# Function and structure of the eye muscles in myasthenia gravis: novel methods to aid in diagnosis and understanding of pathophysiology

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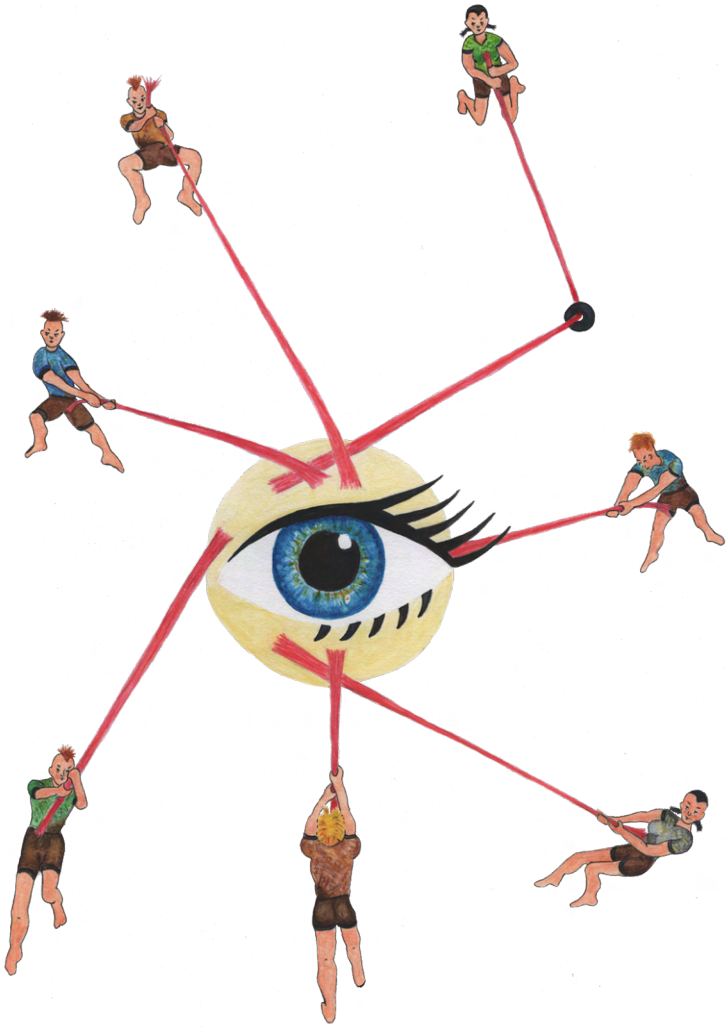

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## Summary, general discussion and future perspectives

The overall aim of the studies described in this thesis was to develop novel methods to improve the clinical care of ocular MG patients. In this thesis the eye muscles were directly studied in patients with myasthenia gravis (MG) using electrophysiology, orthoptics and quantitative MRI. The overall sub aims of this thesis were: **1.** To develop diagnostic tools to aid in the difficult diagnosis of ocular seronegative MG, **2.** To gain a better understanding of eye muscle involvement in MG to better understand the pathogenesis of refractory ophthalmoplegia in MG, and **3.** To develop tools to directly measure eye muscle function in ocular MG to use in clinical trials.

## SUMMARY

In **chapter 2** an extensive literature review was performed into clinical and imaging clues to the diagnosis and follow-up of all diseases with ptosis and ophthalmoparesis. This review combined clinical symptoms, imaging and eye muscle involvement pattern to provide clues for diagnosis of diseases with ophthalmoparesis and ptosis, including acquired and hereditary diseases with pathology located in the brain, nerve, synapse and muscle. The clinical patterns were characterized by the combinations of the presence of ptosis, ophthalmoparesis, diplopia, pain, proptosis, nystagmus, extra-orbital symptoms, symmetry or symptom fluctuations. Additionally, imaging findings of the eye muscles in hereditary muscle, neural and synaptic diseases are atrophy and an increase in fat in the eye muscles. In acquired orbital diseases, inflammation seen as hyperintensity on T2w scans with fat suppression is observed in active stages of disease combined with increases in eye muscle volumes. In diseases with ophthalmoparesis and ptosis, specific patterns of clinical symptoms, the eye muscle involvement pattern and orbital imaging provide valuable information for diagnosis and for understanding of disease pathophysiology.

In **chapter 3** the test-retest reliability was assessed of an electrophysiological test directly measuring decrement in the eye muscles, the *repetitive ocular vestibular evoked myogenic potentials* (RoVEMP) test. The RoVEMP test is a novel diagnostic test to diagnose neuromuscular transmission deficits in the eye muscles in MG. The test-retest reliability of the RoVEMP test was assessed in 19 MG patients and 15 healthy controls. The study showed that in our hands the test-retest reliability of this test is not optimal, with Bland-Altman limits of agreement in decrement of -180% to 139% in MG patients. This reliability correlated significantly with the amplitude of the RoVEMP signal and the amplitude correlated negatively with age. Based on these findings, we believe that the ROVEMP test is especially suitable to detect changes at group level after careful age-matching and we caution against the use of RoVEMP measurements with lower amplitudes in clinical practice.

In **chapter 4** the EOM were functionally assessed with orthoptic tests, measuring maximal duction angles and deviations as proxies for eye muscle strength. We applied the Hess chart in a novel way, by assessing drift during persistent gaze on the Hess chart as a direct measure of eye muscle fatigability. A remarkably high percentage (41%) of MG patients without a measurable ophthalmoparesis had Hess chart deviations. This suggests that subtle differences in contraction force are more prevalent in MG than severe eye muscle weakness causing absolute duction limitations. We studied whether these extended orthoptic tests could aid in the diagnosis of MG. The presence of drift during one minute of persistent gaze had a sensitivity of 81% and a specificity of 100%, compared to our patient control groups. This test could therefore constitute a promising, highly specific diagnostic test for MG, as it is relatively easy to implement in the clinic, affordable and already widely available. In addition to diagnosis, orthoptic measures could also benefit future clinical trials by quantifying the effect of novel treatments on eye muscle weakness in MG patients.

In **chapter 5** and **chapter 6** studies are described into the methodology of quantitative MRI in skeletal muscle and in extra-ocular muscles. In **chapter 5** we developed an extended phase graphs (EPG) based method to measure the T2 of water as a measure of disease activity, for example muscle inflammation. The model assumed a two-component model with calibration of the T2 of fat on subcutaneous fat also including a water component. Additionally, the model includes the slice flip angle profile with corrections for chemical shift displacements. The method was optimized on skeletal muscle in cohorts of healthy controls, Duchenne muscular dystrophy, Becker muscular dystrophy and spinal muscular atrophy patients. In vivo data showed a gradual decline in the T2 of water for increasing fat fractions, with important implications for clinical studies using the T2 of water as an outcome parameter. Using our model, these T2 relaxometry measurements will be more reliable and will allow for better comparison of values between centers and diseases. In **chapter 6** a pilot study was performed to test whether doing quantitative MRI of the eye muscles is feasible using a 7-Tesla MR-scanner. In this pilot study we found an increase in eye muscle volume and FF in MG and Graves' orbitopathy patients. The increase in fat content in the eye muscles in MG has been described in histological studies before, but the increase in muscle volume in the eye muscles of MG patients was unexpected. We also measured water T2 relaxation times comparable to values measured in skeletal muscle, but found a high variability between individuals and between different EOM. We concluded that despite the challenges of size and movement of the eye muscles, quantitative MRI is feasible for measuring fat fraction and muscle volume of individual eye muscles in healthy controls, MG and Graves' orbitopathy patients at 7 Tesla.

In **chapter 7** a quantitative MRI study of the eye muscles is described in a large and well-defined cohort of MG patients, neuromuscular and healthy controls. We included recently diagnosed MG patients, chronic MG patients and seronegative MG patients and compared the eye muscle findings to healthy controls, CPEO, OPMD and Graves' orbitopathy patients. We found no atrophy and limited fat replacement in MG, even in patients with residual ophthalmoplegia. This suggests that the functional impairment of the eye muscles in chronic MG is not due to atrophic muscle fibers or other structural muscle damage on a macroscopic level. These observations suggest that in most MG patients with refractory eye muscle weakness, no structural anatomical changes are present in the eye muscles precluding functional recovery after optimal treatment. Instead of the expected atrophy, volume and fat fraction of the eye muscles were slightly increased in chronic MG patients, which raises pathophysiological questions. Unfortunately, measuring the T2 of water as an inflammatory marker at 7 Tesla turned out to be difficult, due to a high variation in measurements. Diagnostically, orbital MRI did not prove to be useful in differentiating healthy controls from MG patients in our study. Also in the seronegative MG subgroup, which is harder to diagnose, no significant changes of the eye muscles were present as compared to healthy controls. However, differences in eye muscle volume and fat fraction were observed in CPEO, OPMD and GO as compared to MG which could be supportive in differential diagnostics.

## GENERAL DISCUSSION

In this thesis, three different methods were evaluated for directly measuring function or structure of the eye muscles to aid in ocular MG. All methods taught us something about the pathophysiology of eye muscle involvement in MG and about refractory ocular symptoms. The most promising finding was the lack of atrophy in the eye muscles of MG patients, because this could mean that refractory ophthalmoplegia in MG might still be susceptible to other therapeutical options. Additionally, using orthoptic measurements we found that contraction asymmetry is more important than absolute movement limitation of the eyes implying that there might be a role of the central nervous system in the cause of diplopia in MG. Orthoptic measures showed to be particularly useful for diagnosing MG and to quantify the severity of ocular symptoms in MG for clinical trials. MRI turned out to be less useful in diagnosis due to little eye muscle changes in MG, but MG-mimics like CPEO, OPMD and Graves' orbitopathy could be identified due to more severe structural changes like eye muscle swelling or atrophy. Lastly, orthoptic tests could also be relevant as an outcome measure in clinical trials in ocular MG. These tests quantify deviations between the eyes, the amount of ophthalmoparesis and can measure the presence of eye muscle fatigability. All mentioned findings, advantages and disadvantages of the three studied methods are summarized in figure 1 and discussed in more detail below.

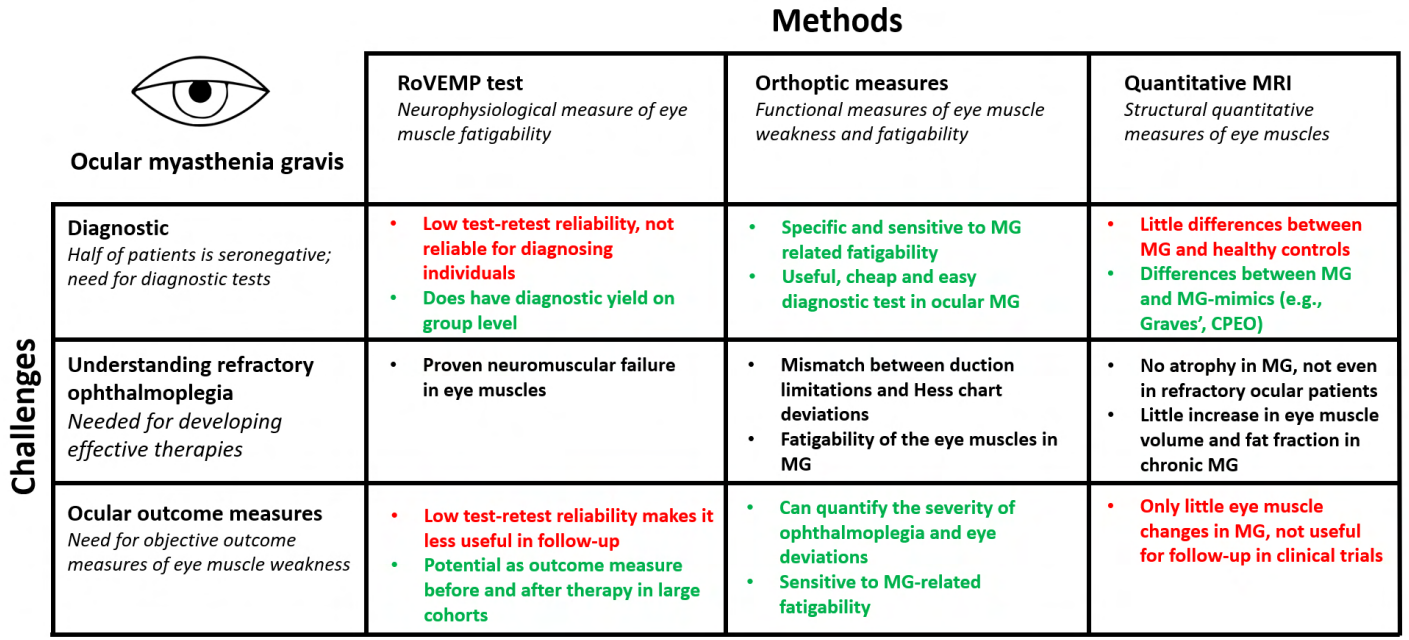


***Figure 1***. *Summary of the three clinical challenges in ocular myasthenia gravis, the three introduced advanced methods to aid in these challenges and the findings. Findings that are useful in diagnostics and as outcome measures are depicted in green and findings that are not or less useful in red.*

The first sub aim was to identify if the mentioned methods might aid in the diagnosis of ocular MG. Firstly, the RoVEMP test showed an average decrement in the eye muscles of MG on group level. However, we found a low test-retest reliability and the RoVEMP test as currently technically implemented is therefore not useful for diagnoses. This low reproducibility is mostly explained by the low signal-to-noise ratio of the measured muscle potential and the presence of blinking artifacts in many patients. Secondly, MRI showed only little differences in the eye muscles between MG and healthy controls, especially in the challenging seronegative patients. MRI is therefore not suitable to differentiate seronegative MG patients form healthy controls. However differences in eye muscle volume and fat fraction were observed in other neuromuscular and orbital diseases as compared to MG, which could be supportive in differential diagnostics in case of clinical doubt. Also in coexisting Graves' orbitopathy and ocular MG, which co-occur frequently, MRI could be supportive when eye muscles are severely swollen.[143] For example in a patient with positive antibodies and severely swollen eye muscles, the presence of both diagnoses is likely. Lastly and most promising, orthoptic tests with added persistent gaze are very specific and sensitive in diagnosing MG. Adding persistent gaze to the Hess chart makes this test very sensitive to MG-related fatigability. In 87% of MG patients a drift in time on the Hess chart was observed. In virtually no other neuromuscular disease controls and healthy controls this phenomenon was observed. Orthoptic tests that are sensitive to MG-related fatigability could therefore be a superior test in diagnosing MG in seronegative (ocular) patients. Comparing our methods, the orthoptic tests show the most promise, are most easily implementable, the fastest and most cost-efficient as compared to other diagnostic tests in MG. Therefore it would be recommended to add an orthoptic evaluation to the clinical workup of every ocular MG patient with the addition of measuring MG-related eye muscle fatigability. With extended research this might even reduce the amount of diagnostic tests needed in the clinical workup.

Measuring the eye muscles directly with three different methods, we learned more about the pathophysiology of ocular symptoms in MG. Using the RoVEMP test, we showed on a group level the presence of decrement of the eye muscles and therefore that neuromuscular transmission is impaired in the eye muscles, as it is in skeletal muscles.[32] It also showed that the myogenic potentials of the inferior oblique muscle are lower in MG than in healthy controls, probably due to fewer fibers being excited. Secondly, using the orthoptic Hess-chart we showed that also eye muscles show the characteristic fatigable weakness as known in the skeletal muscles in MG. MG patients showed less absolute limitations in eye movement as compared to relative deviations. This suggests that a subtle asymmetry in force between the eye muscles is more prevalent in MG than severe ophthalmoparesis. This might be caused by a difference in neuromuscular transmission failure between left and right assuming equal

innervation following Hering's law.[171] Interestingly, these results suggest a possible role for aberrant central nervous system eye muscle coordination in MG. This might indicate that the central nervous system is unable to adapt to the asymmetrical and fluctuating weakness of the eye muscles. Lastly, structural changes in the eye muscles were assessed using MRI. We expected atrophy of the eye muscles, especially in chronic therapy-resistant ocular MG. However, we found virtually no volume decreases in the eye muscles in MG and instead we found a small increase in muscle volume in chronic MG. This might be caused by compensatory muscle hypertrophy or by oedema due to inflammation at the neuromuscular junctions. In histological studies there is sparse evidence of inflammation and knowing whether inflammation is present could aid in the choice of therapy for an individual patient. For example new drugs targeting complement activation might be more effective in the presence of active inflammation. In literature there is evidence of atrophy of the eye muscles in untreated MG patients with MuSK and AChR antibodies. Our cohorts were however treated to the current standard of care, and did not how atrophy. This might stress the importance of timely intervention to preserve muscle. Additionally, the absence of macroscopic eye muscle damage in refractory ocular MG gives hope for the possibility of future therapeutical interventions in this subgroup.

To further speculate on what we learned about the pathophysiological mechanism; Using the RoVEMP test we proved the presence of neuromuscular transmission failure in the eye muscles in MG and that certain muscle fibers are blocked. We showed using adapted orthoptic tests that eye muscles show an increase in weakness during persistent gaze, which makes it probable that direct AChR-receptor blockage is increasing over time due to receptor downregulation and depletion of AChR. Using MRI we showed that chronic eye muscle dysfunction in MG does not lead to atrophy, at least not in patients who are treated. It seems that despite the relative denervation which is present due to the blockage of synaptic transmission, the trophic signaling between the nerve and the muscle fibers stay intact. The sustained trophic signaling would explain the lack of atrophy. It is possible that due to the known multiple innervation[117] of eye muscle fibers, not all synapses fail for one muscle fiber. Also in the distinct contraction-excitation coupling and calcium handling[120] there might be a clue why the eye muscles might be more resilient to atrophy. Currently it remains unknown whether there is inflammation present in the eye muscles in MG. One might speculate that the eye muscles are more prone to inflammation due to complement activation at the neuromuscular junction, because the eye muscles lack complement inhibitory proteins and significantly more neuromuscular junctions are present per relative muscle fiber. Inflammation might possible explain the small volume increases and fat replacement in chronic MG as observed on MR-images.

Given their specialized function and cooperation, large differences exist between the eye muscles and skeletal muscles. As mentioned before on a biophysiological level, but also on a functional and anatomical level. Functionally it should be taken into account that the eye muscles cooperate in a very complex way, via connective tissue pulleys[263] and their combined forces move the eye in all directions. This balance could be very delicate, but also resilient to weakness of one eye muscle. Weakness of one eye muscle might result in an imbalance causing double vision, but feedback loops involving the visual centers of the brain might correct for these imbalances. Therefore the direct translation of EOM weakness as measured with orthoptic tests, or structural changes in MRI, might not translate to symptoms of double vision as expected. The direction of the measured force by a skeletal muscle is easier to predict and well-studied. Anatomically, the eye muscles also have different layers: the orbital and the global layer. The orbital layer initiates eye movement and the global layer activates later is responsible for holding the gaze.[252,264] These layers are normally hard to tell apart using MRI, however in the lateral and medial rectus muscle on our high resolution 7 Tesla images we observed a stripe of fat or connective tissue in the middle which seems to separate these layers. Using high resolution MRI, possibly in different gazing directions, one could study which layer is more affected in MG by assessing deformation and volume changes. Additionally, our high resolution images might aid in the understanding of these layers in several other diseases with eye muscles involvement, and opens an interesting research field in for example strabismus surgery.

Lastly, the studied methods can serve as objective outcome measures in clinical trials for ocular symptoms of MG. Currently, ocular MG is often excluded from clinical trials,[190] also because the degree of ocular weakness has been difficult to quantify.[25,26] The ocular symptoms of MG do however cause significant disability and lower quality of life. Orthoptic measures directly measure the weakness of the eye muscles, therefore being a good objective marker to quantify ocular disease severity of the eye muscles in MG as compared to the QMG for generalized MG. With our addition to implement one minute persistent gaze in the Hess chart, this orthoptic test is also sensitive to MG-related fatigability. Given the small changes we observed in MG with our quantitative MRI parameters, MRI does currently not have a direct role as an outcome measure in clinical trials. Additionally, the RoVEMP test could have an added value in objectifying neuromuscular transmission failure on group levels in clinical trials, for example in drugs that are targeting the neuromuscular junction directly like acetylcholinesterase inhibitors.

## FUTURE PERSPECTIVES

As MG patients with seronegative disease are still regularly misdiagnosed or diagnosed after a considerable delay, additional tests are needed. Combining these multiple diagnostic test to synergistically increase sensitivity and specificity could aid in solving this diagnostic challenge in seronegative MG. For the Hess chart, which has a high specificity and is therefore a promising diagnostic test, I believe the sensitivity could be even further improved by repeated measurements with a focus on variability over time. Both fatigability and fluctuations are hallmarks and specific to MG, therefore measuring fluctuation over time could increase the diagnostic yield even further. I would recommend to perform a prospective diagnostic study including serum auto-antibody testing, neurophysiological tests and orthoptic tests (with additional testing of fatigability and fluctuation). It would be recommended to include both generalized and ocular MG patients, but also patient controls with neuromuscular disease, orbital disease and e.g. strabismus patients, to widely assess and validate the additional diagnostic value of orthoptic tests in both groups. I hypothesize that the orthoptic tests might prove to be the superior diagnostic test in diagnosing seronegative ocular MG.

The RoVEMP test and MRI turned out to be of no use in the direct diagnoses of seronegative ocular MG. The test-retest reliability of our currently implemented RoVEMP test should be improved to increase its reliability to be reliable enough for individual patient diagnosis. The focus should be on technical improvements of the signal to noise ratio. I propose for example to increase the amplifier strength, to increase the sample frequency to sample the short peaks more dense and to develop methods to correct for blinking artefacts. Orbital MRI currently shows promise in differential diagnostics to other disease, but not to separate healthy controls and MG. So far, we have studied the eye muscles while stationary using MRI, however measuring the eye muscles during movement over time in 4D could be a diagnostically interesting step forward. The cooperation of the eye muscles might show a specific pattern in MG, which might aid in diagnosis or understanding of ocular MG. A high sample frequency is however needed, while preserving sufficient resolution, but recent studies have shown it is possible to get to a temporal resolution of up to 35 ms.[265] Additionally, given literature evidence of skeletal muscle atrophy in AChR and MuSK positive MG[18,248], I propose to study the skeletal muscles as well in MG patients. Skeletal muscle MRI could aid in the understanding of refractory symptoms in bulbar and generalized MG.

Currently, $T2_{water}$ measurements are validated in skeletal muscles and turned out to be difficult to measure in the orbital region. Therefore a logical next step would be to perform $T2_{water}$ measurements of the skeletal muscles in MG patients to study whether the inflammatory process driven by complement activation can be measured using MRI at all. If MRI would be

able to identify MG patients where complement activation is the predominant pathophysiological mechanism, this might aid in therapeutic choice. These patients might for example benefit more from complement inhibiting medicine. If $T2_{water}$ does indeed show an increase in skeletal muscles in MG, it is worth improving the $T2_{water}$ measurements of the eye muscles, by for example trying to measure at 3 Tesla with higher magnetic field homogeneity, getting rid of the chemical shift displacement in the slice direction or by increasing the B1 in the orbit. Next to $T2_{water}$, other quantitative MRI parameters reflecting muscle activation profiles or muscle energy metabolism are worth investigating as a diagnostic or prognostic biomarker in MG. For example motor unit MRI shows promising results in measuring the size, shape and position[266,267] of single motor units as well as the width and contraction time of muscle twitches.[268] A portion of the total amount of motor units are known to be blocked in MG and excitation times show different delays, a phenomenon known as jitter. Motor unit MRI could potentially measure the amount of blocked motor units and the excitation contraction delay per motor unit, and could therefore be an interesting diagnostic biomarker in MG patients.

We aimed to explain how the eye muscles change macroscopically in MG, especially in the presence of refractory ocular symptoms, but found no severe atrophy. A next step would be to study the EOM microscopically. Acquiring histological samples of the eye muscles of MG patients with different phenotypes, for example after strabismus surgery or post-mortem, could aid in understanding of refractory ocular symptoms and the presence of inflammation at the neuromuscular junction in MG. We hypothesize that in refractory ocular MG the damage at the neuromuscular junction, for example visible as a decrease in neuromuscular folding, could be different or to another extent than in skeletal muscles. The eye muscles differ from skeletal muscle in that they have distinct fiber type composition[116], multiple innervation[117], smaller motor units[118], increased level of complement activation, higher levels of utrophin expression[119], a distinct contraction-excitation coupling[120], have an increased capability of regeneration and preferentially use glucose-based aerobic metabolic pathways.[84] The neuromuscular junction of the eye muscles might be more prone to damage than skeletal muscles, due to more antibody exposure, more complement activations or the presence of more neuromuscular junctions per muscle volume. On the other hand, due to higher capability of regeneration[84] damage might be more reversible in the eye muscles than in skeletal muscle. Combinations of these properties also explain why the eye muscles are not involved in all neuromuscular disease, for example in Duchenne muscular dystrophy the utrophin expression makes up partly for the lack of dystrophin expression. To conclude, histological research of damage at the neuromuscular junction, complement activation and auto-antibody concentrations might explain why eye muscles are preferentially involved in MG and to explain the pathophysiology of treatment resistant ophthalmoparesis in MG. I propose to start by

comparing histological samples of the eye muscles between treatment responsive ocular MG patients and treatment resistant ocular MG patients (post-mortem or after strabismus surgery), and study if the refractory ophthalmoplegic patients do indeed have less neuromuscular folding and more complement activation.

Ocular MG patients should be more regularly included in therapeutic trials, due to the severity of disability experienced by ocular MG patients and the lack of treatment options in refractory ocular MG. To facilitate the objective evaluation of MG related ocular symptoms in these trials, orthoptic tests should be included in these trials to follow the ocular symptoms over time and before/after treatment. Recently, patient reported outcome measures for ocular MG are being developed in addition to the currently used MG rating scales that focus more on generalized symptoms.[27] These patient reported outcome measures and our proposed orthoptic test should be evaluated over time in a larger cohort of (ocular) MG patients and their relation should be studied. If these patient reported outcome measures and the orthoptic tests show high correlations, they would be very useful in the follow-up of patients in clinical trials like the combination MG-ADL and QMG currently used for generalized MG. I also recommend to implement regular orthoptic measurements in the follow-up of ocular symptoms of MG in clinical care and study the natural course of these symptoms over time in a larger cohort.

To conclude, the most promising next research steps following from the findings in this thesis are 1. Diagnostically, orthoptic tests should be studied prospectively combined with existing diagnostic tests to have a combined higher diagnostic yield for ocular MG. Additionally, utilizing the two hallmarks of muscle weakness in MG, namely the fluctuations and fatigability, new diagnostic tests should be developed. Moreover, I propose to study the skeletal muscle in generalized MG using several quantitative MRI techniques. 2. Pathophysiologically, I propose a histological study of the eye muscles (post-mortem or after strabismus surgery) in ocular MG comparing treatment responsive and refractory patients, focusing on complement activation and destruction of neuromuscular junctions. 3. As an outcome measure in clinical trials, orthoptic measures should be included to quantify therapeutic improvement of ocular symptoms in MG.