctive characteristic of these conditions.[68,69] From the pathophysiologic standpoint all these variants present presynaptic and postsynaptic involvement. Surprisingly, N-terminal mutations in the *AGRN* gene can result in distal limb involvement and a LEMS-like syndrome. The reason for this is unclear, but it may involve a disrupted interaction of agrin and the gamma subunit of laminin with the presynaptic voltage-gated calcium channel.[70,71] The DOK7-CMS is the most common variant of this group, in part due to several recurrent mutations, including c.1124_1127dupTGCC and many other mutations affecting all the protein domains.[72] Treatment is based on sympathomimetic drugs such as albuterol.

DEFECTS OF GLYCOSYLATION (GFPT1, DPAGT1, ALG2, ALG14, GMPPB)

The discovery of the association between limb-girdle myasthenia with tubular aggregates and the gene encoding the enzyme glutamine-fructose-6-phosphate-transaminase 1 (GFPT1) by linkage analysis was surprising but understandable given the heavy glycosylation of proteins of the NMJ.[73] Patients in this group resemble patients with *DOK7* mutations because of the proximal limb weakness. However, the muscle biopsies of these patients often reveal tubular aggregates and patients seldom show bulbar or ocular involvement.[74] In addition, patients with *DPAGT1* and *ALG2* can present with more complex phenotypes that includes mental delay and seizures.[75,76] Patients with mutations in *GMPPB* may present with myopathy, encephalopathy, and intractable seizures.[77] The treatment of this group includes pyridostigmine and albuterol. 3,4 DAP should be avoided because of the possibility of seizures.

DEFECTS OF THE CYTOSKELETON

Plectin deficiency *(PLEC1)*: Mutations in *PLEC1* can cause epidermolysis bullosa simplex, which may associate with muscular dystrophy (EBS–MD) or pyloric atresia (EBS–PA).[78,79] Rare cases may also show neuromuscular transmission failure.[80] Treatment involves pyridostigmine and albuterol. 3,4 DAP should be avoided because of the possibility of an underlying cardiomyopathy and heart arrythmia.

Other genes with possible association with CMS: Several other genes have been suspected to cause CMS, but the genetic mode of transmission and mechanism of failure of neuromuscular transmission have not been completely elucidated. These genes include, *TOR1AIP1*,[81] *PURA*,[82,83] *CHD8*,[84] *SCN8A*[85], and many other genes linked to hereditary myopathies.[86]

Non-pharmacological treatments: In children with

severe forms of CMS the protection of the respiratory function is of paramount importance. Therefore, tracheotomy, mechanical ventilation and gastric tube are all important measures that when indicated, need to be implemented early in the course of the disease to prevent respiratory insufficiency, anoxic brain injury and permanent neurologic damage. Surgical correction of scoliosis is also important to eliminate a potential mechanical impediment of proper respiratory function.

Finally, upcoming molecular therapies based on monoclonal antibodies,[87] AAV-mediated gene therapy and many other target-therapies may expand in the near future the list of treatments available for CMS.[88-90]

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CONFLICT OF INTEREST

None.

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A Promising Antigen-specific Immunotherapy for the Treatment of Myasthenia Gravis

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ABSTRACT

Myasthenia gravis (MG) is a T cell-dependent, antibody-mediated, autoimmune disorder with well-established antigenic targets at the neuromuscular junction. MG autoantibodies mainly target the nicotinic acetylcholine receptor (AChR) and especially epitopes located in the extracellular domain of the α1 subunit (α1-ECD). Today, most therapeutic regimens for MG are non-specific and not curative, requiring chronic treatments that are associated with significant side effects. We aim to develop an antigen-specific therapeutic approach, based on reestablishing tolerance towards the AChR, the dominant autoantigen in MG. To this end, we used a soluble mutated form of the human α1-ECD, which incorporates a major fraction of MG autoreactive T cell epitopes and examined the therapeutic efficiency of intravenous administration in a rat experimental autoimmune MG model. We found that repeated intravenous administration of α1-ECD for up to 12 days led to a robust amelioration of disease symptoms in a dose and time-dependent manner. The observed therapeutic effect of α1-ECD was significantly better than the effect of two current mainstay drugs for MG treatment. There were no signs of toxicity in α1-ECD-treated animals and further studies are underway to fully elucidate the immunological mechanism underlying the treatment effect. In this review we will summarize and discuss our most recently published findings, which strongly suggest that intravenous administration of α1-ECD may represent an efficacious and safe therapeutic approach to treat MG and thus that α1-ECD represents a potential new first in class drug for clinical application in MG.

Key words: autoimmune disease; myasthenia gravis; acetylcholine receptor; antigen specific immune tolerance; intravenous tolerance.

Abbreviations: MG, myasthenia gravis; AChR, nicotinic acetylcholine receptor; NMJ, neuromuscular junction; α1-ECD, extracellular domain of the α1 subunit of the human acetylcholine receptor; α1-ECDm, mutated form of the α1-ECD; α1-ECDmt, mutated and tagged form of the α1-ECD; EAMG, experimental autoimmune myasthenia gravis.

Introduction

Myasthenia gravis (MG) is a prototype organ-specific autoimmune disorder affecting the structure and function of the neuromuscular junction (NMJ), causing weakness and fatigability of skeletal muscles. It is a T cell-dependent antibody mediated disease, primarily caused by autoantibodies against the acetylcholine receptor (AChR). AChR antibodies are found in approximately 85% of MG patients, termed AChR-MG (1). Fewer patients have autoantibodies against other NMJ proteins, such as muscle specific kinase (MuSK) (~9% of patients) or low-density lipoprotein receptor-related protein 4 (LRP4) (~2% of patients) (2). The AChR is a transmembrane pentameric glycoprotein that along with other proteins (including MuSK and LRP4) forms a clustered complex in the post-synaptic membrane of the NMJ. This complex allows transmission of excitatory signals from the axon terminal of motor neurons to the muscle. The AChR is composed of five subunits with an $(\alpha 1)_2\beta 1\varepsilon\delta$ stoichiometry in adult and $(\alpha 1)_2\beta 1\gamma\delta$ in fetal or denervated muscles (3). Each subunit is composed of an N-terminal extracellular domain (ECD), four transmembrane domains (TM1-TM4) and a largely unstructured intracellular domain between TM3 and TM4. The ECDs contain most of the disease relevant autoantibody epitopes. Although, antibodies against the TM and intracellular domains can be found in MG patient sera, they are probably not clinically significant as they cannot bind to their targets in undamaged muscle membranes (4,5). In particular, the ECD of the AChR α1 subunit (α1-ECD) seems to be targeted by most of AChR-specific autoantibodies. It contains the so-called main immunogenic region (MIR), a group of overlapping MG epitopes with a central core located between amino acids 67 and 76 (6,7). AChR-reactive $CD4^+$ T cells have long been identified in MG patients and are essential for T cell dependent production of high affinity autoantibodies by B cells. Analysis of the basis for the T cell activation has identified T cell reactive peptides, most of which are derived from the α1-ECD (4,8–10). Thus, T and B cell epitopes appear to mainly originate from the α1-ECD, indicating its significance in designing AChR-MG therapeutics based on antigen-specific tolerance induction.

Current MG therapeutics are not curative and not antigen-specific. They mostly attain either symptomatic relief for the patients or work by general immunosuppression, potentially leading to significant

side-effects (11). Mainstay treatment options include acetylcholinesterase inhibitors, corticosteroids, intravenous immunoglobulin, plasmapheresis, and thymectomy (12). More recent treatments targeting molecules of the inflammatory response, such as complement, FcRn , proteasome components, and B cell or plasma cell markers, have also been explored with some positive outcomes (13–17). However, response to therapy may differ depending on autoantibody profile, clinical manifestation, and disease onset. For example, MuSK-MG patients do not usually respond well to acetylcholinesterase inhibitors and thymectomy is beneficial mostly for early onset AChR-MG patients (18). Additionally, complement inhibitors usually work better against AChR-MG, while B cell depleting agents such as rituximab are proposed as second in line options for refractory MuSK-MG (19,20).

An ideal therapeutic strategy would only target the autoreactive components of the immune system without impeding normal responses. Such an approach would focus on the regulation of the immune system and promote tolerance reestablishment against the targeted epitopes, in an antigen-specific manner. Therefore, this targeted approach would limit the risk of side-effects and help prevent disease recurrence (21). In this review, key aspects of intravenous antigen-specific tolerance induction are discussed.

Induction of tolerance as a treatment for MG

Induction of tolerance by administration of autoantigens has been addressed in animal models for several autoimmune diseases. In the context of multiple sclerosis, therapeutic tolerance has been achieved in mouse experimental autoimmune encephalomyelitis (EAE) models. Subcutaneous administration of myelin basic protein (MBP) peptide in escalating doses, either prior to or after disease induction, lead to a dose-dependent therapeutic response (22,23). Furthermore, there is evidence that following a repetitive dosing schedule, by either mucosal or non-mucosal routes, immune homeostasis is restored through immunoregulatory transcriptome alterations (22). A more recent study has shown that intradermal injection of a murine myelin oligodendrocyte glycoprotein conjugate led to antigen-specific T cell anergy and peripheral type-2 myeloid response (24). Clinical trials have also provided encouraging data with autoantigens delivered as a peptide-cocktail or as peptide-loaded dendritic cells, following a repetitive dosing schedule (25,26).

With respect to MG, multiple studies have examined tolerance reestablishment in experimental autoimmune MG (EAMG) animal models by administering AChR domains through mucosal routes (27–30). The mechanism behind the therapeutic effect possibly relies on the regulatory role of tissue-resident immune cells in lymphoid organs. For example, oral treatment with a recombinant α1-ECD prevented or ameliorated ongoing EAMG in rats, characterized by a decrease of Th1 response markers and a shift in auto-antibody IgG isotypes from IgG2 to IgG1. Furthermore, the α1-ECD dose affected the response; oral administration of lower doses led to active suppression of the immune response, while higher doses favored clonal anergy, most likely by limiting the proliferation of the autoantigen-specific T cells (31).

Nasal administration of AChR fragments has also shown positive results. Low doses of recombinant human α1-ECD suppressed ongoing EAMG in rats most probably by mechanisms of active suppression rather than clonal anergy, accompanied by a shift of Th1 to Th2/Th3 AChR-specific response (27). Higher antigen doses were necessary to ameliorate disease when treatment was administered after disease induction compared to preventive administration prior to induction (29). Furthermore, a 10-fold lower dose of α1-ECD was needed to achieve a similar therapeutic effect as oral administration (31).

Some studies have made use of AChR-derived peptides and immunodominant T-cell epitopes to reinstate tolerance, as opposed to whole protein domains. Induction of tolerance was reported after oral or nasal administration of immunodominant T cell epitopes derived from the *Torpedo californica* AChR (T-AChR) α-subunit in mice prior to disease induction. This was accompanied by reduced levels of autoantibodies and proinflammatory cytokines expressed by T-AChR reactive T cells, probably via mechanisms of clonal anergy (30,32). However, in other studies nasal administration of AChR-derived peptides in rats failed to have a significant effect on EAMG disease development, despite the fact that tolerization against those specific AChR epitopes was achieved (33,34). This could be due to an inability of tolerance-spreading over a wider bystander epitope range, or due to significant heterogeneity between dominant B and T cell epitope repertoires. Thus, such studies have highlighted that the use of peptides may not always be optimal for clinical application. On the contrary, the use of proteins comprising the majority of epitopes targeted, would not rely on bystander effects and would allow antigen processing and presentation in a native context, therefore, minimizing such limitations (35).

Intravenous α1-ECD as a promising drug candidate for MG therapy

Intravenous delivery of antigen could take advantage of a natural non-inflammatory path, reaching several organs with resident immune cells involved in the induction and

maintenance of tolerance. This mode of treatment delivery has been reported in other autoimmune diseases with promising results (21). In a clinical trial for relapsing multiple sclerosis, a cocktail of 4 MBP tolerogenic epitopes given in repeated escalating doses over 8 to 32 weeks resulted in a significant decrease in new lesions observed (25). Similarly, nanoparticle coated gliadin induced antigen-specific T cell tolerance in celiac disease patients, which also involved a repeated antigen dosing design (36).

Recently, for the first time, we explored antigen-specific tolerance induction by intravenous drug administration in EAMG rats as a therapeutic strategy for AChR-MG (37). We used human α1-ECD, as it contains the majority of AChR-MG-relevant pathogenic B and T cell epitopes. Our team has also previously described the construction of a recombinant human α1-ECD mutant, in which the Cys-loop has been exchanged with that of the acetylcholine-binding protein (AChBP), a homologous soluble protein from the snail *Lymnaea stagnalis*, to improve its hydrophilicity, and consequently its solubility and stability (38). Compared to the *wild* type protein, this mutant was found to have practically identical binding to autoantibodies from MG patient sera (39). The mutant domain (hα1-ECD_{mt}) was expressed in the yeast *Pichia pastoris* as a glycosylated soluble secreted protein with near-native conformation. It contained a C-terminal 6-HIS-tag to facilitate purification via metal-affinity chromatography. A tag free α1-ECD (hα1-ECD_{m}) mutant was also produced in *E. coli,* where it was present in high quantities in inclusion bodies. Following solubilization in urea the protein was allowed to refold overnight at 4°C before final purification by anion exchange and size-exclusion chromatography.

For the *in vivo* studies of therapeutic efficacy, a Lewis rat EAMG model was used. In most cases EAMG was induced in rats by AChR protein extracted from the electric organ of *T. californica* (40). More recently, we described a robust and reproducible EAMG model in female Lewis rats using hα1-ECD_{mt} in CFA (41). Symptoms usually develop 6-8 weeks after induction and, should the rats be left untreated, persist for several weeks allowing for the long-term evaluation of therapeutic interventions. Since the model is induced with the human sequence of α1-ECD, it is well suited for the study of antigen-specific therapeutic approaches (42).

Using the aforementioned tools, we proceeded to evaluate the therapeutic efficacy of intravenous α1-ECD administration. Importantly, all treatment regimens followed a therapeutic rather than a preventive regimen, treatment was always administered after disease induction (Figure 1). EAMG rats were first treated for twelve consecutive days with 100 μg hα1-ECD_{mt} intravenously (tail vein) or intranasally (droplets in nostrils), at seven days post disease induction. Disease progression was then monitored for at least 120 days. We observed that intravenous administration resulted in a highly significant reduction in the rats' EAMG score, representing a huge improvement in therapeutic effect compared to that obtained in rats treated by intranasal administration or in mock (PBS) treated rats (37). A more detailed assessment of intravenous drug-administration demonstrated that the effect was dose-dependent, with higher protein doses yielding a more profound therapeutic effect. These findings were corroborated, in addition to the EAMG scores, by changes in animal body and decrement of the compound muscle action potential in response to repetitive nerve stimulation.

Since the goal of the proposed strategy is to treat active, ongoing disease, we also examined the therapeutic potential of intravenous α1-ECD_{mt} at later time points, when rats display progressive disease at the molecular and the clinical levels (21 and 40 days after disease induction,

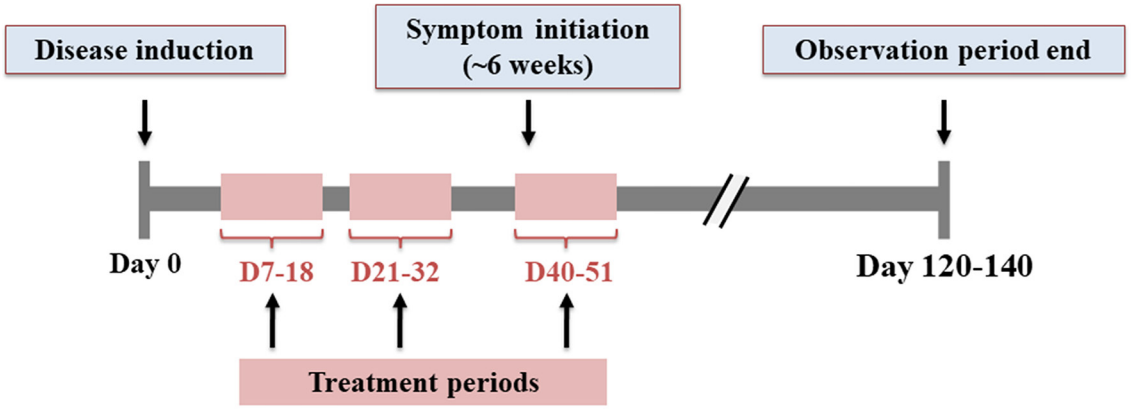

Figure 1. Schematic representation of the treatment regimens implemented in the rat EAMG animal model. Treatments were administered for 12 days starting at different times after disease induction. The animals were followed for at least 120 days after induction of disease to monitor long term effects of treatment.

Table 1: Average EAMG scores at the end of the observation period of rats treated with hα1-ECD$_{mt}$ by intravenous administration initiated at different time points and of their respective control groups. (Derived from data published in ref #31).

Treatment initiation (days after induction)	Treatment regimen (daily doses)	EAMG score (±SEM)
Day 7	PBS (x12)	2.74 (± 0.32)
	5 μg hα1-ECD$_{mt}$ (x12)	2.50 (± 0.72)
	25 μg hα1-ECD$_{mt}$ (x12)	1.05 (± 0.46)
	100 μg hα1-ECD$_{mt}$ (x12)	0.28 (± 0.14)
Day 21	PBS (x12)	3.14 (± 0.40)
	100 μg hα1-ECD$_{mt}$ (x12)	1.52 (± 0.39)
	500 μg hα1-ECD$_{mt}$ (x12)	0.57 (± 0.57)
Day 40	PBS (x12)	2.42 (± 0.49)
	100 μg hα1-ECD$_{mt}$ (x12)	2.06 (± 0.38)
	500 μg hα1-ECD$_{mt}$ (x12)	1.33 (± 0.84)
	1000 μg hα1-ECD$_{mt}$ (x12)	0.33 (± 0.33)

respectively). Treatment initiation at both later time points was found to have a powerful therapeutic effect, lasting at least until day 140 after disease induction. This effect was also dose-dependent (Table 1), where larger overall doses at later time points achieved a similar robust therapeutic effect to smaller doses given at earlier time points. The somewhat larger doses required for effective treatment of active full-blown disease compared to disease prevention could be due to accumulation of damage at the NMJ and/or the establishment of memory cells by the time treatment begins. Interestingly, it appears that overall exposure time was also crucial for optimal response to therapy. Thus, a given total protein amount administered in fewer doses was less effective than the same amount distributed over more frequent administrations. Specifically, daily injections of 100 μg hα1-ECD$_{mt}$ had a more profound effect in EAMG amelioration compared to 400 μg hα1-ECD$_{mt}$thrice (every 4 days) over a 12-day period, even though the total amount of protein administered was the same (1200 μg).

These observations are similar to what has been reported in other EAE models. Intravenous administration of a multi-epitope protein comprised of five different encephalitogenic peptides (75ug per dose for six administrations) offered long-lasting suppression of EAE in mice by downregulating pathogenic T cells and upregulating CD4+ Tregs (43). More recently, Casella et al. showed the therapeutic effect of intravenously injected oligodendrocyte-derived extracellular vesicles containing multiple myelin antigens (such as myelin basic protein, myelin oligodendocyte glycoprotein and myelin proteolipid protein) in EAE mice (44). The suppressive effect involved a mechanisms of autoreactive T cell anergy and apoptosis, rather than T regulatory cell activation. These studies have also utilized a repeated antigen dosing schedule to induce a tolerogenic effect. Indeed, there is evidence from studies in EAE that the dose and administration schedule play a significant role in the observed effect (22).

Investigating the pharmacokinetic properties of α1-ECD$_{mt}$ following intravenous administration revealed a very short plasma half-life (3.6 - 5.5 % of administered protein remained in the circulation 6 hours post injection). This can potentially explain the benefit of repeated dosing, as it prolongs the exposure of relevant cell populations to the protein. Interestingly, the pharmacokinetic profile of α1-ECD$_{mt}$ was not altered by the presence of autoantibodies or the stage of disease development. This has been demonstrated by studies performed in healthy and EAMG rats injected on day 40 after disease induction, when the α1-ECD antibody response is near its peak. As hα1-ECD$_{mt}$ displayed a short plasma half-life, modifications that would

increase its half-life in the circulation may further increase its therapeutic effect. Strategies based on attachment of polyethylene glycol chains (PEG), conjugation to albumin binding domains or an immunoglobulin Fc region and nanoparticle inclusion, have been used extensively by the pharmaceutical industry to improve the pharmacokinetic profile of biotherapeutics (45,46). Such optimization strategies could potentially allow for a dosing strategy with fewer doses. Biodistribution analysis of hα1-ECD$_{mt}$ after 6 hours showed that the majority of the protein was localized in the liver, kidneys and spleen, organs with a known role in tolerance induction and maintenance (47–49). Studies elucidating the involvement of these organs in the therapeutic effect are ongoing and aim to increase our understanding of the mechanism of action. Furthermore, these studies will provide a foundation for the development of next generation therapeutics. In this context, further assessment of immunological mechanisms resulting in EAMG amelioration, such as analysis of cytokine profile and relative frequencies of inflammatory and regulatory T and B cells, are being addressed in ongoing studies.

AChR autoantibodies have been shown to be pathogenic due to their ability to induce EAMG in animal models by passive transfer and because of the clinical improvement of patients after plasmapheresis (50–52). However, AChR antibody titers do not correlate with disease severity in MG patients (53). Furthermore, in our rat model there is poor correlation between EAMG score and rat AChR autoantibody titers, and negligible correlation with α1-ECD antibodies (41). Nonetheless, we sought to examine changes in autoantibody titers in response to treatment. We found that treatment at the earlier time point (day 7) caused a reduction in AChR antibody titers, while administration at the later time points (day 21 or 40) led to an increase in autoantibodies. Similar results were obtained for the α1-ECD antibodies. As mentioned previously, there was no correlation of the autoantibody titers with EAMG scores in rats following treatment. Some previous studies on oral tolerance have also shown an increase in autoantibody titers, despite the fact that disease was ameliorated (54). Therefore, these data underline that disease progression and response to treatment are not correlated to the entire autoantibody pool, but to subsets with specific distinct qualities such as antigen affinity, specificity, antibody isotype, and potential for antigenic modulation or complement activation. To provide insights into the treatment mechanism of action, these characteristics should be addressed to better understand their role in disease manifestation and progression.

Importantly, the potential immunogenicity of the administered protein and its effect on the normal function of the immune system should be investigated. Preliminary non-GLP toxicological studies involving injection of large doses (500 μg) of a1-ECD$_m$ in healthy rats demonstrated that the drug candidate was safe, well tolerated, and no changes in the levels of IL-2, IL-6, IL-10, IFN-γ, TNF-α and C-reactive protein were detected. Furthermore, *in silico* immunotoxicity analyses did not show any increased risk of immunogenicity in humans for a1-ECD$_m$. Nevertheless, further studies, which are underway, are needed to fully elucidate these aspects. The EAMG model in these studies and the therapeutic experimental set up make use of the same protein domain for disease induction and treatment. Studies where the disease is induced with all AChR subunit ECDs or with the torpedo AChR could further elucidate the therapeutic efficacy of α1-ECD$_{mt}$. It should be noted, that in a rat EAMG model induced by α1-ECD immunization, which also included intracellular parts of the receptor, demonstrated significant epitope spreading (55). Furthermore, antibodies against the α1-ECD seem to be the key pathogenic factor in MG. It has been suggested that changes in this class of antibodies is correlated to disease in individual patients, while an increase in antibodies against other subunits did not cause worsening of clinical symptoms (56). This also correlates well with our rat EAMG model in which the α1-ECD is pathogenic while the other AChR subunits weakly induce disease even though they give rise to antibodies (41).

To further establish the value of the novel treatment approach, we compared its efficacy to two commonly used therapies for MG patients in clinical practice, pyridostigmine and methylprednisolone. Pyridostigmine, a cholinesterase inhibitor, was given intraperitoneally (1 mg per rat) and methylprednisolone, a corticosteroid, was given orally (18.5 mg/Kg). Although both doses are higher than what is commonly used for patient treatment, these levels are well tolerated by rats (57). All treatments were initiated 40 days after disease induction. Rats treated with intravenous α1-ECD$_{mt}$ presented with effective reduction of disease symptoms compared to rats treated with the two standard treatments. For comparison, in a study performed by others, rats treated with an experimental anti-rat FcRn monoclonal antibody, a treatment modality recently approved for MG treatment, did not present with reduced disease symptoms compared to rats treated with dexamethasone, another corticosteroid (58). These results underscore the potential of our drug candidate as they demonstrate a superior efficacy of intravenous α1-ECD treatment in our model compared to pyridostigmine and methylprednisolone, two established therapies for MG.

α1-ECD$_{mt}$ contains a 6-HIS-tag which may pose an immunogenicity risk and is thus not ideal for clinical

application. To facilitate the translatability of our approach, we also investigated the therapeutic potential of α1-ECD$_m$, a protein without any tag. Moreover, the α1-ECD$_m$ protein was produced in *E. coli* to allow the potential for manufacturing scale-up purposes. As expected, the two proteins were found to have practically identical therapeutic effect when administered 21 or 40 days after disease induction. Since α1-ECD$_m$ was produced in a prokaryotic expression system, it lacked post-translational modifications, while its yeast counterpart was glycosylated. Their similar efficacy suggests that for our drug candidate glycosylation does not play a major role in its capacity to induce antigen-specific tolerance towards AChR.

Conclusions

Our novel and highly promising drug candidate currently in development, has a strong preclinical foundation as a safe, effective and disease-specific therapeutic option for patients with AChR-MG. It utilizes the organism's own antigen-presenting mechanisms and machinery to skew the autoimmune response towards tolerance without requirement of personalized autoepitopes, since it comprises multiple-epitope presentation in a native context. In our EAMG model, hα1-ECD produces a powerful long-lasting effect in a dose and time-dependent manner, following a short two-week once-daily intravenous dosing regimen. It effectively treated early and late-stage disease, using higher doses for a curative effect in later stages of disease, possibly necessitated by accumulated extensive damage at the NMJ and presence of memory cells. The potential of this antigen-specific tolerance therapy was highlighted by the fact that it greatly surpassed the therapeutic effect of two routinely prescribed treatments for MG. Therefore, it could provide an innovative and alternative route for clinical application with minimal side-effects.

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Conflicts of interest

KL has received research support from Toleranzia AB. 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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Targeting the safety factor for neuromuscular transmission to treat myasthenia gravis

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ABSTRACT

In myasthenia gravis autoantibodies attack the postsynaptic membrane of the neuromuscular junction and cause fatiguing weakness that can wax and wane. Weakness occurs when the safety factor for neuromuscular transmission becomes marginal, meaning that the (postsynaptic) endplate potential is no longer sufficient to reliably trigger action potentials in the muscle fiber. Cholinesterase inhibitor drugs provide temporary relief by increasing the endplate potential amplitude, but additional symptomatic treatment options are needed. Here we review our recent experience in early preclinical testing of candidate compounds. Using an *ex vivo* mouse nerve-muscle contraction assay, followed by endplate potential recordings, we examined the effects of cannabinoids. Our findings highlighted the potentially confounding effects of dimethylsulfoxide (DMSO) when used as a solubilizing agent. They also demonstrate the need to take synaptic homeostasis into account, which can otherwise distort or mask the effects of bioactive agents upon neurotransmission. In all, our studies taught us some hard lessons: pitfalls for the basic scientist seeking to develop a candidate drug.

Keywords: neuromuscular junction disease; myasthenia gravis; experimental myasthenia, synaptic homeostasis

Introduction

In myasthenia gravis (MG) autoantibodies target proteins in the postsynaptic membrane of the neuromuscular junction (NMJ). They reduce the efficacy of neuromuscular transmission by several different pathophysiological mechanisms (reviewed by Huijbers et al. 2022). To understand how impaired neuromuscular transmission leads to weakness it is useful to first briefly review the structure and function of the healthy NMJ.

NMJ structure and function

Under the microscope the human NMJ looks a bit like a bunch of grapes. The motor axon branches to form several terminal swellings, called boutons (Fig 1A, boutons in red). The presynaptic membrane of each bouton is aligned above a portion of the postsynaptic (muscle) membrane that is rich in acetylcholine receptors (AChRs, green labelling). Enlarged under the electron microscope, the postsynaptic membrane is seen to have many deep infoldings. Each infolding marks out a potential site of neurotransmission (Fig 1B). The minimal synaptic unit consists of synaptic vesicles docked on the presynaptic membrane and primed to release their cargo of acetylcholine (Fig 1C). Release of a single such quantum of acetylcholine produces a membrane depolarization known as a miniature end plate potential (MEPP, amplitude ~1mV). MEPPs are thought to occur due to spontaneous release of primed synaptic vesicles and are used as a measure of quantal amplitude.

The endplate potential (EPP) is caused by synchronized release of many such quanta. Every action potential in the motor axon triggers the opening of a small number of voltage-gated calcium channels that are tethered to each primed vesicle. Calcium ions diffuse in through these open channels to produce a brief, local plume of ionic calcium that binds to sensor proteins on the vesicle, triggering exocytosis of acetylcholine. The estimated number of vesicles released to produce the EPP is referred to as 'quantal content'. The EPP activates voltage-gated sodium channels that are concentrated at the base of the postjunctional folds (Fig 1C). The amplitude of the EPP is normally more than sufficient to initiate a muscle action potential, but not so in MG (Fig 1D). Acetylcholinesterase within the synaptic cleft (Fig 1C) rapidly terminates the EPP by breaking down the acetylcholine (MacIntosh et al. 2006; Plomp et al. 2015).

The safety factor and its limitations

The *safety factor for neuromuscular transmission* is typically about two-fold, meaning that synaptic signalling is twice as strong as is needed to trigger an action potential in the muscle fiber (Wood and Slater 2001). In myasthenia gravis, postsynaptic sensitivity to acetylcholine is impaired, causing a reduction in the amplitude of the EPP, and consequently the safety factor. There is some natural (impulse to impulse) variability in the amplitude of the EPP, so when MG reduces the safety factor to unity (approximately 1.0), many nerve impulses will fail to trigger a postsynaptic action potential (Fig 1D; Elmqvist et al. 1964).

Determinants of the safety factor

The safety factor depends upon multiple features of the healthy NMJ. On the presynaptic side the high quantal content at rest is thought to depend upon hundreds of synaptic vesicles that are primed and ready to release

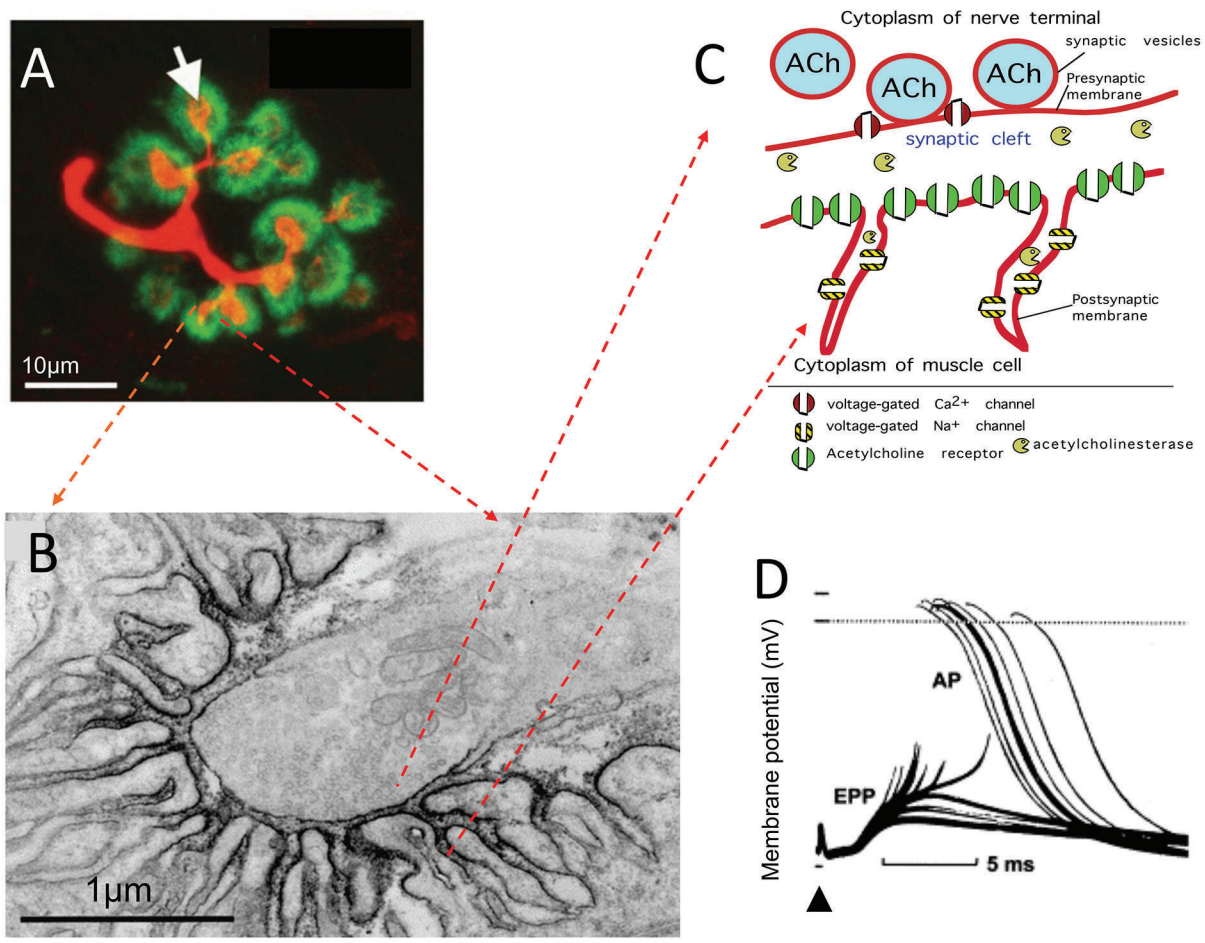

Fig 1. Structure and (dys-)function of the human NMJ. (A) Immunofluorescent image of a human NMJ. Each presynaptic terminal bouton (red, arrow points to one bouton) is aligned above AChR-rich postsynaptic membrane (green; modified from Ding et al 2022; Creative Commons Attribution License, CC-BY 4.0). **(B)** Each bouton forms the core of a 3D calyx-like structure as can be seen from this transverse electron microscope image. The AChRs are concentrated near the tips of the postjunctional folds (dark staining) separated from the overlying presynaptic membrane by the narrow synaptic cleft (modified from Ohno et al. 2002; RightsLink licence # 5493930960934). **(C)** Cartoon representation of a single presynaptic acetylcholine release site aligned above a postsynaptic membrane infolding. **(D)** Multiple superimposed recordings of EPPs and action potentials recorded from a muscle biopsy of an MG patient. Every nerve stimulus should trigger the all-or-none action potential (AP) but in this myasthenic muscle the reduced amplitude of the EPPs often fails to reach the required threshold (figure modified from Elmqvist et al 1964; RightsLink license # 5493921030479).

their contents in response to a nerve impulse. On the postsynaptic side, the normal high quantal amplitude (approximately 1mV) depends upon the dense packing of AChRs at the tips of the postjunctional membrane folds. The deep membrane infoldings of the human NMJ (Fig 1B) funnel synaptic currents from the AChRs to the voltage-gated sodium channels at the base of these folds (Fig 1C). In MG, antibody-mediated loss of AChRs and widening of the synaptic cleft reduce the amplitudes of both the MEPP and the EPP. Complement-mediated damage to the postjunctional folds can also raise the threshold for an action potential (Ruff and Lennon 2008; for a recent review see Huijbers et al. 2022). Both of these changes reduce the safety factor.

Neuromuscular transmission decay

Neuromuscular transmission is vulnerable to fatigue. Muscle contraction force is controlled, in large part, by the frequency of nerve impulses relayed from nerve to muscle through the NMJ (MacIntosh et al. 2006). This can become a problem because during every train of nerve impulses the EPP amplitude declines due to a decline in quantal content ('synaptic depression'; Kamenskaya et al. 1975). During sustained, high-frequency neuromuscular transmission

the decay in quantal content is explained by progressive depletion of the pool of primed synaptic vesicles on the presynaptic membrane (Wang et al. 2016). The rate of vesicle depletion can also be influenced by cholinergic and purinergic autoreceptors on the nerve terminal (Santafe et al. 2015; Sanabria et al. 2022). In healthy muscle, a large safety factor (at rest) ensures that the EPP continues to trigger postsynaptic muscle action potentials despite the natural decay in quantal content during each impulse train. It remains uncertain whether the healthy NMJ ever fails in living, behaving animals. In conditions such as MG, where the safety factor becomes marginal, the intrinsic property of synaptic depression is expressed as fatiguing failure of the muscle action potential (in one muscle fiber after another).

A few difficult lessons about translation

Drugs such as pyridostigmine enhance EPP amplitude by slowing the breakdown of acetylcholine in the synaptic cleft. As first line treatment for MG they provide immediate relief. They can also help minimize corticosteroid dosage when treating chronic MG. However, a substantial subset of patients report loss of efficacy with pyridostigmine, and adverse side effects are common (Remijn-Nelissen et al. 2022). It might be possible to overcome these limitations if we could find a novel drug that would improve the safety factor by targeting a different component of the NMJ. Being new to preclinical translation work we thought we were onto something when a pilot study in our lab suggested that cannabinoids might have the potential to restore EPP amplitude in a mouse model of MG (Morsch et al. 2018). With funding from the Lambert Initiative for Cannabinoid Research we undertook a follow-up study to clarify the pharmacology and mechanism of cannabinoid action at the mouse NMJ. We wanted to see if a cannabinoid therapeutic could be developed.

From mechanism to preclinical translation

The first thing we learned to appreciate was the need for a bioassay to quickly assess the effect of various compounds on the safety factor. Animal models of MG previously used EPP recordings (Morsch et al. 2018). They provide detailed mechanistic information about quantal synaptic transmission, but they are very time consuming and require fairly extensive replication (n=8 preparations). This makes them impractical for screening multiple compounds. Instead, we employed an *ex vivo* mouse phrenic nerve-hemidiaphragm muscle contraction preparation. A train

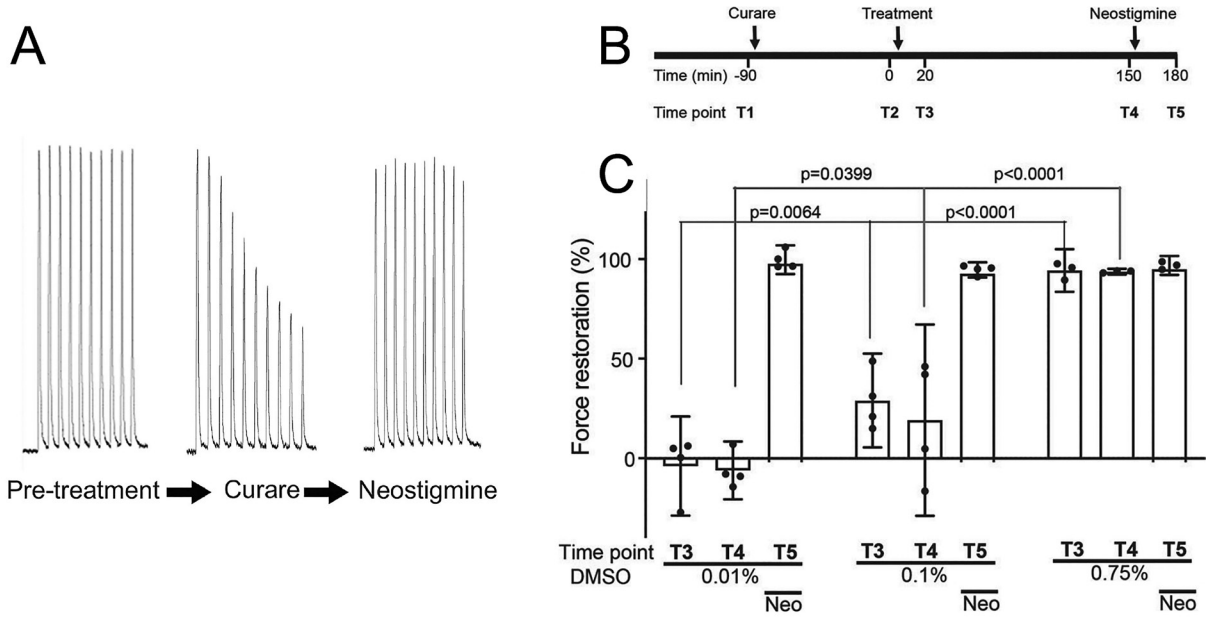

Fig 2. A muscle contraction assay to assess the effect of compounds on the safety factor. **(A)** Contraction force recordings from an isolated section of mouse diaphragm muscle. A train of ten nerve stimuli (3/second) yielded ten twitch contractions of equal force (Pre-treatment). After adding 700 nM tubocurarine (Curare), a decrement in the train reflected progressive failure of neuromuscular transmission. Further addition of the acetylcholinesterase inhibitor drug, neostigmine (Neo), restored consistent twitch force. **(B)** Timeline for a typical assay run. **(C)** Quantitation of force restoration. We measured the degree to which DMSO reversed the curare-induced decrement at the indicated timepoints after adding the test compound (T3=20 min, T4=150 min). Symbols show results from replicate preparations. Bars show means and 95% confidence intervals (P values produced from two-way ANOVA with Tukey multiple comparisons post-test; figure modified from Odierna and Phillips 2021; © 2021 – IOS Press).

of ten stimuli to the nerve (3/sec) normally produces ten brief twitch contractions, all the same amplitude. To mimic myasthenic conditions we used tubocurarine to block the majority of the postsynaptic AChRs. The resulting drop in safety factor became evident as a progressive decrement in twitch force during each train of ten stimuli: analogous to the decrement in the compound muscle action potential in myasthenic muscles (Fig 2A; Plomp et al. 2015). We then measured the percentage decrement in the force from the first (unaffected) twitch to the last twitch in the train to assess the potential of various compounds to restore the safety factor.

The difficult problem presented by bioactive solvents

The second thing that became clear to us was that cannabinoids are very hydrophobic. A solubilizing agent such as dimethylsulfoxide (DMSO) or ethanol is needed to prepare a stock solution from the powdered compound. We found that quite a high molar ratio of DMSO to cannabinoid (a few hundred to one) was needed to prevent the drug from precipitating when the stock solution was subsequently diluted into physiological saline. In practice, a final concentration of 10 μM cannabinoid could only be achieved by including a final concentration of 0.1% DMSO (v/v). Presumably, DMSO forms amphipathic shells around the (hydrophobic) cannabinoid molecules. We are uncertain how the interaction with DMSO might affect the biochemical actions of cannabinoids.

The third thing we discovered was that the real active ingredient for restoring safety factor in our bioassay was the DMSO, not cannabinoids. At a concentration of 0.01% DMSO had no detectable effect on the contraction force decrement, but at concentrations of 0.1% and 0.75% DMSO produced a dose-dependent restoration of force (Fig 2C). We tested two different dual CB1/ CB2 cannabinoid receptor agonists (CP 55,940 and WIN 55,212-2) using the minimum necessary concentration of DMSO to keep them in solution. For each set of experiments, the DMSO component of the treatment was sufficient to explain the observed force restoration. On the contrary, we found that cannabinoids had a negative effect on the safety factor. Follow up contraction experiments using selective agonists suggested that the delayed negative effect of the cannabinoids was mediated by the CB1 receptor, but not the CB2 receptor. Our findings eliminated cannabinoids as potential therapeutic agents to treat MG. Instead, we learned about how organic solvents and cannabinoids affect nerve-muscle function and some challenges facing early preclinical drug development.

Synaptic homeostasis: adapt the assay to the disease context

The NMJ doesn't give up easily. When myasthenic autoantibodies cause a reduction in quantal amplitude, the nerve terminal tries to compensate by increasing quantal content (Plomp et al. 1992, 1995). Acute partial blockade of postsynaptic AChRs can trigger a rapid compensatory increase in the pool of readily-releasable (primed) synaptic vesicles in the nerve terminal (Wang et al. 2016). Our combined electrophysiology results certainly demonstrated this response. From a total of 24 muscle preparations, the average MEPP amplitude was 1.09mV, the mean EPP was 17.7mV, and the mean quantal content was 17.9. When muscles were bathed in 500 nM tubocurarine, the MEPP amplitude fell by 82%, but the (evoked) EPP declined less due to a compensatory 43% increase in quantal content (Odierna and Phillips 2021, supplementary). This illustrates the adaptive presynaptic response that might help mitigate neuromuscular transmission failure in some situations where quantal amplitude is reduced. Evidently, in symptomatic MG patients a gross reduction in quantal amplitude overwhelms the capacity of the nerve terminal to compensate effectively.

Homeostatic plasticity at the NMJ is triggered by increased quantal amplitude

While curare reduced the quantal amplitude, DMSO had the opposite effect. The mechanism by which 0.1% DMSO increased the MEPP amplitude is not certain. At very high concentrations (>1%) DMSO can inhibit acetylcholinesterase, but cholinesterase inhibition would prolong the EPP duration whereas 0.75% DMSO did not (Odierna and Phillips 2021). Irrespective of the mechanism of action, the increase in MEPP amplitude after addition of 0.1% DMSO was not accompanied by the expected rise in EPP amplitude (Fig 3A). A compensatory fall in quantal content prevented any increase in EPP (Odierna and Phillips 2021). This suggests that the homeostatic response can also work in the opposite direction: reducing quantal release in response to an acute rise in quantal amplitude. Interestingly, in the presence of tubocurarine (where MEPP amplitude was 20% of its normal value), DMSO did not provoke a compensatory reduction in quantal content. Under such myasthenic-like conditions, addition of 0.1% DMSO elicited increases in the amplitudes of both the MEPP and the EPP (Fig 3B). There was no opposing reduction in quantal content (Fig 3C, compare filled circles to open circles). Together these results suggest that an increase in quantal amplitude only triggers a compensatory reduction in quantal content if the MEPP amplitude exceeds its normal, physiological level. The results are consistent with the idea that the MEPP has a physiological set point value, below or above which the homeostat will be triggered (Ribchester and Slater 2018). This has practical implications for testing of new drugs to restore safety factor in MG. Their effect upon the myasthenic NMJ must be assessed under myasthenic-like conditions, where MEPP amplitude is suppressed, so that homeostatic compensation will not mask potential positive effects.

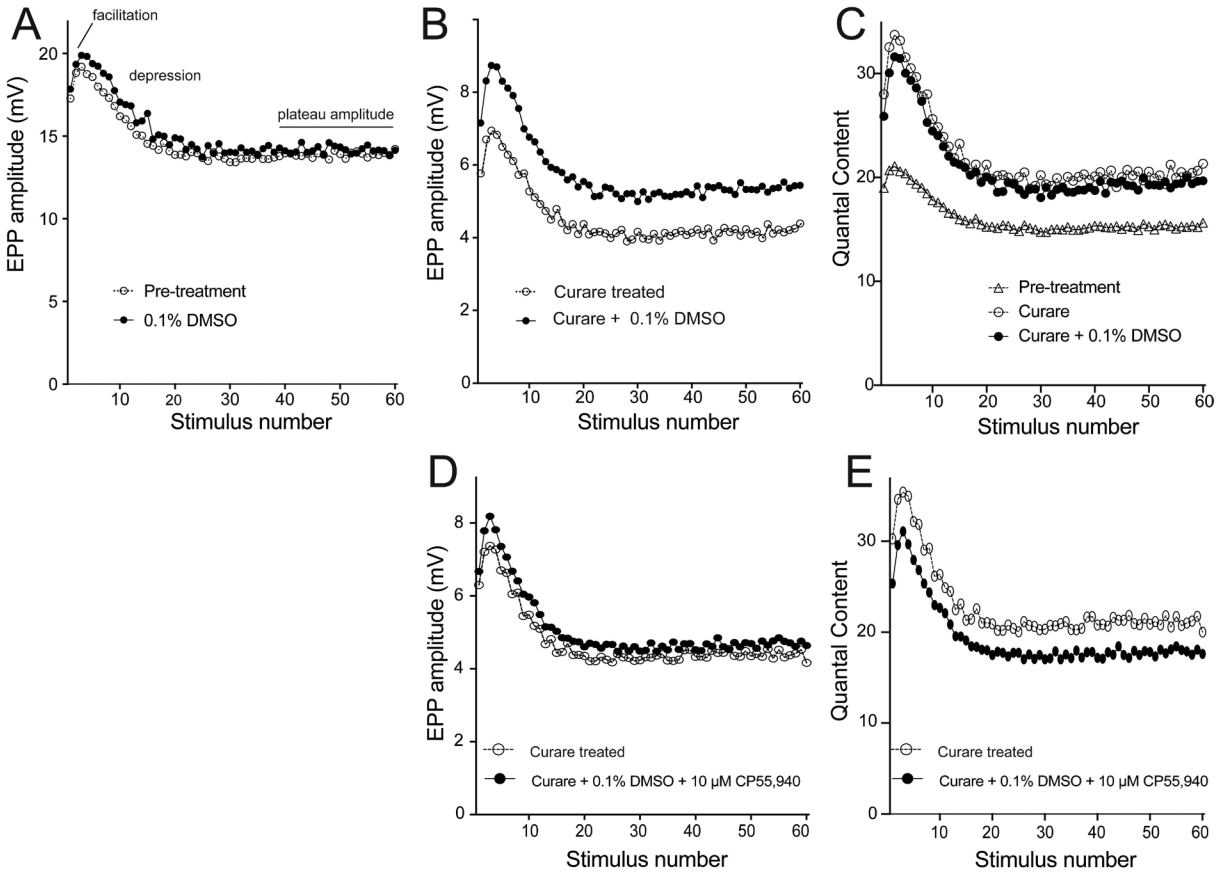

Fig 3. Changes in EPP amplitude and quantal content during trains of 60 nerve stimuli at 40/second. (A) In the absence of curare, EPP amplitude underwent an initial brief facilitation followed by synaptic depression in response to stimulation at 40Hz. Similar results were found with and without 0.1% DMSO. **(B)** Under myasthenia-like conditions (the presence of 500 nM tubocurarine), 0.1% DMSO caused a marked increase in EPP amplitude (note the different amplitude scale compared to panel A). **(C)** Quantal content estimates for the experiments depicted in panels A and B. **(D)** EPP amplitudes in the presence of curare (open circles) are compared to results after treatment with the combination of curare plus 0.1% DMSO and 10 µM CP 55,940 (closed circles). **(E)** Quantal content estimates for the experiments depicted in panel D. Note that our EPPs were not corrected for non-linear summation. In each panel symbols represent the means for n=8 mouse phrenic nerve-hemidiaphragm preparations (modified from Odierna and Phillips 2021; © 2021 – IOS Press).

Effects of cannabinoids on quantal neuromuscular transmission

The homeostatic response seen with DMSO had the potential to mask any beneficial effects of our candidate drugs. To avoid this, we simulated myasthenic conditions in subsequent electrophysiology experiments by including 500 nM tubocurarine in the bath solution. In this way we then tested the effects of a potent dual CB1/CB2 receptor agonist, CP 55,940 (Odierna and Phillips 2021). In the presence of curare, 0.1% DMSO increased both MEPP and EPP amplitudes (Fig 3B). In contrast, the combination of 10 µM CP 55,940 with 0.1% DMSO raised the amplitude of the MEPP by 24% (attributed to the DMSO component) but there was no significant increase in the EPP amplitude (Fig 3D). In these experiments the opposing fall in the quantal content could be attributed to the CP 55,940 component (Fig 3E).

Previous studies have described differing, often contradictory, effects of cannabinoid receptor agonists on MEPP amplitude and quantal content (up, down, or no effect; reviewed in Ge et al. 2020). The seeming inconsistency of the earlier studies might be explained by differences in the specific cannabinoids and concentrations, the solubilizing agents, and muscle preparations. In any event, the large sample sizes we employed (n=8 preparations) give us some confidence that the effects of DMSO and CP 55,940 that we recorded should at least be reproducible. Consistent with our contraction findings, our electrophysiology results under myasthenic conditions suggest that the DMSO-induced increase in quantal amplitude was opposed by the effects of the cannabinoid, which acted to reduce the quantal content. A presynaptic CB1 receptor-mediated

reduction in quantal release at the mammalian NMJ would be consistent with the known actions of cannabinoids upon transmitter release at some synapses in the CNS (Wilson and Nicoll 2001; Kano 2014). These findings show that neurobiological experiments testing the synaptic effects of cannabinoids can be confounded if they include DMSO as a solubilizing agent at concentrations as low as 0.1% (v/v).

Summary

Neuromuscular transmission is generally a safe bet. The safety factor measures the degree to which synaptic signalling exceeds the minimal required to activate the muscle fiber. A healthy safety factor (two or more) ensures the reliability of the NMJ during physiological (tetanic) muscle contractions. In MG the safety factor becomes marginal, and subclinical disease can quickly progress to frank weakness. New drugs to restore a strong safety factor are needed. Development of such drugs will require fast assays that mimic the impaired safety factor at the myasthenic NMJ. Many candidate compounds are hydrophobic, requiring amphipathic solubilizing agents. However, agents such as DMSO have the potential to mask and distort the effects of candidate compounds on synaptic function, in ways that must be taken into account. Finally, a better understanding of the mechanisms behind synaptic homeostasis at the NMJ may also reveal new therapeutic candidates.

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Assay Development and Measurement of Autoantibody-Mediated Complement Activity in Myasthenia Gravis

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ABSTRACT

A major subset of patients with myasthenia gravis (MG) harbor autoantibodies targeting the acetylcholine receptor (AChR) which can directly mediate neuromuscular junction (NMJ) damage through complement activation. Circulating AChR autoantibodies have highly heterogeneous properties that may influence their effector function capacity, including complement activity. In order to measure autoantibody-mediated complement activation in AChR MG patients and determine whether variable efficiency was observed, we developed a live cell-based assay (CBA) that measures AChR autoantibody–mediated complement effector function. The assay involved the expression of AChR on a modified HEK cell line in which the complement regulator genes (CD46, CD55, and CD59) had been knocked out. AChR autoantibody–mediated complement activity was measured using flow cytometry by specifically detecting the membrane attack complex (MAC), the terminal protein assembly in the complement cascade. An association between MAC formation and disease severity as measured by the MGFA classification was found, as well as between autoantibody-mediated complement activity and autoantibody titer. However, outlying samples that included high AChR binders with low complement activity as well as low AChR binders with high complement activity were observed. This mini-review of our previously reported study focuses on complement assay development and the heterogeneity in AChR autoantibody-mediated complement activation.

Introduction

A fundamental pathogenic mechanism of myasthenia gravis (MG) is the activation of complement by acetylcholine receptor (AChR) autoantibodies (1-3). Consequently, this mechanism is a sound target for therapeutic intervention. Indeed, therapeutics that target AChR autoantibody-mediated complement activity limit the capacity of autoantibodies to damage the postsynaptic muscle membrane. Specifically, eculizumab, an anti-C5 monoclonal, and zilucoplan, a peptide, bind to C5 and thereby inhibit C5 cleavage to C5a and C5b and the subsequent generation of the terminal complement complex, C5b-9. Both therapeutics provide benefit to AChR MG patients (4-7). For example, phase III clinical trials of eculizumab have shown efficacy in well over half of treated patients. Unfortunately, 40% of patients did not meet the trial endpoint and some required rescue therapy (5, 6).

The poor responders had measurable circulating AChR autoantibodies, but the titer of the autoantibodies did not associate with response. Given that AChR autoantibodies were present, and a key mechanism of their pathology is complement activation, the trial outcome presents a challenging reconciliation. These results also highlight the limitations of using AChR autoantibody titer as a biomarker. Importantly, it emphasizes the need for further understanding of the variability in AChR autoantibody–mediated pathogenic mechanisms so that the response to treatments can be better anticipated. This mini-review of our work presented at the 14th Myasthenia Gravis Foundation of American (MGFA) International Conference, is focused on describing the development of a novel assay for investigating AChR autoantibody-mediated complement activity, and understanding the observed heterogeneity underlying autoantibody-mediated pathogenic mechanisms in MG.

AChR autoantibody pathogenic mechanisms

AChR autoantibodies elicit tissue damage through three distinct mechanisms (**Figure 1A**) (8-18). The first is receptor internalization (often termed modulation) of AChR, which occurs when an autoantibody divalently binds to two adjacent AChR molecules, causing the cross-linked AChR to be internalized via endocytosis, leading to its degradation. This ultimately reduces the number of AChR molecules present on the cell surface and leads to reduction in neuromuscular transmission. The second mechanism is receptor blocking where autoantibodies prevent acetylcholine (ACh) from binding to AChR by binding close to, or at, the ACh binding site. When ACh is impaired from binding to AChR, the flow of ions across the cell membrane is inhibited (14, 18). It is also reasonable to consider that some blocking antibodies, which do not bind specifically at the ACh binding site, may nonetheless inhibit signaling by altering the conformational state of the AChR such that ACh binding is inefficient. The third mechanism is complement activation, where AChR autoantibodies activate the classical complement pathway (6, 14, 19, 20). The pathway is initiated by the binding of C1q, a component of the complement pathway, to the Fc region of an antibody. This binding promotes subsequent proteolysis of precursor complement proteins that eventually leads to the formation of a membrane attack complex (MAC) or terminal complement complex (TCC). The MAC can cause destruction of the cell membrane, which causes cell death through lysis (21) (**Figure 1B**).

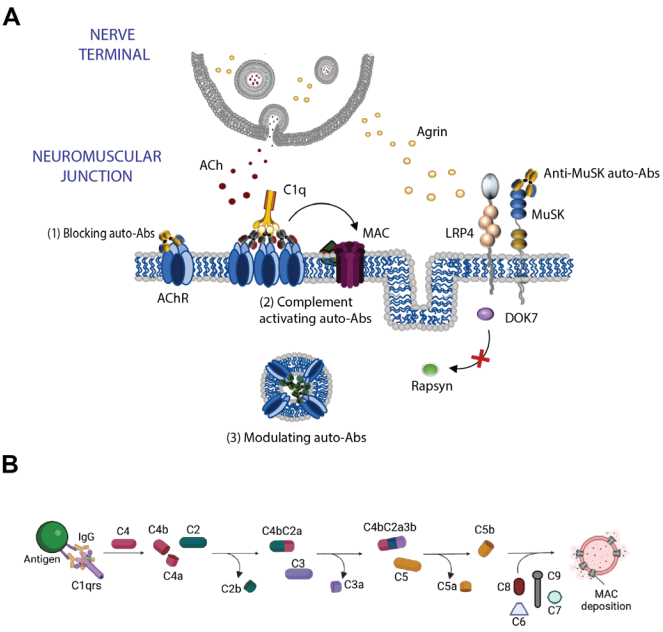

Figure 1. Autoantibody-mediated mechanisms of MG pathology at the neuromuscular junction (NMJ)

A. In a normal NMJ, action potential at the presynaptic nerve terminal releases acetylcholine (ACh) and agrin into the synaptic cleft. ACh binds to acetylcholine receptor (AChR) triggering ion flux and subsequently muscle contraction. Agrin binds to LRP4 leading to MuSK phosphorylation and DOK7 recruitment and rapsyn activation. This leads to AChR clustering and NMJ integrity. In MG, autoantibodies disrupt the NMJ structural integrity and/or neurotransmission. AChR autoantibodies interfere with AChR signaling via (1) blocking ACh, (2) initiating the complement cascade or (3) modulating/internalizing AChR. Anti-MuSK autoantibodies hinder agrin-LRP4-MuSK interaction, thus obstructing AChR clustering, causing reduced clustering and decrease in junctional folds and neuromuscular transmission.
B. The classical complement pathway is activated via C1q binding to an antigen-antibody complex. Following activation, a cascade of protein lysis is initiated that leads to the generation of C3 convertase (C4b2a), which cleaves C3. Following C3 cleavage into C3a and C3b, C3b binds to C4bC2a to generate C5 convertase (C4b2a3b), which initiates the assembly of the membrane attack complex (MAC). The MAC induces cell lysis and death via disruption of the target cell membrane. *NMJ: neuromuscular junction; ACh: acetylcholine; AChR: acetylcholine receptor, MuSK: muscle-specific kinase; LRP4: low-density lipoprotein receptor-related protein 4*

The relative distribution of AChR autoantibodies capable of one (or more than one) of these pathogenic mechanisms in individual patients is not well understood. It is likely to considerably differ between patients and may fluctuate within patients over time and in response to treatment. A means to measure the frequency of unique AChR autoantibody-mediated mechanisms may help in predicting patient response to treatments, especially complement inhibitors. Thus, we sought to examine the relative contribution of the autoantibody-mediated complement mechanism present in serum samples from individual patients. To that end, we adapted the highly sensitive live cell-based assay (CBA) (22), which is usually used to measure AChR autoantibody binding, to quantify autoantibody-mediated complement activation.

Development of an assay to measure AChR autoantibody-mediated complement activation.

Cell-based assays (CBAs) constitute a sensitive method for detecting serum autoantibodies in AChR MG patients. The assay utilizes HEK cells that transiently express the four subunits of the adult AChR receptor, along with rapsyn-green fluorescent protein (GFP) to promote receptor clustering and detection of transfected cells. In the CBA, the native pentameric complex of AChR retains its native structure, whereas other assay formats may disrupt antigen epitopes due to solubilization reagents, purification approaches or antigen immobilization. Furthermore, the CBA allows for the transfection of accessory proteins, which provides a better representation of the *in vivo* NMJ environment. Specifically, the co-expression of the scaffolding protein, rapsyn, with AChR in HEK cells results in the clustering of AChR on the cell surface. This leads to increased assay sensitivity, which was a key development (22) in the detection of AChR autoantibodies in a subset of MG patients thought to be seronegative. These findings has been subsequently confirmed in other independent studies (23, 24).

Accordingly, this platform was leveraged to develop an assay that can measure autoantibody-mediated complement activation (**Figure 2A**). Initial attempts to observe complement assembly on the AChR transfected cells failed. Interestingly, this result stood in stark contrast to what we observed with other autoantigens, including aquaporin-4 (AQP4) and myelin oligodendrocyte glycoprotein (MOG), the targets of autoantibodies found respectively in neuromyelitis optica spectrum disorder (NMOSD) and myelin oligodendrocyte glycoprotein antibody disease (MOGAD). Here, robust complement assembly—mediated by AQP4 and MOG autoantibodies—was readily detectable (25). To address this obstacle, we turned to a previous study that demonstrated increased complement component deposition by disruption of complement regulator/inhibitor expression (26). Accordingly, the genes for the mammalian

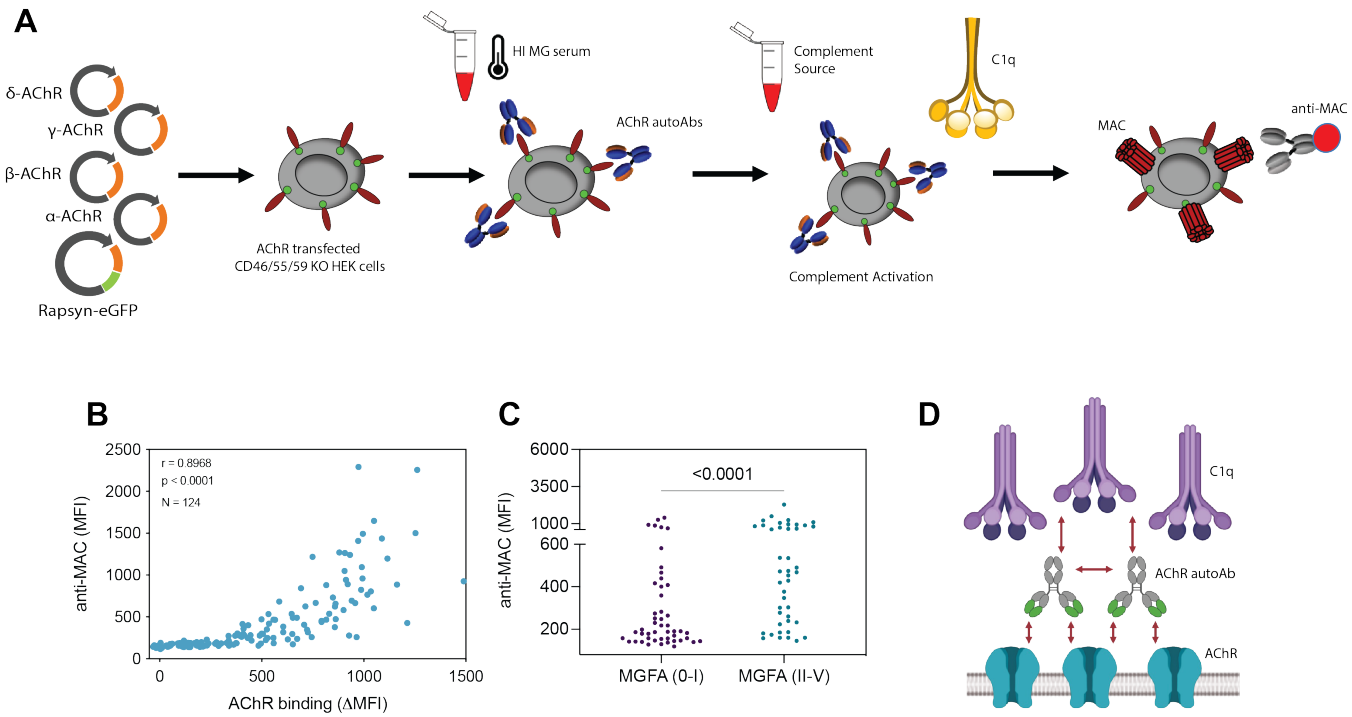

Figure 2. Heterogeneity in Autoantibody-Mediated Complement Activity

A. Schematic of our complement cell-based assay. CD46/55/59 knockout HEK cells are transfected with AChR subunits and rapsyn to express clustered AChR at the cell surface. This is followed by the application of heat-inactivated patient serum and the addition of a consistent complement protein source. MAC formation is measured via staining with anti-MAC antibody and visualization with flow cytometry. **B.** Correlation between autoantibody-mediated MAC formation and AChR binding in AChR MG patients (r=0.8968, p<0.0001). **C.** Differences in MAC formation between samples with low disease severity (MGFA 0/I) and higher disease severity (MGFA II-V). Samples showed a median MAC mean fluorescent intensity (MFI) of 190.3 in samples with MGFA 0-I compared to 468.3 in samples with MGFA II-V (p-value <0.0001). **D.** Schematic of the interactions that support optimum complement activation, which include epitope binding site, spatial arrangement of target antigen, minimum steric interference in the Fc-Fc interactions at the CH3 domain, and C1q-Fc interactions at the CH2 domain. The plots shown in **B** and **C** were constructed from our previously published data (Neurol Neuroimmunol Neuroinflamm. 2022 doi: 10.1212/NXI.0000000000001169. PMID: 35473886)

complement inhibitors CD46, CD55, and CD59 were knocked out in HEK cells using the CRISPR/Cas9 system. Using the modified HEK cells afforded a functional assay to effectively measure AChR autoantibody-dependent complement fixation. Intriguingly, another group also favored the use of a CD46, CD55, and CD59 triple knockout ARPE19 cell line to develop an in vitro assay that allows for testing autoantibody complement activation. They also transfected cells with plasmids encoding AChR subunits and rapsyn, and utilized pooled human serum as a source of complement (27).

It is unclear why measurement of AChR autoantibody-dependent complement fixation required the absence of the CD46, CD55, and CD59 complement regulators, unlike fixation mediated by MOG and AQP4 autoantibodies. However, these regulators have previously been observed to influence MG immunopathology. For example, CD55 knockout mice were shown to be more susceptible to the effects of pathogenic MG autoantibodies (19, 28, 29). In other MG experimental models (mice and rats), complement inhibition has shown efficacy in reducing the effects of the autoantibody response generated by the injection of AChR or peptide fragments of AChR (30, 31). Finally, the extraocular muscle subgroups are highly associated with MG. Interestingly, they express reduced levels of CD55 and CD59, suggesting that diminished complement regulatory activity may contribute to the susceptibility of these muscle groups in MG (19).

During the development of the assay, we also considered how MAC-dependent cell death might influence sensitivity while the assay is being performed, given that the cells must be intact and alive to be measured accurately by FACS. To improve sensitivity, we considered Cr^{52} release to measure cumulative cell death, but we were reluctant to introduce radioactivity into the assay. Instead, we tested an alternative approach with our MOG autoantibody assay to address this concern (25): autoantibody-dependent complement is activated but arrested prior to MAC formation, thus

avoiding cell death. Specifically, a human complement source depleted of C8—a requirement for MAC formation—was used. Complement activity was measured using an antibody specific for C3d (32) which covalently attaches to target cells upon complement initiation. While C3d deposition was detected, no conspicuous increase in sensitivity was observed.

An alternative approach to measuring AChR autoantibody-mediated complement fixation was recently developed which may address some limitations of the CBA approach. This bioassay leveraged intact innervated muscle tissue (33). Here, the authors developed a sophisticated assay that facilitates the visualization of the NMJ using mouse diaphragm-phrenic nerve preparations with physiologically normal characteristics. This methodology eliminates the issues associated with the removal of the complement inhibitory proteins and more accurately reflects the NMJ as it ensures proper density and clustering of AChR. Nevertheless, this approach requires time and resources that does not—at this early stage of its development—allow for the high throughput evaluation of large patient cohorts.

Measuring AChR autoantibody-mediated complement activation in patient serum.

We next used our assay to analyze serum samples from a cohort of MG patients. The assay showed that autoantibody binding was highly correlated with MAC formation (**Figure 2B**). However, heterogeneity was found in the patient cohort, where some cross sectional and longitudinal patients had high AChR autoantibody titers but low complement activity, while others had low titer but high complement activity. These findings suggest that while the majority of AChR autoantibodies can cause tissue damage through complement activation, binding alone does not dictate MAC formation. This was further highlighted when the association between complement deposition and disease severity was examined, and a modest correlation between MAC formation and MGFA classification was found (**Figure 2C**). However, heterogeneity was also observed where there were patients that had high disease severity but low MAC formation while others had relatively elevated MAC formation, but low disease severity scores.

The differences in MAC formation in two subsets of MG, namely early-onset MG (EOMG) and late-onset MG (LOMG), were investigated. No significant differences were observed, which may suggest that there are no major variations in the complement associated properties of the AChR autoantibodies found in the two MG subtypes. Furthermore, there were no differences in MAC formation in patients who had immune modulatory therapy or thymectomy. Given that AChR autoantibodies persist after these treatments (34, 35), it is plausible to conclude that these treatments have minimal effect on the ability of existing autoantibodies to mediate complement activity.

Understanding the heterogeneity of AChR autoantibody-mediated complement activity.

The heterogeneity that we observed in the efficiency of AChR autoantibody-mediated complement activation point to the complexity of the autoantibody repertoire in AChR MG. Patients may harbor AChR autoantibodies; however, whether they mediate MAC formation that contribute to disease severity is subject to multiple factors. These factors may include whether they are tissue resident or in circulation, patient genetics, and the expression levels of complement inhibitors on the muscle tissue. Furthermore, AChR autoantibodies may elicit pathogenicity through other mechanisms, such as blocking of ACh or modulation/internalization of AChR, which results in reduction in neuromuscular transmission. It is also possible that patients with high binding, but low disease severity may have autoantibodies that bind to AChR without effectively causing any tissue damage. The presence of such putative 'binding only' autoantibodies have been reported in autoimmune disorders such as pemphigus (36) and NMO (37).

The disassociation between AChR autoantibody titer and disease severity highlights the complexity of their pathogenic properties. While the detection of circulating AChR autoantibodies can confirm MG diagnosis, the titers can vary widely among individuals and during disease progression. Some patients with a mild phenotype can have very high AChR autoantibody titers, while others with severe disease during a relapse can have very low titers (38-42). Though changes of titer within an individual can be associated with disease severity, it is often observed that AChR autoantibody titer measured at a single point does not correlate well with disease severity or activity and makes it difficult to use titer as a reliable biomarker. The disparity between disease severity and titer may be explained—in part—by the inability of clinical assays to distinguish between AChR autoantibody titer and pathogenic mechanisms.

In addition to variable titers, circulating AChR autoantibodies have highly heterogeneous binding properties that may influence their effector functions. Adult AChR is a pentameric structure consisting of 2α: β: ε: δ subunits while fetal AChR has a similar structure where there is a gamma in lieu of an epsilon subunit (2α: β: γ: δ) (43). AChR autoantibodies are polyclonal in nature; they can bind any of the AChR subunits and various epitopes present on each subunit. The majority of serum AChR autoantibodies bind to the main immunogenic region (MIR) that resides primarily, *but not exclusively*, on the alpha subunit (44, 45); however robust binding to other subunits has also been observed (46).

It is likely that AChR autoantibodies with different subunit and/or epitope targets vary in their efficiency at activating complement. The relationship between epitope binding specificity and complement activation has been

elegantly demonstrated for AQP4-binding autoantibodies. Specifically, AQP4 autoantibody binding alone is not sufficient to induce complement-mediated cell death (47). Instead specific epitope binding and the assembly of multimeric platforms are necessary for optimum complement-mediated cell death (**Figure 2D**)(47). AQP4 autoantibodies that bind epitopes on the extracellular loop C display significantly higher complement activity compared to autoantibodies that target other epitopes. Moreover, AQP4 forms supramolecular orthogonal arrays that organize these epitopes in a manner that enhances the formation of autoantibody multimeric complexes through Fc-Fc interactions and efficient C1q binding, resulting in optimized complement activation (47). In the context of MG, it has been proposed that combinations of recombinant monoclonal antibodies that target specific subunits of AChR increased complement activation in vitro and in a passive transfer-based MG animal model (48). Here, it was hypothesized that the formation of larger AChR clusters and enhanced Fc-Fc interactions increased the magnitude of the autoantibody-mediated complement activation (48). Continued studies of human derived, monoclonal AChR autoantibodies (48, 49) to further understand the relationship between autoantibody binding properties and their effector functions will be necessary to understand these relationships with more granularity.

In addition to binding properties mediated by the variable region of antibodies, the constant region, namely the Fc, can influence effector functions including complement (50, 51). Differences in Fc regions are observed due to IgG subclass usage, constant region polymorphisms, varying glycosylation patterns and post-translational modifications (52, 53). Complement activation is influenced by IgG subclass where IgG3/ IgG1 demonstrates the greatest activation while IgG4 demonstrates negligible activity (54). Furthermore, post-translation modification (PTM) can alter the structure and stability of an antibody as well as its capability to activate complement (50). The IgG Fc domain includes a highly conserved glycosylation site in the constant heavy chain 2 (CH2) domain. Carbohydrate moieties attached to this site can influence the interactions between an antibody and complement proteins. This has been observed in MOGAD, where higher inflammatory profiles were associated with an increase in agalactosylated and asialylated glycovariants on IgGs (55). Furthermore, sialyation of the site can also decrease inflammatory responses by interfering with complement-mediated cytotoxicity (51). The interplay between all these variables can have a major effect on how these autoantibodies elicit tissue damage and understanding this complexity in AChR MG may help develop precisely targeted and personalized therapies.

Conclusions

To understand MG disease course heterogeneity more deeply, future efforts should include the development and application of assays that can accurately measure the composition of the AChR autoantibody repertoire and the varying pathogenic mechanisms they can mediate. These assays should ideally include measures of binding-only, classical pathway complement activation, as well as modulating and blocking functions. Collectively, these measurements may provide valuable insights into disease progression and serve as an improved biomarker for MG compared to autoantibody binding alone. By targeting unique autoantibody-mediated pathogenic pathways, clinicians may be able to develop more individualized and effective treatment plans for their patients.

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MuSK-CAART: A novel precision cellular therapy for muscle-specific tyrosine kinase myasthenia gravis

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ABSTRACT

Chimeric autoantibody receptor (CAAR) T cells are a novel genetically-engineered T cell immunotherapy that aims to durably eliminate antigen-specific B cells while sparing healthy B cells, ideally leading to safe and lasting remission of B cell-mediated autoimmune diseases with a one-time infusion. We describe the preclinical development of muscle-specific tyrosine kinase CAAR T cells (MuSK-CAART) for the treatment of MuSK myasthenia gravis, a debilitating autoantibody-mediated disease that causes potentially life-threatening muscle weakness.

Key words: autoimmunity, neurology, immunotherapy, CAR T cells, neuromuscular junction, MuSK, myasthenia gravis, muscle-specific tyrosine kinase.

Chimeric antigen receptor (CAR) T cells are genetically-engineered cellular immunotherapies that have led to durable remissions of otherwise refractory B cell malignancies. Four CD19-targeted CAR T cell products are clinically approved for the treatment of B cell leukemias and lymphomas, based on pivotal trials in which 53-81% of clinical trial participants achieved complete remission and 40-57% demonstrated long-term remission (1-4), including several that are thought to have achieved cancer cure. However, CAR T cell therapy can cause serious and potentially fatal side effects, including cytokine release syndrome, resulting from the rapid immune activation and tumor cell death that occurs after infusion, immune effector cell-associated neurotoxicity syndrome, and infections from B cell depletion.

Nevertheless, the remarkable success of CAR T cell therapy in B cell-mediated cancers inspired us to consider other B cell-mediated diseases that could be treated with a similar therapeutic approach. Muscle-specific tyrosine kinase (MuSK) myasthenia gravis is a B cell-mediated autoimmune disease in which autoantibodies against the postsynaptic transmembrane protein MuSK interfere with neuromuscular junction signaling, resulting in muscle weakness. Patients with MuSK myasthenia gravis can have difficulty swallowing, speaking, moving, or breathing, which can advance to life-threatening respiratory crisis. Currently, there are no FDA-approved treatments specific for MuSK myasthenia gravis, although corticosteroids and rituximab are considered front-line therapies (5). Anti-MuSK antibody titers drop after rituximab therapy (6), indicating that short-lived plasma cells produce anti-MuSK antibodies (7, 8) and that strategies to deplete anti-MuSK memory B cell precursors should prevent anti-MuSK antibody production.

We therefore re-engineered CAR T cells for antigen-specific B cell depletion in MuSK myasthenia gravis. Anti-CD19 CAR T cells incorporate an anti-CD19 antibody as the extracellular domain of the CAR, linked to cytoplasmic co-stimulatory and activation domains. This approach targets CD19-expressing B cells, both healthy and leukemic, and can lead to B cell cancer remission and potentially lifelong B cell depletion due to the induction of memory CAR T cells (**Figure 1A**). To target only the anti-MuSK B cells in MuSK myasthenia gravis, we expressed the MuSK autoantigen ectodomain on the surface of T cells, linked to CD137 costimulatory and CD3ζ activation domains (**Figure 1B**). This chimeric autoantibody receptor (CAAR) is designed to target the anti-MuSK B cell receptor, which is identical in specificity to the autoantibody the B cell will produce once activated to mature into an antibody-secreting cell. Ideally, MuSK CAAR T cells (MuSK-CAART) will kill all anti-MuSK B cells to achieve complete remission of MuSK myasthenia gravis and also produce memory CAAR T cells to provide potentially lifelong protection against autoimmune disease recurrence.

The MuSK extracellular domain is comprised of three immunoglobulin (Ig)-like and one frizzled (Fz)-like domain, the entirety of which was incorporated into the MuSK CAAR ectodomain. Using in vitro killing assays against B cells engineered to express anti-MuSK B cell receptors targeting all 3 Ig-like and Fz-like MuSK domains, we demonstrated that MuSK-CAART specifically lyses anti-MuSK B cells. We evaluated MuSK-CAART in vivo efficacy in a syngeneic experimental autoimmune myasthenia gravis model induced by immunization of C57BL/6J mice with the human MuSK ectodomain, followed 5 weeks later with non-transduced T cells, anti-CD19 CART, or MuSK-CAART treatment (**Figure 2A**). Anti-CD19 CART treatment fully depleted splenic B cells, whereas MuSK-CAART did not affect splenic B cells relative to non-transduced T cell treatment (**Figure 2B**) since anti-MuSK B cells are rare in these immunized mice (less than 2% of total splenic B cells). Accordingly, anti-CD19 CART reduced both total serum IgG and anti-MuSK IgG, whereas MuSK-CAART reduced anti-MuSK IgG without effect on total serum IgG levels

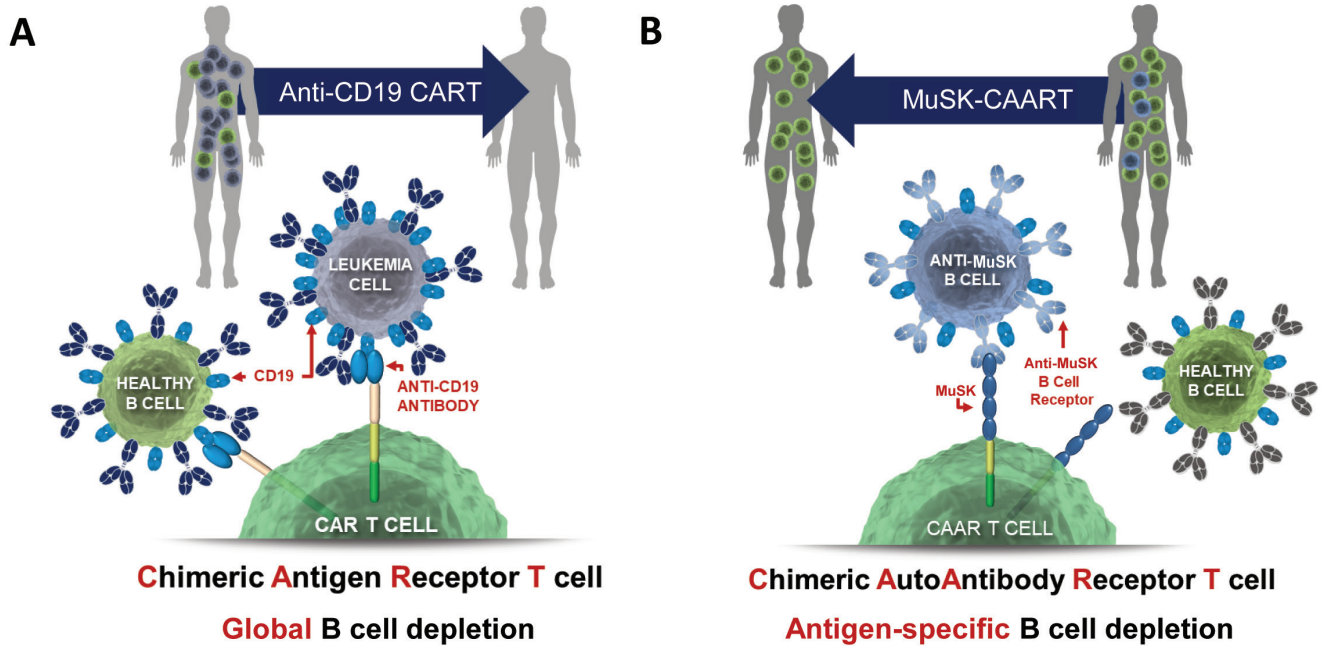

Figure 1. Schematic of chimeric antigen receptor (CAR) T cells versus chimeric autoantibody receptor (CAAR) T cells. A) CAR T cells clinically approved to treat B cell cancers incorporate an anti-CD19 antibody as the extracellular domain of a chimeric immunoreceptor, linked to cytoplasmic co-stimulatory and activation domains. Anti-CD19 CAR T cells kill both healthy and leukemic CD19-expressing B cells, leading to complete and durable cancer remission through global B cell depletion. B) Muscle-specific tyrosine kinase (MuSK) chimeric autoantibody receptor (CAAR) T cells incorporate the MuSK autoantigen targeted in MuSK myasthenia gravis, tethered to cytoplasmic co-stimulatory and activation domains. MuSK CAAR T cells are designed to specifically deplete anti-MuSK B cells that express an anti-MuSK B cell receptor, while sparing healthy B cells, which ideally will lead to durable remission of MuSK myasthenia gravis without global immune suppression. Image credit: Adapted with permission from Cabaletta Bio.

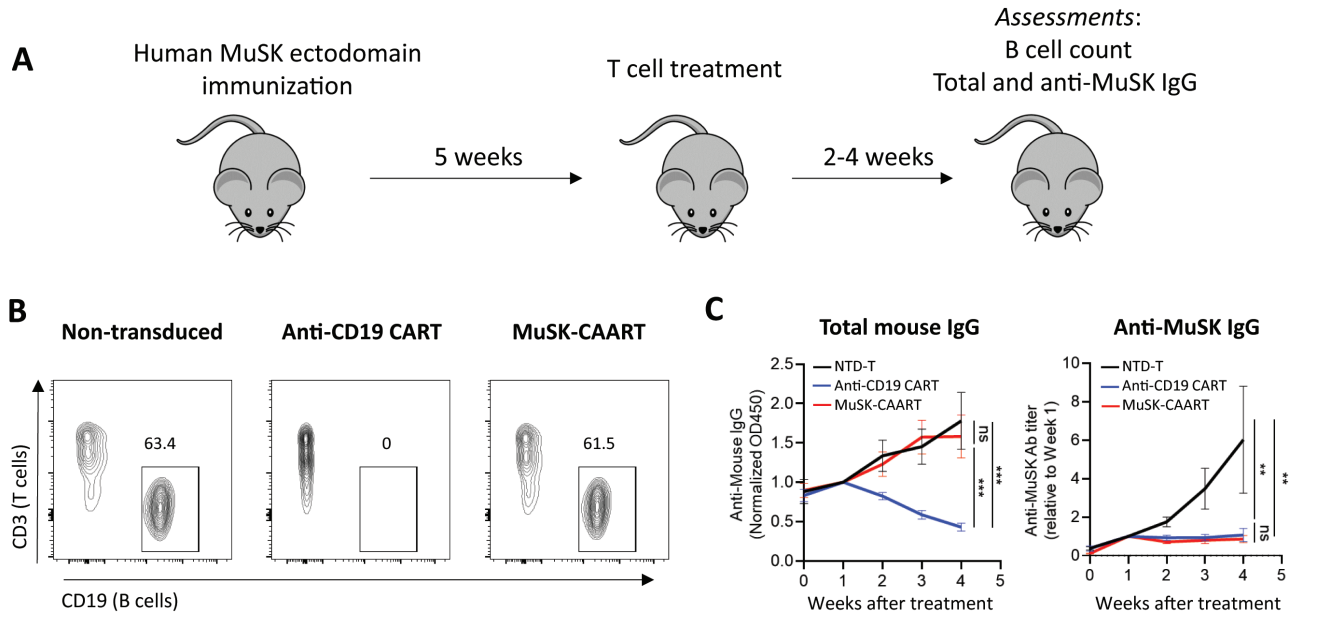

Figure 2. MuSK CAAR T cell therapy of experimental MuSK myasthenia gravis leads to antigen-specific B cell depletion. A) Experimental design: Mice are immunized with human MuSK ectodomain to induce anti-MuSK IgG, followed 5 weeks later by MuSK-CAART treatment. Mice were analyzed 2 weeks later by flow cytometry of spleen and 4 weeks later for total serum IgG or anti-MuSK IgG. B) CD19-expressing B cells are depleted by anti-CD19 CAR T cell treatment (middle panel) but not MuSK CAAR T cell (right panel) or non-transduced T cell treatment (left panel). C) Anti-CD19 CAR T cells (shown in blue) deplete total and MuSK-specific IgG, whereas MuSK CAAR T cells deplete only anti-MuSK IgG (shown in red), while preserving total IgG levels. Non-transduced T cells (NTD-T shown in black) do not deplete total or MuSK-specific IgG.

(**Figure 2C**), indicating antigen-specific B cell depletion.

We examined the potential for unwanted off-target effects of MuSK-CAART through several approaches, including comprehensive organ histology and serum chemistry in MuSK-CAART treated mice (an approach expected to yield information on potential off-target interactions mediated by the MuSK ectodomain given the high homology between mouse and human MuSK), high-throughput screening of membrane proteome arrays expressing greater than 5,300 human membrane proteins, and screening of human primary cell cultures and primary human myotubes for evidence of MuSK-CAART activation after co-incubation. Specific off-target cytotoxic interactions of MuSK-CAART were not identified in these assays.

The complete description of the MuSK-CAART design, as well as evaluations of its efficacy and safety were recently published (9). Collectively, these studies contributed to an Investigational New Drug application for MuSK-CAART and have led to an open label phase 1 study to evaluate the safety and preliminary efficacy of various dosing regimens of MuSK-CAART for MuSK myasthenia gravis (NCT05451212), which is currently recruiting. Participants must be age 18 or older, have active disease (class I-IVa as assessed by the MGFA (Myasthenia Gravis Foundation of America) Clinical Classification), and have a positive anti-MuSK antibody titer. Participants must not have received rituximab in the past 12 months, be on a prednisone dose greater than 0.25 mg/kg/day, or have another disease requiring immunosuppressive therapy. Immunosuppressives used for MuSK myasthenia gravis will be stopped or tapered prior to MuSK-CAART infusion. The primary endpoint of the study will be related adverse events, including dose-limiting toxicities, up to 3 months after MuSK-CAART infusion. Secondary outcomes include MuSK-CAART persistence and change in MuSK autoantibody titer compared to pre-infusion. Exploratory outcomes include frequency and dose of concomitant therapies, clinical disease activity and quality of life measurements.

In summary, MuSK-CAART represents a novel precision cellular immunotherapy for MuSK myasthenia gravis. Ongoing clinical studies will evaluate its potential for safe and durable autoimmune disease remission.

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Conflict of Interest

SO: Cabaletta Bio: Patent licensing

ASP: Cabaletta Bio: equity, payments, research support, patent licensing; Janssen: consultant

Circulating microRNAs in myasthenia gravis (MG)

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ABSTRACT

One of the main difficulties in predicting the clinical course of myasthenia gravis (MG) is the heterogeneity of the disease, where disease progression differs greatly depending on the patient's subgroup. MG subgroups are classified according to the age of onset [early onset MG (EOMG; onset ≤ 50 years) versus late-onset MG (LOMG; onset >50 years]; the presence of a thymoma (thymoma associated MG); antibody subtype [acetylcholine receptor antibody seropositive (AChR+), muscle-specific tyrosine kinase antibody seropositive (MuSK+)]; or presence of autoantibodies against low-density lipoprotein receptor-related protein 4 (Lrp4) or agrin as well as clinical subtypes (ocular versus generalized MG). The diagnostic tests for MG, such as autoantibody titers, neurophysiological tests, and objective clinical fatigue scores, do not necessarily reflect disease progression. Hence, there is a great need for reliable, objective biomarkers in MG to follow the disease course and the individualized response to therapy toward personalized medicine. In this regard, circulating microRNAs (miRNAs) have emerged as promising potential biomarkers due to their accessibility in body fluids and unique profiles in different diseases, including autoimmune disorders. Several studies on circulating miRNAs in MG subtypes have revealed specific miRNA profiles in patient sera. In generalized AChR+ EOMG, miR-150-5p and miR-21-5p are the most elevated miRNAs, with lower levels observed upon treatment with immunosuppression and thymectomy. In AChR+ generalized LOMG, miR-150-5p, miR-21-5p, and miR-30e-5p levels are elevated and decreased by the clinical response after immunosuppression. In ocular MG, higher levels of miR-30e-5p discriminate patients who will later generalize from those remaining ocular. In contrast, in MuSK+ MG, the levels of the let-7 miRNA family members are elevated. Studies of circulating miRNA profiles in Lrp4 or agrin antibody seropositive MG are still lacking. This review summarizes the present knowledge of circulating miRNAs in different subgroups of MG.

Keywords: circulating microRNA, myasthenia gravis, miR-150-5p, miR-21-5p, miR-30e-5p, biomarker.

1. Introduction

Myasthenia gravis (MG) is an autoimmune neuromuscular disorder that causes fatigable skeletal muscle weakness. The global incidence and prevalence of MG are increasing in adults at all ages of onset, with an annual incidence of roughly 10–29 cases per million and a prevalence ranging from 100 to 350 cases per million people (*1*). MG is a heterogeneous disease with different subgroups based on serological status, age at onset, clinical phenotype, and association with thymic pathology. The serological subgroups include patients that have antibodies against the nicotinic acetylcholine receptor (AChR; ~85%), the muscle-specific tyrosine kinase (MuSK; ~7%), and lipodensity related protein 4 (Lrp4;~1-2%) (*2*). Early-onset MG (EOMG) refers to patients with onset of the disease between ages 19-50 years and typically affects women with AChR antibody-positive (AChR+) MG and thymus hyperplasia. Late-onset MG, instead, is more common in men with atrophic thymus. Recently, a group of very-late-onset in the ages above 65 has been described (*3*). These subgroups can be further subdivided according to clinical weakness into MG affecting only the extraocular muscles, known as ocular MG (OMG), or MG affecting skeletal muscle groups outside the ocular area, called generalized MG (GMG). Most patients present with extraocular manifestations alone; however, up to 85% of patients develop the generalized disease within two years of symptom onset. Since MG patients can have different patterns of fatigable muscle weakness over time (*4*) and the disease is very heterogeneous and fluctuating, there is a strong need for prognostic biomarkers of MG progression and treatment outcome. Autoantibodies are valuable diagnostic biomarkers; however, autoantibody titers do not necessarily correlate with disease severity of treatment response (*5*). Circulating microRNAs (miRNAs) are easily measured in blood samples and are changed in different disease states (*6*). Therefore, they have also been suggested as potential prognostic biomarkers in MG (*7, 8*). This review summarizes the data on circulating miRNAs in MG.

2. Extracellular circulating microRNAs (miRNAs)

MiRNAs are short, endogenous non-coding RNA molecules that interact specifically with mRNAs. Due to their specific interaction with different mRNA molecules, they can control the stability and translation of mRNA. Indeed, miRNA interactions with various mRNAs have been shown to regulate critical cellular processes, including differentiation, proliferation, and apoptosis (*9*). It has been estimated that about 2300 true mature miRNAs regulate the expression of more than 60% of protein-coding genes. Altered miRNA expression is found in several disease states, including cancer, cardiovascular and autoimmune diseases (*10-12*). In addition to their intracellular accumulation, mature miRNAs are detectable outside the cells, in the extracellular space.

2.1. Circulating miRNA as potential biomarkers

Circulating miRNAs can be found in human body fluids, including plasma and serum. Notably, circulating miRNAs are stable and can withstand low pH and multiple freeze-thaw cycles (*13*). One of the reasons for this stability is that circulating miRNAs are embedded into membrane-enclosed extracellular vesicles, such as microvesicles and exosomes (*14*). Although the microvesicles and exosomes are structurally similar, they differ in size and cellular origin. Notably, both vesicles contain embedded miRNAs and are released from the cells under physiological and pathophysiological conditions (*15*) .

Circulating miRNAs can be considered paracrine and endocrine signaling molecules that can alter gene expression on nearby and distant target cells (*16*). Furthermore, a correlation between circulating miRNA levels and disease status has highlighted these molecules as potential biomarkers for diagnosis and disease monitoring (*17*). Circulating miRNAs fulfill the requirements for a biomarker as they are specific, very stable, easily accessible in a minimally invasive manner, and their detection is cost-effective. The number of studies showing circulating miRNAs as potential biomarkers is constantly rising. Quantitative reverse transcription PCR (qRT-PCR) is often considered the standard method to evaluate miRNA expression profiles since this method is robust, easy to perform, and quick (*18*). However, normalization of the qRT-PCR data between different circulating miRNA samples is challenging due to the lack of a universal "housekeeping gene" (*17*). However, miR-191 is useful as a housekeeping gene for normalization purposes, both in serum and plasma miRNA studies since it is consistently detected in most patients (*19-21*). Given that most blood samples are stored as serum and there are more RNA degrading enzymes (RNases) present in plasma, miRNA profiles are often analyzed in serum.

2.2. Circulating miRNA profiles in MG subgroups

2.2.1. Acetylcholine receptor antibody seropositive (AChR+) early-onset MG (EOMG)

EOMG primarily affects women and is often associated with thymic hyperplasia. In AChR+ EOMG female GMG patients without immunosuppressive treatment, the serum levels of the immunomiRNAs miR-150-5p and miR-21-5p are elevated, whereas the miR-27a-3p level is reduced, compared to matched healthy control women (*22*) (**Figure 1**). Also, in sera from more heterogenous clinical cohorts of male and female AChR+ and AChR- MG patients, miR-150-5p and miR-21-5p levels are elevated compared to healthy controls and patients with other autoimmune diseases, such as psoriasis and Addison's disease. The levels of miR-150-5p and miR-21-5p are significantly lower in the sera from MG patients on immunosuppressive treatment than those who are immunosuppressive naive (*23*).

Serum levels of miR-150-5p are reduced upon thymectomy in line with clinical improvement in AChR+ patients (*22, 24*). Longitudinal analysis of miR-150-5p and miR-21-5p in the prospective randomized control trial termed MGTX indicated that miR-150-5p levels decreased significantly two years after thymectomy, whereas no significant reduction was found in the group treated with prednisone (*24*). Further, rituximab treatment reduces the serum exosomal miR-150-5p levels in correlation with clinical MG scores and patients' prednisone requirement (*25*). Intriguingly, serum miR-150-5p and miR-21-5p levels are also lowered after a 12-week physical exercise intervention in MG patients (*26*).

The aforementioned circulating miRNAs are not the only reported alterations in AChR+ MG patient biofluids. Another profiling of circulating miRNAs in different AChR+ MG patients [EOMG, LOMG and thymoma associated MG (TAMG)] sera revealed that at least seven miRNAs were downregulated (miR-15b, miR-122, miR-140-3p, miR-185, miR-192, miR-20b, miR-885-5p) compared with healthy controls (*27*). Nevertheless, in this study, miRNA differences were not found between treated and untreated MG patients (*27*). Two other studies confirmed lower serum levels of miR-20b in patients with TAMG (*28, 29*). Serum miR-20b was downregulated both in generalized and AChR+ ocular MG (OMG) patients, and miR-20b expression in generalized MG was much lower than that found in OMG (*28*). Furthermore, miR-20b levels increased after treatment with corticosteroids in this particular study (*28*).

2.2.2. Late-onset MG (LOMG)

In LOMG, most patients are male and often have thymus atrophy, in contrast to EOMG, which primarily affects women and is associated with thymic hyperplasia (*30*). Nevertheless, the majority of LOMG patients also are AChR+. Five miRNAs were found to be elevated in sera from LOMG patients with no immunosuppressive treatment: miR-106b-3p, miR-30e-5p, miR-223-5p, miR-140-5p, and miR-19b-3p (*31*) (**Figure 1**). To assess the prospective influence of these miRNAs in sera of immunosuppressive naïve generalized LOMG patients with immunosuppression, these miRNAs were longitudinally analyzed up to two years after the MG onset (*31*). Since 96% of these LOMG patients were AChR+, the previously found elevated miRNAs miR-21-5p and miR-150-5p (*7*) were also analyzed. After immunosuppression initiation, the steady decline in clinical MGC score at and after one-year follow-up in the LOMG cohort correlated with reduced levels of miR-150-5p, miR-21-5p, and miR-30e-5p (*31*). LOMG patients with generalized disease had higher miR-150-5p and miR-21-5p than those with purely ocular symptoms (*31*) (**Figure 1**).

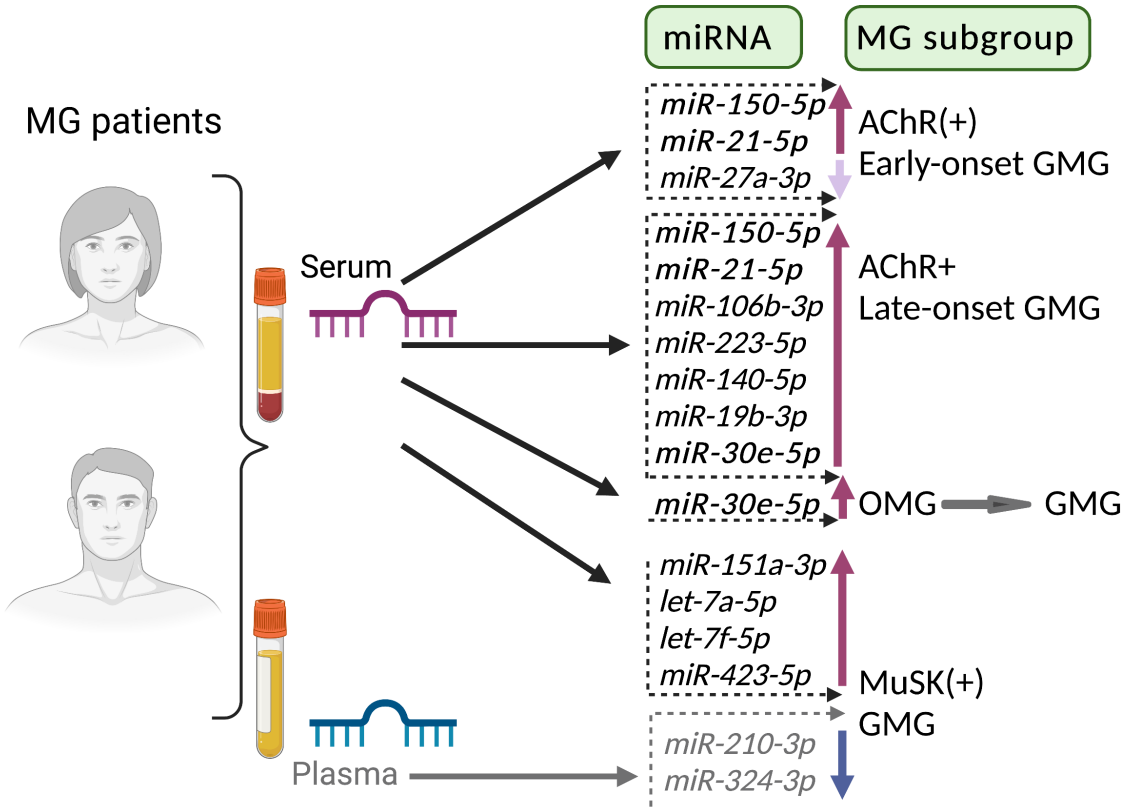

Figure 1. Summary of the circulating microRNAs in serum and plasma that are found associated with the different subgroups of myasthenia gravis. MicroRNAs highlighted in bold have been shown to be reduced upon thymectomy (miR-150-5p) or immunosuppression (miR-150-5p, miR-21-5p and miR-30e-5p). Arrows indicate increased or reduced levels of the miRNAs. MG, myasthenia gravis; GMG, generalized MG; OMG, ocular MG; AChR(+), acetylcholine receptor antibody seropositive; MuSK(+), muscle-specific tyrosine kinase antibody seropositive.

2.2.3 Ocular MG (OMG)

OMG is defined as clinical MG symptoms and signs only in the extraocular muscles, manifesting as ptosis and diplopia. There are no predictive markers for the risk of conversion from OMG to GMG; however, AChR+ MG patients are considered to have a higher risk for generalization than AChR antibody seronegative patients (*32*). Due to differences in some miRNAs in LOMG patients (*31*), one study aimed at determining whether serum miRNAs could be used as potential predictors of the generalization of OMG (*33*). For this purpose, 83 OMG serum samples (82 immunosuppression treatment naïve) were assayed within three months of OMG diagnosis and at a follow-up visit. The miR-30e-5p and miR-150-5p were significantly higher in patients who developed GMG than those who remained with OMG. Of these two miRNAs, miR-30e-5p has 96% sensitivity for differentiating OMG and GMG in all patients and 100% in LOMG patients (*33*) (**Figure 1**). Considering that treatment with corticosteroids could modify the progression of OMG to GMG (*34*) and that half of the OMG patients generalize within one year (*35*), predictive biomarkers would be helpful to tailor the immunosuppressive treatment of individual OMG patients. This could, for example, imply initiating immunosuppressive therapy at an earlier stage if miR-30e-5p levels are higher.

2.2.4. Muscle-specific tyrosine kinase antibody seropositive (MuSK+) MG

MuSK+ MG is considered a more homogenous disease subtype that differs from AChR+ MG by having more bulbar symptoms, no thymic hyperplasia, and different treatment response (*36*). Therefore, it could be suspected that MuSK+ MG has a different profile of circulating miRNAs than AChR+ MG. In sera from MuSK+ MG patients, the profile of miR-151a-3p, let-7a-5p, let-7f-5p, and miR-423-5p are all increased compared to healthy matched control individuals (*37*).

As most blood samples are stored as serum, most studies have analyzed circulating miRNAs in serum; nevertheless, plasma concentrations of miRNAs cannot be presumed to be interchangeable (*38*). Analysis of the miRNA profile in the plasma of MuSK+ MG patients instead suggests lower values of two other miRNAs: miR-210-3p and miR-324-3p (*20*).

2.2.5. Unselected cohort of MG patients compared to other neuroimmune diseases.

Serum miR-30e-5p, miR-150-5p, and miR-21-5p levels correlate with clinical course in specific MG patient subgroups (**Figure 1**). In light of this, another study aimed at better characterizing these three miRNAs, regardless of the MG subgroup, shortly after MG onset and determining their sensitivity and specificity for MG diagnosis, as well as their predictive power for disease relapse (*19*). Serum levels of these miRNAs in 27 newly diagnosed MG patients were compared with 245 healthy individuals and 20 patients with non-MG neuroimmune diseases. Levels of miR-30e-5p and miR-150-5p significantly differed between MG patients and healthy controls; however, no difference was seen compared with patients affected by the other neuroimmune diseases (multiple sclerosis, Lambert-Eaton myasthenic syndrome, chronic inflammatory demyelinating polyneuropathy and inflammatory myelitis) (*19*). In all MG patients, miR-150-5p has a sensitivity of 85% and a specificity of 48%; higher values in EOMG with a sensitivity of 90% and specificity of 58% (*19*). This is in line with a previous study indicating high miR-150 levels are found in other autoimmune conditions, including multiple sclerosis. miR-30e-5p is more specific than sensitive for MG, with a sensitivity of 56% and specificity of 86% (*19*). Intriguingly, high levels of miR-30e-5p predicted MG relapse with a hazard ratio of 2.81 (*19*), in line with higher miR-30e-5p levels in those OMG patients who transitioned to GMG (*21*).

3. The link between circulating miRNAs in MG and intracellular pathophysiology

MiR-150-5p and miR-21-5p are so-called immuno-miRNAs and important regulators for developing and differentiating T cells (*39*). The effector organ in AChR+ EOMG, the thymus, is often characterized by hyperplasia with ectopic germinal centers consisting of infiltrating B cells (*40, 41*). MiR-150 is a marker of lymphocyte activation and regulates proliferation, apoptosis, and differentiation of natural killer (NK), T cells, and B cells (*39, 42, 43*) (*44*). MiR-150 expression is considerably higher in the germinal centers of the thymus of AChR+ EOMG patients compared to healthy controls (*45*). Further, miR-150 levels are lower in peripheral CD4+ T cells of AChR+ EOMG patients than in healthy controls. Thus, increased serum levels of miR-150-5p could result from released miR-150 from activated peripheral CD4+ T cells (*45*). One hypothesis is that miR-150 is regulated by its release into the extracellular space (*46*). There is a positive correlation between the B cell marker *CD19* mRNA and miR-150 expression in the thymus, which could implement an interaction between miR-150-5p and the CD19+ cells involved in the autoimmune response in MG (*45*). Furthermore, miR-150 treatment of PBMCs affects the main proto-oncogene MYB, and thus, miR-150 could play a role in EOMG both at the thymic level and in the periphery by modulating the expression of target genes and peripheral cell survival (*45*). Expression of two pro-apoptotic genes targeted by miR-150: Tumor Protein 53 (P53) and Apoptosis Inducing Factor Mitochondria associated 2 (*AIFM2),* are also increased upon anti-miR-150-5p treatment (*45*).

The other immunomiRNAs, miR-21-5p, is highly expressed in T regulatory cells (*39*) and also associated with other autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (*10, 47*). MiR-21 is induced by several pro-inflammatory molecules, and can regulate the NF-κB and NLRP3 pathways (*48*). NF-κB activation promotes the hyper-expression of target genes involved in pro-inflammatory/stress-like responses, including pro-IL-1β and pro-IL-18 (*49*). MiR-21 orchestrates the fine-tuning of the inflammatory response through direct and indirect activities on these pathways (*48*).

The third miRNA in AChR+ MG, miR-30e-5p, is somewhat contradictorily downregulated in EOMG (*22*) and upregulated in LOMG (*31*). Intriguingly, the low-density lipoprotein receptor-related protein 6 (LRP6), one of the critical co-receptors for Wnts (a family of genes that encode secretory glycoproteins), is a direct target of miR-30e (*50*). Thus, there is a potential role for miR-30e in regulating muscle homeostasis.

The let-7 miRNA family members have been extensively studied because of their broad functional role in various cellular processes, including neuronal development and embryogenesis (*51, 52*). The let-7 miRNAs stimulate the Toll-like receptor 7 (TLR7), thereby activating T cells (*53*). Further, the involvement of TLR7 in CD4+ T cells induces T cell unresponsiveness (*54*). Let-7a-5p and let-7f-5p are upregulated in PBMCs isolated from thymoma-associated MG patients (*55*), whereas let-7f-5p is instead downregulated in the thymus of AChR+ EOMG patients (*56*). Although a key role has been suggested for TLRs in thymic hyperplasia-associated EOMG, through abnormal activation of TLRs, the role of TLRs in MuSK+ MG remains to be defined (*57*).

Neither miR-210-3p nor miR-324-3p have previously been reported to be dysregulated in immune-mediated diseases. MiR-210-3p has been found to be dysregulated in several cancers (*58*), and miR-324-3p has been mentioned as a potential biomarker in osteoporosis (*59*).

4. Conclusion

In summary, circulating miRNAs could serve as potential biomarkers in MG and MG subgroups to monitor the disease course. miR-150-5p is highly sensitive but has low specificity for MG. In contrast, miR-30e-5p has the most significant potential as a predictive biomarker for the disease course in MG, regardless of the subgroup. Multicenter trials for validation of these miRNAs are needed.

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Symptomatic pharmacological treatment of myasthenia gravis

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ABSTRACT

Myasthenia gravis (MG) is a chronic antibody-mediated autoimmune disease. The most frequent form is MG with antibodies directed against the acetylcholine receptor on the postsynaptic membrane. The first step in the treatment of autoimmune myasthenia gravis consists of symptomatic therapy. If this is insufficiently effective, the next step is to start immunosuppressive treatment with corticosteroids, usually prednisolone. A corticoid-sparing agent is often added because of the long long-term side effects of high doses of corticosteroids. The position of emerging immunomodulatory therapies targeting B- and T-cells, the complement cascade, the neonatal Fc receptor, and cytokines associated with antibody production in the treatment of MG is currently unclear. However, it is likely that symptomatic treatment will remain the cornerstone in the management of patients with MG in the foreseeable future. In this review, we provide an overview of currently available symptomatic treatments and recent advances in this field. Pyridostigmine, an acetylcholinesterase inhibitor, is the most commonly used symptomatic drug for MG. Acetylcholinesterase inhibitors prolong and enhance the effect of acetylcholine on muscarinic and nicotinic receptors. In addition, there is evidence that pyridostigmine may also have an anti-inflammatory effect. Pyridostigmine is moderately effective, but side effects are frequently reported by patients. Other therapies include amifampridine and sympathomimetics such as ephedrine, salbutamol, and terbutaline. At present, there is insufficient evidence for the use of amifampridine as monotherapy or as add-on therapy to pyridostigmine. The addition of β2-adrenergic agonists to pyridostigmine may possibly be beneficial in some patients, however, well-designed randomized trials are needed to establish their efficacy. Emerging symptomatic therapies include ClC-channel blockers, fast-skeletal muscle troponin activators, and antisense oligodeoxynucleotides. These therapies appear to be promising, with fewer side effects than pyridostigmine. However, phase III clinical trials are needed to assess their effectiveness and determine their place in symptomatic treatment of MG patients.

Key Words: *myasthenia gravis, symptomatic treatment, pyridostigmine, adrenergic agonist, amifampridine*

Introduction

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction in which autoantibodies bind to the acetylcholine receptor (AChR) or associated structures on the postsynaptic membrane, resulting in impairment of neuromuscular transmission (1). Clinical features are fluctuating weakness in ocular, bulbar, limb, and respiratory muscles. Patients typically experience an increase in weakness with exercise and an improvement after rest of the involved muscles (2, 3). Antibodies are found against the AChR in approximately 80% of patients with generalized MG (3). Less commonly, antibodies against muscle specific kinase (MuSK) or low-density lipoprotein receptor-related protein 4 (LRP4) are formed (4), resulting in different clinical features including an altered response to pharmacologic treatment (5). First line pharmacological treatment consists of symptomatic treatment (6). Patients who do not meet treatment goals with symptomatic drugs, are advised to start corticosteroids often in combination with nonsteroidal immunosuppressive drugs. In recent years, advances in the understanding of the pathophysiology of MG have led to development of new immunomodulatory therapies that act at many different sites of the immune system, including IL-6, CD19, CD20, CD38, CD40, CTLA-4, FcRn, and the complement pathway (7). Although these novel therapies appear to be effective in reducing MG-related muscle weakness, there are some drawbacks. They are associated with high costs, and most of them require intravenous administration, for which hospitalization or infusion in an outpatient setting is often necessary. Furthermore, little is known about their long-term safety, and treatment therefore requires more intensive monitoring. In contrast, the long term risks associated with symptomatic drugs are probably negligible, they are relatively cheap, and they can be used "as needed", allowing the patient a greater degree of control over the management of their disease. It is therefore likely that symptomatic treatment will remain one of the cornerstones of the treatment of patients with MG. However, despite this fact, only limited high-quality data are available regarding their efficacy and safety. In this review, we aim to provide an overview of currently available symptomatic treatments and recent advances in this field (Figure 1).

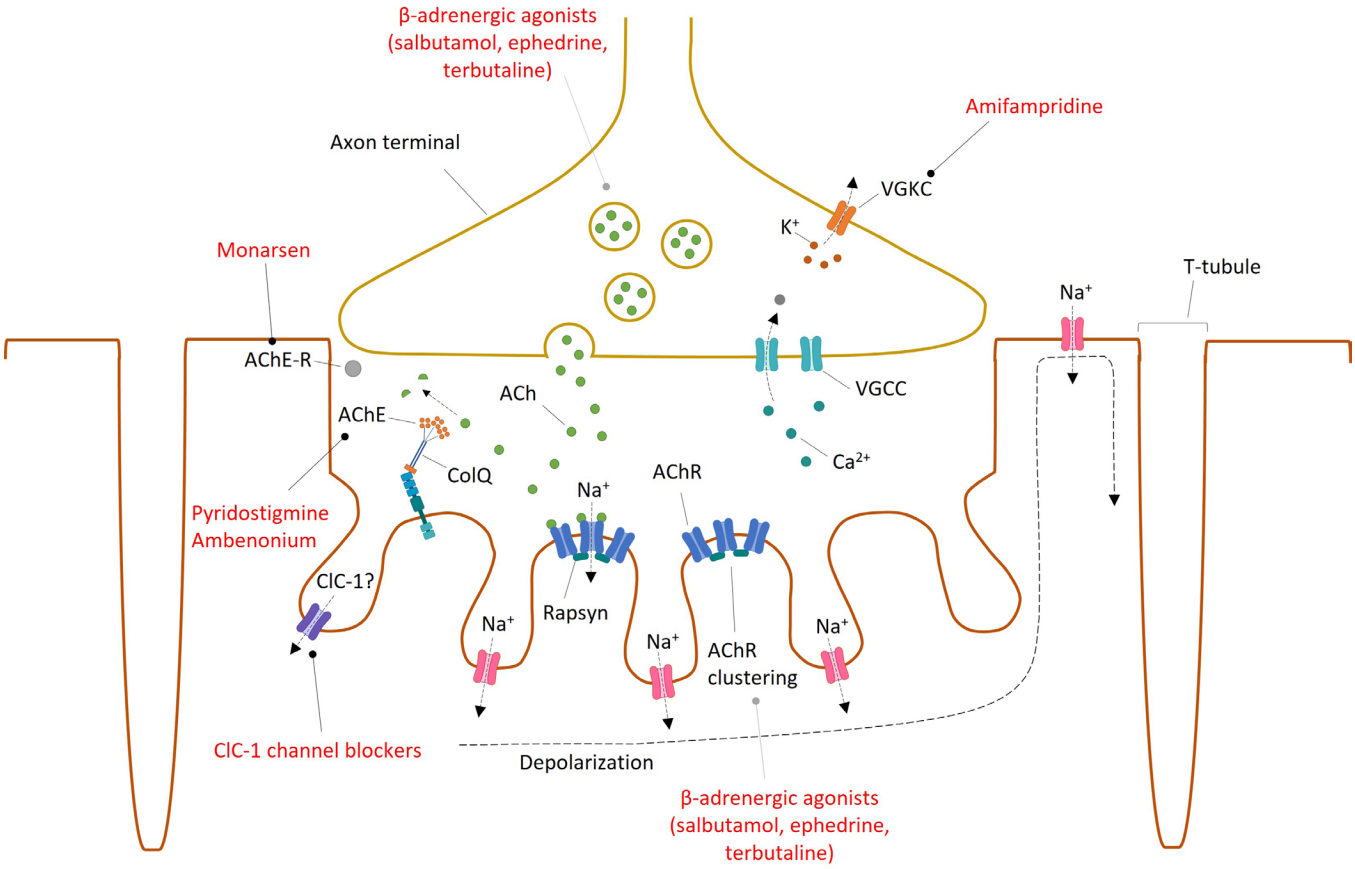

Figure 1. (Assumed) mechanism of action of different drugs in the symptomatic treatment of patients with myasthenia gravis.
Pyridostigmine and ambenonium, both acetylcholinesterase inhibitors, block the enzyme acetylcholinesterase and thereby increase the amount of acetylcholine in the synaptic cleft. Amifampridine blocks potassium efflux, which results in a prolonged action potential of the presynaptic nerve terminal and thereby enhances the release of acetylcholine into the synaptic cleft. The mechanism of action of β-adrenergic agonists (salbutamol, ephedrine, and terbutaline) is not fully understood. There is evidence that β-adrenergic agonists affect post-synaptic AChR clustering. Furthermore, it is hypothesized that β-adrenergic agonists play a role in regulation of quantal acetylcholine content. ClC-1 channel blockers reduce the inhibitory currents that counteract neuromuscular transmission. The precise localization of these channels at the neuromuscular junction is unknown. Monarsen is an antisense oligodeoxynucleotide which inhibits the expression of AChE-R, an isoform of AChE mainly found in patients with myasthenia gravis. Not shown: Fast-skeletal muscle troponin activators (tirasemtiv, reldesemtiv). Abbreviations: ACh Acetylcholine. AChE Acetylcholinesterase. VGCC Voltage-gated calcium channel. VGKC Voltage-gated potassium channel

Existing therapies

Acetylcholinesterase inhibitors

Acetylcholinesterase inhibitors increase the amount of acetylcholine in the synaptic cleft by blocking the enzyme acetylcholinesterase, resulting in enhanced neuromuscular transmission. Their beneficial effects in patients with autoimmune MG have long been recognized; the first application of an acetylcholinesterase inhibitor dates from April 1934 when Dr. Mary Broadfoot Walker treated a patient with physostigmine with dramatic results (8). A year later, neostigmine was introduced, and this was the primary drug for the treatment of MG until the first case reports with the use of pyridostigmine were published in 1947 (9-12). Neostigmine was known to have significant response fluctuations due to a short half-life, which led to patients taking it frequently throughout the day, resulting in high cumulative doses. Furthermore, neostigmine had pronounced side effects, both muscarinic (such as gastrointestinal symptoms, increased salivation, and a marked increase in bronchial secretions) and nicotinic (such as skeletal muscle cramps). These side effects remained and were difficult to control, even with the use of atropine. Pyridostigmine, which had a longer duration of action and had fewer side effects, was developed by Hoffmann-La Roche as a superior alternative (9-13). Since then, pyridostigmine has been the first choice in the symptomatic treatment of myasthenia gravis (6). Other acetylcholinesterase inhibitors include ambenonium chloride, which is used less frequently than pyridostigmine due to a less favorable side effect profile, and hydrophonium or edrophonium, which is only used in the diagnosis of seronegative MG due to a brief duration of action (14). Mouse models suggest that long-term anticholinesterase therapy may have an adverse effect on neuromuscular transmission and motor end-plate structures (15, 16) resulting in a potential decrease in efficacy over time and an increased risk of cholinergic side effects (17). Neurotransmission itself has a dispersal effect on AChR clusters and postsynaptic structures, which is counteracted by the agrin/muscle-specific kinase pathway (i.e. the AChR-clustering pathway). An increase of neurotransmission, through a pharmacological intervention such as acetylcholinesterase inhibitors, will therefore lead to amplification of the disruption of the postsynaptic structures, especially in diseases in which the counteracting AChR agrin/muscle-specific kinase pathway is affected (18). However, in patients with MG, no correlation has been found between the perceived efficacy and age or disease duration, nor between the number and severity of side effects and age or disease duration (19). In addition to the direct effect of acetylcholinesterase inhibitors on the neuromuscular junction, there is evidence that pyridostigmine may also have an anti-inflammatory effect (20, 21) through the cholinergic anti-inflammatory pathway. This pathway can modulate the activity of immune cells through activation of nicotinic acetylcholine receptors on macrophages, dendritic cells, monocytes, and T- and B-lymphocytes. Furthermore, it can inhibit cell proliferation and differentiation, as well as suppress cytokine release (22). The exact role of acetylcholinesterase inhibitors and their interaction with the cholinergic pathway and inflammatory reactions in MG has not been established.

Pyridostigmine

All international guidelines recommend pyridostigmine as the first step in the pharmacological treatment of MG (6, 23-25). In a recent study involving participants in the Dutch national MG registry, 74% reported using pyridostigmine (26). Unfortunately, no precise data are available on its effect, as no randomized controlled studies have ever been performed since the first published case-studies (27). However, in a large-scale cross-sectional study on 410 MG patients, 61% reported that they currently used pyridostigmine, 36% had discontinued pyridostigmine, and 2% reported never using pyridostigmine. On a scale of 0 (no effect at all) to 100 (maximum effect), patients currently using pyridostigmine reported a median effectiveness of 60 (IQR 28-78) and net benefit of 65 (IQR 45-84). In the group of patients who discontinued pyridostigmine, side effects were the reason for discontinuation in 26%. Pyridostigmine monotherapy is used in 22-66% percent of all patients (26, 28, 29), suggesting that it is sufficiently effective to prevent the use of immune suppressant medication in patients with relatively mild symptoms. In an uncontrolled study, pyridostigmine improved symptoms and respiratory function in 9 patients with myasthenia gravis (30). Several studies have evaluated the relationship between plasma pyridostigmine levels and neuromuscular function and clinical effect (31-35). Individual responses vary greatly between patients, probably because of variable pharmacokinetics. In 2018, the first randomized controlled trial began recruiting patients to evaluate the effect of pyridostigmine on muscle strength in two groups: 1) newly diagnosed, treatment-naïve patients treated with 60 mg pyridostigmine administered twice in four hours and 2) patients with MG on stable anti-myasthenic medication treated with the patient's usual dosage also administered twice in four hours (NCT03510546). In 2023, a randomized controlled withdrawal trial in our center will start, comparing the efficacy of pyridostigmine versus placebo over a 5-day period. The primary outcome will be a clinically relevant change on the Myasthenia Gravis Impairment Index (MGII) compared to placebo. Secondary study parameters include change on a 9-item Treatment Satisfaction Questionnaire for Medication (TSQM-9), change on MG-QoL15r, and a clinically relevant change on MG-ADL and QMG. Furthermore, side effects will be recorded. Very few studies have reported on the side effects of pyridostigmine. Current knowledge is mainly based on years of clinical experience. Side effects are due to

overstimulation of the muscarinic receptor, causing symptoms such as abdominal cramping, diarrhea, hyperhidrosis, increased salivation, sweating, lacrimation, and bradycardia. Nicotinic side effects have also been reported due to overdosage of pyridostigmine and include muscle cramps, fasciculations, and muscle weakness (36). Side effects are frequently reported by patients who use pyridostigmine (17, 19). Most frequently reported side effects are gastrointestinal symptoms (flatulence, diarrhea, and abdominal cramps), urinary urgency, muscle cramps, blurred vision, hyperhidrosis, increased salivation, light-headedness, and flu-like symptoms. Diarrhea, abdominal cramps, and muscle twitching are the most frequently cited reasons for discontinuation of pyridostigmine (19). Symptoms of overactive bladder are more common in MG patients compared to healthy controls. The severity of these symptoms is related to the daily dose of pyridostigmine (37). Patients using pyridostigmine appear to have slight airway obstruction compared to non-pyridostigmine treated patients and matched controls (38), although the clinical relevance of this observation is unclear. Cumulative side effects after long-term treatment have not been reported (3). Many patients with MuSK-MG respond poorly to acetylcholinesterase inhibitors with less effect and frequent side effects compared to patients with anti-AChR antibodies (39). Muscarinic side effects of pyridostigmine can be controlled by the addition of muscarinic antagonists such as atropine (3), glycopyrronium bromide (3), propantheline (40), or hyoscyamine (41). Loperamide can be used to treat persistent diarrhea (3). There have been no studies comparing these agents in patients with MG. Their use in clinical practice is therefore based on single case reports, personal experience, and expert opinion. In 2021, a phase II trial began to evaluate the effect of combined therapy of pyridostigmine and ondansetron, an anti-emetic drug which selectively blocks the serotonin 5-HT3-receptor in patients experiencing pyridostigmine-related gastrointestinal adverse events (NCT04226170).In a limited number of countries, a sustained release (SR) formulation of pyridostigmine is available. In a prospective non-interventional multicenter open-label study the usefulness of this agent was evaluated (42). Pyridostigmine was switched from regular acting pyridostigmine to SR pyridostigmine in 72 patients with side effects, drug fluctuations, and/or insufficient efficacy of the regular acting pyridostigmine. In these patients QMG and EuroQol scores improved significantly after switching to SR (42). However, the decrease in QMG score was very low (0.3 points) which is considered to be below the threshold for clinical relevance (43). Adverse events were reported less frequently after switching to SR pyridostigmine (42).

Ambenonium chloride

The first use of ambenonium in patients with MG was reported in 1955. Out of fifty patients treated with oral ambenonium, 41 patients experienced more benefit from it than from neostigmine or pyridostigmine. The main advantages were its longer duration of action and fewer side effects (44). In a later study, patients experienced more side effects with ambenonium than with pyridostigmine, although the duration of action of ambenonium was longer (45). Ambenonium has an unpredictable pattern of bioavailability in MG patients, with a greater risk of accumulation and overdosage, possibly because pharmacokinetics showed no correlation between the daily dose and the area under the curve (46). In current clinical practice, ambenonium is rarely used, although it may be a good alternative for patients for whom pyridostigmine is contraindicated.

Amifampridine

Amifampridine is a well-known treatment for other diseases of the neuromuscular junction such as Lambert Eaton Myasthenic Syndrome (LEMS) and congenital myasthenic syndromes. It is a short-acting potassium channel blocker, which blocks potassium efflux presynaptically. This results in a prolonged action potential of the presynaptic nerve terminal, which enhances release of acetylcholine into the synaptic cleft by an increase in calcium influx into the nerve terminal (47). A preliminary report of a double-blind, placebo-controlled crossover study noted an improvement of at least 3 points on the QMG scale in two of eight MG patients on amifampridine. However, only a limited description of methods and results of this study is available (48). Another randomized controlled crossover trial showed an improvement of 6.9 points on the QMG scale and 5.7 points on the MG-ADL in 7 patients treated with amifampridine monotherapy with MuSK-MG (49). Amifampridine and pyridostigmine act on different parts of the neuromuscular junction, and it is hypothesized that they work in synergy to enhance neuromuscular transmission. Indeed, in a study of LEMS patients, the combination of amifampridine and pyridostigmine had an effect on some pharmacokinetic parameters: the pharmacokinetics of amifampridine were not significantly affected by cotreatment with pyridostigmine, whereas amifampridine caused an increase in the average pyridostigmine serum concentration. However, the average plasma concentrations of pyridostigmine corresponded with clinically therapeutic levels in both the co-treatment and the stand-alone treatment arm (50). The combined use of pyridostigmine and amifampridine in clinical practice is not uncommon in patients with LEMS; 71% of all patients in the Dutch LEMS registry reported using a combination of pyridostigmine and amifampridine (26). The efficacy and tolerability in patients with MG in clinical practice has only been described in a limited number of studies. Two small case studies and one case report provide anecdotal evidence that patients may benefit from the

use of amifampridine as add-on therapy in MG (51-53). A phase II trial provided evidence that amifampridine phosphate was effective in patients with MuSK-MG (49). However, results were not replicated in a phase III trial evaluating the efficacy and long-term safety of amifampridine in 55 patients with MuSK MG and 15 patients with AChRAb MG (NCT03304054). These results are not yet published.

Sympathomimetics

The effects of sympathomimetics on the (myasthenic) neuromuscular junction are complex and insufficiently understood. Therapies acting on the sympathetic nervous system, such as the β2-adrenergic agonist salbutamol and the α- and β-adrenergic agonist ephedrine, have a well-documented effect in subtypes of congenital myasthenic syndromes; a diverse range of genetic disorders in which neuromuscular transmission is impaired at the motor endplate (54). However, the molecular mechanisms underlying the therapeutic effect of sympathomimetics are not understood. It is hypothesized that β2-adrenergic agonists directly influence synaptic organization, and the therapeutic effect may therefore be through morphological restoration of the neuromuscular junction (55). *In vitro* studies have shown that β-adrenergic agonists affect postsynaptic AChR clustering (56). This hypothesis is supported by observations in clinical practice that β-adrenergic agonists are particularly beneficial in disorders in which the endplate structure is disrupted, such as DOK7 and COLQ congenital myasthenic syndromes (CMS) (57). Furthermore, there is evidence that sympathomimetics regulate quantal acetylcholine content and influence the probability of quantal release at the neuromuscular junction (58). Pharmacological stimulation of adrenoceptors, as well as sympathectomy, can affect acetylcholine release from motor nerve terminals (59). Notably, it is uncertain whether the observed effects in these animal studies are relevant for the understanding of the observed effect in clinical practice, since the concentrations of the adrenergic agonists used in animal studies were much higher than those reached in patients. Considerable ambiguities therefore remain regarding the mode of action of sympathomimetics. In addition to their action at the neuromuscular junction, catecholamines play a role in several immune parameters. They affect lymphocyte proliferation and modulate cytokine production and the functional activity of different lymphoid cells (60, 61). However, the role of sympathomimetics on the immune system in MG patients is currently not fully understood.

Ephedrine

Ephedrine is a sympathomimetic drug with a stimulating effect on both α- and β-adrenergic receptors (62). The use of ephedrine in patients with MG was first described in 1930 by Edgeworth, who serendipitously found that the ephedrine she was taking for menstrual cramps was effective for her myasthenic symptoms (63). In a small series of randomized controlled n-of-1 trials with ephedrine as add-on treatment to pyridostigmine or prednisone in MG patients, a small reduction of MG symptoms was seen in both the primary outcome measure (QMG) and the secondary outcome measures (MG-ADL, MGC, and VAS-score). This effect was statistically significant, but below the previously defined cut-off value for a clinically relevant difference (64).

Salbutamol

Salbutamol is a selective β2-adrenergic agonist and is mainly used in patients with CMS. As described above, pyridostigmine may have a long-term adverse effect on the motor end-plate structure and thus on neuromuscular transmission. The addition of salbutamol to pyridostigmine might be beneficial to counteract the long-term adverse effects of pyridostigmine use because of the postulated mechanism of β2-adrenergic agonists to morphologically restore the neuromuscular junction. The functional effect of this combination therapy was explored in acetylcholine receptor deficiency syndrome, the most common form of CMS, in a small long-term cohort of CMS patients and in a mouse model (65). In the cohort of eleven patients with severe AChR deficiency, a sustained response on QMG score was seen: after 4 years of combination therapy with pyridostigmine plus salbutamol, the mean QMG score improved from 17.7 (95% CI 13.25–22.2) at baseline to 12.3 (95% CI 9.1– 15.6), although this effect did not reach statistical significance. Mouse models showed improvement of muscle fatigue which became apparent shortly after starting salbutamol. Furthermore, improved neuromuscular transmission and improved synaptic structure were seen. Whether the addition of salbutamol is useful in patients with MG as well is investigated in an ongoing randomized, controlled cross-over trial to study the efficacy and tolerability of oral salbutamol as adjuvant therapy in patients with MG. This study started in 2019 (NCT03914638).

Terbutaline

Like salbutamol, terbutaline is a β2-adrenergic agonist. In a mouse model, clinical symptoms were suppressed after treatment with terbutaline, and electrophysiological studies showed a significantly larger first compound muscle action potential (66). In a phase II cross-over study in eight patients with generalized MG who were treated with terbutaline or placebo for two weeks, a significant improvement was seen on the QMG score. Five out of eight patients (63%) had a clinically relevant improvement on the QMG score of 3.0 or greater. Pyridostigmine was withheld for at least eight hours before each visit. Terbutaline was well tolerated in all patients (67).

Emerging therapies

ClC-1 channel blockers

In MG, due to the loss of AChR, the excitatory endplate currents are reduced in size. Skeletal muscle-specific ClC-1 chloride ion channels carry the inhibitory currents that counteract neuromuscular transmission. Inhibition of ClC-1 has been shown to reduce the inhibitory current, increasing muscle membrane excitability and strengthening neuromuscular transmission (68). In 2020, a phase I/II randomized, controlled trial was initiated to assess safety and tolerability of NMD670, an inhibitor of the ClC-1 channel (NL8692). Results have yet to be published, although the company announced positive results in a press release (69).

Fast-skeletal muscle troponin activators

Tirasemtiv is a highly selective activator of the troponin complex of fast skeletal muscles. It was developed to increase muscle strength in neuromuscular disease by amplifying the response of the muscle when neuromuscular input is diminished. Binding of tirasemtiv to the troponin complex slows the rate of calcium release from fast skeletal troponin and consequently sensitizes muscle fibers to calcium (70). In a phase II study, the efficacy, safety, and tolerability of single doses of tirasemtiv in patients with AChR MG was investigated. This study showed small dose-related improvements in QMG with tirasemtiv. Furthermore, twice as many patients had clinically significant improvements in QMG (>3 points) at six hours after the 500 mg dose compared to placebo, but this difference did not reach significance due to the small sample size. Two single doses of tirasemtiv were well tolerated, and no serious adverse events occurred (71). A phase III clinical trial in patients with amyotrophic lateral sclerosis did not meet primary or secondary endpoints. Poor tolerability after 48 weeks of double blind treatment may have contributed to this result: 34.2% of all patients treated with tirasemtiv stopped treatment before week 24 vs. 12.2% in the placebo group. Dizziness, fatigue, nausea, weight loss, and insomnia occurred more frequently on tirasemtiv (72). As a result, further development of tirasemtiv has ceased, and the focus will be on reldesemtiv, a next-generation fast skeletal muscle troponin activator. Reldesemtiv advanced into clinical development for its potential to demonstrate increased efficacy relative to tirasemtiv as well as improved tolerability and less potential for drug–drug interactions (73). A phase III trial was initiated in patients with ALS in 2021 (NCT04944784). To our knowledge, no trials in MG are currently planned.

Antisense oligodeoxynucleotides

MG is associated with the production of a rare isoform of acetylcholinesterase which is referred to as the "read-through" transcript (AChE-R) (74, 75). This isoform is found in half of all patients with MG and not in healthy subjects (76). Monarsen (formerly EN101) is an antisense drug which inhibits the expression of AChE-R, potentially resulting in an increase of acetylcholine levels. In a phase II cross-over trial designed to compare three doses of monarsen, a decrease in QMG scores was found compared to baseline. All doses appeared to be effective, but no statistically significant difference between the three doses was found, and the study did not include a placebo control group. Only preliminary results have been published (77). Currently, no plans have been announced to further develop monarsen.

Conclusion

Despite years of experience with symptomatic drugs in the treatment of MG patients, much remains unknown. This makes it challenging to make individually tailored treatment decisions. In the past decades, only 6% of all clinical trials have focused on symptomatic treatment of MG (7). Almost all patients initially start with symptomatic treatment, and approximately two-thirds continue using it throughout their disease (19). Based on the available evidence, it is clear that pyridostigmine should remain the cornerstone of symptomatic treatment of MG patients. Many patients report moderate effectiveness, and a substantial number can be treated with pyridostigmine monotherapy without need for immunosuppressants. Nonetheless, pyridostigmine may cause considerable side effects. A substantial number of patients consider side effects to be moderately, very, or extremely annoying (19), which may impact quality of life. The addition of specific muscarinic antagonists such as atropine may alleviate side effects, but in our experience, the effect of atropine is often insufficient. It can be difficult to find a balance between adequate treatment of muscarinic side effects and inducing signs of atropine overdose. The place of amifampridine and sympathomimetics such as ephedrine, salbutamol, and terbutaline in the treatment of MG remains unclear. The addition of β2-adrenergic agonists to pyridostigmine may possibly be beneficial in some patients, however well-designed randomized trials are needed to establish their efficacy. At present, there is insufficient evidence for the addition of amifampridine to the standard symptomatic treatment with pyridostigmine. New emerging symptomatic therapies, especially the ClC-channel blockers and antisense oligodeoxynucleotides, may be promising therapies with fewer side effects than the current standard. Hopefully, phase III trials can shed more light on their effectiveness and determine their place in the symptomatic treatment of MG. In the future, MG patients would greatly benefit from properly designed trials on symptomatic drugs, as they are likely to remain an important element in achieving symptom relief for a large number of patients. Therefore, exciting developments involving drugs that target the immune system should not overshadow efforts to improve

the quality of life of MG patients by optimizing existing symptomatic treatment.

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Disclosures of Conflicts of interest

LRN and WRB report no disclosures relevant to the manuscript. In the last 3 years TvG has received lecture fees and consulting fees from Roche Diagnostics, Thermo Fisher, Vitaeris, CSL Behring, Astellas, and Aurinia Pharma. In all cases money was transferred to hospital accounts, and none has been paid to his personal bank accounts. JJGMV has been involved in MG research sponsored by the Princes Beatrix Fonds, Health Holland, and consultancies for Argenx, Alexion, and NMD Pharma. Reimbursements were received by the LUMC. He is coinventor on patent applications based on MuSK-related research. The LUMC receives royalties for MuSK antibody assays. He is a member of the Target-to-B! consortium. MRT reports trial support from Argenx and Alexion, consultancies for Argenx and UCB Pharma, and research funding from NMD Pharma, with all reimbursements received by Leiden University Medical Center. LRN, JJGMV and MRT are members of the European Reference Network for Rare Neuromuscular Diseases (EURO-NMD).

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Identification of Rare Membrane Antigen Specific Human B Cells

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ABSTRACT

The experimentally well supported model that MG pathology is caused by antibodies of the IgG class that bind to AChR at the neuromuscular junction, activate complement, and possibly cause internalization of receptors or their functional blockade has enabled the development of a range of reasonably effective treatments. A better understanding of which B cells are responsible for producing these pathogenic antibodies, and why such B cells develop would enable the development of more targeted therapies. Studies of antibodies isolated from single B cells from patients have provided some of this information that was not available from studies of polyclonal antibodies in sera, but perhaps future studies of the B cells themselves will provide deeper insight into the causes of the disease and thereby enable its prevention.

Introduction

The majority of patients (Vincent and Newsom-Davies, 1985) diagnosed with myasthenia gravis (MG) have antibodies against the muscle-type nicotinic acetylcholine receptor (AChR). The receptor is a ligand-gated ion channel built from five protein subunits, each with four transmembrane domains. In the muscles of healthy adults, the AChR is mostly found in dense clusters on the muscle membrane at the neuromuscular junction (NMJ) i.e., the point at which the terminus of the motor neuron contacts the muscle, and each receptor includes one beta subunit, one delta, one epsilon, and two alpha subunits. In fetal muscles, and in denervated muscles, the location of the epsilon subunit is taken by a similar protein encoded by a different gene, the gamma subunit (Gu and Hall, 1988). Reviewing available data in 1981, Engel et al. concluded that there was compelling evidence for a model of the disease based on IgG binding to AChR at the neuromuscular junction, followed by complement-mediated destruction of the postsynaptic membrane and depletion of the receptor. In the forty years since then, this model has been supported by numerous studies and refined in some details, but although much progress has been made in determining how best to treat the disease, our understanding of its cause has not developed extensively. The model from the nineteen seventies predicts that removal of the pathogenic antibodies, inhibition of complement activation, or measures to enhance the effect of the remaining receptors would be clinically effective, and all three predictions have been empirically supported and exploited for treatment. Is there anything more we could know, that could lead to an improvement in patients' lives? Two possibly meaningful avenues of enquiry might be a better understanding of the cells responsible for producing the pathogenic antibodies, which might enable the targeted depletion of these cells, and insight into the original cause of the disease, which might enable its prevention. Focusing narrowly on anti-AChR MG, this review will argue that for both of these goals, the isolation of rare, antigen-specific B cells from patients is a critical step. A great deal of progress has been made in this direction, but at the timepoint of the 14th MGFA conference, technical challenges still remain. Information about the monoclonal antibodies produced by single isolated B cells has already extended what had been deduced from the study of the polyclonal antibody pool in patients' sera, and an important future direction will be the study of the B cells that make these pathogenic antibodies.

Both soluble antibodies and their membrane expressed counterparts (the B cell receptor or BCR) will be discussed, and for readers from non-immunological fields, the relationships between these entities can be summarized as follows.

During its early development, a B cell links together genes encoding sections of protein, thereby generating two new compound genes that between them encode a membrane-expressed antigen receptor, the BCR, with a specificity that is (almost) unique to each B cell. A B cell that has completed this developmental stage but not yet encountered antigen is referred to as "naive". If such a B cell does encounter an antigen that is bound with high enough affinity by its BCR, it will internalize this antigen, cleave it into peptides (assuming the antigen is a protein), and re-externalize these peptide fragments in complex with proteins of the major histocompatibility complex on the extracellular side of its plasma membrane. This event of antigen capture leads to activation of the B cell, and can be followed by one of two possible outcomes. If the presented antigen-fragments are recognized by an activated T cell, the interaction between the B and T cells leads to the series of events described below (Tanaka and Baba, 2020). If no co-cognate T cell is available to provide this signal (T cell

help), then the B cell will die. If T cell help is available, the B cell survives, and undergoes phenotypic development, associated with changes in the structure of the BCR so that it is no longer expressed in the membrane, but secreted as a soluble molecule known as an antibody, with the same binding specificity as the BCR. This process of development can also include changes in the gene sequences that alter the specificity and affinity of the antibody (called somatic hypermutation), and others that do not alter the specificity, but alter other functional properties of the antibody (class switch, i.e., the change from IgM to IgG or other classes). By these developmental processes, naive B cells assume more "effector-like" phenotypes, becoming plasma cells that are specialized for antibody secretion, and memory cells that retain expression of the membrane BCR, and do not produce antibodies initially, but rather contribute to future responses against the same antigen (Suan et al., 2017) . In this review the word "immunoglobulin" will be used to refer to both the membrane-bound BCR and the secreted antibodies.

From the broad questions "Which B cells make the pathogenic antibodies?", and "Why do these cells develop?" we can extract the following narrower questions:

Which B cells?

What are the classes/subclasses, mutation status, and epitope specificity of the pathogenic antibodies?

What is the phenotype (memory, naive, plasma, long- or short-lived) of the pathogenic B cells?

Why?

What was the initial antigen encountered by the naive B cell that led to development into an antibody-secreting phenotype?

These questions have been approached so far by studying serum from patients, and also by a range of cell-oriented techniques, including the immortalization of single B cells using Epstein Barr virus, or hybridoma formation. Considerable information has also been obtained with the technique of phage display (Graus et al., 1997; Farrar et al., 1997; Fostieri et al., 2005), but since this involves the pooling of numerous B cells, rather than the investigation of single cells, this line of enquiry is outside the scope of this review.

Class/Subclass

The question of the class of AChR-binding antibodies in MG can be productively addressed by studying soluble antibodies in serum, since these are thought to contain the pathogenic agent, and subclasses of soluble antibodies can be determined accurately. Tindall (1981) compared abundance of AChR-precipitating antibodies of classes IgG, IgM, and IgA in serum from patients with MG, and reported that (compared with a cutoff at mean + 3 x standard deviations in healthy controls of 0.39, 1.31, and 0.49 units) patients had respectively ranges of 0-1050, 0-13.34, and 0-2.43 units of IgG, IgM and IgA in their serum. Investigation of anti-AChR antibodies in patient sera has accordingly focused on IgG, although unbiased protocols to isolate AChR-specific B cells can also yield cells expressing IgM (Blair et al., 1986; Cardona et al., 1994).

Patients with MG have elevated levels of all four subclasses of IgG compared to healthy controls (Rødgaard et al., 1987; Liu et al., 2010) and although anti-AChR antibodies of all four subclasses can be found, subclass distribution within the AChR-specific fraction is also dominated by IgG1, but differs from the global pattern, with a larger than expected representation in IgG3, and smaller in IgG2 (Lefvert et al., 1987; Rødgaard et al., 1987). An IgG1-dominated, IgG2-poor antibody profile is thought to be typical of a T-cell-dependent humoral response against protein antigens (Barrett and Ayoub, 1986). The question of class could also be answered in theory by examining the sequences of immunoglobulin genes in pathogenic B cells. For example, Cardona et al. (1994), by fusing patient B cells with a mouse-human heterohybridoma cell line, screening the supernatants of resulting hybridomas by TE671 cell ELISA (TE671 is a rhabdomyosarcoma cell line which endogenously expresses the alpha, beta and delta subunits of the AChR and can be made to express complete adult AChR by transfection with the epsilon subunit - Beeson et al., 1996), and limiting dilution, obtained 14 stable clones, of which 5 produced IgM and 9 IgG (2 IgG1, 4 IgG2, 1 IgG3, 1 IgG4, and one unspecified). More recently, Rose et al. (2022) described 6 AChR-specific immunoglobulins of which 3 were IgG1, none was IgG2, 1 was IgG3, and 2 IgG4. These results confirm that anti-AChR immunoglobulins of all four subclasses exist, but draw attention to some of the disadvantages of studying single B cells as opposed to serum. Firstly, there is the question of anatomy - both these studies of single B cells used B cells from peripheral blood, but it is possible that the B cells responsible for producing pathogenic antibodies reside elsewhere, for example the bone marrow, the thymus, or in tertiary lymphoid organs. Secondly, there is the question of B cell subtype - each method of B cell isolation has its own bias regarding which type of B cell is targeted. For example, the MACACS method used by Rose et al. is biased towards memory B cells (Callegari et al., 2022), and these may not faithfully reflect the type of B cells that actually produce the pathogenic antibodies. Thirdly, there is the question of numbers. The human anti-vaccine antibody response is thought to involve of the order of 50-400 clonotypes per individual (Wine et al., 2015), and (assuming that the antibody response is somewhat similar in the autoimmune MG context) while this diversity will be evenly sampled by serum studies, the

numbers of AChR-specific B cells so far obtained in single cell studies, with many fewer than fifty published sequences in the entire literature, are so small that only limited inference about the original population of antibodies from which they were taken can be drawn. Finally it should be noted that these studies concern what class of antibody is found in MG, not what class of antibody causes the problem.

Somatic Hypermutation

It is currently not possible to determine the sequences of soluble serum antibodies with enough precision to measure somatic hypermutation, and therefore what we know about this parameter is derived entirely from the analysis of B cell cDNA sequences. The mutational profile across the entire B cell memory repertoire is similar between patients with MG and healthy individuals (Vander Heiden et al., 2017), with around 3% of bases mutated in IgM heavy chains, and 4-7% in IgG and IgA, depending somewhat on the V gene family and the donor. Naive B cells, almost by definition, have zero somatic hypermutation (Klein et al., 1998). Immunoglobulin gene sequences from single IgG B cells with established specificity for AChR have been consistently mutated (see Table 1). Cardona et al. (1995) analyzed the immunoglobulin gene sequences of four of the AChR-specific B cells they had previously described (Cardona et al., 1995) and report mutation frequencies of 5.7 - 8 %. The

Table 1. Properties of patient derived antibodies from each of four publications. Coumn "source" rports the tissue from which the B cells were taken. Column "% nt mut" is the percentage of nucleotides in the VH genes 5′ of the CDR3, the calculatioin of which may vary slightly between publications. The column PTMG indicates whether the antibody induced cmyasthenic signs in a passive transfer model (yes: behavioral signs and complement activation; EMG: electromyographic signs; "combined"- in combination with another antibody.). Blank fields indicate that the data are not provided or not applicable.

author< (year)	source	mAb ID	subclass	% nt mut	subunit epitope	MIR	PTMG?
Kamo (1982)	thymus				not gamma		EMG
Cardona (1994)	blood						
		M1	1	8	alpha		
		M2	2				
		M3	3				
		M4	2				
		M5	2	8			
		M6	2	7.8	alpha		
		M7	4	5.7			
		M8	1				
Makino (2017)	blood	B12L		mutated	alpha	yes	yes
		3B1					
		1G3					
Vrolix (2014)	thymus	131	1	mutated	gamma		
Rose (2022)	blood	2M18	1	5.9	epsilon		
		5H10	1	4.5	delta		
		3I3	3	5.1	beta		
		5D2	4	5.7	beta		
		6J2	4	7.5	beta		combined
		1J7	1	13.2	alpha	yes	combined

antibodies reported by Vrolix et al. (2014), and Makino et al. (2017) were also mutated. Rose et al. (2022) saw mutation frequencies from 4.5 -13.2 % in the six IgG antibodies they described. From these results it appears that the level of somatic hypermutation in the immunoglobulin genes of AChR-specific IgG B cells from patients with MG is typical of the memory B cell pool. Here too, it should be born in mind that the cells that were sequenced may not be typical of the cells that make the antibodies, but the observed mutation pattern can reasonably be interpreted to imply that these B cells developed their affinity for AChR in the context of an antigen-driven, T cell dependent germinal reaction. This raises the question of what the triggering/driving antigen(s) might be (see Table 1).

Epitope specificity

Early efforts to isolate AChR-reactive B cells were directed towards obtaining monoclonal antibodies, to better understand the relationship between serum antibodies and disease (Kamo et al., 1982; Cardona et al., 1994). These included why anti-AChR titers and disease course are so weakly correlated, and why some murine anti-AChR cause disease when passively transferred, while others do not (Cardona et al., 1994). Broadly, the question was "what makes an anti-AChR antibody pathogenic?". Animal experiments conducted in the nineteen eighties suggested that antibodies targeting a small region on the alpha subunit (known as the main immunogenic region or MIR, because of its immunodominance in rats immunized against *Torpedo* AChR - Tzartos and Lindstrom, 1980) are the pathogenic ones. An obvious question was whether this conclusion could be extended to human patients, but the non-availability of patient-derived monoclonal antibodies meant that this question was mostly addressed using studies of patient sera. Sophianos and Tzartos (1989) looked at whether Fab fragments of rat monoclonals directed against the MIR could protect AChR on TE671 cells from internalization-mediated depletion by patient sera. The results showed clearly that they could, while a control rat monoclonal targeted against the beta subunit could not. This result, however, is far from demonstrating that anti-MIR activity is responsible for pathogenicity in patients, because it looked only at internalization and not at, for example complement activation, and (ii) internalization is dependent on cross-linking which is more extensive when induced by anti-alpha antibodies (which have two binding sites per receptor, rather than the single binding site offered by the other subunits). A number of groups subsequently tackled this question in vivo, where several pathomechanisms are expected to operate, and it was demonstrated that monovalent (Fab or IgG4) versions of a MIR-binding antibody can protect an animal against intact IgG1 monoclonals that would otherwise induce severe myasthenic signs (Panastasiou et al., 2000; Losen et al., 2017). These experiments still did not reveal which kinds of antibodies are pathogenic in patients, because they were conducted with an experimentally constructed antibody as the pathogenic agent, rather than with patient serum. When Namkamura et al. (2018) examined the ability of a Fab fragment of the MIR-targeting mAb35, in the polyclonal autoantibody context of experimental autoimmune myasthenia gravis (EAMG), they found that although the Fab could attenuate the antigenic modulation and complement-activating effects of EAMG serum in vitro, it offered no protection against the passive transfer of such serum in vivo.

A more direct approach would be to isolate anti-AChR antibodies from patients, and determine which antibodies are pathogenic, and which not. Table 1 summarizes reports of AChR-binding antibodies isolated from patients by immortalization with EBV, using hybridoma technology, and more recently by single cell molecular cloning. The earliest reported isolation of a monoclonal human anti-AChR antibody was achieved by immortalizing B cells from a patient's thymus with Epstein Barr virus, and limiting dilution (Kamo et al., 1982). The resulting antibody precipitated AChR from innervated human muscle, suggesting that it targeted a non-gamma subunit. The antibody also induced a reduction in the muscle action potentials evoked by sciatic nerve stimulation, which could be partially rescued by edrophonium chloride administration. This was a good demonstration that patient-derived anti-AChR antibody could cause myasthenic signs without other serum components, but very little information was provided about the characteristics of the antibody. Information about class, subunit specificity, and immunoglobulin sequence was provided by Cardona et al. (1994, 1995) for the anti-AChR antibodies that they isolated, but pathogenicity, other than the potential to mediate antigenic modulation in vitro, was not reported. Using EBV immortalization, the Maastricht group isolated a B cell from the thymus of a patient whose IgG was directed against the gamma subunit (Vrolix et al., 2014; Saxena et al., 2017). These authors reported that the anti-gamma antibody induced neither antigenic modulation nor myasthenic signs by in vivo passive transfer. Makino et al. (2017) sorted memory and plasmablast cells from patients and a healthy donor, and labeled antigen-specific cells with recombinant extracellular domain (ECD) of the human nAChR α-subunit directly conjugated with phycoerythrin. They prepared recombinant IgG antibodies from these cells, and tested them by ELISA or by flow cytometry with AChR-expressing cells. Even without pre-screening the

memory B cells with fluorescent antigen, the authors were able to obtain several recombinant monoclonal antibodies from each of five patients that were AChR-specific by the criterion of binding to recombinant ECD in an ELISA assay. However these antibodies all failed the subsequent test of binding specifically to AChR expressed on live cells. This finding, although reported as more of a nuisance by the authors, is significant because methods relying on denatured proteins (antigen arrays and ELISA, to name but two) are commonplace, and may well be misleading in the context of autoantibody research because there is some evidence that pathogenically relevant autoantibodies are likely to be antigen-conformation-dependent, at least in animal models (Krolick et al., 1994). After pre-screening the B cells with a fluorescently labeled alpha ECD, the authors were able to isolate from 6 donors 8 AChR-specific mAbs that passed the more stringent test of AChR-dependent binding to live cells. Among these was one highly mutated antibody, B12L, that competed with mAb35, and induced myasthenic pathology after transfer into rats. This strategy was clearly an effective one for isolating a pathogenic antibody, but not suitable for screening for a wide variety of potentially pathogenic antibodies. The use of the soluble single subunit extracellular alpha domain as a bait antigen not only restricts the screen to alpha-specific antibody, it also rules out those antibodies whose epitope spans more than one subunit, or those whose conformational epitope is dependent on the interaction between the subunits. Rose et al. (2022) used a different technique, named membrane antigen capture activated cell sorting (MACACS) to isolate AChR-specific B cells and, like Makino et al., cloned the immunoglobulin genes from single cells to prepare recombinant antibodies. Resulting monoclonals were discovered that recognized each of the four adult subunits (alpha, beta, delta and epsilon), and, as expected, the anti-alpha monoclonal was the strongest activator of complement in vitro, although none of the antibodies was as strong as the B12L antibody described by Makino et al. (2017), and none of the antibodies induced myasthenic signs when injected into rats at 4 mg/kg. Unexpectedly, several combinations of antibodies were significantly stronger complement activators in vitro than the individual antibodies, and this was also seen in vivo, where 2 mg/kg each of an anti-alpha and an anti-beta induced clear myasthenic signs, while 4 mg/kg of either given alone did not.

From these results, the postulate derived from animal experiments with animal-derived antibodies that anti-alpha antibodies (and in particular antibodies that react with the MIR) are critical for inducing myasthenic pathology currently can be considered valid with patient-derived antibodies, with the caveat that the only tests of "pathogenicity" we have are either in vitro, or else in animal models, and may differ from the situation in patients. However, the observation that combinations of antibodies show emergent properties that were not predicted from the behavior of single antibodies may require some reevaluation of our model of how anti-AChR antibodies induce pathology. The interaction between two independent anti-AChR antibodies is clearly not an absolute requirement for the induction of pathology, because the single anti-MIR antibody B12L described by Makino et al. (2017) alone induces pathology in rats in a manner very similar to the well-studied pathogenic rat monoclonals such as mAb35. Resolution of this difference will require the isolation of a broader range of patient-derived antibodies, and more systematic assessment of their key properties, notably affinity and fine epitope specificity.

What are the phenotypes of pathogenic B cells?

If the pathogenic agent is considered to be soluble anti-AChR antibodies in circulation, then they may well be derived principally from plasma cells, and it might be argued that none of the patient-derived monoclonal antibodies isolated (which very likely all came from memory B cells) came from a directly pathogenic B cell. On the other hand, since memory and plasma cells are thought to derive from germinal centers that produce both (Elsner and Shlomchik, 2020), information derived from one B cell subtype concerning the specificity and affinity of the immunoglobulins involved is likely to be relevant to the entirety of the AChR-targeted humoral attack. Because memory B cells are thought to differentiate into antibody-secreting plasma cells upon secondary antigen exposure (Kurosaki et al., 2015), it is also possible that the memory B cells themselves are a step in the pathogenic cascade. This possibility is supported by the partial efficacy of CD20-depleting therapies in anti-AChR MG (Brauner et al., 2020), which would be expected to deplete memory but not plasma cells. Assuming then that the memory B cells in the blood to which we have access are in some way representative of the pathogenic population, what information could we usefully gain about them? One question is whether their phenotypes and functions are like the "effector-like" memory B cells that develop in response to infections or vaccines, or whether they can (also) exert a "regulatory" or immunosupressive phenotype (Catalán et al., 2021). A second parameter of interest is their age. Very long-lived memory B cells have particular characteristics that could be used to distinguish them from recently generated counterparts (Chappert et al., 2022), and particularly among newly diagnosed patients, this would have implications for the origin of the disease. Valuable insights into these characteristics could be gained

by state-of-the-art single cell techniques, but unfortunately the original phenotypes of the cells are destroyed, or at the least radically disturbed by the processes used thus far to identify them, including hybridoma formation, EBV immortalization, and MACACS. The technique described by Makino et al. (2017), which only requires labeling the cells with antigen is potentially the least destructive, but is restricted to those B cells that recognize a soluble single subunit, at least in the implementation described. The three other techniques have the advantage that they can be used to screen for B cells whose antigen is dependent on the intact structure of the membrane-expressed AChR. It is possible that some hybrid technique exploiting the best features of more than one of the published methods will be required to obtain this kind of non-sequence information about the pathogenic B cell population.

What was the triggering antigen?

The observations about antibody class and somatic hypermutation discussed above suggest that pathogenic anti-AChR antibodies are the result of a T-cell-dependent B cell response against a protein antigen. This raises the question of what this antigen might be. Both the facts that patient-derived antibodies are found against all four subunits, and that they commonly recognize the human AChR but not the closely related rat AChR (Rose et al., 2022) suggest that the antigen must be something very like the human AChR. In the field of myasthenia research, as in studies of other autoimmune diseases, the notion of "molecular mimicry" (i.e., the idea that an antigen from a pathogen is similar enough to the target autoantigen that the immune immune response against the pathogen gets specifically transferred to the autoantigen) is periodically discussed (e.g., He et al., 2018;), and re-surfaced, not surprisingly in view of the immense numbers of infected people and the resources devoted to detecting and documenting the infections, during the SARS-CoV-2 pandemic of 2020-2022 (Ramdas et al., 2022). Molecular mimicry offers a plausible source of initiating antigen in cases of Guillain Barré syndrome associated with *Campylobacter jenuni* infection, because adequately powered studies have demonstrated an epidmeological connection between the pathogen and the autoimmune syndrome (McCarthy and Giesecke, 2001), and an experimentally supported mechanistic model exists to explain the connection (Yuki et al., 2004). However, no such level of evidence supports the hypothesis that a similar mechanism might be involved in MG.

It might of course, be simply the AChR itself that is the initiating B cell antigen. If this were the case, it would demand that even the germline versions of the mutated AChR-specific antibodies would recognize the AChR, which will hopefully become clear as more antibodies are isolated and characterized. That this can happen has been clearly demonstrated in the context of MG with autoantibodies against muscle-specific kinase (Fichtner et al., 2020). Examining three monoclonal antibodies from two patients, these authors demonstrated that although germline versions of these antibodies had significantly (100-fold or more) lower affinity for the autoantigen, they nonetheless demonstrated clearly specific binding. Even the lower affinities of the germline versions were in the nanomolar Kd range that is thought to be relevant for mediating antigen capture and B cell activation (Abbott et al., 2018).

The major question would then be how such B cells could get T cell help for a self protein, and if this could be answered, we might be a long way towards understanding autoimmunity in general. Our favored hypothesis in this regard is the notion of membrane antigen co-capture (Sanderson et al., 2017). If, on the other hand, the affinity of the germline BCR is too weak to enable capture of the mature AChR, other mechanisms must be envisaged that would generate antigens different enough to be immunogenic, but similar enough to lead to autoimmunity. Some possibilities are discussed by Vincent et al. (1998). This line of enquiry would be greatly facilitated by the availability of more patient-derived antibodies, above all members of expanded, mutated clones. Rose et al. (2022) described two members of a single AChR-binding clone, and this offers the particular opportunity to investigate whether, with additional mutations acquired, affinity for the AChR is increased, as would be predicted if the AChR itself is the driving antigen, or decreased, as is predicted by some other models, for example the idea of molecular mimicry (Burnett et al., 2018).

Summary

The study of single, patient-derived, AChR-specific B cells can yield information that is not available from studies of sera. So far, this has been limited to the study of antibodies derived from such B cells, in particular their epitope specificity, their mutational status, and their ability to induce pathology in passive transfer paradigms, and the results have mostly been consistent with hypotheses developed from studies of sera and animal models. So far unexplored is the study of the phenotypes of these pathogenic B cells, outside of their immunoglobulin products.

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Corticosteroids in Generalized Autoimmune Myasthenia Gravis: A Narrative Review

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Corticosteroids are usually considered for treatment of patients with moderate (*i.e.* class III of the Myasthenia Gravis Foundation America (MGFA) classification), severe (i.e. Class IV), or mechanically ventilated (i.e. Class V)(1) generalized autoimmune myasthenia gravis (MG) that is not controlled by cholinesterase inhibitors (i.e. pyridostigmine)(2, 3). It is usually recommended to ombine prednisone with an immunosuppressant(2, 3), as prednisone will allow relatively rapid control of MG, and the immunosuppressant will allow prednisone tapering without destabilizing the MG. About 80% of individuals with MG are responsive to prednisone(4), irrespective of age and time from MG onset. Prednisone tapering is necessary to avoid corticosteroid side-effects, which are reported in up to 65% of cases (4) depending on its cumulative dose(5). Patients with MG develop Cushingoid symptoms in 30% of patients; weight gain, diabetes, or hypertension in 15%; and bone fracture in 5%(6). Therefore, if the Scylla of prednisone tapering is MG exacerbation, its Charybdis is side effects from continued long-term use. The therapeutic importance of prednisone tapering is supported by the fact that cumulative or final doses of corticosteroids have been considered the primary endpoint of major clinical trials along with MG clinical control(7–11). Rationally, discontinuation of steroids depends on the tapering regimens and on the efficiency of the associated immunosuppressive agent.

There are various means of administering prednisone(12). The most common method proposed in the literature consists of gradually increasing the dose up to 0.75 mg/kg on alternate days and progressively reducing it when the minimal-manifestation status (MMS) is reached(7, 13). Therefore, this increase/tapering strategy was used in two cornerstone randomized controlled trials in which high and prolonged corticosteroid treatment were reported (30 and 20 mg, at 15 and 36 months) (7, 13). Historically, this tapering regimen was initially developed for the trial on the benefit of Azathioprine (13), in 1992, and it was used much later in the thymectomy trial, in 2016(7). We have conducted the multicenter single-blind randomized MYACOR trial to determine whether faster tapering could be achieved in azathioprine-treated generalized MG in comparison with the referent tapering regimen(14) (Table 1). Our rapid-tapering regimen consisted of immediate high-doses of prednisone, daily intake but also rapid or slow-decrease when MMS or improvement was attained (Table 1). MMS attainment without prednisone at 12 months and without relapse or prednisone reintroduction at 15 months was the primary outcome. We found that the proportion of patients who met the primary endpoint was higher in the rapid than in the referent-tapering arm (39 % *versus* 9%) presenting a risk ratio of 3.61 (95% IC [1.64-7.97], $P<0.0001$), after adjusting for center and thymectomy. The reduction of the cumulative dose was 1828 mg (95% CI, -3121 to -461 mg), which corresponds to a clinically relevant sparing of 5 mg per day. Such sparing is particularly important when the daily dosage falls below 20 mg, which is a turning point in our experience with prednisone tapering. The MYACOR trial provided two other interesting findings. First, the rate of serious adverse events (SAEs) was twofold lower than previously reported (6) and did not differ statistically between the two regimens. Infection (10%), diabetes (5%), and osteoporosis (2%) were the most frequent side effects. This indicates that prevention of prednisone side effects has dramatically improved within the last three decades. Second, azathioprine was more efficient than previously reported(13). In the trial by Palace and colleagues, this reduction was not apparent until the eighteenth month and did not become significant prior to the thirty-sixth(13). More than fifty percent of participants were still treated with corticosteroids after one year(15). Since the patients' characteristics and administration of azathioprine were comparable, only the faster tapering of prednisone in the MYACOR trial can account for such a corticosteroid-sparing effect of azathioprine.

The corticosteroid-sparing effect has been assessed with other immunomodulating interventions other than azathioprine. Because it has always been tested against placebo and because of the methodological discrepancies between trials, their corticosteroid-sparing potential cannot be specified. The MGTX trial(7) demonstrated that thymectomy significantly reduced the dose of prednisone at three years, with an average alternate-day prednisone dose of 32 mg (i.e., 16 mg/day). This remaining high dose of prednisone can result from the fact that its tapering was too slow but also from the fact that only 17% of the thymectomized patients had been treated with azathioprine.

Table 1
Tapering regimens

Prednisone	**Slow-tapering regimen**	**Rapid-tapering regimen**
Initial dose	Started 10 mg, then increased by increments of 10 mg every two days up to 1.5 mg/kg (without exceeding 100 mg)	Immediately started at 0.75 mg/kg/day (without exceeding 100 mg)
Intake	Alternate Day	Daily
Tapering criteria	MMS	1) MMS 2) Improvement status
Tapering protocol	1) **<u>If MMS reached</u>** : reduction by 10 mg every 2 weeks until 40 mg, then reduction by 5 mg every month until 0 mg 2) **<u>If MMS not maintained</u>** increase by 10 mg every 2 weeks until MMS , and then tapering as described in 1)	1) **MMS reached at one month :** reduction by 0.1mg/kg every 10 days until 0.45 mg/kg/day, then 0.05 mg/kg every 10 days until 0.25 mg/kg/day, then in decrements of 1 mg every 7 to 15 days 2) **Improved status at one month :** decrease by 0.1 mg/kg every 20 days until 0.45 mg/kg/day then 0.05 mg/kg every 20 days until 0.25 mg/kg/day then 1 mg per kg every 7 to 15 days If <u>MMS is achieved</u>, then tapering is similar to sequence 1). 3) **If MMS and improvement not reached** 0.75 mg/kg maintained for the first 3 months, followed by decrease of 0.1 mg/kg every 20 days until 0.45 mg/kg/day, then by 0.05 mg/kg every 20 days until 0.25 mg/kg/day at 20 days. No further reduction. If <u>improvement is attained</u>, the tapering follows the sequence described in 2)
Severe Side-effects	can be decreased as described in 1)	can be decreased as described in 1)
MG exacerbations	1) Severe: prednisone is doubled 2) Moderate: increase to the previous dose 3) ± IvIg and PE	1) Severe: prednisone is doubled 2) Moderate: increase to the previous dose 3) ± IvIg and PE

Abbreviations : MMS : minimal manifestation status ; IvIg : intravenous immunoglobulins ; PE : plasma exchange

Using the slow-tapering regimen, a very recent open-labeled, randomized trial showed that the 15 month-cumulative dose of prednisone was significantly lower in patients with generalized MG treated with methotrexate(11). Mycophenolate mofetil (MMF) treatment was not superior to placebo in maintaining MG control during a 36-week schedule of prednisone tapering(16). Cyclosporine has also been shown to stabilize MG and to significantly reduce prednisone dosage(17). The corticosteroid-sparing effect of cyclophosphamide has not been reliably assessed, to our knowledge. It must be emphasized that cyclophosphamide, methotrexate, and cyclosporine are usually considered a therapeutic option in refractory MG, although calcineurin inhibitors are considered to be first-line immunosuppressive agents in Japan. It is too early to anticipate how new monoclonal antibody therapies will challenge the position of azathioprine and MMF as first-line immunosuppressants. The cost of these new therapies could preclude their use in a large number of countries.

The corticosteroid-sparing effect of rituximab was initially supported by a retrospective cohort study(18), then recently assessed in two placebo-controlled double-blind randomized trials (i.e. BeatMG Study and RINOMAX trial), with contradictory conclusions(8, 9). The BeatMG study showed that rituximab does not significantly increase the proportion of patients who achieve at least a 75% reduction in prednisone dose at 12 months, with tapering being gradually carried out after 8 weeks but only after confirming that MG symptoms were at least stabilized(9). The RINOMAX trial (8) reported that a single dose of rituximab increased the probability of minimal MG manifestation with less than 10 mg of prednisone at 4 months, given that prednisone was recommended to be reduced up to 8 mg/day at two months. Anti-MuSK MG might be more responsive to rituximab, notably in terms of corticosteroid-sparing(19).

In recent decades, double-blind randomized clinical trials against placebo have shown that new therapies that target complement (i.e., Eculizumab and Zilucoplan) or the neonatal-FC receptor (i.e., Efgartigimod, Batoclimab, and Rozanolixizumab) are effective in controlling MG(10, 20–22). However, the corticosteroid -sparing effect has not been assessed in any of these trials, as prednisone tapering was not allowed during the study period. Finally, intravenous immunoglobulins are not more effective than placebo in reducing corticosteroid dose by 50% at 10 months in adult patients with generalized MG(10).

In conclusion, tapering of prednisone remains a challenge in the therapeutic management of patients with generalized MG, as it is an effective treatment but associated with multiple side effects that prompts determination of its minimal effective dose as soon as possible. Azathioprine and rituximab allow rapid tapering. The corticosteroid-sparing effect of newer therapies must be assessed and compared to azathioprine and rituximab. Given their effect on MG status, it is conceivable that these new treatments will enable a dramatic reduction or even complete discontinuation of corticosteroids. On the other hand, one may argue that treatment with azathioprine and prednisone is effective and well-tolerated in the majority of patients with generalized MG, and also not expensive. Only a clinical trial will determine which immunosuppressant is the most clinically and corticosteroid-sparing agent.

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"Pediatric Myasthenia Gravis", as presented at the MGFA 14th International Conference in Miami, Florida on May 11, 2022.

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ABSTRACT

Pediatric myasthenia gravis (MG) is a relatively rare, but very treatable condition. Prognosis in pediatric myasthenia gravis is favorable for minimal manifestation status (MMS) or remission when compared to adults.[1] Ocular only presentations are more common, though severe refractory generalized MG presentations also occur.[2] An observational examination is key to the diagnosis and follow-up of pediatric MG patients in the clinic setting.[3] Treatment options are limited by side effect and growth considerations, as well as lack of approved MG medications in the pediatric population. Multidisciplinary care should be considered for pediatric MG, as what is common with other neuromuscular conditions seen in specialty care settings.

Key words: pediatric myasthenia gravis, juvenile myasthenia gravis, JMG, pediatric MG

Introduction

Pediatric myasthenia gravis (MG) is thought of as a rare condition. The estimated incidence of myasthenia gravis (adult and pediatric combined) is 3-30/100,000 cases annually. In children, the incidence is estimated to be 1-5/1,000,000 cases annually.[4] In routine practice, it is important to recognize this treatable condition in the pediatric population. Time to treatment is especially important when MG presents early, as later disability can be prevented with a higher chance or remission of symptoms.[1] If the diagnosis of pediatric MG is recognized early and providers become familiar with this diagnosis, this can spur further referrals to the appropriate teams and specialists.

Diagnosis and first presentation of pediatric MG: What do you look for in children?

There is a bimodal distribution in the onset of myasthenia gravis, with increased incidence in younger ages, peaking in the second decade of life, and another peak in the sixth decade of life.[3] Juvenile myasthenia gravis is defined as onset of myasthenia gravis before 18 years of age[5], though some studies place this cut off at 19 years of age.[6]

JMG age of onset can be as young as 12-24 months, up to an adolescent onset. In a Mayo Clinic cohort study of 364 children, median age of onset for JMG in a population including Lambert Eaton Myasthenic Syndrome (LEMS), JMG, and congenital myasthenic syndrome (CMS), was age 5.1 years.[6] For onset in the pre-pubertal ages, there is a prevalence of more ocular cases[4] and a higher chance of spontaneous remission and minimal manifestation status (MMS) when compared to adults.[1] Ocular manifestations can present initially as alternating ptosis, double vision, or blurred vision only in pre-pubertal children.[7] For post-pubertal MG diagnoses, onset is more likely to be generalized, and can present more like the adult-onset MG. Initial generalized presentations are rare in pre-prepubertal children and interpretation of these symptoms may be more challenging.[8] There is a racial predilection of JMG to the non-white population, which includes Asian, Black, and Hispanic patients, the latter two groups with more refractory cases of generalized myasthenia gravis (gMG).[2,5]

Presentation of pediatric MG

Many pediatric MG presentations occur with ocular symptoms of asymmetric ptosis and variable ophthalmoplegia,[5] with the main differential diagnoses being congenital myasthenic syndrome (CMS) and transient neonatal myasthenia. Initial presentations of pediatric MG can be in myasthenic crisis with need for inpatient hospitalization in the pediatric intensive care unit (PICU) for intubation needs. The frequency of myasthenic crises in JMG is unknown but accounted for 10% in population-based cohort study of JMG in Norway.[9] More subtle presentations include ptosis that may have gone unnoticed by the family until the ptosis worsens, or a unilateral onset becomes bilateral ptosis. Fluctuation of symptoms may be difficult to ascertain due to the age of the patient.

Chief complaints often include observations specifically regarding fluctuating ptosis or ophthalmoplegia with dysconjugate gaze. This may be an observation by the parent or the teacher. Other parents may note that their child tires more easily than usual with improved energy in the mornings or after a nap. Other times, the patient is referred directly for an evaluation for MG based on an eye examination at the optometrist or ophthalmologist.

For ocular onset in pediatric MG, it is common for the presentation of ptosis to be asymmetric. This can be a unilateral or bilateral onset. If unilateral, ptosis may become bilateral over time. There is often concern for strabismus from optometrists or ophthalmologists if previously evalu-

ated,[10] though this is a fluctuating ophthalmoplegia. Ocular onset can be isolated to the eyes, or present with subtle generalized symptoms.

For generalized onset in pediatric MG, the areas affected can vary on presentation. This can vary from primarily ocular symptoms with very mild proximal limb symptoms, a bulbar presentation, or a true generalized presentation with all areas affected. It is difficult to ascertain and test the fatigability of these areas in clinic based on the age of the patient, so much of the information on disease onset and progression is dependent on parent and other observer history.

Pertinent history to obtain in pediatric MG

The initial evaluation of pediatric MG includes noting the characteristic fluctuation of symptoms. Younger patients present with acute behavioral problems such as more temper tantrums due to fatigue and the inability to express their symptoms. Also, reviewing video and pictures is helpful. Noting symptoms before and after school, or during homework time in the evenings is helpful. Status in the mornings vs. afternoons and status after naps with regards to symptoms are helpful. Chewing, swallowing, or vocal quality in the evenings around dinnertime are also important to ascertain. Other instances to ask include how the patient does with physical education (PE) classes if they attend, as well as how the patient does in hot weather vs. cold weather.

An autoimmune history must be taken as part of a past medical history evaluation, as pediatric MG often co-occurs with other autoimmune conditions such as diabetes type I, celiac disease, and thyroid disease. Just as important is a careful family history to include autoimmune history, as older family members may have thyroid disease, rheumatoid arthritis, or systemic lupus erythematosus, for example. It is more often to find this history in new diagnoses of pediatric MG, as there is likely a genetic contribution for autoimmune susceptibility.

A careful review of systems can reveal additional information needed, such as vision difficulties such as diplopia, or observation of a new "lazy eye" that was not previously present.[10] There may be report of refusal to walk due to leg or muscle pain, or increased fatigue and desire to rest or sleep.

School and social history can reveal if there has been any impact or change in daily school activities, such as in-person or remote class performance. During the pandemic, the patient's face on screen may demonstrate ptosis or ophthalmoplegia that is visible to the teacher and parent. PE performance may suffer if more fatigue is present, especially if the child participates in timed physical testing. Homework may be challenging if there is eye or muscle fatigue.

Considerations and workup in pediatric MG:

There is a broad differential for pediatric MG given the younger age of patients and other conditions with similar presentations. This includes congenital myasthenic syndromes (CMS), which are genetic in etiology and involve dysfunction at the neuromuscular junction.[3] Congenital myopathy can present with facial weakness and fatigable proximal limb weakness, but often does not have a diurnal or fluctuating pattern of weakness. Mitochondrial myopathy and mitochondrial-related conditions can have onset with significant weakness and fatigability. Chromosomal conditions can appear like pediatric MG but may also co-occur in MG. In the author's clinical practice, there are at least two 22q11 chromosomal abnormality (non-DiGeorge) patients with confirmed co-occurrence of autoimmune MG, presenting similarly with generalized symptoms post-pubertally. Birk-Barel Syndrome (heterozygous KCNK9 mutation on 8q24.3) is considered a chromosomal cause of neuromuscular dysfunction and is treated with Mestinon as part of standard practice. Developmental anomalies can also look like a pediatric MG presentation, such as congenital ptosis or congenital cranial nerve abnormalities (ex. congenital cranial nerve VI palsy, or Duane syndrome).[2]

The workup in pediatric MG, given the broad differential, not only includes autoimmune antibody testing for the binding, blocking, and modulating antibodies to the acetylcholine receptor (AchR) and muscle specific tyrosine kinase (MuSK), but also can include genetic testing for congenital myopathy or congenital myasthenic syndromes. For primarily ocular or bulbar presentations of pediatric MG, magnetic resonance imaging (MRI) of the brain can be considered to rule out cranial nerve abnormalities, perinatal injury, or structural developmental issues. Contrast can be added if there is suspicion for an intracranial autoimmune component; for structural reasons only, the study can be done without contrast.

A broad autoimmune workup can be pursued as directed by family history, to include blood counts, inflammatory markers, and rheumatologic markers as needed. Testing for autoimmunity may be sensitive of an autoimmune process, but not specific. The acetylcholine receptor (AchR) antibody panel would be most specific and helpful in the initial evaluation of patients.

Examination techniques in children for pediatric MG:

Pediatric neurologists often must rely on the observational examination for their patients, and the same applies in the evaluation of a young patient undergoing

evaluation for pediatric MG. General examination of the patient starts when the provider enters the room and even while speaking with the patient's parent or caretaker.

In general examination, often what is observed is spontaneous movement. Is there any antigravity movement of proximal muscles? Is there asymmetry of movement between the upper and lower extremities? Is the child not moving or playing as expected for age?

In the eye examination, using a screen (tablet, phone) or a favorite toy is very useful in maintaining sustained gaze. Fatigable ptosis and ophthalmoplegia can be examined in this manner. Aversion of gaze can indicate fatigue or diplopia.

For the arm examination, overhead movements are important to assess, so reaching for objects such as toys or giving high fives are important to test and observe. For the leg examination, watching the movements in the room (ex. climbing, jumping, rising from the floor) is just as important as attempting formal manual muscle testing. A Gower maneuver can be tested in this population as part of the observational examination.

Examination in older children and adolescents is closer to the adult examination for evaluation of MG. For patients who can cooperate, fatigable examinations are important to distinguish ocular only vs. generalized symptoms, as well as to track treatment progress over time. In addition to sustained upward gaze; arm thrusts, repeated ten times, with testing of shoulder abduction before and after, can be done for arm muscle fatigability, and deep squats, repeated ten times, with testing of hip flexion before and after, can be done to assess leg fatigability.

Examinations can be tracked over time with validated measures such as the Myasthenia Gravis Composite (MGC), the Myasthenia Gravis Activities of Daily Living (MG-ADL), and Myasthenia Gravis Quality of Life (MG-QOL) scores. These have only been validated for the adult population. A pediatric QOL score, the Pediatric Myasthenia - Quality of Life 15 (PM-QOL15) has demonstrated correlation with the MGC in a JMG cohort. [11]

Treatment options for pediatric MG

For management of ocular MG, pyridostigmine treatment alone is a popular and very acceptable treatment for parents facing a new diagnosis in their child. Based on updated consensus guidelines, a trial of low dose steroids in combination with pyridostigmine can be used for more symptom control in ocular pediatric MG.[12]

If the initial onset is generalized, thymectomy should be discussed early.[12] This can even be at the first appointment. Earlier thymectomy may result in reduced medications needs in the future, earlier chance for remission or MMS, and avoidance of NMJ destruction. In addition to thymectomy, discussion of low-dose steroid initiation should also be had. In pediatric MG treatment, steroid doses are not pushed to high doses as they are in adult MG treatment.

Steroids continue to be mainstay of treatment in pediatric MG, though weight gain, acne, decreased bone density, reduced growth velocity, and behavioral changes are specific considerations in treatment of pediatric MG. These are all undesirable side effects for children, especially adolescents. The dose range to aim for is low relative to typical adult dosing: starting 5 to 10mg daily, titrating to no higher than 20mg daily.

For refractory generalized pediatric MG, there are limited steroid sparing therapy options due to lack of pediatric data for these medications in MG.[13] Azathioprine and mycophenolate mofetil have been used in personal practice, but for adolescent patients only due to lack of safety and efficacy data in younger children. The topic of contraception for steroid sparing agents is a necessary discussion and often parents and patients do not agree to an additional prescription as a requirement of treatment.

It is for this reason that intravenous immune globulin (IVIG) and plasma exchange therapy (PLEX) have been used both as acute and chronic treatments for pediatric MG. Chronic IVIG has become one of the widely used treatment options and is approved for use in pediatric MG.[13]

An advantage of IVIG is that it works quickly, has no immune suppression concern, and is an alternative to the oral chemotherapeutic agents with their specific side effect profiles. Unlike steroid therapy, IVIG is weight neutral, and has no effect on growth or teratogenicity.

During the COVID-19 pandemic, IVIG has become one of the treatments of choice for refractory pediatric gMG. There are home infusion options available. There is no immune compromised state and may confer additional protection against infection.

There are ongoing pediatric clinical trials for complement inhibition agents, interleukin-6 antagonists, and neonatal Fc receptor antagonists. However, these treatments are only available by enrolling in a clinical trial. Refractory pediatric MG patients can receive these newer adult gMG approved agents only on an off-label basis.

Thymectomy in pediatric MG

There have been conflicting recommendations in the past regarding thymectomy in MG in general, and especially for pediatric MG patients. There is a now suggestion for thymectomy in the updated guidelines for juvenile MG patients <18 years of age when no suggestion was previously given.[12] The recommendation, however, is stronger for adult patients >18 years and if a thymoma is present, which is

often not the case in pediatric MG. For pediatric MG, thymic hyperplasia is more commonly seen.

Regarding thymectomy in juvenile MG, cohort studies and case series reports have reported favorable outcomes for improvement in symptoms, remission, with low rate of post-operative complications.[14] Thymectomy is recommended in juvenile MG <1 year from onset, so-called "early thymectomy."[15] More than 90% of patients treated with early thymectomy had favorable outcomes, while thymectomy in patients aged <10 years should be performed in specialized centers due to difficulty of perioperative management.[16]

In the author's experience, thymectomy early in the disease course in pediatric MG reduces medication need within 1-2 years after the surgery, hastens and improves the chance for remission or MMS, and results in improved MG-specific scales over time such as the MG-Activities of Daily Living (MG-ADL), and the MG-Quality of Life (MG-QOL). These scales are regularly obtained as part of our clinical practice. Improvements after thymectomy in the adult population as stated have been described for adult MG patients more consistently in the medical literature.[12]

For pediatric MG, there is ongoing work for pediatric-specific validated measures such as the PM-QOL15 to track clinical status longitudinally in this population.[11] Use of the MGC, MG-ADL, and MG-QOL, which are validated only for adults, has been used currently in clinical practice and in ongoing clinical trials in pediatric MG.

Prognosis and management in pediatric MG:

Because there are higher rates of clinical remission or MMS in the pediatric MG population,[1] it is a reasonable goal to aim for minimal or no treatment in the management of pediatric MG. In the author's experience, weaning off chronic IVIG would involve spacing IVIG dosing from every 4 weeks in 2-week intervals, to every 8-10 weeks then discontinuing infusions. This is in line with protocols used at other institutions, in which frequency of chronic IVIG or SCIG for adult MG patients is done at the clinician's discretion.[17] Patients can be weaned to very low dose or completely off steroid therapy with only once daily or as needed pyridostigmine treatment. Some patients remain on steroid sparing agents alone, such as mycophenolate mofetil alone with minimal symptoms.

For chronic symptoms or refractory patients, continued treatment escalation, as in adults, can be tailored for each patient. This may mean more frequent IVIG, up to every 2 weeks, higher doses of steroids or steroid sparing agents, or discussion of rituximab therapy or off-label therapy with new FDA approved adult medications (eculizumab, ravulizumab, efgartigimod).

However, also in the pediatric MG population, the contribution of functional neurologic disorder must also be considered. There is incidence of anxiety, depression, and adjustment disorder due to having childhood onset of a chronic medical condition. Functional neurologic symptoms may masquerade as MG symptoms or MG exacerbation symptoms. Examples include functional ptosis (non-fatigable), globus sensation with subjective dysphagia without aspiration, and give-way weakness with generalized non-fluctuating body and mental fatigue.

In summary, goals of care are to have the pediatric MG patient feel normal and equal in their abilities to their similarly aged peers.

Multidisciplinary care in pediatric MG:

Pediatric MG is not a condition treated in isolation only by the neurologist or neuromuscular specialist.

At Children's Hospital Los Angeles (CHLA), we have a dedicated multidisciplinary team for treatment of pediatric MG, that meets with patients on an annual basis to help with chronic management of pediatric MG. In between, patients are seen in our neuromuscular clinic on average, every 3-6 months depending on clinical status and follow-up needs.

The multidisciplinary care at our institution includes a neuromuscular specialist, (MD or DO), and a neuromuscular nurse care manager (NCM, usually RN level) who coordinates care. Pediatric neuromuscular trained physical therapists and occupational therapists aid in energy conservation techniques and adaptations and recommendations for school and home tasks. A registered dietitian familiar with neuromuscular conditions and consequences of steroid therapy can help with weight management and healthy eating. Lastly, a clinical social worker, familiar with neuromuscular patient needs is part of the clinic to address financial, mental health, and other school and social needs.

The above team can also be found in multidisciplinary muscular dystrophy clinics. This team composition and care coordination is intentional. MG patients have many of the same needs as patients with muscular dystrophy or similar disorders, with exclusion of cardiology and pulmonology evaluation to streamline the clinic.

Based on patient report, this is a very well-liked and valuable clinic, and has resulted in tracking of MG-specific scores (MG-ADL, MG-QOL) and neuromuscular testing such as the 6-minute-walk test (6MWT) and 10-minute walk/run test. While the MG-ADL and MG-QOL are validated only for adults, they are not formally validated for children and adolescents. We still can use these data in the older children who are able to participate or perform partial testing and make note of this across examinations.

A bedside swallow test can substitute for bulbar function items on testing, and a "slurp test" can also be utilized quickly in the clinic setting.[18]

Patient education is done continuously in our clinic, and reputable sources such as the Myasthenia Gravis Foundation of America (MGFA), have been introduced via their website at myasthenia.org, as well as informational physical brochures and handouts to inform patients and parents regarding myasthenia basics as well as specifics on medications recommended at visits.

Summary

Onset of MG in the pediatric population is quite varied, so a careful observational examination is key especially for younger patients who cannot fully cooperate. Thymectomy is recommended early in pediatric MG to improve outcomes such as in reduced medical medication needs and chance for remission of symptoms. Treatment options can be quite limited due to side effect profile of steroids and non-steroidal steroid sparing agents, as well as approval of newer agents limited to adult gMG patients. IVIG and PLEX are maintenance options for pediatric MG. Multidisciplinary care and social considerations in pediatric MG can be practiced as standard of care and is quite helpful to patients and their caregivers. Overall, the treatment goal in pediatric MG is "to feel normal."

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