Myasthenia Gravis and Acetylcholine Receptor-Antibodies

AChR-Antibodies as a Marker for Epidemiological Studies and in the Follow-up of Patients

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List of papers

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Abbreviations

ACh	Acetylcholine
ACh-I	Acetylcholine-inhibitor
AChR	Acetylcholine receptor
AMC	Arthrogryposis multiplex congenita
CI	Confidence interval
CT	Computer tomography
ECG	Electrocardiography
EC	Excitation-contraction
EFNS	European Federation of Neurological Societies
EPP	End plate potential
HLA	Human leukocyte antigen
IgG	Immunoglobuline G
IR	Incidence rate
IVIG	Intravenous immunoglobuline
LRP4	Lipoprotein receptor-related protein 4
MG	Myasthenia gravis
MGFA	Myasthenia gravis Foundation of America
MIR	Main immunogenic region
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MuSK	Muscle-specific tyrosine kinase
MMF	Mycophenolate mofetil
NMJ	Neuromuscular junction
OR	Odds ratio
PE	Plasma exchange
QMG	Quantitative MG
RNS	Repetitive nerve stimulation
RyR	Ryanodine receptor

SFEMG	Single-fiber electromyography
VATS	Video-assisted thoracoscopic

1. Introduction

1.1 Epidemiology

Myasthenia gravis (MG) is a rare neuromuscular autoimmune disease the cause of which remains unknown [87]. However, what is well established is the role of pathogenetic circulating antibodies targeting the nicotinic acetylcholine receptor. Occurring in all races, in both genders of all ages MG has developed from being a severe disease with a mortality of 70% [60], to being, in most cases, a disease that can be effectively treated. As a result of optimal treatment, the disease can be managed satisfactorily in most instances with a good long-term prognosis and a normal life expectancy [135, 175].

A large number of MG epidemiological studies have been performed worldwide over the past 60 years. Norway has contributed to this research from the first nationwide Norwegian published paper on MG epidemiology in 1966 to recent studies [9, 72, 183, 184, 195]. Even though studies from all continents are represented, there is a preponderance of European studies, in particular from Scandinavia, the UK and Italy.

1.1.1 Prevalence and incidence

Reported MG prevalence has increased over time from 15 per million in 1951 [183] to 240 per million in 2012 [123]. Despite variations, there is a clear trend towards an increase in MG prevalence over time. This increase can in part be explained by increased survival for MG patients, in part by population cohorts with more elderly people and less children. In Norway, two recently published studies reported prevalence of 131 and 145 per million respectively [9, 72], and with good concordance

between the two databases used [10]. For publications during the last five years, the MG prevalence has ranged between 117 and 240 per million [27, 41, 54, 123, 137].

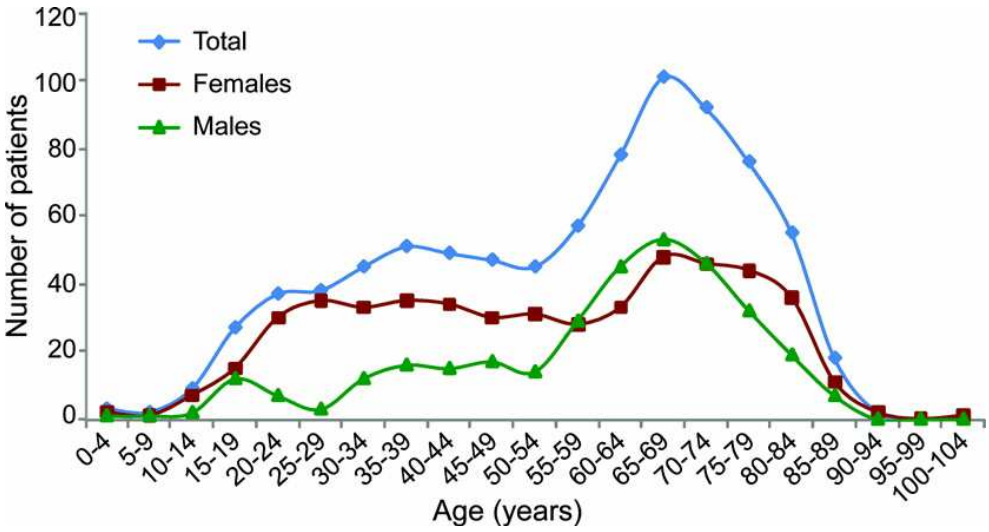
Reported annual MG incidence has also increased from 4.0 in the period 1951-1981 [184], to a report of 24.9 per million in 2012 [54]. Published MG incidence during the last five years has ranged between 14.8 and 24.9 per million [54, 100, 137]. When AChR-antibody assays were introduced in 1976, one could expect the heterogeneity in MG incidence to be reduced, but there is no evidence for this. However, the MG incidence rates increased significantly with approximately a doubling after the introduction of this assay [25].

The increase in MG incidence can partly be explained by improved diagnostics and case ascertainment and the change in demographics with an ageing population and lower birth rates. Nevertheless, it is probably also a genuine increase in MG incidence due to environmental and genetic factors. In Italy, two studies were carried out within the same region and with a similar study design over a twenty-year interval, and found an increase in both incidence and prevalence from 7.4 to 14.8 per million and 82.0 to 129.0 respectively [48, 137]. In two studies from UK [205] and Greece [147], with identical study design and over the same time of period, there was a marked difference in incidence; from 4.8 per million in the Greek study and 18.0 per million in the UK study. The difference might point to genetic and/or environmental causative factors, although medical practice and access to specialist examinations probably also differed between the two countries.

1.1.2 Gender Characteristics

The MG incidence was for many years described with a peak in the 2nd and 3rd decade for women, and peaking in the 6th and 7th decade for men [39, 147]. In recent years, some studies have demonstrated a two-peaked MG incidence for women, and with a

ratio close to 1:1 in the 6th and 7th decade [9, 72, 137], some studies have not identified any early peak for women [54, 100] but a steady increase in MG incidence and a peak for both genders in the 6th and 7th decade. In a systematic review of epidemiological studies of MG, the bimodal pattern for women was observed in 5 of 14 studies [25]. Particularly, this was found in studies from Scandinavia, the UK and Italy. The frequency of older individuals with MG is probably greater than previously thought for both genders [62, 180, 205].



Age at first-time positive AChR-antibody test in the total population, females and males.

Heldal, A.T, 2009; Seropositive myasthenia gravis: a nationwide epidemiologic study [72]

1.2 Diagnosis of MG

1.2.1 Clinical features

The most frequent initial MG symptoms are ptosis or diplopia. The disease becomes generalised in a various proportion of the patients, depending on the population. Predominantly, MG affects oropharyngeal and/or respiratory muscles or limb and/or axial muscles, the latter being the most common [60, 120]. Patients with MG also often describe pathological fatigue, differentiated from normal fatigue because it does not subside with rest [94]. Pathological fatigue is also well documented in patients with other autoimmune diseases, such as multiple sclerosis [151] and rheumatoid arthritis [202]. There are a number of differential diagnoses, including brain stem pathology, amyotrophic lateral sclerosis, multiple sclerosis, polymyositis, muscular dystrophy, mitochondrial myopathies and general fatigue. Especially in very old patients, MG can be overlooked or misdiagnosed. Symptoms such as slurred speech, difficulty in swallowing and dysphagia, have many potential explanations in elderly people [43, 107, 122, 205]. In addition, elderly patients have more comorbidity than younger patients, which can make the symptoms more difficult to identify. Moreover, ocular symptoms as ptosis and diplopia are common clinical signs at MG onset, and are easier to detect in a more youthful appearance. Ptosis can be difficult to spot because of a decrease in the total eyelid area with sagging of the lower eyelids, as a result of ageing. Macular degeneration or cataract formation can impede the detection of diplopia [2]. Pure ocular symptoms may be due to ocular myopathies or neuropathies as neuromyelitis optica [208]. In younger patients, MG could be misinterpreted as multiple sclerosis, brain stem infarction, psychiatric conditions or general fatigue. Other neuromuscular diseases should also be considered, such as Lambert Eaton myasthenic syndrome and genetic myasthenic syndromes.

The most important tool in clinical examination is the patient history and the identification of any fluctuations over the course of the day. A ptosis test may reveal ocular weakness (ability to maintain gaze fixed upwards or laterally for up to 30 seconds). Axial muscles should be tested specifically. A simple test to reveal weakness in proximal limb musculature is by holding an arm stretched out for a defined period of time. Bulbar manifestations including speech and swallowing should be examined. Weight loss can be a sign of bulbar affection. Symptoms of respiratory insufficiency represent severe MG with increased risk for the development of a myasthenic crisis.

1.2.2 AChR-antibody testing

The presence of AChR-antibodies is specific for MG, with some rare exceptions as explained in the section above. MG with a positive AChR-antibody test is in this thesis called anti-AChR MG. AChR-antibody measurements should always be undertaken when MG is suspected. The most common immunological test measures the amount of antibodies in serum that precipitates AChRs. This is detected by radioimmunoassay with ^{125}I-a-bungarotoxin-labeled AChR. Further aspects of the AChR-antibodies and the method of testing will be discussed under the "Pathophysiology of anti-AChR MG" and "AChR database" sections, page 31 and 44 respectively.

1.2.3 Pharmacology

A suspected case of MG can be tested by response to the oral administration of an acetylcholinesterase inhibiting (ACh-I) drug (pyridostigmine). If a marked objective improvement occurs, this will be a rather specific and significant proof of MG. This is diagnostically most important in cases in which no antibodies are detected. Intravenous administration of the ACh-I edrophonium, Tensilon-test [134], was previously an integrated part of the diagnostic procedure, but has recently been phased

out in many clinics as the drug is not easily available, the test is time-consuming, and other methods of equal efficacy are employed, antibody- testing in particular.

1.2.4 Neurophysiology

Neurophysiological tests in the diagnosis of MG include repetitive nerve stimulation (RNS) and single fiber electromyography (SFEMG). In MG, the safety factor (defined as the difference between the end plate potential (EPP) and the threshold potential to initiate an action potential) is reduced, and during RNS, some EPPs may not reach threshold resulting in no action potential to occur. This results in the decrement in amplitude of the compound muscle action potential, thus being the basis for the decremental response in RNS. A positive RNS test is defined as a >10 % decremental response having the patient contract the muscle maximally for 10 to 60 seconds or by delivering a high-frequency train of stimuli with 3-5 Hz repetitive stimulation [78]. The maximal decrease in ACh release occurs after the first four stimuli. RNS is a technically difficult procedure, and the sensitivity and specificity will depend on the operator and the number of muscles tested. However, when affected muscles are tested, and the test properly conducted, the specificity of RNS is approximately 95% for both generalized and ocular MG. The sensitivity is reported as less than 30% in ocular MG and approximately 80% in generalised MG [78].

SFEMG is based on simultaneous recording of two muscle fiber action potentials generated by a single motor neuron. The difference between the firing of one muscle fiber action potential compared to the other in time is termed neuromuscular jitter. The jitter is increased in MG patients. SFEMG is considered as the most sensitive diagnostic test for detecting pathological neuromuscular transmission. For generalised MG, the sensitivity and specificity of SFEMG have been reported as 79% and 97% respectively. In ocular MG, sensitivity has been reported as 29% and specificity 94%

[17, 78]. As for RNS, the sensitivity and specificity will depend on the muscles tested, and how thoroughly the neurophysiological examination is conducted.

1.2.5 Imaging

Thymus pathology is typical for MG, and rarely linked to other autoimmune diseases [26]. All patients diagnosed with MG or suspected to have the disease, should undergo radiological examination of the anterior chest cavity to look for thymus pathology. Most important is it to differentiate thymic hyperplasia (lymphoid hyperplasia) and especially thymoma from the normal thymus. The thymus gland is diffusely enlarged with the tissue similar to normal thymic tissue at both computer tomography (CT)-scan and magnetic resonance imaging (MRI). A normal-sized thymus gland on CT-scan does not exclude hyperplasia, and hyperplasia is difficult, often impossible, to diagnose by imaging. Thymoma is seen as a homogeneous lobulated soft-tissue mass in the anterior mediastinum. It can be difficult to distinguish between thymic hyperplasia and a thymoma. Contrast-enhanced CT-scan is the modality of choice for evaluation of thymomas [116]. The sensitivity of mediastinal CT for undifferentiated thymic pathology is approximately 90%. The sensitivity and specificity of CT-scan for thymus hyperplasia is reported as 36% and 95% respectively. The sensitivity for thymoma is reported as 89-100% and with a specificity of 77% [142, 212].

MRI can give useful additional information if suspicion of tumor spread with local infiltration of surrounding organs, if equivocal information first has been examined on CT [116, 167, 196].

1.3 Subgroups of MG

MG should be separated into several subgroups, reflecting pathogenesis and different considerations prior to therapeutic choices. The subgroups early-onset MG, late-onset

MG, MG with thymoma and ocular MG are relevant for this thesis, as these groups all have AChR-antibodies. The other subgroups either have pathogenetic antibodies against other muscle antigens or no detectable antibodies. These subgroups will be discussed separately in the "MG with low-affinity AChR-antibodies and with non AChR-antibodies"-section, page 33.

1.3.1 Early-onset MG

Up to 1990, the cut off between early- and late-onset MG was set at 35-40 years of age. Based on more precise studies this delineation was increased to 50 years of age [180]. However, there are still studies that argue for early-onset MG to be defined as occurring prior to age 40 years [33, 168]. Our data support onset before or after 50 years of age as the best distinction between the two subgroups early- and late-onset MG [9, 72, 137, 147, 205]. In Scandinavia, there is a preponderance of females in the early-onset MG group [72], however this preponderance has not been recognised in all studies (see "Gender characteristics"- section, page 12).

Thymus hyperplasia with enlarged thymus is most common for early-onset MG with the occurrence of intrathymic lymphoid follicles and germinal centers [118, 155]. Early thymectomy is recommended as treatment, especially if thymus hyperplasia has been identified on their mediastinal CT-scan [61, 98, 175]. Approximately 90% of early-onset MG patients have AChR-antibodies, whereas titin-antibodies are detected only in 10% of such patients. Ryanodine receptor (RyR)-antibodies are seldom found [159].

The main risk allele for early-onset MG is HLA-*B*08 [117], but also HLA class II region DRB1*16 is positively associated with early-onset MG. HLA class II DRB1*0701 is negatively associated with early-onset MG [213].

1.3.2 Late-onset MG

This group is defined as patients with MG onset after 50 years of age. Alternatively, this subgroup is defined as onset after 40 years of age, as discussed in the early-onset section above. Late-onset MG has an equal gender distribution. Thymus is most often normal. Thymectomy is usually not recommended for this group, but may be recommended in patients with thymus hyperplasia [55]. Most often the patients have AChR-antibodies, approximately 60% of whom have titin-antibodies detected, and RyR-antibodies are detected in about 15% of the patients [159]. Late-onset MG is associated with HLA DRB1*15:01[117].

The age limit to distinguish between early- and late-onset MG has implications for sensitivity and specificity for the two subgroups. It has also been suggested a cut-of at 60 years of age for late-onset MG. There is a "grey-zone" in clinical, immunological and pathological terms between 40 and 60 years of age [118]. Age for cut-off is crucial when analysing and comparing data.

1.3.4 MG with thymoma

Thymomas are neoplasms derived from thymic epithelial cells, and these cells are mixed with non-neoplastic T-cells. MG is the most common thymoma-associated autoimmune disease, affecting 30-50% of patients with thymoma [93, 125, 155]. 10-15% of all MG patients have a thymoma [51]. Moreover, an additional 25% of patients with thymoma and no clinical symptoms of MG have circulating AChR-antibodies in their serum [51]. Titin-antibodies are found in up to 95% of MG patients with thymoma, and antibodies against RyRs in 50-60% [118]. There is no typical onset age for MG-thymoma, but it is rarely diagnosed during the two first decades of life.

1.3.5 Ocular MG

Approximately 10-20 % of MG patients have ocular MG, the remaining have a generalised form of MG [53, 60]. Ocular MG is a localised form of MG affecting the ocular and periocular muscles, thereby causing ptosis and diplopia. MG is defined as ocular if no non-ocular symptoms have appeared during the first two years after onset [175]. 30-70% of the patients with ocular symptoms as their debut, develop generalised symptoms during this period [60, 99, 178, 203]. It has been discussed whether there are clinical features at onset which can predict whether an individual patient`s ocular symptoms will convert to a generalised form. Some studies have found that the ocular MG patients usually are below age 65 years, and with milder symptoms than those with conversion to a generalised MG [60, 178]. This is an important issue because of the emerging evidence that early corticosteroid treatment may have a risk-modifying effect. These studies are, however, flawed by suboptimal designs [210].

1.4 Treatment of MG

1.4.1 Symptomatic treatment

The initial treatment of MG is oral ACh-I. Such drugs inhibit the breakdown of ACh at the NMJ, increasing the amount of available ACh and thereby reducing the muscle weakness. Pyridostigmine is the most commonly used drug, but also neostigmine, and to a lesser degree ambenonium is used. Pyridostigmine has a higher bioavailability, slower onset, longer duration and produces fewer side-effects compared to equipotent doses of neostigmine [12]. This treatment is purely symptomatic and usually well-tolerated at standard doses of up to 60 mg five times per day [175]. As the ACh-Is cause increased concentration of ACh at both nicotinic and muscarinic NMJs, typical side-effects occur frequently. These are dose-dependent. Muscarinic side effects are stomach cramps, diarrhea, increased sweating, superfluous respiratory and gastrointestinal secretions [144]. The main nicotinic side effects are muscle fasciculations and cramps [148]. Optimal dosage should be balanced between the clinical improvement of the muscle strength and the degree of side-effects [175].

1.4.2 Immunosuppressive treatment

Corticosteroids: For most patients, pyridostigmine alone is insufficient to reduce the symptoms, and immunosuppressive treatment is needed. The first-line drug of immunosuppressive treatment is prednisolone [175]. To induce remission, it is often required a relatively high-dose (60-80 mg on alternate days) which is slowly tapered to the dose required to maintain the state of remission [175]. High doses every day with prednisolone are given in critically ill patients, in addition to plasma exchange or IVIG to overcome the acute worsening [175]. There is a risk of a temporary worsening at high doses of prednisolone (steroid dip), and close observation of the patient is therefore needed. Steroids are efficient treatment of MG [164] by its immunosuppressive effects, however, the effects are complex and not completely

understood. It is suggested that the therapeutic effect is due to several mechanisms, such as influencing the distribution and trafficking of leukocytes, inhibiting recruitment and migration of lymphocytes to areas of inflammation, and blockade of several functional properties of T-cells [38]. Steroids have in addition a direct effect on the muscle by increasing the number of AChRs on the cell membrane resulting in increased AChR function and improved synaptic transmission [152].

Long-term treatment of prednisolone carries a risk for side-effects and serious health problems. These are reduced glucose tolerance, osteoporosis, increased blood pressure, weight gain, fluid retention and peptic ulcer disease.

While prednisolone is used in most European centers, prednisone is the standard steroid for oral use in the USA, and represents a therapeutically equivalent on weight basis to prednisolone [164]. High-dose parenteral corticosteroids can be given in severe MG and in acute situations in addition to PE or IVIG [129, 175].

Azathioprine: Azathioprine is a first-choice drug for long-term immunosuppressive therapy in MG. It is usually recommended in combination with prednisolone [175]. It inhibits DNA and RNA synthesis and interferes with T-cell function. A main disadvantage of this drug is the delayed onset of action with a maximum effect obtained after 6-24 months. Often azathioprine is therefore started combined with prednisolone to achieve also a more rapid therapeutic effect. Subsequently prednisolone is tapered off over a prolonged time after the clinical effect of azathioprine is achieved. The formal evidence of this is weak even though its extensive use [67]. However, a well-conducted double-blind prospective study showed the long-term superiority of the combination prednisolone and azathioprine as compared with prednisolone alone [136]. Azathioprine is usually well tolerated, but flu-like symptoms

or gastrointestinal disturbances, pancreatitis included, occur in 10%, usually within the first days of treatment. Leukopenia and hepatotoxicity are potential adverse effects, and careful ongoing blood monitoring during the first few months is required. Dosage should be adjusted according to blood cell count and liver enzymes. These adverse effects usually respond to drug withdrawal.

Mycophenolate mofetil (MMF): This drug blocks purine synthesis by selectively inhibiting proliferation of activated B- and T-lymphocytes [4]. MMF has been shown to be safe and beneficial in inflammatory conditions such as psoriasis and systemic lupus erythematosus [171]. Two recent randomized double-blind trials comparing MMF plus prednisone with prednisone alone, failed to prove the benefit of MMF over prednisone in treatment of MG [126, 165]. However, both trials had a short duration of time and may not have taken enough consideration to the great benefit of prednisone, thus masking the additionally and steroid-sparing effect of MMF [166]. In 2010, a retrospective study reviewing 116 MG patients treated with MMF was conducted [70]. When followed for 2-3 years, the authors concluded that MMF was beneficial both with prednisone and as monotherapy, and the effect appeared after 6 months in both groups. MMF is reported to have less side-effects than cyclophosphamide and ciclosporin. The most commonly observed are nausea, headache and diarrhea [119]. Opportunistic infections have been reported [193]. According to the European Federation of Neurological Societies (EFNS) guidelines, MMF should be tried in patients intolerant or unresponsive to azathioprine [175].

Rituximab (Anti-CD20): Rituximab has been reported as a promising agent in MG treatment, especially for the MG subgroup with antibodies to muscle-specific tyrosine kinase (anti-MuSK) MG patients [37, 91, 133]. The efficacy of rituximab in this subgroup may be explained by the disease being mediated by IgG4 antibodies [46]. Patients with anti-MuSK MG have in general a poorer response to standard therapies

than anti-AChR MG patients [138]. In one study from 2012, 17 patients with anti-AChR MG and anti-MuSK MG were treated by rituximab (mean follow-up time 31 months), and compared [37]. Both groups benefited from the treatment, but the effect was dramatically better and longer-lasting in the anti-MuSK MG group. Moreover, the antibody titers in the latter group declined significantly during follow-up. In the anti-AChR MG group, the concentration of AChR-antibodies did not change significantly. Rituximab has, based on the promising studies reported, been proposed as an early therapeutic option especially in anti-MuSK MG patients without response to steroids [37]. The side-effects were minor and related to the infusion of the drug (facial flushing and generalised skin rush). However, there are reports on development of progressive multifocal leukoencephalopathy in patients treated with rituximab, so the patients need to be thoroughly tested and monitored [92, 112, 113].

Ciclosporin: Ciclosporin inhibits T-cell function and is mainly used when azathioprine does not provide sufficient effect. The positive steroid-sparing effect on MG is well documented [30, 130, 131]. However, common adverse effects are nephrotoxicity and hypertension. The drug should therefore only be considered in patients intolerant or unresponsive to azathioprine [175].

Methotrexate: Methotrexate is a structural analogue of folic acid and exerts an anti-proliferative effect on immune cells by inhibiting DNA synthesis. Both safety and efficacy of this drug is well documented in other autoimmune diseases such as rheumatoid arthritis, but poorly investigated in MG [69]. According to the EFNS guidelines, methotrexate should be considered in MG patients who do not respond to first choice immunosuppressive drugs [175].

Cyclophosphamide: Cyclophosphamide is used in severe MG. It adds an alkyl group to the guanine base of DNA, and thereby interferes with DNA replication. It is a strong suppressor of B-cell activity and antibody synthesis, and at high doses it also affects T-cells. The effect on MG is well-documented [35, 40] but with a risk of toxicity leading to bone-marrow suppression, opportunistic infections, bladder toxicity, sterility and neoplasms. The medication should therefore be limited to those patients with MG intolerant or unresponsive to steroids plus azathioprine, methotrexate, cyclosporine and/or mycophenolate mofetil [175]

Tacrolimus (FK506): This agent inhibits proliferation of activated T-cells via the calcium-calcineurin pathway. It potentiates excitation-contraction coupling in skeletal muscle [80]. Tacrolimus may have an additional effect in RyR-antibody positive patients through enhancing RyR-related sarcoplasmic calcium release that in theory might be blocked by RyR-antibodies, hence inducing symptom relief [82, 175]. Tacrolimus is recommended for MG patients with poorly controlled disease, and especially in RyR-antibody positive patients [175].

Eculizumab: Eculizumab is a recombinant humanised monoclonal $IgG_{2/4}$ antibody that blocks the formation of complement complex. A double-blind, placebo controlled crossover trial of anti-AChR MG patients with severe refractory disease, has recently been reported [79]. In this study they found a significant improvement of the patients on eculizumab. The trial demonstrated a promising result with a possible new approach for the management of severe and refractory MG.

There are other emerging therapy options acting on various elements of the immune system, such as etanercept, bortezomib, belimumab and granulocyte-macrophage colony-stimulating factor. Some of these drugs are approved for treatment for other

autoimmune diseases but not validated for use in MG, and should therefore be used only as part of open studies. Establishing treatment with new and more selective immunoactive drugs, and a more tailored treatment for the different MG subgroups and even individual patients should be aimed for in the coming years.

Plasma exchange (PE) and intravenous immunoglobulin (IVIG): During PE, AChR-antibodies are removed from sera by membrane filtration or centrifugation. IVIG affects the function and/or the production of antibodies by complex mechanisms that are not entirely understood. It acts on the immune system through various ways including suppressing antibody production and by anti-idiotypic autoantibodies neutralizing the autoantibodies [34]. Both PE and IVIG have a rapid effect, occurring after a couple of days and lasting for 1-3 months. Both are short-term treatments. They are used in severe cases to induce remission, as a lifesaving procedure in myasthenic crisis, and as a preoperative preparation [175]. PE and IVIG have been shown to be equally effective [13, 42, 52]. PE has a higher frequency of severe side effects, and represents a more complicated procedure. For that reason, IVIG treatment is the preferred option in most centers [52, 56]. A report from the American Academy of Neurology concluded that evidence is insufficient to distinguish between IVIG and plasmapheresis regarding efficiency in treating MG [139].

1.4.3 Thymectomy

Thymectomy is an integral part of MG treatment. Data from several class III observational studies show that thymectomy is beneficial in MG for non-thymomatous MG patients. Moreover, early thymectomy in the course of MG improves the chance of remission. Thymectomy is therefore a recommended treatment for MG, and an early intervention is preferred compared to later in the course of MG [175, 177, 181, 192].

Thymectomy can be performed either transsternally or by a video-assisted thoracoscopic (VATS) approach. One review from 2011 concluded VATS to be the preferred method because of better cosmetic result, reduced need for postoperative medication and equivalent disease resolution [214]. The effect of thymectomy is expected to occur within two years. Immunotherapy is often started before thymectomy and continued and tapered off as the effect of surgery appears.

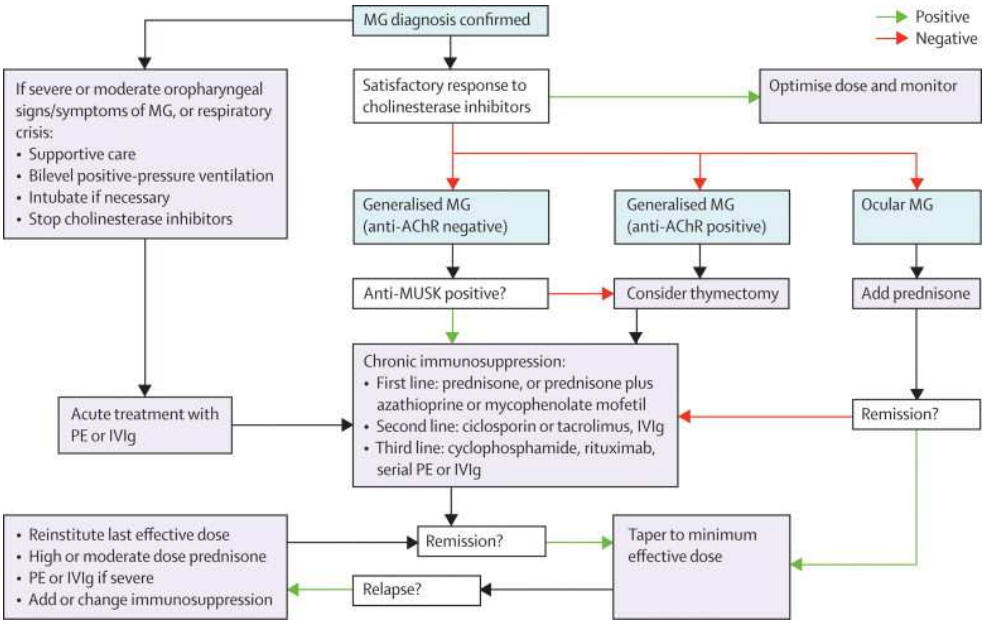
Indication for thymectomy is related to subgroup of MG:

Early-onset MG: Thymus in these patients is enlarged, and is probably the site where the autoimmune response develops and arises [26]. Thymectomy should be undertaken early, before disease-specific T-cells seed in the periphery [55]. Most centers will recommend early thymectomy in all early-onset MG cases without full clinical remission on ACh-I treatment [55, 156, 174, 175]. Surgery is usually uncomplicated.

Late-onset MG: Thymectomy is not recommended for late-onset MG with an atrophic thymus. A proportion of late-onset MG patients have, however, a hyperplastic thymus, usually those with an early-onset within this group. Late-onset MG patients with hyperplastic thymus most probably have a positive effect of thymectomy [57]. Patients with titin- or RyR-antibodies are believed to be less likely to benefit from a thymectomy than those without such antibodies [157].

Thymoma-MG: Thymectomy is the mainstay treatment for all thymoma-MG patients aiming to remove a potentially infiltrating tumor [175]. Surgical removal is sufficient for non-invasive thymomas. If not possible with surgical removal, chemotherapy and radiotherapy are also effective treatment for a thymoma [55, 96].

Ocular MG: There is no evidence for a better clinical outcome after thymectomy. Thymectomy is not recommended for this group [29, 57].



Treatment flowchart. Meriggioli, M.N, 2009; Autoimmune myasthenia gravis [120]

1.4.4 Supplementary treatment

Respiratory muscle training has been shown to improve lung function and respiratory muscle strength and/or endurance in MG patients [150]. Physical training can be carried out safely in MG and produces some improvement in muscle force [175]. Weight reducing or -control, training and other life style modifications are suggested as important, but there is no solid scientific evidence to support these recommendations [175]. Seasonal flu vaccination should be recommended in MG patients. Aggressive treatment of infections is recommended.

1.4.5 MG and giving birth

MG represents a risk factor for pregnancy and delivery. Several pregnancy complications occur with slightly increased frequency, and also operative interventions during delivery are undertaken more often than expected [77]. Women with MG should be followed up by a neurologist during a pregnancy, and an active cooperation between obstetrician, neurologist and pediatrician is recommended. 10-20% of the children born to mothers who have MG develop transient neonatal MG because of transplacental transmission of IgG antibodies. The antibodies diminish naturally postpartum with clearance. The symptoms include weak sucking, dysphagia, weak cry, hypotonia, and more rarely, respiratory problems. The symptoms are usually evident within the two first days of life and can last for two to four weeks [45, 76]. Arthrogryposis multiplex congenita (AMC) is defined as nonprogressive congenital contractures that generally results from lack of fetal movement in utero [145]. Maternal MG can be a rare cause of AMC. There is no correlation between the severity of maternal disease and the occurrence of neonatal MG or AMC [64, 75].

Pregnancy does not worsen the long term outcome of MG, and women should therefore not be discouraged from conceiving [175]. Breast feeding should be encouraged [132].

1.4.6 Treatment myasthenic crisis

Approximately 15-20% of MG patients experience a myasthenic crisis during lifetime [7]. Myasthenic crisis is defined as weakness severe enough to necessitate intubation or delayed extubation following surgery [16, 90]. Approximately 70% of myasthenic crisis are precipitated by infections [194]. Surgery and administration of different medications are other risk factors. In many cases, there is no obvious cause which led to crisis. In patients with a confirmed MG-disease, deterioration to a myasthenic crisis

is easy to identify. Sometimes MG can start with severe respiratory failure, requiring an extended diagnostic work-up.

The life-saving treatment of a myasthenic crisis is promptly to recognise it and to establish adequate mechanic respiratory support, either by non-invasive positive pressure ventilation or intubation [162]. ACh-Is are discontinued as this medication increases bronchial secretion, the patient has often used high doses during the MG worsening leading to the crisis [55]. PE and IVIG are comparable in terms of efficacy on the basis of clinical evidence preceding controlled trials [19, 52, 149, 175]. However, PE is probably slightly more effective than IVIG in myasthenic crisis. There is a general agreement that PE or IVIG should be combined with high doses of steroids during recovery from myasthenic crisis, and the treatment is required for several weeks [28, 85]. The mortality rate of myasthenic crisis has improved greatly from above 40% in the 1960s to less than 5% today [31, 209].

Long term immunosuppressive treatment is recommended to maintain the effect of PE or IVIG [175]. Gradually tapered dose of prednisolone combined with azathioprine is usually chosen. Ach-Is should be introduced again.

1.4.7 MG and the heart

Nearly 50% of all MG patients and approximately 97% of all MG thymoma patients have antibodies against titin and RyR, autoantibodies targeting both proteins in striated and cardiac muscle [127]. Antibodies targeting adrenergic ß-receptors [211], muscarinic AChRs [191] and voltage-gated K+ channel (anti-Kv1.4) [186] has later been identified. That myocardial pathology may occur in association with thymoma MG, is established knowledge [198]. This is possibly due to thymoma infiltration and invasion of the pericardium, myocardium, large vessels and other local structures, and thereby a possible alteration of the cardiac function. Myocarditis is the heart condition most often thought to be related to MG [189]. This is a potentially lethal disorder in

which symptoms such as shortness of breath, exercise intolerance or fatigue can be misinterpreted as myasthenic symptoms [189]. Findings of electrocardiography (ECG) and conventional ultrasound echocardiography are unspecific [1].

Anti-Kv1.4 antibodies have been reported as a possible marker for cardiac involvement [185, 190]. One study from 2013 found patients with antibodies against anti-Kv1.4 in 70 of 650 MG patients. Of these, 60% had abnormal ECG findings with high frequencies of T-wave abnormality and QT-prolongation. Clinically suspected myocarditis was found in eight MG patients, and none in the MG patients without anti-Kv1.4 antibodies [185]. Heart examination is necessary in MG patients with antibodies to RyR, titin or anti-Kv1.4.

1.5 Pathophysiology of anti-AChR MG

The NMJ consists of three main parts: the presynaptic nerve terminal where acetylcholine is made, stored and released; the synaptic space, and the postsynaptic membrane. When a nerve action potential enters the nerve terminal, it triggers an exocytosis of synaptic vesicles containing ACh. The ACh diffuses across the synaptic cleft and interacts with the AChRs clustered on the ridges of the postsynaptic folds of the muscle membrane. This leads to depolarization of the membrane, and a muscle action potential is induced.

In approximately 85% of cases, MG is caused by AChR-antibodies with affinity for the nicotinic AChRs in skeletal muscle [111, 206]. These antibodies were first discovered in 1976 [111]. AChR-antibodies are polyclonal, mainly immunoglobulin G (IgG), which in humans consist of four isotypes. These isotypes (IgG 1-4) have similar amino acid sequences, but differ in their ability to activate complement. IgG 1 and IgG 3 are effective complement activators, IgG 2 activates poorly and IgG 4 does not

activate complement at all. In anti-AChR MG, the isotypes IgG 1 and IgG 3 predominate, and IgG 1 and IgG 4 are present only in low concentrations [59, 153].

The AChR is an oligomeric membrane protein composed of five subunits. In the fetal form, the receptor consists of two alphas, one beta, one gamma and one delta subunit. In the adult form, the gamma is substituted with an epsilon unit [84]. This switch is accompanied by a decrease in channel opening time and a longer receptor half-life [74]. AChR-antibodies can bind to both fetal and adult types of the receptors, but often with different affinity. Most AChR-antibodies bind to the main immunogenic region at the alpha1-subunit [115].

There are three main mechanisms by which the AChR-antibodies reduce the number of functional receptors:

1. Complement-mediated lysis of the postsynaptic membrane resulting in distortion [44, 158]
2. Cross-linking of adjacent AChRs resulting in their accelerated internalisation and degradation[110]
3. Direct blocking of the acetylcholine binding site, this being less important than mechanisms 1. and 2. [24]

AChR-antibodies are very specific antibodies for MG. They are not detected in healthy individuals, and rarely in patients with other neuromuscular or autoimmune disorders.

1.6 MG with low-affinity AChR-antibodies and with non AChR-antibodies

1.6.1 Low-affinity AChR-antibodies

These antibodies are identified in approximately 60% of patients negative for both AChR- and MuSK-antibodies in radioimmunoprecipitation assays (RIAs). The antibodies do not have the high affinity required to bind to AChRs in solution, but bind divalently to adjacent AChRs expressed in dense clusters, and can be detected using cell-based assays and direct immunofluorescence [207]. These methods have unfortunately not yet become commercially introduced. The antibodies are still likely to be pathogenic because of the high density of AChRs at the NMJ, and because the antibodies are of the IgG1 class and activate complement [104]. Patients with low-affinity AChR MG have the same pathogenesis, treatment and treatment response as patients with ordinary AChR-antibodies. They have, as expected, in general a good response to therapy, to ACh-I as well as to immunosuppressive treatment. It is suggested that early-onset MG without AChR- and MuSK-antibodies should have thymectomy as recommended for MG with AChR-antibodies, especially if they have an enlarged thymus on imaging [175].

1.6.2 Anti-MuSK antibodies

In Europe, anti-MuSK MG patients are most frequently found between the latitudes 30° and 50° N [204]. Up to 64% of non-AChR MG patients in Italy have a detectable concentration of MuSK-antibodies [172]. In contrast, there have been few reports of anti-MuSK MG patients in northern countries such as Norway (unpublished). An association between MuSK-antibody concentration and disease severity has been found according to a study from Italy [14].

MuSK is a NMJ protein co-localised with AChR at the postsynaptic membrane. The protein is important in the maintenance of the normal functionality of the NMJ by mediating clustering of AChRs [120]. MuSK-antibodies are not detected in AChR-antibody positive patients [63]. While complement activation plays a major role in anti-AChR MG, this is not the case in anti-MuSK MG. These antibodies are of IgG-4 type which does not activate complement. In anti-MuSK MG, a correlation between titer of IgG4 subclass and disease severity was found [143]. It is suggested that the myasthenogenic effect of these antibodies is through a decrease in postsynaptic AChR density [102]. There has been reported low-affinity MuSK-antibodies detected by cell-based assays only [218]. The full understanding of the pathological mechanisms in anti-MuSK MG remains elusive.

Clinically, anti-MuSK MG differs from anti-AChR MG by predominantly cranial- and bulbar involvement and a higher risk of respiratory crisis. Maximum disease severity is usually seen a short time after disease onset [163]. The response to ACh-Is is poorer than in anti-AChR MG. The effect of immunosuppressive drugs is usually favourable [170]. Rituximab has been recommended as an early therapeutic option if no response to corticosteroids [106]. In general, no thymic pathology is observed in this MG group [47, 105], and therefore no expected effect of thymectomy.

1.6.3 Anti-LRP4 antibodies

LRP4-antibodies were initially found in 8% of the patients with no antibodies to MuSK or AChR. One large study screened approximately 800 MG sera from 10 countries, and identified LRP4-antibody positive patients among those negative for AChR- and MuSK antibodies varying between 7% (Norway) to 32.7% (Poland) [219]. LRP4 antibodies were surprisingly also found in some sera positive for AChR-antibodies or MuSK-antibodies in the same study. LRP4 is a NMJ transmembrane

protein localised at the postsynaptic membrane where it binds to agrin-receptor in skeletal muscle [215]. LRP4 interacts with MuSK and plays a role both in the clustering AChR and in other synaptic tasks to maintain the formation of the membrane [216]. The majority of anti-LRP4 MG patients have a mild ocular or generalised MG, milder than most patients positive to AChR- or MuSK-antibodies. Bulbar symptoms are more frequent at onset than limb or axial muscle-affection [18, 219]. The role of thymus in this MG subgroup is still unknown.

1.6.4 Ryanodine receptor (RyR)-antibodies

RyR-antibodies in MG were first identified by Mygland et. al in 1992 [128]. The receptor is a Ca^{2+} -release channel located intracellularly in the sarcoplasmic reticulum. RyR-antibodies have an essential role in excitation-contraction (EC)-coupling in striated muscle. A pathogenic role for striational antibodies has not been proven in vivo. One recent study found that an impaired EC-coupling contributed to muscle weakness in patients with MG [82], but another study by the same authors could not reproduce this finding [81]. RyR-antibodies serve as a marker for a more severe and prolonged disease, and indicate the presence of a thymoma [154].

1.6.5 Titin-antibodies

Titin-antibodies were first discovered by Aarli et. al in 1990 [3]. Titin is a giant muscle protein, and one of the largest components of the skeletal and cardiac sarcomere [160]. Titin-antibodies are associated with late-onset MG patients and thymoma. In late-onset MG, titin-antibodies are detected in 30-50% of the patients, and most frequently found in patients older than 60 years of age [157, 187]. These antibodies are very uncommon

in early-onset MG, and absent or borderline in patients without AChR- or MuSK-antibodies and in healthy controls [23]. 95% of patients with thymoma-MG have titin-antibodies [159]. Testing for RyR- and titin-antibodies combined gives a 95% sensitivity and specificity, and a 70% positive predictive value for a thymoma in MG [159]. Presence of titin-antibodies correlates with MG severity [176]. RyR- and titin antibodies can be detected by enzyme-linked immunosorbent assay (ELISA) or immunoblot.

1.6.6 Voltage-gated potassium channel (VGKC) Kv1.4 antibodies

Kv 1.4 is a transmembrane protein, located mainly in the brain, peripheral nerves and skeletal- and heart muscles. Kv1.4 antibody is a marker for the potential development of severe autoimmune myocarditis and response to calcineurin inhibitors (see "Myasthenia gravis and the heart"-section [185, 188]. Kv1.4 antibodies can be detected by an immunoprecipitation assay using 35S-labeled rhabdomyosarcoma cellular extract as the antigen source [185].

1.7 MG and epidemiological research

MG is a rare disease, in Europe defined as “not more than 5 in 10 000 persons”[32] . Epidemiological research seeks to: “1) describe health status by measuring disease frequency, distribution and trends; 2) know who becomes ill, what are the specific characteristics of cases, where and when these cases occur; 3) explain disease etiology; and 4) control the spread of specific diseases and their effects, by setting up preventive measures, improving prognosis and/or quality of life, and reducing mortality and other devastating complications” [36]. In my research, all points have been applied: I have measured disease frequency by calculating prevalence and incidence of MG, I have studied the distribution of the disease and trends of incidence over decades, and thereby tried to contribute to explain its etiology. I have also studied specific characteristics of MG patients and especially studied whether AChR-antibody test can be used as a biomarker to support the clinical assessments.

Two concepts are essential in epidemiological research as well as in my research; prevalence and incidence:

Prevalence: Prevalence can be defined as “the probability that an individual in a population will be a case at time t” [73]. A more practical definition of prevalence, the proportion of the population that has a disease at a set time point, point prevalence, has been applied in my research. Prevalence is a crucial estimator to gain an understanding of how many patients live with MG at a given time, and enables comparing frequency of a disease among regions or countries. To know the prevalence of a certain disease is also useful for long-range health care planning, organising health services for the disease, and for how to distribute research- and clinical competence of the disease.

Incidence: Incidence can be expressed as a rate providing a measure of the occurrence of new disease cases per person-time unit [73]. Another incidence measure, which was implied in my research, is the incidence proportion, which gives the proportion of the population that develops the disease during a specific period of time. Incidence per year is the most common way to refer to time when studying MG either as a rate or as a proportion. A reason for that is that life-expectancy in MG is now approximately the same as in the general population in Norway [146]. Thus the denominator, which for the incidence rate is the number of persons under surveillance multiplied by one (1 year), equals the general population resulting in the incidence rate and incidence proportion being two concepts measuring the same number of new incident cases.

Prevalence-incidence ratio is interesting to study over time because it can reveal a change in the disease dynamics: if incidence is high relative to prevalence of a disease, it tells us that this disease has a low survival rate or the patients are cured. If incidence is low compared to the prevalence, it tells us that the survival rate is high, or it can be a result of improved detection of the disease. For MG, the latter is the case, having a high prevalence and a low incidence. The prevalence-incidence ratio has increased [25] over the last decades, suggesting an improving MG treatment and survival with subsequently increasing life-expectancy. The other explanation is the improved detection; it is likely that case-ascertainment has improved in the recent epidemiologic studies conducted in Norway. Moreover, there have been demographic changes in most western countries with an ageing population and lower birth rates.

It requires robust methods and high-quality data to make accurate epidemiological estimates of MG-incidence. Accurate estimates of MG-incidence allow studying the actual occurrence of MG, capturing the dynamic of the disease over time and also the geographical distribution of MG. Accurate estimates are necessary to compare incidences of MG between populations and thereby provide clues to the etiology of the disease.

An important way of creating robust methods and good-quality data is to develop large population-based registries. In Norway, several national health registries have been established. National health registries provide an opportunity to conduct epidemiological research on a complete, nationwide population. The Norwegian Prescription Database is one example of a national health register from which we have obtained data in paper III. The AChR-database is not a registered national health register. It is not obliged by law to take an AChR-antibody test for all suspected MG cases, as it is for the pharmacies to register all prescription medications dispensed. However, we believe that AChR-antibody status is investigated in every suspected new MG-case in Norway. Thus, the AChR-antibody database should serve as a nationwide and specific proxy to identify new cases of MG.

The health care registries are regulated by the Personal Health Data Filing System Act (2002). The importance of an individual´s right to privacy is emphasised, and access to the registries presupposes an approval from the Regional Ethics Committee.

Clinical epidemiology is a term derived from two parent disciplines: clinical medicine and epidemiology. It is clinical as it endeavors to answer clinical questions and support clinical and therapeutic decisions. The methodology to answer the clinical questions is epidemiologic. The purpose of clinical epidemiology is to develop and apply sound methodology to validate conclusions based on clinical observations and thereby avoiding systematic errors. It is an important approach to guide doctors in making the best available decisions for the patients [50].

1.7.1 Myasthenia gravis Foundation of America Clinical Classification

Myasthenia gravis Foundation of America (MGFA) Clinical Classification was designed to classify patients with MG with similar clinical features and severity of the disease, enabling comparative analysis of the various therapeutic interventions for MG. It may be hard to classify patients with MG because of the fluctuations and the variable predominance of affected muscle groups, thus even more important to develop universally accepted standards to grade patients and methods to evaluate patients undergoing MG-treatment. The Medical Scientific Advisory Board of MGFA thus formed a Task Force in May 1997 to address these issues [86]. Existing classifications at that time was mostly Osserman´s classification from 1958 or modifications of it [134].

MGFA Clinical Classification

Class I	Any ocular muscle weakness; may have weakness of eye closure. All other muscle strength is normal.
Class II	Mild weakness affecting muscles other than ocular muscles; may also have ocular muscle weakness of any severity.
	IIa. Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal muscles.
	IIb. Predominantly affecting oropharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both.
Class III	Moderate weakness affecting muscles other than ocular muscles. May also have ocular muscle weakness of any severity.
	IIa. Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal muscles.
	IIb. Predominantly affecting oropharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both.
Class IV	Severe weakness affecting muscles other than ocular muscles. May also have ocular muscle weakness of any severity.
	IIa. Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal muscles.
	IIb. Predominantly affecting oropharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both.
Class V	Defined as intubation, with or without mechanical ventilation, except when employed during routine postoperative management. The use of feeding tube without intubation places the patient in class IVb.

It is an inherent imprecision of classifying MG as with mild, moderate or severe weakness, as is done in the MGFA classification. What will be defined as mild by one doctor, can be defined as moderate by another. The grading will also depend on how the patient formulates his or her symptoms. The MGFA classification represents a subjective assessment with a lack of quantification. A standard classification is, however, needed to achieve a meaningful comparison of data. The Task Force recommends that the most severely affected muscles should define the score.

A quantitative MG-scoring system (QMG-score) was developed by the same Task Force to more objectively evaluate therapeutic interventions of MG. This score is

recommended used in conjunction with the Clinical Classification and the Post intervention status, and should not primarily be used to compare severity between patients. QMG should be used in all prospective studies of MG therapy. This scoring system was not applicable for our study as it was retrospective and the scores being based on free text medical notes. To reduce parts of the imprecision in the MGFA Clinical Classification, we validated the score by two independent observers, as explained in the statistical methods-section.

2. Aims of study

i. To determine the prevalence and incidence of anti-AChR MG in Norway.
ii. To study anti-AChR MG incidence in the different geographical regions in Norway, and thereby identify factors that may contribute to the development of MG.
iii. To compare incidence and prevalence data from the AChR database and the national prescription database to evaluate the two nationwide registries and find correct estimates.
iv. To examine the association between MG clinical severity and concentration of AChR-antibodies in individual patients over time to evaluate if repeated AChR-antibody measurements have a value for therapeutic decisions.

3. Materials and methods

3.1 AChR database

In 1974, AChR-antibodies were discovered to be present in the majority of MG patients [5, 6]. Subsequently, an assay to quantify the AChR-antibody concentration was developed [108]. The method was based on extracts containing human AChRs prepared from amputated legs. The receptors were labeled with ^{125}I-α-bungarotoxin. Concentration of AChR was estimated by measuring ^{125}I-α-bungarotoxin binding to AChRs. The toxin-receptor complex was then precipitated with a possible myasthenic serum. Different preparations of AChR from human muscle could vary in the capacity to bind both the ^{125}I-α-bungarotoxin and the receptor antibody. To adjust for this, the concentration of AChR-antibodies was measured in arbitrary units/L. The patient sample was compared with a healthy serum, and values above a 95% confidence limit, was considered as abnormal. A standard positive patient serum with a known AChR-antibody concentration was also added. By extrapolating from this information, all new positive sera got their AChR-antibody concentration. This method was used at the laboratory of Haukeland University Hospital from 1983 to 1994 as the only laboratory in Norway offering this analysis. From 1994, the laboratory incorporated a commercial kit (IBL-Hamburg GmbH, Germany) for the method to analyse concentration of AChR-antibodies. Using a radio-receptor assay for the in-vitro-diagnostic semi-quantitative determination of AChR-antibodies in serum ensured high sensitivity and reproducibility. In this method, ACh from human muscle is used as antigen. The receptors are prelabelled with ^{125}I-α-bungarotoxin. This snake venom binds to the receptors specifically and almost irreversibly. Autoantibodies present in the patient´s serum attach to the labelled receptors. The resulting immune complexes are precipitated with anti-human IgG. The amount of radioactivity of the sediment is proportional to the AChR-antibody concentration in the sample. AChR-antibody concentration is measured in nmol/L with a cut- off value set to 0.5 nmol/L, in line with literature recommandations [147, 179] and “instruction for use” from the

producer. Four times a year, the laboratory participates in a control trial assessment to ensure the analytic quality. These assessments have confirmed a high analytical quality, further explained in the "internal validity"-section, page 64.

Sensitivity and specificity of the AChR-antibody test is of clinical importance. The AChR-antibody test measured by RIA technique is nearly 100 % specific as a diagnostic test for MG [49]. Even though the antibodies are very specific for MG, they may very rarely be detected in patients with celiac disease [21], systemic lupus erythematosus [21], rheumatoid arthritis on penicillamine [124], in allogeneic bone marrow transplantation patients who develop graft-versus-host disease [103], in patients with thymoma without MG, and in neuromyelitis optica [207]. The sensitivity of the RIA is difficult to quantify because it depends on the gold-standard used. The producer of the test claims a 100% sensitivity using a predicat RIA method as a comparison. This is highly questionable, and the producer has certainly not taken into account the presence of low-affinity AChR-antibodies.

From 1983, name of patient, date of birth, date of sample- acquisition and name of referring doctor/hospital have been registered at the Haukeland University Hospital laboratory. All samples from the whole of Norway were sent to this laboratory. In the first years, the information was handwritten in books, resulting in some information being missed. From 1995 and onwards, all information was computerised and regarded as complete. From 1983 to 2008, approximately 12 000 samples were analysed with an annual increase from 47 in 1983 to more than 1000 tests in 2008. From 2008, a laboratory at Oslo University Hospital has offered analysing AChR-antibody concentration.

The three first papers in the present thesis were based on the AChR-antibody tests for the period 1983-2008. This was the fundament for a nationwide calculation on prevalence, incidence and geographical distribution of anti-AChR MG. In the fourth paper, tests from the period 2008-2013 were also included.

3.2 Norwegian Prescription database

All pharmacies in Norway register all prescription medication dispensed. They have been obliged by law to do so since 2004. The Norwegian prescription database (NorPD) contains thus information on drug consumption for the entire Norwegian population. Individual patients are anonymous, but can be identified by a unique person identifier. For MG and other chronic conditions with a confirmed diagnosis, medication is reimbursed, and the reimbursement code for MG served as a proxy for the MG diagnosis in paper III.

The inclusion criteria for the study population drawn from the NorPD were as follows: Minimum two prescriptions of pyridostigmine during the study period (January 1st, 2004 - December 31st, 2007), a prescription from a specialist in neurology, or prescription for MG as specified in NorPD. Patient's age, gender and county of residence were identified for each prescription. Drug information included the Anatomical Therapeutic Chemical code (N07AA02) and date of expedition.

3.3 Population and health regions of Norway

On January 1st, 1995, Norway had a population of 4,348,410, increasing to 4,737,171 on January 1st, 2008. In the same period the number of first or second generation of immigrants roughly doubled from approximately 200,000 (4.6%) to 460,000 (9.7%) (Statistics Norway - SSB, www.ssb.no). Norway has been a rather homogenous population, the one exception being the Sami population in the northern region. All prevalence and incidence calculations for the first two papers were based on the entire Norwegian population.

In the period 1995-2007, the Norwegian Healthcare System was divided into five geographical health regions: east-, south-, west, mid- and north region. The

geographical distribution of anti-AChR MG examined in paper II, was based on the differences in incidence of anti-AChR MG between these five health regions. The number of inhabitants in the different health regions varied from 470 000 (north) to 1 600 000 (south). Geographically, the northern region constitutes the largest area in Norway.

3.4 Western Norway

Paper IV was based on the population in western Norway. Western Norway constitutes the counties of Sogn and Fjordane, Hordaland, and Rogaland, comprising approximately 1 000 000 people in 2011 (Statistics Norway - SSB, www.ssb.no). In this study we used the AChR database to identify all patients from western Norway with two or more AChR-antibody tests taken. Then we compared the AChR-antibody concentrations with the MGFA scores to evaluate if there was an association between the antibody concentration and clinical severity derived from the score.

3.5 Study populations in the four papers

Paper I: The study population was obtained from 1983 to 2008 and comprised 11 926 assays registered in the AChR database. The data obtained was used to calculate the prevalence. Incidence calculations were based on assays from 1995 to 2008 comprising 8 742 samples. The Norwegian National Registry was used to determine whether the patients were alive at the prevalence day.

Paper II: The study population comprised 419 individuals with first time positive AChR-antibody tests from 1995 to 2007 obtained from the AChR database. The total population, counted by gender and age in each health region, was recorded per year

from 1995 to 2007 to calculate annual incidence in each of the five geographical regions.

Paper III: For the AChR database, the population was identical to the population in paper I and II. For NorPD, prevalence calculation was based on the number of patients who had received prescriptions of pyridostigmine according to the inclusion criteria and were residents in Norway on prevalence day. 677 (94%) out of the 723 with prescription of pyridostigmine met the criteria for being included in the prescription database. The selected prevalence day was January 1st 2008, the same as the prevalence day in paper I. Incidence of MG was defined as new users of pyridostigmine, with no prescription of pyridostigmine dispensed during the years of the study period preceding 2007.

Paper IV: 185 patients from western Norway had two or more AChR-antibody tests taken. Eighty-five of these were deceased, and 98 patients were alive at study start (November 1st 2012). Two patients were impossible to trace. The patients alive were sent written information about the study and a request to participate. We received signed consent from 82 patients (positive response rate 84%). The final inclusion comprised the deceased patients and those with a signed consent form. 24 of the deceased and 43 of those alive had two or more AChR-antibody tests combined with a medical note including a possible MGFA score.

3.6 Statistical methods

Paper I: Incidence and prevalence were calculated per 1 million inhabitants per January 1st each year 1995–2008. 95% CIs were calculated assuming the Poisson distribution. Statistical software SPSS (15.0) and StatXact 7 were used for statistical analysis.

Paper II: The odds ratio with 95% CI was estimated to quantify relative risk for a positive AChR-antibody test in the patients over 50 years of age relative to patients younger than 50 years of age. To assess variation in incidence, Multiple Poisson regression analysis was used. This was done using the GENLIN procedure of SPSS with a logarithmic link-function and population size as an offset.

Paper III: MG patients receive symptomatic treatment with pyridostigmine regardless of AChR-antibody. In Caucasian populations, 85% of the MG patients are regarded to be AChR-antibody positive [109, 206]. Thus a 15% stipulated share of AChR-antibody negative patients was included in the calculations from the AChR database to compare the two databases.

Age and sex specific incidence rates among MG patients were determined by calculating the standardised incidence ratios (SIRs) of observed number of patients identified in NorPD compared to the number of MG patients calculated from the numbers in the AChR database. 95% CIs were calculated assuming a Poisson's distribution. All statistical analyses were performed in Microsoft Excel (2010).

Paper IV: Two observers independently classified the first medical note of 33 randomly picked included patients to validate the MGFA score. Inter-examiner agreement achieved was Kappa (K) 0.84 (almost perfect agreement). Time since first test was analysed both as a continuous variable and by dividing tests into four quartiles for each patient to adjust for the AChR-antibody measurements having been performed at irregular time intervals. The 1st quartile comprised 0.24 years (0-3 months), the 2nd quartile comprised 0.24-1.85 years (3-19 months), the 3rd quartile comprised 1.85-4.93 years (19-37 months), and the 4th quartile comprised the rest of the time period (37 months-21 years). Correcting for repeated measures in the same individuals, multiple ordinal logistic regression using generalised estimating equations was used to estimate a possible predictive effect of AChR-antibody concentration and time since onset on MGFA score. We also investigated if the effect of AChR-antibody concentration on MGFA score depended on longer time since onset (test of time × AChR-antibody concentration interaction) using the Generalised Linear Model option

in SPSS 21. Results were reported as odds ratios (OR) and 95% CI. SPSS 21 was also used for all other statistical analyses.

4. Summary of results

4.1 Seropositive myasthenia gravis: a nationwide epidemiologic study (Paper I)

In this study, comprising data on AChR-antibody tests taken 1983-2008, we investigated incidence and prevalence of AChR-antibody positive MG patients for a nationwide population spanning over decades. The number of assays taken increased from 47 in 1983 to 1007 in 2007. The number of individual patients tested was 8628, 5521 females and 3100 males (female:male ratio 1.8). We identified 898 AChR-antibody positive MG patients: 543 females and 350 males (female:male ratio 1.6). Of these patients, 308 (34.2%) had their first-time positive AChR-antibody test before age 50 years and 525 (58.4%) at age 50 years or later. For 65 (7.2%) patients, this age was unknown. 420 new AChR-antibody positive MG patients were identified 1995-2008. The total crude yearly incidence for 1995–2007 was 7.2 per million (95% CI: 6.5, 7.9). The prevalence of AChR-antibody positive MG was 115.5 per million (95% CI: 106.1, 125.7). On January 1st, 2008, 547 of the 898 AChR-antibody positive MG patients were alive, 274 were dead, and the status of 77 patients was unknown. Presuming the same survival ratio among these 77 patients as in the rest of our MG population (66.6%), the prevalence of AChR-antibody positive MG would be 126.2 per million.

4.2 Geographical distribution of a seropositive myasthenia gravis population (Paper II)

In this study, we examined the geographical distribution of MG in Norway. Norway was divided according to the five previous health regions, and all calculations were based on this. 419 first-time AChR-antibody positive patients were identified (231 females, 188 males), numbers varying between 80 and 212 in the five geographical

regions. Unadjusted average incidence per million inhabitants per year ranged from 5.96 (north) to 9.05 (mid-Norway). Average annual incidence for all five regions combined was 7.04, ranging from <1 to 14 per million in the various regions.

The main finding in this study was that the average incidence per million inhabitants did not vary significantly between the five regions ($P = 0.091$). There was no significant linear change in annual MG incidence in the period from 1995 to 2007 ($P = 0.308$) adjusted for gender, region and age.

Overall, it was a slightly higher probability to develop MG for females than for males (female: male ratio 1.32). In contrast, the odds of being late-onset was 2.25 times higher for a male than for a female (95% CI: 1.45, 3.48), and this did not vary between the regions.

138 first-time AChR-antibody positive patients were 50 years or younger, while 278 were over 50 years of age. Mean age at first positive AChR-antibody test in the early-onset group varied from 30.2 years (north) to 36.1 years (west) with an average of 32.5 years in all regions combined. In the late-onset group, mean age at first positive AChR-antibody test varied from 69.0 years (south) to 71.3 years (west) with an average of 70.2 years.

4.3 Myasthenia gravis epidemiology in a national cohort; combining multiple disease registries (Paper III)

In this study, we compared prevalence and incidence calculated from the NorPD and the AChR database. While the AChR-antibody test provided an opportunity to monitor all tests taken in the study period and thereby identify all incident and prevalent cases of MG, NorPD provided the same opportunity by monitoring individual drug use of pyridostigmine.

Both a positive AChR-antibody test and the use of pyridostigmine are regarded as specific markers for MG. Prevalence January 1st 2008 was 131 per million by using the NorPD database, and 145 per million by using the AChR database (an estimated 15% share of AChR-antibody negative patients was added).

Analysing prevalence data, we found no significant difference between the NorPD and AChR databases (SIR 1.1; 95% CI 1.0, 1.2). Moreover, there was no difference in the number of females in the two cohorts. However, more men were found in the NorPD. The number of early-onset MG patients did not differ between the two cohorts, while in the late-onset groups more male patients were identified through NorPD compared to the AChR database.

The incidence rate based on NorPD data was 16.0 per million in 2007, compared to 8.8 per million per year in the AChR database, calculated as mean annual rates. This means a doubled incidence for the NorPD group compared to the AChR group (SIR 1.8; 95% CI 1.4, 2.3). Nearly a threefold more female MG patients was found in the NorPD compared to the patients from the AChR-antibody database, while no differences in the number of male MG patients. Analysing the incidence rates according to early- and late-onset MG subgroups, the number of patients was doubled for the early-onset group in the NorPD compared to the AChR database. For females, twice as many were identified through the NorPD, while no differences for males between the NorPD and AChR database. There was an approximately doubling in the MG incidence rate for the late-onset group in the NorPD compared to AChR database. A threefold more females were identified through NorPD compared to AChR database, while the number of new male MG patients did not differ significantly in the two cohorts.

4.4 Acetylcholine receptor antibody concentration and association to myasthenia gravis development (Paper IV)

This retrospective clinical study was based on patients from western Norway with two or more AChR-antibody tests and with corresponding medical notes. The study population was divided into those treated with immunosuppressive drugs (immunosuppressed MG group), and those treated with pyridostigmine only (pyridostigmine MG group). In total, 67 patients were included with 309 measurements of AChR-antibodies. The immunosuppressed MG group constituted 56 of the 67 patients, with 272 AChR-antibody measurements with a corresponding MGFA score. The pyridostigmine MG group constituted 11 patients with 37 AChR-antibody measurements with a corresponding MGFA score.

The concentration of AChR-antibodies was associated with the longitudinal development of clinical severity in individual patients treated with immunosuppressive drugs. This showed an effect of AChR-antibody concentration on the MGFA score for the whole study period in this group, though not significant for the last quartile (37 months to 21 years). In the pyridostigmine group, this association was weakened considerably, and no longer significant. We found a diminished effect of AChR-antibody concentration on MGFA score after the first three months. In the last quartile, the effect seemed to be the opposite of what was expected. The results of this study indicate that AChR-antibody measurements may be a valuable tool in the follow-up for the MG group treated with immunosuppressive drugs, and that an increase or decrease should influence therapeutic decisions.

5. General Discussion

We found the annual incidence of AChR-antibody positive MG patients in Norway 1995-2008 to be 7.2 per million. The point prevalence was 126.2 per million January 1st 2008. In 1984, the total MG incidence and prevalence in Norway was reported as 4.0 and 90 per million per year respectively [184]. Our study demonstrates an increase in both incidence and prevalence in Norway compared with the study from 1984.

The increasing incidence can be explained by several factors. First, case-finding has probably improved due to the advent of the AChR-antibody assay. Worldwide, the MG incidence doubled after 1976, when the first version of the modern AChR-antibody assay was described and introduced [25]. Our data were obtained from a national database, with a positive AChR-antibody test as a proxy measurement for anti-AChR MG. The epidemiological data from 1984 was based on data sent from neurological departments and practising neurologists in Norway. Furthermore, the healthcare system and neurological services have improved greatly from the study period of the 1984 study (inclusion period 1912-1981) and to our study (inclusion period 1983-2013). This has probably led to a lower threshold for neurological examinations and a subsequent increased case ascertainment.

There is evidence for MG having been underdiagnosed in the elderly in particular. The clinical signs in MG are more difficult to assess correctly in this group, as explained in the "Clinical features"- section, page 14 [2]. More patients are diagnosed with MG today than previously because of enhanced awareness of MG in the elderly [2, 205].

With an ageing population, an increase in new MG cases is expected as this group are at higher risk of acquiring MG. In our studies, twice as many patients had their first-time positive AChR-antibody test after 50 years of age. In the Norwegian study from 1984, calculations based on incidence rates demonstrated a higher rate for those with onset before 50 years versus those with onset after 50 years of age (2.9 per million versus 1.1 per million) [184]. The higher incidence among the elderly in recent years clearly shown in two epidemiological studies from Italy conducted within the same

region, with a similar study design and with a twenty years interval between the studies. The increase in incidence was mainly because of an increase of male patients with MG onset after age 50 years [137]. Therefore, enhanced awareness about late-onset MG and increased longevity in the general population with more MG patients diagnosed in the elderly are causes of the increased MG incidence [71, 72, 137, 140, 141, 205].

It is a striking observation that in several studies the MG incidence peaks in the 70-80 year old, and then fall off abruptly [9, 72, 147, 205]. This can partly be explained by underdiagnosing in the very elderly. In addition, Vincent (2003) discusses two other explanations: The peak can be a cohort effect, where people represented in the peak has been exposed to a particular environmental exposure, increasing their risk for developing MG. However, this explanation requires a very short time of duration of susceptibility of the exposure and a very long fixed duration from exposure to MG development. This explanation fits poorly as the short time of rise and fall for the incidence peak happens in several populations. It could also be that the steep fall in the incidence represents a competing risk for those patients at risk for developing MG. It could be that these patients are at higher risk than the general population for developing some other fatal disease. This is unlikely as it has to be very strongly associated with the risk of developing MG and a risk strictly limited to the very elderly [205].

It could also be that there has been a genuine general increase in incident cases of MG from 1984 to 2008 caused by unknown environmental triggers. However, there is no evidence for an increase in MG incidence in Norway1995-2008 based on the AChR-antibody database [71].

The increase in MG incidence demonstrated in other studies are mainly caused by an increase in the proportion of patients with onset after 50 years of age [25]. The

immunological differences between early- and late-onset MG, suggest that these subgroups are different disease entities. The majority of early-onset anti-AChR MG patients have hyperplastic thymus with lymphocytic infiltrates and germinal centres similar to those found in lymph nodes. The thymuses contain all components necessary for a development of an immune response, supporting an intrathymic pathogenesis involved in the immune response in early-onset MG [95]. Late-onset MG usually lacks the thymic abnormalities, and therefore, the mechanisms of autosensitisation to AChRs in this subgroup are unclear. Their HLA-profile differs, and titres of circulating AChR-antibodies are lower in late-onset MG than in early-onset MG. Antibodies to striated muscles are more often found in late-onset MG, while autoimmune comorbidity is more uncommon in this subgroup [120]. These aspects may explain why a genuine increase in late-onset MG may occur even if this is not true for early-onset MG.

The MG incidence in our study was in the lower range compared to what was found in 8 other studies on anti-AChR MG from other populations. The range in these studies spanned from 4.3 to 18.0 per million per year [25]. The observed heterogeneity may be explained by either biological and environmental factors, that incidence actually vary in different populations, or that the variation is due to methodological problems and study quality. There is a trend towards decreasing heterogeneity with increasing study quality [25]. A genuine geographical difference in anti-AChR MG epidemiology remains to conclude on. One exception is childhood onset MG in Asian populations appearing to be much higher compared to Caucasian populations [217].

There was a marked variety in the prevalence found in previous studies, ranging from 70 per million to 163 per million. There is linear trend of increasing prevalence with year of study [25]. The increased prevalence is for a large part related to longer life spans due to improved MG treatment [42]. An increased prevalence- incidence ratio has been demonstrated over time, reflecting the reduced MG mortality.

Our study was based on a nationwide population, a time period spanning over decades, very specific inclusion criteria, and the belief that we included nearly all cases of AChR-antibody positive MG patients in the study population through the study period. Accurate epidemiological analysis of low frequency diseases such as MG requires a large population to identify sufficient numbers of affected people to disclose patterns of the disease. The majority of published studies are limited in geographical scope, typically yielding small samples where estimates of incidence and prevalence cannot always be generalised beyond the study population. We have a sufficiently large population to draw realistic conclusions about the incidence and prevalence of anti-AChR MG in Norway. Selection bias is a concern in most studies, but the risk of this type of bias should be low in our population.

In paper III we evaluated the AChR database as a tool for epidemiological studies by comparing the results with those obtained through the NorPD database. We found a good concordance for the prevalences in the two databases, but the incidence calculated from the NorPD database was much higher. However, the incidence from NorPD was calculated only for 2007, while the incidences for data obtained from the AChR database were calculated annually from 1995 to 2007. The higher MG incidence found in NorPD can be explained by the inclusion of relapsing cases of MG because of a short inclusion period for this registry. If so, we will expect to find a lower incidence in future studies in NorPD. It is also unknown to what degree pyridostigmine have been prescribed as ex juvantibus treatment or how frequent prescribing practice without proper reimbursement coding occurs. There is a possibility that pyridostigmine prescriptions for other purposes than reducing MG symptoms in some cases have been included. An effect of pyridostigmine for diagnoses, such as gastrointestinal dysmotility in idiopathic pseudo-obstruction, has been inconclusive [182].

To be able to compare the two databases, a stipulated 15% share of MG patients without AChR-antibodies was added to the population from the AChR database. Even though there is a consensus about the anti-AChR MG constituting 85% of the total MG population, we have to consider whether this assumption is correct. Most studies performed to calculate this 85% proportion were conducted in the early 1980s. This proportion may have changed during the decades. There is also a possibility that not every diagnosed MG case actually has taken the AChR-antibody test, especially for the first years after the test was introduced in Norway in 1983. This would lower the incidence based on the AChR database. Norway has a public welfare system which is mainly free of charge for the patient, and the AChR-antibody test is of no extra cost. The test is well-known for its very high specificity, and regarded as a first diagnostic step to confirm a suspected MG case.

The incidence for anti-AChR MG did not differ significantly between the five geographical regions of Norway (paper II). However, it tended to be lower in the northern region. We hypothesised that one reason for this tendency could be the existence of the Sami population in the north. It is known that multiple sclerosis (MS) occurs less frequently in this population, perhaps as a result of a different HLA profile [65, 66]. However, there is no official Sami registry, which hampers an estimation of the actual Sami population. We have not been able to disclose any epidemiological differences between the Norwegian and Sami population in our study.

Another explanation for the trend towards a lower incidence in northern Norway could be the lower sun exposure and subsequently lower concentrations of vitamin D in this population. Vitamin D deficiency has been associated with several autoimmune diseases [15]. On the other hand, it is known that the intake of vitamin D through the diet is higher in northern Norway compared to the southern regions, compensating for the lower sun exposure [22]. There is a high prevalence of vitamin D deficiency in the adult population of Norway in general, and it is associated with season, body mass index and lifestyle. Body mass index has increased markedly in the last decades in

Norway [11]. An increase in body mass index, and a changed lifestyle, with lower intake of cod fish liver, could affect the prevalence of vitamin D deficiency, and should be a topic for future studies. However, there is no evidence to support a higher frequency of vitamin D deficiency in northern Norway compared to southern parts [89, 101]. For MS, it has been found an effect of month and season of birth for disease risk. In the northern hemisphere, patients born in the spring have a higher risk for developing MS than those born in the autumn. The suggested reason for this is the lower concentration of vitamin D in the winter season, thus being an environmental factor in the intrauterine period affecting the MS susceptibility [197]. This effect seems to be more prominent in high-risk areas for MS, especially in areas where exposure to sun is low. Recently, it was revealed that after controlling for vitamin D concentration, decreased sunlight exposure still remained significantly associated with MS. This implicated that vitamin D is not the only mediator of the beneficial influence of sunlight on MS [68, 114]. Melatonin is another hormone with immunomodulatory properties, and it is light-dependent. This hormone may be a potential mediator of the neuro-immunomodulatory effects of vitamin D in patients with MS [58]. This could explain why there is a lower incidence of MS in the north even though the vitamin D levels do not differ from levels in the southern part of Norway. The month of birth-effect has not been investigated for MG, and should be a topic for a future study.

A distinct geographical distributional pattern for MS-incidence worldwide has been defined, with a significantly higher incidence with increasing latitude [173]. So far, it has not been possible to identify any differences in epidemiology worldwide explained by environmental factors for anti-AChR MG, as discussed previously in the general discussion. In Norway, we did not identify any differences in anti-AChR MG incidence in the different regions. Numerous epidemiological studies on MS in Norway have been conducted, spanning over decades. These studies have revealed a lower incidence in the northern region, and persistently the highest MS incidence in the inland in southern Norway [121]. For MS, genes associated with the immune system play a major role in the pathogenesis [83]. But as for MG, environmental

factors are of significance. Smoking is thought to elevate the risk of MS, while factors as sun exposure and food high in vitamin D are thought to decrease the risk. Less smoking and people spending more time in sunny parts of the world during the winter, thus increasing the levels of vitamin D, should decrease the frequency of MS. It is still not known how a change in diet will influence this risk [121]. However, MS still tends to increase in frequency. This illustrates how complex the interaction between genes and environmental factors is on the individual level. Both MG and MS are immunoactive disorders with autoimmunity, but most etiological properties are thought to differ. Our study indicates that geographically-related factors are among those that differ.

For the anti-MuSK MG subgroup, which was not examined in our study, prevalence clearly differs among populations. In Norway, there are five known cases of anti-MuSK MG (unpublished), four of these being alive. This gives a prevalence of approximately 0.8 per million in Norway. The prevalence is also low in Poland [97] but high in Greece [201]. Why this MG subgroup shows so distinct differences in incidence and prevalence while the anti-AChR MG subgroup does not remains to be resolved.

In paper IV, we showed that patients with and without immunosuppressive treatment differed significantly according to AChR-antibody concentration and longitudinal association to clinical severity. While the group treated with immunosuppressive drugs had a significant association, no association was found in those patients treated with pyridostigmine only. This latter group was, however, small, constituting only 11 patients. This indicates that it will be valuable to monitor the patients treated with immunosuppressive drugs for AChR-antibody concentration. AChR-antibody concentration probably reflects the amount of drug response, and changes in this concentration may indicate further treatment regarding drug or drug dose. The effect immunosuppressive drugs has on the AChR-concentration can be due to a reduction of B-cell synthesis and for activation, acting directly, or indirectly through inhibiting T-cell mechanisms. We did not monitor the effect of individual immunosuppressive

drugs. It could well be that the effects on AChR-antibody concentration vary for the different drugs. However, we adjusted for thymectomy in the analysis, and this did not change the results. This means that the effect of immunosuppressive drugs on the AChR-concentration appears regardless of whether the patient is thymectomised or not.

Prior to this study, statistical power was calculated. We managed to achieve the minimum sample size required to detect an effect of AChR-concentration on clinical state. Nevertheless, it would have been feasible to imply more variables in the regression model if we had a larger sample size, including subgrouping according to the different immunosuppressive drugs. This could be obtained by a multicentre study. On the other hand, the results from a multicentre study could be biased by different treatment approaches and variation in documenting the clinical state. By having a single center study, fewer doctors were involved and the MGFA score from the clinical assessment in the medical notes more homogenous. Indications for treatment and treatment changes would also be similar. To monitor the disease with AChR-antibody tests does not seem to have any value for the patients treated with pyridostigmine only.

No consistent association between concentration of AChR-antibodies and MG disease severity has previously been found in. Most studies were performed in the early 1980s. Our study was retrospective. All clinical assessments were based on structured and validated medical notes. A prospective design would have included direct MGFA-scoring and regular intervals between the clinical assessments and the AChR-antibody tests taken. This was not possible in our study and represents a major weakness.

Based on the results from this study, it may be helpful to monitor MG patients on immunosuppressive drugs with repeated AChR-antibody tests, and especially if they do not respond clinically.

6. Methodological considerations

6.1 Study design

The studies were all retrospective cohort studies, and based on the AChR database. The database contains patient information (Patient name, date of birth, data of sample acquisition, and name of referring doctor/hospital) for all AChR-tests taken from the very beginning of performing the analysis of AChR-antibody concentration. For paper I, II and III there was no clinical information available, while paper IV was based on clinical information from the anti-AChR MG cohort of western Norway. The inclusion criteria in Paper I, II and III were entirely based on a positive AChR-antibody test. Because of the high specificity of AChR-antibody test, it is regarded as a first diagnostic step for MG. Therefore there is reason to believe that case ascertainment approximated 100% in the three first studies. In paper IV, we included all AChR-antibody tests up till 2013. In 2008, another Norwegian laboratory (in Oslo) started to offer AChR-antibody analysis. This means that some AChR positive MG cases may be missed in paper IV. However, in this study we only included patients treated at Haukeland University hospital. We believe that most AChR-antibody tests in this region were analysed at this regional hospital, not at a regional hospital in Oslo. In paper IV we did no epidemiological calculations.

For MG being a rare disease, it is always a challenge to have a sample size large enough enabling to disclose detailed epidemiological patterns. One solution to this problem would have been to establish a multinational study. However, we prioritised a homogenous national population and a study based on one database to increase reliability and validity. There were also practical reasons for choosing a single center study.

In all clinical studies, a researcher has two concerns: the internal and external validity of the study. The internal validity of epidemiological studies can be affected by

random and systematic errors. External validity describes to what extent of a study can be generalised to other populations than the one studied [161].

6.2 Internal validity

6.2.1 Random errors

Random error reflects a problem of precision in assessing a given exposure-disease relationship and can be reduced, among others, by increasing the sample size [199]. Random errors may also occur as a result of measurements not performed according to the procedures, misreading of AChR-antibody results, and incorrect coding of patient data and/or AChR-antibody result when typing it into the database. The database contains data of 15 000 tests, and with such a large study size, the effect of random errors will be reduced [161], yielding a more precise result of the study. The commercial RIA was introduced in 1994, and the analytical technique has been used since then at Haukeland University Hospital. The laboratory has built up extensive experience in using this particular test. Four times a year, the laboratory participates in an external quality assessment to promote best practice. The result from the quality assessment is measured as an overall misclassification score (OMIS), with three categories of performance: good, adequate and poor. The laboratory at Haukeland University Hospital has always performed in the best category since they joined the quality assessment program. Both the large population size and the high quality of the analyses performed at the laboratory reduce the effect of random error.

6.2.2 Systematic errors

Systematic errors or bias arise as a result from the research methods used by the investigator, from factors affecting the study participation (selection bias) or from

systematic distortions when collecting information about exposures and outcomes (information bias) [169].

6.2.2.1 Research methods

P-value: A p-value expresses "the probability that results at least as extreme as those obtained in an analysis of sample data are due to chance"[20]. When our estimates included use of a p-value in the analysis, we used the common standard that the p-value for our results had to be less than 0.05 (the significance level) to reject the null hypothesis. There is always a probability of error in this type of reasoning. The possibility that significant results can be due to chance, as for example sampling error, has to be taken into consideration, as for all statistical analysis. 0.05 is somehow an arbitrary choice of level; this has been some of the critics of p-value. A low p-value reduces the risk of rejecting a null hypothesis that is actually true (type 1 error). A p-value set to 0.1 reduces the risk of a type II error (the failure to reject a false null hypothesis), but then again increases the risk of a type I error, considered to be more serious.

Regression model: The concept of a regression model is to predict or explain a dependent variable (response variable) by using a set of independent variables (explanatory variables). In paper II and IV, regression models were used to study if the dependent variable was the function of one or more independent variables. In paper II, incidence was defined as the response variable, and gender, onset age and health region were defined as explanatory variables. Our null hypothesis was that anti-AChR MG incidence did not vary between the regions. The null hypothesis was not rejected, as we found no significant differences between the regions. In paper IV, MGFA score was defined as a response variable, and AChR-antibody concentration and time since onset defined as explanatory variables. Our null hypothesis for this study was that there was no association between MGFA score and AChR-antibody concentration.

The null hypothesis was rejected, as we found an association between the two variables for the group treated with immunosuppressive drugs.

As for most statistical procedures, linear regression makes certain assumptions about the data used in the analysis. The assumptions are data appropriateness, independence, linearity, distribution and homoscedasticity. In both paper II and paper IV these assumptions were met in an acceptable manner. If these assumptions are not met properly, it may reduce the validity of the studies [20].

The theory associated with linear regression is well understood, and allows rather complex statistics to be easily interpretable. However, even if there is an association between the predictor and outcome, that is no proof for an underlying causal mechanism. In paper IV, we found an association between AChR-antibody concentration and MGFA score, but the model says nothing about why there is an association. Regression models are sensitive for outliers because outliers pull the regression line towards itself. This can result in a solution more accurate for the outlier, but less accurate for all other cases in the data set. As discussed above, we did not have any extreme outliers in our data set.

A general thumb rule in multiple regression is that one should have at least 10 times as many observations as variables; otherwise the estimates of the regression line may be unstable and difficult to replicate [8]. In paper II, the number of anti-AChR MG cases was 419. With such a large number of cases, the estimates should be stable and reproducible. In paper IV, the number was 67 anti-AChR MG cases. In this model, we had 6 independent variables, near the limit for the general rule.

Poisson regression, the method applied in paper II, assumes that the observed counts are generated from a Poisson distribution. This model is often used to model count when the events being counted are somewhat rare. The probability of disease occurrence was low relative to total population size. In paper IV, multiple ordinal logistic regression was applied by using generalised estimating equations. We corrected for repeated measures in the same individuals, by assuming an exchangeable correlation structure. In this way, we estimated a possible predictive effect of AChR-antibody concentration and time since onset on MGFA score.

In paper III we compared MG incidence and prevalence using two different Norwegian nationwide databases by calculating the standardised ratios. The two databases used different markers to identify MG cases. A positive AChR-antibody test was used as a marker in the one database, while prescription of pyridostigmine was used as a marker in the other (NorPD). These databases have different sensitivity and specificity which complicates the comparison. The AChR database has a high specificity, but lacks the capacity to identify MG patients without AChR-antibodies. Therefore, we had to estimate a 15% share of MG patients negative to AChR-antibodies to enable comparison between the databases. This lowers the sensitivity of the AChR database. The MG diagnosis is not available in NorPD, but the indication for treatment with pyridostigmine covered by reimbursement regulations is exclusive for MG. However, pyridostigmine prescriptions can be reimbursed for other diagnosis, for example congenital myasthenic syndromes or Lambert-Eaton myasthenic syndrome. This renders a lower specificity for NorPD. Such differences between the databases have to be taken into consideration when comparing the data. Thus, the two databases should be complementary and together elucidate the true MG prevalence and incidence in Norway. Both databases are nationwide and the data were collected within the same period of time.

The choice of research method has implications for the results from the studies, and it is important to carefully consider the methods before the study process begins. This became evident when conducting paper IV. Initially, we found no association between AChR-antibody concentration and MGFA score because we did not distinguish between those treated by pyridostigmine only and those treated with immunosuppressive drugs. When we did so, the results and the subsequent conclusion changed.

6.2.3 Selection bias

The AChR-database is a nationwide database with an expected coverage of all tests performed from the whole country during the period 1983-2008. In 2008, the laboratory at Oslo University Hospital also started to offer analysing AChR-antibody concentration, but for the three first papers, the inclusion stopped December 31st 2007. It is possible that some samples were sent abroad to be tested during the study period, however, this is probably a very small number. It could also be that some patients are diagnosed without taking AChR-antibody test. This is especially a concern for those diagnosed during the first years after the introduction of the test in 1983. There has been a steady increase in number tests taken from 47 in 1983 to over 1000 tests annually analysed in the recent years. This illustrates how the test is incorporated as a diagnostic tool [88]. Our calculations of incidence had a starting point in 1995, 12 years after the introduction of the test. This reduces the risk of missing patients diagnosed without an AChR-antibody test. Prevalence was calculated 1st January 2008, and included all anti-AChR MG patients diagnosed the past 25 years and alive. In paper I, II and III, the only inclusion criteria was a positive AChR-antibody test taken, with cut-off at 0.5 nmol/L. This very strict inclusion criteria and the nationwide inclusion, reduce the chances of selection bias significantly.

In paper II, we studied the geographical distribution of MG in Norway. We did not have the precise address of the patients, but used the address of the referring hospital

or doctor as a marker of the health region the patient belonged to. Norway has a public health care system funded from the national budget and run by five (in the study period, now four regions) Regional Health Authorities. Every health region has hospitals with high competence for neurological service. The citizens have free choice of hospital within Norway and citizens are eligible for treatment free of charge in the public health system. Therefore, it may be that some samples were sent from one region whereas the patient belonged to another region. However, only approximately 1% of patients are treated by other hospitals than default hospital within their health region, one-third representing minor surgery. When conducting paper the IV study, this assumption was also confirmed as no patients belonging to Haukeland University Hospital had chosen other than default hospital.

Another challenge of defining the patients' health region was migration within Norway. The majority group of migration is those from 20 to 29 years of age. In paper II, we consistently defined the patient´s region based on the first positive AChR-antibody test from the region of the referring hospital or doctor (defined as MG onset). If a patient has been exposed to a possible environmental exposure for developing MG, but is registered with a first positive test in another region than the region for the potential exposure, we miss the opportunity to eventually identify this mechanism. This could represent a selection bias for those developing the disease as early-onset MG, and the distribution can be biased because more patients can be registered with their first positive test in another region than the region for exposure. However, even if we had known the exact address and migration pattern of the patients, we still had had to define one health region as the belonging health region. In this study we were consequent in the definition, and we think that more information about young adults' migration would not change the results substantially.

In paper IV, the inclusion criteria were more complex because only patients from western Norway with two or more AChR-antibody tests and a medical note written by a neurologist corresponding in time to the AChR-antibody test were included. This introduces a bias because we do not know why some patients took more tests than

others. Some doctors take more AChR-antibody tests than others. Some patients have a more severe disease or a more fluctuating disease or some may have had relevant comorbidity. Gender and age are thought not to be determining factors. Most probably, those with two or more test taken, had a more severe disease than those without. We concluded in the study that repeated AChR-antibody measurements are valuable to monitor response to immunosuppressive treatment in individual MG patients.

6.2.4 Information bias

The most important type of information bias is the misclassification bias [199]. In this study, classification first of all depended of the accuracy of the diagnostic test (AChR-antibody test). As discussed in other sections, the AChR-antibody test is very specific for MG, even without clinical information available. With a cut-off value at 0.5 nmol/L, there is, however, still a small risk for false positive test. The risk of a false negative test is probably higher, but difficult to determine (as discussed in the "AChR database-section", page 44). In this thesis, we have defined patients with low-affinity AChR-antibodies as a separate subgroup.

This study is limited to one MG subgroup. We have not investigated MG with low-affinity AChR-antibodies or other antibodies such as against MuSK or LRP4. As discussed in "Pathophysiology of anti-AChR MG"-section, the AChR-antibody subgroup constitutes about 85% of all MG cases in Norway. To include just one subgroup of MG is both strength and a weakness of the study. The study has a strict inclusion criteria and it is based on one database with tests performed with high quality. On the other hand, these results cannot be generalized to all patients with MG in Norway.

Misclassifications can also be introduced by the observer (interviewer bias, biased follow up), or by the study participants (recall bias). In paper IV all clinical information was entirely based on what the neurologist reported in the structured

medical notes. Our MGFA score was depended on what the patient told the doctor (recall bias), and how the doctor obtained the answers from the patient (interviewer bias), and finally how the observation was formulated in the medical note. Even though all doctors were experienced doctors on MG, these biases represent the weakness of a retrospective clinical study. Another bias was introduced when the investigator evaluated the medical notes and scored them according to MGFA score. To minimise this bias, the first medical notes from 33 randomly selected patients were validated by two independent observers, and with very good agreement between the observers. This validation did not reduce the recall- or interviewer bias, but ensured the best possible score based on the medical notes.

6.2.5 Confounding

Confounding occurs when the relationship between a given exposure and a specific disease/outcome is misinterpreted because of the influence of a third variable or group of variables that has not been taken into account (confounder) [200]. Controlling for confounders can be done both in the study design (as randomization, restriction and matching), and/or during data analysis. In the data analysis, the most effective way to controI confounders, is by stratification.

In paper I, we calculated annual incidence of anti-AChR MG in the period 1995-2008 based on a positive AChR-antibody test. We had no clinical information, except for age and gender. The test for homogeneity of rates, used to compare the annual number of new cases, was based on the assumption that this number followed an independent Poisson distribution. This test was performed to evaluate whether the variation in estimates from one stratum to another (age or gender) was compatible with the assumption that the dependent variable, incidence, was uniform independent of age or gender.

In Paper II, multiple Poisson regression analysis was used to assess variation in incidence depending on year, gender, onset age, and geographical region. Multiple Poisson regression analysis was used because the probability of disease occurrence was low relative to the total population size. All incidence analyses were adjusted for gender, region and age.

In paper IV, we cannot be sure to have all relevant information about the patients included in the medical notes. It could be that the patient or the doctor leave out information relevant for the MGFA scoring. The information about medication could have been incomplete. This may have resulted in uncontrolled confounders. In paper IV, all calculations were adjusted for gender, thymectomy and subclass (early-onset MG, late-onset MG, ocular MG and thymoma MG).

6.2.6 External validity

Epidemiological MG studies report a wide variation in prevalence and incidence in various populations. Since we do not know whether the variation is due to study quality or actual biological differences, the results of paper I, II and III, cannot automatically be generalised to all other populations. However, we will argue that our calculated prevalence and incidence reflect accurate epidemiological data for MG in Norway since we expect our case ascertainment to be close to 100%, nationwide, and by one database. Our data can therefore be used as comparison for high quality epidemiological data from other populations. Such comparisons should hopefully elucidate genetic or environmental causative factors.

We believe that the results for paper IV are generalisable. This assumption is based on the pathogenesis of anti-AChR MG being the same independently of the population.

We conclude that AChR-antibody measurements should be included as a decision-making tool, valuable to monitor response to immunosuppressive treatment.

7. Conclusions

This study has provided important epidemiological clues to anti-AChR MG: a nationwide incidence, prevalence and gender characteristics. Moreover, the study has yielded knowledge of the association between clinical anti-AChR MG status and concentration of AChR-antibodies intra-individually over time.

We found a total crude yearly anti-AChR MG incidence of 7.2 per million in the period 1995-2007, and a prevalence of 126.2 per million January 1st 2008.

The incidence of anti-AChR MG in the five geographical regions of Norway did not differ significantly. This means that the incidence of anti-AChR MG is the same in the different regions of Norway.

We validated the AChR-database using NorPD, and found good concordance for the prevalence calculated in the two databases. The prevalences were calculated as 145 and 131 per million for the two databases respectively. However, the incidence for 2007 was twice as high in NorPD than in the AChR-database, 16.0 and 8.8 per million respectively. This may be explained by a shorter study period for NorPD (2004-2007).

We found an association between clinical MG state and concentration of AChR antibodies intra-individually over time in patients with immunosuppressive drug treatment. In the group treated with pyridostigmine only, such an association was not found. Repeated AChR-antibody tests in MG patients treated with immunosuppressive drugs have an additional value and can be a support for therapeutic decisions.

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AChR-antibodies as a marker for epidemiological studies and in the follow-up of patients

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