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► **To cite this version:**

Maartje Huijbers, Alexander Marx, Jaap Plomp, Rozen Le Panse, William Phillips. Advances in the understanding of disease mechanisms of autoimmune neuromuscular junction disorders. *The Lancet Neurology*, 2022, 21 (2), pp.163-175. 10.1016/S1474-4422(21)00357-4 . hal-03563023

**HAL Id: hal-03563023**

**<https://hal.science/hal-03563023v1>**

Submitted on 7 Feb 2023

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## Advances in the understanding of disease mechanisms of autoimmune neuromuscular junction disorders

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**Declaration of interests:** MGH and JJP are co-inventors on patent applications based on MuSK-related research. MGH, JJP and LUMC receive royalties from these patents. LUMC receives royalties from TECAN/IBL. All other authors report no disclosures.

**Author contributions:** All authors wrote, revised and approved the manuscript. All authors contributed equally.

**Funding sources:** MGH receives financial support from the LUMC (OIO, 2017), Top Sector Life Sciences & Health to Samenwerkende Gezondheidsfondsen via the Target to B! consortium (LSHM18055-SGF), Prinses Beatrix Spierfonds (W.OR-17.13 and W.OR-19.13) and the Dutch Science Organization NWO (VENI 0915016181 0040). MGH and JJP are members of the Netherlands Neuromuscular Center and the European Reference Network for rare neuromuscular diseases EURO-NMD. MGH and JJP are co-inventors on two patent applications on MuSK-related research. LUMC, MGH, JJP receive license income from these patents. LUMC receives royalties for a diagnostic MuSK ELISA. RLP receives financial support from the “Association Française contre les Myopathies” (AFM). WDP received financial support from the Lambert Initiative for Cannabinoid Therapeutics and Bridging Grants from The University of Sydney (no grant numbers available). Funders had no role in the writing of this review.

### Abstract

Autoimmunity at the neuromuscular junction (NMJ) can be classified into three categories based on the location of the antigenic targets. Autoantibodies can be directed at: 1) post-synaptic components of the NMJ, defined as myasthenia gravis (MG), 2) a pre-synaptic component of the NMJ, defined as Lambert-Eaton myasthenic syndrome (LEMS), and 3) an unknown component of the NMJ, classified as seronegative MG or LEMS. All these diseases are hallmarked by fatigable muscle weakness. Although a plethora of (immunosuppressive) treatments exists, no cure is available to date and many patients are left with debilitating muscle weakness. Recent advances in understanding the physiology and structure of the NMJ have been instrumental in unravelling the pathophysiology of these autoimmune diseases, and provide the rationale for developing new therapeutic strategies. Here, we review current understanding of NMJ physiology and the latest paradigms around autoimmune NMJ disease mechanisms. Furthermore, we highlight exciting new directions in the field, such as

development of novel *in vitro* cellular model systems for human NMJs and single-cell analyses of pathogenic autoantibody producing lymphocytes.

## **Introduction**

Early recognition in the 1970s of the acetylcholine receptors (AChR) at the neuromuscular junction (NMJ) as the major auto-antigen in myasthenia gravis (MG), together with the easy experimental accessibility of the NMJ, made AChR(+) MG a paradigm for study of antibody-mediated autoimmunity. Recent insights into the aetiology and pathophysiological mechanisms of autoimmune NMJ disorders have improved diagnosis for the patients (see accompanying review by Punga et al. (1)) and stimulated the development of novel therapeutics (see accompanying review by Verschuuren et al. (2)).

This review provides state-of-the-art insights on pathogenic mechanisms of antibody-mediated diseases of the NMJ, focusing on research progress in the last 5 years, based on key publications. We discuss recent insights into immunological and neurophysiological disease mechanisms and relevant technological advances, such as single cell RNA sequencing, new animal models and neuromuscular organoid cultures.

## **Structure, physiological function, and maintenance of the neuromuscular junction**

Correct skeletal muscle function is essential for body posture, movement and respiration. Each motor neuron controls the contraction of multiple muscle fibres, together forming a motor unit (Figure 1A). Structure and function of the NMJ, the synapse through which a motor axon interacts with a muscle fibre, is summarized in figure 1B. Neuromuscular transmission depends upon a series of essential processes, including the opening of pre-synaptic voltage-gated calcium channels (VGCCs), subsequent release of acetylcholine (ACh) and its binding to the closely packed post-synaptic AChRs, causing their opening (figure 1B). Spontaneous release of single ACh quanta produces brief depolarizations of approximately 0.5-1 mV, i.e. miniature endplate potentials (MEPPs), which by themselves have no known physiological function. Approximately 30 quanta are released simultaneously in response to a nerve action potential. This produces an endplate potential (EPP) of ~20-30 mV, which amply exceeds the firing threshold, triggering a post-synaptic action potential which induces muscle fibre contraction (3). The post-synaptic action of ACh is terminated by its hydrolytic breakdown by acetylcholine esterase (AChE) in the synaptic cleft. In myasthenic diseases of the NMJ, the safety factor for neuromuscular transmission (the degree by which the EPP exceeds the threshold (4)) becomes reduced. During tetanic contractions, ACh release is subject to some degree of physiological rundown. In MG, this results in progressive failure of muscle fibre activation/contraction, and thus fatigable muscle weakness (3).

Two post-synaptic mechanisms help establish and maintain the integrity of the NMJ. Signalling mediated by muscle-specific kinase (MuSK) is synapse-promoting, driving specialization of both the post-synaptic (directly) and pre-synaptic structures (indirectly). MuSK is a transmembrane tyrosine kinase that acts locally to stabilize post-synaptic clusters of AChRs (5). Agrin, released by the motor nerve terminal, binds low-density lipoprotein receptor-related protein 4 (Lrp4), which then forms a complex with MuSK (figure 1B). This causes dimerization and activation of the MuSK kinase and leads to recruitment of cytoplasmic proteins such as downstream of tyrosine kinase 7 (Dok7) and rapsyn, ultimately inducing AChR clustering. The cluster site seems to be determined by a localized high level of MuSK expression (5). Lrp4 serves an additional pro-synaptic role by acting back on the motor axon to induce pre-synaptic differentiation (6). Another important positive regulator of MuSK is Dok7. By binding cytoplasmic domains of MuSK, Dok7 increases the kinase activity, stimulating AChR clustering and thereby increasing NMJ size and EPP amplitude (7). A second developmental/maintenance system opposes the MuSK signalling pathway. It is driven by ACh, which induces a weak calcium signal entering via AChRs. This signal is amplified by calcium release mediated by inositol tris-phosphate receptors located immediately beneath the post-synaptic membrane (8). Activated caspase-3 then acts on the MuSK complex to de-stabilize AChR clusters and suppress NMJ function.

Interference with any of these critical processes can result in myasthenia. Autoimmunity against post-synaptic factors causes MG, while autoimmunity targetting pre-synaptic VGCCs causes Lambert-Eaton myasthenic syndrome (LEMS).

### **Immunological dysregulation**

MG and LEMS are multifactorial diseases with subtypes based on their clinical presentation and antibody status (table 1). Development of MG may depend on genetic predispositions found on HLA genes and other genes (9), epigenetic factors including miRNA dysregulation (10), female gender (9, 11), and immune dysregulations (12) (table 1). MG and LEMS can also be of paraneoplastic origin typically with thymoma in MG and small-cell lung cancer (SCLC) in LEMS.

#### *Role of the thymus*

In early-onset AChR(+) MG (EOMG), the thymus shows lymphoid follicles and germinal centers, i.e. thymic follicular hyperplasia (table 1, figure 2), associated with active neoangiogenesis processes and the overexpression of chemokines as for tertiary lymphoid organs (13). This indicates abnormally active immune responses in the thymus. In MG, the thymus represents: i) the start site of autoimmunization, ii) the place where AChR antibodies are produced, and iii) the origin of autoreactive T, B and plasma cells spreading to the extrathymic immune system to perpetuate EOMG there, even after thymectomy (14, 15). In line with this, the 2015 MGTX thymectomy trial (an international, multicentre, randomized, controlled study of therapeutic thymectomy in 126 MG patients aged 18 to 65 years) demonstrated improved clinical outcomes (e.g. lower quantitative MG scores and reduced need of prednisone and azathioprine) in MG patients after thymectomy. This further emphasized the key role of the thymus in non-thymomatous AChR(+) MG (15). Female predominance in most autoimmune diseases may be explained by estrogens that reduce the transcription in the thymus of the autoimmune regulator (*AIRE*) gene and its targets, (e.g. *CHRNA1*: the gene encoding the AChR  $\alpha$ -subunit (16)). Reduced thymus expression of such genes prevents proper tolerization and may result in autoimmunity.

Thymoma-associated MG (TAMG) generally is a subtype of AChR(+) MG (17). Most patients exhibit an AB, B1 or B2 thymoma. Almost all thymomas produce and pre-prime AChR autoreactive T cells, but mature CD4+ T cells leave the thymoma only in TAMG patients, providing help to AChR-directed B cells in the extra-tumorous immune system, including the remnant thymus (18). Three recent studies showed a higher proportion of T follicular helper cells (19), B cells and CXCL13 (20), and germinal centers (18) in the tumour-adjacent thymic tissue of TAMG patients. Of note, LEMS is an exceptionally rare complication among the many autoimmune diseases that thymomas can be associated with (21).

Late-onset, non-thymomatous AChR(+) MG (LOMG) mostly occurs in males (table 1). The thymus shows normal-for-age atrophy, but the presence of anti-striational and anti-cytokine autoantibodies, normally associated with TAMG, strongly suggest a role of the thymus in LOMG (17). Specifically, the atrophic thymus and thymomas share paucity of muscle-like myoid cells and *AIRE*(+) thymic epithelial cells. Myoid cell deficiency is thought to contribute to generation of muscle-directed autoantibodies, while the paucity of *AIRE*(+) cells might elicit anti-cytokine autoantibodies (17). The mechanisms remain to be resolved.

In MuSK(+) MG, thymic pathology is rare (22) and likely of minor relevance. In a multicentre, retrospective blinded review of rituximab treatment in 55 patients (85% women) 8 to 69 years of age, it was observed that thymectomy did not cause clinical improvement (23).

In Lrp4(+) MG, involvement of the thymus remains unclear. One international, multicentre, retrospective study of clinical charts of 42 MG patients with isolated anti-Lrp4 autoantibodies reported that 31% had been diagnosed with "thymic hyperplasia" (24). Detailed immunohistochemical analysis of 4 cases (2 females, 2 males, age 28-53 years) revealed no thymic pathology, but clinical improvement after thymectomy in 2 cases suggest that a role of the thymus in Lrp4(+) MG cannot be excluded (25).

### *Role of small cell lung cancer*

SCLC and (rarely) other cancers, can elicit LEMS and, exceptionally, AChR(+) MG (26). This reflects immune responses against tumor proteins that cross-react with neuromuscular autoantigens. For example, SCLC cells from an MG patient expressed native AChR and major histocompatibility complex I/AChR-peptide complexes, while SCLCs from non-MG patients did not (27).

Immune checkpoint inhibitors (ICI) exacerbated pre-existing AChR(+) MG and LEMS, demonstrating the importance of the PD-1/PD-L1 immune checkpoint for the prevention of these disorders (28, 29). Moreover, in neurologically asymptomatic SCLC patients (with or without pre-existing VGCC autoantibodies), ICI treatment elicited *de novo* AChR(+) MG (29) or LEMS (28). Whether failure of tolerance in ICI-induced MG and LEMS occurs at thymic level remains uncertain.

### *Immune cell dysregulation*

The last five years brought expanded insights into immune dysregulations in MG. Levels of circulating molecules (e.g. IL-17, IL-21, BAFF, APRIL) and microRNAs (miRNAs) have proven biomarkers (1). Some of these dysregulations reflect differences in the proportions or activation states of lymphocyte subpopulations (Supplementary table, appendix p4) (30, 31). Reduced immunosuppressive function of regulatory T cells in MG and LEMS has been suggested (32, 33). In AChR(+) MG, the functional impairment is more pronounced in thymic than peripheral regulatory T cells, and these regulatory T cell subpopulations are phenotypically different (33). Decreased functionality of thymic regulatory T cells is partially due to MG thymic epithelial cells (TECs) (33).

Increased proportions of Th17 cells and IL-17 are observed in AChR(+) MG thymi due to a higher secretion of IL-23 by MG TECs (34). In AChR(+) MG, abnormal development of T cells, leading to an imbalance between regulatory and pathogenic cells, originates in the inflamed thymus. IFN- $\beta$  might play an upstream role here (34). The proportion of thymic and circulating follicular helper T (Tfh) cells is also increased (19). They might promote thymic germinal center development, but also B cell activation and antibody production (19). Tfh cells have also been claimed to be increased in MG-associated thymomas (35). In MuSK(+) MG, among Tfh cell subsets, a specific increase in Tfh17 cells is observed (36).

B cells produce the autoantibodies in MG, but regulatory B cells possess immunosuppressive functions. A decreased number and altered functionality of regulatory B cells is observed in untreated AChR(+) MG patients (37). They seem sequestered in the thymus, because their number is restored after thymectomy (37). Activation of innate signaling pathways and an IFN-I signature are clearly detected in EOMG and TAMG thymi (38, 39). Viral infections that induce IFN-I expression have long been suspected MG triggers, but so far no specific pathogen has been linked (40). Immunological dysregulation is a clear hallmark of autoimmunity, but the exact trigger and progression factors in MG and LEMS are yet to be discovered.

### **Pathophysiology of AChR(+) MG**

Most MG patients (80-85%) have antibodies against the NMJ AChR, an ion channel composed of two  $\alpha$ 1 subunits, one  $\delta$ 1, one  $\beta$ 1, and one  $\epsilon$  (adult) or  $\gamma$  (foetal) subunit. Most antibodies bind to an extracellular domain of the  $\alpha$ 1 subunit and are of the IgG1 or IgG3 subtypes. They reduce the number and/or function of AChRs via three main mechanisms (figure 3A and below). Insight into the pathophysiology of MG has come from animal models, involving either active immunization or passive transfer of AChR antibodies (appendix, p1).

### *Antigenic modulation of AChR degradation*

Bivalent IgG1/3 AChR antibodies deplete AChRs by cross-linking adjacent AChRs, accelerating their endocytosis and lysosomal proteolysis (41). Most AChR antibodies bind the main immunogenic region (MIR) on the  $\alpha$ 1 subunit, as does mab35 (a rat monoclonal IgG). When passively transferred into rats, mab35 causes acute MG via a combination of antigenic modulation and complement-mediated damage (42). Recent X-ray crystallography showed that mab35 Fab fragment forms a relatively rigid interface with the MIR and N-terminus of the  $\alpha$ 1 AChR subunit (43).

### *Complement-mediated damage to the muscle membrane*

Bound AChR antibodies activate the complement cascade that eventually forms membrane attack complex (MAC). Rodent models of AChR(+) MG proved a central role for complement/MAC in causing neuromuscular transmission failure. Mice lacking complement factors (C3, C4, C6) are resistant to EAMG, whereas mice lacking protective regulators, DAF1 and CD59a, are more prone to it (44). MAC causes loss of AChRs and widening of the synaptic cleft, reducing EPP amplitudes. Moreover, the damaged post-synaptic membrane infoldings have fewer voltage-gated sodium channels, increasing the post-synaptic firing threshold (45). Both changes reduce the safety factor of neuromuscular transmission.

### *AChR-channel block*

Some MG AChR antibodies bind at or near the ACh binding site and thereby block AChR channel opening. However, their clinical relevance remains elusive. A recent electrophysiological study assessed the prevalence of channel-blocking antibodies in 11 MG sera (46). Six of them caused an immediate reduction of ACh-induced whole-cell current in cells expressing human AChRs, apparently due to AChR-channel blockade. Their effect was more pronounced in cells with rapsyn-clustered AChRs. Thus, while based on only 11 MG patients, channel-blocking antibodies might contribute more to the pathogenesis than previously thought.

### **Pathophysiology of MuSK(+) MG**

About 5-8% of MG patients have autoantibodies against MuSK (47). Although sharing many features with AChR(+) MG, MuSK(+) MG seems a separate disease entity on three different levels. First, it is hallmarked by ocular, facial and prominent bulbar muscle weakness (48), with great inter-individual variety in severity, timing and muscles affected. Typically, ocular muscle weakness fades with time and profound bulbar weakness or generalized weakness remain. Second, MuSK(+) MG generally responds poorly to AChE inhibitor therapy (48). Third, the disease is thought to be caused predominantly by IgG4 autoantibodies, which are anti-inflammatory and do not activate complement (49). Instead, IgG4 MuSK antibodies cause NMJ failure by blocking MuSK function, leading to AChR cluster loss (figure 3B).

### *The role of IgG4 in MuSK(+) MG*

Evidence of pathogenicity of MuSK autoantibodies first came from active immunisation of rabbits with MuSK, causing MG symptoms (50). Human disease severity correlates with plasma levels of IgG4 MuSK antibodies (51). Some patients have low titres of MuSK antibodies of other isotypes, but their contribution remains unclear. IgG1-3 fractions from MuSK(+) MG patients impaired AChR clustering in myotube cultures, but did not induce a phenotype in mice (49, 52). IgG4 from the same patients clearly induced myasthenic weakness, proving the direct pathogenic nature of IgG4 MuSK antibodies (49). IgG4 cannot bind C1q and is thereby unable to activate complement. It has low affinity for activating Fc receptors on immune cells and, therefore, generally considered anti-inflammatory (53). Thus, the pathogenicity of MuSK antibodies must be related to binding and influencing MuSK. The MIR of MuSK is the N-terminal Ig-like 1 domain (54, 55), which is critical for interaction with Lrp4 and MuSK's own dimerization (56). Patient antibodies thus block the interaction between Lrp4 and MuSK and thereby the activation/phosphorylation of MuSK (52, 57). Prolonged loss of MuSK signalling results in fragmentation of post-synaptic AChR clusters and synaptic disintegration which causes impaired neurotransmission and thus muscle weakness (58). After prolonged period, atrophy may occur. MuSK antibodies may also block the interaction between MuSK and collagen Q, which is important for anchoring AChE (59). This perhaps explains the hypersensitivity of some MuSK MG patients to AChE inhibitor therapy.

IgG4 undergoes Fab-arm exchange with IgG4s of other specificities, which is thought to occur stochastically and continuously in blood. The resulting IgG4 molecule is bi-specific and interacts with its antigen in a monovalent fashion. Indeed, >99% of MuSK autoantibodies is functionally monovalent

(60). Recombinant monoclonal MuSK antibodies have so far been derived from four patients. The limited number of clones captured likely reflects the relatively low number of circulating MuSK-specific B cells (61, 62). These antibodies bind either the Ig-like 1 domain or Ig-like 2 domain and use a variety of VDJ genes. The immune response is thought to require affinity maturation to become fully pathogenic as the parental antibody clone displayed lower binding and pathogenic effects in myotube cultures (63). Experiments with patient-derived monoclonal antibodies revealed that monovalent (Fab-arm exchanged) MuSK antibodies blocked AChR clustering in myotube cultures, whereas bivalent antibodies partially activated MuSK signalling and AChR clustering (62). In mice, monovalent MuSK antibodies induced myasthenia, whereas bivalent MuSK antibodies either had no effect or induced delayed and milder muscle weakness (64). This suggests that MuSK antibody valency (and thus isotype) influences their pathogenicity. Whether other isotypes (i.e. monospecific IgG1 or IgG3) MuSK antibodies contribute to the disease (e.g. via complement activation) remains unclear.

### **Pathophysiology of LEMS**

Antibodies against Ca<sub>v</sub>2.1 (also called P/Q-type) VGCCs are detected in >90% of LEMS patients (65, 66). Their pathogenic actions at the NMJ are less well understood than those of AChR and MuSK antibodies in MG. The tiny dimensions of motor nerve terminals preclude direct measurement of Ca<sub>v</sub>2.1 function. Therefore, any dysfunction must be inferred from secondary parameters, particularly from post-synaptic electrophysiological measurement of EPPs and MEPPs (3). NMJ studies in LEMS muscle biopsies and muscles from passive transfer mouse models showed EPPs that are too small, failing to reach the muscle fibre's firing threshold, thereby inducing muscle weakness (67-69). This is caused by a pre-synaptic defect, quantified as a severe reduction of quantal content (the number of ACh quanta released per nerve impulse).

LEMS IgG binds to pre-synaptic active zones where it disrupts intramembrane particles, presumably VGCCs (70, 71). More direct evidence for Ca<sub>v</sub>2.1 VGCC came from studies in which LEMS IgG reduced Ca<sub>v</sub>2.1-mediated currents in SCLC or neuronal cell lines and in Ca<sub>v</sub>2.1-transfected human embryonic kidney cells (72-75). Furthermore, LEMS patient IgG reduced neurotransmitter release from cultured neurons, but not if they were genetically deficient for Ca<sub>v</sub>2.1 (76). Several cellular and serological studies identified co-existing antibodies against other active zone proteins, as well as against Ca<sub>v</sub>2.2 (N-type) VGCCs (65, 66, 77). In LEMS patients with SCLC, these likely result from an immunological response to the SCLC, expressing these antigens in addition to Ca<sub>v</sub>2.1 (78, 79). However, such antibodies are thought to play a pathogenic role in only a minority of LEMS patients (77).

Ca<sub>v</sub>2.1 VGCCs are the predominant autoantigens in LEMS, but the mechanism by which the autoantibodies affect them is unclear. Their subclasses are unknown, but are assumed to be IgG1 and IgG3 (as in AChR(+) MG), capable of cross-linking and complement activation. Similar to AChR(+) MG, three conceivable modes of action can be considered. First, direct block or functional change of Ca<sub>v</sub>2.1 channels. However, no indications for such effects exist (72, 73). Second, cross-linking and reshuffling/depletion of channels. Ultrastructural studies indeed form strong evidence for such a mechanism (70, 71). Third, there is no direct evidence for local pre-synaptic NMJ damage through complement activation. LEMS biopsy NMJs do not display complement deposits (80). Furthermore, LEMS NMJ phenotypes can be induced by passive transfer of LEMS patient IgG to complement C5-deficient mice, as well as by transfer of F(ab)<sub>2</sub> fragments of LEMS patient IgG (i.e. incapable to activate complement) into normal mice (68, 70, 81). More detailed information on the molecular pathogenic mechanisms of Ca<sub>v</sub>2.1 VGCC antibodies awaits further study.

### **Pathophysiology of non-AChR(+)/non-MuSK(+) MG**

Roughly 5-10% of clinically diagnosed MG patients have no detectable antibodies against either AChR or MuSK and are therefore termed 'double seronegative'. However, autoimmune aetiology in these patients is most likely and their sera probably harbour pathogenic antibodies against other NMJ proteins (82).

#### *Antibodies to Lrp4*

Within the double seronegative group, antibodies against Lrp4 have been detected in highly variable proportions (1-50%), depending upon sample origin and assay used (83-86). Lrp4 antibodies can co-exist with antibodies against agrin (87). Furthermore, they are present in a small proportion of AChR(+) or MuSK(+) MG patients (24, 86). Lrp4 MG antibodies are of the IgG1 subclass, but many patients have additional IgG2 and IgG3 reactivity (24, 83).

In view of the crucial participation of Lrp4 and agrin in the AChR clustering pathway at the NMJ, antibodies to these antigens are hypothesized to eventually reduce the post-synaptic AChR density, explaining the myasthenic muscle weakness. Indeed, Lrp4 MG sera/IgGs obstruct the interaction between agrin and Lrp4 in ELISAs (83, 85), and inhibit agrin-induced AChR clustering in the C2C12 myotube assay (84, 85). First *in vivo* evidence of pathogenicity of Lrp4 antibodies came from mice actively immunized with the extracellular domain of rat Lrp4 (88). They developed myasthenic weakness and NMJs showed fragmented AChR clusters with myasthenic electrophysiological features (i.e. small MEPPs and EPPs). Direct pathogenicity of Lrp4 antibodies was further shown through passive transfer of IgG from Lrp4-immunized rabbits to mice, which developed myasthenic weakness, with associated fragmentation of AChR clusters at their NMJs (89). Interestingly, no compensatory increase in ACh release was present at NMJs of Lrp4 MG mice. Rather, quantal content was decreased (88, 90), suggesting primary or secondary pre-synaptic effects of Lrp4 antibodies and a possible role of Lrp4 in synaptic homeostasis (see below). Complement activation by bound Lrp4 antibodies may be one of the contributing mechanisms (88, 89), but is not absolutely required for induction of experimental MG (90). No passive transfer studies have been published with human IgG from Lrp4 MG patients to date.

#### *Antibodies to agrin*

Antibodies to agrin are detected in 10-15% of MG sera, often in co-existence with AChR, MuSK or Lrp4 antibodies (87, 91, 92). *In vivo* pathogenicity of agrin antibodies has so far only been experimentally explored in one study, in which mice were actively immunized with either the neural or muscle variant of agrin (93). Mice injected with neural agrin developed myasthenic muscle weakness, with an associated NMJ phenotype of AChR cluster fragmentation and small MEPPs and EPPs. Interestingly, mice immunized with muscle agrin developed antibodies, but no muscle weakness and NMJ deficits.

#### **Autoantibodies directed against other antigens in MG**

In addition to autoantibodies against AChR, MuSK, Lrp4 and agrin in MG variants, several types of other autoantibodies can be present that target other NMJ proteins with extracellular epitopes. It is unclear whether they contribute to MG symptoms or have diagnostic value and their mechanisms of emergence are unknown. In addition, autoantibodies to intracellular muscular proteins can be present in a proportion of the MG patients. They are not causing MG, but some of them are of diagnostic value and associated with more severe disease. For details on these other antibodies to extra- and intracellular proteins see (appendix, p1).

#### **Muscle weakness in myasthenic disorders**

Not all muscles are equally affected in patients with myasthenic disorders. Within the AChR(+) MG population there is high variability, ranging from restricted extraocular to generalized weakness (94). Furthermore, frequent shifting of the affected muscle groups occurs during the disease course, with a tendency to progress in a craniocaudal direction. In LEMS, this direction is opposite, with proximal leg muscles being affected first. Extraocular muscles are often spared and, if affected, almost never in a pure way (95). MuSK MG differs from AChR MG with extraocular muscle weakness being less prominent, and instead distinct bulbar weakness with frequent progression to respiratory muscles (48).

The pathophysiological basis of inter-muscle, inter-patient, and inter-MG syndrome weakness heterogeneity is a longstanding and yet largely unresolved question. Several (subtle) physiological and molecular differences between muscle groups and their NMJs may underlie: 1) The access of pathogenic IgG to NMJs via the interstitial fluid of muscle and its lymphatic removal. This depends on the level of vascularization, FcRn-mediated endothelial transcytosis and usage intensity of a muscle (96). 2) The density, characteristics and turnover rate of antigenic proteins at NMJs. 3) The magnitude of the safety factor of neuromuscular transmission at the NMJ. NMJs on slow and fast skeletal muscle fibre types have been shown to differ in morphological and transmission characteristics (97). 4) The level of protection against complement activation (98). For instance, extraocular muscles contain relatively low levels of complement protective molecules. 5) Inter-muscle and inter-patient differences in muscle regeneration processes, possibly influenced by impaired satellite cells in MG (99). Neuromuscular transmission in myasthenic disorders is in a critical state, with peri-threshold EPPs. Thus, small variations in these five factors between muscle types and patients may have large consequences for the regional distribution and weakness severity. More study is needed to elucidate these complex relationships.

### **Synaptic adaptations to autoantibody attack at NMJs**

The NMJ is a highly plastic synapse, capable of structural and functional adaptation (within certain boundaries) to changing physiological conditions and pathological disturbances (100, 101). Basic studies in invertebrate NMJs identified several candidate molecules and pathways involved, and some of them may be of relevance for the mammalian NMJ (101). An important homeostatic aspect for the post-synaptic myasthenic disorders is that NMJs can increase pre-synaptic ACh release in response to loss of post-synaptic ACh sensitivity (102). We know this is regulated at the level of individual NMJs because in rodent and human AChR MG, NMJs display an inverse relationship between AChR density and quantal content (103, 104). Most likely, retrograde signalling occurs from post- to pre-synapse. Interestingly, the extracellular domain of Lrp4 has been implicated in retrograde signalling during NMJ development (6). It can be shed from the post-synaptic membrane through enzymatic cleavage (105). Furthermore, overexpression of Dok7 in muscles of mice causes enlargement of the pre-synaptic nerve terminal (7). Moreover, in MG caused by agrin, Lrp4 and MuSK antibodies, the normal compensatory upregulation of quantal content fails to occur at NMJs (49, 88, 93, 103). Together this suggests a role for Lrp4 and the agrin/Lrp4/MuSK/Dok7 pathway in transsynaptic homeostasis at the NMJ. Thus far, there are no indications that NMJs in LEMS patients or animal models develop post-synaptic compensations in response to their pre-synaptic defect (102). Further detailed study of homeostatic mechanisms at myasthenic NMJs may identify pharmacological targets to improve neurotransmission.

### **Novel tools for the study of autoimmune NMJ disorders**

Our understanding of MG pathophysiology is increased each time a new technology allows for more in-depth investigations. Major advances in recent years have been made possible due to (single-cell) RNA sequencing analyses and the development of novel *in vitro* and *in vivo* models.

Very recently, single-nucleus RNA-sequencing demonstrated regional transcriptional diversity within multinucleated skeletal myofibers (106, 107). The technology enabled purification of NMJ-specific nuclei, based on their unique gene expression pattern, and demonstrated novel, NMJ-specific genes (106, 107). This may lead to identification of novel autoantigens and therapeutic targets in MG and LEMS.

Single-cell sequencing methodologies have also been used to investigate the role of antigen-specific B cells and T cells from MG patients (14, 108-110). Thymus-derived peripheral B cells from AChR(+) MG patients persisted in the circulation after thymectomy, to a degree that correlated with poorer symptom resolution (14). Single-cell analyses of lymphocytes from MuSK(+) MG patients treated with rituximab (a B cell depleting biological) identified resistant B cell clones emerging from failed depletion of pre-existing clones. These clones are composed of plasma cells and memory B cells that express lower levels of CD20 and increased genes associated with B cell survival (109). CyTOF

based analysis of blood and thymi from MG patients furthermore identified two novel dysregulated subsets of memory T helper cells providing insight into the immune aetiology of MG and identifying potential therapeutic immunological targets (110).

In addition, study of MuSK(+) MG patient-derived monoclonal antibodies has led to a better understanding of the mechanisms of action of MuSK antibodies. Two recent studies demonstrate that such monoclonal antibodies can impair AChR clustering by distinct mechanisms, leading either to the induction or inhibition of MuSK phosphorylation (61, 62). These antibodies could be employed as reproducible, long-acting tools to generate *in vitro* and *in vivo* MG models for preclinical testing of potential therapeutics.

The development of novel *in vitro* cellular model systems for human NMJs is another important recent advancement (111). Using human pluripotent stem cells, it was shown that the cell types required for the formation of functional NMJs can be cultured and self-organized in a 3D *in vitro* nerve-muscle model, and stimulated through use of optogenetics (112-114). Exposure of such neuromuscular organoids to AChR antibodies from MG patients induced severe defects of their integrity and reduced their contractile activity, recapitulating key aspects of the disease phenotype. These models may help reducing experimental use of animals and provide clean high-throughput platforms for screening potential therapeutics in a (diseased) human genetic background.

### **Conclusions and future directions**

MG and LEMS pathophysiology depends greatly on the autoantibody subtype. While many aspects of the pathophysiology at the NMJ have been unravelled, the primary autoimmune aetiology of these diseases is still little understood. These disorders, therefore, remain chronic autoimmune diseases with no definite cure. Future research should aim at identifying the triggering event(s) leading to MG or LEMS. In a context of personalized medicine this is of major interest to be able to develop preventive approaches or avoid environmental triggers for predisposed individuals. The pathophysiological actions of a number of myasthenia-associated autoantibodies has not yet been (completely) elucidated. Especially the pathogenic actions of antibodies against Ca<sub>v</sub>2.1, Lrp4 and agrin await further clarification. With respect to immunology, restoring the immune imbalance in parallel to symptomatic therapeutic approaches at the NMJ will be crucial to obtain long-term remission or even cure. While a number of useful disease models already exists, generation of faithful and clinically relevant preclinical models of all subtypes of MG and LEMS is needed to further clarify the pathophysiology and facilitate development of new therapeutics. Finally, the inter-patient, inter-muscle and inter-MG syndrome heterogeneity is a long-standing issue which deserves further study. This review might also be also relevant to the rapidly expanding field of other antibody-mediated non-myasthenic (neurological) autoimmune diseases. Much of what we have learnt about autoantibody pathophysiology and related treatments of myasthenic autoimmune disorders might be translated to these new patient groups and thereby speed up processes of diagnosis and development of new therapeutics.

### **Search strategy**

This review was written by including landmark papers from all decades and recent publications from the NCBI Pubmed database using the search terms described in Supplementary appendix, p3. In short, we used key terms describing the main topics of this review including MG, LEMS and SNMG in combination with terms describing the specific subtopic, e.g. pathophysiology, NMJ. For recent technological advances we focussed on publications specifically describing new models e.g. organoids or sequencing methodologies.

### **Acknowledgements**

We thank Roy Augustinus for help with drafting Figure 1 and 3 and Rian Schopmeijer for help with designing Figure 3.

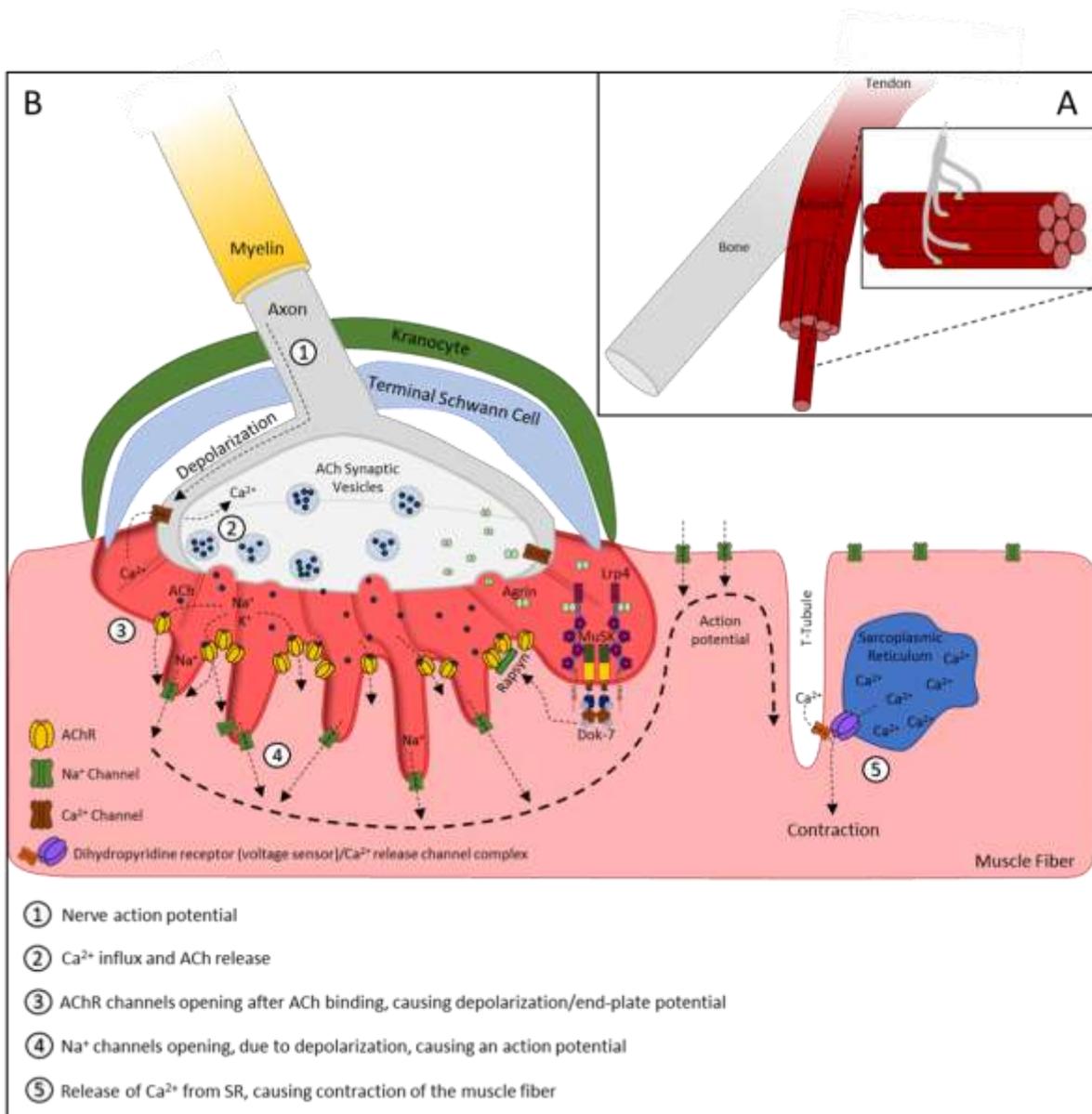


Figure 1: Structure and physiology of the neuromuscular junction (NMJ). (A) At the human NMJ, the terminal branches of the motor axon form a spray of synaptic endings (boutons) on the midline of the muscle fibre surface. (B) Each pre-synaptic bouton sits in a cavity formed by the post-synaptic membrane of the muscle fibre. Specialized terminal Schwann cells cap the terminal bouton and are thought to provide structural protection, trophic support and play a role in synaptic homeostasis. Effective neuromuscular transmission depends upon the following series of essential processes: 1) the pre-synaptic action potential depolarizes the nerve terminal membrane; 2) this rapidly opens voltage-gated calcium channels (VGCCs) located at vesicle release sites in the pre-synaptic membrane; 3) the burst of calcium ions entering the nerve terminal acts via calcium sensor proteins on the synaptic vesicle to trigger release of a quantum of ACh; 4) the ACh rapidly diffuses across the synaptic cleft to activate AChR cation channels that are closely packed together within the post-synaptic membrane. When sufficient AChR have opened and depolarisation of the membrane is achieved an action potential starts running along the muscle membrane. 5) The action potential propagates into t-tubuli throughout the muscle fibre. Activation of L-type VGCCs in the t-tubular membranes then triggers release of calcium from intracellular stores, which acts on the myofibrils to cause muscle fibre contraction.

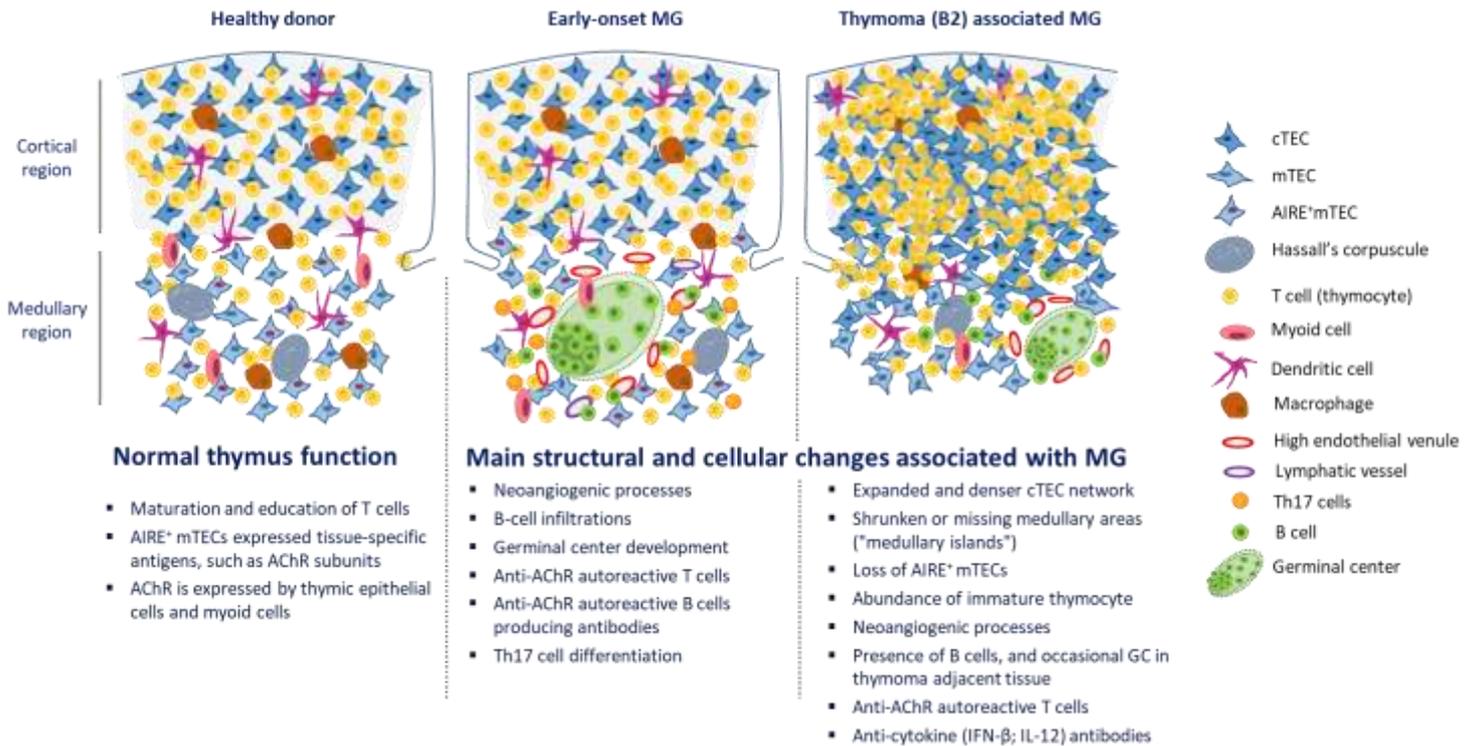


Figure 2: Thymic changes in MG. The normal thymus shows cortical regions that are mainly composed of cortical epithelial cells and abundant T cells, and medullary regions, in which T cells complete their maturation process. These T cells include regulatory T cells, and are accompanied by immunological tolerance-inducing dendritic and epithelial cells (including AIRE<sup>+</sup>-positive epithelial cells and Hassall corpuscles) and by sparse B cells and rare myoid cells that are supposedly involved in the induction of immunological tolerance. In early-onset MG, the thymus shows normal cortical regions, while the inflamed medullary regions are expanded by abundant B cells, lymphoid follicles adjacent to myoid cells, activated T cells and numerous blood vessels (high endothelial venules). In thymoma-associated MG, the cortical regions are mostly expanded through increased numbers of neoplastic epithelial cells that are still intermingled with numerous T cells. By contrast, the medullary regions show rudimentary development, only rare B cells, rare lymphoid follicles and Hassall corpuscles and near-absence of AIRE-positive epithelial cells, regulatory T cells and myoid cells.

\*AIRE, autoimmune regulator (a transcription factor of medullary thymic epithelial cells that is key for the induction of immunological tolerance) (17). (B2) denotes the highly MG-prone 'Type B2 thymoma' histological subtype.

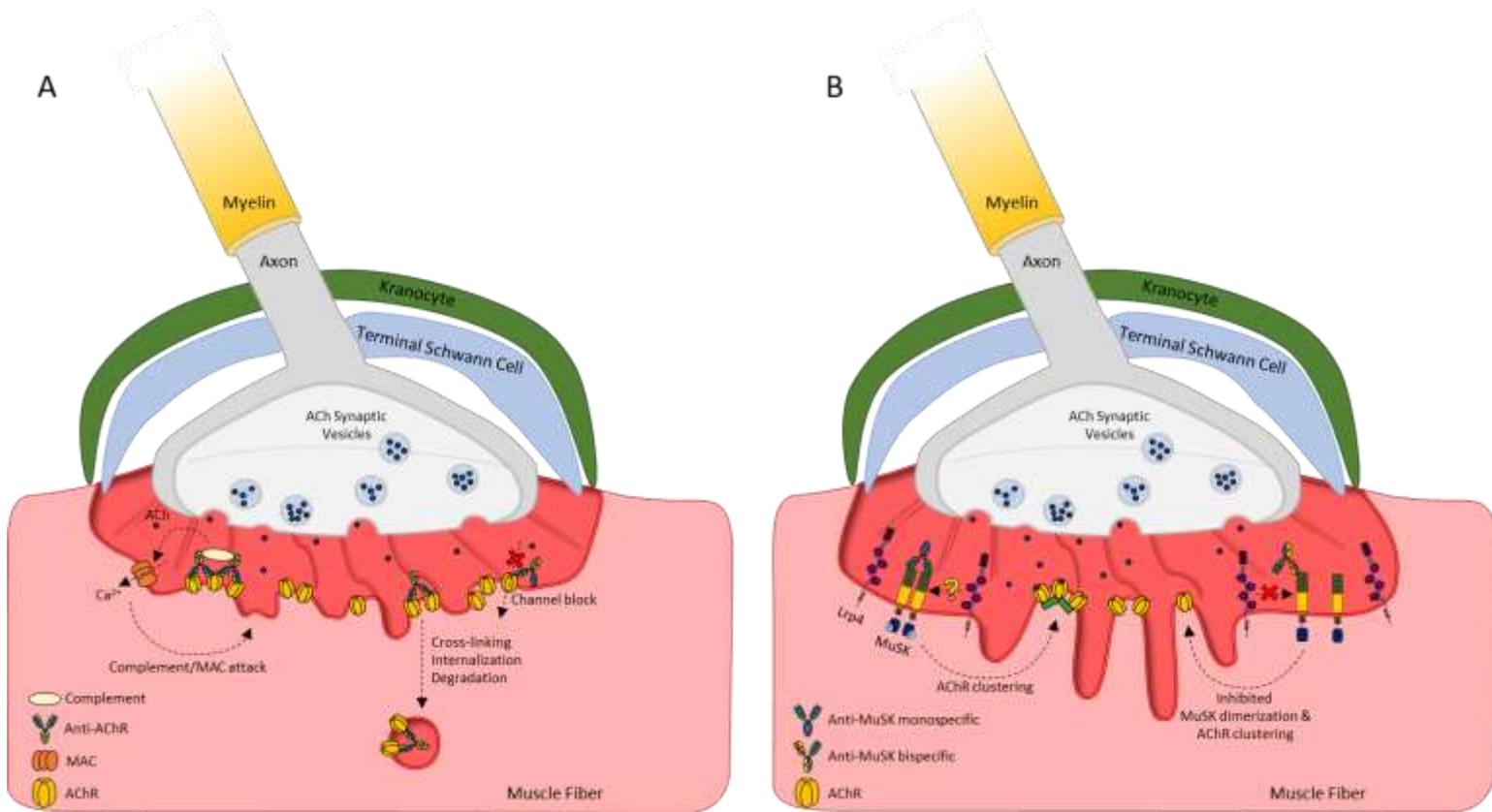


Figure 3: Pathophysiology at the neuromuscular junction in MG patients. (A) In AChR(+) MG, IgG1/3-type AChR autoantibodies activate complement pathways with assembly of destructive MAC cation channels and loss of NMJ tertiary structure. These autoantibodies also cross-link AChRs, causing accelerated lysosomal degradation of AChRs and thereby loss of signal transduction. Some patients have AChR autoantibodies that block the ACh-binding site preventing AChR channel opening. (B) In MuSK(+) MG, IgG4 MuSK autoantibodies block Lrp4 from activating the MuSK tyrosine kinase, which results in a loss of post-synaptic AChR clustering and synaptic fragmentation.

	AChR MG			MuSK MG	Lrp4 MG	Agrin MG	SNMG	LEMS		SNLEMS
Antigenic target	AChR			MuSK	LRP4	Agrin	?	Ca <sub>v</sub> 2.1 (P/Q-type) VGCCs		?
Frequency among MG or LEMS	~85%			~5%	2%	<1%	8%	~85%		~15%
Subcategory	EOMG (associated with thymic follicular hyperplasia)	TAMG (Thymoma)	LOMG	-	-	-	-	~40-50% Non-paraneoplastic	~50-60% Paraneoplastic (SCC)	?
Age onset	Before ~50	Peak age 50	After ~50	Before ~50	Early onset	?	?	Bimodal (young females older males)	After ~50	?
Gender bias	Yes (Female)	No	Male	Yes (Female)	Yes (Female)	?	Yes(Female)	No	Yes (Male)	?
Dominant IgG isotype	IgG1 – IgG3	IgG1 – IgG3	IgG1 – IgG3	IgG4	IgG1 – IgG3	?	?	Potentially IgG1 – IgG3		?
Role of complement	Yes	Yes	Yes	No	Yes	?	?	Potentially yes		?
HLA association	DR3-B8	No	No consistent association	DR14-DQ5	?	?	?	DR3-B8 and DQ2	No	?
Tumor association	No	Thymoma	No	No	?	?	?	No	Small cell lung cancer	?
Class level of evidence for pathogenicity*	I, II			I,II	II	III		I,II		

\*Based on Witebsky's postulates and Rose *et al.* Immunology today 1993

Class I direct evidence: pathogenicity elicited by passive transfer in animal models or in vitro models by use of patient antibodies

Class II indirect evidence: active immunization with the antigen causes an MG phenotype in animals or transplacental transfer causes a temporary phenotype in child

Class III circumstantial evidence: pathogenicity is expected based on the biological role of the antigen or positive response to immunosuppressive treatments although direct experimental evidence is lacking

Table 1: Pathophysiological characteristics of the MG subtypes. Gender bias refers to overrepresentation of the syndrome in one of two sexes.

\*Based on Witebsky's postulates and Rose *et al.* Immunology today 1993

Class I direct evidence: pathogenicity elicited by passive transfer in animal models or in vitro models by use of patient antibodies

Class II indirect evidence: active immunization with the antigen causes an MG phenotype in animals or transplacental transfer causes a temporary phenotype in child

Class III circumstantial evidence: pathogenicity is expected based on the biological role of the antigen or positive response to immunosuppressive treatments although direct experimental evidence is lacking

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## Supplementary appendix

### Animal models for studying MG

Passive immunoglobulin transfer models of MG typically involve injecting laboratory rodents with autoimmune IgG sourced from MG patients, experimental animals or monoclonal hybridomas. Passive transfer of purified AChR antibodies from MG patients into rats caused acute myasthenic weakness within a few days (1). By contrast, EAMG generally refers to the active immunization of laboratory animals with purified AChRs or peptide fragments of an AChR subunit from another species. It is necessary to co-inject an adjuvant in order to break self-tolerance of the healthy immune system. After booster injection/s animals will generally develop sustained high serum titres of AChR antibodies, a reduced safety factor for neuromuscular transmission and muscle weakness. While passive transfer allows study of pathophysiology at the level of NMJ, EAMG is a better model for studying issues related to chronic MG and immunological characteristics (2). Unfortunately, the thymic pathophysiological events leading to human EOMG are not recapitulated in EAMG (3).

### Autoantibodies directed against other antigens in MG

#### *Autoantibodies directed against other extracellular antigens*

In addition to AChR, MuSK, and Lrp4, autoantibodies in MG can target other post-synaptic proteins with extracellular epitopes. Antibodies to the  $\alpha$ -subunit of the Kv1.4 potassium channel occur in 10-20% of AChR(+)MG patients and are often associated with a thymoma, a severe clinical course, myositis, myocarditis and cardiac complications among the Japanese (4), but mainly LOMG and mild symptoms in White populations (5). Kv1.4 antibodies are furthermore associated with conduction disorders and arrhythmias like long QT syndrome (6). Antibodies to AChE (in up to 50% of MG patients), the AChE anchor protein, collagen Q (in 3%), and collagen XIII (in 7%, including 16% of SNMG patients) are not MG-specific (5). Whether they cause MG symptoms or have diagnostic value needs clarification. Mechanisms driving the emergence of these autoantibodies are unknown.

#### *Autoantibodies directed against intracellular antigens*

Autoantibodies to intracellular autoantigens such as titin, ryanodine receptors (RYR), cortactin and rapsyn do not cause MG, but those to titin and RYR are of diagnostic value and associated with a more severe form of MG (5, 7). Titin autoantibodies mainly recognize epitopes in a 30 kD domain (MGT30) within this "striational" protein. Using MGT30-based ELISAs, Titin-autoantibodies are detectable only in AChR(+) MG patients: in 50-95% with a thymoma, 40-80% with non-thymomatous LOMG over 60 years of age, but almost never in non-thymomatous EOMG patients. Accordingly, a positive anti-titin ELISA hints to a thymoma in AChR(+) MG patients under 60 with ~90% sensitivity and specificity (7). By contrast, more sensitive radio-immunoprecipitation assays (RIPA), reveal anti-titin antibodies in all types of MG, including up to 6% of EOMG and 13% of triple-SNMG patients, but not healthy controls (8, 9). Thus, against an appropriate clinical and electrophysiological background, a positive (usually low-titer) anti-titin RIPA may support a diagnosis of MG in seronegative patients (5). Titin autoimmunity in TAMG patients is thought to be initiated by proteins with titin-like epitopes that are abnormally expressed in thymoma epithelial cells (10). No such trigger is evident in atrophic thymi of titin autoantibody-positive LOMG patients; vice versa, the reported HLA-association of LOMG is not observed in TAMG (table 1). Despite these differences, the microenvironments of thymomas and atrophic thymi in LOMG resemble each other in two respects: both largely lack tolerogenic, Aire(+) medullary epithelial cells and titin-expressing myoid cells, while both cells types are present in hyperplastic EOMG thymi (11). Titin (as well as Kv1.4) autoantibodies can also arise in patients with various cancers following PD1-directed immune checkpoint inhibitor (ICI) therapies, typically in conjunction with myositis (12) and eventually with ICI-induced AChR(+) MG (12) or SNMG (13).

Muscle-type RYR (encoded by *RYR1* and *RYR2*) are calcium release channels of the sarcoplasmic reticulum in skeletal muscle (RYR1) and cardiac muscle (RYR2). High-titer autoantibodies are

detectable in up to 75% of TAMG and 14% of LOMG patients and are associated with MG severity (5). Using highly sensitive cell-based assays, low-titer RYR autoantibodies are also detectable in healthy individuals (4). The pathogenesis of RYR autoimmunity is unknown, but expression of highly homologous neuronal *RYR3* transcripts in thymomas was associated with TAMG in The Cancer Genome Atlas (TCGA) cohort of patients, suggesting intratumorous autoimmunization and/or positive selection of T cells through a 'cross-reacting autoantigen' (10).

Autoantibodies to cortactin and rapsyn (both involved in AChR clustering) occur in a minority of AChR(+) MG and SNMG patient and are not associated with MG prognosis. Due to their lack of MG-specificity and occurrence in some healthy controls, they are not of diagnostic relevance (5). Their pathogenesis is unknown.

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## Search strategy

References for this review were identified by searching NCBI PubMed for landmark papers from all decades and relevant recent publications dating from between 1/1/2015 to 1/1/2021 using the search terms:(((myasthenia gravis) AND (acetylcholine receptor)) AND (pathophysiology)) NOT (MuSK) NOT ("muscle specific kinase"[All Fields]) NOT (review[Publication Type]), (MuSK myasthenia gravis), (MG and cytokines), (MG and Th17 / regulatory T cells /Tfh), (LEMS and Th17 or Treg/Tfh), (MG and B cells), (MG and miRNA), (MG and HLA genetic predisposition), (LEMS), (Thymectomy and myasthenia and acetylcholine receptor and trial), (Thymoma and myasthenia gravis (2016-2021)), (Thymoma and Lambert-Eaton), (Thymus and myasthenia and MuSK), (Thymectomy and myasthenia and MuSK and trial LRP4 and myasthenia), (Small cell lung cancer and autoimmunity), (Thymoma and Lambert-Eaton), (Small cell lung cancer and autoimmunity), (Thymus and AIRE and autoimmunity and gender), (Myasthenia and immune checkpoint), (Thymoma and myasthenia and 2019), (Thymic hyperplasia and immune checkpoint inhibitor), (Thymic hyperplasia and myasthenia and 2021), (Thymoma and myasthenia and 2018), (Thymoma and myasthenia and 2020), (LEMS and Cav2.1) (LEMS and P/Q-type calcium channel), (LEMS and voltage-gated calcium channels), (LEMS and active zone), (LEMS and IgG1), (LEMS and IgG3), (LEMS and complement), (myasthenia gravis and LRP4), (myasthenia gravis and agrin), (myasthenia gravis and ColQ), (NMJ and homeostasis), (NMJ and plasticity), (Striational + autoantibodies + myasthenia), (Myasthenia + thymus pathology +ryanodine), (Myasthenia + muscle + ryanodine receptor), (Myasthenia + intracellular antigens + muscle), (Myasthenia + intracellular + titin + ryanodine), (Myasthenia + intracellular antigen + thymus), (Myasthenia + intracellular antigen + pathophysiology), (Myasthenia + seronegative + titin), (Myasthenia + seronegative + autoantibodies +diagnosis), (Myasthenia + titin + thymoma + sensitivity), (Myasthenia + titin + radioimmunoassay), (Myasthenia + thymoma + genetic + transcriptional), (Myasthenia + thymoma +TCGA), (Myasthenia thymus autoimmunity mechanisms), (fatal MG+myositis Myasthenia + immune checkpoint inhibitor + titin), (Myasthenia + ICI + fatal), (Myasthenia + pembrolizumab + titin), (Myasthenia + thymus + autoimmunity + mechanisms), (Myasthenia + thymus + single-cell profiling).

Only references in the English language were included. The final reference list was generated on the basis of relevance to the review, with priority given to publications within the last 5 years.

## Supplementary table

Summary of lymphocyte dysregulation reported in the respective MG syndromes.

	AChR-MG	AChR-TAMG	MuSK-MG
<b>Effector T cells (Th1, Th2, Th17)</b>	<ul style="list-style-type: none"> <li>• ↑ in Th1 and Th17 cytokines in periphery (a)</li> <li>• ↓ % of Th2 in periphery (b)</li> <li>• ↑ % of Th17 in periphery (b)</li> <li>• ↑ % of Th17 in the thymus (c).</li> </ul>	<ul style="list-style-type: none"> <li>• ↑ in Th17 cytokines in periphery (d)</li> <li>• ↑ number in periphery (e)</li> </ul>	<ul style="list-style-type: none"> <li>• ↑ in Th1 and Th17 cytokines (f, g)</li> </ul>
<b>T follicular helper cells (Tfh, Tfh<sub>r</sub>)</b>	<ul style="list-style-type: none"> <li>• ↑ frequency in Tfh in periphery (g, h, i, j) especially in generalized MG (k)</li> <li>• ↑ % of Tfh17 in periphery (b)</li> <li>• ↓ frequency of regulatory Tfh (tfhr) (k, j)</li> <li>• ↑ frequency of Tfh in the thymus (l)</li> </ul>	<ul style="list-style-type: none"> <li>• ↑ frequency of Tfh in periphery (m)</li> <li>• ↑ frequency of Tfh in thymoma (n)</li> </ul>	<ul style="list-style-type: none"> <li>• ↑ frequency of Tfh17(o)</li> <li>• ↑ ratio of Tfh17/Tfh1(o)</li> <li>• ↑ ratio of Tfh/Tfhr (o)</li> </ul>
<b>Regulatory T cells</b>	<ul style="list-style-type: none"> <li>• Controversial data on Treg proportion in periphery (d, p)</li> <li>• ↓ immunosuppressive function (p)</li> <li>• Functional impairment more pronounced in thymic Treg (p)</li> </ul>	<ul style="list-style-type: none"> <li>• ↓ number in periphery (d, e)</li> <li>• ↓ number in thymomas (q, r)</li> </ul>	<ul style="list-style-type: none"> <li>• No change in proportion (f)</li> </ul>
<b>B cells</b>	<ul style="list-style-type: none"> <li>• No increase (s), except a decrease in patients with long-term disease or with immunosuppressors (s)</li> <li>• ↑ frequency of plasma cells (k, s)</li> </ul>		<ul style="list-style-type: none"> <li>• No change for the percentages of total B cells and B cell subsets (naive, memory, isotype-switched, plasmablast, transitional B-cells) (t)</li> </ul>
<b>Breg cells</b>	<ul style="list-style-type: none"> <li>• ↓ proportion of Breg in periphery (u)</li> <li>• ↓ functionality of peripheral Breg (u)</li> <li>• ↑ in IL-10<sup>+</sup> B cells in the thymus (u)</li> </ul>	<ul style="list-style-type: none"> <li>• ↑ % of Breg in periphery (v)</li> <li>• ↓ of IL-10 producing B cells in periphery (v)</li> <li>• ↑ in IL-10<sup>+</sup> B cells in the thymus (v)</li> </ul>	<ul style="list-style-type: none"> <li>• ↓ of IL-10 producing B cells in periphery (t)</li> </ul>

(a) Gradolatto 2014 - doi: 10.1016/j.jaut.2013.12.015

(b) (31) Cebi 2020 - doi: 10.3389/fimmu.2020.00809

(c) (34) Villegas 2019 - doi: 10.1016/j.jaut.2018.11.005

(d) Wang 2012 - doi: 10.1111/j.1365-3083.2012.02703.x

(e) Chen 2021 - doi: 10.1016/j.molimm.2021.02.011

(f) Yi 2014 - doi: 10.1016/j.jaut.2013.12.005

(g) Yilmaz 2015 - doi: 10.1371/journal.pone.0123546

(h) Luo 2013 - doi 10.1016/j.jneuroim.2012.12.001

(i) Zhang 2016 - doi - 10.4049/jimmunol.1500725

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(s) Kohler 2013 - doi: 10.1016/j.jneuroim.2013.09.006

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(u) (37) Yilmaz 2018 - doi: 10.1002/acn3.645

(v) Lu 2017 - doi: 10.18632/oncotarget.17908