

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Investigations of the Neuromuscular Junction under Normal and
Pathological Conditions

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF CLINICAL MEDICINE

DEBRECEN, 2026

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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, on March 31, 2026, at 13:00.

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1. INTRODUCTION

Numerous diseases are associated with impaired function of the neuromuscular junction (NMJ), leading to muscle weakness, hypotonia, and atrophy. These disorders cover a broad spectrum, ranging from genetic conditions to autoimmune and inflammatory diseases, each requiring distinct diagnostic and therapeutic approaches in both pediatric and adult populations.

In neonates and infants, hypotonia and impaired skeletal muscle function may indicate neuromuscular disorders such as congenital myasthenic syndromes (CMS). When myasthenia is clinically suspected, serological testing for acetylcholine receptor (AChR) and muscle-specific kinase (MuSK) antibodies is essential. In antibody-negative cases, or when CMS is suspected, clinical neurophysiological testing is crucial to confirm NMJ dysfunction.

Repetitive nerve stimulation (RNS) is recommended as the first diagnostic step, as it is a valuable tool for distinguishing between pre- and postsynaptic dysfunction of the NMJ and for guiding further genetic testing in suspected CMS cases. An alternative technique is single-fiber electromyography (SFEMG), which, although more sensitive, provides less information on the exact localization of abnormalities and requires considerable expertise from the neurophysiologist.

One of the main challenges in pediatric cases is that the NMJ is still undergoing development during the neonatal and early childhood period. Morphometric studies have shown that postsynaptic membrane length, postsynaptic area size, and the ratio of postsynaptic to presynaptic membrane length are significantly lower in children aged 2 months to 3 years compared with adults.

In addition, the subunit composition of the acetylcholine receptor changes during human development: while in the fetal, neonatal, and infant forms the complex consists of two α , one β , one δ , and one γ subunit, in the adult type the γ is replaced by an ϵ subunit. It remains unclear to what extent these structural differences influence functional properties of the junction. Studies have demonstrated that train-of-four ratios, post-tetanic facilitation, and twitch-to-tetanus ratios vary significantly during the first months and years of life. Other investigations have shown that infant NMJs are more resistant to depolarizing blockers, such as succinylcholine, compared with non-depolarizing blockers, such as tubocurarine.

There is no consensus on age-specific normal values for RNS. Several neurophysiology textbooks and reviews still recommend using adult thresholds (10% decrement) to detect abnormalities. To date, only five studies have reported RNS data in healthy children, and some of these used techniques no longer applied in clinical practice.

In the first part of my dissertation, I present our results on RNS findings in children, with particular emphasis on the relationship between decrement values and age.

Myasthenia gravis (MG) and idiopathic inflammatory myopathies (IIM) are rare autoimmune diseases affecting skeletal muscle. Both cause muscle weakness, which may complicate differential diagnosis when the two conditions coexist. Although very rare, cases of MG and IIM occurring simultaneously have been reported.

In MG, autoantibodies typically target NMJ components, most commonly the acetylcholine receptor (present in 85% of patients). In AChR-negative cases, antibodies to MuSK (6%), low-density lipoprotein receptor-related protein 4 (LRP4), ryanodine receptor (RyR), or titin can be detected. Thymic pathology plays a key role in MG pathogenesis, present in more than 50% of patients.

Idiopathic inflammatory myopathies are classified into four major clinical subgroups: dermatomyositis (DM), polymyositis (PM), immune-mediated necrotizing myopathy (IMNM), and sporadic inclusion body myositis (IBM). The clinic-serological classification additionally distinguishes antisynthetase syndrome (ASys) as a separate subgroup, which represents a frequently occurring and clinically distinct phenotype. In myositis, myositis-specific (MSA) and myositis-associated (MAA) antibodies greatly aid classification. These autoantibodies are detectable in approximately 75% of classic DM/PM cases but are usually absent in MG. MSAs predominantly target cytoplasmic ribonucleoproteins; they include anti-Jo-1, anti-PL-7, anti-PL-12, anti-SRP, anti-Mi-2 β , and anti-Mi-2 α . MAAs include anti-PM-Scl-75, anti-PM-Scl-100, anti-Ku, and anti-Ro-52. Unlike MG, thymic involvement is not a feature of IIM.

Clinically, MG and IIM show overlapping features, but the most important distinction is the presence of ocular manifestations, which are characteristic of MG but virtually absent in IIM. In both conditions, weakness can be generalized or localized, affecting bulbar, respiratory, axial, or proximal muscles. In MG, weakness fluctuates throughout the day and worsens with activity, whereas in IIM it remains constant in intensity, progressively worsens over weeks or

months, and is often accompanied by myalgia. Serum creatine kinase (CK) levels are typically elevated in IIM but usually normal in MG.

Coexistence of MG and IIM is exceedingly rare; only ~50 cases have been reported in the literature, mainly case reports. Therefore, data on the frequency of IIM among MG patients remain scarce. Recognition of such cases is crucial, as therapeutic approaches differ: pyridostigmine, an acetylcholinesterase inhibitor that enhances NMJ transmission, is the first-line treatment in MG, while corticosteroids are first-line in IIM. Both conditions may require immunosuppressive therapy; however, unlike in IIM, initiation of high-dose corticosteroids in MG can paradoxically exacerbate weakness and trigger myasthenic crisis.

Low serum vitamin D levels play an important role in the activity of several autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS). Patients with IIM also tend to have lower vitamin D levels. Several reports have described reduced serum vitamin D in MG as well.

In the second part of my dissertation, I present data from our neuromuscular center on MG patients, focusing on elevated CK levels, the presence of MSA and MAA, reduced vitamin D levels, and the frequency of definitive myositis or other overlapping neuromuscular disorders.

2. OBJECTIVES

1. The primary objective of our first study was to characterize the neuromuscular junction (NMJ) in neonates, infants, and young children using repetitive nerve stimulation (RNS). Since NMJ disorders predominantly manifest in proximal muscles, we performed stimulation of the axillary nerve. The decrement of the amplitude and area of the evoked compound muscle action potentials (CMAPs) was calculated between the first and fourth responses. Post-tetanic facilitation and the twitch-to-tetanus ratio were also determined. Furthermore, we aimed to assess the potential bradycardic effect of supraclavicular stimulation, for which heart rate was continuously monitored.
2. The second objective was to investigate, among patients with myasthenia gravis followed at the tertiary regional Neuromuscular Outpatient Clinic of the Department of Neurology, University of Debrecen, the frequency of elevated serum creatine kinase (CK) levels, the presence of myositis-specific (MSA) and myositis-associated antibodies (MAA), as well as the prevalence of concomitant myositis and low serum vitamin D levels.

3. PATIENTS AND METHODS

3.1 NEUROPHYSIOLOGICAL CHARACTERIZATION OF THE NEUROMUSCULAR JUNCTION IN CHILDHOOD

3.1.1 PARTICIPANTS

A total of 54 children were enrolled in the study. All examinations were performed with the written informed consent of the parents and after providing complete information. The children underwent elective surgical procedures (e.g., urethroplasty, pyeloplasty, palatoplasty, inguinal hernia repair, orchidopexy, ureteral reimplantation, or ureterocele surgery). Due to time constraints (surgery always had priority over electrophysiological testing), the RNS test could not be fully completed in 20 cases. Consequently, data from 34 children (23 boys and 11 girls, aged 0–48 months) were included in the analysis. All participants were age-appropriate in development and had no known neuromuscular disorder. The study was approved by the Ethics Committee of the University of Debrecen and the National Institute of Pharmacy and Nutrition (OGYÉI/38595/2017). The investigation complied with the ethical standards of the Declaration of Helsinki.

3.1.2 REPETITIVE NERVE STIMULATION (RNS) PROCEDURE

The procedure was performed prior to surgery. Before the RNS test, the children were sedated with intravenous propofol (3 mg/kg), midazolam (0.1 mg/kg), and fentanyl (1.9 µg/kg) under the supervision of an anesthesiologist experienced in pediatric anesthesia. Following the test, the depth of anesthesia was adjusted according to surgical requirements. Blood pressure, ECG, and SpO₂ were continuously monitored throughout the procedure. Compound muscle action potentials (CMAPs) were recorded using surface electrodes while the axillary nerve was subjected to repeated supramaximal stimulation at 3 Hz above the clavicle (7 times on the right and 27 times on the left side). The distance between the stimulating electrodes was 1.5 cm. The active electrode was placed over the belly of the deltoid muscle, the reference electrode over the acromion, and the ground electrode over the lateral epicondyle. Stimulation consisted of controlled current pulses with a duration of 0.2 ms. To achieve supramaximal stimulation, intensity was increased until the CMAP reached its maximum, after which it was raised by an additional 25% (typically around 20 mA). Participants were examined

in a supine position with their upper limbs fixed, and skin temperature was maintained above 32 °C.

Tests were performed using a DANTEC Keypoint Portable EMG device (Middleton, WI, USA). Initially, 10 supramaximal stimuli at 3 Hz were delivered at rest, followed by a 1-second 30 Hz tetanic stimulation. Thereafter, further 3 Hz stimulation trains were administered immediately (within 2 s), 20 s, and 1 min after the tetanic stimulus. The amplitude and area of the negative peak of the CMAPs were measured, and the decrement between the first and fourth responses was determined. The percentage change in the first post-tetanic CMAP amplitude compared to the pre-tetanic CMAP was also calculated. An increase in CMAP amplitude after tetanic stimulation was defined as post-tetanic facilitation, while a decrease was considered post-tetanic exhaustion. The ratio of the highest CMAP amplitude during tetanic stimulation to the first pre-tetanic CMAP amplitude (“twitch-to-tetanus ratio”) was also determined. Heart rate was continuously monitored at rest and during both 3 Hz and 30 Hz stimulation.

3.1.3 STATISTICAL ANALYSIS

A convenience sample was used without a priori sample size calculation. Normality of data distribution was tested with the Shapiro–Wilk test. Correlations between age and CMAP amplitude and area decrement, post-tetanic facilitation, twitch-to-tetanus ratio, and procedure-related heart rate changes were analyzed with Spearman’s correlation test. Data are presented as median $\pm$ IQR (interquartile range), as most groups did not follow normal distribution. CMAP area decrements were analyzed using the Kruskal–Wallis test followed by Dunn’s multiple comparison test. Periprocedural heart rate values (measured before and during 3 Hz and 30 Hz stimulation) were assessed with Friedman’s test followed by Dunn’s multiple comparisons. A p-value < 0.05 was considered statistically significant. The strength of correlation was categorized based on correlation coefficients (moderate correlation if $\rho = 0.40\text{--}0.69$).

3.2 PREVALENCE OF MYOSITIS AND MYOSITIS-ASSOCIATED AUTOANTIBODIES IN MYASTHENIA GRAVIS

3.2.1 PARTICIPANTS

We enrolled 101 patients with myasthenia gravis (MG) who attended the regional tertiary Neuromuscular Outpatient Clinic of the Department of Neurology, University of Debrecen, between December 3, 2019, and March 17, 2022. The diagnosis of MG had been established previously according to international guidelines, based on clinical, immunological, neurophysiological, and pharmacological criteria. The study was approved by the Ethics Committee of the University of Debrecen (DE RKEB/IKEB 5313-2019) and by the relevant governmental authority. The research was conducted in compliance with the Declaration of Helsinki. All examinations were performed following detailed patient information and with written informed consent.

3.2.2 NEUROPHYSIOLOGICAL EXAMINATIONS

Electromyography was performed in cases with suspected myositis. At least three muscles were assessed: the deltoid, vastus lateralis, and tibialis anterior. Electrophysiological assessment of NMJ dysfunction (repetitive nerve stimulation and/or single-fiber EMG) was conducted as part of the diagnostic evaluation for MG.

3.2.3 SEROLOGICAL EXAMINATIONS

Serum creatine kinase (CK) and vitamin D levels were measured in the Institute of Laboratory Medicine, University of Debrecen Clinical Center. CK levels were determined by automated enzymatic assay (reference range: 40–200 U/L). The lower cutoff for vitamin D was 75 nmol/L. Myositis-specific and myositis-associated autoantibodies were tested using a line blot assay (Euroimmun, Lübeck, Germany), performed in the laboratory of the Department of Clinical Neurophysiology, University of Debrecen. The test panel included 16 autoantigens (Mi-2 α , Mi-2 β , TIF1 γ , MDA5, NXP2, SAE1, Ku, PM-Scl100, PM-Scl75, Jo-1, SRP, PL-7, PL-12, EJ, OJ, and Ro-52). Antibody reactivity was graded according to band intensity as negative (–), borderline (+/–), weakly positive (+), moderately positive (++), or strongly positive (+++). For the analyses, results were classified as positive if reactivity was at least weakly positive (+, ++, +++), while negative (–) and borderline (+/–) results were considered negative. All tests were performed with a standardized methodology and independently evaluated to ensure data reliability and comparability. In addition, most patients had been tested previously for

antibodies against AChR and MuSK during the routine diagnostic evaluation for MG. Antibody testing was performed using ELISA. Anti-AChR antibodies were measured with the EUROIMMUN AG assay, while anti-MuSK antibodies were determined using the Tecan IBL International MuSK-Ab ELISA kit. The analyses were carried out at the Immunology and Biotechnology Institute of the University of Pécs Clinical Centre and in the SYNLAB laboratory. However, these tests were not performed in all patients, as they are not fully reimbursed by the National Health Insurance Fund; therefore, testing was limited to those patients who covered the cost themselves.

3.2.4 IMAGING EXAMINATIONS

MRI of the proximal muscles of the upper and lower limbs was performed using a 3 Tesla scanner, following standard international protocols. Radiological evaluation primarily focused on T1-weighted turbo spin-echo (T1-TSE) and fat-suppressed short-tau inversion recovery (STIR) sequences. Pathological findings assessed included:

- Fibrofatty replacement of muscle tissue
 - Muscle atrophy, indicating chronic changes
 - Edema or inflammatory signs, observed as hyperintensity on STIR sequences, suggesting active inflammation
- These findings contributed to the differential diagnosis of myositis and other inflammatory or degenerative myopathies.

3.2.5 MUSCLE BIOPSY

Muscle biopsy was performed only in cases with clinical, laboratory, electrophysiological, or imaging (MRI) findings suggestive of myositis or another neuromuscular disorder. The biopsy site was determined individually, based on current symptoms, physical examination, and MRI results. The deltoid and vastus lateralis were most frequently sampled. Tissue specimens were processed using standard histopathological techniques, including routine staining and microscopic evaluation, to identify structural abnormalities, inflammatory changes, or other pathological processes.

3.2.6 CLASSIFICATION OF PATIENTS WITH SUSPECTED IDIOPATHIC INFLAMMATORY MYOPATHY (IIM)

In patients with suspected myositis, the diagnosis and classification were established according to the criteria of the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR). The official online calculator for adult and juvenile idiopathic inflammatory myopathies, developed by the Karolinska Institute (Stockholm, Sweden), was used (<http://www.imm.ki.se/biostatistics/calculators/iim>). This tool integrates clinical, laboratory, imaging, and histopathological data into a score that supports accurate classification and diagnosis of IIM.

3.2.7 CLINICAL SCALES FOR ASSESSING THE SEVERITY OF MYASTHENIA GRAVIS

At enrollment, MG severity was determined according to the Myasthenia Gravis Foundation of America (MGFA) clinical classification system, which stratifies patients into five categories (I–V) based on the extent and distribution of muscle weakness. Disease burden was further assessed using the Myasthenia Gravis Composite (MGC) scale, a validated instrument combining objective findings from clinical examination with patient-reported symptoms. The MGC score evaluates multiple domains, including ocular, bulbar, respiratory, and limb involvement, thereby providing a comprehensive measure of disease severity. It is particularly useful for monitoring clinical status and objectively evaluating treatment response.

3.2.8 STATISTICAL ANALYSIS

MGC scores were compared across groups stratified by serum CK levels (normal vs. elevated), MSA/MAA antibody status (positive vs. negative), and vitamin D levels (low vs. normal). Data distribution was tested with the Kolmogorov–Smirnov test. As none of the groups demonstrated significant normality (α not <0.05 in any comparison), intergroup differences were analyzed using the nonparametric Mann–Whitney U test.

4. RESULTS

4.1 NEUROPHYSIOLOGICAL CHARACTERIZATION OF THE NEUROMUSCULAR JUNCTION IN CHILDHOOD

In total, 34 subjects were included in the study; the right axillary nerve was stimulated in 7 cases and the left in 27 cases. One subject was 5 weeks old, and two additional infants had been born prematurely. One was delivered at 24 weeks of gestation and was 5 months old at the time of the study (corresponding to a corrected age of 28 days), while the other was born at 33 weeks of gestation and was 1 month and 12 days old at the time of testing (estimated gestational age: 40 weeks). Based on corrected gestational age, all three participants were classified as normal neonates. The remaining participants were between 2 and 48 months of age (9 between 2–6 months, 6 between 6–12 months, 9 between 12–24 months, and 7 between 24–48 months). Accordingly, all participants were considered developmentally mature ($n = 34$; 23 boys, 11 girls; age: 0–48 months).

First, we examined the relationship between CMAP amplitude decrement and age. Statistical analysis showed no significant correlation between age and CMAP amplitude decrement values at rest, immediately after tetanic stimulation, or at 20 seconds and 1 minute post-tetanus ($p > 0.05$ in all cases). Based on this, we did not create separate age subgroups for further analyses. The median CMAP amplitude decrement during RNS was 1% (interquartile range: 1–3%; total range: 0–7%).

A moderate positive correlation was observed between age and post-tetanic facilitation. No facilitation was observed below 4 months of age; instead, exhaustion was most commonly detected in this age group. Facilitation appeared in older age groups. The twitch-to-tetanus ratio also showed a moderate positive correlation with age, with lower values observed in younger children.

Previous studies demonstrated that CMAP amplitude and area decrement values during low-frequency RNS are similar in adults. To investigate this in young children, we analyzed the relationship between CMAP area decrement and age. Statistical analysis revealed a moderate positive correlation between CMAP area decrement and age. The median CMAP area decrement during RNS in the 0–5 month age group was 11% (interquartile range: 5–15%). In this group, 5 of 9 infants exhibited a CMAP area decrement greater than 10%. In contrast, no

child in the 6–48 month group showed a decrement exceeding 10%; the median was 4% (interquartile range: 2–5%). This moderate positive correlation was observed immediately, 20 seconds, and 1 minute after tetanic stimulation.

Statistical analysis also revealed significant differences between CMAP area decrement values recorded at different time points ($p < 0.0005$). Dunn's multiple comparison test showed that CMAP area decrement values were significantly higher at 1 minute post-tetanus compared with baseline, immediately post-tetanus, and 20 seconds post-tetanus ($p = 0.0015, 0.0011, \text{ and } 0.0174$, respectively).

Changes in heart rate were not associated with age during either 3 Hz or 30 Hz stimulation. Furthermore, there were no significant differences between heart rate values measured before and during 3 Hz or 30 Hz stimulation. No clinically significant bradycardia was observed in any child.

Importantly, no safety concerns (e.g., restricted limb movement, bruising, or local tenderness at the stimulation site) arose during or after the procedure, and recordings were generally reproducible. However, in some cases, establishing and maintaining a stable baseline proved challenging, particularly when sedation was insufficient. A key practical lesson was that obtaining high-quality recordings required sufficient time investment, which was sometimes difficult to achieve in this clinical setting.

4.2 PREVALENCE OF MYOSITIS AND MUSCLE AUTOANTIBODIES IN MYASTHENIA GRAVIS

We included 101 patients with myasthenia gravis (MG) who attended the Neuromuscular Outpatient Clinic of the University of Debrecen Clinical Center between December 3, 2019 and March 17, 2022. The cohort comprised 27 men (26.7%) and 74 women (73.2%). Median age at inclusion was 57 years (IQR 45–70), and median age at MG onset was 43 years (IQR 30–57). Median disease duration was 7 years (IQR 2.0–17).

Regarding clinical subtypes, generalized MG was most frequent ($n=68, 67.33\%$), followed by bulbar ($n=18, 17.82\%$) and purely ocular MG ($n=15, 14.85\%$).

Serology demonstrated AChR antibodies in 67 patients and MuSK antibodies in 4; 14 patients were seronegative for both AChR and MuSK. In 16 patients, the diagnosis of MG was

based on electrophysiology, and antibody testing had not been performed. Routine chest CT during work-up identified thymoma in 6 patients and thymic hyperplasia in 39.

4.2.1 ELEVATED CK LEVELS

At study entry, elevated serum CK was found in 10/101 patients (9.9%). Of these, seven were AChR-antibody positive, two were seronegative, and none was MuSK-antibody positive. One patient had neither AChR nor MuSK testing.

In six patients CK did not exceed twice the upper limit of normal (ULN); in three of them CK normalized on follow-up (patients 3, 6 and 7). Patients 8 and 10 had mildly increased CK, while patient 9 later exceeded 2×ULN.

EMG showed mild myopathic changes in patient 3; all others were normal. In patient 9, MRI was negative and the online IIM classification tool did not support myositis; therefore, muscle biopsy was not performed. No MRI or biopsy was performed in the remaining patients. None of these patients had detectable MSA or MAA autoantibodies.

Four additional patients (patients 1, 2, 4 and 5) had repeatedly elevated CK >2×ULN. EMG suggested myositis in patients 1 and 5, and mild myopathy in patients 2 and 4.

On MRI, inflammatory involvement was suspected in patients 1 and 4; patient 2 had fibrofatty replacement; patient 5 had no MRI abnormalities. Muscle biopsy was performed in all four: histology confirmed myositis in patient 1; dystrophic changes in patient 2; myopathic changes in patient 4; and mild, nonspecific alterations compatible with MG in patient 5.

Serology in patient 1 revealed anti-Jo-1, anti-PM/Scl-75 and anti-Ro52 antibodies. No MSA/MAA antibodies were detected in the others, although patient 4 was positive for anti-GAD65.

Using the EULAR/ACR IIM online calculator, a definite IIM diagnosis was established for patient 1, while patients 4 and 5 were classified as probable myositis.

Median MGC scores did not differ between patients with normal vs. elevated CK (median [IQR] 4.0 [1.0–7.0] vs. 3.5 [2.25–7.0]; $p=0.9841$).

Summary of patients with persistently and significantly elevated CK:

Patient 1. A 32-year-old woman with polyarthritis, Raynaud phenomenon, myalgia, and CK up to 10,000 U/L since 2017. EMG, limb MRI and vastus lateralis biopsy confirmed myositis. Serology: anti-Jo-1, anti-PM/Scl-75, anti-Ro52; diagnosis: antisynthetase syndrome. She improved on high-dose steroids and methotrexate. HRCT showed persistent thymus without alveolitis/fibrosis. Serum AChR Ab 0.56 nmol/L (cut-off 0.4). Neurologic exam and RNS in 2017 were normal while on steroids/methotrexate. Due to myositis flare in 2018, cyclosporine replaced methotrexate. In 2019, neck flexor and proximal limb weakness responded to pyridostigmine 60 mg b.i.d.; chest CT again showed persistent thymus. Because of frequent flares, IVIg was started in 2019. Thymectomy was performed in Jan 2020 (histology: persistent thymus, small paraganglioma). Postoperatively she developed ptosis, facial weakness and fatigability with normal CK; dose escalation of pyridostigmine improved symptoms. MG has since remained stable. Owing to cyclosporine adverse effects, therapy was switched to azathioprine (2021) and then mycophenolate mofetil (2022). In 2024, active myositis and HRCT-proven pulmonary fibrosis prompted initiation of rituximab. Current therapy: pyridostigmine 60 mg t.i.d., methylprednisolone 8 mg, mycophenolate mofetil 1,000 mg b.i.d., monthly 60 g IVIg, and rituximab; she is in remission.

Patient 2. A 70-year-old woman with four years of proximal lower-limb weakness and, over the past three years, fluctuating bilateral ptosis and mild dysphagia. Serum AChR Ab 4.08 nmol/L; partial response to pyridostigmine. CK persistently ~600 U/L. EMG: myopathic changes. Acid α -glucosidase activity normal; serum lactate exercise test negative. Limb MRI showed fibrofatty transformation in gluteal and adductor muscles. Vastus lateralis biopsy revealed dystrophic changes. Targeted testing for common LGMD hotspot mutations (SGCG c.848G>A p.Cys283Tyr; FKRP c.826C>A p.Leu276Ile; CAPN3 c.550delA) was negative. An extended LGMD gene panel subsequently identified a heterozygous ORAI1 c.551C>T variant.

Patient 4. A 59-year-old man with diabetes mellitus and diabetic polyneuropathy. Late-onset generalized MG was diagnosed by RNS; AChR Ab negative. He also had anti-GAD65-positive stiff-person syndrome predominantly affecting distal upper-limb and tibialis anterior muscles. CK 393–544 U/L. Chest CT: thymic hyperplasia. MSA/MAA serology negative. EMG showed myogenic damage; vastus lateralis biopsy, performed on moderate-dose steroids, showed myopathic changes. Limb MRI in 2018, prompted by worsening proximal upper-limb weakness, suggested myositis.

Patient 5. A 60-year-old man with AChR-positive (119.3 nmol/L) late-onset generalized MG and markedly elevated CK (649–1008 U/L). CK repeatedly fell with steroid dose escalation. No other autoimmune disease or MSA/MAA antibodies. EMG in 2011 suggested chronic myositis. Limb MRI (2018) and deltoid biopsy (2012) were unremarkable, both on lower-dose steroids. In 2023, after tapering methylprednisolone to 4 mg/day, he developed myalgia and weakness; EMG then showed acute myositis in tibialis anterior.

Patient 9. A 64-year-old man with AChR-positive (>8 nmol/L) bulbar MG presenting with transient dysarthria and dysphagia. RNS and SF-EMG confirmed NMJ transmission defect. Chest CT: no thymic pathology. CK mildly elevated (253–433 U/L). EMG, limb MRI, and MSA/MAA serology were negative. CK remained elevated during a short steroid course. The IIM online calculator did not support IIM; biopsy was not performed. No other autoimmune disorder was detected.

4.2.2 PREVALENCE AND CLINICAL RELEVANCE OF MSA/MAA ANTIBODIES

MSA or MAA antibodies were detected in 19/101 patients (18.8%). MSA positivity (n=7) comprised anti-PL-7 (n=2), anti-Mi-2 α (n=1), anti-Mi-2 β (n=4), and anti-Jo-1 (n=1). MAA positivity (n=13) included anti-Ro52 (n=5), anti-PM-Scl-100 (n=6), anti-PM-Scl-75 (n=1), and anti-Ku (n=4). Co-positivity for multiple antibodies was observed in 4 patients.

Clinically relevant CK elevation occurred only in patient 1; CK remained within normal limits in the other MSA/MAA-positive patients. Electrophysiology was performed in 15 patients: myositis-suggestive findings were present in 1 (patient 1) and myopathic features in 4 (patients 13, 16, 23 and 26).

MRI was performed in 5 patients and was negative in 4; in patient 1, imaging supported myositis. Muscle biopsy was undertaken in 4 patients: myositis was histologically confirmed in patient 1; an earlier biopsy (1980) in patient 13 showed myopathic changes; biopsies in patients 17 and 25 were normal.

Among the 19 MSA/MAA-positive patients, 7 had concomitant systemic autoimmune disease (e.g., Sjögren's syndrome, Raynaud phenomenon, rheumatoid arthritis, autoimmune thyroiditis, or undifferentiated connective tissue disease). The IIM online classification tool indicated a high probability of IIM only in patient 1.

There was no statistically significant difference in MGC scores between patients with and without MSA/MAA antibodies (median [IQR] 4.0 [2.0–6.5] vs. 4.0 [1.0–7.0]; $p=0.8296$).

4.2.3 SERUM VITAMIN D CONCENTRATIONS

Vitamin D was measured in all participants. Normal levels were found in 32.7% ($n=33$), while 67.3% ($n=68$) had low levels (<75 nmol/L). Vitamin D deficiency was present in 5/10 patients with elevated CK, in 5/7 MSA-positive patients, and in 7/19 MAA-positive patients. Comparison of MGC scores between patients with normal vs. reduced vitamin D showed no significant difference (median [IQR] 4.0 [2.0–6.5] vs. 3.5 [1.5–5.8]; $p=0.514$).

5. DISCUSSION

5.1 NEUROPHYSIOLOGICAL CHARACTERIZATION OF THE NEUROMUSCULAR JUNCTION IN CHILDHOOD

In our study, the median CMAP amplitude decrement recorded during repetitive stimulation in young children was 1% at rest (range 0–7%). Although CMAP amplitude decrement showed no association with age, we found moderate positive correlations between age and CMAP area decrement, post-tetanic facilitation, and the twitch-to-tetanus ratio.

Only a limited number of studies have assessed the neuromuscular junction (NMJ) of healthy children using repetitive nerve stimulation (RNS). Churchill-Davidson and Wise examined five children and reported an 11% amplitude decrement in preterm infants with 2.5 Hz needle stimulation of the ulnar nerve. Between 0 and 26 months of age they detected no significant decrement at low frequency, whereas high-frequency stimulation (50 Hz for 20 s) produced a marked decrement. Wise and McQuillen stimulated the ulnar nerve in three healthy neonates (age 1–3 days) at 3, 10, or 50 Hz and found no significant CMAP amplitude decrement. Koenigsberger et al. examined the median nerve in 17 neonates and observed no decrement at low frequency (1–2 Hz), while higher frequencies (20–50 Hz) induced significant amplitude reductions. Crumrine and Yodlowski stimulated intramuscular motor nerves in 25 children aged 1 day to 5 years and recorded responses from tibialis anterior; no decrement occurred at low frequencies, but in infants <12 weeks high-frequency stimulation (50–100 Hz) revealed transmission failure. Maselli et al. reported no amplitude decrement during 2 Hz ulnar nerve stimulation in three healthy pediatric controls (age not specified).

Multiple reports show that congenital myasthenic syndromes (CMS) may present at birth or shortly thereafter; nevertheless, diagnosis is often delayed by years, exposing children to severe, sometimes life-threatening complications. When myasthenia is suspected, RNS should be the first-line test because it is widely available in electrophysiology laboratories and is a sensitive, specific functional assessment of NMJ transmission; single-fiber EMG may be added in selected cases. In myasthenic disorders, RNS is typically more sensitive in proximal than in distal muscles an important consideration in generalized myasthenia gravis, where weakness often involves proximal girdle muscles. In contrast, some limb-girdle forms of CMS primarily affect distal muscles; in such cases, RNS should preferentially target distal muscles.

In adult practice, repetitive stimulation of the accessory nerve is technically simpler and more comfortable than axillary nerve testing when proximal muscles are evaluated. In small children, however, the cervical course of the accessory nerve poses a risk of inadvertent vagus stimulation, and recording from the trapezius is technically difficult because the stimulation and recording sites are very close. By contrast, the supraclavicular region is readily accessible, and the size of the deltoid allows reliable CMAP recording; therefore, we chose axillary nerve testing.

Textbooks and neurophysiology guidelines generally regard a decrement $>10\%$ between the first and fourth responses as evidence of NMJ dysfunction, and in adults amplitude and areacbased decrements are considered clinically interchangeable. Some laboratories accept lower cut-offs ($\geq 7\%$ or even $\geq 5\%$) for facial muscles, provided technical artifact is excluded. Importantly, these thresholds derive exclusively from adult cohorts. In our series, CMAP amplitude decrement $>7\%$ did not occur, suggesting that, in young children, amplitude decrements fall within the adult range. Consistent with this, amplitude decrement did not correlate with age.

Interestingly, we observed a moderate positive correlation between CMAP area decrement and age. Although the 0–5-month subgroup was small ($n=9$), more than half of the infants showed an area decrement $>10\%$, exceeding the adult normal threshold, whereas all children ≥ 6 months had area decrements $<10\%$.

These findings may be relevant for quantitative intraoperative neuromuscular monitoring in infants. While in adults amplitude and area based assessments are broadly interchangeable for grading neuromuscular block, this equivalence may not hold in neonates, infants, and young children.

The larger CMAP area decrement seen in children <6 months suggests age-specific NMJ physiology. CMAP amplitude reflects the peak of the summed action potentials and may remain stable when most fibers are activated; CMAP area is more sensitive to synchrony among fibers. Increased temporal dispersion can cause phase cancellation and reduce the area under the curve. In young children, immaturity of nerve and muscle conduction velocities may augment area decrement even when peak amplitudes appear stable. Minor fluctuations in resting intracellular calcium may further impair synchronization of muscle fiber responses.

In healthy adults, post-tetanic facilitation is not expected, and any increase in CMAP amplitude is considered “pseudo-facilitation.” In contrast, we observed immediate post-tetanic exhaustion in infants younger than four months. This aligns with previous observations that high-frequency stimulation in both preterm and term neonates tends to produce decrement rather than facilitation, indicating distinct presynaptic NMJ behavior in very young children.

Use of light sedation for neurophysiological testing varies widely across countries. Unlike conventional EMG where a single CMAP is typically recorded RNS requires that the limb remain immobile so that successive responses are comparable. Some experts perform RNS in awake, cooperative toddlers, but in practice only resting trains are usually feasible; fatigue and post-tetanic series are seldom obtainable. Light sedation enables tetanic stimulation and post-tetanic recordings. In our experience, without adequate sedation children frequently withdraw the limb around the 3rd–5th stimulus of a 3 Hz train, creating artifacts and rendering studies uninterpretable. While sedation is not generally feasible in routine outpatient settings, tertiary pediatric neuromuscular centers usually can provide the necessary sedation and analgesia.

We used an anesthesiologist-supervised protocol (propofol 3 mg/kg, midazolam 0.1 mg/kg, fentanyl 1.9 µg/kg), which did not significantly affect NMJ transmission. In contrast, nitrous oxide (N₂O) is not advised because it may depress NMJ function in infants younger than 12 weeks.

In summary, our work offers a new approach to understanding NMJ function in pediatric populations, especially very young infants. While CMAP amplitude decrements conform to adult norms, CMAP area decrements frequently exceed adult thresholds in children <6 months, implying distinct physiology during early development. The observed moderate positive correlation between area decrement and age underscores maturational effects on the NMJ and argues for age-specific reference values in RNS. Overall, in neonates, infants, and young children, amplitude-based decrement appears preferable to area-based measures for evaluating NMJ transmission.

Our study has limitations. It is a single-center investigation with a relatively small, Central-European Caucasian sample, which may limit generalizability. All children were tested under sedation, potentially influencing neurophysiological responses. We focused exclusively on the axillary nerve, a less common target in RNS, so extrapolation to other nerves or to awake

testing should be cautious. We acknowledge the risk of type II error with Spearman rank correlations when sample size is limited; however, in our dataset p-values were well above 0.05 and rho values were low, indicating weak positive and negative correlations e.g., for CMAP amplitude decrement consistent with lack of association.

Further studies are warranted, including other peripheral nerves and pediatric cohorts of different ages and backgrounds, ideally without sedation, to confirm and extend these findings.

5.2 PREVALENCE OF MYOSITIS AND MUSCLE AUTOANTIBODIES IN MYASTHENIA GRAVIS

Although several case reports and small series have previously described the coexistence of myasthenia gravis (MG) and idiopathic inflammatory myopathies (IIM), data on the frequency of elevated creatine kinase (CK) levels and the occurrence of IIM among MG patients treated in tertiary centers remain limited. In a Japanese study of 924 MG patients, the prevalence of IIM was 0.9%; in a Portuguese series it was 2.3%, and in an Italian cohort of 441 patients it was 2.9%.

In our cohort, 9.9% of patients had elevated CK at the time of inclusion. Definite myositis was confirmed in only one patient, and in two additional cases the findings were consistent with probable myositis, yielding an overall frequency of 2.9% in our population. All three affected patients were anti-AChR antibody positive. In a fourth patient with persistent CK elevation, muscle biopsy and genetic testing confirmed a concomitant limb-girdle-type muscular dystrophy.

Systematic reviews of MG–IIM overlap have shown that thymoma is present in more than 60% of patients, suggesting a pathogenic role of thymic abnormalities in autoimmune processes. In contrast, such a high rate was not observed in our series: two patients had thymic hyperplasia, and imaging in the third patient revealed no thymic abnormality.

The temporal relationship between MG and IIM also varied. In one case, the diagnosis of MG preceded the onset of myopathy by four years, representing late-onset MG. In the remaining two patients, the two conditions manifested simultaneously, one with early-onset and one with late-onset MG. This observation is consistent with previous studies, which have reported that MG and IIM frequently present concurrently.

The prevalence of myositis-specific autoantibodies (MSA) in patients with concomitant MG and IIM has generally been found to be low. In our patient with definite polymyositis, anti-Jo-1, PM/Scl-75, and Ro52 antibodies were detected, and the clinical picture was dominated by antisynthetase syndrome, necessitating close immunological follow-up. In the literature, we identified only two cases where anti-Jo-1 positivity was reported in association with both MG and IIM. Diaco et al. described a 47-year-old woman with anti-AChR-positive MG and thymic hyperplasia who later developed IIM with anti-Jo-1 positivity and antisynthetase syndrome; her symptoms improved with prednisone and cyclosporine, indicating a favorable prognosis. Huang et al. reported a 74-year-old woman with anti-AChR-positive MG, IIM, and thymic carcinoma. Her serology revealed anti-Jo-1, anti-Ro52, anti-titin, and anti-ryanodine receptor antibodies, indicating broad autoimmune activation, and she improved with plasmapheresis and tacrolimus.

In our cohort, neither MSA nor MAA were detected in the two probable myositis cases or in the other patients with elevated CK. One probable myositis patient, however, tested positive for anti-GAD65, with clinical features consistent with stiff-person syndrome, another autoimmune neurological disorder. Importantly, none of our patients with MG-IIM overlap had evidence of malignancy.

In recent years, the clinical significance of detecting MSA and, to a lesser extent, MAA has increased considerably. In 2017, EULAR and ACR developed new classification criteria for both adult and juvenile IIM, recommending routine testing for anti-Jo-1 autoantibodies in addition to clinical features, and incorporating anti-Jo-1 status into their online diagnostic calculator. In 2018 and 2020, ENMC experts revised the earlier predominantly clinicopathological, consensus-based classification of IIM into a clinico-seropathological approach, updating the classification of immune-mediated necrotizing myopathy (IMNM) and dermatomyositis (DM). According to these recommendations, when the clinical phenotype is characteristic, the presence of anti-SRP or anti-HMGCR antibodies in IMNM, or anti-Mi2, anti-NXP2, anti-MDA5, anti-SAE, or anti-TIF1 γ antibodies in DM, may render muscle biopsy optional.

Although previous studies have examined the presence of MSA and MAA in MG-IIM overlap, their prevalence within the broader MG population remains unknown. Interestingly, in our cohort, 18.8% of MG patients tested positive for MSA/MAA. With the exception of the single definite polymyositis case, all MSA/MAA-positive patients had normal CK levels, and

none were diagnosed with myositis. In 37% of positive cases, especially those with strong antibody reactivity an associated systemic autoimmune disease was present, such as Sjögren's syndrome, Raynaud's phenomenon, rheumatoid arthritis, autoimmune thyroiditis, or undifferentiated connective tissue disease. Of note, one patient with strong anti-PL-7 positivity had no autoimmune comorbidity at the time of testing but was diagnosed with diffuse large B-cell lymphoma two years later. Another patient with high anti-Ro52 levels had no symptoms suggestive of systemic autoimmune disease.

In 63% of our MSA/MAA-positive cases, only low-titer autoantibodies (anti-Ku, anti-Mi2 β , anti-PM-Scl-100, or anti-PL-7) were observed. Among anti-Ku-positive patients, one had Raynaud's phenomenon, while another with anti-PM-Scl-100 antibodies was diagnosed with autoimmune thyroiditis. In the remaining cases, neither systemic autoimmune disease nor IIM was confirmed, and none showed multiple antibody positivity. The clinical significance of low-titer MSA/MAA remains uncertain. A study of 727 patients with diverse neurological conditions reported that MAA and MSA could be present in 6.7–30.0% of cases, highlighting the relatively high rate of false positives in clinical practice. Furthermore, several studies have noted the limited specificity of line/dot blot assays, as 9–16% of healthy controls tested positive. These false positives were typically low-titer and often showed multiple antibody reactivities. To improve the clinical utility of MSA/MAA testing, further validation, standardization, and harmonization are required.

The immunomodulatory role of vitamin D has been widely studied. In several autoimmune diseases, loss of tolerance to autoantigens has been associated with lower vitamin D levels. Vitamin D receptors are also present in muscle cells and play a role in regeneration; deficiency may lead to weakness and myalgia. Thus, vitamin D may exert dual effects in MG by regulating autoimmunity and maintaining muscle function. A study of 151 MG patients reported an association between disease risk, clinical features, and vitamin D receptor polymorphisms. Several publications have shown lower serum vitamin D levels in MG patients, and a meta-analysis of five studies including 219 MG patients and 231 healthy controls confirmed this, recommending routine monitoring and supplementation when needed. In contrast, a two-sample Mendelian randomization analysis found no causal effect of circulating vitamin D levels on MG risk. Nevertheless, supplementation with cholecalciferol (800 IU/day) improved muscle weakness in MG patients with vitamin D deficiency.

In our large Central–Eastern European MG cohort, 67.3% of patients had low vitamin D levels, consistent with published data. MGC scores did not differ significantly between patients with normal and low vitamin D levels. Prospective follow-up after vitamin D correction is needed to determine the impact of supplementation on disease activity and quality of life.

This study has several limitations. First, the sample size was relatively small, limiting generalizability. Second, the cohort consisted of Central–Eastern European Caucasians, restricting applicability to other ethnic groups. MG phenotype is likely influenced more by genetic variability than ethnicity per se, but genetic testing was not performed. Third, the study design combined cross-sectional analysis and case series, which carries a risk of selection and observation bias; retrospective data collection may have added further distortion. Not all clinical parameters or comprehensive MG antibody profiles were available for every patient. Being a single-center, non-randomized study with sequential recruitment, sampling bias may also limit external validity. Finally, vitamin D levels were assessed only once at study inclusion, providing a snapshot rather than accounting for temporal or seasonal fluctuations or the effects of supplementation. The extent and regularity of supplementation were not systematically assessed. Longer-term, prospective studies are needed to clarify the effects of vitamin D status and supplementation on MG severity and disease course.

In conclusion, the coexistence of MG and IIM is rare but clinically relevant, affecting approximately 1–3% of MG patients according to the literature. In MG, serum CK measurement is recommended in cases of myalgia, non-fatigable weakness, or phenotypes suggestive of inherited myopathy or muscular dystrophy. Markedly elevated CK ($\geq 2\times$ upper limit of normal) or the requirement for prolonged steroid therapy should prompt further evaluation for associated myopathies, particularly myositis. In cases of mild CK elevation ($< 2\times$ ULN) detected incidentally, identification of predisposing factors (e.g., thyroid disease, statin use) and regular monitoring is advisable. MSA/MAA testing does not appear suitable as a first-line screening tool for suspected IIM in MG, given the relatively high rate of low-titer false positives. Our results support the importance of routine monitoring and correction of vitamin D levels in MG management. Finally, it is important to emphasize that the MG population is not homogeneous; other neuromuscular disorders, such as inherited muscular dystrophies, may occasionally be present and should be considered in the differential diagnosis.

6. SUMMARY

In our first study, we investigated the age-specific characteristics of neuromuscular junction function in healthy neonates, infants, and young children using repetitive nerve stimulation. Stimulation of the axillary nerve with surface electrodes was performed in 34 subjects (23 boys, 11 girls; age range: 0–48 months). The median compound muscle action potential amplitude decrement was 1%, showing no significant correlation with age. In contrast, post-tetanic facilitation and the twitch-to-tetanus ratio demonstrated a moderate positive correlation with age: exhaustion was more common under 4 months, while facilitation was observed in older children. CMAP area decrement also proved to be age-dependent, with a median of 11% in the 0–5 month group compared to 4% in the 6–48 month group. Significant differences were found between time points, with the greatest decrement occurring at 1 minute post-stimulation. No clinically significant adverse events occurred, and high-quality, analyzable recordings were obtained in most cases. These results highlight specific age-related patterns of RNS in early childhood, underlining the need for age-adjusted reference values.

In the second series of investigations, we analyzed the clinical and serological characteristics of myasthenia gravis and idiopathic myositis overlap, with a particular focus on autoantibody profiles (MSA, MAA), creatine kinase (CK) levels, and vitamin D status. Data from 101 MG patients (74 female, 27 male; median age: 57 years) were prospectively evaluated. Generalized MG was present in 67%, bulbar in 18%, and ocular in 15%. Antibody testing revealed AChR-positivity in 66%, MuSK-positivity in 4%, and seronegativity in 14%. CK elevation occurred in 10 patients, with four exceeding $2\times$ ULN; one had anti-Jo-1 positive polymyositis, two were considered probable myositis, and one had muscular dystrophy. Overall, 19 patients (18.8%) showed MSA or MAA positivity, of which 37% had high titers with concomitant systemic autoimmune disease, while 63% had low titers without such associations. Vitamin D deficiency was detected in 67.3% of patients, but no significant correlation with the MGC score was observed. Our findings emphasize that MG–IIM overlap, although rare, represents an important diagnostic and therapeutic challenge, and that the frequent occurrence of vitamin D deficiency warrants further clinical and immunological investigations.

7. NOVEL SCIENTIFIC FINDINGS

1. In our study, we demonstrated for the first time that in healthy children aged 0–48 months, the CMAP amplitude decrement recorded during repetitive nerve stimulation (RNS) remains within the adult reference range (median: 1%, IQR: 1–3%, total range: 0–7%), while CMAP area decrement values in younger children (particularly under 6 months of age) often exceed the adult reference range, indicating age-specific physiological differences.
2. We were the first to identify a moderate positive correlation between age and CMAP area decrement, post-tetanic facilitation, and the “twitch-to-tetanus” ratio, whereas CMAP amplitude decrement showed no correlation with age. This finding highlights quantitative features of neuromuscular junction (NMJ) maturation.
3. In children younger than 4 months, we observed post-tetanic exhaustion, in contrast to the pseudofacilitation typically seen in adults, pointing to differences in presynaptic NMJ immaturity.
4. We demonstrated that CMAP area decrement may be more sensitive to synchronization disturbances due to temporal dispersion than amplitude, which may result in false positive findings in children, particularly under 6 months of age.
5. The applied axillary nerve stimulation with surface recording from the deltoid muscle produced high-quality RNS curves and proved technically feasible in young children, representing a suitable alternative to the more commonly used accessory nerve.
6. The sedation protocol applied in our study (propofol, midazolam, fentanyl) did not significantly influence NMJ function and enabled reliable quantitative RNS examinations even in young children.
7. Our study is among the first in the literature to provide age-specific, quantitative RNS data in healthy children, underscoring the need for age-related reference values and providing a basis for future clinical and diagnostic applications.
8. For the first time, we determined the prevalence of overlap between myasthenia gravis (MG) and idiopathic inflammatory myopathy (IIM) in a Central–Eastern European cohort. Among 101 examined patients, three cases (2.9%) of definite or probable myositis were identified, consistent with previously reported international prevalence rates of 0.9–2.9%.

9. Elevated serum CK levels were found in 9.9% of MG patients; however, clinically relevant myositis was confirmed in only a minority of these cases. This finding highlights the nonspecific nature of CK elevation and confirms the need for further diagnostic evaluation.
10. We identified MSA or MAA antibody positivity in 18.8% of MG patients; however, most cases were not associated with clinically manifest myositis. This observation supports that MSA/MAA antibodies alone are not suitable for IIM screening in MG, particularly at low titers.
11. Low serum vitamin D levels were detected in 67.3% of MG patients, but no significant association was found between vitamin D status and disease severity (MGC score). This confirms the high prevalence of vitamin D deficiency reported in the literature, while its clinical significance requires further prospective investigation.

These novel observations may contribute to the standardization of pediatric neuromuscular investigations and to a more comprehensive immunological characterization of MG patients.

8. PUBLICATIONS



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Registry number: DEENK/533/2025.PL
Subject: PhD Publication List

Candidate: Márk Kozák
Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Kozák, M.**, Szatmári, K., Németh, E., Fülesdi, B., Nagy, A. C., Tóth, A., Boczán, J.: Function of the Normal Neuromuscular Junction in Young Children.
Muscle Nerve. 72 (2), 306-312, 2025.
DOI: <http://dx.doi.org/10.1002/mus.28445>
IF: 3.1 (2024)
2. **Kozák, M.**, Kovács, E., Nagy-Vincze, M., Tóth, A., Boczán, J.: Prevalence of Elevated CK Levels, Myositis-Specific and Myositis-Associated Antibodies, Myositis, and Other Neuromuscular Diseases in Myasthenia Gravis Patients - Experience from an Eastern European Tertiary Center.
J Clin Med. 14 (7), 1-13, 2025.
DOI: <http://dx.doi.org/10.3390/jcm14072449>
IF: 2.9 (2024)

Total IF of journals (all publications): 6

Total IF of journals (publications related to the dissertation): 6

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

29 September, 2025



9. ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, **Dr. Judit Boczán**, for her devoted guidance, continuous support, and trust invested in me throughout my research, from its very beginning.

I am grateful to the present and former heads of the **Department of Neurology**, **Prof. Dr. László Oláh** and **Prof. Dr. László Csiba**, for their professional support and for providing the opportunity to conduct my work at the Department of Neurology.

My thanks go to all the physicians and healthcare staff at the **Neuromuscular Outpatient Unit of the Department of Neurology** for their dedicated care of the patients who participated in these studies.

I would also like to thank **Prof. Dr. Béla Fülesdi** and the colleagues of the **Department of Anesthesiology and Intensive Therapy** for their valuable assistance in the examinations. I am further indebted to **Prof. Dr. Attila Tóth** and **Prof. Dr. Attila Nagy** for their extensive help with the statistical analysis of the data.

I am grateful to our TDK students (**Patrícia Hogeá-László** and **Gréta Szilvia Major**) for their contribution to data collection and analysis.

Special thanks are due to all the patients who participated in the studies, thereby contributing to the advancement of science.

Finally, and most importantly, I owe heartfelt gratitude to my parents and friends for their continuous support and encouragement throughout the years.

