

*Digital Comprehensive Summaries of Uppsala Dissertations  
from the Faculty of Medicine 2269*

# Circulating Biomarkers and Complement-Associated Skeletal Muscle Pathology in Myasthenia Gravis

YU-FANG HUANG



ACTA UNIVERSITATIS  
UPSALIENSIS  
2026



UPPSALA  
UNIVERSITET

Dissertation presented at Uppsala University to be publicly examined in H:son-Holmdahlsalen, Ingång 100, 2 tr., Akademiska sjukhuset, Uppsala, Friday, 5 June 2026 at 09:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Researcher Inga Koneczny (Department of Neurology, Medical University of Vienna, Austria).

### Abstract

Huang, Y.-F. 2026. Circulating Biomarkers and Complement-Associated Skeletal Muscle Pathology in Myasthenia Gravis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 2269. 58 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-2835-5.

Myasthenia gravis (MG) is an autoimmune neuromuscular disorder characterized by pathogenic autoantibodies targeting components of the neuromuscular junction, most commonly the nicotinic acetylcholine receptor (nAChR). In AChR seropositive (AChR+) MG, autoantibody binding to nAChRs can activate the complement cascade, leading to membrane attack complex (MAC) formation, loss of nAChRs, and impaired neuromuscular transmission. MG is heterogeneous with multiple subgroups, and reliable circulating biomarkers and mechanistic insights into skeletal muscle pathology remain limited. This thesis investigates MG-associated circulating blood biomarkers and complement-associated pathogenic mechanisms using an *in vitro* human skeletal muscle model.

Papers I-II demonstrated that serum miR-150-5p and miR-30e-5p were elevated in MG, with miR-30e-5p correlating with disease course. Additionally, they exhibited good temporal stability. Paper III identified an altered inflammatory protein profile in AChR+ MG, in which CCL28, FGF-23, FGF-5, TGF- $\alpha$ , TNFSF14, and uPA exhibited the highest differences between MG and HC. Papers IV-V demonstrated complement activation in MG. Increased C1s/C1-INH complexes indicated proximal classical pathway activation, while elevated plasma C3a and soluble C5b-9 reflected downstream and terminal pathway activation. C3a exhibited the highest diagnostic performance. Papers V-VI established a human skeletal muscle model of AChR+ MG, in which pathogenic antibodies bound to nAChRs, causing receptor loss, MAC deposition, and impaired cholinergic calcium signaling. Similar effects induced by AChR  $\alpha$ -subunit-specific monoclonal antibodies were restored by C3 inhibition, indicating complement activation as a key driver of antibody-mediated pathogenic effects.

Taken together, these studies identify candidate circulating miRNA, inflammatory, and complement-related biomarkers in MG and demonstrate the pathogenic effects *in vitro*. These findings provide a broader view of immune and inflammatory activation in MG, as well as mechanistic insights into complement-associated skeletal muscle pathology, including proximal complement C3 inhibition as a promising therapeutic strategy.

*Keywords:* Myasthenia gravis, nicotinic acetylcholine receptor, biomarker, muscle cell, complement activation, calcium signaling

*Yu-Fang Huang, Clinical Neurophysiology, Ingång 85, 3 tr, Akademiska sjukhuset, Uppsala University, SE-751 85 Uppsala, Sweden.*

© Yu-Fang Huang 2026

ISSN 1651-6206

ISBN 978-91-513-2835-5

URN urn:nbn:se:uu:diva-582857 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-582857>)

*Dedicated to my family and friends*



# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. Beretta, F., **Huang, Y.-F.**, Punga, A. R. (2022). Towards Personalized Medicine in Myasthenia Gravis: Role of Circulating microRNAs miR-30e-5p, miR-150-5p and miR-21-5p. *Cells*, 11(4), 740.
- II. **Huang, Y.-F.\***, Bhandage, A. K.\*, Adeström, L. D., Punga, A. R. (2024). Short-term changes in serum miRNA levels and patient-reported clinical outcomes in myasthenia gravis. *Muscle & Nerve*, 70(2), 284–289.
- III. Bhandage, A. K., Kenina, V., **Huang, Y.-F.**, Roddate, M., Kauke, G., Grosmane, A., Žukova, V., Eriksson, N., Gabrysch, K., Punga, T., Punga, A. R. (2024). Serum protein biomarker profile distinguishes acetylcholine receptor antibody seropositive myasthenia gravis patients from healthy controls. *iScience*, 27(8), 110564.
- IV. **Huang, Y.-F.**, Briggs, C. M., Gokhale, S., Punga, A. R. (2024). Elevated C1s/C1-INH in serum and plasma of myasthenia gravis patients. *Journal of Neuroimmunology*, 396, 578447.
- V. **Huang, Y.-F.**, Sandholm, K., Persson, B., Nilsson, B., Punga, A. R. (2024). Visualization and characterization of complement activation in acetylcholine receptor antibody seropositive myasthenia gravis. *Muscle & Nerve*, 70(4), 851–861.
- VI. **Huang, Y.-F.**, Bhandage, A. K., Fichtner M., Punga, A. R. (2026). Complement C3 Inhibition Restores Myasthenia Gravis AChR Antibody-Mediated Muscle Pathophysiology. *Submitted manuscript*.

\*Equal first authors.

Reprints were made with permission from the respective publishers.

# Publications Not Included in This Thesis

- I. Bhandage, A. K., Hoffmann, S., Dusemund, C., Stascheit, F., **Huang, Y.-F.**, Eriksson, N., Gabrysch, K., Meisel, A., Punga, A. R. (2025). AXIN1, STAMBP, ST1A1, CDCP1, and SIRT2 Validated as Myasthenia Gravis Biomarkers: A Comparative Proteomic Study With MS, CIDP, and Controls. *European Journal of Neurology*, 32(11), e70426.
- II. Bhandage, A. K., **Huang, Y.-F.**, Punga, T., Punga, A. R. (2025). On the road to blood biomarkers in myasthenia gravis (MG): Beyond clinical scales. *Journal of Neuromuscular Diseases*, 22143602251348753.
- III. **Huang, Y.-F.**, Verpalen, R. L. K., Punga, A. R., Huijbers, M. G. (2025). Cell models for studying myasthenia gravis. *International Review of Neurobiology*, 182, 121–143.

# Contents

Introduction .....	13
Myasthenia gravis.....	15
Epidemiology.....	15
Diagnosis.....	15
Subgroups .....	15
MG outcome measurements .....	16
Immunopathogenesis .....	17
Pathophysiology.....	18
Treatment .....	20
Biomarkers.....	21
Disease models.....	22
Skeletal muscle.....	23
Postsynaptic component of the neuromuscular junction .....	23
Mechanisms of skeletal muscle contraction .....	24
Nicotinic acetylcholine receptors.....	24
Voltage-gated calcium channels .....	25
Aims of the Thesis.....	27
Methods.....	28
Ethics .....	28
Study participants .....	28
Papers I-V .....	28
Papers V-VI .....	29
Overview of study design and experimental approaches .....	29
Papers I-V .....	30
Papers V-VI .....	31
Handling and processing of blood samples .....	31
RNA isolation and qPCR.....	32
Serum and plasma protein analysis .....	32
Human skeletal muscle cell culture and MG induction.....	32
Cellular protein expression analysis .....	33
Immunocytochemistry.....	33
Live-cell imaging.....	33
Image analysis .....	34
Cell viability assay.....	34
Statistical analysis.....	35

Results and Discussion.....	36
Papers I-III.....	36
Results.....	36
Discussion.....	37
Papers IV-V.....	38
Results.....	38
Discussion.....	39
Papers V-VI.....	41
Results.....	41
Discussion.....	44
Conclusion.....	46
Future Perspective.....	47
Acknowledgments.....	49
References.....	51

# Abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AChR	Acetylcholine receptor
AChR+	Acetylcholine receptor seropositive
APRIL	A proliferation-inducing ligand
BAFF	B cell-activating factor
C1-INH	C1 inhibitor
CBA	Cell-based assay
CD6	T cell surface glycoprotein CD6 isoform
cDNA	Complementary DNA
CCh	Carbamoylcholine chloride
CCL	C-C motif chemokine
Ct	Cycle threshold
DHPR	Dihydropyridine receptor
EAMG	Experimental autoimmune myasthenia gravis
ECC	Excitation-contraction coupling
ECD	Extracellular domain
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EN-RAGE	Protein S100-A12
EOMG	Early-onset myasthenia gravis
FGF	Fibroblast growth factor
FSS	Fatigue Severity Scale
FcRn	Neonatal Fc receptor
GDNF	Glial cell line-derived neurotrophic factor
gMG	Generalized myasthenia gravis
HEK	Human embryonic kidney
IgG	Immunoglobulin G
IL	Interleukin
IL-22RA1	Interleukin-22 receptor subunit alpha-1
IVIg	Intravenous immunoglobulin
KCl	Potassium chloride
LOMG	Late-onset myasthenia gravis

LRP4	Low-density lipoprotein receptor-related protein 4
LRP4+	Low-density lipoprotein receptor-related protein 4 seropositive
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MBSI	Magnetic bead-based sandwich immunoassay
MCP-3	Monocyte chemotactic protein 3
MGC	Myasthenia gravis Composite
MG	Myasthenia gravis
MG-ADL	Myasthenia gravis Activities of Daily Living
MG-QoL15	Myasthenia gravis Quality of Life 15-item questionnaire
miRNA	MicroRNA
MIR	Main immunogenic region
mAbs	Monoclonal antibodies
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MuSK	Muscle-specific kinase
MuSK+	Muscle-specific kinase seropositive
mTOR	Mechanistic target of rapamycin
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NMJ	Neuromuscular junction
NPX	Normalized protein expression
nAChR	Nicotinic acetylcholine receptor
OND	Other neuroimmune disease
oMG	Ocular myasthenia gravis
OPG	Osteoprotegerin
OSM	Oncostatin-M
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEA	Proximity extension assay
PLEX	Plasma exchange
QMG	Quantitative myasthenia gravis
RIA	Radioimmunoassay
RNS	Repetitive nerve stimulation
ROC	Receiver operating characteristic
RyR	Ryanodine receptor
SERCA	Sarcoendoplasmic reticulum calcium ATPase
SFEMG	Single-fiber electromyography
SIRT2	SIR2-like protein 2
SNMG	Seronegative myasthenia gravis
SR	Sarcoplasmic reticulum
ST1A1	Sulfotransferase 1A1
TGF	Transforming growth factor

Th17	T helper 17
Tfh	Follicular helper T
TNF	Tumor necrosis factor
Treg	Regulatory T cell
T-tubule	Transverse tubule
uPA	Urokinase-type plasminogen activator
VEGF-A	Vascular endothelial growth factor A
VGCC	Voltage-gated calcium channel
VGSC	Voltage-gated sodium channel
VLOMG	Very late-onset myasthenia gravis



# Introduction

Skeletal muscle contraction requires efficient neuromuscular transmission at the neuromuscular junction (NMJ), a specialized synapse that transmits the signal from the motor neuron to initiate muscle contraction. Disruption of the postsynaptic components of the NMJ impairs neuromuscular transmission and leads to fatigable muscle weakness.

Myasthenia gravis (MG), characterized by such NMJ disruptions, is an autoimmune disorder caused by pathogenic autoantibodies that target postsynaptic proteins, most commonly the nicotinic acetylcholine receptor (nAChR; Figure 1), as well as muscle-specific kinase (MuSK) and low-density lipoprotein receptor-related protein 4 (LRP4). These proteins are essential for neuromuscular transmission and NMJ organization and maintenance. The autoimmune attack impairs neuromuscular transmission, leading to insufficient depolarization of the postsynaptic membrane, and resulting in clinical muscle weakness and fatigability<sup>1</sup>. This thesis focuses on AChR seropositive (AChR+) MG, investigating potential circulating blood biomarkers and pathogenic mechanisms at the postsynaptic muscle compartment.

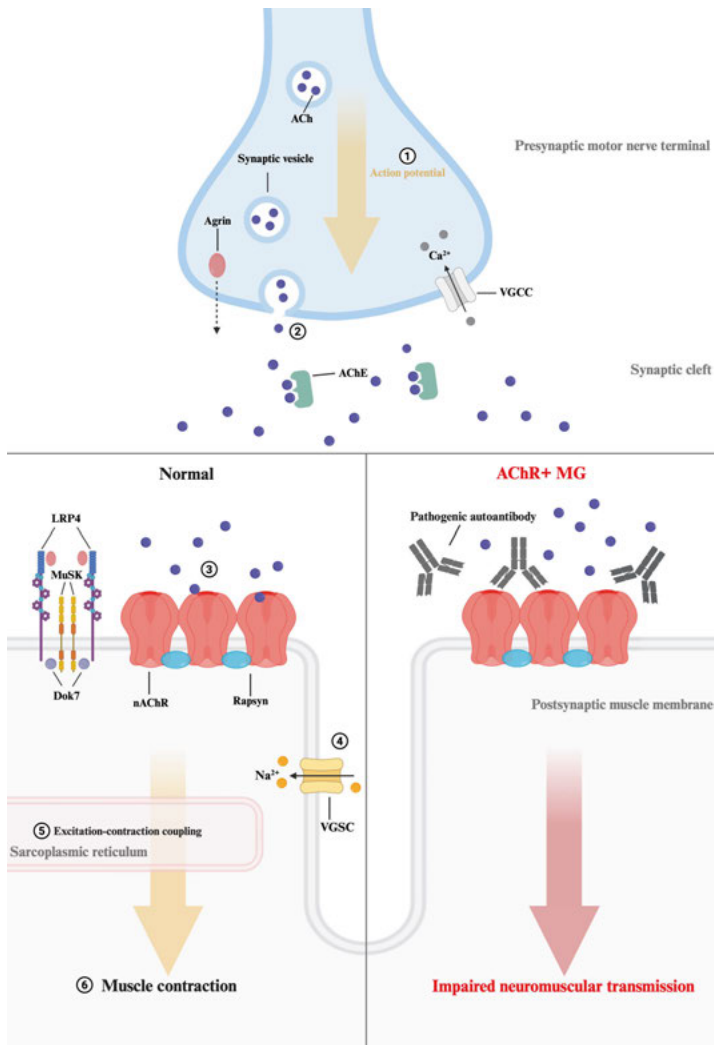


Figure 1. Schematic illustration of neuromuscular transmission in normal conditions and acetylcholine receptor seropositive (AChR+) myasthenia gravis (MG).

Under normal conditions (left panel), the neuromuscular transmission proceeds as follows: (1) an action potential reaches the presynaptic motor nerve terminal, triggering (2) calcium ( $\text{Ca}^{2+}$ ) influx through voltage-gated calcium channels (VGCCs), and release of acetylcholine (ACh) into the synaptic cleft. (3) ACh binds to nicotinic acetylcholine receptors (nAChRs), which are clustered at the postsynaptic membrane via the agrin–low-density lipoprotein receptor-related protein 4 (LRP4)–muscle-specific kinase (MuSK) signaling complex, leading to postsynaptic depolarization. This activates (4) voltage-gated sodium channels (VGSCs), initiates (5) excitation-contraction coupling via  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum, and results in (6) muscle contraction. Acetylcholinesterase (AChE) rapidly hydrolyzes ACh in the synaptic cleft, preventing prolonged nAChR activation. In AChR+ MG (right panel), pathogenic autoantibodies target nAChRs, reducing receptor density and blocking receptor function, thereby impairing neuromuscular transmission and causing muscle weakness.

This figure was created with BioRender.

# Myasthenia gravis

## Epidemiology

The incidence of MG is approximately 20 cases per million person-years, with a prevalence of around 25 cases per 100,000 population<sup>2</sup>. The increasing number of cases worldwide reflects improved diagnostic tools, disease awareness, ageing populations and improved survival associated with advances in immunosuppressive and supportive therapies<sup>2,3</sup>. The mortality rate is approximately 5-9% and has decreased over the past two decades owing to advancements in diagnosis, treatment, and respiratory care<sup>4</sup>. MG can occur at any age; however, females are more frequently affected than males, with an estimated female-to-male ratio of 1.6:1<sup>5</sup>.

## Diagnosis

Diagnosis begins with a neurological examination identifying characteristic fluctuating and fatigable skeletal muscle weakness affecting ocular, bulbar, limb, or respiratory muscles, and is further supported by serological and electrophysiological investigations<sup>3</sup>.

Radioimmunoassay (RIA) is a widely used method for detecting antibodies against the acetylcholine receptor (AChR), MuSK, and LRP4<sup>3</sup>. In addition to RIA, enzyme-linked immunosorbent assay (ELISA) has been employed to detect AChR and MuSK antibodies and offers a non-radioactive alternative<sup>6,7</sup>. More recently, cell-based assays (CBAs) using human embryonic kidney cells expressing clustered AChRs have improved diagnostic sensitivity. CBAs can identify AChR antibodies in a substantial proportion of patients previously classified as seronegative (SNMG), in whom MG-related antibodies are not detectable by RIA<sup>8</sup>.

Electrophysiological studies provide functional confirmation of neuromuscular transmission failure and include repetitive nerve stimulation (RNS) and single-fiber electromyography (SFEMG)<sup>3</sup>. RNS typically demonstrates a decremental reduction in compound muscle action potential amplitudes with low-frequency stimulation. SFEMG detects increased jitter, defined as the fluctuation in the time interval between action potentials of two muscle fibers belonging to the same motor unit, and represents the most sensitive indicator of NMJ dysfunction in MG<sup>3,9</sup>.

## Subgroups

Patients with MG can be classified into subgroups based on the distribution of clinical weakness, age at disease onset, thymic pathology, and serological status<sup>10</sup>. These classifications reflect the heterogeneity of MG.

### **Clinical distribution**

Ocular MG (oMG) is characterized by weakness limited to extraocular muscles, presenting as ptosis, which is usually asymmetrical, or diplopia<sup>3</sup>. Approximately 15-20% of patients present with purely ocular symptoms<sup>11</sup>, and up to 80% subsequently progress to generalized MG (gMG), most commonly within the first two years of disease<sup>4,11</sup>. gMG involves bulbar, limb, axial, or respiratory muscles, with limb weakness typically symmetrical and predominantly proximal<sup>10,12</sup>.

### **Age at onset**

Based on age at onset, MG is classified as juvenile-onset ( $\leq 18$  years), early-onset MG (EOMG; 19-50 years), late-onset MG (LOMG; 51-65 years), and very late-onset MG (VLOMG;  $\geq 65$  years)<sup>3,12</sup>. EOMG predominantly affects women and is associated with thymic hyperplasia and AChR seropositivity, whereas LOMG occurs more frequently in men<sup>10</sup>.

### **Thymoma-associated MG**

Thymoma-associated MG accounts for approximately 10-20% of all MG cases and occurs predominantly in older patients<sup>3,12,13</sup>. Most patients have detectable AChR antibodies and present with generalized weakness. Approximately 30% of patients with thymoma develop MG, while up to 50% exhibit AChR antibodies without clinical manifestations<sup>4,12</sup>.

### **Serological subgroups**

Serological classification is based on the presence of disease-specific auto-antibodies. AChR antibodies are detected in approximately 75-85% of MG patients and constitute the largest serological subgroup, including most cases of EOMG, LOMG, and thymoma-associated MG<sup>12,14</sup>. MuSK seropositive (MuSK+) MG accounts for 5-8% of cases and is frequently associated with bulbar weakness and a higher risk of myasthenic crisis, a life-threatening exacerbation characterized by severe respiratory and/or bulbar weakness<sup>15</sup>. LRP4 seropositive (LRP4+) MG is rare (1-3%) and typically associated with milder symptoms<sup>14,16</sup>. Patients who test negative for AChR, MuSK, and LRP4 antibodies are classified as SNMG, representing approximately 5-15% of cases and including patients with undetectable or as-yet-unidentified antibodies<sup>12,14</sup>.

### **MG outcome measurements**

Quantitative assessment of disease severity in MG is challenging due to fluctuating muscle weakness and fatigability. To this end, several validated outcome measures are widely used in clinical practice and therapeutic trials, including patient-reported and clinician-assessed scales<sup>3,17</sup>.

MG Activities of Daily Living (MG-ADL) and MG Quality of Life 15-item questionnaire (MG-QoL15) are commonly used patient-reported outcome measures. MG-ADL assesses symptom burden and its impact on daily activities, with higher scores indicating greater disease severity<sup>18</sup>. MG-QoL15 evaluates a patient's quality of life related to MG, with higher scores reflecting poorer quality of life. Fatigue Severity Scale (FSS), although not specific to MG, is used to evaluate general fatigue. It is a 9-item scale developed for use in various neurological disorders, including MG<sup>18</sup>.

Clinician-assessed outcome measurements include the MG Composite (MGC) and Quantitative Myasthenia Gravis (QMG). MGC combines findings from clinical examination and patient-reported symptoms to assess disease severity, with higher scores indicating more severe disease<sup>19</sup>. QMG requires clinician assessment and specialized equipment. It provides an objective, quantitative evaluation of neuromuscular weakness<sup>20</sup>.

## Immunopathogenesis

MG is a thymus-associated, T-cell-dependent, and B-cell-mediated disease, in which a series of immunological events leads to production of pathogenic autoantibodies.

### Defects in immune tolerance

Immune tolerance prevents immune responses against self-antigens by eliminating self-reactive immune cells. These mechanisms occur in the thymus and bone marrow, as well as in secondary lymphoid tissues, where regulatory T cells (Tregs) support this process<sup>21</sup>. In MG, defects in these mechanisms lead to the escape of self-reactive immune cells<sup>22,23</sup>.

### Thymic abnormalities

MG is often associated with thymic abnormalities, including follicular hyperplasia and thymoma. Follicular hyperplasia is particularly prevalent in AChR+ cases. It is characterized by the abnormal expansion of ectopic lymphoid follicles with active germinal centers, which facilitate pathogenic autoantibody production. Thymoma occurs in a small subset of patients and is associated with disruption of central immune tolerance<sup>24</sup>.

### T cells and related cytokines

The imbalance among CD4+ T-cell subsets and their associated cytokines plays a key role in the immunopathogenesis of MG. Increased frequencies of T helper 17 (Th17) cells in MG promote chronic inflammation through the secretion of interleukin-17 (IL-17)<sup>25</sup>. In addition, elevated thymic follicular helper T (Tfh) cell frequencies support B-cell activation and differentiation into antibody-secreting plasma cells by secreting interleukin-21 (IL-21)<sup>25</sup>. Tregs, which are responsible for the production of immunosuppressive

cytokines such as interleukin-10 (IL-10) and transforming growth factor (TGF)- $\beta$ , are reduced in frequency and/or function in MG <sup>25,26</sup>.

### **B cells and related cytokines**

Pathogenic autoantibody production is driven by dysregulated B-cell activation. In MG, B cell homeostasis is altered, with increased frequencies of memory B cells and reduced ratios of regulatory B cells <sup>25</sup>. Cytokines that promote B cell survival and maturation, particularly B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL), are upregulated in MG <sup>25,27</sup>.

## **Pathophysiology**

The predominant autoantibody target is the nAChR, which is located on the postsynaptic muscle membrane and mediates synaptic transmission by binding neurotransmitter acetylcholine (ACh) and initiating postsynaptic membrane depolarization. Additional autoantibody targets include MuSK and LRP4, which are essential for NMJ development and maintenance <sup>28</sup>. Antibodies against these targets are mainly of non-complement-activating immunoglobulin G (IgG) subclasses and are not the focus of this thesis.

### **Pathophysiological mechanisms of AChR autoantibodies**

AChR autoantibodies are predominantly of the IgG1 and IgG3 subclasses and recognize epitopes on the extracellular domain (ECD) of the nAChR <sup>16,29,30</sup>. The pathogenic effects of AChR autoantibodies are mediated through at least three mechanisms: antigenic modulation, direct functional blockade of nAChRs, and complement-mediated postsynaptic membrane damage <sup>31</sup>.

Antigenic modulation occurs when autoantibodies bind to adjacent nAChRs. This cross-linking triggers accelerated internalization of nAChRs via endocytosis, followed by degradation of the receptor-antibody complexes <sup>32</sup>. As a consequence, the number of functional nAChRs on the postsynaptic membrane is reduced. In addition, autoantibodies can directly block nAChRs by competing with ACh for binding sites on the receptor. This blockade prevents nAChR activation <sup>31,33</sup>.

Complement-mediated destruction of the postsynaptic membrane is a major pathogenic mechanism in AChR+ MG <sup>34</sup>. Binding of IgG1 and IgG3 antibodies to nAChRs triggers activation of the classical complement cascade <sup>35</sup>. Sequential activation of downstream complement components leads to the assembly of the membrane attack complex (MAC; C5b-9) on the postsynaptic membrane <sup>35,36</sup>. MAC formation damages the postsynaptic membrane and causes the loss of functional nAChRs and voltage-gated sodium channels (VGSCs), all of which compromise neuromuscular transmission <sup>35</sup>.

## Complement activation

The complement system forms part of the innate immune response and operates through a coordinated cascade of complement components involved in the clearance of pathogens and immune complexes. Activation of the complement cascade promotes opsonization, generation of pro-inflammatory mediators, and formation of MAC (C5b-9), leading to damage of target cell membranes. Under normal conditions, complement activity is tightly regulated to prevent damage to autologous tissues<sup>35</sup>.

Complement can be activated through three pathways: the classical, lectin, and alternative pathways (Figure 2). In AChR+ MG, activation occurs predominantly via the classical pathway, which is initiated by binding of IgG-antigen immune complexes to the C1 complex. The C1 complex consists of the recognition subunit C1q and the enzymatic subunits C1r and C1s. Activation of the classical pathway is tightly regulated by the C1 inhibitor (C1-INH), which restricts complement activation by binding to C1r and C1s. Upon C1-INH binding, the C1 complex dissociates, releasing free C1q together with covalent C1r/C1-INH and C1s/C1-INH complexes<sup>37</sup>. When regulatory mechanisms are insufficient, C1r autoactivates and then activates C1s. C1s leads to formation of the classical C3 convertase. Downstream complement activation involves cleavage of C3 into C3a and C3b. C3b participates in the formation of the C5 convertase, which subsequently cleaves C5 into C5a and C5b. C5b then associates with C6, C7, and C8, followed by binding of multiple C9 molecules, resulting in formation of the MAC (C5b-9)<sup>35,37</sup>. Protection against aberrant complement activation is mediated by membrane-bound regulatory proteins, including CD55 and CD59, which limit C3 convertase activity and inhibit MAC formation, respectively<sup>35</sup>.

Early pathological studies provided important evidence for a direct role of complement activation in MG by demonstrating deposition of IgG and complement components at the NMJ in both human biopsies and animal models<sup>38</sup>. Deposition of complement component C3 at postsynaptic region was associated with reduced nAChR density, implicating complement-mediated NMJ destruction<sup>36,39</sup>. This pathogenic role has been further supported by studies using C3-, C4-, C5-, and C6-deficient rodent models, which demonstrated attenuated disease severity and reduced postsynaptic damage<sup>40-42</sup>.

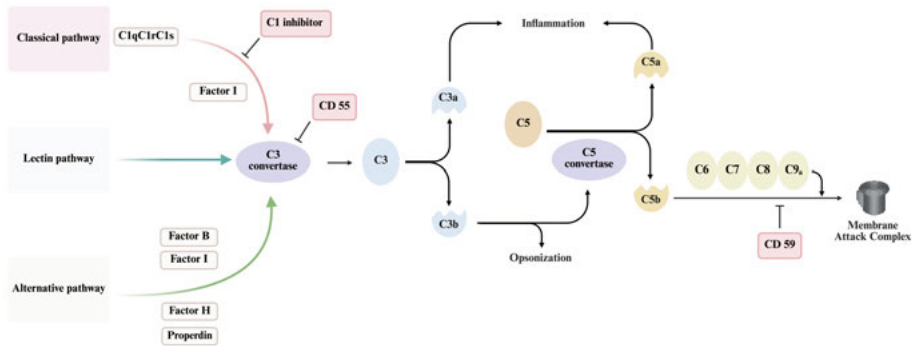


Figure 2. Overview of complement activation pathways and regulatory mechanisms.

The classical, lectin, and alternative complement pathways converge at the formation of the C3 convertase. The classical pathway is initiated by the C1 complex (C1q, C1r, and C1s) and regulated by C1 inhibitor. The lectin pathway activates C3 convertase through pattern recognition molecules, while the alternative pathway amplifies complement activation and is regulated by Factor B, Factor H, Factor I, and properdin. C3 convertase cleaves C3 into C3a and C3b, mediating inflammatory signaling and opsonization, respectively. C3b also contributes to C5 convertase formation, leading to cleavage of C5 into the anaphylatoxin C5a and C5b. C5b initiates assembly of the terminal complement components (C6-C9) to form the membrane attack complex (MAC). Complement activity is regulated by CD55, which limits C3 convertase activity, and CD59, which inhibits MAC formation.

This figure was created with BioRender.

## Treatment

Therapeutic strategies are determined by disease severity, antibody status, thymic pathology, and treatment response.

### Standard treatment

Symptomatic treatment with acetylcholinesterase inhibitors (AChEIs), most commonly pyridostigmine, represents the initial treatment in MG. By inhibiting the enzymatic degradation of ACh, AChEIs enhance neuromuscular transmission and provide rapid symptomatic relief. However, as they do not modify the underlying autoimmune process, most patients require additional therapy<sup>14,43</sup>.

Immunosuppressive treatment with corticosteroids, most commonly prednisone, is the first-line therapy for patients who remain symptomatic despite AChEI therapy, although long-term use is limited by side effects<sup>43</sup>. Steroid-sparing immunosuppressants, such as azathioprine and ciclosporine, are therefore commonly used to reduce steroid exposure. These steroid-sparing drugs suppress immune responses through various mechanisms, including inhibition of immune cell proliferation<sup>14,43</sup>.

Thymectomy, the surgical removal of the thymus, is mandatory in thymoma-associated MG. In addition, thymectomy is recommended for patients with non-thymomatous AChR+ gMG who are younger than 65 years, ideally within the first two years after diagnosis. Thymectomy is associated with improved clinical outcomes, including reduced immunosuppressive requirements, lower QMG scores, and decreased hospitalization rates<sup>14,43</sup>.

For patients experiencing acute disease exacerbations or myasthenic crisis, intravenous immunoglobulin (IVIg) and plasma exchange (PLEX) are used to achieve prompt clinical improvement. These interventions act by reducing circulating pathogenic autoantibodies <sup>43</sup>.

Despite the availability of multiple standard therapeutic options, approximately 30-50% of patients fail to achieve satisfactory clinical improvement and disease stability <sup>14</sup>.

### **Approved biological therapies**

Approved biological therapies are designed to target key pathogenic mechanisms, including complement activation, IgG autoantibody recycling, and B-cell-mediated autoantibody production. Complement inhibition, particularly through targeting of complement component C5, represents a major therapeutic advance in AChR+ MG, as it prevents terminal complement activation and the formation of MAC. Additional strategies target the neonatal Fc receptor (FcRn) to reduce IgG recycling and accelerate degradation of pathogenic autoantibodies. B-cell-targeted approaches limit autoantibody production by reducing circulating B-cell populations <sup>14</sup>.

### **Biomarkers**

Biomarkers are measurable indicators of normal or abnormal biological states, pathogenic processes, or pharmacological responses to a therapeutic intervention. Depending on their intended use, biomarkers can be classified into categories, including diagnostic, prognostic, predictive, monitoring, and pharmacodynamic biomarkers <sup>44</sup>. In MG, SFEMG and antibodies to AChR and MuSK are diagnostic biomarkers; however, reliable markers that reflect disease severity or treatment response remain limited <sup>45</sup>. Ideally, biomarkers should be minimally invasive, easily accessible, and capable of distinguishing patients from healthy individuals. In this context, circulating blood-based molecules, such as microRNAs (miRNAs), complement factors, and proteins have been proposed as potential biomarkers in MG <sup>46</sup>.

### **Circulating miRNAs**

miRNAs are small non-coding RNA molecules, typically ~22 nucleotides in length, that regulate gene expression at the post-transcriptional level by targeting the messenger RNA (mRNA). Circulating miRNAs are released into the extracellular space and can be detected in various biological fluids, including blood <sup>47</sup>. Several studies have reported altered serum miRNA profiles in MG, particularly in AChR+ MG. Elevated levels of miR-150-5p, miR-21-5p, and miR-30e-5p are observed in AChR+ patients compared with healthy individuals <sup>48-50</sup>. Thymectomy and immunosuppressive treatment are associated with reduced levels of miR-150-5p and miR-21-5p <sup>49,51</sup>. Increased miR-30e-

5p levels are detected early in the disease course of oMG patients who subsequently generalize<sup>52</sup>.

### **Complement components**

As complement activation plays a central role in the pathophysiology of AChR+ MG, circulating complement components in serum or plasma have been investigated as potential biomarkers. Reported alterations include C2, C3 (C3a and C3b), C4a, C5a, and C5b-9<sup>46</sup>. However, plasma is the preferred sample type for complement assessment, since chelation of divalent ions (Ca<sup>2+</sup> and Mg<sup>2+</sup>) by EDTA prevents *ex vivo* complement activation, a process that is not inhibited in serum<sup>53</sup>.

### **Proteins**

Proteins involved in immune regulation and inflammation have emerged as potential biomarkers in MG. In AChR+ MG, several interleukins associated with pro-inflammatory and regulatory pathways, including IL-6, IL-17, IL-21, and IL-10, are dysregulated. Additionally, changes in tumor necrosis factor (TNF) family proteins and elevated matrix metalloproteinase (MMP) levels have been reported<sup>46</sup>.

### **Disease models**

Disease models of MG enable the study of disease mechanisms and support preclinical therapeutic development.

#### **Animal models**

Experimental autoimmune myasthenia gravis (EAMG) can be induced in rodents by either active immunization with MG-associated autoantigens or passive transfer of patient-derived IgG or monoclonal antibodies. In studies of AChR+ MG, EAMG reproduces several pathogenic features, including the generation of anti-AChR antibodies, complement activation at the NMJ, loss of postsynaptic nAChRs, and muscle weakness<sup>54</sup>. However, thymic pathology is not recapitulated<sup>55</sup>, and disease induction is variable<sup>54</sup>.

#### **Cell-based models**

The myasthenic phenotype can be recapitulated *in vitro* by exposure of cells to patient-derived serum or purified autoantibodies<sup>56</sup>. Muscle cell cultures, neuromuscular co-culture models, and transfected cell systems constitute the main *in vitro* models of MG.

Muscle cell cultures enable focused investigation of postsynaptic pathological mechanisms. In these systems, innervated human muscle can be mimicked by application of neural agrin, which induces nAChR clustering in differentiated myotubes<sup>57</sup>. The immortalized murine C2C12 cell line is the most extensively used experimental system; however, species-specific differences

between murine and human muscle cells limit the translational relevance of animal-derived models. Human muscle cell models therefore offer greater advantages. The TE671 cell line, a human rhabdomyosarcoma-derived myogenic line, expresses functional nAChRs and has been used to study antibody-induced nAChR downregulation. In addition, primary human myoblasts, isolated from muscle biopsies or obtained commercially, can be differentiated into myotubes and provide high physiological relevance. These cells respond to MG autoantibodies with morphological alterations, nAChR loss, and complement activation<sup>57</sup>.

Neuromuscular co-culture models incorporate motor neurons with muscle cells to form functional NMJ and typically use primary cells, immortalized cell lines, or pluripotent stem cell derivatives. Transfected cell models, such as human embryonic kidney (HEK 293) cells expressing MG-related antigens, are used to investigate antibody binding and antigen-specific pathogenic mechanisms<sup>57</sup>.

## Skeletal muscle

### Postsynaptic component of the neuromuscular junction

Skeletal muscle contraction is initiated by neuromuscular transmission at the NMJ. Structurally, the NMJ comprises a presynaptic motor nerve terminal, a synaptic cleft, and the postsynaptic muscle membrane (Figure 1). The presynaptic motor nerve terminal contains synaptic vesicles filled with neurotransmitter ACh, which is released in response to action potential-evoked calcium influx. The synaptic cleft is a narrow extracellular space separating the nerve terminal from the muscle fiber and contains a specialized basal lamina that supports signal transmission and enzymatic breakdown of ACh. The postsynaptic muscle membrane is the primary functional component and is organized for efficient neuromuscular transmission<sup>58,59</sup>.

The postsynaptic muscle membrane is characterized by deep junctional folds that increase surface area and cluster nAChRs at high density, reaching up to  $10^4$  receptors per  $\mu\text{m}^2$ . nAChR clustering is induced by agrin released from motor neurons. The organization of the postsynaptic domain is maintained by coordinated interactions among agrin, nAChRs, and other proteins, including MuSK, LRP4, rapsyn, and associated cytoskeletal components<sup>59-61</sup>. VGSCs are concentrated at the depths of deep junctional folds, facilitating efficient initiation and propagation of muscle action potentials along the specialized muscle plasma membrane, termed sarcolemma.

The binding of ACh to nAChRs generates an endplate potential that, if sufficient, triggers a muscle action potential and subsequent contraction. Disruption of postsynaptic molecular organization or receptor density compromises neuromuscular transmission, leading to muscle weakness<sup>58,62</sup>.

## Mechanisms of skeletal muscle contraction

Skeletal muscle contraction is driven by coordinated molecular events occurring within the sarcomere, the fundamental contractile unit of muscle fiber (also referred to as muscle cells) <sup>63</sup>. Sarcomeres consist of thin (actin) and thick (myosin) filaments arranged in a highly ordered, repetitive pattern along myofibrils, enabling efficient force generation and transmission. Muscle force is generated by the sliding of actin filaments past myosin filaments, resulting in sarcomere shortening <sup>63</sup>. This process depends on cyclic interactions between myosin heads and actin filaments, termed cross-bridge cycling <sup>64,65</sup>.

Actin-myosin interactions are tightly regulated by the troponin-tropomyosin complex. Under resting conditions, tropomyosin blocks myosin-binding sites on actin, preventing cross-bridge formation. Upon elevation of intracellular calcium ( $\text{Ca}^{2+}$ ) levels,  $\text{Ca}^{2+}$  binds to troponin C, inducing a conformational change that shifts tropomyosin away from the binding sites. This permits myosin attachment, cross-bridge cycling, and force generation within the sarcomere <sup>66</sup>.

The rise in intracellular  $\text{Ca}^{2+}$  that initiates contraction is controlled by excitation-contraction coupling (ECC), the process that links depolarization of the muscle fiber membrane to sarcomere contraction <sup>67</sup>. ECC occurs at specialized triad junctions formed by transverse tubules (T-tubules) and the sarcoplasmic reticulum (SR). Propagation of an action potential along the sarcolemma and into the T-tubules depolarizes voltage-gated calcium channel (VGCC), specifically dihydropyridine receptors (DHPRs), which in turn activate ryanodine receptors (RyRs) on the SR membrane, triggering rapid  $\text{Ca}^{2+}$  release into the cytosol <sup>67</sup>. The resulting transient  $\text{Ca}^{2+}$  elevation enables actin-myosin interactions and sarcomere contraction. When stimulation ceases,  $\text{Ca}^{2+}$  is actively pumped back into the SR by sarcoendoplasmic reticulum calcium ATPase (SERCA) pumps, lowering cytosolic  $\text{Ca}^{2+}$  and ending contraction <sup>67</sup>.

## Nicotinic acetylcholine receptors

The nAChRs are broadly classified into neuronal and muscle types based on their subunit composition and tissue distribution; in this thesis, the focus is on muscle-type nAChRs localized at the postsynaptic muscle membrane.

nAChRs are pentameric ligand-gated ion channels that mediate neuromuscular transmission by binding ACh and permitting the flux of cations across the muscle membrane <sup>68</sup>. nAChRs are heteropentameric receptors assembled from a pool of homologous subunits ( $\alpha 1-10$ ,  $\beta 1-4$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ ), with each subunit comprising a large N-terminal ECD, four transmembrane segments, a cytoplasmic loop, and an extracellular C-terminal region <sup>29,68</sup>.

The subunit composition of nAChRs differs between developmental stages. In fetal muscle, receptors consist of two  $\alpha 1$  subunits together with  $\beta 1$ ,  $\gamma$ , and  $\delta$  subunits, arranged in a stoichiometry of 2:1:1:1 (Figure 3). During postnatal

maturation, the  $\gamma$  subunit is replaced by the  $\epsilon$  subunit, forming the adult-type receptor<sup>68</sup>. Among the subunits,  $\alpha 1$  plays a particularly crucial role, as ligand binding occurs at the interfaces between  $\alpha 1$  subunits and their adjacent subunits,  $\epsilon/\gamma$  and  $\delta$  subunits<sup>69</sup>. The  $\alpha 1$  subunit also mediates the conformational changes required for channel opening following agonist binding<sup>70</sup>. Importantly, the ECD of  $\alpha 1$  subunit contains the main immunogenic region (MIR), which comprises the epitopes recognized by pathogenic autoantibodies in MG<sup>29,30</sup>.

The  $\beta$ ,  $\gamma$ , and  $\delta$  subunits contribute to receptor assembly, trafficking, and stabilization. Agrin-induced clustering of nAChRs at the postsynaptic membrane is mediated through MuSK activation and recruitment of the scaffold protein rapsyn, which anchors receptors to the cytoskeleton and promotes phosphorylation of  $\beta$  subunits<sup>71</sup>. Efficient receptor assembly depends on phosphorylation of  $\gamma$  subunits and glycosylation of  $\delta$  subunits<sup>72,73</sup>.

## Voltage-gated calcium channels

VGCCs represent a link between electrical excitation and downstream cellular processes, including contraction. In skeletal muscle, VGCCs act as key transducers of membrane depolarization, activating in response to action potentials and subthreshold depolarizing signals to mediate  $\text{Ca}^{2+}$  influx, maintain intracellular calcium homeostasis, and initiate calcium-dependent signaling pathways<sup>74,75</sup>.

VGCCs are multi-subunit protein complexes composed of up to four subunits, including a central pore-forming  $\alpha 1$  subunit and auxiliary subunits that regulate channel trafficking and stability (Figure 3). The  $\alpha 1$  subunit is essential for channel function and determines the fundamental properties of VGCCs, including ion conductance and voltage sensitivity. Auxiliary subunits include the intracellular  $\beta$  subunit, the extracellular  $\alpha 2$  subunit, and the transmembrane  $\delta$  and  $\gamma$  subunits, with  $\alpha 2$  and  $\delta$  linked by a disulfide bond. Distinct  $\alpha 1$  subunits define VGCC subtypes.  $\text{Ca}_v1$  and  $\text{Ca}_v2$  form high-voltage-activated channels (L-, P/Q-, N-, and R-type) activated at relatively depolarized membrane potentials (typically above  $-40$  mV), whereas  $\text{Ca}_v3$  forms low-voltage-activated T-type channels activated near the resting membrane potential (approximately  $-60$  to  $-70$  mV)<sup>74,75</sup>.

In skeletal muscle, L-type calcium channels containing the subunit  $\text{Ca}_v1.1$  act as voltage sensors for ECC by coupling membrane depolarization to calcium release from the SR<sup>74</sup>. T-type calcium channels, particularly those formed by subunit  $\text{Ca}_v3.2$ , contribute to the regulation of basal intracellular calcium levels<sup>76</sup>.

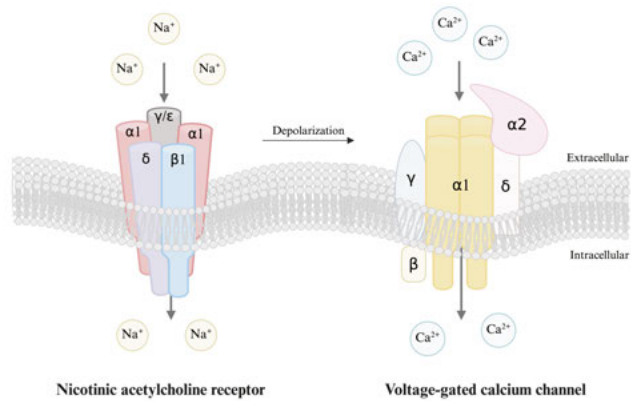


Figure 3. Schematic illustration of nicotinic acetylcholine receptor (nAChR)-mediated depolarization and activation of voltage-gated calcium channel (VGCC) on the skeletal muscle membrane.

The nAChR is a pentameric ligand-gated ion channel composed of two  $\alpha$  subunits and  $\beta$ ,  $\delta$ , and  $\gamma/\epsilon$  subunits, mediating sodium ( $\text{Na}^+$ ) influx upon acetylcholine binding and subsequent membrane depolarization. This depolarization activates VGCCs, multi-subunit channel complexes with a pore-forming  $\alpha 1$  subunit tetrameric composition and auxiliary  $\alpha 2$ ,  $\beta$ , and  $\gamma$  subunits, allowing calcium ( $\text{Ca}^{2+}$ ) influx into the cytosol. This figure was created with BioRender.

# Aims of the Thesis

The aim of this thesis was to explore disease mechanisms in MG by analyzing of circulating biomarkers in the blood and complement-associated pathology in an *in vitro* human skeletal muscle model. Specific aims were:

- I. To evaluate the potential utility of circulating miRNAs and inflammatory proteins as biomarkers in MG sera, distinguish MG from healthy individuals, including their stability assessment (**Papers I-III**).
- II. To investigate complement components and activation products in MG sera and plasma, with the objective of assessing their potential as circulating biomarkers (**Papers IV-V**).
- III. To investigate the effects of MG-associated pathogenic AChR antibodies and the role of complement activation in a human skeletal muscle cell model of MG, including the functional consequences for cholinergic calcium signaling, nAChR distribution, and MAC deposition (**Papers V-VI**).

# Methods

## Ethics

This study was approved by the Swedish Ethical Review Authority and Latvian Riga Stradiņš University. The permits were: 2020-03049, 2023-02129-02, 2-PEK-4/70/2023, 2018/446, 2023-01455-02, and 2024-02500-01. Written informed consent was obtained from all participants before sample collection.

## Study participants

### Papers I-V

Study participants included patients with MG, HC, and, where applicable, other neuroimmune diseases (ONDs), including multiple sclerosis, Lambert-Eaton myasthenic syndrome, chronic inflammatory demyelinating polyneuropathy, and inflammatory myelitis. Characteristics are summarized in Table 1.

Table 1. Overview of study population characteristics and sample types in Papers I-V.

Group	Paper	I	II	III	IV		V	
	Sample type	Serum	Serum	serum	Serum	Plasma	Serum	Plasma
MG	N	27	39	98	73	23	23	23
	Age (IQR)	58 (39-70)	67 (56-76)	61 (44-72)	66 (44-74)	66 (42-71)	66 (42-71)	66 (42-71)
	Sex (F:M)	15:12	21:18	60:38	41:32	14:9	14:9	14:9
	IS naïve	19 (70.4%)	15 (38.5%)	28 (28.6%)	33 (45.2%)	11 (47.8%)	11 (47.8%)	11 (47.8%)
HC	N	245	-	77	74	57	24	140
	Age	44 (31-55)	-	55 (41-64)	62 (44-69)	54 (38-66)	55.5 (32-64)	*
	Sex (F:M)	129:116	-	46:31	41:33	39:18	15:9	*
OND	N	20						
	Age	46 (33-60)						
	Sex (F:M)	13:7						
	IS naïve	20						

Age is presented as median with interquartile range (IQR), and sex as female-to-male ratio (F:M). \* indicates that information on sex and age at sample collection was not available. Abbreviations: MG, myasthenia gravis; HC, healthy control; OND, other neuroimmune disease; N, number of individuals; IQR, interquartile range; F, female; M, male; IS, immunosuppression.

## Papers V-VI

Serum samples from MG patients and HC were used for *in vitro* experiments. Sample characteristics are summarized in Table 2.

Table 2. Characteristics of serum samples used for *in vitro* experiments in Papers V-VI.

Group	Paper	V	VI
	Sample type	Serum	Serum
AChR+ MG	N	9	3
	Age (IQR)	39 (29-48)	38 (18-45)
	Sex (F:M)	9:0	3:0
	IS naïve	3	0
HC	N	3	3
	Age	34 (29-48)	38 (18-45)
	Sex (F:M)	3:0	3:0

Age is presented as median with interquartile range (IQR), and sex as female-to-male ratio (F:M). Abbreviations: AChR+, acetylcholine receptor seropositive; MG, myasthenia gravis; HC, healthy control; N, number of individuals; IQR, interquartile range; F, female; M, male; IS, immunosuppression.

## Overview of study design and experimental approaches

The overall study design for the analysis of potential circulating biomarkers (**Papers I-V**) is summarized in Figure 4, and the *in vitro* studies of complement-mediated effects of MG-associated pathogenic antibodies (**Papers V-VI**) are outlined in Figure 5.

## Papers I-V

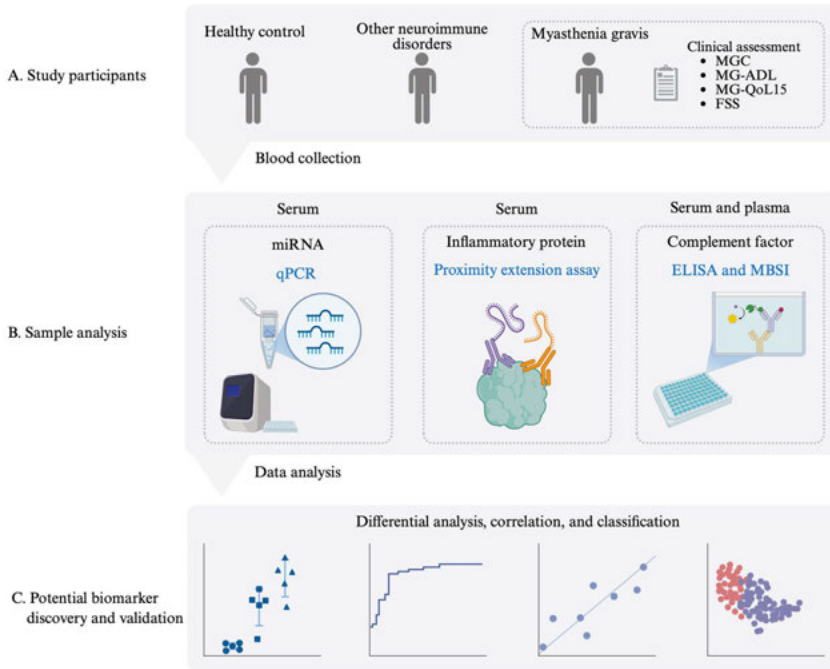


Figure 4. Schematic illustration of the experimental workflow for circulating biomarker analysis. (A) Study participants included healthy controls, patients with other neuroimmune disorders, and patients with myasthenia gravis (MG). MG patients were assessed using MG Composite (MGC), MG Activities of Daily Living (MG-ADL), MG Quality of Life 15-item questionnaire (MG-QoL15), and Fatigue Severity Scale (FSS). (B) Blood samples were processed into serum and plasma for analyses of miRNAs, inflammatory proteins, and complement components using qPCR, proximity extension assay, enzyme-linked immunosorbent assay (ELISA), and magnetic bead-based sandwich immunoassay (MBSI). (C) Data were analyzed using differential expression, correlation with clinical parameters, and classification approaches to identify potential circulating biomarkers.

This figure was created with BioRender.

## Papers V-VI

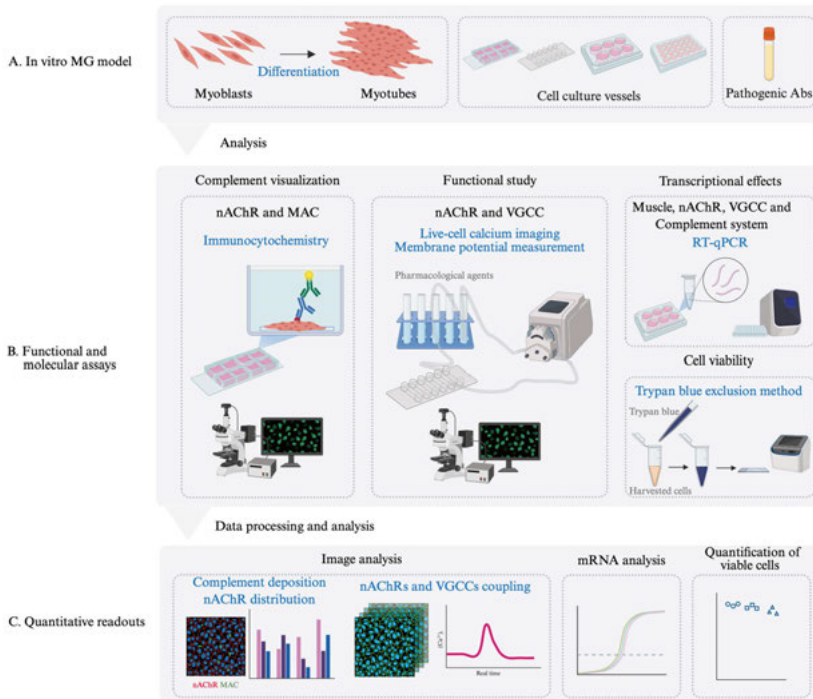


Figure 5. Schematic illustration of the *in vitro* human skeletal muscle model of myasthenia gravis (MG) and experimental workflow. (A) An *in vitro* MG model was established by differentiation of myoblasts into myotubes cultured in appropriate cell culture vessels and exposed to pathogenic antibodies (Abs). (B) Functional and molecular assays were performed to investigate pathophysiological mechanisms. Nicotinic acetylcholine receptor (nAChR) distribution and complement deposition were assessed by immunocytochemistry targeting nAChR and membrane attack complex (MAC). Functional coupling between nAChRs and voltage-gated calcium channels (VGCCs) was examined using live-cell calcium imaging and membrane potential measurement in the presence of pharmacological agents. Transcriptional effects on muscle markers, nAChRs, VGCCs, and components of the complement system were evaluated by RT-qPCR. Cell viability was assessed using the trypan blue exclusion method. (C) Quantitative outcomes were obtained by image analysis, mRNA expression analysis, and cell viability was calculated as the percentage of viable cells to the total number of cells counted. This figure was created with BioRender.

## Handling and processing of blood samples

Serum samples were collected in tubes without additives, and plasma samples in tubes containing EDTA. Serum samples were kept at room temperature for 20 min before centrifugation at  $2200 \times g$  for 10 min, while plasma samples were centrifuged immediately under the same conditions. The serum or plasma was transferred to new polypropylene tubes and stored at  $-80^\circ\text{C}$ .

## RNA isolation and qPCR

In **Papers I-III**, total RNA, including miRNA, was isolated from serum samples using the miRNeasy Serum/Plasma Advanced Kit. The RNA was reverse-transcribed into complementary DNA (cDNA) using miRCURY LNA RT Kit. miRNA expression levels were analyzed by quantitative PCR (qPCR) using pre-designed panels containing primers for selected target miRNAs. qPCR was performed using miRCURY LNA SYBR Green PCR kit. miR-191-3p was used as reference miRNA. miR-23a-3p and miR-451a were used for hemolysis controls. Synthetic RNA Spike-in standards were included for quality control (UniSp2, UniSp4, UniSp5, and UniSp6), and for interplate control (UniSp3). miRNA expression levels were presented as relative expression.

In **Paper VI**, total RNA was extracted from cells using RNeasy Mini Kit and treated with DNase I to remove contaminating genomic DNA. The RNA was reverse-transcribed into cDNA using iScript cDNA Synthesis Kit, and mRNA expression was analyzed using real-time qPCR with gene-specific custom-designed primers. qPCR was performed using KAPA SYBR FAST qPCR Master Mix. GAPDH, TBP, IPO8, and RPLP0 were used as reference genes. Data are presented as relative mRNA expression or normalized expression. All qPCR reactions were performed in duplicate.

## Serum and plasma protein analysis

Protein levels in serum and plasma were quantified using proximity extension assay (PEA), enzyme-linked immunosorbent assay (ELISA), and magnetic bead-based sandwich immunoassay (MBSI), depending on the target analytes and study design. In **Paper III**, inflammatory proteins in serum samples were analyzed using the Olink Target 96 Inflammation panel based on multiplex PEA technology. Briefly, pairs of DNA oligonucleotide-labeled antibodies bind their specific target proteins, and upon dual binding, the oligonucleotides hybridize and are extended and amplified by DNA polymerase. The resulting DNA sequences were quantified by quantitative real-time PCR, and protein levels were reported as normalized protein expression (NPX) values calculated from normalized Ct values. In **Papers IV and V**, complement components were measured using commercially available or in-house-validated ELISA and MBSI. All samples were analyzed in duplicate.

## Human skeletal muscle cell culture and MG induction

In **Papers V and VI**, primary human skeletal muscle myoblasts from Lonza were cultured and differentiated into myotubes according to the manufacturer's instructions. Recombinant neural agrin was added to the culture three

days post-differentiation to mimic neuronal innervation in the myotubes. For *in vitro* MG induction, differentiated myotubes were incubated with sera from MG patients or purified recombinant monoclonal antibodies (mAbs) targeting specific nAChR subunits for 0.5, 2, or 24 hours, depending on the experimental design. Serum was diluted 1:1 in horse serum-free differentiation medium, whereas mAbs were used at a concentration of 2  $\mu\text{g/ml}$  in differentiation medium supplemented with 2% non-heat-inactivated horse serum.

## Cellular protein expression analysis

In **Paper VI**, protein expression was analyzed using a capillary-based western assay. Cells were lysed in RIPA buffer, and protein concentration was determined before analysis. Lysates and recombinant human nAChR  $\alpha 1$  protein (positive control) were loaded together with blocking reagent, primary antibody, biotin-conjugated secondary antibody, streptavidin-HRP, and chemiluminescent substrate. A biotinylated molecular weight ladder was included. Plates were processed using the Jess Automated Western Blot System with a 12-230 kDa fluorescence separation module for automated separation, electrophoresis, and immunodetection.

## Immunocytochemistry

In **Papers V and VI**, tetramethylrhodamine-conjugated  $\alpha$ -bungarotoxin was used to label nAChRs prior to fixation. Cells were permeabilized, and non-specific binding was blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS). Primary antibodies targeting desmin and sarcomeric  $\alpha$ -actinin were applied to identify muscle cells, while antibodies against human IgG and complement C5b-9 (MAC) were applied to assess antibody binding and complement deposition. Fluorescently labeled secondary antibodies were then applied, and nuclei were stained with DAPI. Fluorescence imaging and Z-stack acquisition were performed using a Zeiss LSM700 confocal microscope.

## Live-cell imaging

In **Paper VI**, live-cell imaging was performed to assess calcium signaling and membrane potential changes in myotubes. Myoblasts were cultured and differentiated on chambered slides prior to imaging. For calcium imaging, cells were incubated with fluorescent calcium indicator Fluo-8H, followed by washing with phenol red-free medium prior to recording. Real-time

fluorescence images were acquired every 2.5-second interval using a Leica Stellaris 5 confocal microscope. Baseline fluorescence was recorded during the initial 3 min of each experiment. Pharmacological agents, including ACh, carbamoylcholine chloride (CCh), tubocurarine hydrochloride pentahydrate, potassium chloride (KCl), benidipine, nifedipine, and NNC 55-0396 dihydrochloride were applied using a peristaltic pump system at defined time points during the experiment.

Membrane potential was assessed using the voltage-sensitive fluorescent dye DiBAC<sub>4</sub>(3), which enters and accumulates in depolarized cells. Changes in DiBAC<sub>4</sub>(3) fluorescence intensity reflect alterations in membrane potential, with a sensitivity of ~1% fluorescence change per millivolt<sup>77,78</sup>. In electrically excitable cells, DiBAC<sub>4</sub>(3) exhibits a linear correlation between fluorescence intensity and membrane potential<sup>79</sup>. Myotubes cultured on chambered slides were loaded with DiBAC<sub>4</sub>(3). Image acquisition and pharmacological agent application were performed using the same imaging setup and protocol as described for calcium imaging.

## Image analysis

In **Papers V and VI**, image processing and quantification were performed using ImageJ software. For analysis of nAChR distribution and MAC (C5b-9) deposition, fluorescence channels corresponding to nAChR, C5b-9, and desmin were separated and thresholded using the Otsu method to distinguish signal from background. Thresholded images were converted into binary masks, and signal areas were quantified. Overlapping areas were determined using the Image Calculator function. For the calcium imaging analysis, fluorescence intensity was extracted and quantified by manually marking individual myotubes as regions of interest from time-lapse image series. Data were expressed as normalized fluorescence intensity ( $F/F_B$ ), where baseline fluorescence ( $F_B$ ) was defined as the mean signal during the initial recording period prior to stimulation, or as maximal normalized intensity ( $(F/F_B)_{\max}$ ). Pseudocolor images were generated to visualize changes in fluorescence intensity.

## Cell viability assay

In **Paper VI**, cell viability was assessed using the trypan blue exclusion assay. Myotubes were treated according to the experimental conditions and subsequently harvested. Cell suspensions were mixed with trypan blue solution and loaded onto counting slides. Viable and non-viable cells were quantified using a Countess 3 Automated Cell Counter. Cell viability was calculated as the percentage of viable cells to the total number of cells counted.

## Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Normality of datasets was assessed using the D'Agostino-Pearson or Shapiro-Wilk tests. For comparisons between two groups, unpaired or paired t-tests were used for normally distributed data, while Mann-Whitney or Wilcoxon signed-rank tests were applied when normality was not met. For comparisons involving more than two groups, one-way ANOVA was used for normally distributed data, whereas non-normally distributed data were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc correction. Correlation analyses were performed using Spearman's rank correlation coefficient, with strength interpreted as weak ( $r = 0.10-0.39$ ), moderate ( $r = 0.40-0.69$ ), or strong ( $r = 0.70-1.00$ )<sup>80</sup>. Receiver operating characteristic (ROC) curve analysis was applied using the Wilson-Brown method to evaluate biomarker performance. For multiple comparisons, the Benjamini-Hochberg method or Bonferroni correction was applied. Statistical significance was defined as  $p < 0.05$  unless otherwise stated.

# Results and Discussion

## Papers I-III

### Results

#### **Elevated serum miR-150-5p and miR-30e-5p in MG**

Serum levels of miR-150-5p and miR-30e-5p were significantly elevated in MG patients, whereas miR-21-5p did not differ between MG patients and HCs. ROC analysis demonstrated that miR-150-5p had high sensitivity but low specificity for MG. Elevated miR-30e-5p levels were associated with disease relapse, indicating predictive potential for disease course. However, none of these miRNAs were specific to MG, as their expression overlapped with ONDs.

Short-term stability of miR-150-5p, miR-30e-5p, and miR-21-5p was evaluated using serum samples collected from MG patients with unchanged medication over a one-month follow-up period. Levels of miR-30e-5p and miR-150-5p exhibited greater stability over time, whereas miR-21-5p showed significant differences across sampling weeks and in subgroup analyses by immunosuppressive treatment and sex. These findings demonstrated that miR-30e-5p and miR-150-5p maintain stable expression over short time intervals in MG patients.

#### **Serum inflammatory protein profiles in MG**

Logistic regression analysis adjusted for age and sex identified 23 proteins that differed between MG patients and HCs. Of these, 16 were increased, including CCL28, CCL20, CCL11, CCL7 (MCP-3), TNF- $\alpha$ , IL-7, IL-6, IL-17C, FGF-23, FGF-21, FGF-5, VEGF-A, GDNF, IL-10, 4E-BP1, and EN-RAGE, whereas 7 were decreased, including TNFSF14, uPA, TGF- $\alpha$ , ST1A1, SIRT2, OSM, and CD6. Among these, CCL28, FGF-23, FGF-5, TGF- $\alpha$ , TNFSF14, and uPA exhibited the most pronounced differences. A volcano plot based on odds ratios and statistical significance across the full dataset highlighted proteins that differed between MG patients and HCs. In addition, principal component analysis (PCA) revealed a clear separation between MG patients and HCs. Subgroup analysis revealed that inflammatory protein profiles varied according to age of onset, disease severity, treatment status, and thymic pathology.

Table 3 summarizes miRNAs and inflammatory proteins reported in Papers I-III.

Table 3. Summary of miRNAs and inflammatory proteins identified from Papers I-III.

Serum miRNAs (Papers I-II)	
Analyte	Key findings
miR-150-5p	Sensitivity: 85.2% Specificity: 48.2% Short-term stability
miR-30e-5p	Sensitivity: 55.6% Specificity: 85.7% Short-term stability Predictive potential of disease progression
miR-21-5p	Low short-term stability
Serum inflammatory proteins (Paper III)	
Family	Key findings
Chemokine	↑ : CCL28, CCL20, CCL11, CCL7 (MCP-3)
Tumor Necrosis Factor	↑ : TNF- $\alpha$ ↓ : TNFSF14, uPA
Interleukin	↑ : IL-7, IL-6, IL-17C
Growth Factor/Hormone	↑ : FGF-23, FGF-21, FGF-5, VEGF-A, GDNF
Anti-inflammatory Cytokine	↑ : IL-10
Transforming Growth Factor	↓ : TGF- $\alpha$
Other pathways	↑ : 4E-BP1, EN-RAGE ↓ : ST1A1, SIRT2, OSM, CD6

Symbols indicate the direction of change in myasthenia gravis (MG) compared with healthy controls (HC):  
 ↑ , increased; ↓ , decreased; =, no significant difference. Key findings highlight major analytical outcomes.

## Discussion

Serum miR-150-5p and miR-30e-5p levels were significantly elevated in MG patients compared to HCs, consistent with previous reports<sup>48-50</sup>. The altered expression of miR-150-5p likely reflects its role as an immuno-miR involved in T-cell maturation, as well as its regulatory functions in B cells and natural killer (NK) cells<sup>81,82</sup>. miR-150-5p and miR-30e-5p both exhibited short-term stability. miR-30e-5p was associated with disease progression, with previous reports identifying increased levels early in oMG patients who later generalized<sup>52</sup>, supporting its predictive potential. miR-21-5p showed a distinct pattern, exhibiting marked short-term instability and no significant difference between MG patients and HCs. Given its known associations with Tregs, T-cell activation, and apoptosis resistance<sup>81</sup>, miR-21-5p may reflect dynamic, short-term immune states.

All three miRNAs are linked to several pro-inflammatory pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B)<sup>83</sup>, a central mediator of inflammatory gene expression<sup>84</sup>. Dysregulation of these miRNAs has been reported in other autoimmune diseases, including

multiple sclerosis (MS)<sup>85,86</sup>, Sjögren's syndrome<sup>87</sup>, systemic lupus erythematosus<sup>88</sup>, and inflammatory arthritis<sup>89</sup>. Notably, all three miRNAs were reported to be increased in MS patients; this may explain the overlap observed between MG and OND patients in Paper I.

The inflammatory protein profiling revealed a broad activation of immune pathways. Among the candidates, elevated levels of the chemokines CCL28 and CCL20 point to sustained recruitment of B cells and T cells, supporting ongoing immune activation<sup>90</sup>.

Members of the tumor necrosis factor (TNF) superfamily play a central role in the regulation of immune cell survival, activation, and inflammatory signaling<sup>91,92</sup>. Accordingly, TNFSF14 has been shown to promote differentiation and activation of immune cells and to regulate cytokine production, including IL-6<sup>93</sup>. Moreover, TNF family cytokines act as key upstream regulators of inflammatory signaling and can trigger multiple downstream pathways, most prominently NF- $\kappa$ B and the mitogen-activated protein kinase (MAPK) pathway<sup>84</sup>, with MAPK regulating inflammatory responses by controlling the intensity and duration of cytokine-driven gene expression<sup>94</sup>. Crosstalk between NF- $\kappa$ B signaling and MAPK pathways has been reported to regulate multiple biological processes, including immunity and inflammation<sup>95</sup>, and therefore may contribute to the chronic pro-inflammatory immune milieu in MG.

There are some limitations to consider. The relatively small sample sizes may limit generalizability. In Paper I, clinical assessments were not consistently available for all patients. In Paper II, clinical assessments were subjective, and external factors, including sleep, stress, and environmental influences, may have influenced both symptom scores and miRNA levels. The lack of healthy individuals may limit interpretation. In Paper III, most patients were receiving immunosuppressive treatment, which may have influenced inflammatory protein levels.

## Papers IV-V

### Results

#### **Complement activation in MG**

The proximal classical complement pathway component complex C1s/C1-INH was analyzed in both serum and plasma. Levels of the C1s/C1-INH complex were significantly increased in patients with MG compared to HCs. No significant differences in serum C1s/C1-INH were observed between subgroups stratified by immunosuppression, disease severity, AChR serostatus, age at onset, or sex.

Downstream complement activation products and components were further assessed. Plasma levels of C3a and sC5b-9 were significantly higher in MG patients than in HCs. In addition, altered levels of other plasma complement

components were observed, including increased levels of C5 and Factor I, as well as decreased levels of properdin.

ROC analysis demonstrated that plasma C3a showed the highest sensitivity and specificity, followed by sC5b-9. In contrast, plasma C5 exhibited lower sensitivity despite comparatively high specificity. Plasma levels of Factor B, Factor I, and Factor H positively correlated with MGC scores. In serum, Factor I levels were significantly higher in MG patients compared with HCs, whereas other measured complement components did not differ between MG patients and HCs. Together, these findings demonstrated the activation of both early and terminal components of the complement cascade in MG patients.

Table 4 summarizes the plasma complement components analyzed.

Table 4. Summary of plasma complement components reported from Papers IV-V.

Plasma complement components (Papers IV-V)		
Analyte	MG vs HC	Key findings
C1s/C1-INH	↑	-
C1q	=	-
C3a	↑	Sensitivity: 95.7% Specificity: 100%
sC5b-9	↑	Sensitivity: 82.6% Specificity: 96.6%
C5	↑	Sensitivity: 56.5% Specificity: 86%
Factor B	=	Positively correlated with MGC ( $r = 0.6748$ )
Factor I	↑	Positively correlated with MGC ( $r = 0.5837$ )
Factor H	=	Positively correlated with MGC ( $r = 0.4874$ )
Properdin	↓	-

Symbols indicate the direction of change in myasthenia gravis (MG) compared with healthy controls (HC): ↑, increased; ↓, decreased; =, no significant difference. Key findings highlight major analytical outcomes, including diagnostic performance and correlations. A dash (-) indicates that no additional significant findings were identified.

## Discussion

Complement analysis provides a link between autoimmune activation and effector mechanisms responsible for tissue damage in MG. Elevated levels of the C1s/C1-INH complex in MG patients indicate activation of the proximal classical complement pathway, extending findings from previous studies that primarily focused on downstream complement components. The finding that AChR seronegative patients exhibited comparable C1s/C1-INH levels suggested that classical pathway activation may not be restricted to AChR+ MG,

consistent with the histopathological evidence of IgG1, complement C1q and C5b-9 depositions at the skeletal muscle biopsy of SNMG <sup>96</sup>.

In addition to proximal pathway activation, increased plasma levels of C3a and sC5b-9 demonstrate activation of downstream and terminal complement components. Importantly, these complement activation products are recognized not only as mediators of membrane damage but also as potent pro-inflammatory effectors <sup>97</sup>. C3a functions as an anaphylatoxin that promotes inflammatory cytokine production, and activation of inflammatory signaling pathways, including NF- $\kappa$ B and MAPK pathways<sup>98</sup>. Moreover, sC5b-9 has been shown to induce inflammation and cytokine production <sup>97</sup>. Therefore, complement activation in MG may contribute to both local skeletal tissue damage at the NMJ and the maintenance of a broader pro-inflammatory state.

Altered levels of complement regulatory components further support the presence of sustained complement activation in MG. Increased levels of Factor I, a regulator that inactivates C3b, suggest complement activation accompanied by active regulatory control. In parallel, decreased levels of properdin, a positive regulator that stabilizes the alternative pathway C3 convertase<sup>99</sup>, may reflect consumption during alternative pathway activation.

The lack of correlation between complement activation products and disease severity is consistent with a previous study reporting no association between complement activity and disease severity <sup>100</sup>. This likely reflects that the complement-mediated damage in MG occurs primarily at the skeletal muscle, which may not be accurately captured by systemic measurements.

The relatively small plasma sample size in this study may limit generalizability, and the absence of non-MG disease controls restricts the assessment of the specificity of the observed complement alterations.

Differences across studies in reported complement profiles include findings such as no difference in plasma sC5b-9 between MG and HC, and lower serum C5 levels observed in MG <sup>101,102</sup>. Such differences are likely due to methodological differences, including sample type, handling, and analytical approaches <sup>53</sup>, as well as small sample sizes, which may contribute to variability in the findings. The use of EDTA plasma for complement activation products is critical to prevent ex vivo activation, whereas serum remains suitable for functional complement analysis <sup>53</sup>. These methodological considerations are essential for interpreting and comparing complement data across studies.

## Papers V-VI

### Results

#### **MG alters nAChR distribution and induces complement deposition in human myotubes**

Human skeletal muscle cells were used to model MG *in vitro*, with recombinant neural agrin pre-incubation applied to mimic innervated human muscle. Differentiation of human skeletal muscle myoblasts into elongated, multinucleated myotubes was confirmed by expression of  $\alpha$ -actinin, a marker of sarcomere formation. Further, agrin-induced nAChR clustering was observed. Following incubation with AChR+ MG patient serum, human IgG binding to nAChRs was detected by immunocytochemistry.

Incubation with sera from non-immunosuppressed MG patients resulted in reduced nAChR levels on myotubes at 0.5 h and 2 h compared with those exposed to HC sera. In addition, MAC deposition was detected and colocalized with nAChRs. Myotubes treated with sera from patients receiving azathioprine exhibited reduced MAC-nAChR colocalization. Confocal Z-stack imaging was used to examine the spatial relationship between MAC deposition and nAChRs after incubation with serum from a non-immunosuppressed MG patient with high disease severity (MGC score of 17), revealing ring-shaped MAC deposition surrounding nAChR clusters.

#### **nAChR-mediated depolarization and calcium influx in human myotubes**

Cholinergic stimulation with ACh and CCh, a stable agonist for prolonged stimulation that activates nAChRs, both induced robust intracellular calcium transients, as assessed by real-time calcium imaging. CCh induced a sigmoidal dose-response calcium transients in myotubes. This result validated CCh as a physiologically relevant and experimentally stable substitute for ACh. mRNA analysis confirmed a muscle-type nAChR subunit profile, with the *CHRNA1* ( $\alpha 1$ ) exhibiting the highest expression, followed by *CHRN1* ( $\beta 1$ ). Expression of  $\alpha 1$  protein was confirmed by capillary-based Western immunoassay. The observed calcium responses upon CCh stimulation were confirmed to be mediated through nAChR activation by co-application of CCh with tubocurarine, a specific nAChR antagonist. CCh-induced membrane depolarization in myotubes was assessed using the voltage-sensitive fluorescent probe DiBAC<sub>4</sub>(3), which showed a 21.6% increase in fluorescence, corresponding to approximately 21.6 mV of depolarization.

Pharmacological inhibition of VGCCs demonstrated that membrane depolarization induced by nAChR activation triggers the opening of VGCCs, resulting in calcium influx in myotubes. Co-application of T-type antagonist caused 59.0% inhibition of CCh-induced calcium influx, whereas L-type antagonist resulted in 18.4% inhibition. Consistent with this, the VGCC  $\alpha$ -

subunit expression profile exhibited higher expression levels of *Cacna1H* (Cav3.2), suggesting a major role for T-type channels in this calcium influx.

### **MG-associated antibodies impair nAChR-dependent calcium signaling**

Robust CCh-induced calcium transients were observed in HC serum-treated myotubes, whereas AChR+ MG serum markedly suppressed these responses. As MG patient-derived antibodies are polyclonal—capable of binding various epitopes of nAChR<sup>103</sup>, subunit-specific effects were examined using recombinant mAbs targeting the nAChR  $\alpha$ - or  $\beta$ -subunits. Exposure to the  $\alpha$ -subunit-specific mAb completely abolished CCh-induced calcium responses, whereas mAbs targeting the nAChR  $\beta$ -subunit had no effect on it. These findings demonstrated that MG-associated antibodies impair nAChR-dependent calcium responses in human myotubes and indicated that targeting of the  $\alpha$ -subunit is critical for this functional disruption.

### **MG-associated antibodies alter transcriptional profiles of nAChRs, VGCCs, and muscle markers**

Exposure to mAbs induced more pronounced transcriptional changes in myotubes compared with MG sera. nAChR subunits *CHRNA1* ( $\alpha$ 1), *CHRN1* ( $\beta$ 1), and *CHRN3* ( $\gamma$ ) showed a trend toward increased expression following treatment with both  $\alpha$ - and  $\beta$ -subunit-specific mAbs, whereas *CHRNA5* ( $\alpha$ 5), *CHRNA9* ( $\alpha$ 9), and *CHRN4* ( $\delta$ ) subunits tended to be upregulated in  $\beta$ -subunit-specific mAb-treated myotubes. In addition, transcripts of *ACTA1* ( $\alpha$ -actin) and *ACHE* (acetylcholinesterase; AChE) were increased in  $\alpha$ -subunit-specific mAb treated myotubes, whereas *DES* (desmin) was elevated in both mAb-treated myotubes. VGCC subunits exhibited trends toward increased *Cacna1S* (Cav1.1) and *Cacna1C* (Cav1.2) expression following MG serum exposure, and increased *Cacna1H* (Cav3.2) expression after  $\beta$ -subunit-specific mAb treatment.

### **$\alpha$ -Subunit-specific MG antibodies upregulate complement-related gene expression**

Exposure to the  $\alpha$ -subunit-specific mAb significantly increased expression of early classical pathway components, including *C1S* (C1s), *C3A1* (C3a receptor), *C5* (C5), and the terminal MAC component *C9* (C9), as well as complement regulators *CD55* (CD55) and *CD59* (CD59). In contrast, the  $\beta$ -subunit-specific mAb resulted in similar but less pronounced upregulation, most notably of *C1R* (C1r). These results demonstrated that  $\alpha$ -subunit-specific mAb exposure induces upregulation of complement-related genes in myotubes, in line with the complement deposition observed in MG serum-treated myotubes.

### **Complement C3 inhibition ameliorates $\alpha$ -mAb-mediated pathogenic effects**

Upregulation of early classical complement pathway components induced by the  $\alpha$ -subunit-specific mAb led to the investigation of whether inhibition of

complement activation could restore impaired cholinergic signaling. The  $\alpha$ -subunit-specific mAb was co-applied with compstatin, a C3-binding peptide that prevents C3 cleavage and downstream MAC formation<sup>104,105</sup>. After 24 h of exposure, compstatin significantly restored the complete inhibition of cholinergic calcium signaling induced by the  $\alpha$ -subunit-specific mAb, whereas the partial recovery was observed after 2 h of treatment. The impaired cholinergic calcium signaling induced by the  $\alpha$ -subunit-specific mAb was associated with reduced nAChR distribution and increased MAC deposition after 24 h of exposure. These effects were partially restored by compstatin. No significant changes in cell viability were observed after 24 h of exposure to the  $\alpha$ -subunit-specific mAb compared to the untreated group, indicating that the impaired calcium signaling reflects complement-mediated sublytic functional effects rather than loss of cell death or membrane integrity. Figure 6 summarizes the findings of the *in vitro* study from Papers V-VI.

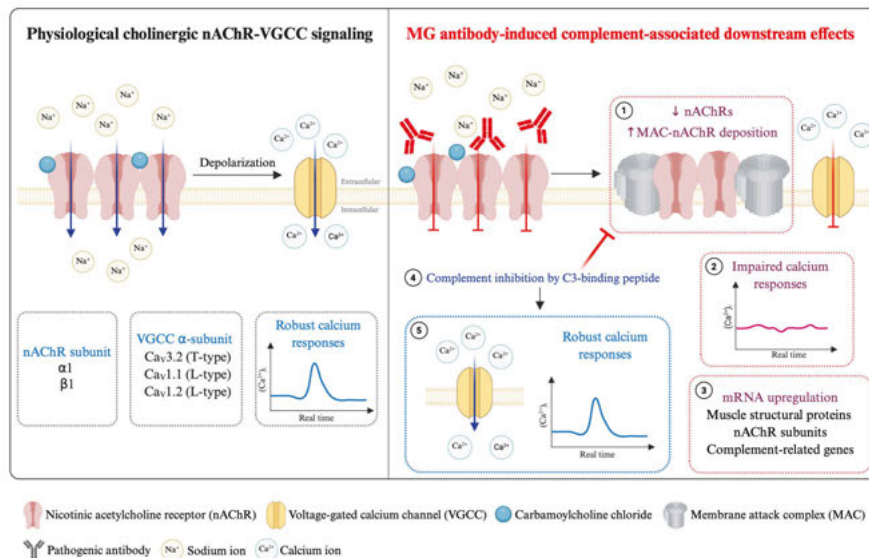


Figure 6. Schematic summary of functional and molecular findings.

In human myotubes under normal conditions (left panel), activation of nicotinic acetylcholine receptors (nAChRs) by cholinergic stimulation induces membrane depolarization, which subsequently activates voltage-gated calcium channels (VGCCs), predominantly T-type ( $Cav3.2$ ). This results in robust intracellular calcium transients. In the presence of myasthenia gravis (MG)-associated antibodies (right panel): (1) pathogenic antibodies bind to nAChRs, leading to reduced nAChR levels, deposition of the membrane attack complex (MAC) and (2) impaired nAChR-dependent calcium signaling. (3) Antibody exposure is accompanied by transcriptional upregulation of muscle structural proteins, nAChR subunits, and complement-related genes. (4) Pharmacological inhibition of complement activation using a C3-binding peptide restores (5) cholinergic calcium responses, preserves nAChR distribution, and reduces MAC deposition. This figure was created with BioRender.

## Discussion

These studies provide mechanistic insight into how pathogenic antibodies in MG affect human skeletal muscle cells at both molecular and functional levels, and into the role of complement activation in mediating these effects. Serum from AChR+ MG patients reduced nAChR levels, induced MAC deposition on myotubes, and suppressed nAChR-mediated calcium signaling, demonstrating that this model recapitulates key pathogenic features of AChR+ MG. MAC deposition was detected following short incubation periods, consistent with the rapid enzymatic cascade of complement activation that leads to MAC assembly once triggered<sup>106</sup>. Activation of nAChRs induced membrane depolarization and calcium influx through VGCCs, recapitulating a physiologically relevant signaling pathway underlying *in vivo* muscle contraction and homeostasis. VGCCs subtype-specific inhibition and transcript profiling identified T-type *Cacna1H* (Cav3.2) as a predominant contributor to cholinergic calcium responses in this model, followed by L-type *Cacna1S* (Cav1.1). This supports previously known roles for Cav3.2 channels in the integrity and functionality of skeletal muscle, calcium homeostasis, and late-stage myotube maturation, and for Cav1.1, exclusively expressed in skeletal muscles, as the voltage sensor for ECC<sup>76,107,108</sup>.

Exposure to MG patient sera markedly suppressed the cholinergic calcium responses, representing a functional consequence of nAChR loss and MAC deposition. The prolonged serum exposure closely mimics the chronic immune attack experienced *in vivo* and likely involves multiple pathogenic mechanisms. Moreover, the subunit-specific pathogenic effects on cholinergic stimulation highlight the dominant role of the  $\alpha$ -subunit in receptor activation and align with known structural features of muscle-type nAChRs, in which ligand binding and channel gating are mediated at interfaces involving  $\alpha$  subunits as well as the pathogenic antibody binding to the MIR on the  $\alpha$ -subunit. These findings indicate that AChR antibodies differ in pathogenic potential, with subunit specificity determining functional impairment, consistent with previous studies showing that  $\alpha$ -subunit-targeting antibodies are more pathogenic than  $\beta$ -subunit antibodies in EAMG and cell-based systems<sup>109,110</sup>. Recent structural studies demonstrate that AChR antibodies exhibit heterogeneity in subunit specificity, epitope location, and mechanism of action, indicating that pathogenicity depends not only on the targeted subunit but also on the precise epitope and binding orientation<sup>111</sup>.  $\alpha$ -subunit-specific antibodies can trigger all major pathogenic mechanisms in MG<sup>103</sup>; however, inhibition of complement activation alone was sufficient to restore nAChR-mediated calcium signaling after 24 h of exposure. This observation demonstrates that complement activation is the key driver of functional neuromuscular impairment. Accordingly, preservation of neuromuscular transmission can be achieved despite continued presence of pathogenic antibodies when the complement cascade is interrupted. This is supported by the clinical efficacy of

C5-targeting therapies, which prevent MAC formation, in patients with AChR+ MG<sup>14</sup>. The present results demonstrate that inhibition at the proximal end of the complement cascade, specifically at the level of C3 cleavage, can prevent nAChR loss, MAC formation, and functional effects in human muscle cells. This observation aligns with findings from rat passive transfer MG model, where C3 knockdown by siRNA reduced nAChR loss, decreased complement deposition, and improved muscle function<sup>112</sup>. As C3 is the convergence point of all complement activation pathways, its modulation may provide broader protection by preventing both MAC formation and pro-inflammatory fragment generation, suggesting proximal complement components as promising therapeutic targets.

In addition to functional effects, the transcriptional responses observed in myotubes exposed to MG sera and subunit-specific antibodies suggest that muscle cells actively respond to immune-mediated stress. Upregulation of muscle structural proteins and complement components, along with trends toward increased expression of nAChR and VGCC subunits, may represent compensatory mechanisms in response to complement-driven damage; however, these changes are likely insufficient to achieve effective functional restoration. Similar compensatory responses, including increased  $\alpha 1$ -subunit mRNA expression, have been reported in both EAMG and muscle biopsies from MG patients<sup>113-115</sup>. While such upregulation does not restore neuromuscular function in EAMG models<sup>114</sup>, its functional relevance in human muscle remains to be elucidated.

This *in vitro* study has some limitations. As the developmental  $\gamma$ -to- $\epsilon$  nAChR subunit switch is regulated by motor neuron innervation and electrical activity<sup>116</sup>, this model may not fully reflect mature myotubes *in vivo*. The relatively small number of patient sera and the use of samples from a single clinical site may limit generalizability. Finally, the interventions were primarily pharmacological and antibody-based.

# Conclusion

This thesis identified potential circulating biomarkers in MG and investigated the complement-associated effects, including molecular and functional, of pathogenic MG antibodies *in vitro*.

In **Papers I-II**, serum miR-150-5p and miR-30e-5p were elevated in MG and exhibited short-term stability, whereas miR-21-5p did not differ between MG patients and HCs and showed unstable short-term expression. miR-30e-5p exhibited the greatest predictive potential. However, these miRNAs were not specific to MG, as their expression overlapped with ONDs.

In **Paper III**, inflammatory serum proteins CCL28, FGF-23, FGF-5, TGF- $\alpha$ , TNFSF14, and uPA showed the greatest differences between MG and HC. Subgroup differences highlighted the heterogeneity within MG.

**Papers IV-V** demonstrated activation of the complement cascade in MG, with increased C1s/C1-INH levels indicating proximal classical pathway activation and elevated plasma C3a and sC5b-9 reflecting downstream and terminal complement activity.

**Papers V-VI** established an *in vitro* human skeletal muscle cell model of MG, induced by application of MG sera or pathogenic mAbs, that recapitulated key pathogenic features, including antibody binding to nAChRs, nAChR reduction, MAC deposition, and impaired nAChR-dependent calcium signaling.  $\alpha$ -subunit-specific mAb induced transcriptional upregulation of nAChR subunits, muscle structural proteins, and complement components. Notably, subunit-specific differences were observed, as  $\beta$ -subunit-specific antibodies had no functional impact. Inhibition of complement activation at C3 cleavage preserved nAChR distribution, reduced MAC deposition, and restored cholinergic calcium responses, indicating complement activation as a driver of antibody-mediated neuromuscular dysfunction and highlighting proximal complement inhibition as a promising therapeutic strategy.

## Future Perspective

Although this thesis provides insight into potential circulating biomarkers and complement-associated mechanisms in MG, it raises further research questions that should be addressed in future.

For biomarker studies, validation of candidate biomarkers in larger and multi-center cohorts, is an important next step. Longitudinal studies with longer follow-up periods and the inclusion of disease control groups would strengthen the interpretability of these findings and would be valuable for studying the dynamics of these candidate biomarkers, particularly during disease progression and treatment response. Such studies could help determine the temporal dimensions of these candidates and whether they are responsive to therapeutic interventions, thereby improving their potential utility.

Findings from the human muscle cell model demonstrate that complement inhibition at the level of C3 is a promising therapeutic strategy, supporting further exploration of upstream complement inhibition alongside currently available C5-targeted treatments. Moreover, whether targeting C3 exerts additional effects, such as anti-inflammatory mechanism, remains to be elucidated. In addition to complement components in the main activation pathway, complement regulatory proteins, particularly CD55 and CD59, are also an important area for future study. MG pathogenic antibodies have been shown to increase transcriptional expression in this thesis, suggesting a potential compensatory response. Given their roles in restricting complement activation and preventing MAC formation, further investigation of their regulation and functional role in MG may provide important insights into the protective mechanisms. Genetic approaches, including siRNA-mediated knockdown or CRISPR-based knockout of CD55 and CD59, as well as plasmid-mediated overexpression, can be used to investigate the regulatory roles of these proteins in complement-associated processes.

The altered distribution of nAChRs observed in muscle cells following exposure to MG pathogenic antibodies motivates further investigation into nAChR dynamics under complement activation conditions. Approaches such as transfection with fluorescently tagged nAChR subunits could enable real-time visualization of receptor trafficking, helping to elucidate the surface expression or endocytosis of the receptors.

The muscle cell model used in this thesis provides a platform for studying skeletal muscle pathology in MG; however, the nAChR subunit composition

may not fully reflect the adult type. Introducing motor neurons to enable neuronal innervation may strengthen the physiological relevance of the model. Moreover, future work integrating electrophysiological functional readouts, such as patch-clamp recordings and microelectrode array (MEA) systems, could provide deeper insights into ion channel activity and network-level electrical activity.

# Acknowledgments

I am deeply grateful to all who have supported me throughout my PhD journey and would like to express my sincere appreciation.

I would like to express my sincere gratitude to my supervisor, **Anna Rostedt Punga**, for giving me the opportunity to begin as an intern, continue as a research assistant, and ultimately pursue my PhD under your supervision. I am deeply thankful for your guidance and consistent support throughout this journey. Your mentorship and the knowledge you shared have been essential to my development. I am grateful for your encouragement and positivity along the way, as well as for your constructive feedback. I also appreciate your guidance in helping me navigate challenges and grow.

I am also thankful to my co-supervisor, **Maria Lindskog**, for the valuable discussions and input for my study.

I would like to extend my special thanks to **Amol Bhandage**, not only for being my co-supervisor but also a great colleague. You introduced me to the fascinating world of calcium imaging and provided guidance throughout my experimental work, and shared your knowledge. I am grateful for your support and encouragement during challenging times, and for always helping me regain my calm when I felt overwhelmed.

Sincere thanks to **Bo Nilsson** for sharing your expertise in the complement system, for your guidance and valuable input in experimental design, and for your support of my experimental work in your lab.

I am thankful to **Barbro Persson** for valuable discussions and for your assistance in designing and refining the experiments. I am also grateful for your kindness and support during challenging moments.

I would like to thank **Lisa Monie** for your great help with carrying out experiments and for the many enjoyable moments we shared in the lab. Your support during difficult times meant a lot to me, and it has always been a pleasure working with you.

I would like to thank **Tanel Punga** for your expertise and support in plasmid construction.

I would like to thank the former and present members of the Clinical Neurophysiology group. Special thanks to **Milos Radivojevic** for introducing me to the MEA field, for your inspiration, creativity, and encouragement, and for the time we worked together. Many thanks to **Francesca Beretta** for the time we spent together, for the occasional Zoom catch-ups, and for your support. I

would also like to thank **Anton Tjust, Maja Norling, Laura O'Connor, Pirkko Hynninen, Sara Ekberg** and **Artor Pogosean** for your company and encouragement.

I would like to thank **Jeremy Adler** and **Ross Smith** for valuable discussions on imaging and image analysis.

Special thanks to **Hanzhao** for your company and for all the ways you have helped me, and **Menghan** for all the moments we have shared together and for always bringing positivity. Special thanks to **Susie, Kim, Tianqi, Yongsheng,** and **Mehran** for the warm and inspiring conversations and encouragement.

A very special thank you to Ursula, my closest group of friends since high school in Taiwan—**Chih-Hsuan, Cian-Yu, Hui-Ting, Jo-Ping, Ju-Chun, Ping-Chih,** and **Yao**—for your unwavering emotional support across the years and across distances. Thank you for always being there for me, for answering my calls in the middle of the night in Taiwan, and for making every visit back home so special. You have always meant so much to me.

I would like to express my heartfelt gratitude to **Carlos** for your constant support, positivity, and for always believing in me. Thank you for your emotional support and comfort during difficult times, as well as for the thoughtful surprises that brought me joy. Your presence has meant more to me than words can express. I am also thankful to the **Carlos' family** for your support and encouragement. I am grateful that you always made sure I joined you for holidays and celebrations, and for making these occasions so special that I truly felt at home.

To my family, I am forever grateful. To my mother, **Yue-Ling Hu;** my sister, **Chien-Ju,** and her husband, **Hsin-Yu;** and my brother, **Chien-Hsiang,** with his wife, **Ying-Tung,** and their lovely children, **Pin-Wei** and **Pin-Tse**—thank you for your love and support. Thank you for visiting me after I moved to Sweden, and for always planning thoughtful trips and activities whenever I return home, creating many meaningful and cherished memories together. I am grateful for our constant phone calls and messages, and for sharing our daily lives through pictures, which have helped me through moments of homesickness.

I want to thank **Momo,** my beloved dog, who was by my side for ten years and came with me to Sweden, accompanying me during my first year here. In difficult times, the memory of his companionship continues to give me comfort and strength.

Finally, I would like to especially remember my father, **Chin-Liang Huang,** a kind, gentle, and warm person. The love and the quiet strength he gave me continue to guide and support me. His positive influence remains with me in many ways, and I am deeply grateful for everything he has given me.

Thank you all for being part of this journey.

# References

- 1 Romi, F., Hong, Y. & Gilhus, N. E. Pathophysiology and immunological profile of myasthenia gravis and its subgroups. *Curr Opin Immunol* **49**, 9–13 (2017). <https://doi.org/10.1016/j.coi.2017.07.006>
- 2 Gilhus, N. E. & Breiner, A. Epidemiology of myasthenia gravis. *Int Rev Neurobiol* **182**, 161–196 (2025). <https://doi.org/10.1016/bs.irm.2025.04.028>
- 3 Punga, A. R., Maddison, P., Heckmann, J. M., Guptill, J. T. & Evoli, A. Epidemiology, diagnostics, and biomarkers of autoimmune neuromuscular junction disorders. *Lancet Neurol* **21**, 176–188 (2022). [https://doi.org/10.1016/S1474-4422\(21\)00297-0](https://doi.org/10.1016/S1474-4422(21)00297-0)
- 4 Dresser, L., Wlodarski, R., Rezaia, K. & Soliven, B. Myasthenia Gravis: Epidemiology, Pathophysiology and Clinical Manifestations. *J Clin Med* **10** (2021). <https://doi.org/10.3390/jcm10112235>
- 5 Sciancalepore, F. *et al.* Prevalence, Incidence, and Mortality of Myasthenia Gravis and Myasthenic Syndromes: A Systematic Review. *Neuroepidemiology* **59**, 579–592 (2025). <https://doi.org/10.1159/000539577>
- 6 Luong, K. *et al.* Comparison of three methods for the detection of antibodies against muscle-specific kinase. *J Immunol Methods* **526**, 113627 (2024). <https://doi.org/10.1016/j.jim.2024.113627>
- 7 Gambino, C. M. *et al.* Detection of Antibodies against the Acetylcholine Receptor in Patients with Myasthenia Gravis: A Comparison of Two Enzyme Immunoassays and a Fixed Cell-Based Assay. *J Clin Med* **12** (2023). <https://doi.org/10.3390/jcm12144781>
- 8 Leite, M. I. *et al.* IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. *Brain* **131**, 1940–1952 (2008). <https://doi.org/10.1093/brain/awn092>
- 9 Tannemaat, M. R., Huijbers, M. G. & Verschuuren, J. Myasthenia gravis-Pathophysiology, diagnosis, and treatment. *Handb Clin Neurol* **200**, 283–305 (2024). <https://doi.org/10.1016/B978-0-12-823912-4.00026-8>
- 10 Meriggioli, M. N. & Sanders, D. B. Autoimmune myasthenia gravis: emerging clinical and biological heterogeneity. *Lancet Neurol* **8**, 475–490 (2009). [https://doi.org/10.1016/S1474-4422\(09\)70063-8](https://doi.org/10.1016/S1474-4422(09)70063-8)
- 11 Axelsen, K. H., Kjaer Andersen, R., Andersen, L. K., Vissing, J. & Witting, N. Ocular versus generalized myasthenia gravis: a continuum associated with acetylcholine receptor antibody titers. *Neuromuscul Disord* **43**, 39–43 (2024). <https://doi.org/10.1016/j.nmd.2024.07.002>
- 12 Gilhus, N. E. & Verschuuren, J. J. Myasthenia gravis: subgroup classification and therapeutic strategies. *Lancet Neurol* **14**, 1023–1036 (2015). [https://doi.org/10.1016/S1474-4422\(15\)00145-3](https://doi.org/10.1016/S1474-4422(15)00145-3)
- 13 Aarli, J. A. Myasthenia gravis in the elderly: Is it different? *Ann N Y Acad Sci* **1132**, 238–243 (2008). <https://doi.org/10.1196/annals.1405.040>

- 14 Gerischer, L., Dokhani, P., Hoffmann, S. & Meisel, A. New and Emerging Biological Therapies for Myasthenia Gravis: A Focussed Review for Clinical Decision-Making. *Biodrugs* **39**, 185–213 (2025). <https://doi.org/10.1007/s40259-024-00701-1>
- 15 Rodolico, C., Bonanno, C., Toscano, A. & Vita, G. MuSK-Associated Myasthenia Gravis: Clinical Features and Management. *Front Neurol* **11**, 660 (2020). <https://doi.org/10.3389/fneur.2020.00660>
- 16 Gilhus, N. E. *et al.* Myasthenia gravis - autoantibody characteristics and their implications for therapy. *Nat Rev Neurol* **12**, 259–U291 (2016). <https://doi.org/10.1038/nrneurol.2016.44>
- 17 Thomsen, J. L. S. & Andersen, H. Outcome Measures in Clinical Trials of Patients With Myasthenia Gravis. *Front Neurol* **11** (2020). <https://doi.org/10.3389/fneur.2020.596382>
- 18 Barnett, C., Herbelin, L., Dimachkie, M. M. & Barohn, R. J. Measuring Clinical Treatment Response in Myasthenia Gravis. *Neurol Clin* **36**, 339–353 (2018). <https://doi.org/10.1016/j.ncl.2018.01.006>
- 19 Burns, T. M., Conaway, M. R., Cutter, G. R., Sanders, D. B. & Grp, M. S. Construction of an Efficient Evaluative Instrument for Myasthenia Gravis: The Mg Composite. *Muscle Nerve* **38**, 1553–1562 (2008). <https://doi.org/10.1002/mus.21185>
- 20 Guptill, J. T. *et al.* Addressing Outcome Measure Variability in Myasthenia Gravis Clinical Trials. *Neurology* **101**, 442–451 (2023). <https://doi.org/10.1212/Wnl.0000000000207278>
- 21 Waldmann, H. Mechanisms of immunological tolerance. *Clin Biochem* **49**, 324–328 (2016). <https://doi.org/10.1016/j.clinbiochem.2015.05.019>
- 22 Squire, J. M. Architecture and function in the muscle sarcomere. *Curr Opin Struct Biol* **7**, 247–257 (1997). [https://doi.org/10.1016/s0959-440x\(97\)80033-4](https://doi.org/10.1016/s0959-440x(97)80033-4)
- 23 Lee, J. Y. *et al.* Compromised fidelity of B-cell tolerance checkpoints in AChR and MuSK myasthenia gravis. *Ann Clin Transl Neurol* **3**, 443–454 (2016). <https://doi.org/10.1002/acn3.311>
- 24 Wang, Z. & Yan, Y. Immunopathogenesis in Myasthenia Gravis and Neuromyelitis Optica. *Front Immunol* **8**, 1785 (2017). <https://doi.org/10.3389/fimmu.2017.01785>
- 25 Uzawa, A. *et al.* Roles of cytokines and T cells in the pathogenesis of myasthenia gravis. *Clin Exp Immunol* **203**, 366–374 (2021). <https://doi.org/10.1111/cei.13546>
- 26 Thirupathi, M. *et al.* Functional defect in regulatory T cells in myasthenia gravis. *Ann N Y Acad Sci* **1274**, 68–76 (2012). <https://doi.org/10.1111/j.1749-6632.2012.06840.x>
- 27 Mackay, F., Schneider, P., Rennert, P. & Browning, J. BAFF AND APRIL: a tutorial on B cell survival. *Annu Rev Immunol* **21**, 231–264 (2003). <https://doi.org/10.1146/annurev.immunol.21.120601.141152>
- 28 Fichtner, M. L., Jiang, R., Bourke, A., Nowak, R. J. & O'Connor, K. C. Autoimmune Pathology in Myasthenia Gravis Disease Subtypes Is Governed by Divergent Mechanisms of Immunopathology. *Front Immunol* **11**, 776 (2020). <https://doi.org/10.3389/fimmu.2020.00776>
- 29 Kalamida, D. *et al.* Muscle and neuronal nicotinic acetylcholine receptors. Structure, function and pathogenicity. *FEBS J* **274**, 3799–3845 (2007). <https://doi.org/10.1111/j.1742-4658.2007.05935.x>
- 30 Tzartos, S. J. *et al.* Anatomy of the antigenic structure of a large membrane autoantigen, the muscle-type nicotinic acetylcholine receptor. *Immunol Rev* **163**, 89–120 (1998). <https://doi.org/10.1111/j.1600-065x.1998.tb01190.x>

- 31 Huijbers, M. G. *et al.* Pathogenic immune mechanisms at the neuromuscular synapse: the role of specific antibody-binding epitopes in myasthenia gravis. *J Intern Med* **275**, 12–26 (2014). <https://doi.org/10.1111/joim.12163>
- 32 Kaminski, H. J., Sikorski, P., Coronel, S. I. & Kusner, L. L. Myasthenia gravis: the future is here. *J Clin Invest* **134** (2024). <https://doi.org/10.1172/JCI1179742>
- 33 Drachman, D. B., Adams, R. N., Josifek, L. F. & Self, S. G. Functional activities of autoantibodies to acetylcholine receptors and the clinical severity of myasthenia gravis. *N Engl J Med* **307**, 769–775 (1982). <https://doi.org/10.1056/NEJM198209233071301>
- 34 Koneczny, I. & Herbst, R. Myasthenia Gravis: Pathogenic Effects of Autoantibodies on Neuromuscular Architecture. *Cells-Basel* **8** (2019). <https://doi.org/10.3390/cells8070671>
- 35 Howard, J. F. Myasthenia gravis: the role of complement at the neuromuscular junction. *Ann Ny Acad Sci* **1412**, 113–128 (2018). <https://doi.org/10.1111/nyas.13522>
- 36 Sahashi, K., Engel, A. G., Lambert, E. H. & Howard, F. M., Jr. Ultrastructural localization of the terminal and lytic ninth complement component (C9) at the motor end-plate in myasthenia gravis. *J Neuropathol Exp Neurol* **39**, 160–172 (1980). <https://doi.org/10.1097/00005072-198003000-00005>
- 37 Zeerleder, S. C1-Inhibitor: More Than a Serine Protease Inhibitor. *Semin Thromb Hemost* **37**, 362–374 (2011). <https://doi.org/10.1055/s-0031-1276585>
- 38 Tuzun, E. & Christadoss, P. Complement associated pathogenic mechanisms in myasthenia gravis. *Autoimmun Rev* **12**, 904–911 (2013). <https://doi.org/10.1016/j.autrev.2013.03.003>
- 39 Engel, A. G., Lambert, E. H. & Howard, F. M. Immune-Complexes (Igg and C3) at Motor Endplate in Myasthenia-Gravis - Ultrastructural and Light Microscopic Localization and Electrophysiologic Correlations. *Mayo Clin Proc* **52**, 267–280 (1977).
- 40 Engel, A. G. *et al.* Passively transferred experimental autoimmune myasthenia gravis. Sequential and quantitative study of the motor end-plate fine structure and ultrastructural localization of immune complexes (IgG and C3), and of the acetylcholine receptor. *Neurology* **29**, 179–188 (1979). <https://doi.org/10.1212/wnl.29.2.179>
- 41 Tuzun, E., Scott, B. G., Goluszko, E., Higgs, S. & Christadoss, P. Genetic evidence for involvement of classical complement pathway in induction of experimental autoimmune myasthenia gravis. *J Immunol* **171**, 3847–3854 (2003). [https://doi.org/DOI 10.4049/jimmunol.171.7.3847](https://doi.org/DOI%2010.4049/jimmunol.171.7.3847)
- 42 Chamberlain-Banoub, J., Neal, J. W., Mizuno, M., Harris, C. L. & Morgan, B. P. Complement membrane attack is required for endplate damage and clinical disease in passive experimental myasthenia gravis in Lewis rats. *Clin Exp Immunol* **146**, 278–286 (2006). <https://doi.org/10.1111/j.1365-2249.2006.03198.x>
- 43 Alhaidar, M. K., Abumurad, S., Soliven, B. & Rezanian, K. Current Treatment of Myasthenia Gravis. *J Clin Med* **11** (2022). <https://doi.org/10.3390/jcm11061597>
- 44 Califf, R. M. Biomarker definitions and their applications. *Exp Biol Med* **243**, 213–221 (2018). <https://doi.org/10.1177/1535370217750088>
- 45 Kaminski, H. J. *et al.* Biomarker development for myasthenia gravis. *Myasthenia Gravis and Related Disorders II* **1275**, 101–106 (2012). <https://doi.org/10.1111/j.1749-6632.2012.06787.x>

- 46 Bhandage, A. K., Huang, Y. F., Punga, T. & Punga, A. R. On the road to blood biomarkers in myasthenia gravis (MG): Beyond clinical scales. *J Neuromuscul Dis*, 22143602251348753 (2025). <https://doi.org/10.1177/22143602251348753>
- 47 O'Brien, J., Hayder, H., Zayed, Y. & Peng, C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol* **9** (2018). <https://doi.org/10.3389/fendo.2018.00402>
- 48 Punga, T. *et al.* Circulating miRNAs in myasthenia gravis: miR-150-5p as a new potential biomarker. *Ann Clin Transl Neur* **1**, 49–58 (2014). <https://doi.org/10.1002/acn3.24>
- 49 Punga, A. R., Andersson, M., Alimohammadi, M. & Punga, T. Disease specific signature of circulating miR-150-5p and miR-21-5p in myasthenia gravis patients. *J Neurol Sci* **356**, 90–96 (2015). <https://doi.org/10.1016/j.jns.2015.06.019>
- 50 Sabre, L., Maddison, P., Sadalage, G., Ambrose, P. A. & Punga, A. R. Circulating microRNA miR-21-5p, miR-150-5p and miR-30e-5p correlate with clinical status in late onset myasthenia gravis. *J Neuroimmunol* **321**, 164–170 (2018). <https://doi.org/10.1016/j.jneuroim.2018.05.003>
- 51 Molin, C. J., Sabre, L., Weis, C. A., Punga, T. & Punga, A. R. Thymectomy lowers the myasthenia gravis biomarker miR-150-5p. *Neurol Neuroimmunol Neuroinflamm* **5**, e450 (2018). <https://doi.org/10.1212/NXI.0000000000000450>
- 52 Sabre, L. *et al.* miR-30e-5p as predictor of generalization in ocular myasthenia gravis. *Ann Clin Transl Neurol* **6**, 243–251 (2019). <https://doi.org/10.1002/acn3.692>
- 53 Ekdahl, K. N. *et al.* Interpretation of Serological Complement Biomarkers in Disease. *Front Immunol* **9**, 2237 (2018). <https://doi.org/10.3389/fimmu.2018.02237>
- 54 Mantegazza, R., Cordiglieri, C., Consonni, A. & Baggi, F. Animal models of myasthenia gravis: utility and limitations. *Int J Gen Med* **9**, 53–64 (2016). <https://doi.org/10.2147/IJGM.S88552>
- 55 Meinel, E., Klinkert, W. E. & Wekerle, H. The thymus in myasthenia gravis. Changes typical for the human disease are absent in experimental autoimmune myasthenia gravis of the Lewis rat. *Am J Pathol* **139**, 995–1008 (1991).
- 56 Steinbeck, J. A. *et al.* Functional Connectivity under Optogenetic Control Allows Modeling of Human Neuromuscular Disease. *Cell Stem Cell* **18**, 134–143 (2016). <https://doi.org/10.1016/j.stem.2015.10.002>
- 57 Huang, Y. F., Verpalen, R. L. K., Punga, A. R. & Huijbers, M. G. Cell models for studying myasthenia gravis. *Int Rev Neurobiol* **182**, 121–143 (2025). <https://doi.org/10.1016/bs.irn.2025.04.026>
- 58 Hughes, B. W., Kusner, L. L. & Kaminski, H. J. Molecular architecture of the neuromuscular junction. *Muscle Nerve* **33**, 445–461 (2006). <https://doi.org/10.1002/mus.20440>
- 59 Rodriguez Cruz, P. M., Cossins, J., Beeson, D. & Vincent, A. The Neuromuscular Junction in Health and Disease: Molecular Mechanisms Governing Synaptic Formation and Homeostasis. *Front Mol Neurosci* **13**, 610964 (2020). <https://doi.org/10.3389/fnmol.2020.610964>
- 60 Willmann, R. & Fuhrer, C. Neuromuscular synaptogenesis: clustering of acetylcholine receptors revisited. *Cell Mol Life Sci* **59**, 1296–1316 (2002). <https://doi.org/10.1007/s00018-002-8509-4>
- 61 Burden, S. J., Huijbers, M. G. & Remedio, L. Fundamental Molecules and Mechanisms for Forming and Maintaining Neuromuscular Synapses. *Int J Mol Sci* **19** (2018). <https://doi.org/10.3390/ijms19020490>

- 62 Martyn, J. A., Fagerlund, M. J. & Eriksson, L. I. Basic principles of neuromuscular transmission. *Anaesthesia* **64** Suppl 1, 1–9 (2009). <https://doi.org/10.1111/j.1365-2044.2008.05865.x>
- 63 Frontera, W. R. & Ochala, J. Skeletal muscle: a brief review of structure and function. *Calcif Tissue Int* **96**, 183–195 (2015). <https://doi.org/10.1007/s00223-014-9915-y>
- 64 Mukund, K. & Subramaniam, S. Skeletal muscle: A review of molecular structure and function, in health and disease. *Wiley Interdiscip Rev Syst Biol Med* **12**, e1462 (2020). <https://doi.org/10.1002/wsbm.1462>
- 65 Fitts, R. H. The cross-bridge cycle and skeletal muscle fatigue. *J Appl Physiol (1985)* **104**, 551–558 (2008). <https://doi.org/10.1152/jappphysiol.01200.2007>
- 66 Galinska-Rakoczy, A. *et al.* Structural basis for the regulation of muscle contraction by troponin and tropomyosin. *J Mol Biol* **379**, 929–935 (2008). <https://doi.org/10.1016/j.jmb.2008.04.062>
- 67 Calderon, J. C., Bolanos, P. & Caputo, C. The excitation-contraction coupling mechanism in skeletal muscle. *Biophys Rev* **6**, 133–160 (2014). <https://doi.org/10.1007/s12551-013-0135-x>
- 68 Albuquerque, E. X., Pereira, E. F., Alkondon, M. & Rogers, S. W. Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiol Rev* **89**, 73–120 (2009). <https://doi.org/10.1152/physrev.00015.2008>
- 69 Sine, S. M. & Claudio, T. Gamma- and delta-subunits regulate the affinity and the cooperativity of ligand binding to the acetylcholine receptor. *J Biol Chem* **266**, 19369–19377 (1991).
- 70 Unwin, N., Miyazawa, A., Li, J. & Fujiyoshi, Y. Activation of the nicotinic acetylcholine receptor involves a switch in conformation of the alpha subunits. *J Mol Biol* **319**, 1165–1176 (2002). [https://doi.org/10.1016/S0022-2836\(02\)00381-9](https://doi.org/10.1016/S0022-2836(02)00381-9)
- 71 Gillespie, S. K., Balasubramanian, S., Fung, E. T. & Haganir, R. L. Rapsyn clusters and activates the synapse-specific receptor tyrosine kinase MuSK. *Neuron* **16**, 953–962 (1996). [https://doi.org/10.1016/s0896-6273\(00\)80118-x](https://doi.org/10.1016/s0896-6273(00)80118-x)
- 72 Green, W. N., Ross, A. F. & Claudio, T. Acetylcholine receptor assembly is stimulated by phosphorylation of its gamma subunit. *Neuron* **7**, 659–666 (1991). [https://doi.org/10.1016/0896-6273\(91\)90378-d](https://doi.org/10.1016/0896-6273(91)90378-d)
- 73 Ramanathan, V. K. & Hall, Z. W. Altered glycosylation sites of the  $\delta$  subunit of the acetylcholine receptor (AChR) reduce  $\alpha\delta$  association and receptor assembly. *J Biol Chem* **274**, 20513–20520 (1999). <https://doi.org/DOI 10.1074/jbc.274.29.20513>
- 74 Catterall, W. A. Voltage-gated calcium channels. *Cold Spring Harb Perspect Biol* **3**, a003947 (2011). <https://doi.org/10.1101/cshperspect.a003947>
- 75 Neumaier, F., Dibue-Adjei, M., Hescheler, J. & Schneider, T. Voltage-gated calcium channels: Determinants of channel function and modulation by inorganic cations. *Prog Neurobiol* **129**, 1–36 (2015). <https://doi.org/10.1016/j.pneurobio.2014.12.003>
- 76 Bijlenga, P. *et al.* T-type alpha 1H Ca<sup>2+</sup> channels are involved in Ca<sup>2+</sup> signaling during terminal differentiation (fusion) of human myoblasts. *Proc Natl Acad Sci U S A* **97**, 7627–7632 (2000). <https://doi.org/10.1073/pnas.97.13.7627>
- 77 Adams, D. S. & Levin, M. Measuring resting membrane potential using the fluorescent voltage reporters DiBAC4(3) and CC2-DMPE. *Cold Spring Harb Protoc* **2012**, 459–464 (2012). <https://doi.org/10.1101/pdb.prot067702>
- 78 Haupt, A. *et al.* Electrochemical regulation of budding yeast polarity. *PLoS Biol* **12**, e1002029 (2014). <https://doi.org/10.1371/journal.pbio.1002029>

- 79 Epps, D. E., Wolfe, M. L. & Groppi, V. Characterization of the steady-state and dynamic fluorescence properties of the potential-sensitive dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol (Dibac4(3)) in model systems and cells. *Chem Phys Lipids* **69**, 137–150 (1994). [https://doi.org/10.1016/0009-3084\(94\)90035-3](https://doi.org/10.1016/0009-3084(94)90035-3)
- 80 Schober, P., Boer, C. & Schwarte, L. A. Correlation Coefficients: Appropriate Use and Interpretation. *Anesth Analg* **126**, 1763–1768 (2018). <https://doi.org/10.1213/ANE.0000000000002864>
- 81 Kroesen, B. J. *et al.* Immuno-miRs: critical regulators of T-cell development, function and ageing. *Immunology* **144**, 1–10 (2015). <https://doi.org/10.1111/imm.12367>
- 82 Sabre, L., Punga, T. & Punga, A. R. Circulating miRNAs as Potential Biomarkers in Myasthenia Gravis: Tools for Personalized Medicine. *Front Immunol* **11**, 213 (2020). <https://doi.org/10.3389/fimmu.2020.00213>
- 83 Fiorillo, A. A. *et al.* Estrogen Receptor, Inflammatory, and FOXO Transcription Factors Regulate Expression of Myasthenia Gravis-Associated Circulating microRNAs. *Front Immunol* **11**, 151 (2020). <https://doi.org/10.3389/fimmu.2020.00151>
- 84 Liu, T., Zhang, L., Joo, D. & Sun, S. C. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther* **2**, 17023– (2017). <https://doi.org/10.1038/sigtrans.2017.23>
- 85 Bergman, P. *et al.* Circulating miR-150 in CSF is a novel candidate biomarker for multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm* **3**, e219 (2016). <https://doi.org/10.1212/NXI.0000000000000219>
- 86 Keller, A. *et al.* Multiple sclerosis: microRNA expression profiles accurately differentiate patients with relapsing-remitting disease from healthy controls. *Plos One* **4**, e7440 (2009). <https://doi.org/10.1371/journal.pone.0007440>
- 87 Alevizos, I., Alexander, S., Turner, R. J. & Illei, G. G. MicroRNA expression profiles as biomarkers of minor salivary gland inflammation and dysfunction in Sjogren's syndrome. *Arthritis Rheum* **63**, 535–544 (2011). <https://doi.org/10.1002/art.30131>
- 88 Kim, B. S., Jung, J. Y., Jeon, J. Y., Kim, H. A. & Suh, C. H. Circulating hsa-miR-30e-5p, hsa-miR-92a-3p, and hsa-miR-223-3p may be novel biomarkers in systemic lupus erythematosus. *HLA* **88**, 187–193 (2016). <https://doi.org/10.1111/tan.12874>
- 89 Wade, S. M., McGarry, T., Wade, S. C., Fearon, U. & Veale, D. J. Serum MicroRNA Signature as a Diagnostic and Therapeutic Marker in Patients with Psoriatic Arthritis. *J Rheumatol* **47**, 1760–1767 (2020). <https://doi.org/10.3899/jrheum.190602>
- 90 Mohan, T., Deng, L. & Wang, B. Z. CCL28 chemokine: An anchoring point bridging innate and adaptive immunity. *Int Immunopharmacol* **51**, 165–170 (2017). <https://doi.org/10.1016/j.intimp.2017.08.012>
- 91 Croft, M. The role of TNF superfamily members in T-cell function and diseases. *Nat Rev Immunol* **9**, 271–285 (2009). <https://doi.org/10.1038/nri2526>
- 92 Ware, C. F. The TNF superfamily. *Cytokine Growth Factor Rev* **14**, 181–184 (2003). [https://doi.org/10.1016/s1359-6101\(03\)00032-7](https://doi.org/10.1016/s1359-6101(03)00032-7)
- 93 Ikawa, T. *et al.* The Contribution of LIGHT (TNFSF14) to the Development of Systemic Sclerosis by Modulating IL-6 and T Helper Type 1 Chemokine Expression in Dermal Fibroblasts. *J Invest Dermatol* **142**, 1541–1551 e1543 (2022). <https://doi.org/10.1016/j.jid.2021.10.028>

- 94 Kyriakis, J. M. & Avruch, J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. *Physiol Rev* **92**, 689–737 (2012). <https://doi.org/10.1152/physrev.00028.2011>
- 95 Guo, Q. *et al.* NF-kappaB in biology and targeted therapy: new insights and translational implications. *Signal Transduct Target Ther* **9**, 53 (2024). <https://doi.org/10.1038/s41392-024-01757-9>
- 96 Hoffmann, S. *et al.* Complement deposition at the neuromuscular junction in seronegative myasthenia gravis. *Acta Neuropathol* **139**, 1119–1122 (2020). <https://doi.org/10.1007/s00401-020-02147-5>
- 97 Morgan, B. P. The membrane attack complex as an inflammatory trigger. *Immunobiology* **221**, 747–751 (2016). <https://doi.org/10.1016/j.imbio.2015.04.006>
- 98 Li, K. *et al.* Functional modulation of human monocytes derived DCs by anaphylatoxins C3a and C5a. *Immunobiology* **217**, 65–73 (2012). <https://doi.org/10.1016/j.imbio.2011.07.033>
- 99 Dunkelberger, J. R. & Song, W. C. Complement and its role in innate and adaptive immune responses. *Cell Res* **20**, 34–50 (2010). <https://doi.org/10.1038/cr.2009.139>
- 100 Fichtner, M. L. *et al.* Myasthenia gravis complement activity is independent of autoantibody titer and disease severity. *Plos One* **17**, e0264489 (2022). <https://doi.org/10.1371/journal.pone.0264489>
- 101 Ozawa, Y. *et al.* Activation of the classical complement pathway in myasthenia gravis with acetylcholine receptor antibodies. *Muscle Nerve* **68**, 798–804 (2023). <https://doi.org/10.1002/mus.27973>
- 102 Iacomino, N. *et al.* Complement Activation Profile in Myasthenia Gravis Patients: Perspectives for Tailoring Anti-Complement Therapy. *Biomedicines* **10** (2022). <https://doi.org/10.3390/biomedicines10061360>
- 103 Pham, M. C. *et al.* Individual myasthenia gravis autoantibody clones can efficiently mediate multiple mechanisms of pathology. *Acta Neuropathol* **146**, 319–336 (2023). <https://doi.org/10.1007/s00401-023-02603-y>
- 104 Pedersen, E. D. *et al.* CD59 efficiently protects human NT2-N neurons against complement-mediated damage. *Scand J Immunol* **66**, 345–351 (2007). <https://doi.org/10.1111/j.1365-3083.2007.01959.x>
- 105 Ricklin, D. & Lambris, J. D. Compstatin: a complement inhibitor on its way to clinical application. *Adv Exp Med Biol* **632**, 273–292 (2008). [https://doi.org/10.1007/978-0-387-78952-1\\_20](https://doi.org/10.1007/978-0-387-78952-1_20)
- 106 Parsons, E. S. *et al.* Single-molecule kinetics of pore assembly by the membrane attack complex. *Nat Commun* **10**, 2066 (2019). <https://doi.org/10.1038/s41467-019-10058-7>
- 107 Chen, M. *et al.* T-type calcium channel blockade induces apoptosis in C2C12 myotubes and skeletal muscle via endoplasmic reticulum stress activation. *FEBS Open Bio* **10**, 2122–2136 (2020). <https://doi.org/10.1002/2211-5463.12965>
- 108 Flucher, B. E. Skeletal muscle Ca(V)1.1 channelopathies. *Pflugers Arch* **472**, 739–754 (2020). <https://doi.org/10.1007/s00424-020-02368-3>
- 109 Kordas, G., Lagoumintzis, G., Sideris, S., Poulas, K. & Tzartos, S. J. Direct proof of the in vivo pathogenic role of the AChR autoantibodies from myasthenia gravis patients. *Plos One* **9**, e108327 (2014). <https://doi.org/10.1371/journal.pone.0108327>

- 110 Sideris, S. *et al.* Isolation and functional characterization of anti-acetylcholine receptor subunit-specific autoantibodies from myasthenic patients: receptor loss in cell culture. *J Neuroimmunol* **189**, 111–117 (2007). <https://doi.org/10.1016/j.jneuroim.2007.06.014>
- 111 Li, H. *et al.* Autoimmune mechanisms elucidated through muscle acetylcholine receptor structures. *Cell* **188**, 2390–2406 e2320 (2025). <https://doi.org/10.1016/j.cell.2025.03.004>
- 112 Schöttler, A. K. *et al.* Complement inhibition by C3-siRNA treatment prevents AChR loss and reduces complement activation in the rat Passive Transfer Myasthenia Gravis (PTMG). *bioRxiv*, 2025.2008.2031.673367 (2025). <https://doi.org/10.1101/2025.08.31.673367>
- 113 Asher, O., Neumann, D. & Fuchs, S. Increased levels of acetylcholine receptor alpha-subunit mRNA in experimental autoimmune myasthenia gravis. *FEBS Lett* **233**, 277–281 (1988). [https://doi.org/10.1016/0014-5793\(88\)80442-3](https://doi.org/10.1016/0014-5793(88)80442-3)
- 114 Sheng, J. R., Li, L. C., Prabhakar, B. S. & Meriggioli, M. N. Acetylcholine receptor-alpha subunit expression in myasthenia gravis: a role for the autoantigen in pathogenesis? *Muscle Nerve* **40**, 279–286 (2009). <https://doi.org/10.1002/mus.21371>
- 115 Guyon, T. *et al.* Regulation of acetylcholine receptor gene expression in human myasthenia gravis muscles - Evidences for a compensatory mechanism triggered by receptor loss. *J Clin Invest* **102**, 249–263 (1998). <https://doi.org/10.1172/Jci1248>
- 116 Cetin, H., Beeson, D., Vincent, A. & Webster, R. The Structure, Function, and Physiology of the Fetal and Adult Acetylcholine Receptor in Muscle. *Front Mol Neurosci* **13**, 581097 (2020). <https://doi.org/10.3389/fnmol.2020.581097>



# Acta Universitatis Upsaliensis

*Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 2269*

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)

Distribution: [publications.uu.se](http://publications.uu.se)  
urn:nbn:se:uu:diva-582857



ACTA UNIVERSITATIS  
UPSALIENSIS  
2026