Vaccinations in autoimmune myasthenia gravis

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Chapter 1

General introduction

MYASTHENIA GRAVIS

Myasthenia gravis (MG) is an acquired autoimmune disease of the neuromuscular junction. The disease is characterized by fluctuating weakness and fatigability of the skeletal muscles [1, 2]. The pattern and severity of clinical symptoms can vary widely between patients and the distribution of muscle weakness can help to recognize a subtype, which can be related to the type of antibody that is present [3]. The majority of MG patients have acetylcholine receptor (AChR) antibodies. These were already described in 1973 in rabbits and in 1975 in humans [4, 5]. Other antibodies, are found less frequently and are directed to muscle-specific kinase (MuSK) in MuSK MG or to voltage-gated calcium channels (VGCC) in the Lambert-Eaton myasthenic syndrome (LEMS) [6, 7]. In rare cases also antibodies to Lrp4, or agrin have been described [8-10].

The initial trigger for developing these autoantibodies is unknown, but both T- and B-cells have to be involved in this process. There is evidence that the thymus has a crucial role in AChR MG, as many patients have either thymic lymphoid hyperplasia or a thymic tumor [3, 11]. In healthy individuals the thymus will start to show atrophy from early adulthood. In the hyperplastic thymus often lymphocytic infiltrates and germinal centers are found, and these are thought to play a role in the initiation or continuation of the immune response against the AChR. AChR expression can be activated in thymic epithelial cells through cytokine and receptor signaling, potentially triggered by a virus. However, no specific virus has been identified so far [1]. Autoreactive T cells, specific for AChR, escape the normal intrathymic surveillance and are exported to the periphery where they stimulate B cells to produce antibodies. Differences in autoantibody pattern, HLA associations, thymic pathological changes, cytokine intrathymic pattern, and T-cell subsets and clones all point to differences in induction mechanisms for early-onset, late-onset, and thymoma-associated myasthenia gravis [1]. A frequently used therapy is a thymectomy in patients with thymic hyperplasia or a thymic tumor, which has a favorable effect on the disease course and provides additional evidence that abnormal thymic function contributes to the development of MG [12].

ROLE OF AUTOANTIBODIES

Next, a closer look will be taken at the pathophysiological effect of the two most frequently found antibodies, i.e., AChR and MuSK antibodies. Serum antibodies to the AChR are usually of the IgG1 or IgG3 subclass. These antibodies can cross-link because they are bivalent and they can activate serum complement to cause complement-dependent damage to the neuromuscular junction (NMJ) [13, 14]. The latter is the most important mechanism in most patients and results in morphologic damage to the NMJ with loss of AChRs [13]. This damage to the normally highly folded NMJ postsynaptic muscle membrane results in a reduction in the number of voltage-gated sodium channels, increasing the threshold for activation of the

action potential and further impairing the efficacy of signal transmission [2, 13, 14]. Furthermore, accelerated internalization of the AChRs induced by polyvalent antibody cross-linking further reduces the AChR numbers. Direct blocking of AChR function by a variety of possible mechanisms is rarely a major mechanism [2].

Serum antibodies against MuSK are predominantly of the IgG4 subclass. Antibodies of the IgG4 subclass do not activate complement, and are considered to be monovalent for binding to MuSK, as IgG4 antibodies can “exchange” arms with other IgG4 antibodies. Thus, complement activation and antigenic modulation are not thought to play a major role in the pathogenesis of MuSK MG (compared with AChR MG). IgG4 MuSK antibodies block the agrin-induced binding of LRP4 to MuSK, which activates multiple signaling pathways that lead to aggregation of AChR and transition from the plaque-to-pretzel form of the neuromuscular synapse [14, 15]. Thus, in MuSK MG the disease mechanism leading to clinical weakness is clearly different from that in AChR MG.

IMMUNOSUPPRESSIVE MEDICATION

A large part of the patients needs long-term immunosuppressive medication, because symptomatic treatment with cholinesterase inhibitors (such as mestinon) is insufficient. Even despite immunosuppressive medication, in 10-15% of the patients full control of the disease is not achieved. This is possibly associated with severe side-effects of the medication [1].

The most frequently used medication is azathioprine combined with prednisolone, followed by mycophenolic mofetil and cyclosporine. The latter two are also frequently combined with prednisolone and are also used to reduce the dosage of prednisolone. Less frequently used medication is rituximab and eculizumab, which is mainly used in patients with more severe myasthenia gravis. In this thesis we mainly describe patients with mild to moderate and stable disease, therefore rituximab and eculizumab will not be further discussed here.

Prednisolone or prednisone is a corticosteroid that has a broad suppressing effect on the immune system. For patients who need to take long-term corticosteroids, specific precautions should be taken to reduce the risks of glucose intolerance, gaining excess bodyweight, hypertension, and development of osteoporosis.

Azathioprine is a purine antagonist, which suppresses the increase and proliferation of B- and T-lymphocytes and damages DNA by uptake of thiopurine. The most encountered side-effects of azathioprine are leucopenia and hepatotoxic effects, mainly during the first months of treatment [1]. Mycophenolic mofetil (MMF) is a second-line medicine. MMF is a prodrug that after conversion blocks purine synthesis and interferes with B-cell and T-cell proliferation. For MMF side-effects are rare, with mild headache, nausea, and diarrhea as the most commonly reported.

Ciclosporine and methotrexate seem to be as effective as azathioprine. Ciclosporine suppresses specifically (reversibly), the proliferation of T-cells, leaving phagocytic cells unaffected. Patients should be monitored for potential side-effects, especially nephrotoxic effects and hypertension [1].

In this thesis we often compare within the above mentioned treatment groups patients with and without immunosuppressive medication and those with and without a thymectomy in the past. This in order to find or exclude an effect of a treatment.

PRESENCE OF ANTI-ACETYLCHOLINE RECEPTOR ANTIBODIES AND CLINICAL ONSET

As described above, despite that the pathogenic AChR antibodies are already described decades ago, it is still unknown what the initial trigger for making these antibodies is in individual patients.

In chapter 2 of this thesis, we describe a case report of presence of anti-acetylcholine receptor antibodies 2 years before clinical onset. In this case, onset of clinical symptoms was during pregnancy, which could be considered as a immunological event. Pregnancy is a well-known trigger for the clinical onset of MG, and there is a considerable risk for clinical onset during the post-partum period [16]. We studied the anti-AChR levels before onset of clinical symptoms and during treatment in the years thereafter (Chapter 2).

Evolution of the symptoms in individual patients has a variable pattern and, because the disease can be difficult to recognize, the diagnosis can be delayed until the second year after symptom onset [17]. It is unknown how long the presence of anti-acetylcholine receptor antibodies may precede clinical symptoms. Furthermore, titers of anti-AChR antibodies as such are not a reliable biomarker for the clinical severity or the clinical change at group level, but can show a reasonably good correlation in one and the same patient [18]. Prospective monitoring of the onset of anti-AChR antibodies in a group of individuals at risk for MG would be helpful, but is not feasible due to the low incidence of the disease.

A PATIENT REPORTED OUTCOME MEASURE FOR QUALITY OF LIFE

Optimal treatment aiming to achieve mild disease manifestations or remission often requires the use of immunosuppressive medication [1]. Despite treatment, patients can experience restrictions in their daily activities and health related quality of life (HRQOL) [19]. This can be due to side effects of medication or to the disease itself. The impact of the disease on quality of life is best reported directly by the patient through Health-Related Patient Outcomes (HR-PROs) [19]. PROs are measurements

of any aspect of a patient's health status that are evaluated from the patient's perspective without interpretation of the response by a clinician or anyone else [20]. Nowadays, the focus in the clinical setting is mainly on the effect of treatment in terms of clinical symptoms. Previously, outcome measures like the MG composite (MGC) and the Quantitative Myasthenia Gravis (QMG) score have been used. These outcome measures do not assess health-related quality of life. The 15-item myasthenia gravis quality of life scale (MG-QOL15) was constructed to measure the patient's perceived HRQOL, which covers broad domains like physical, social, and psychological well-being [21, 22]. In order to use the MG-QOL15 as an outcome measure in clinical trials and standard care, it must be validated. At the moment, the MG-QOL15 has been validated in several languages [21, 23-26]. In preparation of the tetanus and influenza vaccination trials, we translated and validated it into Dutch and evaluated its measurement properties in terms of test-retest reliability and construct validity (Chapter 3).

TETANUS AND INFLUENZA VACCINATION

In case of vaccinations, which can also be considered as an immunological event, little was known in myasthenia gravis on the efficacy of vaccination and its possible effect on disease activity. This is described in the chapters 4-6.

Patients with an autoimmune disorder are believed to be more prone to infection, due to their immunosuppressive therapy or due to the immune abnormalities associated with their disease [27, 28]. In myasthenia gravis, an infection has been associated with an aggravation of the symptoms, sometimes resulting in a myasthenic crisis. Specific data on infection rates in myasthenic patients do not exist [29]. For some of these infections vaccines are available and some of them, such as the annual influenza vaccination, are recommended for patients with an autoimmune disease as MG. However, an adequate immune response to vaccination could be hampered by the dysregulation of the immune system which is evident from the development of autoimmunity or by the effect of the immunosuppressive medication on the immune system. Little is known about safety and effectiveness of vaccination in myasthenic patients and this remains a matter of debate. For the clinician, it is also important to be able to inform their patients about the risks and benefits of a vaccination. Also, because people travel more to areas for which prophylactic vaccinations are recommended.

In this thesis we therefore describe two randomized clinical trials investigating the effect of two vaccinations. The first trial investigated tetanus revaccination. We choose tetanus because it a frequently used vaccine with a well-known safety profile and antibody response in healthy individuals as well as in immunocompromised individuals with HIV or after stem cell transplantation [30, 31]. Furthermore, knowledge of tetanus revaccination in myasthenia gravis is also practical for both clinicians as patients, because patients that attend the emergency department often

need a tetanus revaccination because of the trauma they suffered (Chapter 4). The second vaccine we describe in this thesis is the annual influenza vaccination. As already mentioned above, the annual influenza vaccination is recommended for all patients with MG. No specific guidelines regarding (influenza) vaccinations in patients with MG exist, but a small number of observational studies suggest that influenza vaccination is safe [32, 33] and recently a randomized controlled trial confirmed this by the finding that influenza vaccination has no influence on the AChR antibody titre [34].

In our personal experience and as earlier described [32], many patients express concern that influenza vaccination may lead to an exacerbation and a substantial number declines vaccination each year based on this concern. This is unfortunate, as seasonal vaccination against influenza is highly effective in reducing laboratory-confirmed influenza illness, hospital admissions and risk of death, especially in elderly and frail patients [35]. This is relevant, as this age group has currently the highest incidence of autoimmune MG [36]. Therefore, we performed a double-blind, placebo-controlled trial to investigate the efficacy and safety of the seasonal (2016/2017) influenza vaccine in patients with AChR MG, with and without immunosuppressive medication (Chapter 5).

THE EFFECT OF A VACCINATION ON THE T- AND B-CELLS COMPARTMENT

Performing the above described tetanus study gave the opportunity to also investigate the cellular response to tetanus revaccination. The humoral response to tetanus is T cell dependent and the immunosuppressive medication has a general suppressing effect, also on the T cell compartment. Therefore, we investigated the *in vitro* tetanus-specific T cell responsiveness in the same MG cohort pre and post revaccination, with a focus on the effect of immunosuppressive medication and the influence of a preceding thymectomy. Furthermore, we investigated a broad spectrum of (functional) subsets in the B- and T cell compartments pre and post vaccination (Chapter 6)

The results and conclusions of these thesis are summarized and discussed in Chapter 7.

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Chapter 2

Presence of AChR antibodies before clinical onset myasthenia gravis

Ellen Strijbos, Jan J.J.G.M. Verschuuren, Jan B.M. Kuks

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1. INTRODUCTUION

Myasthenia Gravis is a disorder of the neuromuscular junction characterized by fluctuating muscle weakness influenced by exercise. The onset of auto-immune myasthenia and evolution in the individual patient has a variable pattern and the diagnosis is often made in the second year after symptom onset [1]. All voluntary muscles may be involved, but many patients start with ocular symptoms like double vision and drooping eyelids. Antibodies to the acetylcholine receptors (anti-AChR) on the muscle membrane [2] are the antibodies that are most frequently found. Finding these antibodies is highly specific (>99%) for the diagnosis whereas the sensitivity of the test is usually around 85% [3, 4]. In seronegative patients or in case a rapid diagnosis is mandatory, neurophysiologic tests may be helpful [5]. Anti-AChR antibodies are not a reliable biomarker for the clinical severity or the clinical change at group level, but can show a reasonably good correlation in one and the same patient [6]. Although there is conclusive evidence that the anti-AChR antibodies are pathogenic [7, 8], it is unknown what triggers the start of the anti-AChR immune response and how long seroconversion may precede clinical symptoms. Prospective monitoring of the onset of anti-AChR antibodies in a group of individuals at risk for MG would be helpful, but is not feasible due to the low incidence of the disease.

We encountered a unique case of a young female with MG in whom serum samples were available over a period of at least 2 years before the onset of clinical symptoms. We studied the anti-AChR levels before onset of clinical symptoms and during treatment in the years thereafter.

2. CASE REPORT

A 22 years-old female experienced symptoms of unspecific fatigue, muscle pain and arthralgia after her first pregnancy in 1986. She was surmised from having an auto-immune disease and thus seen by an experienced immunologist. Although a-specific symptoms of fatigue and arthralgia were reported, no rheumatic arthritis and SLE associated antibodies could be found and no diagnosis could be made for the time being. A wait and see policy was followed and the patient consented to participate in an SLE research programme for which blood samples were collected and stored at regular intervals. By the end of 1988 she got pregnant for the second time and delivered in august 1989. Her new-born daughter experienced problems with drinking, for which she was fed through a tube, and had a feeble cry during the first days after birth. In retrospect, she might have been suffering from neonatal myasthenia gravis. The day after delivery the patient herself developed swallowing problems, dysarthria, loss of strength in her hands and weakness in the neck muscles. By then, the diagnosis of MG was made based on clinical symptoms and repetitive nerve stimulation (decrement 17% in the ulnar muscles). She was treated successfully with anticholinesterases. Anti-AChR serum antibodies were found positive one week later (68 nmol/l; normal values <1 nmol/l (9)).

Being asked for specifically she remembered periods of a tired feeling of the eyelids by watching television in February 1989 after 3 months of pregnancy. She experienced no other clinical symptoms, suspect for myasthenia. Although she was interviewed on a regular base, her rheumatologist, being a specialist in auto-immune diseases, did not recognize her eyelid symptoms as being abnormal and did not notice other symptoms that would suggest a clinical diagnosis of MG.

Serum samples, collected in the two years before the apparent symptoms, were analysed for anti-AChR antibodies. The first sample in 1986 showed a titer of 13 nmol/l and there was a gradual increase towards 82 nmol by the end of her pregnancy 2.5 years later, without any clinical suspicion of myasthenia. In retrospect, at time of the first symptoms of 'tired eyelids' the serum level was 48 nmol/l.

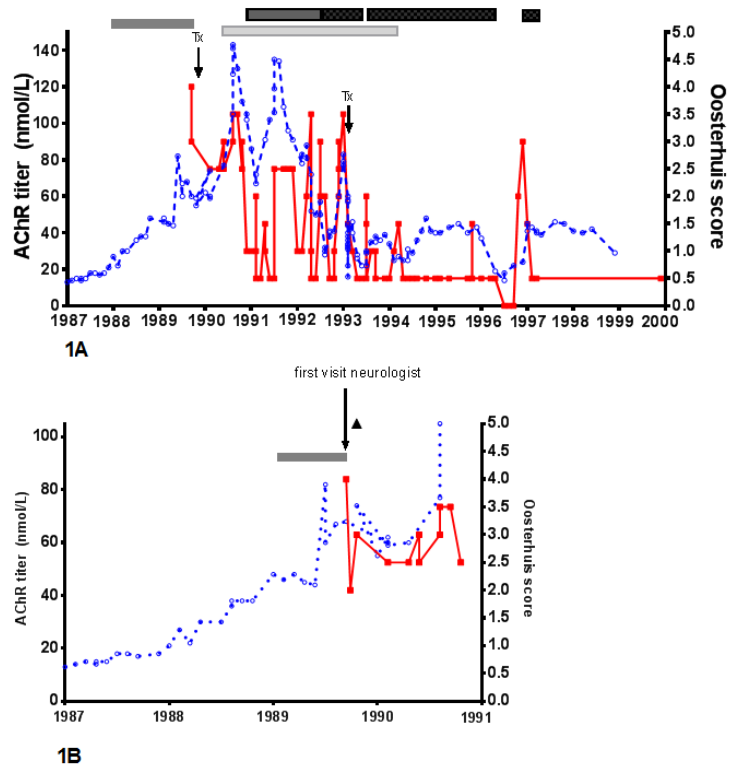

Figure 1A: Course of anti-AChR antibodies, clinical score and medication over a period of 13 years. Oosterhuis score: 0 remission, no medication use, 1 minimal signs and symptoms, 2 mildly disabled, 3 moderately disabled, 4 severely disabled, 5 respiratory support needed. Anti-AChR antibodies (), Oosterhuis score (), thymectomy (Tx), Pregnancy (), Azathioprine dose of 50-150mg/day(), Cyclosporine dose of 100-400mg/day (), mean daily prednisolone dose of 5-60mg/day (). **1B:** Enlargement of course during the first 4 years.

She was followed clinically until she went into clinical remission of myasthenia after intensive medical and surgical treatments: in 1990, half a year after delivery she underwent a thymectomy, later on was treated with prednisolone, azathioprine and cyclosporine. In 1993 she underwent another 'extended' thymectomy, at which some thymic remnants were found. Hereafter, the MG seemed to be milder with occasionally a mild upbeat of symptoms that could be treated with increasing the cyclosporine or adding some steroids. Another three years later, in 1996, the diagnosis of SLE was made based on positive ANA, anti-dsDNA, pleuritis, cutaneous manifestations, renal disorder and joint disease and for this, immunosuppressive therapy was intensified. Myasthenic symptoms subsided and no further antibody-titers were determined from the end of 1998. A survey over clinical course, therapies and antibody-titers is depicted in figure 1a and an enlargement of the course in the beginning years is depicted in figure 1b. The patient died in 2012 at the age of 47 years because of complications of the SLE.

3. DISCUSSION

This patient showed a gradual increase of anti-AChR antibodies in a period of more than two years before becoming symptomatic of myasthenia. Over these years she was followed by an experienced immunologist, being familiar with myasthenia gravis, who did not recognize any suspect symptom for this disease.

Pregnancy is a well-known trigger for the clinical onset of MG, and there is a considerable risk for clinical onset during the post-partum period [10]. Myasthenia might be associated with other auto-immune disease like rheumatic arthritis and SLE and a puerperal onset of this disease has been described as well [11]. To the best of our knowledge there is no description of a long course of anti-AChR titers before the onset of myasthenia but the presence of autoantibodies several years before clinical symptoms appeared has been described in inflammatory arthritis [12].

During the more than 10-year follow-up period in this patient anti-AChR antibody titers significantly correlated with the Oosterhuis score [13] (Spearman rank correlation coefficient: $r=0.52$; $p<0.0001$). That this correlation is only 'reasonable' [6] is obvious: at several time points in the symptomatic period of the study, anti-AChR titers were at the same level as before onset of clinical symptoms. This stresses the complex relationship between anti-AChR antibodies and disease severity, even in an individual patient.

In conclusion, anti-AChR antibodies can be present at least two years before patients experience overt clinical symptoms of MG. The data suggest that in our patient pregnancy triggered the clinical manifestation of a smouldering autoimmune antibody response, but is not the primary trigger in itself.

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Chapter 3

Translation, cross-cultural adaptation, and validating the Myasthenia Gravis Quality of Life Questionnaire (MG-QOL15)

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1. INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disorder of the neuromuscular junction in which patients experience fluctuating weakness that most often affects specific muscle groups. In the majority of patients, MG is caused by antibodies against the acetylcholine receptor (AChR) or the muscle-specific receptor tyrosine kinase (MuSK). Optimal treatment aiming to achieve mild disease manifestations or remission often requires the use of immunosuppressive medication [1]. Despite treatment, patients can experience restrictions in their daily activities and health related quality of life (HRQOL) [2]. This can be due to side effects of medication or to the disease itself. The impact of the disease on quality of life is best reported directly by the patient through Health-Related Patient Outcomes (HR-PROs) [2]. PROs are measurements of any aspect of a patient's health status that are evaluated from the patient's perspective without interpretation of the response by a clinician or anyone else [3]. Nowadays, the focus in the clinical setting is mainly on the effect of treatment in terms of clinical symptoms. Previously, outcome measures like the MG composite (MGC) and the Quantitative Myasthenia Gravis (QMG) score have been used. These outcome measures do not assess health-related quality of life. The 15-item myasthenia gravis quality of life scale (MG-QOL15) was constructed to measure the patient's perceived HRQOL, which covers broad domains like physical, social, and psychological well-being [4,5]. In order to use the MG-QOL15 as an outcome measure in clinical trials and standard care, it must be validated. At the moment, the MG-QOL15 has been validated in several languages [6,4,7-9]. We translated and validated it into Dutch and evaluated its measurement properties in terms of test-retest reliability and construct validity.

2. PATIENTS AND METHODS

2.1 Design

This study had a cross-sectional design. It was executed at the outpatient clinic of the Department of Neurology of a Dutch academic medical center between March 2015 and January 2016. Ethical approval was obtained from the Medical Ethics Committee of this university hospital. Informed consent was obtained of all patients. The Dutch version of the MG-QOL15 was tested among 50 patients with acetylcholine-receptor antibody positive (AChR) MG who participated in a study of tetanus revaccination in patients with MG. This study was performed in order to investigate the effectiveness and safety of a tetanus revaccination in patients with MG. To validate and evaluate the measurement properties of the Dutch MG-QOL15, we used the data from the 2 time points over a 1-week interval before the revaccination.

2.2 Translation and adaptation of the MG-QOL15

The English version of the MG-QOL15 consists of 15 items with a 5-point response scale (0= not at all, 4 = very much). The response categories represent how applicable

the statement is for the patient in the past few weeks. The total scale score is the sum score of all 15 items, ranging from 0 to 60, with higher scores indicating less quality of life. The questionnaire was translated independently by 2 persons, a native Dutch-speaking translator who was a non-medical, lay person and a translator who works in the biomedical research field. The 2 translations were compared and combined into 1 Dutch version by the investigators (Figure 1). No significant modifications were required.

2.3 Sampling and questionnaire administration

The most important inclusion criterion for the study was a confirmed diagnosis of MG, based on clinical symptoms and a positive serological test for AChR antibodies. Patients had to be age 18 years or older, with a maximum of 65 years at time of vaccination. The dosage of their immunosuppressive medication had to be stable over the preceding 3 months, with a maximum of 30mg prednisolone per day. The use of IVIg or plasmapheresis was not allowed in the 3 months before participation. The patient was excluded from the vaccination study in the patient had tetanus revaccination in the past year, had a thymoma, or if the patient had undergone thymectomy in the preceding year.

We recruited patients through the outpatient clinic of a Dutch university hospital and the national patient organization. Included patients received the questionnaires either by mail or during their hospital visits and returned them in person at the hospital.

2.4 Measurement instruments

2.4.1 SF-36

The SF-36 Health Survey is a generic patient-reported quality of life measure. It is composed of 36 items organized into 8 multi-item scales: physical functioning (PF), role limitations due to physical health problems (RP), bodily pain (BP), general health perceptions (GH), vitality (VT), social functioning (SF), role limitations due to emotional problems (RE), and mental health (MH). From these scales, 2 sum scores can be calculated: the physical component score (PCS) and the mental health component score (MCS). All scale scores are converted to a 0 to 100 scale, with higher scores indicating higher levels of functioning or well-being10. In this study we used the 2 component scores.

2.4.2 MG-ADL

The Myasthenia gravis activities of daily living (MG-ADL) profile is an 8-item patient-reported scale that is administered by a physician to assess MG symptoms and their effects on daily activities11. It has a 4-point response scale ranging from 0 (normal) to 3 (constant/gastric tube/ventilator dependence). The total score of the MG-ADL ranges from 0 to 24, and higher scores indicate more impact of MG on daily activities [11].

2.4.3 MG composite
The Myasthenia Gravis Composite (MGC) scale, a physician-administrated scale, consists of 10 items that measure symptoms and signs of MG, with weighted response options [12,13]. These 10 items were selected from existing MG-specific scales [MG-ADL, QMG, and the Manual Muscle Test (MMT)] [12,13]. For each item there are 4 response options ranging in general from 0 (normal) to 9 (severe). The sum score of the MGC ranges from 0 to 50, with higher scores indicating greater clinical severity of MG.

2.4.4. QMG
The Quantitative myasthenia gravis score (QMG) is a 13-item (3 ocular, 2 bulbar, 1 respiratory, 1 neck, and 6 limb) scale that measures muscle strength and endurance [14,15]. This scale is a physician-administrated scale, which contains 4 response categories ranging from 0 (none) to 3 (severe). The QMG score ranges from 0 to 39, with higher scores indicating more severe MG.

2.5 Analyses

2.5.1 Internal consistency of scale
Internal consistency is the degree of interrelatedness among items [2,16], indicated by the Cronhbach alpha. A Cronhbach alpha of 0.70 or above is regarded as sufficient [17].

2.5.2 Test-retest reliability
Reliability is the degree to which the measurement is free from measurement error [3, 14]. To evaluate the test-retest reliability, we assessed the level of agreement and measurement error. Test–retest reliability is the extent to which results for patients who have not changed are the same for repeated measurements over time [2,16]. One assumption for the test-retest reliability is that respondents have to be stable in their symptoms during the 2 measurement points. To guarantee stability of the disease symptoms as much as possible, we chose a 1 week interval between the 2 measurement points [2,16] .To assess test-retest reliability, the intraclass correlation coefficient (ICCagreement) was calculated for data from time 1 (T1) and time 2 (T2). The formula used was: ICCagreement = $\sigma 2p / (\sigma 2p + \sigma 2time + \sigma 2error)$. Good test-retest reliability was assumed for an ICC ≥0.70 [17].

The measurement error is the systematic and random error of a patient's score that is not attributed to true changes in the construct to be measured [2,16]. Based on the Standard Error of Measurement (SEM), it is possible to conclude whether changed scores within 1 subject over the time is based on a real difference or based on measurement error (difference score<SEM [18]). The SEM was calculated using the formula: SEMagreement= $\sqrt{\sigma 2time + \sigma 2error}$.

2.5.3 Construct validity
Construct validity is the degree to which the scores of an HR-PRO instrument are consistent with hypotheses based on the assumption that the HR-PRO instrument is a valid measures of the measured construct [2,16]. We made the following hypotheses:

1. We hypothesized that the correlation of the MG-QOL15 with the MG-ADL is medium [5, 12], because this scale measures the influence of the disease on activities of daily living. Therefore, it overlaps in its content a fair amount with measures of HRQOL.

2. We hypothesized that the correlation of the MG-QOL15 with the QMG is medium [5], because the QMG measures only physical strength and function, but no factors of HRQOL.

3. We hypothesized that the correlation of the MG-QOL15 with the MGC is medium [5, 12].

4. We hypothesized a high negative correlation of the MG-QOL15 with the physical component score of the SF-36, because the measured construct overlaps to a great extent [19].

5. We hypothesized that the correlation of the MG-QOL15 with the SF-36 mental component score was lower than with the physical component score based on findings in an earlier study [19].

To test these hypotheses we calculated Pearson correlations between the MG-QOL15, MG-ADL, QMG, and MG-composite and the SF-36 scores (table 1). A correlation coefficient of r<0.3 was considered as low, between 0.3 and 0.5 as medium and >0.7 as high [20].

Hypotheses		Confirmed
1.	The MG-QOL15 has a medium correlation with the MG-ADL	Yes
2.	The MG-QOL15 has a medium correlation with the QMG	No
3.	The MG-QOL15 had a medium correlation with the MGC	Yes
4.	The correlation of the MG-QOL15 with the SF-36 PCS is negative high.	Yes
5.	The correlation of the MG-QOL15 with the SF-36 PCS is higher negative than with the SF-36 MCS	Yes

Table 1. Hypothesis for the construct validity of the MG-QOL15

3. RESULTS

3.1 Study sample

Fifty AChR MG patients were enrolled. They had a median age of 56 years, and 37 (74%) were women. Almost half of the patients used some kind of immunosuppressive medication (46%). Four patients were scored as in remission (MGFA 0), 4 as ocular (MGFA 1), 40 as mild generalized (MGFA 2), and 2 as moderate-severe (MGFA 3). The mean disease duration was 14.6 years (SD 13 years). See Table 2 for an overview.

	N	(%)
Number of patients	50	-
Diagnosis AChR MG	50	(100)
Gender, women	37	(74)
Age, median (SD)	56	(11,5)
Duration of disease (SD)	14.6	(13)
MGFA classification*		
0	4	(8%)
1	4	(8%)
2	40	(80%)
3	2	(4%)
Use of immunosuppressive medication	23	(46%)
Thymectomy	29	(58%)

Table 2. Participant characteristics.
MGFA Myasthenia Gravis Foundation of America

3.2 Descriptive statistics of measurement outcomes

The mean MG-QOL15 score at T1 was 20.4 (SD 11.2). The mean MG-QOL15 score at T2 was 19.4 (SD 11.6). The mean score of the MGC, MG-ADL, and QMG were 5.5 (SD 4.9), 3.9 (SD 3.2), and 6.7 (SD 4.3), respectively.

3.3 Internal consistency

The internal consistency proved to be sufficient, as the Cronbach alpha was 0.928.

3.4 Test-retest reliability

We had a 100% response rate of these 50 AChR MG patients. Based on this stable sample, the ICC between measurements T1 and T2 was good: ICC (95% confidence interval) = 0.866 (0.776-0.922). The SEM was 4.1 (6.8% of the scale range of 0-60) with a 95% CI of 1.4 to 6.9.

3.5 Construct validity

The MG-QOL15 had a medium high correlation with the MG-ADL (r = 0.501) and MGC (r = 0.388). The correlation with the QMG (r= 0.224) was low. The hypothesis of a medium correlation with the QMG could therefore not be confirmed. The high

negative correlation of the MG-QOL15 with the SF-36 PCS score (r = -0.832) was confirmed. Also, the hypothesis that the correlation of the MG-QOL15 with the SF-36 PCS was stronger than with the SF-36 MCS (r= -0.743) was confirmed. See table 3 for the correlations.

MG-QOL15	Correlation	*P*-value
MGC	0.388	0.005
MG-ADL	0.501	<0.001
QMG	0.224	0.117
SF-36 PCS	-0.832	<0.001
SF-36 MCS	-0.743	<0.001

Table 3. Correlation of the MG-QOL15 with the MGC, MG-ADL and QMG and the SF-36 component scores at 4 weeks.

4. DISCUSSION

The original English version of the MG-QOL15 was translated into Dutch and evaluated in a test-retest design with 2 measurement points separated by a 1-week interval. The sample consisted of patients who had stable disease, based on the MGFA classification and the requirement for a stable medication regimen over in the preceding 3 months. The requirement for good test-retest reliability was fulfilled with an ICC of 0.866. From our predefined hypotheses, 4 of 5 (80%) were confirmed, which points to good construct validity [17].

We predefined hypotheses about the correlations with 3 frequently used MG-specific outcome measures, the MGC, QMG, and MG-ADL. As expected, we found medium correlations of the MG-QOL15 with the MGC and MG-ADL. However, its correlation with the QMG was low instead of the expected medium correlation. When formulating the hypothesis, we focused on the relationship between symptoms and HR-QOL, which would lead one to expect a medium-high correlation. The QMG objectively measures muscle strength and endurance, but strongly depends on patient effort during only a short time window. A patient can obtain a low score on the QMG, suggesting mild symptoms of MG, while in everyday life mild weakness might lead to a highly variable degree of limitations in different patients with MG. Also, the QMG does not take emotional or mental aspects into account. These differences in the measurement construct between the QMG and the MG-QOL15 might explain the low instead of medium correlation.

The MQ-QOL15 aims to measure HR-QOL, and therefore we hypothesized a strong relationship with a generic HR-QOL measure, the SF-36. The high correlations with the SF-36 component scores we found are opportune, because they prove that the intended construct is indeed what the MG-QOL15 measures. In line with results of an earlier study that describes the development of the MG-QOL1519, we expected

a lower correlation for the mental component score compared to the physical component score. This hypothesis is confirmed, and although the correlation with the mental component score is lower than with the physical component score, it still is high (r=-0.74). The high correlation we found can be explained by the 3 items of the MQ-QOL15 (items 1, 11, and 14) that focus on emotions and distress experienced by the patient, which clearly overlap with the content of the items of the SF-36 mental component score.

These results allow us to assume that that it is necessary to pay attention to psychological distress that MG patients can experience, such as frustration, depression, or an overwhelmed feeling due to the disease. The MG-QOL15 might be suitable to signal any distress in MG patients, but its discriminative ability for this aim should be studied further. Signalling any signs of distress in MG patients is a prerequisite for helping the patient and improving these complaints. The role of psychological distress in MG patients has not been studied. For the future it would be important to study the prevalence of distress, its causes, and possible interventions for this patient group, as well as the role that treatment with corticosteroids or other immunosuppressive drugs plays in the psychological well-being of MG patients.

Overall, the low to medium correlations of the MG-QOL15 with the 3 MG-specific outcome measures and the high correlations with the generic QOL measure, the SF-36, confirms that QOL is measured well by the Dutch MG-QOL15. At the same time these results confirm the additional value of this MG specific quality of life outcome measure. As is typical for a HR-QOL outcome measure, its score is based on patient self-report, and it takes the physical and mental limitations in everyday life due to the disease into account. A benefit of the MG-QOL15 compared to existing generic HR-QOL measures, such as the SF-36, is that it is disease specific and therefore provides more detailed information about MG relevant limitations. The total score of the MG-QOL15 is easier to calculate than the SF-36, since calculation of the total scores are less complex. Furthermore, the MG-QOL15 is shorter than the SF-36, which makes it more feasible and less burdensome for patients to complete the questionnaire.

With its good test-retest reliability and construct validity, the MG-QOL15 is suitable as a MG- specific quality of life measure for research purposes. The MQ-QOL15 might be suitable for monitoring individual patients as well. There is a trend in healthcare to use patient reported outcomes in clinical practice to inform the patient and clinician about development of symptoms and limitations in individual patients [21]. The scores on the MG-QOL 15 might provide a starting point for the clinician and patient to discuss factors that contribute to the burden of disease in MG patients and to subsequently adapt patient care to these factors. With the Cronbach alpha exceeding 0.90 it fulfils the requirement for use on the individual level, [22] and with its short length and disease-specific character, we consider it to be very feasible for application in clinical practice. When comparing MG with other diseases, the SF-36 can be used, because it is a generic quality of life questionnaire, and it showed a high correlation with the MG-QOL15.

A limitation of our study is the relatively small number of included patients. Myasthenia gravis is a rare disease, which makes it challenging to establish a larger homogeneous population. Furthermore, in our study, we aimed to sample a stable population. Our inclusion and exclusion criteria were quite narrow , which challenged patient recruitment even more. The requirement for stable dosing of the immunosuppressive medication and the prednisone were the main recruitment challenges. However, we were able to include 50 patients, which is considered to be the least number of patients needed for a questionnaire validation17. A strength of our study is that we used predefined hypotheses to test the construct validity of the MG-QOL15, by which we tried to make the risk of bias as small as possible17. Another strength is that we included 4 comparison measures in the hypothesis testing, based on 3 frequently used MG-specific outcome measures.

To use the Dutch MG-QOL15 as an outcome measure in intervention studies, changed scores need to be interpreted well. For this, the smallest detectable change value is relevant, which can be based on the SEM we have calculated in this study. Additionally, the minimal clinically important change (MCIC) score for improvement would be of relevance. The MCIC is a score on the scale range of the instrument that indicates the lowest change score that is regarded as high enough to be considered clinically relevant. This score is crucial in indicating change. For calculating the MCIC, it is necessary to have a patient sample that experiences improvement in quality of life [23]. Therefore, it was not possible in our study. The MCIC calculation should be focus of further evaluation studies of the MG-QOL15.

5. CONCLUSION

The Dutch version of the MG-QOL15 demonstrates good test-retest reliability and good construct validity. This version of the MG-QOL15 now can be used in the research setting to measure disease-specific health related quality of life in MG patients. Furthermore, it may be suitable for follow-up of disease-specific quality of life in individual MG patients.

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Chapter 4

Tetanus revaccination in patients with myasthenia gravis

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1. INTRODUCTION

Myasthenia gravis (MG) and the Lambert-Eaton myasthenic syndrome (LEMS) are acquired autoimmune diseases of the neuromuscular junction. The clinical hallmark of MG and LEMS is fluctuating muscle weakness, often in specific muscle groups [1]. The majority of MG patients have acetylcholine receptor (AChR) antibodies. Other antibodies, are found less frequently and are directed to muscle-specific kinase (MuSK) in MuSK MG or to voltage-gated calcium channels (VGCC) in LEMS. A large part of MG and LEMS patients need long-term immunosuppressive medication, because symptomatic treatment is insufficient. Due to the immunosuppressive therapy, patients have an increased risk of infection [2], which can aggravate the symptoms, sometimes resulting in myasthenic crisis. For some of these infections vaccines are available. An example is the annual influenza vaccination which is recommended for all patients with an autoimmune disease. However, safety and efficacy of vaccination remain matter of debate [2]. Prospective studies in systemic lupus erythematosus and autoimmune vasculitis suggest that vaccination in these autoimmune diseases is effective [3, 4] and safe [5]. Little is known about safety and effectiveness of vaccination in myasthenic patients. Tetanus toxoid is a frequently used vaccine with a well-known safety profile and antibody response in healthy individuals as well as in immunocompromised individuals with HIV or after stem cell transplantation [6, 7]. Therefore, we choose this vaccine to prospectively investigate the clinical safety and humoral immune response in patients with MG or LEMS.

2. MATERIALS AND METHODS

2.1 Patients

This study contained 51 patients with AChR MG, 6 patients with MuSK MG, 9 patients with LEMS, a historical control group of 20 healthy individuals (HC group) revaccinated with tetanus toxoid and 23 AChR MG patients injected with a placebo (placebo AChR MG group).

2.2 Prospective tetanus vaccination study protocol

This single-centre, prospective, placebo-controlled study was performed at the Leiden University Medical Centre. A group of 66 patients, of whom 51 with AChR MG, 6 with MuSK MG, and 9 with LEMS were revaccinated with tetanus toxoid and 23 AChR MG patients received a placebo, i.e., saline. At day 1 serum was obtained and clinical tests were performed before revaccination. Four weeks thereafter a second serum sample was obtained and the clinical tests were repeated.

Inclusion criteria were a confirmed diagnosis of MG or LEMS, age between 18 and 65 years and stable disease during the past 3 months. Diagnosis of MG or LEMS was based on clinical signs or symptoms suggestive of MG or LEMS and a positive serological test for AChR, MuSK or VGCC antibodies. Patients continued their medication during the study. A maximum daily dose of 30 mg of prednisolone (+/- 5 mg) was allowed as well as the use of other immunosuppressive medication (see Table 1). Time from last pyridostigmine dose to clinical testing was kept constant in one and the same patient on the two test days, but was allowed to vary between patients. Dosage of the immunosuppressive medication had to be stable in the 3 months before revaccination till at least 4 weeks after tetanus revaccination.

The exclusion criteria were: instable disease based on medication use or a Myasthenia gravis Foundation America classification (MGFA) classification of 4 or 5, presence of a thymoma, use of vitamin K antagonist or new oral anti-coagulants (NOACs), other relevant immunosuppressive/secondary immunodeficiency conditions (not applicable on screened patients), pregnancy, no previous tetanus vaccination or tetanus revaccination in the past year.

	AChR MG	MuSK MG	LEMS	Total	(%)
Number of patients	50	6	9	65	
Gender, female (%)	37	3	6	46	(70.7)
Age, median years (range)	56	44.5	49.3	55	(21-65)
Duration of disease, mean years (SD)	14.6	5.5	9.7	13.1	(11.9)
MGFA classification*					
0 (%)	4	3	2	9	(13,8)
1 (%)	4	1	0	5	(7,7)
2 (%)	40	2	5	47	(72,3)
3 (%)	2	0	2	4	(6,2)
Use of immunosuppressive medication, %	46	83.3	44.4	49.2	
Prednisolone, %	14	16.7	33.3	16.9	
Mean daily dose, mg (range)	10.3	7.5	7.5		(0-15)
Azathioprine, %	30	33.3	22.2	29.2	
Mean daily dose, mg (range)	108	75	125		(25-200)
Mycophenolic acid, %	4	33.3	11.1	7.7	
Mean daily dose, mg (range)	1250	750	1500		(500-2000)
Cyclosporine, %	6	0	0	4.6	
Mean daily dose, mg(range)	140	0	0		(75-200)
Combination of immunosuppressive medication, %	18	16.7	33.3	20	
Thymectomy in the past (>1 year ago, N) (%)	29	0	0	29	(44.6)
Last tetanus vaccination, years ago (SD)	26.4	13.5	24.1	24.9	(19.5)

Table 1. Baseline characteristics

* MGFA classification: Myasthenia gravis foundation America classification

2.3 Placebo AChR MG group

Twenty-three AChR MG patients were intramuscularly injected with a placebo (saline). These patients fulfilled the same in- and exclusion criteria and completed the same clinical outcome scores (Quantitative Myasthenia Gravis (QMG) score, MG composite (MGC) score and the MG specific activities of daily living (MG-ADL)) at the same time points, before and 4 weeks after receiving placebo.

2.4 Sampling protocol and clinical scoring

The QMG, MGC and the MG-ADL are the clinical outcome measures that were used. The QMG is a 13-item scale that measures muscle strength and endurance. The MGC is a composite scale selected from existing MG-specific scales (MG-ADL, QMG and Manual Muscle Test). The MG-ADL is a scale to assess MG symptoms that patients experience in their daily activities. For all three outcome measures, higher scores indicate more severe clinical MG [8-12]. These three clinical outcome scores were performed before and 4 weeks after tetanus revaccination. The MG-ADL was repeated by the physician by telephone at 12 weeks after revaccination.

2.5 Tetanus vaccine

A commercially available tetanus vaccine was used, manufactured by Bilthoven Biologicals (tetanus vaccine, RVG 17639) [13]. One dose of 0.5 mL contains ≥40 IU tetanus toxoid (TT), 1.5 mg aluminium phosphate and 0.05 mg thimerosal. Administration was intramuscularly, as a bolus, in the non-dominant upper arm.

2.6 Tetanus antibody response

IgG1, IgG4 and IgG total tetanus antibodies were quantified using a previously described ELISA[7], with the exception of using tetanus toxoid (NIBSC 02/232, National Institute for Biological Standards and Control, London, UK) for coating and the World Health Organization (WHO) 1th international standard for tetanus immunoglobulin (NIBSC TE3) for calibrating of the quantification. Titers were measured in serum samples taken at the same day of, but prior to, tetanus revaccination and 4 weeks thereafter. Criteria for a significant response against the tetanus booster were defined as either a ≥ 1.25-fold increase in IgG total TT antibodies and reaching a minimum titre of 5 µg/mL or a twofold increase in antibody concentration and a minimum titre of 1 µg/mL IgG total TT antibodies [7]. The pre immunization titre is considered protective above ≥0.1 IU/ml, which is equal to 0.05 µg/mL [14, 15]. The TT antibody response of a historic control group of 20 TT revaccinated healthy adults served as a reference for the normal range of anti-TT titr.

2.7 Antibodies against AChR, MuSK and VGCC

The AChR, MuSK and VGCC antibody titres were measured with a commercially available radio immunoprecipitation assay (RIA)(RSR ltd.)[16]. Titres were measured using multiple dilutions of each serum sample taken before and 4 weeks after tetanus revaccination.

2.8 Standard protocol approvals, registrations, and patient consents

The study was approved by the Local Committee on Medical Ethics of the Leiden University Medical Centre. Subjects provided written informed consent for participation in the study and received reimbursement of travel costs.

2.9 Statistical analysis and power

The study is powered for an expected response rate of 75% with a 95%-confidence interval of 63-87%. Statistical analysis was performed with Graph-Pad Prism software (version 7) and SPSS version 23. In all tests $p<0.05$ was considered statistically significant. Tetanus IgG titres were log transformed. Comparison for normally distributed numerical variables was done with the ((un)paired) T-test or a one-way analysis of variance (ANOVA). Anti-TT antibody responses were compared between the AChR MG patients, the LEMS patients and the MuSK MG patients, respectively, and the healthy controls. Within the AChR MG group, responses were compared between patients with and without immunosuppression and between patients with and those without thymectomy.

3. RESULTS

3.1 Patient characteristics

Fifty-one AChR MG patients (74% female, median age 56 years, range 21-65 years) were revaccinated with tetanus toxoid in the period from March 2015 to November 2015. One patient was excluded from analysis because of receiving other vaccinations (Diphtheria/tetanus/polio (DTP) and typhoid), before the control time point 4 weeks after tetanus revaccination. Also, 6 patients with MuSK MG and 9 with LEMS, representing more rare myasthenia subtypes, were included. There were no significant differences in baseline characteristics between patients with and without immunosuppressive medication (IM). Patients characteristics are given in Table 1.

3.2 Response to Tetanus revaccination

The AChR MG group had a significantly lower geomean titre (GMT) of IgG total anti-TT before ($p=0.003$) and after ($p= 0.03$) tetanus revaccination compared to healthy controls (HC) (Figure 1A). The AChR MG group also had a significantly ($p=0.02$) lower mean IgG1 titre before revaccination than the HC group, but not 4 weeks after revaccination. No significant difference in IgG4 titres before and after revaccination was found between the AChR MG and HC groups (Figure 1A). Nevertheless, even before revaccination all patients had a protective IgG total anti-TT titre (> 0.05 µg/mL according to the World Health Organization (WHO)[15]). To investigate the effect of immunosuppressive medication (IM), we divided the AChR MG group in a subgroup with (n=23, Table 1) and one without (n=27) IM (IM+ and IM-, respectively). Both subgroups had a significant lower GMT before revaccination compared to HC (IM-, $p=0.02$; IM+, $p<0.01$), but only the IM+ group had a significantly ($p<0.01$) lower GMT 4 weeks after revaccination. There was no significant difference between

the IgG total anti-TT GMT of the IM- and the IM+ subgroups (Figure 1B). The increase factor of the IgG total anti-TT titre after revaccination was not significantly different between the HC group (mean 23-fold increase, range 1.25-313), the IM- (mean 31-fold, range 1.51-445) and the IM+ subgroups (mean 14-fold, range 0.68-70), although patients with a lower increase factor were mostly IM+ patients. The increase factor was lower in individuals with higher pre-vaccination titres (Figure 2A). Four weeks after tetanus revaccination, 46 AChR MG patients did significantly respond to tetanus revaccination (Figure 2B). Thus, the response rate in the AChR MG group is 92% (95%CI 81-98%).

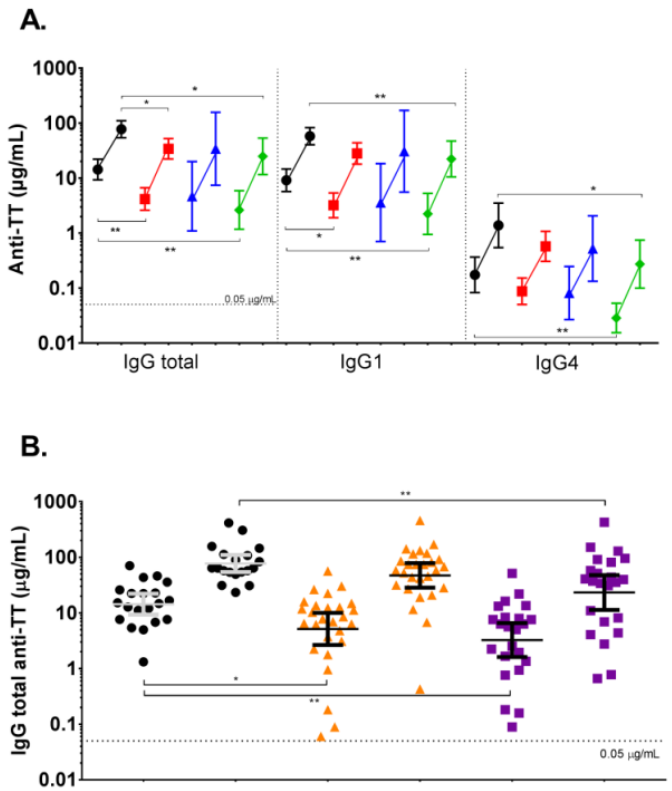
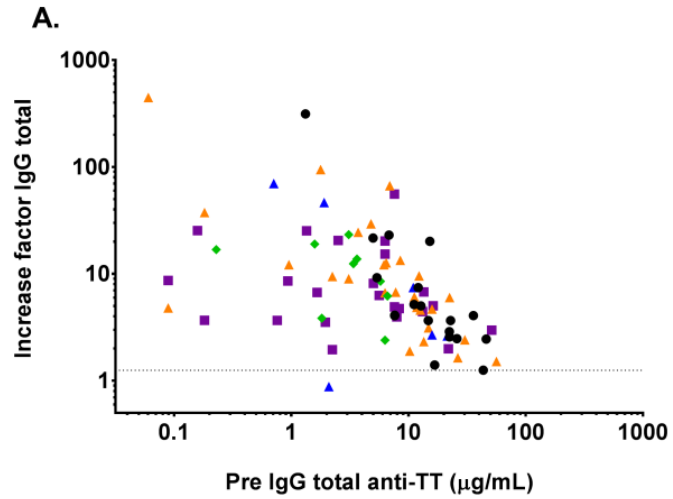


Figure 1

A. Response to tetanus revaccination. Geomean titres (GMT) of tetanus toxoid (TT) specific IgG total, IgG1 and IgG4, pre and 4 weeks post-vaccination with a 95%CI. Groups consist of: 20 healthy controls (●), 50 patients with AChR MG (■), 6 patients with MuSK MG (▲) and 9 with LEMS (◆). The dotted line is the minimal IgG total anti-TT titre that is considered as protective (0.05 µg/mL). Anti-TT titres were log transformed. * p<0.05, ** p<0.01.

B. Effect of immunosuppressive medication. Geomean titres of IgG total anti-TT in HC (●) and AChR MG with (■) and without (▲) immunosuppressive medication (IM).

The dotted line is the minimal IgG total anti-TT titre that is considered as protective (0.05 µg/mL). Anti-TT titres were log transformed. *p<0.05, **p<0.01.



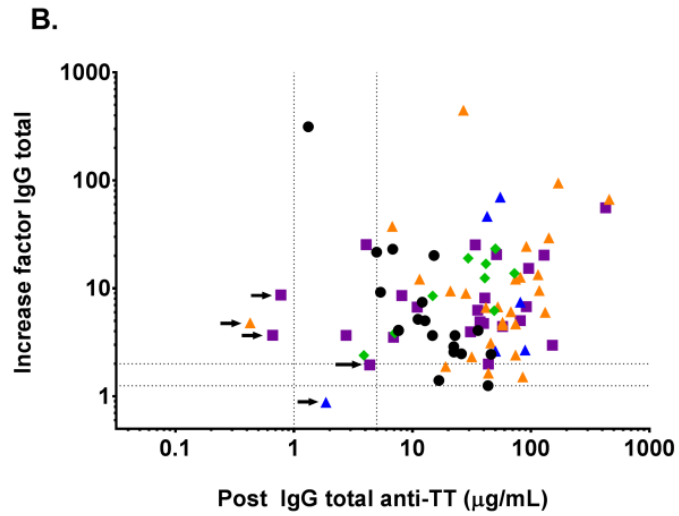


Figure 2

A. The factor increase of the IgG total anti-tetanus toxoid (TT) titre in the healthy controls (●), in patients with AChR MG with (■) and without immunosuppressive medication IM (▲) and in the patients with MuSK MG (▲) and LEMS (◆) is dependent on the pre revaccination IgG total anti-TT titre.

B. To fulfil the criteria of a significant response, a factor increase of 1.25 or 2 times the pre revaccination IgG total anti-TT titre (horizontal dotted lines) and a post IgG total anti-TT titre > 1 µg/mL or 5 µg/mL (vertical dotted lines), respectively. The arrows indicate patients who don't meet one of these criteria.

The healthy controls had a significantly (p<0.0001) lower median age (median age 33 years, range 20-55 years) than AChR MG patients (median age 56 years, range 21-65 years). From the HC group 55% is female vs. 74% in the AChR MG group. In the HC group the TT response showed a tendency (p=0.07) to be age-dependent. The controls in the age category >50 years had a lower post IgG total TT titre (mean GMT 43.4 µg/mL, 95%CI 20.7-90.4) than the controls <30 years of age (mean GMT 109.9 µg/mL, 95%CI 61.5-196.3). In the AChR MG group, containing only a few young patients, such a difference based on age groups was not observed. The years passed since the last tetanus revaccination did not affect the increase factor of the TT titre.

Since the response to tetanus toxoid is T-cell dependent and almost half of our AChR MG group (58%) underwent a thymectomy in the past (Table 1), we tested whether a thymectomy impacted the antibody response. We found no significant difference in pre (p=0.8) and post IgG total TT titre (p=0.2) between the groups with (pre mean GMT 4.0 µg/mL, 95%CI 2.1-7.5; post mean GMT 27 µg/mL, 95%CI 14.9-49) and without (pre mean GMT 4.5 µg/mL, 95%CI 2.1-9.5; post mean GMT 46.8 µg/mL, 95%CI 25-87.5) a thymectomy.

In the LEMS group, the mean GMT of pre and post-revaccination IgG total (pre, p<0.01; post, p<0.01), IgG1 (pre, p<0.01; post, p<0.01) and IgG4 (pre, p<0.01; post, p=0.03) TT titre was significantly lower, compared to that of healthy controls (Figure 1A). There was no significant difference in pre and post-revaccination IgG total, IgG1 and IgG4 anti-TT titre in the MuSK MG group, compared to healthy controls (Figure 1A).

3.3 Antibodies against AChR, MuSK and VGCC

To investigate if tetanus revaccination affects auto-antibody levels, AChR, MuSK and VGCC antibody titres were measured. No changes in all these antibody titres were observed 4 weeks after revaccination compared to the day of revaccination (Figure 3).

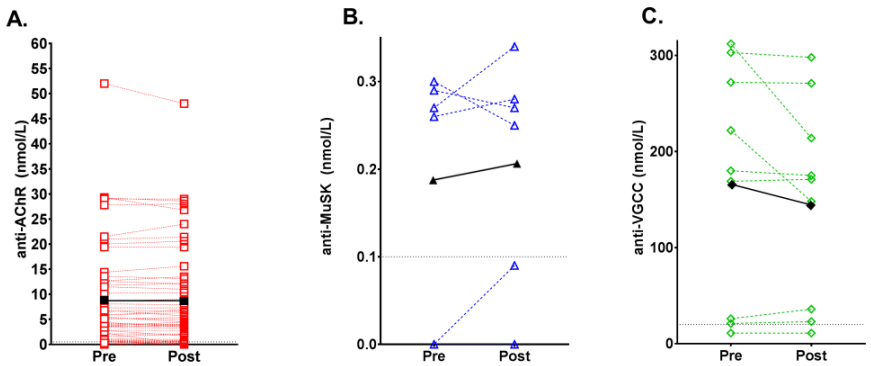


Figure 3. Anti-AChR (A), anti-MuSK (B) and anti-VGCC (C) antibody concentrations before and 4 weeks after revaccination with tetanus. The dotted lines indicate the minimal titre that is considered as positive (anti-AChR: 0.5 nmol/L; anti-MuSK: 0.1 nmol/L and anti-VGCC: 20 nmol/L). Black: mean titres of the group, in colour individual titres are depicted.

3.4 Clinical scores

The MGC score, QMG score and MG-ADL were obtained at the visit of the revaccination and 4 weeks after revaccination to measure the impact of revaccination on disease severity. Individual scores and the delta of the scores of the AChR MG group (n=50) and the placebo AChR MG group (n=23) are shown in Figure 4. Total scores for these 3 outcome measures pre-revaccination were comparable between the tetanus revaccination group and the placebo group. There was no significant change of the mean score of the MGC and MG-ADL after revaccination / placebo administration in these respective groups. The QMG score showed a significant increase (p<0.01) at 4 weeks in the AChR MG revaccinated group (Figure 4D). The delta of the QMG in the AChR MG revaccinated group also showed a statistically significant increase (p=0.01) compared with the delta of the placebo group. Mean increase of the QMG in the AChR MG revaccinated group was 1.08 points, 95%CI 0.5-1.7 (Figure 4F). The MG-ADL was also evaluated after 12 weeks in the tetanus revaccination group. The mean MG-ADL score showed a significant decrease of 0.86 point (95%CI 1.6-0.2) after 12 weeks compared with the MG-ADL score before revaccination (data not shown). At individual level there was a large variation between the three clinical outcome scores. Only one patient showed a clinical relevant increase in all tests. But all patients who had a worse score on the MG-ADL after 4 weeks, normalised to the pre-vaccination MG-ADL score after 12 weeks. We also obtained clinical outcome scores for the MuSK MG and LEMS patients before and 4 weeks after revaccination. The clinical scores were not statistically different in these two groups (data not shown).

3.5 Non-responders

There were 5 non-responders to tetanus upon revaccination, 4 with AChR MG and 1 with MuSK MG (arrows in Figure 2B). One AChR MG patient did not reach the required factor of increase (1.95-fold instead of 2-fold increase) of the IgG total TT titre and reached a post TT titre of 4.36 µg/mL. This patient used cyclosporine A (a daily dose of 200 mg) and mycophenolic acid (daily dose of 2000 mg) and was the only one with this combination of IM. Three other non-responsive AChR MG patients showed an adequate ratio between post and pre IgG total TT titre (> 2-fold), but the post titre was below the lower threshold of 1 µg/mL. These patients received their last tetanus boost at the age of 9, which was >50 years ago. Two of them used prednisolone at a dose of 10 and 15 mg every other day, without other immunosuppressive agents. These conditions may have interfered with their response to tetanus. The fifth non-responder was a patient with MuSK MG who received rituximab 22 months before vaccination.

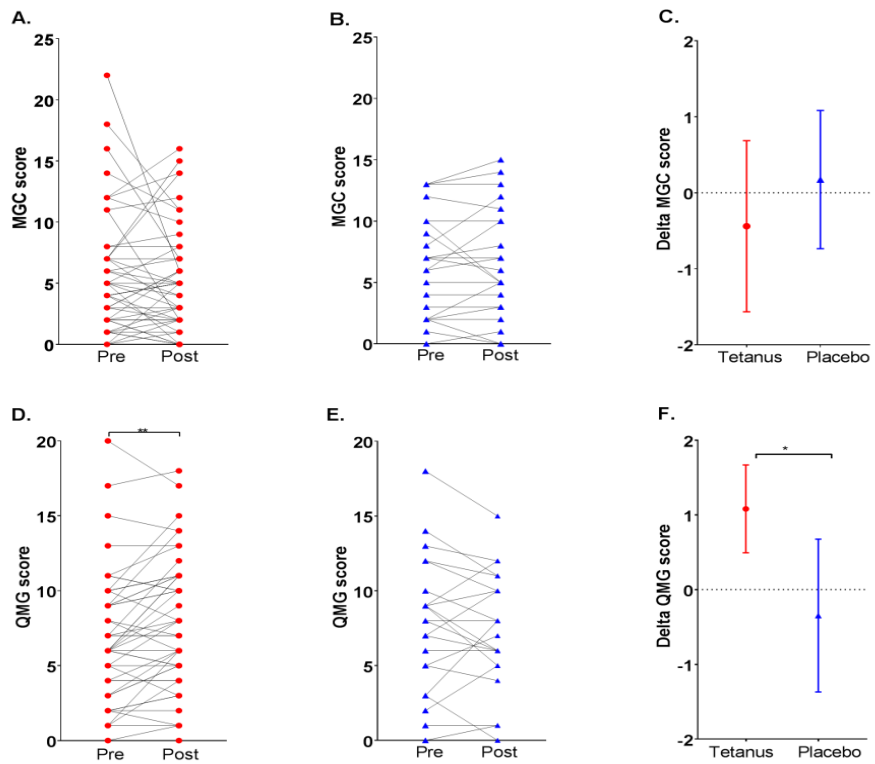


Figure 4. Individual clinical scores of MG Composite score (MGC), (A: AChR MG with tetanus revaccination, B: AChR MG with placebo), Quantitative Myasthenia Gravis score (QMG), (D: AChR MG with tetanus revaccination, E: AChR MG with placebo) and Myasthenia Gravis Activities of Daily Living (MG-ADL) (G: AChR MG with tetanus revaccination, H: AChR MG with placebo) at the day of tetanus revaccination or placebo administration and 4 weeks thereafter. Delta of the scores for the group of AChR MG with a tetanus revaccination and the AChR MG group who received placebo is shown (C, F, I). *p<0,05, ** p<0.01

4. DISCUSSION

In this prospective study we showed that tetanus revaccination is safe and effective in patients with MG. The vaccinated population consisted of patients who had a stable disease, based on the MGFA classification and a stable medication regime in the past 3 months. Tetanus revaccination evoked a significant antibody response in 92.3% (60/65) of the study cohort. We found that immunosuppressive medication slightly lowered pre and post tetanus antibody titres. However, in the subgroups with and without immunosuppressive medication there was a median 6-fold increase factor of the tetanus antibody titre. No immunological exacerbation was found as the AChR, MuSK or VGCC antibody titres did not change after revaccination. Overall, tetanus revaccination proved to induce a significant humoral response and to be safe in this study cohort with stable disease. Patients with more severe or instable disease or receiving a higher dose of immunosuppressive medication might respond differently.

Although pre and post titres are lower in a part of our patients, all patients were protected for a tetanus infection according to WHO guidelines [15]. This is similar to our historic control group of healthy controls. It also corresponds with a previous study that measured the IgG level of diphtheria and tetanus antibodies in patients with MG, without revaccination [14]. In the latter study no significant difference in the protection rate between healthy controls, patients with systemic lupus erythematosus (SLE) or MG was found [14]. Other prospective vaccination studies in patients with autoimmune diseases were performed in SLE and ANCA+ vasculitis, which also suggest that vaccination is safe and effective [3-5]. However, lower response rates than in healthy controls were observed. This differs from our study, but might be due to the type of vaccine (Pneumococcal polysaccharides, a T-cell independent vaccine, and Influenza, respectively) and the kind of treatment that patients received.

Part of our patients with immunosuppressive medication had lower levels of tetanus antibody titres, but immunosuppressive medication did not affect the ability to respond to revaccination. Due to small size of treatment subgroups it was not possible to investigate the effect of specific treatment modalities on tetanus antibody titres. Studies in other autoimmune diseases described the effect of immunosuppressive medication, like rituximab, azathioprine or TNF-α blockers [17-19]. These studies showed only a modestly impaired immune response in patients with TNF-α blockers, but a long-term effect of rituximab [17, 18]. Indeed, one of our non-responding patients was treated with rituximab 22 months before revaccination. A study in inflammatory bowel disease patients reported that azathioprine limits the immune response to hepatitis B vaccine. In the group without azathioprine, 88% (103/117 patients) reached protective titres (anti-HBs titres >10 IU/L) compared to only 55% (47/86 patients) in the group with azathioprine [19]. Prednisone has a dose-dependent effect on the immune system; a daily dose of less than 10 mg is considered non-immunosuppressive [20]. Overall, the results of our study add to these observations that immunosuppressive medication influences the height of the

humoral immune response, but affects tetanus toxoid responsiveness as such in only a very limited number of cases.

The primary clinical outcome measure in our study was the MGC, which showed no change 4 weeks after revaccination compared to the day of revaccination. The QMG was the only secondary clinical outcome measure that suggested some worsening of the MG. This showed a statistically significant increase of 1 point at 4 weeks, which is less than the minimal clinically relevant difference of 2.3 points described in literature, and fits within normal fluctuation of MG [8, 9]. In contrast, the MG-ADL showed a marginal improvement at 4 weeks, and even a statistically significant improvement at 12 weeks after revaccination, compared to the MG-ADL score before revaccination. Our placebo group did not show a statistically significant difference for any outcome measure at 4 weeks. Therefore, after revaccination an individual patient might experience a temporarily, clinically insignificant worsening of symptoms, which in all cases recovered 12 weeks after revaccination. These conclusions are supported by the observation that tetanus revaccination has no impact on titres of disease-specific antibodies. At an individual level, variation between pre- and post- revaccination clinical outcome scores was quite large in both the revaccination and the placebo group. This likely reflects characteristic disease fluctuation in MG, but also demonstrates limitations of the use of these clinical outcome scores as primary outcome measures. Of note, in our study anti-TT antibody responses were determined at 4 weeks and clinical outcome measures at 4 and 12 weeks after revaccination. In most clinical trials of new vaccines data are collected up to 6 weeks after vaccination [21]. Currently, a similar study is performed on the antibody response upon the yearly influenza virus (re)vaccination and its safety in MG patients.

In conclusion, patients with AChR MG are able to mount an antibody response to a tetanus revaccination, irrespective of immunosuppressive medication. Tetanus revaccination does not induce an immunological exacerbation of AChR MG. At group level, clinical relevant worsening is absent, and does not impair daily activities of patients.

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Chapter 5

Influenza vaccination in patients with myasthenia gravis

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1. INTRODUCTION

Myasthenia gravis (MG) is an acquired autoimmune disease of the neuromuscular junction and is characterized by fluctuating weakness and fatigability of skeletal muscles [1]. In the majority of MG patients acetylcholine receptor (AChR) antibodies are found [1]. Symptomatic treatment is often insufficient, and a considerable proportion of patients need long-term immunosuppressive medication (IM). Patients with an autoimmune disorder are generally believed to be at an increased risk of infection, either due to their immunosuppressive therapy or due to immune abnormalities associated with their disease [2, 3]. Conversely, an infection can cause exacerbation of symptoms, potentially resulting in a myasthenic crisis. Specific data on infection rates in myasthenic patients are not available [4].

Little is known about the efficacy and safety of vaccination in patients with autoimmune diseases. No specific guidelines regarding vaccinations in patients with MG exist, but a small number of observational studies suggest that influenza vaccination is safe [5-7] and recently a randomized controlled trial showed that influenza vaccination has no influence on AChR antibody titres[8]. In a recent study we found a small, temporary, but significant increase in Quantitative Myasthenia Gravis scores (QMG) after tetanus vaccination. However, this was far less than what is generally considered clinically relevant [9]. In the Netherlands, annual vaccination against influenza is recommended for all patients with an autoimmune disease [10]. However, in our personal experience and as described earlier [5], many patients express concern that vaccination may lead to an exacerbation and a substantial number decline vaccination each year based on these concerns. This is unfortunate, as seasonal vaccination against influenza is highly effective in reducing laboratory-confirmed influenza illness, hospital admissions and risk of death, especially in elderly and frail patients [11]. This is relevant, as this age group has the highest incidence of autoimmune MG [12]. Another concern is that IM may hamper the development of protective antibody levels. Therefore, we performed a double-blind placebo-controlled trial to investigate the efficacy and safety of the seasonal (2016/2017) influenza vaccine in patients with AChR MG with and without IM.

2. MATERIALS AND METHODS

2.1 Standard protocol approvals, registrations, and patient consents

This study was approved by the Local Committee on Medical Ethics of the LUMC. Subjects provided written informed consent and received reimbursement of travel costs. The trial is listed on clinicaltrialsregister.eu under 2016-003138-26.

2.2 Patients

We included 47 patients with AChR MG and 47 healthy controls at the start of the flu season (October 2016). AChR MG patients were recruited from the neurology outpatient clinic of Leiden University Medical Center (LUMC) and through the national patient organization. Seasonal influenza vaccination was offered at the start of the flu season to all LUMC employees; healthy controls were recruited from this population.

Inclusion criteria for the patient group were a diagnosis of AChR MG, age ≥ 18 years and stable disease in the past 3 months. Diagnosis of AChR MG was based on clinical signs or symptoms consistent with MG and a positive serological test for AChR antibodies. A maximum daily dose of 30mg of prednisolone, with a variation of +/- 5mg during 3 months before participation was allowed as well as use of other immunosuppressive medication.

During the study, patients were on a stable dose of their medication (see Table 1). Time from last pyridostigmine dose to clinical testing was kept constant for each patient on test days, but was allowed to vary between patients. Inclusion criteria for healthy controls were an age ≥18 years and no autoimmune disease or immunosuppressive medication.

Exclusion criteria for the AChR MG group were: instable or severe disease as evidenced by recent changes in medication or an MGFA classification of 4 or 5, presence of a thymoma, use of vitamin K antagonist or new oral anti-coagulants (NOACs), pregnancy and other diseases of the immune system that may affect the efficacy of vaccination.

2.3 Study protocol

This single-center, prospective, double-blind, randomized, placebo- controlled study was performed at the LUMC. Randomization was performed by a randomization list created by the hospital pharmacy. Patients and physicians performing clinical tests were blinded for treatment allocation until the end of T1. Research nurses, who administered the vaccination, were not blinded, because the placebo was provided in a different syringe than the commercial influenza vaccine. Patients were randomized to receive either an intramuscular injection with the influenza vaccine or a placebo (0.5 mL 0.9% NaCl) (T0). At T0 age, sex, disease duration, use of medication, MGFA classification, thymectomy and seasonal influenza vaccinations in the previous 3 years were recorded. Prior to injection (T0) and four weeks later (T1), serum and several clinical outcome measures were obtained. Four weeks (T1) after this first vaccination, patients were unblinded and patients in the placebo group were vaccinated with the influenza vaccine (Figure 1). At T2, 4 weeks after the flu vaccination, a third blood sample and MG specific activities of daily living (MG-ADL) score were obtained from the (initial) placebo group. In all patients, an MG-ADL was obtained by phone by a research nurse, twelve weeks after influenza vaccination (T3). At T1, T2 and T3 AChR MG patients were asked for side effects and exacerbation of their MG symptoms. Healthy controls were asked for side effects at T1. Figure 1 shows an overview of the study design.

	AChR MG Vaccination	AChR MG Placebo	HC	Total
Number of patients – n	24	23	47	94
Gender, female (%)	11 (45.8)	14 (60.9)	36 (76.6)*	61 (64.9)
Age, median years (range)	61.5 (32-72)	63 (22-74)	54 (24-65)*	
Duration of disease, mean years (SD)	14.3 (13.9)	10.7 (9.9)	-	
MGFA classification**			-	
0 – n (%)	10 (41.7)	8 (34.8)	-	
1 – n (%)	0	5 (21.7)	-	
2 – n (%)	13 (54.2)	9 (39.1)	-	
3 – n (%)	1 (4.2)	1 (4.3)	-.	
Use of immunosuppressive medication, n (%)	15 (62.5)	14 (60.9)	-	
Prednisolone, n (%)	9 (37.5)	11 (47.8)	-	
Mean daily dose, mg (range)	9.2 (5-20)	6.8 (1-10)	-	
Azathioprine, n (%)	13 (54.2)	10 (43.5)	-	
Mean daily dose, mg (range)	131.2 (50-200)	116.7 (50-200)	-	
Mycophenolic acid, n(%)	0	2 (8.7)	-	
Mean daily dose, mg (range)	-	2000 (2000)	-	
Cyclosporine, n (%)	3 (12.5)	0	-	
Mean daily dose, mg(range)	166.7 (150-200)	-	-	
Combination of immunosuppressive medication, n (%)	8 (33.3)	8 (34.8)	-	
Thymectomy in the past (>1 year ago) – n (%)	15 (62.5)	14 (60.9)	-	
Past seasonal trivalent inactivated influenza vaccination - n (%)			39 (83)	78 (83)
2015-2016	15 (62.5)	16 (69.6)	28 (59.6)	59 (63)
2014-2015	16 (66.7)	17 (73.9)	33 (70.2)	66 (70)
2013-2014	16 (66.7)	15 (65.2)	31 (66)	62 (66)

Table 1. Baseline characteristics. The AChR MG group is divided in the in vaccination and placebo group. *Healthy controls are significantly younger (p=0.001) than the AChR MG group en consist out of significantly more females (p=0.02). ** MGFA classification: Myasthenia gravis foundation America classification.

2.4 Influenza vaccine

We used the commercially available influenza vaccine manufactured by Sanofi Pasteur (Vaxigrip, RVG 22306) for the season 2016/2017. One dose of 0.5 mL contains 15 µg haemagglutinin of each of the influenza virus strains in the split inactivated influenza vaccine: A/California/7/2009 (H1N1)pdm09, A/Hong

Kong/4801/14 (H3N2) and B/Brisbane/060/08 (B/Victoria/2/87- line). The vaccine was administered intramuscularly, as a bolus, in the non-dominant upper arm.

2.5 Influenza antibody response

The primary endpoint of this study was change in titre of antibodies to the flu vaccine strains. A secondary endpoint was the effect of IM on the humoral response. Antibodies to the vaccine strains A/California/7/2009 (H1N1)pdm09, A/Hong Kong/4801/14 (H3N2) and B/Brisbane/060/08 were measured using the hemagglutination-inhibition (HI) assay, according to standard methods at the national influenza center at the Erasmus Medical Center[13]. Titres below the detection limit (i.e. ,≤1:10) were assigned a value of 1:5. Geometric mean titres (GMTs) and seroprotection rates (defined as HI titres ≥1:40) were chosen as the main outcome measures. Seroconversion was defined as a post-vaccination HI titre of at least 1:40 combined with at least a four-fold increase in titre. A non-responder was defined as a post vaccination HI-titre of <1:40.

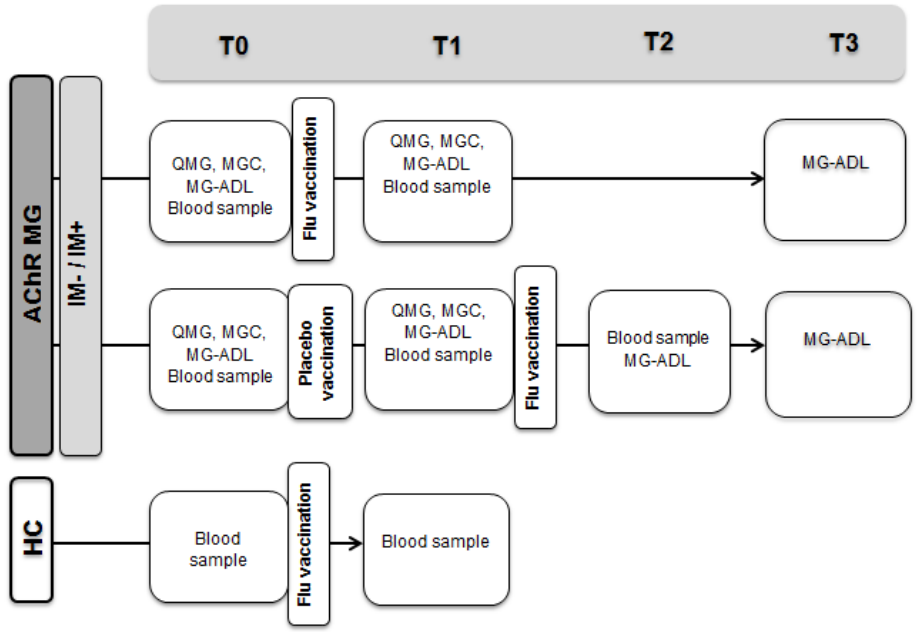
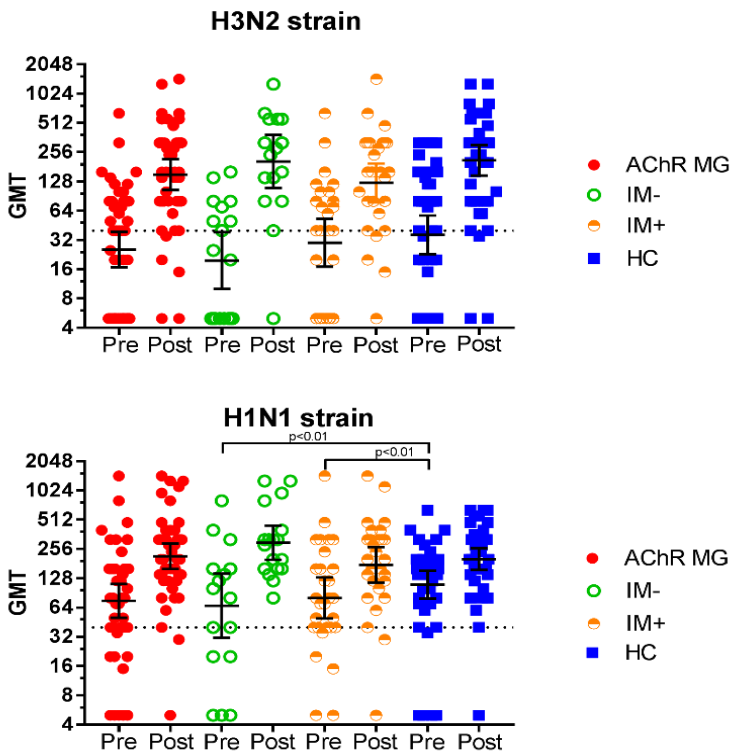


Figure 1. Study flowchart. T0: baseline, T1: 4 weeks after influenza (flu) or placebo vaccination, T2: 4 weeks after vaccination in placebo group, T3: 12 weeks after vaccination with influenza. In the AChR MG vaccination group a blood sample was taken at T0 and T1 and in the AChR MG placebo group at T0, T1 and T2 in the placebo group. HC: Healthy controls . QMG: Quantitative Myasthenia Gravis score, MGC: Myasthenia Gravis Composite score, MG-ADL: myasthenia gravis activities of daily living score.



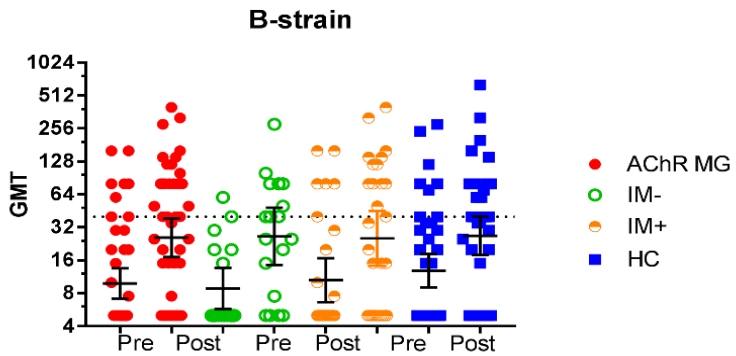


Figure 2. Response to influenza vaccination. Geomean titres (GMT) of H3N2, H1N1 and B-strain, pre and 4 weeks post-vaccination with a 95%CI. Groups consist of: 47 AChR MG patients, 18 AChR MG patients without immunosuppressive medication (IM-), 29 AChR MG patients with IM+ and 47 healthy controls. The dotted line is the minimal GMT that is considered as protective (HI-titre 1:40).

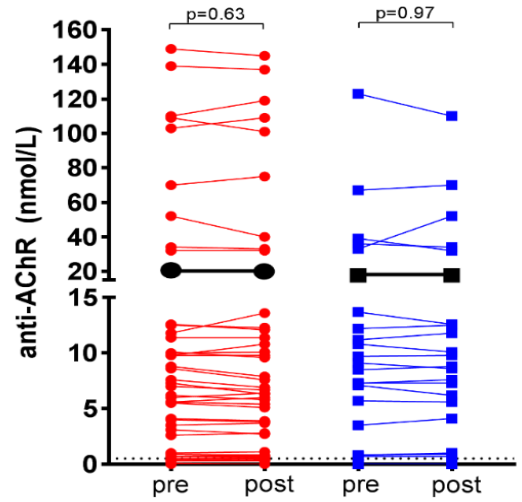


Figure 3. Anti-AChR antibody concentrations before and 4 weeks after vaccination with influenza in all MG patients and before and 4 weeks after placebo administration. The dotted line indicate the minimal titre that is considered as positive (0.5 nmol/L). Black: mean titre of the group, individual titres are depicted in colour (● Vaccination group (n=47); ■ Placebo group (n=23)).

2.6 Sampling protocol and clinical scoring

Another secondary endpoint was a clinical relevant change in clinical scores. We used the QMG, MG Composite (MGC) and the MG-ADL scores as clinical outcome measures. The QMG is a 13-item scale that measures muscle strength and endurance, ranging from 0 to 39. The MGC is a composite scale selected from existing MG-specific scales (MG-ADL, QMG and Manual Muscle Test (MMT)), ranging from 0 to 50. The MG-ADL is a scale to assess MG symptoms that patients experience in their daily activities, ranging from 0 to 24. A change of 2.3 points for the QMG, 3 for the MGC and 2 points for the MG-ADL was considered clinically relevant [14-16]. For all three outcome measures, higher scores indicate a higher clinical severity of MG[14-18].

2.7 Antibodies against AChR

The last secondary endpoint was a change in antibodies against AChR. AChR antibody titres were measured with a commercially available radio immunoprecipitation assay (RIA)(RSR Ltd., Cardiff, UK)[19]. Absolute titres were measured using multiple dilutions of each serum sample.

2.9 Statistical analysis and power

The study was powered for an expected response rate (i.e. seroprotection rates) of 75% with a 95%-confidence interval of 63-87% in MG patients. Herefore, 50 patients with MG were needed. Statistical analysis was performed with Graph-

Pad Prism software version 7 and SPSS version 23. In all tests p<0.05 was considered statistically significant. Influenza titres were log transformed in order to normalize the data. Comparison for normally distributed numerical variables was done with paired or unpaired T-tests or a one-way analysis of variance (ANOVA). Influenza virus specific antibody responses were compared between AChR MG patients (with and without immunosuppression) and healthy controls. Within the AChR MG group, patients with and those without thymectomy (Tx) were compared. The AChR antibody titres before vaccination of all AChR MG patients were compared to titres 4 weeks after influenza vaccination. The clinical outcome measures were compared between the AChR MG vaccination and placebo group.

3. RESULTS

3.1 Patient characteristics

Forty-seven patients (53.2% female, median age 62 years, range 22-74 years) and 47 healthy controls (76.6% female, median age 54 years, range 24-66 years) were vaccinated with the seasonal influenza vaccine from October to December 2016. Healthy controls were significantly younger (p=0.001) and were more frequently female (p=0.02) than the MG group. In the MG group, 23 patients randomly received a placebo injection followed by flu vaccination 4 weeks later. Baseline characteristics did not differ between the two MG patients groups that either received first the flu vaccination or the placebo vaccination. The MG group consisted of 29 patients with (IM+) and 18 without (IM-) immunosuppressive medication. The IM+ group was significantly older (p<0.01) than the IM- group and contained more female patients (p=0.04). Disease duration and whether a patient underwent a thymectomy in the past was not significantly different between IM- and IM+ groups (p=0.4 and p=0.16, respectively). Baseline characteristics are given in Table 1.

3.2.1 Serological response to Influenza vaccination

Upon vaccination the MG group (n=47) developed a geomean titre (GMT) for all three vaccine strains that was similar to the HC group (H3N2, p=0.2; H1N1, p=0.7; and B-strain, p=0.9) (Figure 2). The post-vaccination seroprotection and seroconversion rates were comparable between the MG group and HC group for all strains. In the MG group, 40.4% of all patients (19/47) reached a seroprotective titre for all three strains. In the HC group this was 51% (24/47) (Table 2).

3.2.2 Influence of use of immunosuppressive medication and thymectomy

No significant effect on the serological response to influenza vaccination was observed between the IM- (n=18) and IM+ group (n=29) (H3N2, p=0.2; H1N1, p=0.1; and B-strain, p=0.9). The pre-vaccination H1N1 GMT was significantly lower in both the IM- and IM+ groups (p<0.01 for both), but there was no significant difference in post-vaccination GMT compared to the HC group. Seroconversion and post-vaccination seroprotection rates were also similar between HC and the IM- and IM+ groups (Table 2).

	AChR MG (n=47)	IM- (n=18)	IM+ (n=29)	HC (n=47)
H3N2 strain				
Pre HI titre ≥1:40 – n (%)	25 (53.2)	8 (44.4)	17 (58.6)	26 (55.3)
Post HI titre ≥1:40 – n (%)	42 (89.4)	17 (94.4)	25 (86.2)	44 (93.6)
Pre GMT – value (95% CI)	26 (17-39)	20 (10-39)	30 (17-53)	36 (23-57)
Post GMT – value (95% CI)	150 (104-216)	205 (109-384)	124 (78-196)	210 (147-301)
Seroconversion – n (%)	22 (46.8)	11 (61.1)	11 (37.9)	26 (55.3)
H1N1 strain				
Pre HI titre ≥1:40 – n (%)	37 (78.7)	13 (72.2)	24 (82.7)	42 (89.4)
Post HI titre ≥1:40 – n (%)	45 (95.7)	18 (100)	27 (93.1)	46 (97.9)
Pre GMT – value (95% CI)	75 (50-112)	67 (31-143)	80 (49-131)	110 (79-153)
Post GMT – value (95% CI)	215 (159-291)	297 (198-446)	176 (115-268)	201 (156-259
Seroconversion – n (%)	15 (31.9)	6 (33.3)	9 (31)	9 (19.1)
B- strain				
Pre HI titre ≥1:40 – n (%)	8 (17)	2 (11.1)	7 (24.1)	10 (21.3)
Post HI titre ≥1:40 – n (%)	22 (46.8)	9 (50)	13 (44.8)	24 (51)
Pre GMT – value (95% CI)	10 (7-14)	9 (6-14)	11 (7-17)	13 (9-18)
Post GMT – value (95% CI)	26 (17-39)	26 (15-48)	25 (14-45)	27 (18-40)
Seroconversion – n (%)	12 (25.5)	7 (38.9)	5 (17.2)	13 (27.7)

Table 2. Humoral response to seasonal influenza vaccine 2016-2017. Chi-square tests showed no significant difference in pre and post HI titres between HC and AChR MG groups and between HC and IM-/IM+ groups.

Since the antibody response to influenza is T-cell dependent and a large portion of our patients (42.6%) underwent a thymectomy in the past (Table 1), we tested whether a thymectomy impacted the antibody response. We found no significant difference in pre- (H3N2, p =0.7; H1N1, p=0.6; B-strain, p=0.5) and post-vaccination GMT (H3N2, p =0.2; H1N1, p=0.4; B-strain, p=0.5), neither between patients with and without thymectomy, nor between patients and healthy controls (data not shown).

Both IM use and thymectomy can influence the absolute cell counts of T- and B-cells, therefore, we performed an immunophenotyping in all patients pre- and post-vaccination. Patients of the IM+ group had significantly lower absolute cell counts of CD19+ B-lymphocytes (mean 73x10^6/L, p<0.001), CD4+ T-lymphocytes (mean 621x10^6/L, p=0.02), CD8+ T-lymphocytes (mean 245x10^6/L, p=0.04) and NK-cells (mean 97x10^6/L, p<0.001) than patients of the IM- group. However, these values are in the range of healthy controls, except for the CD8+ T-lymphocytes

(normal values 260-990x10^6/L). There was no difference in absolute cell counts between the groups with and without thymectomy.

3.3 Non-responders

There were 5 non-responders in the MG group to H3N2 vaccination vs. 3 in the HC group, 2 to H1N1 vs. 1 in the HC group, 25 to the B-strain vs. 23 in the HC group. In the IM- group and IM+ group there were 1 and 4 non-responders respectively to H3N2, 0 and 2 respectively to H1N1, 9 and 16 respectively to the B- strain. The largest difference in response between IM- and IM+ groups was found for the B-strain: 9 non-responders in the IM- group and 16 in the IM+ group, although this apparent difference did not reach statistical significance: p=0.73. Of the 16 non-responders to the B-strain in the IM+ group, 12 used prednisone, 14 used azathioprine and 2 used three types of immunosuppressive medication (prednisone, azathioprine and cyclosporine). Only 1 MG patient and 1 HC were non-responders for all three strains.

3.4 Clinical scores

Figure 4 shows individual clinical scores and changes of the MG vaccination group (n=24) and MG placebo group (n=23) from T0 to T1. Use of IM was comparable (Table 1). Total scores of the three outcome measures were the same before and after vaccination between both groups. In addition, there was no significant change in the mean score or delta of all three outcome measures between T0 and T1. The MG-ADL also showed no significant difference 12 weeks (T3) after vaccination in the MG vaccination group compared to T0 and T1 (p=0.12). In the placebo group there was no significant difference between any of the 4 time points at which the MG-ADL was performed (T0-T3) (data not shown).

3.5 Antibodies against AChR

No change in antibody titre was observed 4 weeks after influenza vaccination (Figure 3).

3.6 Side effects

The MG vaccination group reported side effects in 30.4% (7/23) at T1, the placebo group in 37.5% (9/24) at T1 (p=0.6). At T2, 4 weeks after unblinded influenza vaccination of the placebo group 52% (12/23) reported side effects. At T1 healthy controls reported significantly more side effects (70%; 33/42) than the MG vaccination or placebo group (p<0.01). The most commonly reported side effects for MG or HC were local redness and soreness at the injection site. No change in MG symptoms was reported in the MG group at T1. In the placebo group, 3 patients reported a mild exacerbation of their MG symptoms during the T1-T2 period. Exacerbation of symptoms lasted 1 day to 1 week after vaccination and did not lead to a change in medication.

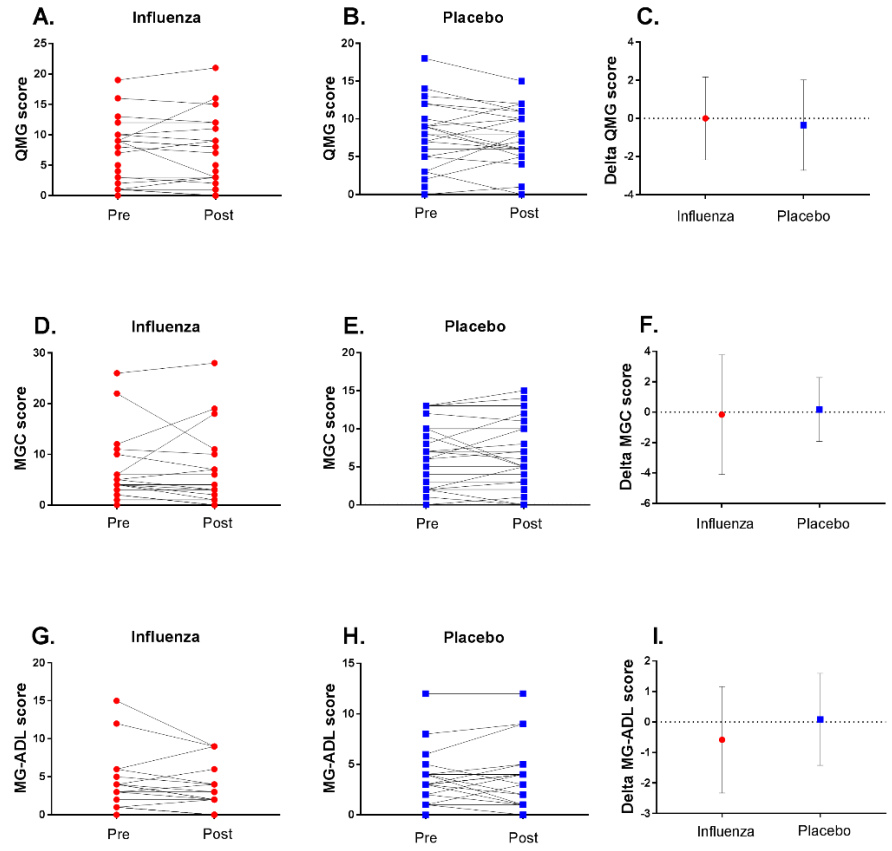


Figure 4. Clinical outcome measures for the AChR MG vaccination group ( ) and placebo group ( ) pre- and 4 weeks post-vaccination of the Quantitative Myasthenia Gravis score (QMG), Myasthenia Gravis Composite score (MGC) and Myasthenia Gravis Activities of Daily Living (MG-ADL) in A, B, D, E, G and H. The delta of the clinical outcome scores are shown in C, F and I. No significant differences were found.

4. DISCUSSION

In this prospective, double blind, randomized, placebo-controlled study we show that in AChR MG patients influenza vaccination is safe and induces an immune response comparable to that of healthy controls. The study population consisted of patients with stable disease and a stable medication regime in the past 3 months. A seroprotective titre for all three strains of the seasonal influenza vaccine was reached in 40.4% (19/47) of the AChR MG group and in 51% (24/47) of the HC group. IM or thymectomy status did not significantly influence post vaccination GMT titres. No clinical or immunological exacerbation was found as

clinical outcome scores and AChR antibody titres showed no significant changes. It is generally assumed that patients with an autoimmune disease are more prone to infections, resulting in increased morbidity and mortality [2]. In autoimmune inflammatory rheumatic disease, influenza-vaccinated patients have a lower incidence of pneumonitis, acute bronchitis and viral infections than unvaccinated patients [3]. To our knowledge no such studies have been performed in patients with MG. Recently a randomized controlled trial on influenza vaccination showed that influenza vaccination is safe, based on QMG scores and AChR antibody titres, but without including an healthy control group[8]. Studies on the efficacy of influenza vaccination in rheumatic disease also found that achievement of seroprotection (post HI-titre ≥1:40) is similar to healthy controls, irrespective of medication [3]. In patients with SLE, the response to influenza vaccination is comparable to that of healthy controls [3]. Two studies showed a trend towards a lower response to vaccination in patients who used azathioprine [20, 21], which is also commonly used in MG next to corticosteroids. In this study we did not find a significant effect of IM on the humoral response. Due to small size of treatment subgroups and because of frequent combinations of IM, we could not investigate specific effects of a single drug. In a study on the efficacy and safety of a tetanus vaccination in MG, we found that IM lowers pre- and post-vaccination GMTs, but did not affect the efficacy of the response [9]. This difference might be explained by the type of vaccine that is investigated and the vaccination history of the patients.

Some MG patients chose not to participate out of concern for an exacerbation of their symptoms. Even in our trial participants, only two-thirds had obtained an influenza vaccination in previous years, similar to the frequency of our healthy controls. The tetanus revaccination study in AChR MG patients showed a small but statistically significant increase of the QMG score of 1 point at 4 weeks, which is far less than the 2.3 points that is generally accepted as the minimal clinically relevant difference. A recent study indicated that an exacerbation of MG is more likely after an influenza-like infection or a common cold, than following an influenza vaccination (10/25 (40%) and 15/96 (15.6%) vs. 2/133 (1.5%) [7]. In line with our results, no clinical exacerbation was found in patients with RA and SLE following influenza vaccination [3]. Interestingly, unblinded influenza vaccination of MG patients in T1-T2 resulted in more reported side effects and a higher incidence of self-reported aggravation of MG symptoms than blinded vaccination or placebo injection. This may be explained by the presence of a prejudice among MG patients that vaccination might be harmful, leading to increased reporting of subjective complaints.

4.1 Strengths and limitations

The main strengths of this study are its placebo-controlled, double blind, randomized design and the systematic assessment of multiple relevant measures of clinical disease severity at multiple time points up to twelve weeks.

Limitations are the exclusion of patients with severe or unstable MG and patients using high doses of corticosteroids. Therefore, we cannot draw a conclusion on

the safety and efficacy of vaccination in these groups. Although the study was not powered to detect small changes in clinical outcomes, none of these measures show a trend indicating a possible negative effect.

Theoretically, the unblinded nurses may have caused unblinding of patients, but they specifically ensured that patient blinding was maintained during injection. Furthermore, clinical outcome measures, which are likely the most susceptible to unblinding were taken before unblinding the patients 4 weeks after the injection. Median age of healthy controls was lower, which might result in an stronger humoral response. However, no significant post-vaccination differences were observed between MG and HC groups.

5. CONCLUSION

The antibody response to an influenza vaccination in patients with mild to moderate MG is similar as in healthy subjects, and not affected by the use of immunosuppressive medication. Influenza vaccination did not induce any immunological or clinical exacerbation of MG.

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Chapter 6

In vitro antigen-specific proliferative response and composition of the circulating B- and T-cell compartments before and after tetanus vaccination in patients with myasthenia gravis

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Submitted

1. INTRODUCTION

Safety and efficacy of vaccinations have recently been heavily debated. For patients with autoimmune diseases, especially if they use immunosuppressive medication, vaccination or revaccination might be vital to protect them for severe infections. We studied the effect of tetanus revaccination in patients with a well-defined autoimmune disease, e.g. myasthenia gravis. Myasthenia gravis (MG) is an acquired autoimmune disease of the neuromuscular synapse. Clinical hallmarks are fluctuating muscle weakness of skeletal muscles and increased muscle fatigability. The pattern and severity of clinical symptoms can vary widely between patients and this pattern of muscle weakness can help to recognize a subtype, which can be related to the type of serum antibody present [1].

MG is one of the few autoimmune diseases in which the pathogenic antibodies are well known. The most frequently present antibodies are those against the acetylcholine-receptor (AChR). The role of T cells in the development of MG is well established. Evidence for this comes from the following observations: a) pathogenic antibodies are mainly of the IgG isotype, b) a strong association with HLA-DR3 and HLA-B8 (8.1 haplotype) in early onset MG in Caucasians [2, 3] and c) frequent presence of thymic abnormalities in patients and a beneficial effect from thymectomy [4]. Studies on the effect of a thymectomy on circulating T-cells did neither reveal a significant difference in the absolute numbers of naïve, memory or total T-cells [5], nor on regulatory T-cells (Treg) [6, 7]. Treg are believed to play an important role in MG, but results of studies on frequency of Treg, compared to healthy controls (HC), are contradicting. In some studies [6, 8] no difference in frequency of Treg was found [6], while another study has reported a lower frequency of Treg in peripheral blood of patients with MG [9]. Less functionality of Treg has also been described [8, 10, 11]. These studies provided little data on the effect of immunosuppressive medication (IM). One study excluded patients with IM [6], while others did not discriminate on IM use [5, 7, 9]. This could be a possible explanation for inconsistent findings.

A large part of MG patients need long term IM. Most frequently this consists of prednisolone, often in combination with other IM like azathioprine (AZA), cyclosporine or mycophenolic acid. IM has been described in MG to affect the composition of the B- and T-cell compartments [12-14]. A lower frequency of CD4+ T-cells [12, 13] and a higher frequency of CD19+ and CD27+ B-cells in IM treated patients [14] have been reported. This makes investigation of B- and T-cell compartments even more relevant, because it could have consequences for side-effects or other concomitant treatments, like vaccinations.

Recently, we reported that tetanus revaccination in MG patients with stable disease is safe and induces a protective antibody response [15]. This humoral response to this T-cell dependent recall antigen was not affected by thymectomy, but immunosuppressive medication was associated with lower antibody titres although the response remained significant [15]. In the present study we investigated in

detail subsets of the B- and T-cell compartments and NK-cells as well as the *in vitro* tetanus-specific T cell responsiveness in the same MG cohort, with a focus on the effect of IM and of a preceding thymectomy.

2. MATERIALS AND METHODS

2.1 Standard protocol approvals, registrations, and patient consents

The study was approved by the Local Committee on Medical Ethics of the Leiden University Medical Centre. Subjects provided written informed consent for participation in the study and received reimbursement of travel costs.

2.2 Study protocol

This single-centre, prospective study was performed at the Leiden University Medical Centre. A group of 51 patients with AChR MG was revaccinated with tetanus toxoid. At the day of, but prior to, revaccination and 4 weeks thereafter a blood sample was obtained. One of the AChR MG patients was excluded from analysis because of receiving other vaccines (diphtheria/tetanus/polio (DTP) and typhoid), before the control time point 4 weeks after tetanus toxoid (TT) revaccination, resulting in a study cohort of 50 patients. A group of 28 historical healthy age- and gender -matched individuals without MG was used as a control cohort for comparison of the pre vaccination lymphocyte subsets; 15 of these healthy individuals were also investigated in the proliferation assays.

Inclusion criteria for patients were a confirmed diagnosis of MG, age between 18 and 65 years and stable disease during at least 3 months before participation. Diagnosis of MG was based on clinical signs or symptoms suggestive of MG and a positive serological test for AChR antibodies. Stable disease was defined as an unchanged dosage of IM in the 3 months before revaccination till at least 4 weeks after tetanus revaccination. A maximum daily dose of 30 mg of prednisolone (+/- 5 mg, in the 3 months before participation) was allowed as well as the use of other immunosuppressive medication (IM, see Table 1). Patients continued their medication during the study

The exclusion criteria were instable disease, evidenced by a change in immunosuppressive medication, a Myasthenia gravis Foundation America (MGFA) classification of 4 or 5, presence of a thymoma, other relevant immunosuppressive/secondary immunodeficiency conditions, pregnancy, no previous tetanus vaccination or a tetanus revaccination in the past year. Healthy controls had neither immunosuppressive medication nor any auto-immune disease.

	IM-	IM+	Total AChR MG	HC
Number of patients	27	23	50	28
Gender, female (%)	21 (78)	16 (70)	37 (74)	20 (71)
Age at entering the study, median years (range)	54 (21-65)	57 (22-65)	56 (21-65)	57 (24-67)
Duration of disease, median years (range)	7.0 (0.3-39)	14.0 (2-47)	9.5 (0.3-47)	-
MGFA* classification				-
0, N (%)	4 (15)	0	4 (8)	
1, N (%)	2 (7)	2 (9)	4 (8)	
2, N (%)	21 (78)	19 (83)	40 (80)	
3, N (%)	0	2 (9)	2 (4)	
Prednisolone, N (%)	-	13 (57)	13 (26)	-
Mean daily dose, mg (range)	-	10.4 (5-15)		-
Azathioprine, N (%)	-	15 (65)	15 (30)	-
Mean daily dose, mg (range)	-	108.3 (25-200)		-
Mycophenolic acid, N (%)	-	2 (9)	2 (4)	-
Mean daily dose, mg (range)	-	1250 (500-2000)		-
Cyclosporine, N (%)	-	3 (13)	3 (6)	-
Mean daily dose, mg(range)	-	141.7 (75-200)		-
Combination of immunosuppressive medication, N (%)	-	18 (78)	18 (36)	-
Thymectomy in the past (>1 year ago), N (%)	12 (44)	17 (74)	29 (58)	-
Last tetanus vaccination, years ago, median (range)	24 (2-57))	20 (2-57)	22.5 (2-57)	-

Table 1. Characteristics of the AChR MG cohort and the controls
*MGFA classification: Myasthenia gravis foundation America classification, IM-: without immunosuppressive medication, IM+: with immunosuppressive medication, HC: healthy controls

2.3 Tetanus vaccine
A commercially available tetanus vaccine was used, manufactured by Bilthoven Biologicals (tetanus vaccine, RVG 17639)(16). One dose of 0.5 mL contains ≥40 IU tetanus toxoid (TT), 1.5 mg aluminium phosphate and 0.05 mg thimerosal. Administration was intramuscularly, as a bolus, in the non-dominant upper arm.

2.4 Patient samples
Leukocyte and lymphocyte counts were determined on a hematology analyser. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation and stored in liquid nitrogen until further use.

PBMC were thawed in AIM-V (Invitrogen, Thermofisher Scientific, Waltham, MA, USA) supplemented with penicillin/streptomycin (PS) and 20% heat inactivated foetal calf serum (FCS, Greiner, Kremsmünster, Austria) and incubated for 5 minutes at 37°C with DNase (1600 IU/mL; Sigma, St. Louis, MI, USA) and washed twice. Next PBMC were incubated for one hour at 37°C to let viable cells recover, and subsequently live cells were counted and used in lymphocyte proliferation assays and/or flowcytometry.

2.5 Lymphocyte proliferation
Triplicate cultures of 1x105 PBMC per well were performed in 96 well round-bottom microtiter plates (Corning, Corning, NY, USA) in a final volume of 200 µl RPMI 1640 glutamax 1 (Invitrogen) supplemented with 10% heat-inactivated pooled human AB serum (Sanquin Bloodbank, Amsterdam, the Netherlands) and penicillin/streptomycin (PS; 100 U/ml /100 µg/ml; MBL, Woburn, MA, USA). Cells were cultured in medium only or stimulated with TT (0.2 or 20 Lf/ml; NIBSC, Potters Bar, Hertfordshire, United Kingdom) for 5 days at 37 °C and 5% CO2. Subsequently, 1 µCi/well 3H-thymidine (Perkin Elmer, Waltham, MA, USA) was added 18 hours before harvesting. 3H-thymidine uptake of cultured PBMC was measured as counts per minute (cpm) of triplicate cultures by liquid scintillation counter (Perkin Elmer) and expressed as a stimulation index (SI) (ratio geometric mean cpm antigen-stimulated / geometric mean cpm medium control). SI ≥3.0 was considered as evidence of antigen-induced proliferation. As positive control, the proliferative capacity of the PBMC was tested after mitogenic or polyclonal stimulation, using phytohemagglutinin (PHA, 5 µg/ml Murex, Dartford, England) and IL-2 (100 IU/mL, Chiron, Emeryville, CA, USA) or CD3 (coat 1 µg/ml OKT3, muromab; Janssen-Cilag, Beerse, Belgium), followed by 3H-thymidine incorporation at day 4.

2.6 Flow cytometry
For lymphocyte subset determination and investigation of B-cell differentiation, PBMC were stained for 30 minutes on ice with a mixture of antibodies (see supplementary Table 1). For analysis of T-cell differentiation PBMC were stained for 15 minutes at 37°C with a mixture of antibodies. Samples for investigation of functional T helper cells were preincubated for 20 minutes at 37°C with FcR blocking reagent and subsequently stained for 15 minutes at 37°C with a mixture of antibodies (see supplementary Table 1). After staining, the PBMC were washed twice before analysis.

T regulatory cells (Treg) were first stained with fixable vitality dye UV on ice and washed twice, subsequently preincubated for 20 minutes at 37°C with FcR blocking reagent and next stained for 15 minutes at 37°C with a mixture of antibodies

staining surface membrane markers. Cells were fixed and permeabilized with FoxP3 bufferkit according to the manufacturer's protocol. After permeabilization cells were preincubated for 20 minutes at 37°C with FcR blocking reagent, FoxP3 was stained intracellularly and next the samples were washed three times before analysis.

Samples were measured on a BD LSRII flowcytometer (BD Biosciences, San Jose, CA, USA) and data were analyzed using Kaluza software (Beckman Coulter, Brea, CA, USA).

2.7 Statistical analysis and power

Lymphocyte proliferative responses are expressed as stimulation index (SI), geometric mean ± 95% confidence interval. Lymphocyte subsets and differentiation stages, determined using flowcytometry data, are expressed as absolute cell counts, geometric mean with 95% confidence interval. Unpaired T-test was used to compare two groups. Comparisons between 3 groups were conducted using one-way ANOVA test. Correction for multiple testing is done in a one-way ANOVA with the Tukey multicomparison test and thereafter/and overall with a Bonferroni correction for the number of figures.

All analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., USA). In all tests a two-tailed $p<0.05$ after correction for multiple testing was considered statistically significant. All data were log transformed. Within the AChR MG cohort, responses were compared between patients with (IM+) and those without (IM-) immunosuppressive medication and, within the IM-group, between patients with and those without thymectomy. The patients with immunosuppression were also further divided in IM containing AZA and IM without AZA (IM+ other).

3. RESULTS

3.1 Patient characteristics

Fifty AChR MG patients (74% female, median age 56 years, range 21-65 years) with a median disease duration of 9.5 years were included and revaccinated with TT. Fifty-eight percent of this cohort underwent a thymectomy in the past, which was always >1 year before entering the study. IM was given to 23 (46%) of the patients and included AZA in 15 (65%) of them. There were no significant differences in baseline characteristics between patients with and without immunosuppressive medication (IM- versus IM+).

The age- and gender matched healthy controls (HC, n=28) had a median age of 57 years, range 24-67 years. Baseline characteristics of the AChR MG cohort and the healthy controls are given in Table 1.

3.2 Lymphocyte proliferation in response to TT

All PBMC samples showed a proliferative response to PHA, indicating cell viability. To allow for an effect of antigen dose in the proliferation assay, we used two concentrations of TT for stimulation, i.e., 0.2 and 20 Lf/mL. Upon in vitro TT stimulation with either concentration, a significant increase of the stimulation index (SI) was observed when comparing the post vaccination to the pre vaccination SI in patients with and without IM (Figure 1A and 1B). The IM+ group had significantly lower pre and post vaccination SI than the patients who received no IM, but proliferation was still significantly increased after vaccination. (Figure 1A and 1B). The post vaccination SI was comparable to that of cells from healthy controls, of whom the date of the most recent vaccination was unknown.

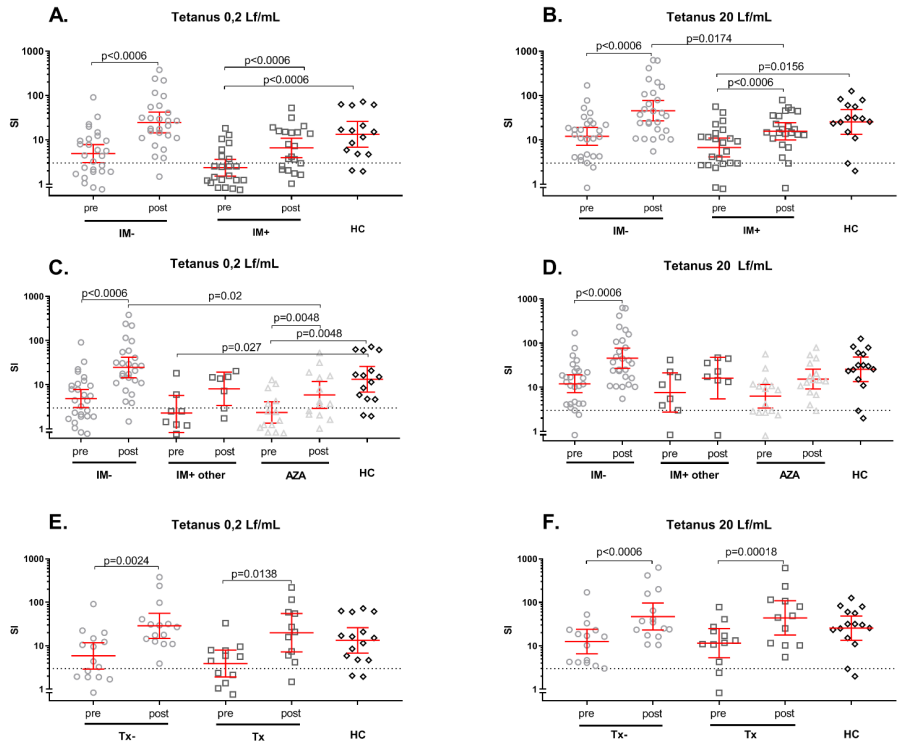


Figure 1

Proliferative response of peripheral blood mononuclear cells (PBMC) after *in vitro* stimulation with tetanus toxoid. Responses of PBMC taken pre and 4 weeks post tetanus revaccination from myasthenia gravis patients were presented and expressed as stimulation index (SI). Panel A and B: comparison of patients treated with immunosuppressive medication (IM+) and those without (IM-). Panel C and D: comparison of IM- patients and IM+ patients, who are divided in patients receiving IM containing azathioprine (AZA) or not (IM+ other). Panel E and F: comparison of patients without (Tx-) and with (Tx+) thymectomy amongst the IM- group.

Two doses of tetanus toxoid were used for stimulation, *i.e.*, 0.2 (panel A, C and E) and

20 Lf/mL (panel B, D and F). HC: healthy age-matched controls who are not recently revaccinated.

AZA is commonly used as treatment in MG and is known to affect the B- and T-cell compartments (1, 17, 18). We investigated whether AZA was the cause for the above described differences in SI between the IM- and IM+ groups. By dividing the IM+ group into patients with and without AZA (AZA/IM+ other), it became apparent that AZA use did not account for this difference (Figure 1C and 1D) because SI's were comparable between use of AZA and other IM. The SI pre and post vaccination per individual also showed a significant increase of SI in the AZA group (Figure 2). To date, thymectomy is an accepted therapeutic intervention in AChR MG (4). The thymus is essential for the development of T-cells. We investigated whether thymectomy affected the proliferative response. Our results show that a preceding thymectomy in patients without IM (to exclude an effect of medication) was not associated with a lower SI before revaccination, and the increase in SI after revaccination was similar in patients with and without thymectomy (Figure 1E and 1F). The time since the previous tetanus revaccination before inclusion in the study or since thymectomy had no influence on the magnitude of the tetanus-specific proliferative response (data not shown).

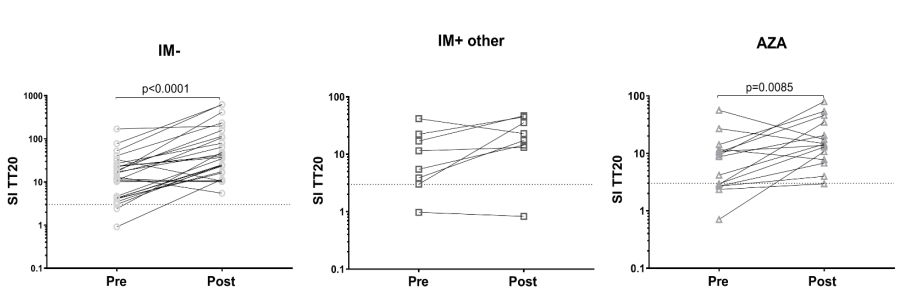


Figure 2
Proliferative response of peripheral blood mononuclear cells (PBMC) after *in vitro* stimulation with tetanus toxoid. Responses of PBMC taken pre and 4 weeks post tetanus revaccination from myasthenia gravis patients were presented on an individual basis and expressed as stimulation index (SI). IM-: patients not receiving immunosuppressive medication; IM+ other: patients treated with medication not containing azathioprine; AZA: patients treated with medication containing azathioprine.

3.3 Lymphocyte populations and B- and T-cell differentiation stages

Because we found lower SI in patients treated with IM and IM is known to affect the composition of the B- and T-cell compartments (12-14), we investigated the absolute numbers of cells in subsets of the B- and T-cell compartments. AZA treated patients had slightly lower geomean counts of lymphocytes, T cells and T-cell

subsets, whereas NK cell and B cell counts were significantly lower in this group compared to the HC, IM- and IM+ not receiving AZA (IM+ other) groups (Figure 3). Geomean counts of leucocytes, lymphocytes, T cells, CD4+ and CD8+ T-cell subsets, TCRγδ+ T cells NK cells and B cells in the IM- group and in the IM+ other group were within the range of healthy adult controls. TT revaccination by itself had no effect on cell numbers (Figure 3). Thymectomy status in patients without immunosuppression had no significant impact on the composition of the lymphocyte subpopulations (supplementary Figure 1).

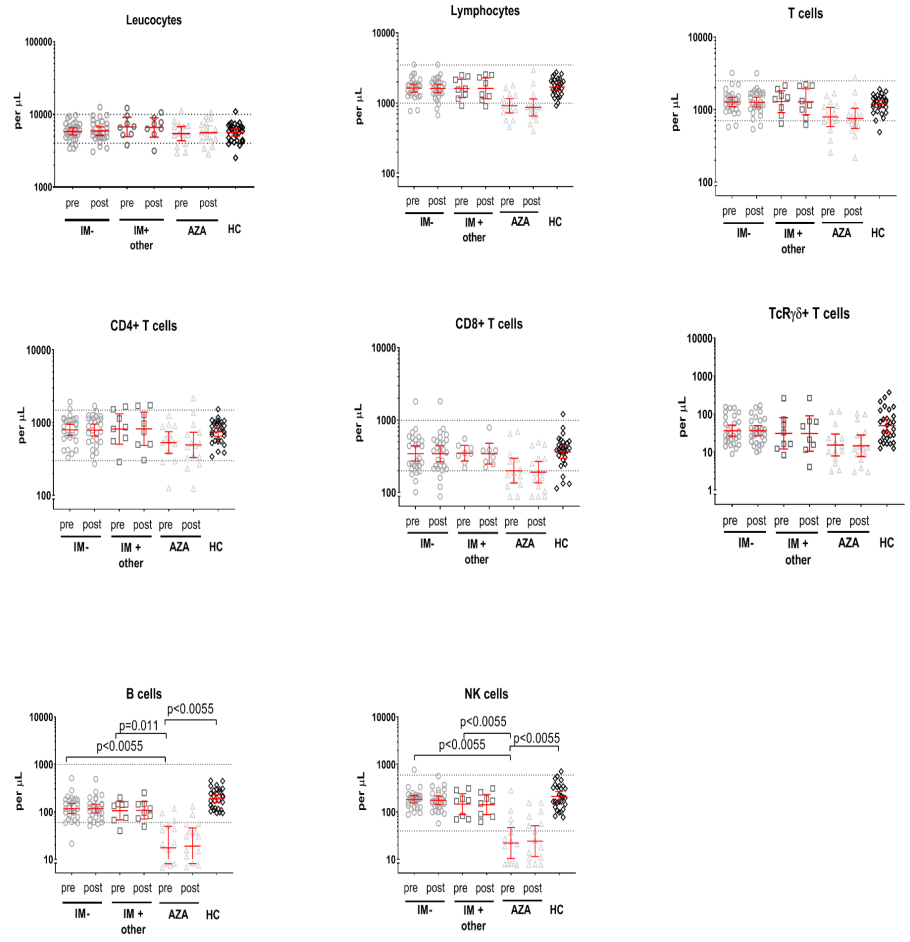


Figure 3
Numbers of leukocytes, lymphocytes, T cells and CD4+, CD8+ and TCRγδ+ T-cell subsets, B cells and NK cells. Blood of myasthenia gravis patients was taken pre and 4 weeks post tetanus revaccination.
IM-: patients not receiving immunosuppressive medication; IM+ other: patients treated

with medication not containing azathioprine; AZA: patients treated with medication containing azathioprine. HC: healthy age-matched controls who are not recently revaccinated.

Analysis of the differentiation stages of the B-cells indicated that AZA treated patients had significantly lower counts in all stages analysed (Figure 4 and supplementary Figure 2). Because of the trend of lower counts of T-cells, CD4+ and CD8+ T-cell subsets in AZA treated patients, we also analysed the differentiation stages in the T-cell subsets. In the CD4+ T-cell subsets no significant differences between AZA treated patients compared to the patients without IM (IM-) or with IM not containing AZA (IM+ other) were observed, although a trend to lower counts was mostly observed in the memory subsets rather than in the naïve cells (supplementary Figure 3). The same holds for the CD8+ T-cell subset, except that there was a significant effect of azathioprine for the CD8+ central memory (CM) stage when comparing the AZA group with the IM- group (supplementary Figure 4). Overall, we found that AZA usage is associated with a trend towards reduction of the numbers of memory T cells. The pattern pre TT revaccination was similar to that post revaccination.

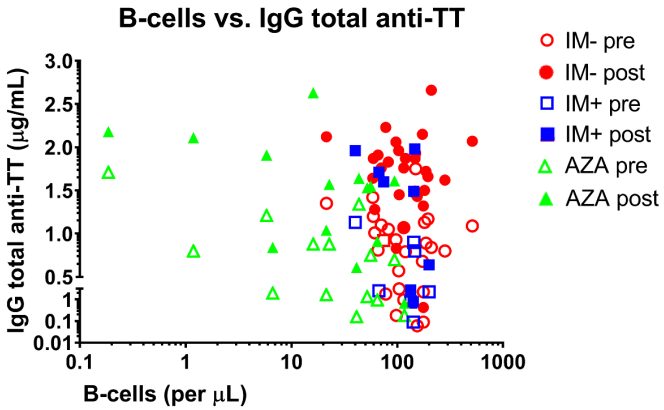


Figure 5
Relation between numbers of B cells and levels of IgG anti-Tetanus toxoid (TT) antibodies. Samples were taken from myasthenia gravis patients pre and 4 weeks post tetanus revaccination. IM- (red circles): patients not receiving immunosuppressive medication; IM+ (blue squares): patients treated with medication not containing azathioprine; AZA (green triangles): patients treated with medication containing azathioprine. Open symbols: pre revaccination; closed symbols: 4 weeks post revaccination.

3.4 Functional T-cell subsets

Since we found a trend towards reduced numbers of memory T cells in AZA treated patients, we investigated whether this trend was also observed in functional T-cell subsets. Defined on the basis of their discriminative phenotypical characteristics,

various functional subpopulations in the memory CD4+ T-cells , i.e., helper T-cells type 1 (Th1), type 2 (Th2), type 17 (Th17), regulatory T-cells (Treg) and follicular T-helper cells (Tfh) were identified (supplementary Table 1) and quantified. Again there was a trend, but not a significant difference, to lower counts across all of these functional CD4+ T-cell subsets in the group of AZA treated patients in comparison with the IM-, IM+ other and HC groups (supplementary Figure 5). Counts pre and post TT revaccination were similar. In comparison with HC, the ratios of Treg versus Th17 and Th1 versus Th2 tended to be higher in AChR MG patients, with exception of the ratio of Th1 versus Th2 in the AZA group. This difference was only significant for the Th1 versus Th2 ratio when comparing the IM- and the IM+ other group and the HC (supplementary Figure 6).

3.5 Absolute numbers of B-cells compared to IgG total anti-TT titres

We previously reported that MG patients demonstrated significant antibody responses after TT revaccination, and in the present study we observed that AZA is associated with lower B-cell numbers. Therefore, we investigated whether there was a correlation between numbers of B-cells and total IgG anti-TT levels pre and post revaccination. Interestingly, the results indicate that the absolute number of B-cells was not associated with levels of antibodies before or after revaccination. (Figure 5).

4. DISCUSSION

In the current study, we investigated the proliferative response of T cells to a tetanus revaccination and the circulating numbers of B- and T-cell subsets and NK-cells. In a previous study we described the safety aspects and humoral immune response in this patient group [15]. The study was performed in a cohort of AChR MG patients with stable disease, defined as having an unchanged dose of immunosuppressive medication or no immunosuppressive treatment for at least 3 months. Prior to revaccination, PBMCs of the majority of the MG patients showed a specific proliferative response after *in vitro* stimulation with 20 Lf/mL tetanus. Patients without IM showed a higher increase of the proliferation post revaccination than patients with IM. Furthermore, tetanus revaccination had neither influence on the composition of the B-cells and T-cell subsets nor on the number of NK cells. Cell numbers in this AChR MG cohort were comparable to healthy controls, except in AZA treated patients, in whom B- and NK-cell numbers were significantly decreased. Despite lower absolute numbers of circulating B-cells in the AZA group, this was not associated with lower titres of IgG total anti-TT antibodies upon revaccination. Thymectomy status did neither impact the tetanus-specific proliferative response nor the differentiation stages in CD4+ and CD8+ T-cell subsets. Treg/Th17 and Th1/Th2 ratios tended to be higher in patients than in controls. One could hypothesize that timing since thymectomy, in combination with IM, can influence the differentiation stages of the T-cell subsets.

Fattorossi et al. found higher numbers of Treg pre thymectomy in patients with IM and described normalization of Treg numbers 12-16 months after thymectomy in these patients [19]. The patients without IM in their study had significantly lower Treg than both HC and patients with IM. On the other hand, Sun et al. reported higher Treg in thymectomized patients, but they did not differentiate between IM and no IM [20]. In our study, we did not find an effect of thymectomy on Treg in both the IM+ and IM- groups (data not shown).

As shown in our study, the usage of azathioprine is associated with a strong decrease of B- and NK-cell counts in blood and, to a lesser extent, of T-cell differentiation stages and subpopulations of functional CD4+ Th-cells. IM (prednisolone alone or in combination with azathioprine and other IM) has also been described to have an influence on the functionality of cells [11, 14] and this is confirmed by the lower TT induced proliferative response of cells from patients who received IM in our study. We did not analyse the function of Treg. The effect of AZA on B- and NK-cells has also been reported in other autoimmune diseases like lupus and chronic glomerulonephritis, Bechet's disease and inflammatory bowel disease [21-23]. In MG the effect of azathioprine resulting in lower NK-cell numbers has not been described before. Kohler et al. reported a lower frequency of CD27− IgD+ naïve B cells and a higher frequency of CD27+ IgD− memory B cells in MG patients with IM, but which IM other than steroids was given is not specified (24). The most known effect of AZA is lymphopenia [1, 18].

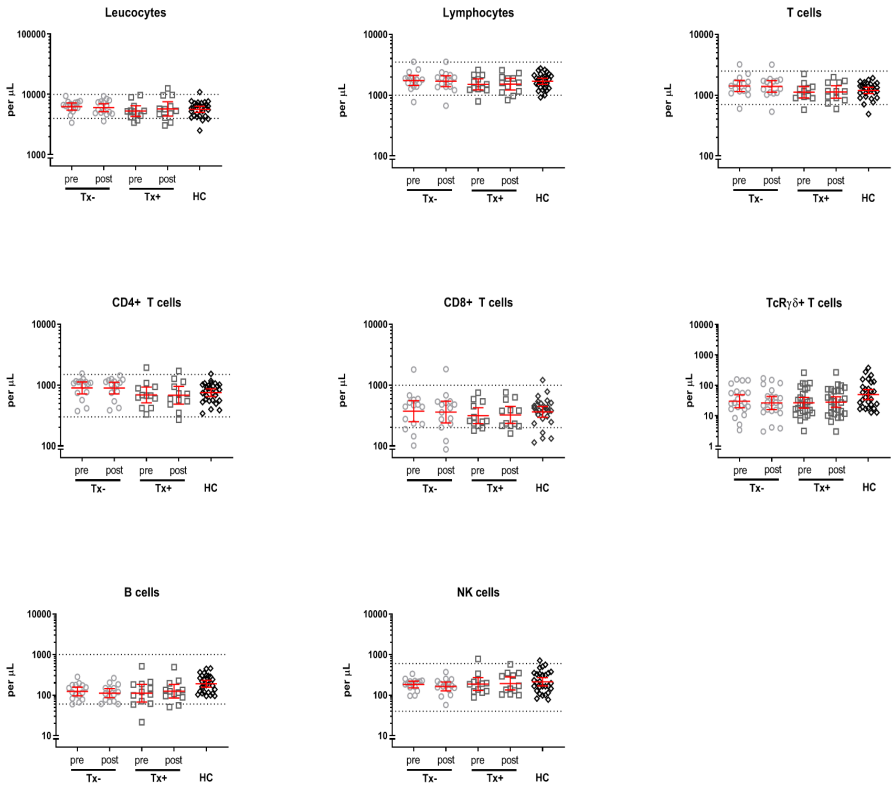
Overall, studies in MG on differences in the B- and T-cell compartments are often performed in heterogeneous patient populations, making it difficult to compare the results of these studies and to point out a specific cause for differences in immune parameters when compared to healthy controls. Patient selection is of great importance to compare these types of studies.

Our previous studies in MG patients on the humoral response to tetanus and influenza showed that (re)vaccination is effective and safe, based on stable AChR antibodies and stable clinical outcome measures [15, 25]. Such studies on vaccination in MG are rarely performed, but are of practical relevance for both patients and medical specialists, in order to relieve concerns about worsening or exacerbation of symptoms [15, 25]. To our knowledge, this study is the first to describe the proliferative response to revaccination with a T-cell dependent antigen and the effect of this revaccination on the composition of the T- and B-cell compartments in autoimmune MG.

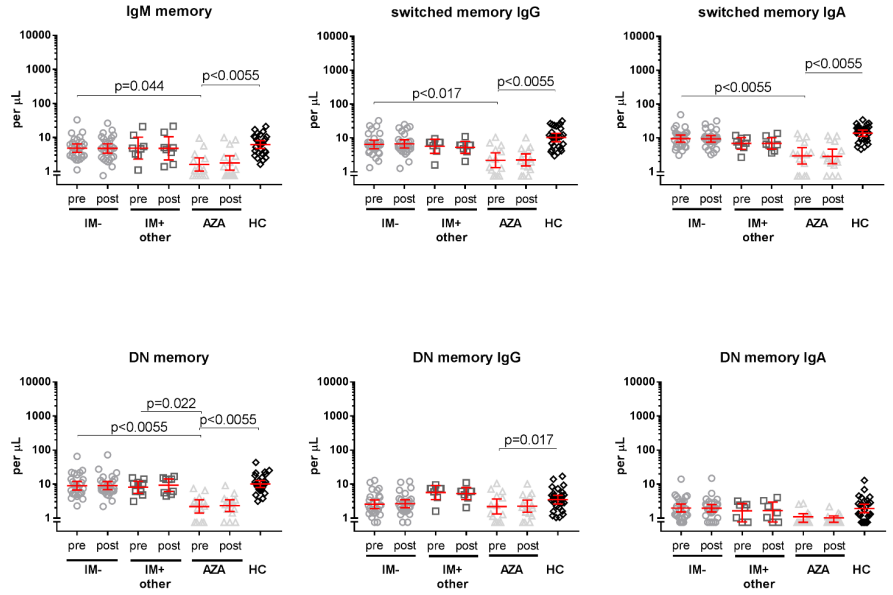
CONCLUSION

In conclusion, revaccination of MG patients with stable disease leads to a clear increase of the *in vitro* tetanus-specific proliferative response of T cells in both MG patients with or without IM, although the SI's in patients treated with IM are lower. Surprisingly, we could neither detect a significant effect of a preceding thymectomy on the proliferative response nor on composition of the T- and B-cell compartments. A significant effect of IM on both the proliferative response as well as the numbers of B-cell subsets and NK cells was found. The latter was in particular associated with azathioprine. However, this azathioprine associated decrease in B-cell numbers had no impact on the IgG anti-tetanus response upon revaccination. Together with our previous study on the humoral response to tetanus revaccination in this cohort, our data supports the notion that tetanus revaccination is safe and effective in MG patients with stable disease and does neither affect B- and T-cell responsiveness nor absolute numbers of T- and B-cell subsets.

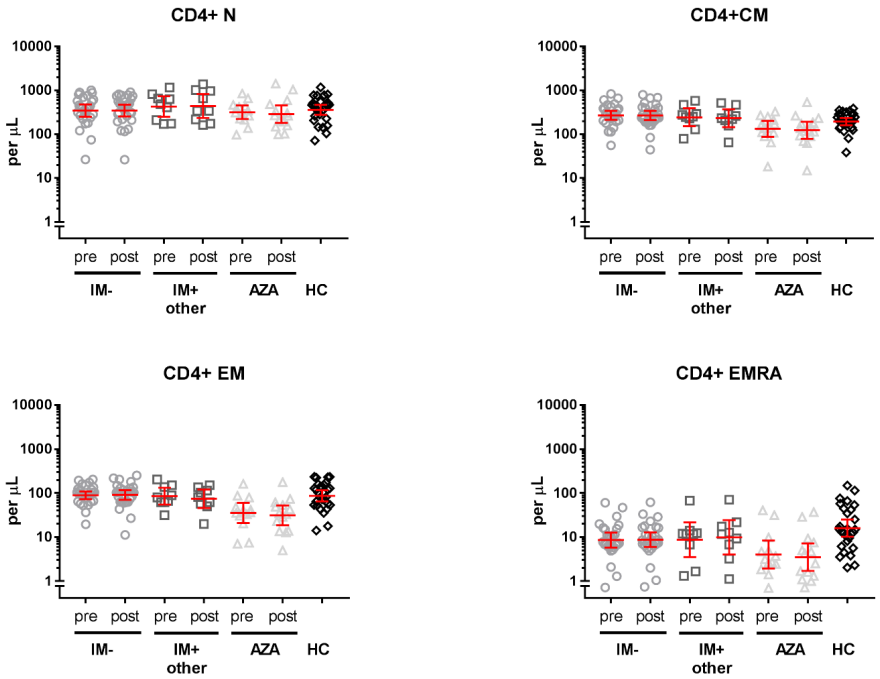
SUPPLEMENTARY FIGURES



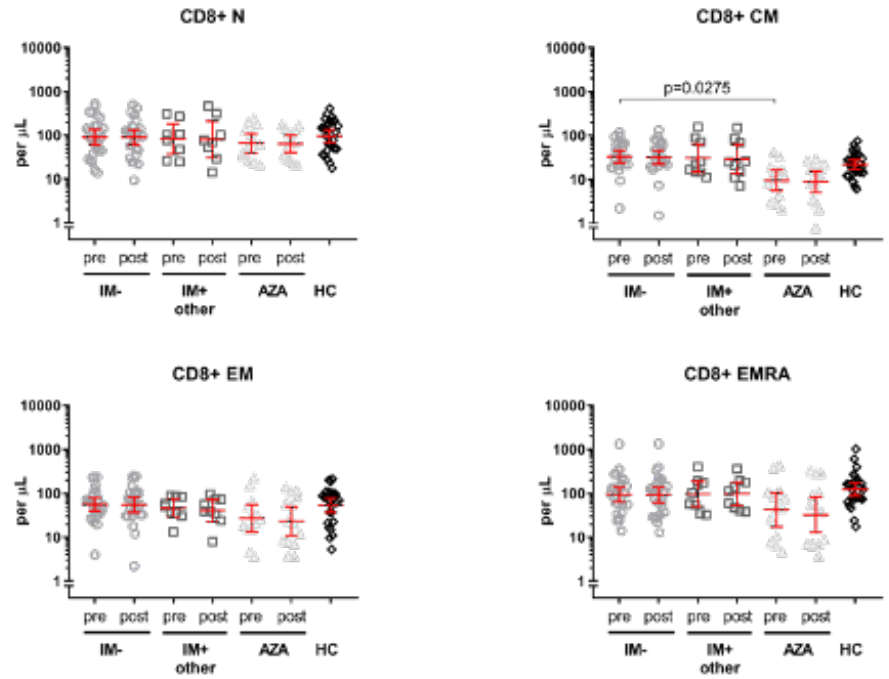
Supplementary Figure 1
Effect of thymectomy on the numbers of leukocytes, lymphocytes, T cells and CD4+, CD8+ and TCRγδ+ T-cell subsets, B cells and NK cells. Blood of myasthenia gravis patients was taken pre and 4 weeks post tetanus revaccination. Only patients not receiving immunosuppressive medication were included. Tx-: patients without thymectomy in the past; Tx+: patients with thymectomy in the past.
HC: healthy age-matched controls who are not recently revaccinated.



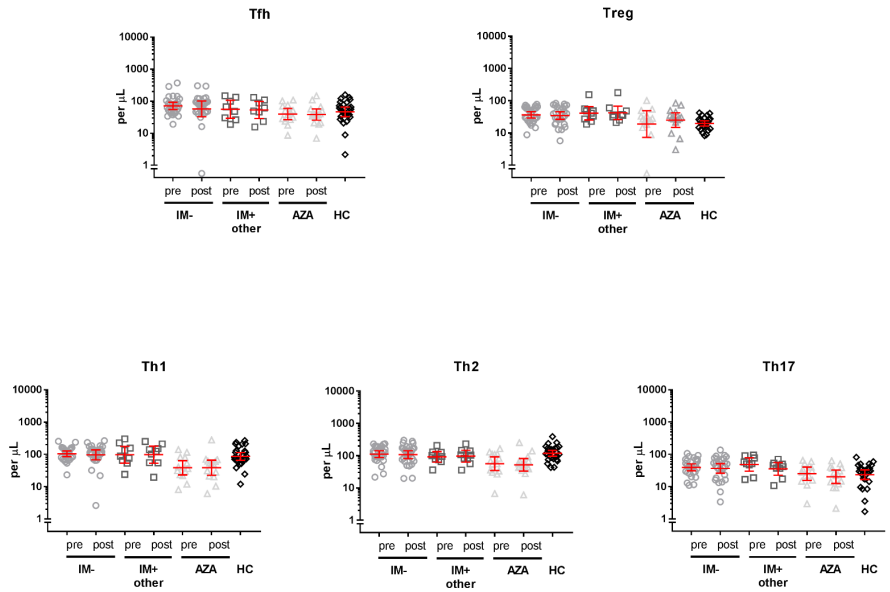
Supplementary Figure 2
Numbers of IgM memory, switched memory IgG, switched memory IgA, double negative (DN) memory, DN memory IgG and DN memory IgA differentiation stages within the B-cell population. Blood of myasthenia gravis patients was taken pre and 4 weeks post tetanus revaccination.
IM-: patients not receiving immunosuppressive medication; IM+ other: patients treated with medication not containing azathioprine; AZA: patients treated with medication containing azathioprine. HC: healthy age-matched controls who are not recently revaccinated.



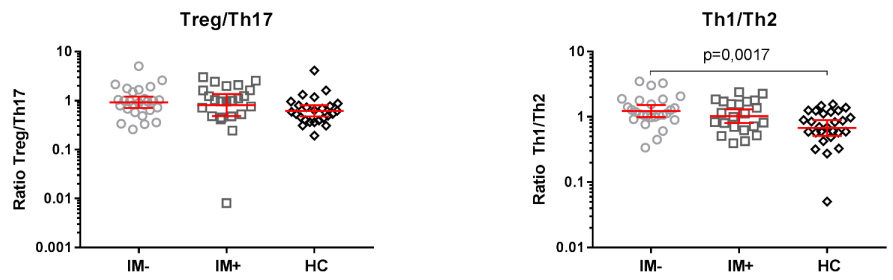
Supplementary Figure 3
Numbers of CD4+ T-cells in the differentiation stages. Blood of myasthenia gravis patients was taken pre and 4 weeks post tetanus revaccination. IM-: patients not receiving immunosuppressive medication; IM+ other: patients treated with medication not containing azathioprine; AZA: patients treated with medication containing azathioprine. HC: healthy age-matched controls who are not recently revaccinated. N: naive; CM: central memory; EM: effector memory; EMRA: end-stage effector cells.



Supplementary Figure 4
Numbers of CD8+ T-cells in the differentiation stages. Blood of myasthenia gravis patients was taken pre and 4 weeks post tetanus revaccination. IM-: patients not receiving immunosuppressive medication; IM+ other: patients treated with medication not containing azathioprine; AZA: patients treated with medication containing azathioprine. HC: healthy age-matched controls who are not recently revaccinated. N: naive; CM: central memory; EM: effector memory; EMRA: end-stage effector cells.



Supplementary Figure 5
Numbers of follicular T helper cells (Tfh), regulatory T cells (Treg), helper T cells type 1 (Th1), helper T cells type 2 (Th2) and helper T cells type 17 (Th17). Blood of myasthenia gravis patients was taken pre and 4 weeks post tetanus revaccination. IM-: patients not receiving immunosuppressive medication; IM+ other: patients treated with medication not containing azathioprine; AZA: patients treated with medication containing azathioprine. HC: healthy age-matched controls who are not recently revaccinated.



Supplementary Figure 6
Ratio of regulatory Tcells (Treg) versus helper T cells type 17 (Th17) and helper T cells type 1 (Th1) versus helper T cells type 2 (Th2). Data from blood of myasthenia gravis patients taken pre tetanus revaccination are presented . IM-: patients not receiving immunosuppressive medication; IM+ other: patients treated with medication not containing azathioprine; AZA: patients treated with medication containing azathioprine. HC: healthy age-matched controls who are not recently revaccinated.

Company	CD code	target or description	clone	fluoro-chrome	isotype	catalogue number
BD	CD3		SK7	APC-H7	mouse IgG1	560176
BL	CD3		UCHT1	BV510	mouse IgG1	300448
BC	CD3		UCHT1	PE-TxR	mouse IgG1	A07748
BL	CD4		SK3	A700	mouse IgG1	344622
BL	CD4		SK3	BV421	mouse IgG1	344632
BC	CD4		13B8.2	PE-Cy5.5	mouse IgG1	B16491
BD	CD7		M-T701	APC-R700	mouse IgG1	659124
BD	CD8		SK1	APC-H7	mouse IgG1	560179
BD	CD8		SK1	BV605	mouse IgG1	564116
BD	CD8		SK1	PE-Cy7	mouse IgG1	335822
BL	CD16		3G8	APC-Cy7	mouse IgG1	302018
BD	CD19		SJ25C1	BV510	mouse IgG1	562947
BD	CD20		2H7	BV605	mouse IgG2b	563783
BC	CD20		B9E9	PE-Cy5.5	mouse IgG2a	B23134
TF	CD24		SN3	PE-A610	mouse IgG1	MHCD2422
BD	CD25		M-A251	PE	mouse IgG1	555432
BD	CD27		M-T271	BV421	mouse IgG1	562513
BD	CD27		L128	BV605	mouse IgG1	562655
BD	CD28		L293	PE	mouse IgG1	348047
BD	CD33		P67.6	PE	mouse IgG1	345799
BD	CD38		HIT2	PerCP-Cy5.5	mouse IgG1	551400
TF	CD45RA		MEM-56	PE-TxR	mouse IgG2a	MH-CD45RA17
BC	CD56		N901	APC	mouse IgG1	IM2474
BL	CD95		DX2	A647	mouse IgG1	305618
TF	CD127		RDR5	PE-Cy7	mouse IgG1	25-1278-42
BL	CD183	CXCR3	G025H7	A647	mouse IgG1	353712
BL	CD185	CXCR5	J25D4	BV421	mouse IgG1	356920
BL	CD194	CCR4	L291H4	BV510	mouse IgG1	359416
BD	CD196	CCR6	11A9	PE-CY7	mouse IgG1	560620
RD	CD197	CCR7	150503	FITC	mouse IgG2a	FAB197F-100
TF	CD278	ICOS	ISA-3	PE	mouse IgG1	12-9948-42
BC	CD235a	Glycophorin A	11E4B-7-6	PE	mouse IgG1	A07792
BD	CD45/14	Leucogate	2D1, МФР9	FITC	mouse IgG1/ mouse IgG2a	342408
TF		foxp3	PCH101	APC	rat IgG2a	17-4776-42

table continues

Comp-any	CD code	target or description	clone	fluoro-chrome	isotype	catalogue number
DK		IgA	polyclonal	FITC	polyclonal Rabbit IgG F(ab')2	F031601
BD		IgD	IA6-2	PE-Cy7	mouse IgG2a	561314
BD		IgG	G18-145	APC-H7	mouse IgG1	561297
BD		IgM	G20-127	APC	mouse IgG1	551062
BD		TcR gamma/ delta	11F2	APC-R700	mouse IgG1	657706
TF		Fixable vitality dye 455 UV				65-0868-18
TF		Fc Block reagent				14-9161-73
TF		FoxP3 bufferkit				00-5523-00

Supplemental Table 1A: Antibodies and reagents for flow cytometry of cell subsets
BC: Beckman Coulter (Brea, CA, USA); BD: BD Biosciences (san Jose, CA, USA); BL: BioLegend (San Diego, CA, USA); DK: Dako (Glostrup, Denmark); RD: R&D systems (Minneapolis, MN, USA); TF: Thermo Fisher Scientific (Waltham, MA, USA).

			Figure
Lymphocytes	T cells	$CD3^+$	3, S1
	$CD4^+$ T cells	$CD3^+CD4^+$	3, S1
	$CD8^+$ T cells	$CD3^+CD8^+$	3, S1
	NK cells	$CD3^-CD56^+$	3, S1
	B cells	$CD19^+$	3, S1,5
	$TCR\gamma\delta^+$ T cells	$CD3^+TCR\gamma\delta^+$	3, S1
B cell differention	transitional	$CD19^+CD27^-CD24^{high}CD38^{high}$	4
	naïve	$CD19^+CD27^-CD24^{dim}CD38^{dim}IgD^+$	4
	unswitched memory	$CD19^+CD27^+IgD^+IgM^+$	4
	switched memory	$CD19^+CD27^+IgD^-IgM^-$	4
	IgM memory	$CD19^+CD27^+IgD^-IgM^+$	S2
	switched memory IgG	$CD19^+CD27^+IgD^-IgM^-IgG^+$	S2
	switched memory IgA	$CD19^+CD27^+IgD^-IgM^-IgA^+$	S2

table continues

			Figure
CD4 and CD8 T cell differentiation	DN memory	$CD19^+CD27^-CD24^{dim}CD38^{dim}IgD^-IgM^{-0r+}$	S2
	DN memory IgG	$CD19^+CD27^-CD24^{dim}CD38^{dim}IgD^-IgM^-IgG^+$	S2
	DN memory IgA	$CD19^+CD27^-CD24^{dim}CD38^{dim}IgD^-IgM^-IgA^+$	S2
	$CD4^+$ N	$CD3^+CD4^+CCR7^+CD45RA^+$	S3
	$CD4^+$ CM	$CD3^+CD4^+CCR7^+CD45RA^-$	S3
	$CD4^+$ EM	$CD3^+CD4^+CCR7^-CD45RA^-$	S3
	$CD4^+$ EMRA	$CD3^+CD4^+CCR7^-CD45RA^+$	S3
	$CD8^+$ N	$CD3^+CD8^+CCR7^+CD45RA^+$	S4
	$CD8^+$ CM	$CD3^+CD8^+CCR7^+CD45RA^-$	S4
	$CD8^+$ EM	$CD3^+CD8^+CCR7^-CD45RA^-$	S4
	$CD8^+$ EMRA	$CD3^+CD8^+CCR7^-CD45RA^+$	S4
Fuctional T cell subsets	Tfh	$CD3^+CD4^+CXCR5^+$	5,6
	Treg	$CD3^+CD4^+FoxP3^+$	5,6
	Th1	$CD3^+CD4^+CXCR3^+CCR6^-$	5,6
	Th2	$CD3^+CD4^+CXCR3^-CCR6^-$	5,6
	Th17	$CD3^+CD4^+CXCR3^-CCR6^+$	5,6

Supplemental Table 1B: Phenotypical definition of lymphocyte subsets (based on live single events within the lymphocyte gate)

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Chapter 7

General discussion and future perspectives

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GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Within the neurological field there is a broad spectrum of autoimmune diseases that affect the central or the peripheral nervous system. This range includes disorders like autoimmune encephalitis up to autoimmune-mediated myopathies. In the case of an autoimmune disease, two problems can arise in the context of a vaccination: 1) The vaccine stimulates the immune system and thereby aggravates the pre-existing autoimmune disease. 2) The vaccine is less effective due to the often (necessary) use of immunosuppressive medication or due to the underlying immune dysregulation underlying the autoimmune disease. Another association between vaccination and autoimmune diseases is the occurrence of autoimmunity de novo after vaccination, such as Guillain-Barré syndrome or narcolepsy [1, 2]. This latter possibility is not a topic of this thesis and will not be discussed here.

Treatment with immunosuppressive medication makes patients more prone to infections. Therefore, they are eligible for prophylactic vaccinations, such as influenza vaccination. It is also known that infections can (temporarily) aggravate the symptoms of autoimmune diseases such as myasthenia gravis (MG) and multiple sclerosis (MS)[3, 4]. On the other hand, an adequate immune response to vaccination could be hampered by the dysregulation of the immune system which is evident from the development of autoimmunity or by the effect of the immunosuppressive medication on the immune system. This thesis discusses autoimmune mediated MG and the indication, effectiveness and safety of vaccinations for this condition.

Autoimmune mediated MG is the most well-known neuromuscular junction disorder [5]. It is the first neurological disorder that has been identified as an antibody-mediated disease [6]. The initial trigger for making the pathogenic acetylcholine receptor (AChR) antibodies, which cause MG, still has to be elucidated. AChR antibodies can be present long before clinical onset, as we described in chapter 2 of this thesis. This supports the idea that development of autoimmunity takes time and becomes evident when titres reach a critical threshold. Which triggers facilitate the increase of autoantibody titres are not known. As described in chapter 2, a possible contributing trigger for onset of clinical symptoms can be pregnancy. Vaccinations could also be a trigger, that was why we investigated two frequently used vaccines and found no immunological, neither humoral nor cellular, or clinical exacerbations.
Treatment of MG consists of symptomatic treatment with cholinesterase inhibitors, but immunosuppressants are also often required to adequately control the symptoms. A disadvantage of treatment with immunosuppressive medication is the increased risk of infections and a more serious course of infections. This is because of a decrease in the number of B and T cells or an immune system malfunction due to this medication (7, 8). Therefore, due to immunosuppressants the desired immune response, following a vaccination, can be elicited less efficiently. This applies to a greater extent for a primary immune response after a first vaccination than for a secondary response after a revaccination / booster. Corticosteroids, as well as azathioprine, or a combination thereof are widely used for the treatment of

MG. To illustrate the effect of immunosuppressive medication: from a daily dose of prednisolone of ≥10 mg, a person should be considered as immune compromised [8]. From a daily dose at 20 mg, a person can be classified as seriously immune compromised [7]. Eculizumab, a recent addition to the treatment options of MG, inhibits the formation of the terminal complement complex. The recommendation is to vaccinate for Neisseria meningitidis prior to the start of treatment, because the complement system is especially important for the immune response to this specific bacteria. In case of rituximab, another recently added treatment option for MG, a patient needs to complete any vaccination that is needed, 4 weeks prior to the treatment. This is because of the depletion of CD20+ B-cells by rituximab. Patients can't be vaccinated with live vaccines during, or in the months after treatment with rituximab. In chapters 4 and 6 we saw a clear effect of treatment with immunosuppressive medication, azathioprine in particular. In chapter 4 we describe that patients with immunosuppressive medication had a significantly lower pre and post titre compared to healthy controls, but their humoral response was still significant. In chapter 6, a significant effect on both the proliferative response as well as the number of B-cell subsets and NK cells was described. However, this azathioprine associated decrease in B-cell numbers had no impact on the IgG anti-tetanus response upon vaccination in our cohort.

Importantly, in the immunocompromised patient, the titre does not necessarily need to be as high as in healthy controls, as long as it falls within the range that is considered protective. It should be noted, however, that the height of the titre can influence the duration of the protection [8].

Vaccinations can prevent some infections or make the course less serious. The best known example is the annual influenza vaccination. This vaccination is recommended for a number of patient groups, including patients with an autoimmune disease or to patients with immunosuppressive medication. In addition, patients with immunosuppressive medication or an autoimmune disease also increasingly want to travel abroad, often also to regions for which vaccinations are recommended. Important points to consider, as a treating physician, are the effectiveness and safety of prophylactic vaccinations for this population. In this assessment, the indication and necessity of a vaccination also need to be taken into account. These can differ between vaccinations. Potential side effects, both local and a potential flare-up of the disease, must outweigh the benefits. There are currently no specific guidelines for vaccinations in patients with MG or other neurological autoimmune diseases.

Prior to the studies described in this thesis, little research on the effectiveness and safety of vaccinations in patients with autoimmune MG was performed. In the 60s and 70s, two studies on vaccinations in MG were reported. These studies were performed in light of the thymectomy that was introduced since recently at that time. The aim was to investigate the humoral response in thymectomized patients compared to healthy controls. Adner et al. included 48 MG patients and 21 healthy controls and used the vaccine for Pasteurella Pestis [9]. Kornfeld et al. included 38

MG patiens and 29 healthy controls and used the vaccine for typhus [10]. Both studies found an acceptable primary response, but Kornfeld et al. found a relatively less secondary response to a booster [9, 10]. They didn't investigate a possible effect of a vaccination on the disease symptoms. Nor did they obtain information on the effect of immunosuppressive medication, since this medication wasn't used yet in patients with MG. Furthermore, the influence of a vaccination on the pathological antibodies couldn't be investigated, as they weren't known at that time. In our studies in chapter 4 and 5 we do describe that there is no effect on the disease symptoms or pathological antibodies, but that there is an effect of immunosuppressive medication. A later conducted study investigated the titre of antibodies to diphtheria and tetanus in healthy controls, and in patients with SLE or MG [11]. No difference in the coverage ratio was found between these groups [11]. However, most of these patients were already vaccinated prior to onset of the disease. Neither effectiveness of the immune response to the vaccination nor the safety of a vaccination was studied prospectively. Usage of medication in the study population was not described.

Two other studies investigated the number of hospital admissions of MG patients in the period of the annual influenza vaccination [12, 13]. No increase of the number of admissions due to an exacerbation of the symptoms of MG was found [12, 13].

Aside from immunological and physician-reported clinical outcome measures, also patient-reported outcome measures are increasingly important tools. We validated a patient-reported questionnaire in Dutch during the tetanus study: the Dutch MG-QoL15 [14] (chapter 3). This makes it possible to monitor a patient, based on a patient-reported outcome score instead of a physician reported outcome score. This is important, because a physician can interpret good or improving scores on the QMG or MG composite (physician-reported), but this can differ from the health-related quality of life that a patient experience.

Tetanus revaccination in myasthenia gravis

As described in this thesis in chapter 4, we prospectively investigated the efficacy and safety of a tetanus revaccination in 50 AChR MG patients, 6 MuSK MG and 9 LEMS patients [15]. These patients had a 'stable disease' and used daily prednisolone dosages up to 30 milligrams, which could be combined with other immunosuppressive medication. Stable disease was defined as a stable dosage of immunosuppressive medication at least 3 months prior to the study and a maximum MGFA classification of 3 (mild severe MG). Our findings showed that the patients had an adequate humoral immune response, independently of the type of medication they used. Neither an increase of the pathological antibodies (AChR, MuSK, VGCC) nor a change of the clinical outcome measures was found.

We also investigated the cellular immune response to tetanus vaccination and found a lower pre and post vaccination stimulation index in patient with immunosuppressive medication compared to those without IM (chapter 6). Despite this, both groups reached a significant post vaccination response. Tetanus revaccination did not affect

cell counts of lymphocyte subpopulations and B- and T-cell differentiation stages. A preceding thymectomy showed no effect on lymphocyte compartments. However, immunosuppression, azathioprine in particular, was associated with strongly decreased natural killer (NK) cell and B-cell counts, but did not affect levels of anti-tetanus antibodies before or after revaccination. Therefore, a tetanus revaccination seems to be safe in a patient with (stable) MG.

Influenza vaccination in myasthenia gravis

As mentioned above, the annual influenza vaccination is recommended for patients with an autoimmune disease like MG or patients who use immunosuppressive medication. In our own experience and as described by others, patients with MG are concerned that this vaccination can give a exacerbation of their disease and, therefore, don't take the annual influenza vaccination [12]. This is most likely unnecessary, as there are indications from previous research that influenza vaccination can be effective in reducing (laboratory confirmed) influenza disease, hospital admissions and the risk of death, especially in vulnerable and elderly patients [16, 17]. Furthermore, we already reported that tetanus revaccination, as described in chapter 4, is safe and effective, and decided that providing evidence for the safety and efficacy of the influenza vaccination would be practical for both patient and clinician. In order to investigate this, we conducted a double-blind, placebo-controlled, randomized study in 47 patients with MG in the 2016-2017 influenza season [18] (chapter 5). Our study demonstrated an effective response comparable to healthy controls. Also, no clinical or immunological (AChR antibodies) exacerbation was found 4 weeks after vaccination. It was striking that patients even reported less frequently adverse reactions to the influenza vaccination than healthy controls [18]. Thus, the results of the tetanus and influenza vaccinations studies were very comparable.

Since we found that relatively little research is conducted in neurological autoimmune diseases and vaccinations, except for MS, it is interesting to compare our results with other groups of autoimmune diseases, MS, Guillain-Barré syndrome (GBS), Chronic Inflammatory Demyelinating Polyneuropathy (CIDP) or rheumatoid arthritis (RA).

Multiple sclerosis

MS is the only neurological autoimmune disease in which a lot of vaccination research has been conducted and for which guidelines are published. In MS there are studies focusing on safety of vaccination by looking at the frequency of relapses or radiological changes of scans [19-25]. Vaccination with a live weakened yellow fever vaccine resulted in an increased relapse in a small study of seven patients [26]. However, the clinical relevance of this finding is probably limited, since vaccination with live attenuated vaccine is not recommended in patients taking immunosuppressive drugs, due to an increased risk of infection. Furthermore, there are studies that investigated the efficacy (the specific increase in titre) of vaccinations, mostly of influenza vaccination, during the use of immunosuppressive or immunomodulating medication. Several studies show that the frequency of

relapse or the radiological image of MS does not change due to a vaccination [19-25]. No adverse effect of teriflunomide (an NF-κB inhibitor) or interferon treatment on increase of the titre following influenza vaccination is described [24, 27]. Findings for natalizumab (monoclonal antibody against α4-integrin) and fingolimod (causing internalization of S1P receptors) are varying and often involve small studies, making it difficult to draw conclusions [19, 20, 22, 23, 25]. For fingolimod a larger placebo-controlled, randomized study reported that there is a lesser increase of titre after influenza vaccination, 3 and 6 weeks after vaccination (vaccinated 6 weeks after starting fingolimod), compared to healthy controls. For tetanus revaccination this only applies at 3 weeks, not at 6 weeks [19, 20]. In a small study (23 patients with natalizumab), Natalizumab does not appear to have a significant influence on the response to influenza [25]. A study published in 2018 found lower titres in patients who used natalizumab. However, only 8 patients used natalizumab in this study [28]. For glatiramer (a myelin basic protein analogue) and mitoxantrone (type II topoisomerase inhibitor), an influence on the increase of titres after influenza vaccination has been described [21, 22]. A later, observational study, on the other hand, found a good response to influenza, despite glatiramer use [29].

Hepatitis B, BCG, tetanus and varicella vaccinations do not seem to give an increased relapse rate [30, 31]. Vaccinations are recommended in a stable phase of the disease and preferably 4-6 weeks after a relapse. Tetanus vaccination is indicated in case of a wound after an outdoor accident. The influenza vaccination is recommended, because it is assumed that an influenza infection itself has greater adverse effects than the possible side effect of the vaccination itself [29]. A smaller increase of titre can still offer sufficient protection. An option is to determine the height of the titre. If the titre is too low, one can consider repeating the influenza vaccination. This principle can also be applied to other vaccinations.

Guillain-Barré syndrome and CIDP

Studies on vaccination in the Guillain-Barré syndrome (GBS) mostly investigate the incidence of a primary episode of GBS following a vaccination. There are two retrospective studies that investigated by questionnaires whether a new episode of GBS or an increase of symptoms occurred in CIDP patients after vaccinations. One of these studies found no relapse in the group with GBS-patients (n=106) after one or more influenza vaccinations (total 775 vaccinations in GBS-group) in the years after diagnosis. In the CIDP-group, 5 out of 24 patients who got an influenza vaccination after the diagnosis, reported an increase of symptoms after influenza vaccination [32]. The other study investigated the occurrence of relapse or an increase of symptoms by questionnaires and found a risk of 3.5% for the GBS patients and of 8% for the CIDP patients [30]. Overall, both studies reported a relative low risk. It is important to take a possible recall bias in account for both studies.

Inflammatory rheumatic conditions and vaccinations

For the group of inflammatory rheumatic disorders, more research on the efficacy and safety of vaccinations is conducted. The European League against Rheumatism

(EULAR) published recommendations for this group of patients [33]. They recommend to vaccinate patients in a stable phase of their disease. There are some small studies that included patients with mild to severe disease (activity), which found no increased risk for side effects or flares of the disease. However, based on a theoretical higher risk of flares, they recommend to vaccinate during stable disease. A distinction is made for the type of vaccination. Life-attenuated vaccines are discouraged in patients with immunosuppressive medication, because of the increased risk of conversion to an active infection. The question remains to what extent the dosage of the immunosuppressive medication relates to a higher risk. Based on the conducted studies, also it was stated that it can be necessary to repeat a vaccination in order to reach an adequate immune response [34]. The EULAR strongly advices to vaccinate patients for influenza, based on the increased risk of morbidity and mortality in case of an actual influenza infection or pneumonia in this population [33]. Finally, they conclude that it remains necessary to make the assessment per individual patient, based on the indication and necessity of the vaccination [33].

Conclusions and recommendations

Patients with AChR MG can make an effective immune response to tetanus revaccination and influenza vaccination, irrespective of their immunosuppressive medication. Immunosuppressive medication does cause a lower anti-tetanus pre and post titre in patients, compared to healthy controls. In case of influenza vaccination, immunosuppressive medication only influences the pre vaccination titre. Influenza vaccination and tetanus revaccination do neither result in an immunological exacerbation nor in any clinically significant exacerbation of symptoms of AChR MG. In case patients experience an increase of their MG symptoms, this increase is mild and of short duration.

Generalization of these results to other vaccinations can't be done with certainty. A tetanus vaccine can differ from other vaccines in immunogenicity. Also, a primary immune response to a vaccination can differ from a boost of the immune response with a recall antigen for which a patient already has memory B-cells [8]. However, an influenza or tetanus (re)vaccination in patients with MG neither cause an exacerbation of clinical symptoms nor an immunological exacerbation in patients with MG.

We suggest to provide an advice on vaccinations for the individual patient, based on the indication and necessity of a vaccination. Preferably vaccinate in a stable phase of the disease and advise against live attenuated vaccines in the immune compromised patient. Consider checking the efficacy of the immune response after vaccination, by measuring antibody titres. If necessary, the vaccination can be repeated in order to achieve an adequate, protective titre. At last, we recommend the influenza vaccine to all patients with an autoimmune disease or who use immunosuppressive medication, given the increased risk of morbidity and mortality in infections.

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ENGLISH SUMMARY

Myasthenia gravis (MG) is an acquired autoimmune disease of the neuromuscular junction. The initial trigger for making the pathogenic anti-AChR antibodies which cause myasthenia gravis still has to be elucidated. As described in **Chapter 2**, anti-AChR antibodies can be present long before clinical onset of the disease. We described a unique case of a young female with MG in whom serum samples were available over a period of at least 2 years before the onset of clinical symptoms. This patient showed a gradual increase of anti-AChR antibodies in a period of more than two years before becoming symptomatic of myasthenia. Our data suggest that pregnancy triggered the clinical manifestation of a smouldering autoimmune antibody response, but was not the primary trigger that started the production of anti-AChR antibodies in itself.

In all human clinical trials, good, validated, clinical outcome measures are of great importance. One of these outcome measures in myasthenia gravis is the 15-item Myasthenia Gravis Quality of Life (MG-QoL15). The MG-QoL15 scale has been developed to assess the health-related quality of life of patients with MG. The aim of this study was to translate the original English version into Dutch and to test the test-retest reliability and construct validity **(Chapter 3)**. Fifty patients with MG were included. Test-retest reliability and internal consistency were assessed using the intraclass correlation coefficient (ICC) and the Cronbach α. Construct validity was assessed by testing 5 predefined hypotheses, which were defined based on previous literature and the content of the outcome measure. A good test-retest reliability was confirmed with an ICC of 0.866. The Cronbach α was 0.93. The predefined hypotheses were confirmed in 80% of cases, which points to good construct validity. Since the questionnaire is validated in Dutch, it can be used for research in a Dutch-speaking population. It is also suitable for monitoring individual patients in clinical practice **(Chapter 3)**.

Patients with an autoimmune disorder are believed to be at an increased risk of infection, due to their immunosuppressive therapy or due to the immune abnormalities associated with their disease. Therefore, patients with an autoimmune disease, like myasthenia gravis (MG), are recommended to use preventive vaccinations, in particular the influenza vaccination. Since MG is a rare disease, specific studies on the effect of vaccinations in MG were not yet performed.

In **Chapter 4** we described a prospective study on the efficacy and safety of tetanus revaccination in patients with stable MG or Lambert-Eaton myasthenic syndrome. The aim of this study was to investigate the humoral immune response to and safety of a tetanus revaccination in these patients. A tetanus revaccination was administered to 66 patients. Before and 4 weeks after revaccination a blood sample and clinical outcome scores were obtained. Anti-tetanus IgG total, IgG1 and IgG4 titres were measured with an ELISA and disease-specific antibody titres (AChR, MuSK or VGCC) with a radio-immunoprecipitation assay. A historic healthy control group was used

for comparing tetanus antibody titres with that of our patients. A placebo (saline) vaccination group was used to investigate the variability of clinical outcome scores with a 4 weeks interval.

In 60 of 65 patients, we found a significant increase of the anti-tetanus antibody response. Thymectomy did not have an impact on this responsiveness. Patients with immunosuppressive medication had a significantly lower pre and post titre compared to healthy controls, but their response was still significant. The titrers of disease-specific antibodies were unchanged 4 weeks after revaccination. The clinical outcome scores showed no exacerbation of symptoms of the disease.

Therefore, we concluded that a tetanus revaccination in patients with myasthenia gravis or Lambert-Eaton myasthenic syndrome is safe and induces a significant immune response, irrespectively of their immunosuppressive medication. We observed neither immunological nor clinical relevant exacerbations associated with the tetanus revaccination.

A small number of observational studies suggest that influenza vaccination is safe(1-3) and recently a randomized controlled trial showed that influenza vaccination has no influence on AChR antibody titres.

In the Netherlands, annual vaccination against influenza is recommended for all patients with an autoimmune disease. However, in our personal experience and as described earlier, many patients express concern that vaccination may lead to an exacerbation and a substantial number decline vaccination each year based on these concerns. This is unfortunate, as seasonal vaccination against influenza is highly effective in reducing laboratory-confirmed influenza illness, hospital admissions and risk of death, especially in elderly and frail patients. This is relevant, as this age group has the highest incidence of autoimmune MG. In Chapter 5 we describe our prospective, placebo-controlled study on influenza vaccination in AChR MG. The aim of this study was to investigate the efficacy and safety of an influenza vaccination in patients with AChR MG. An influenza vaccination or placebo was administered to 47 AChR MG patients. Before and 4 weeks after administration blood samples and clinical outcome scores were obtained. Antibodies to the vaccine strains A/California/7/2009 (H1N1) pdm09, A/Hong Kong/4801/14 (H3N2) and B/Brisbane/060/08 were measured using the hemagglutination-inhibition (HI) assay and disease-specific AChR antibody titres were measured with a radio-immunoprecipitation assay. Forty-seven healthy controls (HC) were vaccinated with the same influenza vaccine to compare antibody titres. A post-vaccination, seroprotective titre (HI≥1:40) was achieved in 89.4% of MG patients vs. 93.6% in healthy controls for the H3N2 strain, 95.7% vs 97.9% for the H1N1 strain and 46.8 vs 51% for the B-strain. A seroprotective titre for all three strains of the seasonal influenza vaccine was reached in 40.4% (19/47) of the MG group and in 51% (24/47) of the HC group. Immunosuppressive medication did not significantly influence post geomean titres (GMT). The titres of disease-specific AChR antibodies were unchanged 4 weeks after vaccination. The clinical outcome scores

showed no exacerbation of MG symptoms. Concluding, the antibody response to an influenza vaccination in patients with AChR MG was not different from that in healthy subjects, even in AChR MG patients using immunosuppressive medication. Influenza vaccination does not induce an immunological or clinical exacerbation of AChR MG.

In **Chapter 6** we describe the cellular response to a tetanus revaccination, combined with broad subsets of T- and B-cells before and after vaccination. The fifty, included patients are the same as the study that investigated the humoral response to tetanus vaccination in Chapter 4. Before and 4 weeks after revaccination a blood sample was obtained. Lymphocyte subsets and B- and T-cell differentiation stages in isolated peripheral blood mononuclear cells (PBMC) were investigated by flowcytometry. PBMC were in vitro stimulated with TT (0.2 or 20 Lf/mL) and, after 3H-thymidine uptake, a stimulation index (SI) ≥3.0 was considered as evidence of antigen-induced proliferation.

Patients showed a significant tetanus induced proliferative response. Lower pre and post vaccination SI was found in patient with immunosuppressive medication (IM+) compared to those without IM (IM-). Despite this, both groups reached a significant post vaccination response. TT revaccination did not affect cell counts of lymphocyte subpopulations and B- and T-cell differentiation stages. A preceding thymectomy showed no effect on lymphocyte compartments. However, IM, in particular azathioprine, was associated with strongly decreased NK cell and B-cell counts, but did not affect levels of anti-TT antibodies before or after revaccination.

Overall, we concluded that, TT revaccination resulted in an increase of the in vitro tetanus-specific proliferative response and did not affect the composition of lymphocyte compartments. Whereas thymectomy had no significant influence, significant effect of immunosuppressive medication, azathioprine in particular, i.e. a decrease of numbers of B-cell subsets and NK cells, was found. However, this had no impact on the IgG anti-tetanus response upon revaccination. In conclusion, revaccination is effective in adult AChR MG patients with stable disease irrespective of their thymectomy status and actual immunosuppressive medication.

NEDERLANDSE SAMENVATTING

Myasthenia gravis (MG) is een verworven auto-immuunziekte van de neuromusculaire overgang. De initiële trigger voor de start van de productie van de pathogene AChR-antistoffen is onbekend. Zoals beschreven in hoofdstuk 2 kunnen de AChR-antistoffen al lang aanwezig zijn voordat zich klinische symptomen voordoen. We beschrijven een unieke casus van een jonge vrouw met MG van wie er tot 2 jaar voor het begin van de klinische symptomen, serummonsters beschikbaar waren. In deze serummonsters werd een langzame stijging van de AChR-antistoffen gevonden, voordat er klinische symptomen waren. Onze data suggereert dat de zwangerschap een trigger is geweest voor het klinisch manifest worden van een sluimerende productie van auto-immuun antistoffen, maar de zwangerschap was niet de primaire trigger voor deze productie.

In alle klinische trials zijn goede, gevalideerde, klinische uitkomstmaten van groot belang. Een van deze uitkomstmaten in MG is de 15-item Myasthenia Gravis Quality of Life (MG-QoL15). De MG-QoL15-schaal is ontwikkeld om de gezondheid gerelateerde kwaliteit van leven van patiënten met MG te beoordelen. Het doel van deze studie was om de originele Engelse versie in het Nederlands te vertalen en de test-hertest betrouwbaarheid en constructvaliditeit te testen (hoofdstuk 3). Vijftig patiënten met MG werden geïncludeerd. Test-hertest betrouwbaarheid en interne consistentie werden beoordeeld met behulp van de intra-klasse correlatiecoëfficiënt (ICC) en de Cronbach α. De constructvaliditeit werd beoordeeld door 5, vooraf gedefinieerde hypotheses te testen. Deze hypotheses werden gedefinieerd op basis van eerdere literatuur en de inhoud van de uitkomstmaat. Een goede test-hertest betrouwbaarheid werd bevestigd met een ICC van 0.866. De Cronbach α was 0,93. De vooraf gedefinieerde hypotheses werden in 80% van de gevallen bevestigd, wat wijst op een goede constructvaliditeit. Aangezien de vragenlijst nu in het Nederlands is gevalideerd, kan deze worden gebruikt voor onderzoek in een Nederlandstalige populatie. De MG-QoL15 is ook geschikt voor het monitoren van individuele patiënten in de klinische praktijk (hoofdstuk 3).

Patiënten met een auto-immuunziekte lopen een verhoogd risico op infectie, vanwege hun immunosuppressieve therapie of vanwege de immuun afwijkingen die verband houden met hun ziekte. Daarom wordt patiënten met een auto-immuunziekte, zoals myasthenia gravis (MG), aanbevolen preventieve vaccinaties te gebruiken, met name de griepvaccinatie. Aangezien MG een zeldzame ziekte is, waren er nog geen specifieke onderzoeken naar het effect van vaccinaties bij MG uitgevoerd.

In hoofdstuk 4 hebben we een prospectieve studie beschreven naar de effectiviteit en veiligheid van tetanusrevaccinatie bij patiënten met stabiele MG of het Lambert-Eaton myastheen syndroom (LEMS). Het doel van deze studie was om de humorale immuunrespons en veiligheid van een tetanusrevaccinatie bij deze patiënten te onderzoeken. Een tetanusrevaccinatie werd toegediend aan 66 patiënten.

Vóór en 4 weken na revaccinatie werd er bloed en de klinische uitkomstscores afgenomen. Anti-tetanus IgG-totaal, IgG1- en IgG4-titrs werden gemeten met een ELISA en ziekte-specifieke antistoffen (AChR, MuSK of VGCC) met een radio-immunoprecipitatieassay. Een historische, gezonde controlegroep werd gebruikt om tetanus-specifieke antistoftiters te vergelijken met die van onze patiënten. Een placebo vaccinatiegroep (zoutoplossing) werd gebruikt om de variabiliteit van klinische uitkomstscores te onderzoeken met een interval van 4 weken.

Bij 60 van de 65 patiënten vonden we een significante toename van de anti-tetanus antistoffen. Thymectomie had geen invloed op deze respons. Patiënten met immunosuppressieve medicatie hadden een significant lagere pre- en posttiter vergeleken met gezonde controles, maar hun respons was nog steeds significant. De titers van ziekte-specifieke antistoffen waren 4 weken na revaccinatie onveranderd. De klinische uitkomstscores toonden geen verergering van de symptomen van de ziekte.

Daarom hebben we geconcludeerd dat een revaccinatie van tetanus bij patiënten met MG of LEMS veilig is en er een significante immuunrespons wordt geïnduceerd, ongeacht hun immunosuppressieve medicatie. We hebben noch immunologische noch klinische relevante exacerbaties waargenomen die verband houden met de revaccinatie van tetanus.

Een klein aantal observationele studies suggereert dat de griepvaccinatie veilig is in MG (1-3) en recent toonde een gerandomiseerde gecontroleerde studie aan dat griepvaccinatie geen invloed heeft op AChR-antistoftiters.

In Nederland wordt de jaarlijkse griepvaccinatie aanbevolen voor alle patiënten met een auto-immuunziekte. In onze persoonlijke ervaring en zoals eerder beschreven, uiten veel patiënten met MG echter de bezorgdheid dat een griepvaccinatie kan leiden tot een verergering van de ziekte en haalt daarom een aanzienlijk aantal geen griepvaccinatie. Dit is jammer, omdat een griepvaccinatie zeer effectief is bij het verminderen van laboratorium-bevestigde influenza, ziekenhuisopnames en het risico op overlijden, vooral bij oudere en kwetsbare patiënten. Dit is relevant, omdat deze leeftijdsgroep de hoogste incidentie van auto-immuun MG heeft. In hoofdstuk 5 beschrijven we onze prospectieve, placebogecontroleerde studie naar griepvaccinatie bij AChR MG. Het doel van deze studie was om de effectiviteit en veiligheid van een griepvaccinatie bij patiënten met AChR MG te onderzoeken. Een griepvaccinatie of placebo werd toegediend aan 47 patiënten met AChR MG. Vóór en 4 weken na toediening werd er bloed afgenomen en de klinische uitkomstscores verkregen. Antistoffen tegen de 3 stammen; A /California / 7/2009 (H1N1) pdm09, A / Hong Kong / 4801/14 (H3N2) en B / Brisbane / 060/08, werden gemeten met behulp van de hemagglutinatie-inhibitie (HI) -assay en AChR-antistoffen werden gemeten met een radio-immunoprecipitatieassay. Zevenenveertig gezonde controles (HC) werden gevaccineerd met hetzelfde vaccin om antistoffen te vergelijken. Een post-vaccinatie, seroprotectieve titer (HI> 1: 40) werd bereikt bij 89,4% van

de MG-patiënten versus 93,6% bij gezonde controles voor de H3N2-stam, 95,7% versus 97,9% voor de H1N1-stam en 46,8 versus 51% voor de B-stam. Een seroprotectieve titer voor alle drie de stammen werd bereikt in 40,4% (19/47) van de MG-groep en in 51% (24/47) van de HC-groep. Immunosuppressieve medicatie had geen significante invloed op post-geomean titers (GMT). De titers van de AChR-antistoffen waren 4 weken na vaccinatie onveranderd. De klinische uitkomstscores toonden geen verergering van MG-symptomen. Concluderend, de humorale respons op een griepvaccinatie bij patiënten met AChR MG is niet anders dan die bij gezonde personen, zelfs niet bij patiënten die immunosuppressieve medicatie gebruikten. Griepvaccinatie veroorzaakt geen immunologische of klinische verergering van AChR MG.

In hoofdstuk 6 beschrijven we de cellulaire respons op een tetanusrevaccinatie, gecombineerd met een breed aantal subsets van T- en B-cellen voor en na revaccinatie. De vijftig beschreven patiënten zijn dezelfde als in de studie die de humorale respons na tetanusrevaccinatie in hoofdstuk 4 beschrijft. Vóór en 4 weken na revaccinatie werd er bloed afgenomen. Lymfocytensubsets en B- en T-celdifferentiatiestadia in geïsoleerde, perifere mononucleaire cellen (PBMC) werden onderzocht met flowcytometrie. PBMCs werden in vitro gestimuleerd met TT (0,2 of 20 Lf / ml) en na opname van 3H-thymidine werd een stimulatie-index (SI)> 3,0 beschouwd als bewijs voor door antigeen geïnduceerde proliferatie.

Patiënten vertoonden een significante door tetanus geïnduceerde proliferatieve respons. Lagere pre- en post-vaccinatie-SI werd gevonden bij patiënten met immunosuppressieve medicatie (IM +) in vergelijking met patiënten zonder IM (IM-). Desondanks bereikten beide groepen een significante respons na vaccinatie. TT-revaccinatie had geen invloed op celtellingen van lymfocytsubpopulaties en B- en T-celdifferentiatiestadia. Een voorafgaande thymectomie vertoonde geen effect op lymfocytencompartimenten. IM, in het bijzonder azathioprine, was echter geassocieerd met sterk verlaagde aantallen NK-cellen en B-cellen, maar had geen invloed op de niveaus van anti-TT antistoffen vóór of na hervaccinatie.

In het algemeen hebben we geconcludeerd dat TT-hervaccinatie resulteerde in een toename van de in vitro tetanus-specifieke proliferatieve respons en geen invloed had op de samenstelling van lymfocytencompartimenten. Terwijl thymectomie geen significante invloed had, werd een significant effect van immunosuppressieve medicatie, in het bijzonder azathioprine, dat wil zeggen een afname van het aantal B-cel subsets en NK-cellen gevonden. Dit had echter geen invloed op de IgG-anti-tetanusrespons bij hervaccinatie. Concluderend is tetanus revaccinatie effectief bij volwassen AChR MG-patiënten met stabiele ziekte, ongeacht hun thymectomiestatus en gebruik van immunosuppressieve medicatie.

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CURRICULM VITAE

Ellen Strijbos werd op 17 april 1987 geboren in Nuenen. In 2005 behaalde ze haar Gymnasiumdiploma aan het Lorentz Casimir Lyceum in Eindhoven en startte met de studie Geneeskunde aan de Universiteit van Leiden. Tijdens haar 2e studiejaar ging ze voor keuzevakken naar China om meer te leren over Traditional Chinese Medicine. In 2009 deed ze tijdens haar wetenschappelijke stage onderzoek naar de mond- en gezichtsbetrokkenheid bij sclerodermie. Als onderdeel van haar co-schap liep ze stage in het Academisch Ziekenhuis van Paramaribo op de afdeling oogheelkunde en deed ze hier haar co-schap sociale geneeskunde en huisartsgeneeskunde in Suriname.

Na het afronden van de studie geneeskunde in november 2011, werkte ze als ANIOS neurologie in het Reinier de Graaf Gasthuis in Delft. In oktober 2013 begon ze haar opleiding tot neuroloog in het Leids Universitair Medisch Centrum (opleiders Prof. dr. R.A.C. Roos, Prof. dr. J.J. van Hilten en dr. C.S.M. Straathof). Ze startte met haar promotieonderzoek 'Vaccinations in autoimmune myasthenia gravis' in oktober 2014, zoals beschreven in dit proefschrift, onder leiding van Prof. J.J.G.M. Verschuuren. Ze hoopt haar opleiding tot neuroloog in 2022 af te ronden.

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