



## Research Paper

# Acetylcholine Receptor Antibody Titers and Clinical Course after Influenza Vaccination in Patients with Myasthenia Gravis: A Double-Blind Randomized Controlled Trial (ProPATient-Trial)



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## ABSTRACT

**Background:** It is a continuous matter of discussion whether immune activation by vaccination in general and Influenza vaccination in particular increases the risk for clinical deterioration of autoimmune diseases. This prospective study investigated the serological and clinical course of autoimmune Myasthenia gravis (MG) after a seasonal influenza vaccination.

**Methods:** This randomized, placebo-controlled, double-blind study enrolled MG patients with antibodies against acetylcholine-receptors (AChR-ab). They were allocated to receive seasonal influenza vaccine or placebo. The primary endpoint was the relative change of AChR-ab-titer over 12 weeks. A relative increase of 20% was set as non-inferiority margin. Secondary endpoints were clinical changes in the modified Quantitative Myasthenia Gravis Score (QMG), increase of anti-influenza-ELISA-antibodies, and changes of treatment. The study is registered with Clinicaltrialsregister.eu, EudraCT number 2006-004374-27.

**Findings:** 62 patients were included. Mean  $\pm$  standard deviation (median) in the vaccine and placebo group were AChR-ab-titer changes of  $-6.0\% \pm 23.3\%$  ( $-4.0\%$ ) and  $-2.8\% \pm 22.0\%$  ( $-0.5\%$ ) and QMG score changes of  $-0.08 \pm 0.27$  (0.17) and  $0.11 \pm 0.31$  (0.00), respectively. The difference between groups (Hodges-Lehmann estimate with 95% CI) was - for the AChR-ab-titer change  $4.0\% [-13.3\%, 4.5\%]$  ( $p = 0.28$  for testing a difference,  $p < 0.0001$  for testing non-inferiority) and for the QMG change  $0.00 [-0.17, 0.00]$  ( $p = 0.79$  for testing a difference). The occurrence of 74 adverse events (AE) was comparable between groups. The most common AE was flu-like symptoms. One serious AE (hospitalisation following gastrointestinal haemorrhage) in the verum group was not related to the vaccine.

**Interpretation:** Influenza vaccination in MG is safe. Uprating the potential risk of a severe course of MG exacerbation during influenza infection compared to the 95% CI differences for the endpoints, vaccination is principally indicated in this patient population.

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## 1. Introduction

Myasthenia gravis (MG) is an antibody-mediated autoimmune neuromuscular disorder. In the vast majority of cases, T-cell dependent

autoantibodies against the nicotinic acetylcholine receptor (AChR-ab) cause exaggerated fatigability of striated skeletal muscles with amelioration after periods of rest. Pathophysiologically, MG is a heterogeneous disease with an ocular manifestation, an early or late generalized onset, thymoma associated, seronegative for AChR-ab or associated with other autoantibodies like anti-MuSK or anti-LRP4 (Sommer et al., 2008; Binks et al., 2016). Although incidence and prevalence are increasing, myasthenia gravis remains a rare disease, affecting about 78 per 100,000 people world-wide (range 15–179) (Carr et al., 2010). However,

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myasthenia gravis is considered as an index disease, from which general pathophysiological principles of antibody-mediated autoimmunity have been deduced. Clinical signs include exercise-induced fatigue either of the ocular muscles alone (ocular myasthenia) or striated skeletal muscle and the ocular, facial and bulbar musculature (generalized myasthenia). Changes in AChR-ab titers correlate intra-individually with the severity of symptoms (Tzartos et al., 1982). The thymus is altered in the majority of patients with early-onset or thymoma associated MG, but data from recently published clinical trials suggest, that patients with late-onset could also benefit from thymectomy (Sommer et al., 2008; Wolfe et al., 2016). In most cases anticholinesterase drugs, immunosuppressive treatment and thymectomy result in effective disease control (Wolfe et al., 2016). Omission of anticholinesterase drugs or immunosuppressants, administration of drugs interrupting neuromuscular transmission, and infections, in particular of the upper respiratory tract and pneumonias, can cause acute exacerbations (Hohlfeld et al., 1985). The consequential myasthenic crisis is characterized by life-threatening complications with severe weakness, swallowing difficulties and respiratory failure, which requires intensive care treatment (Thomas et al., 1997).

Influenza infections are common in the general population, affecting about 5% to 20% during winter months (RKI, Robert-Koch-Institut, 2012). Patients receiving immunosuppressive treatment, including those with myasthenia gravis, are at increased risk of influenza infections. In Germany, the Standing Committee on Vaccination at the Robert Koch Institute (STIKO) recommends seasonal influenza vaccination for people over age 60 and for those with chronic diseases, including neurologic disorders (RKI, Robert-Koch-Institut, 2012). Other countries recommend influenza vaccination for the general public starting 6 months after birth (Fiore et al., 2010).

The effectiveness of vaccinations can be impaired by several factors, such as age, comorbid conditions, and concomitant medication. Conflicting results in terms of effectiveness were found in a meta-analysis of studies examining immunological response to influenza vaccination in patients who are at particular high risk for serious post-influenza complications and for whom immunization against this virus is strongly recommended. However, there was consensus that influenza vaccines were well tolerated in high risk patients, and all adverse reactions were generally mild and similar to those observed in healthy people (Brydak and Machala, 2000). The analysis included patients with pulmonary diseases, renal diseases, diabetes mellitus, cancer and haemophilia, and HIV infection. Controlled studies in patients with autoimmune disorders, however, are sparse and none have been performed in patients with myasthenia gravis. Although patients with autoimmune disorders are at increased risk of influenza infections due to immunosuppressive treatment, there is concern that vaccinations may trigger the immune system and lead to exacerbation of the underlying disease. So far, the issue of clinical or paraclinical deterioration following vaccination remains unclear and has not been systematically investigated in the setting of autoimmune disorders. Myasthenia gravis is well suited for this investigation because the AChR-ab causing the exercise-induced muscle weakness can be precisely determined. In consequence, myasthenia gravis is characterized as an index disease for all T-cell-dependent antibody-mediated autoimmune diseases. The present randomized controlled trial was the first to be conducted in order to investigate the effect of seasonal influenza vaccinations on AChR-ab-titers in patients with myasthenia gravis. Due to the index nature of MG, the results obtained in this setting may then stimulate further clinical research in other antibody-mediated autoimmune disorders.

## 2. Methods

### 2.1. Study Design

This phase IIIb prospective, double-blind, randomized, placebo-controlled study was conducted in a single centre in Germany during

a period of three consecutive winter seasons. The study was done in compliance with the Declaration of Helsinki, Good Clinical Practice guidelines, the approval of all relevant institutional review boards and ethics committees, and local regulatory requirements. Ethics approval was obtained from the IRB of the Faculty of Human Medicine at the University of Marburg (Eudra-CT-Number: 2006–004374–27). Fig. 1 gives an overview of the study recruitment. The study protocol is available at <https://www.clinicaltrialsregister.eu/2006-004374-27/DE>.

### 2.2. Participants

The main eligibility criteria for the inclusion of patients were age 18–80 years, diagnosis of generalized myasthenia gravis (ICD10GM2006: G70.0), positive acetylcholine receptor antibodies, stable clinical course for at least 4 months before inclusion in the study (i. e. no impact on 'activities of daily living' with or without immunosuppressive and/or symptomatic treatment), and written informed consent. The main exclusion criteria were any vaccination in the last 9 months prior to study entry, history of serious or acute heart disease, severe cardiac dysrhythmias during the ECG at the screening visit, history of cancer, current infection or current pyrexia, known allergy to chicken proteins, severe adverse event in earlier vaccination, any contraindication for Mutagrip® according to the summary of product characteristics (SmPC), and current participation in another clinical trial. Participants were recruited from the patient pool of the neuro-immunological outpatient department at the university hospital Marburg, Germany. All patients signed written informed consent before entry into the study. Table 1 shows the demographic, clinical and therapeutic baseline characteristics of both groups.

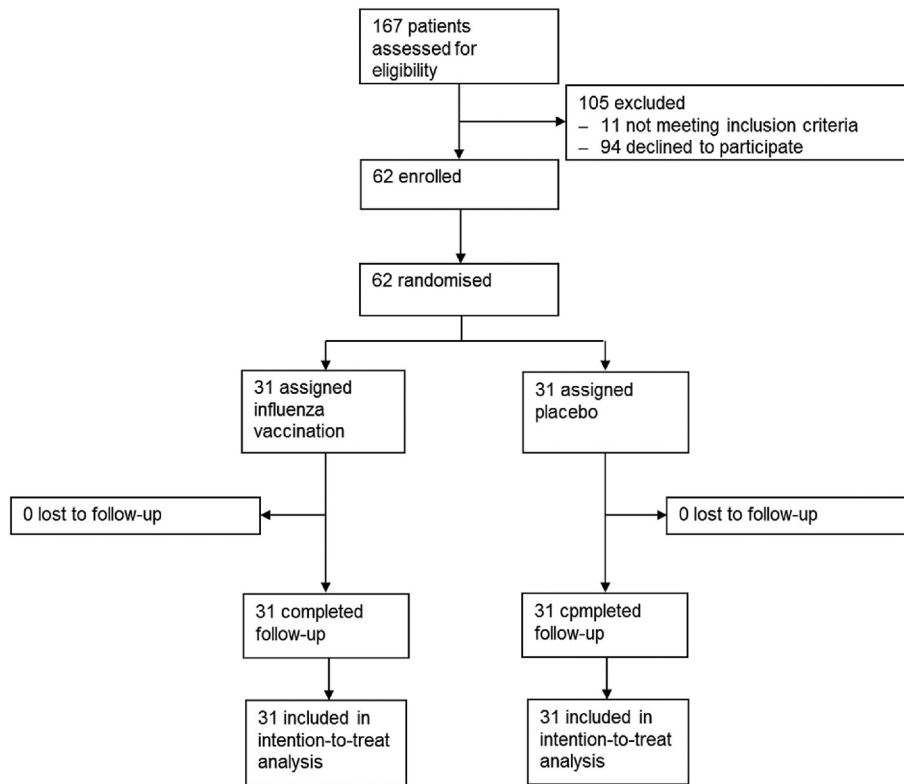
### 2.3. Randomization and Masking

Patients were randomly allocated in a ratio of 1:1 to receive intramuscular injection of non-adjuvanted seasonal influenza vaccination (Mutagrip®) or placebo (0.9% NaCl solution). Absence or presence of immunosuppressive treatment was considered by stratified randomization. The Coordinating Centre for Clinical Trials at the Philipps-University in Marburg, Germany, did a centralised, concealed randomization to either DP or IP (1:1) by fax at visit 1, after the patients were enrolled into the study. The randomization sequence was computer generated. The randomization procedure was a covariate-adaptive procedure according to Rosenberger and Lachin.

In order to maintain masking of all investigators, a study nurse was employed for documentation, preparation and administration of injections. The randomization result was exclusively known by the nurse. In order to maintain masking of patients, patients were equipped with sleep masks during the injection procedure. The vaccine was provided from the hospital pharmacy in commercial pre-filled glass syringes. Since no pre-filled syringes identical in appearance were available, the placebo was provided in polycarbonate/polypropylene syringes.

### 2.4. Procedures

Commercially available influenza vaccine was used (Mutagrip®, Sanofi Pasteur MSD GmbH). In concurrence with the annual recommendations provided by the World Health Organization (WHO), different combinations were used for each season. The pre-filled syringes contained 0.5 mL vaccine. 0.9% NaCl solution was used as placebo. Patients received injections into the deltoid muscle. The follow-up period was 12 weeks per patient and included four visits. Patients had to return to the centre 3 (visit 3) and 12 weeks (visit 5) after the baseline visit (visit 1, screening and vaccination), respectively. Visits 2 and 4 were conducted via phone call 1 and 8 weeks after visit 1, respectively. Each patient underwent a 3-year post-study follow-up observational period by phone-call whereby participants were asked whether they had experienced influenza infections since the end of the study.



**Fig. 1.** Trial profile. After randomization all patients underwent 5 visits each over a period of 12 weeks. After visit 1 (vaccination), in-house visits followed at week 3 (V3) and 12 (V5 – end of study). For safety reasons two telephone calls at week 1 (V2) and week 8 (V4) were performed.

## 2.5. Outcomes

The primary outcome endpoint was the relative change of AChR-ab-titer at week 12 compared to baseline values. Secondary endpoints were AChR-ab-titer at week 3, clinical change assessed by a modified quantitative myasthenia gravis score (QMG), increase of anti-influenza-ELISA-antibodies (Influenza A/B IgA and IgG) 3 and 12 weeks after immunization, and changes of immunosuppressive or anticholinesterase treatment after 12 weeks. The standard QMG was slightly modified because during the planning phase of the study one has to keep in mind, that e. g. due to concomitant diseases like stroke, peripheral nerve injury et cetera, not every single patient is able to perform every single test. Therefore we divided the QMG score result by the total number of performed items. Safety assessments were the occurrence of adverse events recorded at every visit. Adverse events of special interest were influenza infection or fever.

## 2.6. Statistical Analysis

The primary endpoint (change of AChR-ab-titer) was taken into account for sample size calculation. In an own case series of 13 clinically stable MG patients we obtained during a mean observation period of 6 months a mean change of the AChR-ab-titer of  $12.7\% \pm 8.7\%$ . Initially, based on the assumption of a SD of 9% from this observation, a 5% drop-out rate at week 12, and the assumption that a 5% difference to be detected in the change of AChR-ab-titer between groups was immunologically relevant, (Besinger et al., 1983) a total number of 80 patients per treatment group was needed to show a significant effect for the primary endpoint with a two-sided comparison type I error rate controlled at 0.05 and a power of 90%. However, data on the clinically relevant difference in literature are controversial, ranging from 5% to 50% (Besinger et al., 1983; Seybold and Lindstrom, 1981). After three years of recruitment and randomization of  $n = 62$  patients we observed neither a

single clinical deterioration nor any serious or severe adverse events due to vaccination for prevention of influenza. The ProPATIENT study was designed as a monocenter study and was conducted independently from industrial funding or support. Due to economic restrictions an extension to a multi-center design at that time to overcome recruitment difficulties was not possible. We therefore obtained advice from an external expert panel of eight international MG experts yielding an increase of the AChR-ab-titer of  $<20\cdot0\%$  as clinically irrelevant and an increase of the AChR-ab-titer of  $20\cdot0\%$  or more as potentially clinically relevant (class 5 evidence). Hence the objective of the trial was bridged from a strict immunologically driven perspective to a clinical one. Inferiority should no longer be demonstrated, but non-inferiority of influenza vaccination. The sample size was recalculated expecting no change in the AChR-ab-titer and adapting the SD of the titer change to a SD of 23.3%, the observed titer change at an interim look in which the effect on the AChR-ab-titer change was withheld to the investigators. Recalculation resulted in a total number of 28 patients needed to be analyzed per treatment group in order to claim a significant non-inferiority for the primary endpoint with a two-sided comparison type I error rate controlled at 0.05 and a power of 80%. Since 31 patients had already been recruited in each group, all of them not dropping out for the primary analysis, recruitment was stopped.

The primary efficacy analyses were done in the intention-to-treat population (ITT), defined as all randomized patients who received one injection. The primary endpoint relative percentage change of AChR-ab-titer was calculated as the ratio of AChR-ab-titers at visit 5 and visit 1 minus 1. Success of vaccination was defined as a negative difference between the relative changes of antiAChR-ab-titer in the verum group and the placebo group. A difference of 20% was set as the non-inferiority margin. The change of the modified quantitative myasthenia gravis score (QMG) was the secondary outcome of most importance and was calculated as the absolute difference of the score at visit 5 minus the score at visit 1. An advantage of vaccination was a negative difference between the changes of QMG in the verum group and the

**Table 1**  
Baseline characteristics.

Baseline characteristics of the patients	Vaccinated group (n = 31)	Placebo group (n = 31)	Total (n = 62)
Age in years – mean (range)	58.48 (24–77)	58.19 (30–74)	58.34 (24–77)
Age > 60 years n, (%)	17 (54.8)	18 (58.1)	35 (55.6)
Male n, (%)	18 (58.1)	14 (45.2)	32 (51.6)
Female n, (%)	13 (41.9)	17 (54.8)	30 (48.4)
MG-Subtype n, (%)			
OMG	7 (22.6)	2 (6.5)	9 (14.5)
EOMG	9 (29.0)	11 (35.5)	20 (32.2)
LOMG	11 (35.5)	16 (51.5)	27 (43.6)
TOMG	4 (12.9)	2 (6.5)	6 (9.7)
MGFA Clinical Classification n, (%)			
I	6 (19.4)	3 (9.7)	9 (14.5)
II a	11 (35.5)	15 (48.4)	26 (41.9)
II b	9 (29.0)	10 (32.2)	19 (30.7)
III a	2 (6.5)	3 (9.7)	5 (8.1)
III b	3 (9.7)	0	3 (4.8)
MGFA Therapy-Status n, (%)			
NT	3 (9.7)	2 (6.5)	5 (8.1)
CH	3 (9.7)	4 (12.9)	7 (11.3)
PR	1 (3.2)	0	1 (1.6)
IM	6 (19.4)	5 (16.1)	11 (17.7)
CH, IM	14 (45.2)	12 (38.7)	26 (41.9)
CH, PR, IM	4 (12.9)	5 (16.1)	9 (14.5)
CH, PR, IGc	0	1 (3.2)	1 (1.6)
CH, PR, IM, IGc	0	1 (3.2)	1 (1.6)
CH, IM, IGc	0	1 (3.2)	1 (1.6)
Specification IM n, (%)	n = 24	n = 24	n = 48
Aza	18 (58.1)	22 (71.0)	40 (64.5)
MMF	2 (6.5)	2 (6.5)	4 (6.5)
CsA	2 (6.5)	0	2 (3.2)
MTX	1 (3.2)	0	1 (1.6)
Tac	1 (3.2)	0	1 (1.6)
Thymectomy n, (%) SPT	13 (41.9)	15 (48.4)	28 (45.2)

OMG: ocular MG; EOMG: early-onset MG; LOMG: late-onset MG; TOMG: Thymom-associated MG. NT: no therapy; CH: Cholinesterase inhibitors PR: Prednisone IM: Immunosuppression therapy other than Prednisone; IGc: IVIg-Therapy, chronic; SPT: Status post-thymectomy. Specification of immunosuppression therapy other than prednisone: Aza: Azathioprin; MMF: Mycophenolatumofetil; CsA: Cyclosporin A; MTX: Methotrexat; Tac: Tacrolimus. Using  $X^2$  analysis, there were no statistical differences between the verum and placebo group, neither for MG subtype nor for MGFA classes.

placebo group. For both, the relative change of AChR-ab-titers and the absolute change of the QMG, treatment groups were compared using the Mann-Whitney *U* test, and the difference between groups was estimated with 95% confidence interval (CI) by the Hodges-Lehmann (HL) method (HL estimate and 95% CI). Anti-influenza titers were determined by enzyme-linked immunosorbent assay (ELISA). For comparison of anti-influenza titer at baseline and at visit 3 the mean fold increase was calculated. Treatment groups were compared using Student's *t*-test. Changes in the modified myasthenia gravis score were used to assess the clinical course. Absolute changes from baseline to visit 3 and 5 were calculated, respectively. The number of patients with dose changes in pyridostigmine and immunosuppressive medication was recorded over the course of the study and compared between groups using the chi-square-test. *P*-values of two-sided tests were presented, for confirmatory, explanatory, and for descriptive purposes. The significance level for confirmatory analysis of the primary endpoint was 0.05. Data were recorded on an electronic case report form (eCRF). Data entered into the eCRF were monitored periodically. The collected data were analyzed using the SPSS (IBM Deutschland GmbH, Ehningen, Germany) Cytel Studio (Cytel Software Corporation, Cambridge, MA, USA) for Windows programme packages.

### 2.7. Role of the Funding Source

The funder and the corresponding author were jointly involved in the study design, data gathering, data management, and statistical

analysis. The funder had no role in the interpretation of the data or in the decision to submit the manuscript for publication. The funder funded editorial assistance provided during the writing of the manuscript. All authors had full access to all the data in the study and final responsibility for the decision to submit the paper for publication.

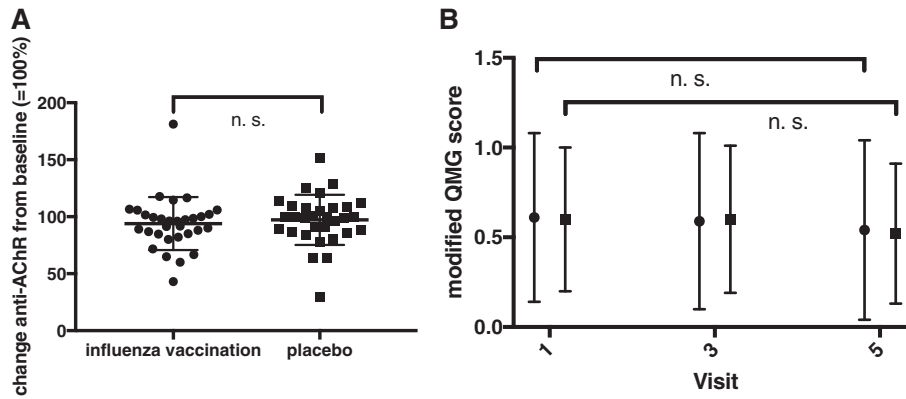
### 3. Results

During a period of three consecutive winter seasons, 167 patients were assessed for eligibility. Of those, 11 did not meet the inclusion criteria and 94 declined to participate. Patients declined participation predominantly because they did not want to lose the vaccine protection by receiving placebo or they had concerns regarding safety and tolerability of the vaccination. The remaining 62 AChR-ab-positive patients were randomly allocated, 31 to each treatment group (Fig. 1). No acute influenza infection occurred either in the verum or in the placebo group during the study. However, 97% of the verum group patients and all patients in the placebo group were pre-vaccinated at baseline. One patient of the verum group was vaccinated during the incubation period of an acute influenza infection, and showed no signs or symptoms of an acute infection when he was randomized. The overall benefit risk ratio was considered to be positive, though sample size was not calculated for proving efficacy of the vaccination. Hence, recruitment was discontinued when no clinical deterioration after 3 seasons in either of the still unblinded groups was observed after vaccination. From a clinical point of view, it was considered unethical to withhold vaccination from patients by continuing allocation to the placebo group.

All patients completed the follow-up period and were included in the analysis. Baseline characteristics, including the numbers of MG subgroups and MGFA classes were of no statistical significant difference between verum and placebo treated patients ( $X^2$  test; Table 1), including the proportion of patients receiving immunosuppressive therapy.

Over 12 weeks, a decrease of AChR-ab-titers was found in both groups ( $6.0\% \pm 23.3\%$  and median  $4.0\%$  in the verum group of 31 patients,  $2.8\% \pm 22.0\%$  and median  $0.5\%$  in the placebo group of 31 patients, Fig. 2A). The difference between the relative change of AChR-ab-titer in the verum group and the placebo group in terms of the Hodges-Lehmann-estimate with 95% CI was  $-4.0\%$  [ $-13.3\%$ ,  $4.5\%$ ]. There was no difference in the AChR-ab titer between week 3 and week 12 (Table 2). Thus, the vaccination did not induce a clinically relevant AChR-ab boost ( $p < 0.0001$  for testing non-inferiority). Although the one outlier in the verum group in Fig. 2A showed a titer increase of  $81.2\%$ , this was not associated with a worsening of clinical symptoms or signs, as indicated by a stable modified QMG. Five patients had a clinically relevant titer increase of  $\geq 20\%$ . Of those, one immunosuppressed patient with late-onset MG was allocated to the verum group and four received placebo. A clinically irrelevant increase of the AChR-ab-titers could not be excluded ( $p = 0.28$  for testing differences). However, even the immunological relevance of an increase of  $4.5\%$  is questionable. Among the subgroup of 12 patients not receiving immunosuppressive treatment, those 6 patients in the verum group had a mean decrease in AChR-ab-titer of  $8.7\% \pm 16.3\%$  while those 6 patients in the placebo group had a mean increase of  $3.0\% \pm 12.8\%$ . However, the difference between the groups was not clinically relevant.

Increases in the modified QMG indicative of clinical deterioration were not observed in any treatment group (Table 2B). Over the course of 12 weeks a decline was observed in both groups, a decline of  $0.08 \pm 0.27$  (median  $0.17$ ) in the verum group and of  $0.11 \pm 0.31$  (median  $0.00$ ) in the placebo group. The difference in terms of the Hodges-Lehman estimate (HL-estimate) with 95% CI was  $0.00$  [ $-0.17$ ,  $0.00$ ],  $p = 0.79$ . Pyridostigmine or immunosuppressive medication remained unchanged over the course of the study in 52 of the 62 participants. The dosage of pyridostigmine or immunosuppressants could be decreased in 6 patients of the verum group and 3 patients of the placebo group. Pyridostigmine dosage was increased in one patient of the placebo group.



**Fig. 2.** Relative change of AChR-ab-titer (A) and modified QMG score, i. e. the total sum score of the standard QMG divided by the absolute number of the performed examination items (B) from baseline (visit 1) to week 12 (visit 5). Shown are single values, means, and SD.

At baseline, 98% of all included patients showed a seroprotection titer of  $34.4 \pm 16.6$  for anti-Influenza A IgG and  $13.5 \pm 5.2$  for anti-Influenza B IgG, respectively. Three weeks after vaccination, the verum group showed an increase in either in anti-influenza A (IgA  $1.55 \pm 1.17$  fold; IgG  $1.08 \pm 0.39$  fold) or anti-influenza B antibodies (IgA  $1.10 \pm 0.44$  fold; IgG  $1.41 \pm 0.81$  fold). By contrast, there was no increase of these serum titers in the placebo group (0.97–1.00 fold titer change 3 weeks after placebo injection). However, the sample size of the ProPATient trial was not calculated to prove efficacy of the vaccination. Interestingly, for anti-influenza A IgG and anti-influenza B IgG baseline anti-influenza titers correlate inversely with mean fold increase after 3 weeks (Fig. 3). During the post-study follow-up 33 (53.2%) of the 62 participants had been successfully contacted by phone. Of those, none had experienced influenza since end of study. 8 of them (24.2%) reported influenza vaccination during the 3-year follow-up period.

One serious adverse event (SAE) occurred during the course of the study. The patient from the verum group was hospitalized following gastrointestinal haemorrhage. According to a study-independent physician, this event was not related to the study drug. In addition to the one SAE, 74 non-serious adverse events (AE) were documented during the course of the study, 42 AE in the verum group and 32 AE in the placebo group (Table 3). There were no obvious statistical differences between the frequency of either infectious or non-infectious AEs between the immunosuppressed ( $n = 50$ ) and non-immunosuppressed ( $n = 12$ ) MG patients. Flu-like symptoms occurred in 61.3% in the verum group and in 41.9% in the placebo group, however this difference was not statistically significant. Most patients experienced the flu-like symptoms within the first three weeks after the injection. Three patients described subjective deterioration of myasthenia symptoms. This was correlated

with a significant increase in the modified QMG from 0.17 to 1.17 in only one patient from the verum group. He was randomized and vaccinated during his asymptomatic incubation period of an acute influenza infection. Three days after vaccination he developed fever of  $>39^\circ\text{C}$  with cough. At this time point an influenza screening test was positive. The infection took a benign course and the patients recovered completely. The AChR-ab-titer was not affected. In the other two patients, the subjective deterioration of myasthenia symptoms was not correlated either with changes in the modified QMG or in AChR-ab titers.

**4. Discussion**

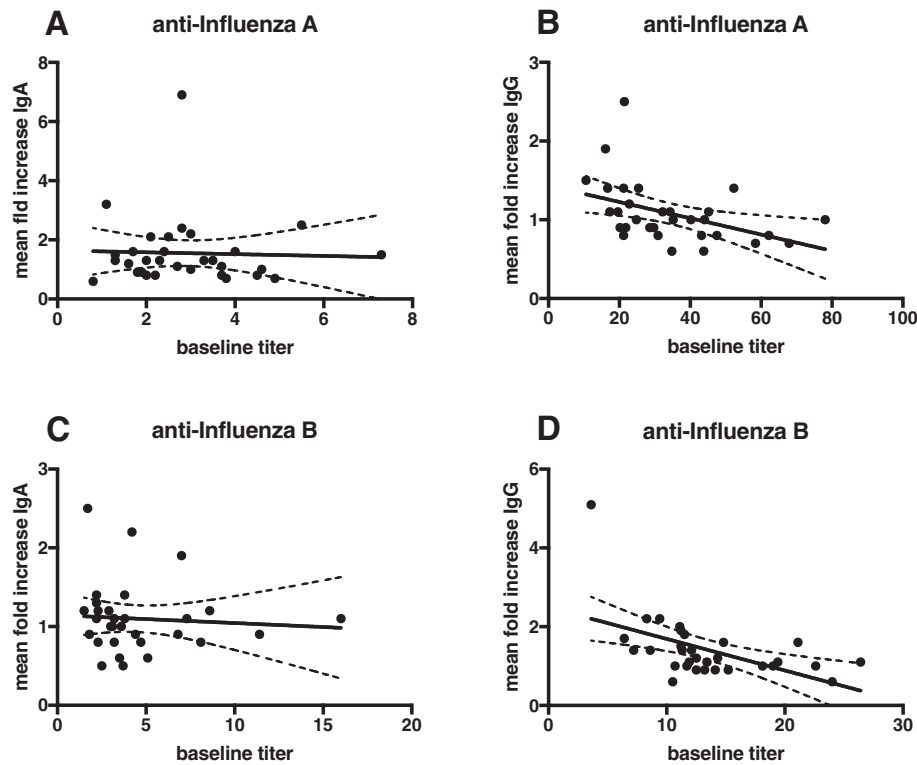
The results from this study show that seasonal influenza vaccination of myasthenia gravis patients does not lead to a clinically relevant increase of AChR-ab-titer, does not aggravate clinical signs of myasthenia gravis or require changes in pyridostigmine or immunosuppressant dosage. Beyond a slightly higher incidence of flu-like symptoms in the verum group (19:13), no additional increase in adverse events was observed. One serious adverse event occurred in the verum group which was not related to vaccination.

To date, investigations about the effect of vaccination on triggering a boost of auto-antibodies are sparse and inconsistent. So far only three randomized controlled trials have been conducted in the setting of granulomatosis with polyangiitis (GPA) and multiple sclerosis, the latter not being an antibody-mediated autoimmune disease (Holvast et al., 2009; Jeffs et al., 2015; Miller et al., 1997). Hence, the present study is the first randomized controlled trial investigating influenza vaccination in myasthenia gravis.

**Table 2**  
Changes in the AChR-ab titer and the modified myasthenia gravis score over the course of the study.

AChR-ab titer	Vaccinated group (n = 31)	Placebo group (n = 31)	p
Visit 1	28.7 ± 52.7	20.2 ± 20.0	n. s.
Visit 3	26.6 ± 49.0	18.6 ± 18.7	n. s.
Visit 5	25.8 ± 45.8	19.6 ± 20.1	n. s.
Change AChR-ab titer (% baseline)			
Visit 5 – Visit 1	–6 ± 23.3	–4.2 ± 23.4	n. s.
Patients with clinical deterioration, n (%)	0	0	n. s.
MG-score			
Visit 1	0.61 ± 0.47	0.60 ± 0.40	n. s.
Visit 3	0.59 ± 0.49	0.60 ± 0.41	n. s.
Visit 5	0.54 ± 0.50	0.51 ± 0.40	n. s.
Change MG-score			
Visit 5 – Visit 1	–0.08 ± 0.27	–0.11 ± 0.31	n. s.
Patients with clinical deterioration, n (%)	0 (0.0)	0 (0.0)	n. s.

Mean ± SD or n (%), and p-value for comparison of groups.



**Fig. 3.** Correlation between baseline influenza-ab-titers (ELISA) of anti-influenza A IgA (3A), anti-influenza A IgG (3B), anti-Influenza B IgA (3C), anti-influenza B IgG (3D) and mean fold increase after 3 weeks in patients from the verum group.

In the present study influenza vaccination did not lead to a boost in AChR-ab production during the 3-month observation period. On the contrary, titers in the verum group decreased over the course of the study. However, this is not of any clinical meaning, because a group effect on the myasthenia score could not be observed, and therefore it seems to be in the normal range of titer fluctuation. Of note, the decrease in the AChR-ab-titer was particularly prominent in the subgroup of patients not receiving immunosuppressive therapy. In addition to the more pronounced AChR-ab reduction, patients without immunosuppressive treatment had a more pronounced increase in three of the four examined anti-influenza antibody Ig classes, which might lead to the question whether the influenza vaccination inhibits an AChR-ab boost via its protective effect on subclinical infections, and whether suppression of auto-immune-effective B and T cells occurs simultaneously with the vaccine-induced immune response. By presentation of

inactivated haemagglutinin-/neuraminidase-surface antigens via antigen-presenting cells, influenza vaccination activates CD4+ T helper cells via MHC II. Activated CD4+ T helper cells stimulate and regulate the B cell response within the process of plasma cell and B memory cell differentiation and the switch in antibody class production from IgM to IgG (Holvast et al., 2007). AChR-ab production also depends on T helper cells (Sommer et al., 2008). Hypothetically and in theory, triggering an immune response to the vaccination could lead to a reduction of auto-immunity-mediating antibodies. However, by sharp contrast to this hypothesis, up to 15% of healthy subjects experienced de-novo production or increase of auto-antibodies after influenza vaccination. (Toplak et al., 2008) Similarly in systemic lupus erythematoses (SLE), transient increases of anti-cardiolipin-antibodies were found after influenza vaccination, (Vista et al., 2012) so that the finding in non-IS treated patients in the ProPATlent-trial cannot be clearly explained.

**Table 3**  
Summary of adverse events.

Adverse events	Vaccinated group n (%)	Placebo group n (%)	p-value	RR (95%-CI)
Local symptoms				
Pain	5 (16.1)	1 (3.2)	n.s.	5.00 (0.62–40.37)
Pruritus	0	1 (3.2)	n.s.	0.00 (0.00–∞)
Flu-like-symptoms	19 (61.3)	13 (41.9)	n.s.	1.46 (0.89–2.41)
Until V3	13 (41.9)	8 (25.8)	n.s.	1.63 (0.79–3.36)
Fatigue	2 (6.5)	5 (16.1)	n.s.	0.40 (0.08–1.91)
Headache	3 (9.7)	1 (3.2)	n.s.	3.00 (0.33–27.29)
Nausea and vomiting	2 (6.5)	1 (3.2)	n.s.	2.00 (0.19–20.94)
Vertigo	3 (9.7)	1 (3.2)	n.s.	3.00 (0.33–27.29)
Subjective deterioration of MG	1 (3.2)	2 (6.5)	n.s.	0.50 (0.48–5.23)
Gastrointestinal symptoms	3 (9.7)	5 (16.1)	n.s.	0.60 (0.16–2.30)
EBV-infection	1 (3.2)	0	n.s.	∞ (0.00–∞)
Urinary tract infection	0	1 (3.2)	n.s.	0.00 (0.00–∞)
Herpes labialis	0	1 (3.2)	n.s.	0.00 (0.00–∞)
Onychomycosis	1 (3.2)	0	n.s.	∞ (0.00–∞)
Visual impairment	1 (3.2)	0	n.s.	∞ (0.00–∞)
Fall	1 (3.2)	0	n.s.	∞ (0.00–∞)
Gastrointestinal haemorrhage	1 (3.2)	0	n.s.	∞ (0.00–∞)

In general, a sustained response to the vaccine can be expected after 12 weeks (Saiki et al., 2013). Therefore the primary endpoint was assessed at week 12 too. Theoretically, an earlier increase of the AChR-ab-titer is possible. However, the the AChR-ab-titer at week 3 showed no difference in comparison to the baseline results. Consequently, there is no evidence either for an early nor a late autoantibody boosting after influenza vaccination. Additionally this is in line with stable concurrent QMG results. Since the first report of a possible clinical meaning of an intraindividual increase of AChR-ab-titers, (Besinger et al., 1983) evidence from prospective studies either for a predictive value of AChR-ab-titers in general or a defined threshold for a clinical meaning is still lacking. However class 5 evidence suggests an intraindividual increase of the autoantibody being a useful sign to detect disease activity (Sommer et al., 2008; Deutsche Gesellschaft für Neurologie, 2012).

Our results are in line with the three available randomized controlled trials conducted in autoimmune disorders which demonstrated that influenza vaccination had no negative impact on the clinical course of multiple sclerosis and GPA (Holvast et al., 2009; Jeffs et al., 2015; Miller et al., 1997). Another phase III study in which patients with rheumatoid arthritis were randomized concurred with these observations (Chalmers et al., 1994). Other studies supporting the safety of influenza vaccinations in the setting of SLE and rheumatoid arthritis were of class II evidence. A meta-analysis of these studies found no evidence of compromised patient safety, disease deterioration or increased occurrence of severe adverse events after influenza vaccination of immunocompromised patients (Beck et al., 2011).

The effect of influenza vaccinations on myasthenia gravis patients has been investigated previously in two retrospective studies. A population-based study examined the association between hospitalisation due to disease exacerbation and previous influenza vaccination in 513 myasthenia gravis patients in Ontario between 1992 and 2006 (Zinman et al., 2009). Although no significant increase in hospitalisation was identified, the validity was limited by the study design, which considered only disease aggravations leading to hospitalisation, while mild and moderate exacerbations that may potentially affect a significant percentage of patients were not taken into account. Furthermore, the study does not allow conclusions about the safety of vaccinations because the analysis was limited to cases in which myasthenia gravis was coded as the primary reason for hospitalisation, thus omitting those patients that may have been hospitalized in consequence of the vaccinations without recognising the association to myasthenia gravis.

A second retrospective case-control study investigated patient compliance to vaccinations as well as side effects (Auriel et al., 2011). Seventy-four myasthenia gravis patients completed a questionnaire during the vaccination season 2009/2010, documenting whether they received influenza vaccination and if they experienced any side effects after the injection. 54.4% and 32.4% received seasonal influenza vaccination and pandemic anti-H1N1 vaccination, respectively, and 27% received both. The main reasons for refusing vaccination were fear of general side effects (42.6%) and fear of deterioration of myasthenia symptoms (31.5%). Although patients did not report any specific impairments following influenza vaccination, the validity is limited by recall bias (Okasha, 2007).

Due to the limitations owing to the design of both retrospective studies, caution had to be observed with the interpretation of these results. However, with the evidence derived from the ProPATient trial, it can now be concluded that patients with myasthenia gravis do not experience a clinically relevant increase in AChR-antibody titers. Furthermore the slightly higher incidence of flu-like symptoms in the verum group does not change risk-benefit ratio in MG patients. In this context the positive benefit-risk ratio of an influenza vaccination is strongly supported by upper respiratory infections (URI, e. g. influenza) being a major risk factor for the life threatening myasthenic crisis (Wendell and Levine, 2011; Kalita et al., 2014) or exacerbation (Seok et al., 2017). Hence, the ProPATient-trial provides class 1b evidence, that the

risk-benefit ratio of influenza vaccination in autoimmune MG is positive. Therefore vaccination can be recommended in this patient group. Since myasthenia gravis is an index disease for antibody-mediated autoimmunity, this finding can be postulated for other antibody-mediated autoimmune disorders. Presumably, the use of other attenuated vaccines may be similarly safe, but should be studied further.

Among the vaccinated participants, none experienced influenza infections during the study period or during the post-study follow-up. However, the study population appears to have an immune response of reduced quantity with most of the patients achieving seroprotection but only one patient achieving seroconversion. These results are consistent with a meta-analysis of 209 studies indicating lower odds of achieving seroconversion in all influenza subtypes in immunocompromised patients compared with healthy subjects (Beck et al., 2011). Similarly, a randomized controlled trial in patients with multiple sclerosis receiving immunizations and concomitant fingolimod treatment in comparison to placebo showed decreased vaccination-induced immune response (Kappos et al., 2015). Consistently, patients receiving immunosuppressive treatment in our study had a tendency towards anti-influenza-ab increase in three of the four Ig classes compared to patients not receiving immunosuppressants. In concordance with the observation that high influenza-ab-titers before vaccination correlate with the odds to achieve seroprotection while baseline titers and seroconversion are inversely correlated, (Seidman et al., 2012; Feng et al., 2009) patients in our study having a high influenza-ab-titer at baseline showed an inferior increase in the subtypes influenza A and B IgG and vice versa. In order to ensure a protective immune response, anti-influenza-ab titers should be determined after vaccination, and a second vaccine dose should be considered in patients receiving immunosuppressants.

In this context, enhancing the immune response by a second vaccine dose requires further investigation. While a second dose increased the rates of seroconversion and seroprotection in the general public (Seidman et al., 2012) and in liver transplant recipients, (Soesman et al., 2000) studies failed to demonstrate a positive effect on HIV patients, (Kroon et al., 1994) SLE patients, (Holvast et al., 2009b) and patients on dialysis. (Tanzi et al., 2007) Also the use of adjuvants has shown benefits by accelerating and enhancing an immune response in the elderly and in children, (Tsai TF, 2011; Haas, 2009) however, to date, the use of adjuvants in the setting of auto-immune diseases has not been investigated.

In addition to the double-blind, randomized, placebo-controlled design, potential strengths of this study include the choice of AChR-ab-titer changes as the primary endpoint, which has been identified to be a reliable and objective surrogate marker of clinical changes in myasthenia gravis patients (Tindall, 1981). The patient-reported incidence of influenza-like illness during the post-study follow-up poses a potential source of recall bias. The 3-month observation period was considered sufficient in order to detect any changes in myasthenia gravis symptoms based on the observation that changes in AChR-ab-titer may precede changes in clinical symptoms by 2 to 4 months (Schumm et al., 1984). Comparing patient demographics with real world data indicates that patients included in this study are representative of myasthenia gravis patients in general in terms of age, symptom severity, and immunosuppressive comedication (Kalb et al., 2002; Hart et al., 2009).

Overall, the ProPATient-trial increases class 1b evidence for a positive risk-benefit ratio of influenza vaccination in autoimmune MG. This is of clinical relevance for patients and physicians alike because many are reluctant to obtain vaccinations due to the absolute contraindication of administering attenuated vaccines to immunocompromised patients, (Deutsche Gesellschaft für Neurologie, 2012) which was mirrored during the recruiting period. The poor vaccination compliance of myasthenia gravis patients (Auriel et al., 2011) indicates the need for education about the risks and benefits of influenza vaccinations. Because of the low influenza antibody response, a second vaccine dose should be considered in patients receiving immunosuppressive drugs.

In this context further investigation concerning vaccine effectiveness and efficacy and changes in vaccine strategy is needed.

## Contributors

BT provided the initial ideas presented in the introduction and discussion of this article, was the author of the study protocol, provided guidance about the analysis and presentation of the data, and drafted most sections of the manuscript. MS, FB, CE, AW, ME, DK, HHM, NS made substantial contributions to the analysis and interpretation of the data and to the writing and revising of the manuscript. HHM was the statistician for this study and critically reviewed the manuscript for data accuracy. MS, CSB, FB, AW, ME, DK, MD, HHM were involved in the study design, data interpretation, and critical review of the manuscript. All authors approved the submitted version of the manuscript.

## Declaration of Interests

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MS, FB, CSB, AW, ME, HDK, HHM, NS have nothing to declare.

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## Appendix A. Supplementary data

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## References

- Auriel, E., Regev, K., Dori, A., Karni, A., 2011. Safety of influenza and H1N1 vaccinations in patients with myasthenia gravis, and patient compliance. *Muscle Nerve* 43, 893–894.
- Beck, C.R., McKenzie, B.C., Hashim, A.B., et al., 2011. Influenza vaccination for immunocompromised patients: systematic review and meta-analysis from a public health policy perspective. *PLoS One* 6, e29249.
- Besinger, U.A., Toyka, K.V., Hömberg, M., Heining, K., Hohlfeld, R., Fateh-Moghadam, A., 1983. Myasthenia gravis: long-term correlation of binding and bungarotoxin blocking antibodies against acetylcholine receptors with changes in disease severity. *Neurology* 33, 1316–1321.
- Binks, S., Vincent, A., Palace, J., 2016. Myasthenia gravis: a clinical-immunological update. *J. Neurol.* 263, 826–834.
- Brydak, L., Machala, M., 2000. Humoral immune response to influenza vaccination in patients from high risk groups. *Drugs* 60, 35–53.
- Carr, A.S., Cardwell, C.R., McCarron, P.O., McConville, J., 2010. A systematic review of population based epidemiological studies in Myasthenia gravis. *BMC Neurol.* 10 (46).
- Chalmers, A., Scheifele, D., Patterson, C., et al., 1994. Immunization of patients with rheumatoid arthritis against influenza: a study of vaccine safety and immunogenicity. *J. Rheumatol.* 21, 1203–1206.
- Deutsche Gesellschaft für Neurologie, 2012. Leitlinien für Diagnostik und Therapie in der Neurologie. 5. Auflage. Thieme, Stuttgart.
- Feng, J., Gulati, U., Zhang, X., et al., 2009. Antibody quantity versus quality after influenza vaccination. *Vaccine* 27, 6358–6362.
- Fiore, A.E., Uyeki, T.M., Broder, K., et al., 2010. Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2010. *MMWR Recomm. Rep.* 59, 1–62 (RR-8).
- Haas, W., 2009. Influenza: Prävention, Diagnostik, Therapie und öffentliche Gesundheit. Urban & Fischer in Elsevier, München.
- Hart, I.K., Sharshar, T., Sathasivam, S., 2009. Immunosuppressant drugs for myasthenia gravis. *J. Neurol. Neurosurg. Psychiatry* 80, 5–6 (discussion 6).
- Hohlfeld, R., Toyka, K.V., Besinger, U.A., Gerhold, B., Heining, K., 1985. Myasthenia gravis: reactivation of clinical disease and of autoimmune factors after discontinuation of long-term azathioprine. *Ann. Neurol.* 17, 238–242.
- Holvast, B., Huckriede, A., Kallenberg, C.G., Bijl, M., 2007. Influenza vaccination in systemic lupus erythematosus: safe and protective? *Autoimmun. Rev.* 6 (5), 300.
- Holvast, A., Stegeman, C., Benne, C., et al., 2009. Wegener's granulomatosis patients show an adequate antibody response to influenza vaccination. *Ann. Rheum. Dis.* 68, 873–878.
- Holvast, A., van Assen, S., de Haan, A., et al., 2009b. Effect of a second, booster, influenza vaccination on antibody responses in quiescent systemic lupus erythematosus: an open, prospective, controlled study. *Rheumatology (Oxford)* 48, 1294–1299.
- Jeffs, L.S., Peh, C.A., Jose, M.D., Lange, K., Hurtado, P.R., 2015. Randomized trial investigating the safety and efficacy of influenza vaccination in patients with antineutrophil cytoplasmic antibody-associated vasculitis. *Nephrology (Carlton)* 20, 343–351.
- Kalb, B., Matell, G., Pirskanen, R., Lambe, M., 2002. Epidemiology of myasthenia gravis: a population-based study in Stockholm, Sweden. *Neuroepidemiology* 21 (5), 221.
- Kalita, J., Kohat, A.K., Misra, U.K., 2014. Predictors of outcome of myasthenic crisis. *Neurol. Sci.* 35, 1109–1114.
- Kappos, L., Mehling, M., Arroyo, R., et al., 2015. Randomized trial of vaccination in fingolimod-treated patients with multiple sclerosis. *Neurology* 84, 872–879.
- Kroon, F.P., van Dissel, J.T., de Jong, J.C., van Furth, R., 1994. Antibody response to influenza, tetanus and pneumococcal vaccines in HIV-seropositive individuals in relation to the number of CD4+ lymphocytes. *AIDS* 8, 469–476.
- Miller, A.E., Morgante, L.A., Buchwald, L.Y., et al., 1997. A multicenter, randomized, double-blind, placebo-controlled trial of influenza immunization in multiple sclerosis. *Neurology* 48, 312–314.
- Okasha, M., 2007. Epidemiological research. *Allg. Med.* 83, 452–454.
- RKI, Robert-Koch-Institut, 2012. Impfempfehlungen der Ständigen Impfkommission (STIKO) am Robert Koch-Institut/Stand: Juli 2012. *Epidemiol. Bull.* 30, 283–310.
- Saiki, I., Koizumi, K., Goto, H., et al., 2013. The long-term effects of a kampo medicine, juzentaihoto, on maintenance of antibody titer in elderly people after influenza vaccination. *Evid. Based Complement. Alternat. Med.* 568074.
- Schumm, F., Fatehmoghadam, A., Dichgans, J., 1984. Correlation between serum anti-acetylcholine-receptor antibody-levels and clinical state under immunosuppressive therapy in Myasthenia-gravis. *Eur. Arch. Psychiatry Clin. Neurosci.* 234, 224–230.
- Seidman, J.C., Richard, S.A., Viboud, C., Miller, M.A., 2012. Quantitative review of antibody response to inactivated seasonal influenza vaccines. *Influenza Other Respir. Viruses* 6, 52–62.
- Seok, H.Y., Shin, H.Y., Kim, J.K., et al., 2017. The impacts of influenza infection and vaccination on exacerbation of myasthenia gravis. *J. Clin. Neurol.* 13, 325–330.
- Seybold, M.E., Lindstrom, J.M., 1981. Patterns of acetylcholine receptor antibody fluctuation in myasthenia gravis. *Ann. N. Y. Acad. Sci.* 377, 292–306.
- Soesman, N.M., Rimmelzwaan, G.F., Nieuwkoop, N.J., et al., 2000. Efficacy of influenza vaccination in adult liver transplant recipients. *J. Med. Virol.* 61, 85–93.
- Sommer, N., Tackenberg, B., Hohlfeld, R., 2008. The immunopathogenesis of myasthenia gravis. *Handb. Clin. Neurol.* 91, 169–212.
- Tanzi, E., Amendola, A., Pariani, E., et al., 2007. Lack of effect of a booster dose of influenza vaccine in hemodialysis patients. *J. Med. Virol.* 79, 1176–1179.
- Thomas, C.E., Mayer, S.A., Gungor, Y., et al., 1997. Myasthenic crisis: clinical features, mortality, complications, and risk factors for prolonged intubation. *Neurology* 48, 1253–1260.
- Tindall, R.S., 1981. Humoral immunity in myasthenia gravis: biochemical characterization of acquired antireceptor antibodies and clinical correlations. *Ann. Neurol.* 10, 437–447.
- Toplak, N., Kveder, T., Trampus-Bakija, A., Subelj, V., Cucnik, S., Avcin, T., 2008. Autoimmune response following annual influenza vaccination in 92 apparently healthy adults. *Autoimmun. Rev.* 8, 134–138.
- Tsai TF, 2011. MF59 adjuvanted seasonal and pandemic influenza vaccines. *Yakugaku Zasshi* 131, 1733–1741.
- Tzartos, S., Seybold, M., Lindstrom, J., 1982. Specificities of antibodies to acetylcholine receptors in sera from myasthenia gravis patients measured by monoclonal antibodies. *Proc. Natl. Acad. Sci.* 79, 188–192.
- Vista, E.S., Crowe, S.R., Thompson, L.F., Air, G.M., Robertson, J.M., Guthridge, J.M., James, J.A., 2012. Influenza vaccination can induce new-onset anticardiolipin but not  $\beta$ 2-glycoprotein-I antibodies among patients with systemic lupus erythematosus. *Lupus* 21, 168–174.
- Wendell, L.C., Levine, J.M., 2011. Myasthenic crisis. *Neurohospitalist* 1, 16–22.
- Wolfe, G.I., Kaminski, H.J., Aban, I.B., et al., 2016. Randomized trial of thymectomy in Myasthenia gravis. *N. Engl. J. Med.* 375, 511–522.
- Zinman, L., Thoma, J., Kwong, J.C., Kopp, A., Stukel, T.A., Juurlink, D.N., 2009. Safety of influenza vaccination in patients with myasthenia gravis: a population-based study. *Muscle Nerve* 40, 947–951.