JUVENILE MYASTHENIA GRAVIS IN NORWAY

Epidemiological, clinical, genetic and immunological studies

Trine Haug Popperud

Department of Neurology, Oslo University Hospital

Faculty of Medicine, University of Oslo

Oslo 2018

UiO : **Faculty of Medicine**
University of Oslo

© Trine Haug Popperud, 2018

*Series of dissertations submitted to the
Faculty of Medicine, University of Oslo*

ISBN 978-82-8377-313-2

All rights reserved. No part of this publication may be
reproduced or transmitted, in any form or by any means, without permission.

Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.

Table of Contents

1 PREFACE.....	5
1.1 Acknowledgements.....	5
1.2 List of abbreviations	7
1.3 Publications included	9
2 INTRODUCTION AND BACKGROUND	11
2.1 A brief history of juvenile myasthenia gravis	11
2.2 Epidemiology	13
2.3 Clinical presentation	15
2.4 Subgroup classification.....	16
2.4.1 MG with AChR ab.....	17
2.4.2 MG with MuSK ab	18
2.4.3 MG with LRP4 ab.....	18
2.4.4 Seronegative MG.....	19
2.4.5 MG with thymoma.....	19
2.4.6 Ocular MG (OMG)	20
2.4.7 Neonatal myasthenia gravis.....	20
2.5 Diagnostics	20
2.5.1 Clinical evaluation	21
2.5.2 Serum antibodies.....	21
2.5.3 Neurophysiological testing	22
2.5.4 Pharmacological testing (Tensilon test).....	24
2.5.5 "Ice pack" test.....	25
2.5.6 Chest image.....	25
2.5.7 MicroRNAs.....	25
2.6 Differential diagnosis.....	26
2.6.1 Congenital myasthenic syndrome (CMS)	26
2.6.2 Other differential diagnoses to have in mind are:	27
2.7 The immune system	28
2.8 Autoimmunity.....	32
2.9 Pathogenesis of juvenile MG.....	34
2.9.1 The neuromuscular junction	34
2.9.2 Immunopathogenesis	34
2.9.3 Genetics aspects in JMG.....	38
2.9.4 Epigenetic mechanisms in JMG.....	40
2.9.5 Environmental aspects in JMG.....	40
2.9.6 Infections.....	40
2.9.7 Pharmacological drugs.....	41
2.9.8 Hormones.....	42
2.10 Treatment	42
2.10.1 Symptomatic.....	42
2.10.2 Immunotherapies	43
2.10.3 Thymectomy.....	44
2.10.4 Supportive therapy	46
2.11 Course of disease	46
2.11.1 Comorbidity	47
3 AIMS OF THE THESIS.....	48
4 METHODS	50
4.1 Study design	50
4.2 Study population	50
Paper I.....	52

<i>Paper II</i>	54
<i>Paper III</i>	54
<i>Paper IV</i>	54
4.3 Data collection	54
4.4 Medical records	55
4.5 Clinical evaluation with standardized interview	55
4.6 MGFA clinical classification	55
4.7 MGFA Postintervention Status	56
4.8 Blood samples and laboratory measures	57
4.9 HLA genotyping (Paper III)	57
4.10 Immunophenotyping (Paper IV)	58
4.11 TREC analysis (Paper IV)	59
4.12 Ethical aspects	60
4.13 Statistical considerations	60
4.13.1 Type I and Type II error	61
4.13.2 Confounding	61
4.13.3 Internal and external validity	62
4.13.4 HLA association analyses	63
5 SUMMARY OF RESULTS	64
5.1 Paper I	64
5.2 Paper II	65
5.3 Paper III	66
5.4 Paper IV	66
6 GENERAL DISCUSSION	68
6.1 Incidence of juvenile MG in Norway	68
6.2 Clinical characteristics and outcome	70
6.2.1 What is the typical Norwegian JMG patient?	70
6.2.2 What is the prognosis?	71
6.2.3 What about comorbidity?	72
6.3 Thymectomy in juvenile MG	74
6.4 HLA associations in Norwegian juvenile MG – a novel finding	76
7 CONCLUSION	80
8 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVE	82
9 REFERENCES	84

1 PREFACE

1.1 Acknowledgements

The work presented in this thesis was carried out at the Department of Neurology, Oslo University Hospital (OUS), and the Institute for Clinical Medicine, Faculty of Medicine, University of Oslo, between 2012 and 2018. The project was financially supported by the Institute for Clinical Medicine through a scholarship while working as a research fellow and clinical teacher in neurology at the University of Oslo. I am grateful for having this opportunity.

Research is teamwork, and I would like to express my sincere gratitude and appreciation to all of you who have contributed and supported me to these years.

First of all, my principal supervisor and mentor Emilia Kerty, who initiated this project. This work would not have been possible without you. You have guided, motivated and inspired me through these years. Your knowledge is endless. Also, my sincerest thanks to my co-supervisor Magnhild Rasmussen for your guidance and valuable contributions.

The JMG patients who made this research possible by participating, allowing me to read their medical journals, donating blood and travel to Rikshospitalet from all around the country. Meeting these patients was inspiring. I am grateful.

My fellow MG investigators at OUS; Marion Boldingh, Ahmed Elsaïs and Angelina Maniaol, I am grateful for your contributions and for giving me access to the adult MG database. Also, thank you for encouragement and support.

All colleagues at the Paediatric and Neurologic Departments in Norway, who have contributed to the collection of patients and clinical data, thank you for all help. A special thank to Nils Erik Gilhus and my co-author Anne Heldal, for giving me access to the AChR antibody database at Haukeland University Hospital, and to my co-authors Kai I Müller, Kashif W Faiz, Knut Øymar.

Benedicte A Lie, my gratitude for your contributions, for sharing your knowledge on HLA, for help on the manuscript preparation, and for your enthusiasm.

Marte K Viken for all your assistance and for performing the HLA analyses, a sincere thanks.

Cathrine Brunborg, thank you for statistical support and guidance in epidemiological research.

Tore Abrahamsen for your experience and thoughts, and for contributions in manuscript preparations. I am grateful.

Kiran Gul, my gratitude for the TREC analyses and for critically reading the manuscript.

Liv Toril Osnes and your technicians, thank you for your help and assistance in the T and B cell quantification.

Richard W Olaussen, thank you for your knowledge and contribution.

While conducting this Ph.D. project, I have partially worked as a teacher and physician at the Department of Neurology OUS Rikshospitalet, and I am grateful to all my colleagues for a supportive working environment. I would particularly like to thank the former head of the Department of Neurology, Espen Dietrichs and the present, Sigrid Svalheim, for supporting and facilitating this research.

Anne Sofie Molandsveen, thank you for reading my thesis.

Last, but not least, my warmest thank to friends and family. I appreciate you all. Thank you for your endless support, understanding and motivation; and for sharing all these other important experiences in life that have kept me going while working on my Ph.D. I will never forget.

Trine Haug Popperud
Oslo, June 2018

1.2 List of abbreviations

AChR	acetylcholine receptor
AChE	acetylcholine esterase
ab	antibodies
CBA	cell-based assays
CD	clusters of differentiation
CHRNA1	cholinergic receptor nicotinic alpha 1 subunit
CK	creatine kinase
CMS	congenital myasthenic syndrome
CSR	complete stable remission
CT	computed tomography
CTLA4	cytotoxic T lymphocyte-associated 4
ELISA	enzyme-linked immunosorbent assay
EMG	electromyography
EOMG	early onset myasthenia gravis
GWAS	genome wide association studies
HLA	human leucocyte antigens
ICD	International Classification for Disease
Ig	immunoglobulin
INF- α	interferon alfa
IVIg	intravenous immunoglobulin
JMG	juvenile myasthenia gravis
LD	linkage disequilibrium
LOMG	late onset myasthenia gravis
LRp4	low-density lipoprotein-related receptor 4
MG	myasthenia gravis
MGFA	Myasthenia Gravis Foundation of America
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
MuSK	muscle specific tyrosine kinase
NGS	next generation sequencing
OMG	ocular myasthenia gravis
PCR	polymerase chain reaction

PIS	Post intervention Status Scale
PLEX	plasma exchange
PR	pharmacological remission
PTPN22	Protein Tyrosine Phosphatase non-receptor type 22
RA	rheumatoid arthritis
RCT	randomized controlled studies
REK	Regional Committee for Medical and Health Research Ethics
RIPA	radio immunoprecipitation assay
RNS	repetitive nerve stimulation
SFEMG	single-fiber EMG
SLE	systemic lupus erythematosus
TCR	T cell receptor
TLR	toll like receptor
TNF	tumor necrosis factor
TNIP1	TNF- α -induced protein 3 interacting protein 1
TREC	T cell receptor rearrangement excision circles
Tx	thymectomized

1.3 Publications included

The thesis is based on the following original articles:

- I **Popperud TH**, Boldingh MI, Brunborg C, Faiz KW, Heldal AT, Maniaol AH, et al. Juvenile myasthenia gravis in Norway: A nationwide epidemiological study. *Eur J Paediatr Neurol*. 2017;21(2):312-7.

- II **Popperud TH**, Boldingh MI, Rasmussen M, Kerty E. Juvenile myasthenia gravis in Norway: Clinical characteristics, treatment, and long-term outcome in a nationwide population-based cohort. *Eur J Paediatr Neurol*. 2017;21(5):707-14.

- III **Popperud TH**, Viken MK, Kerty E, Lie BA. Juvenile myasthenia gravis in Norway: HLA-DRB1*04:04 is positively associated with prepubertal onset. *PLoS ONE*. 2017;12(10)

- IV **Popperud TH**, Gul KA, Brunborg C, Olaussen RW, Abrahamsen TG, Osnes LT, Kerty E. Thymectomy in juvenile myasthenia gravis- any long term effect on immunosenescence markers?
Submitted.

2 INTRODUCTION AND BACKGROUND

Juvenile myasthenia gravis (JMG) is a rare autoimmune disease affecting the neuromuscular endplate, thus impairing the neuromuscular transmission. The clinical hallmark is fatigable and fluctuating muscle weakness. The aggressiveness of the disease varies and it is potentially lethal due to respiratory failure if not diagnosed and treated properly. Fortunately, mortality has been reduced due to more knowledge about the disease and available symptomatic and immunomodulating treatment.

Figure 1

Twins, aged 26 months, with seropositive-MG. One (left) has asymmetric lid ptosis, the other (right) holds his head back to see beneath bilaterally ptotic lids (1). *Clin. Neurol.*, 91 (2008) 229-252. With permission

2.1 A brief history of juvenile myasthenia gravis

The anatomist Thomas Willis (1621-1675) is usually credited with the first written clinical description of myasthenia gravis (MG) published in 1672, where he describes a fluctuating "spurious palsy" affecting limbs, tongue and speech (2, 3). However, the first description of MG in children was by Samuel Wilks (1824-

1911) in 1877, a girl with fluctuating bulbar paralysis (2). In 1879 Wilhelm Erb gave a more detailed description including the typical ocular symptoms (3, 4). Friedrich Jolly where the one to identify the neuromuscular transmission abnormality in MG and introduced the name myasthenia gravis pseudo-paralytica in 1895 (2). He also described two cases in boys. Myasthenia is derived from Greek "mys" meaning muscle and "asthenes" meaning weak. Gravis is Latin for serious (5). The term juvenile myasthenia gravis (JMG) was coined by Osserman in 1956 (6) to describe the autoimmune disease in children and adolescents as opposed to congenital myasthenic syndromes (CMS) (7).

At the beginning of the 19th century the connection between MG and the thymus was established when EF Buzzard found an increase of lymphocytes in the thymus (8) and C Weigert a thymic lymphoma in patients with myasthenia (3). The first thymectomy in a patient with MG was performed by E F Sauerbruch in 1911 (2). Subsequently, thymectomy was introduced by A Blalock in 1939 (9) and established as a treatment option in MG. Its role has remained somewhat contentious; however, the first randomized controlled study on efficacy in adults was published in 2016 by Wolfe et al and showed beneficial effect (10). Definitive data on efficacy and safety in juvenile myasthenia gravis is still not available.

From 1930 medical therapy for MG started to evolve. First, ephedrine and glycine were described to have beneficial effect, and then Mary B Walker introduced the acetylcholine esterase (AChE) inhibitors in 1934 (11).

DW Smithers and J Simpson suggested an autoimmune disease mechanism in MG in 1958 (2) and this was further supported by the identification of the acetylcholine receptor (AChR) as the auto antigen in 1973 (12) and the identification of AChR antibodies (ab) in 1976 by J Patrick and JM Lindstrom et al (13). When autoimmunity was established as mechanism, immunosuppressant agents were introduced; first corticosteroids in the 1950s followed by azathioprine in 1967 and plasma exchange in 1976 (2).

In the last decades, advances in myasthenia gravis have been on immunological aspects where novel autoantibodies like MuSK ab (14) and LRp4 ab (15) have been discovered, and our knowledge about the immune pathogenesis has increased.

2.2 Epidemiology

The prevalence of JMG varies in different countries and races (16). A recent review showed an overall MG incidence in Europe estimated to 30/1,000,000/year and an incidence of JMG (age 0-19 years) of 1.0-5.0/1,000,000/year (17). Other epidemiologic studies on JMG show similar numbers (18-21). Different methodology and inclusion criteria might explain some of the variation in the incidence rates. See Table 1.

Incidence and prevalence of MG have increased over time (22). This is mainly in the LOMG group and has not been shown for JMG (18, 21). It has been argued that improved diagnostic and epidemiological methodology may explain some of the increase (22).

	Methodology	JMG incidence*	MG incidence*	JMG prevalence**	MG prevalence**	Female:Male
McGrogan, 2010 (17)	Review article	1.0-5.0 (0-19 y)	30.0	na	na	
Cetin, 2012 (Austria) (18)	Hospital discharge register			2.2 (0-14 y)	80.0	F=M 1:1
Gattellari, 2012 (Australia) (19)	Pyridostigmin prescription database	4.5 (0-24 y)	24.9	6.2 (0-14 y)	87.8	F<M 1:1.5 (0-14 y)
VanderPluym, 2013 (Canada) (20)	Canadian Pediatric Surveillance Program	0.9 -2.0 (0-17 y)	na	na	na	F>M 1.4:1 (<12 y) 2:1 (12-17 y)
Parr, 2014 (UK) (21)	AChRab database	1.5 (0-17 y)	na	na	na	F>M 2.1:1 (< 10 y) 1.4:1 (< 7 y)
Mombaur, 2014 (South-Africa) (23)	AChRab database	4.3 (<20 y) 2.5 (0-4 y) 3.1 (5-9 y) 2.8 (10-14 y) 6.0 (15-19 y)	8.5 4.1 (< 50 y) 24.0 (>50y)	na	na	K=M (< 10 y) 5:1 (15-20 y)
Lai, 2010 (Taiwan) (24)	National Health Insurance Research database	8.9 (0-4 y) 6.9 (5-9 y) 3.7 (10-14 y) 8.3 (15-19 y)	22	na	140	2:1 (0-4 y) F=M (5-14 y) 2:1 (15-19 y)
Heldal, 2009 (Norway) (25)	AChRab database	na	7.2	na	126	
Andersen, 2014 (Norway) (26)	Pyridostigmin prescription database	na	16.0 6.7 (<50 y) 34.0 (>50y)	na	131	

Table 1

Showing studies on MG and JMG incidence and prevalence

*Annual incidence rate per million.

**Prevalence per million

y = years of age

na = not applicable

Presentation and prevalence of JMG correlate with racial origin. In Chinese and Japanese populations, JMG is more frequent and constitutes up to 50% of MG patients (27, 28) as opposed to 10-15% in European populations (29). Asian JMG populations have more often pure ocular symptoms, reported in up to 80% of cases (30, 31).

2.3 Clinical presentation

JMG does not differ from the adult disease in pathophysiology, but has some different clinical features, signs and presentation.

The impaired neuromuscular transmission in MG results in muscle weakness that is typically fatigable and fluctuating. Physical activity and exertion of a muscle group may aggravate the weakness, while examination after rest can appear normal (1, 16). All skeletal muscles may be involved, but usually some muscle groups are more affected. Most frequent are ocular symptoms, ptosis typically asymmetric, or diplopia (1, 32). The child may not always identify and complain about the specific symptoms. Double vision can present as difficulties in climbing stairs. When ptosis, the child may recline the head to better be able to see (16). In children it is especially important to be aware of ptosis and intervene on this symptom to prevent amblyopia. Bulbar involvement gives slurred, nasal speech, and difficulties chewing and swallowing. The facial expression changes due to facial weakness. Smiling gets difficult, and the child may look sad or angry. Neck muscles, respiratory muscles and limb muscles, proximal more than distal, may be affected. Muscle pain is uncommon, but patients may complain of generalized fatigue (1). Localized muscle atrophy is detectable in a few, especially in bulbar muscles in MuSK MG (32, 33).

Several factors may aggravate myasthenic symptoms: systemic illness like infections, vaccinations, insect bites, drugs, menstrual cycle, increased body temperature and emotional upset (1, 34).

Myasthenic crisis with need of intubation or ventilatory support may occur if there is rapid increase in bulbar and respiratory muscle involvement. Such severe symptoms have been reported to occur more frequent early in the illness

(1), and more frequent in prepubertal juvenile MG compared with postpubertal (35).

A cholinergic crisis due to overdosing in AChE inhibitor medication can be difficult to distinguish from myasthenic crisis. Here, instead of the tachycardia and obstipation often present in myasthenic crisis, there is bradycardia, diarrhea, increase bronchial secretion, muscular fasciculation and warm skin.

2.4 Subgroup classification

MG may present at any age. When onset is in childhood or adolescents the term JMG is used. JMG is further subdivided as prepubertal or postpubertal according to age of first symptoms, before the age of 12 years or after respectively (16). A similar subdivision applies to adult MG being divided into early onset (EOMG) and late onset (LOMG) according to onset before the age of 50 years or later (36). However, subgroup classification in MG, including age classification and the definite age cut-off, is on debate (37, 38) and there are still no consensus on the border between JMG and EOMG. One could also ask whether a cut-off at age 12 for the prepubertal and postpubertal sub classification is meaningful. Age of sexual maturity vary interindividually and also with race and geographical latitude REF. In studies on JMG the upper age cut-off varies from age 14 to 20 years (31, 39, 40). This challenges the comparison of studies and a uniform understanding of JMG. Due to variations in clinical picture, immune pathogenesis and response to treatment, both research and clinical practice would benefit from an appropriate subgroup classification. In addition to age at onset, MG patients are often sub classified according to antibodies present (AChR ab, MuSK

ab, LRP4 ab), thymus histology (hyperplasia, thymoma, normal) or affected muscles (ocular vs. generalized).

Figure 2 Showing how MG may be classified into different subgroups

2.4.1 MG with AChR ab

AChR ab are the most frequently found ab, occurring in 85% of all MG cases (41). Among JMG patients and especially in the prepubertal group, the rate of AChR ab positivity is reported lower at 56-74% (35, 42). This is also the case among pure ocular MG patients, both juvenile and adult, were approximately 50% are AChR ab positive (41, 43).

2.4.2 MG with MuSK ab

JMG with MuSK ab is rare, especially in the prepubertal group although it has been reported with onset down to age 2 years (44). Prevalence vary with ethnicity and geography (45), and is more frequent in Southern than in Northern Europe (46, 47). Onset in the two first decades accounted for 3% of MuSK MG cases in Greece and 20.7% in the United States (48, 49) and females are predominately affected (48, 50). MuSK MG in children presents with the same clinical picture as in adults, with prevalent facial and bulbar weakness, associated with a more severe disease and frequent respiratory crisis (16).

Figure 3

Weakness and relative frequencies of myasthenia. The color intensity indicates the distribution of the weakness in the different forms of myasthenia. The numbers below indicate the relative frequencies of the different forms of autoimmune myasthenia gravis (MG) or Lambert–Eaton myasthenic syndrome (LEMS) within a group of 100 patients with one or another form of myasthenia. Thus, 85 out of 100 patients with myasthenia will have acetylcholine receptor (AChR) MG, while only 4 out of 100 will have LEMS. MuSK, muscle-specific kinase. (51) *Handb Clin Neurol.* 2016;133:447-66. With permission.

2.4.3 MG with LRP4 ab

The occurrence of LRP4 ab vary considerable between populations examined, however shown to be present also in juvenile onset MG (52). LRP4 MG appears

to have milder symptoms both at onset and as the disease progresses. Test for LRP4 ab are not yet commercially available.

2.4.4 Seronegative MG

Initially a term used about the 10-20% of MG patients without AChR ab. After the discovery of MuSK ab (14) and LRP4 ab (15, 53), this group has become smaller (54). Seronegativity is reported more frequent among JMG and among pure ocular MG. Lately, low affinity ab and low concentrations of ab against AChR, MuSK and LRP4, are identified by new cell-based methods (55, 56). In addition, new antibodies against agrin (57) and cortactin (58, 59) have been identified, although their pathogenicity is still unknown. And finally, some MG patients probably have pathogenic antibodies against yet unidentified antigens. Currently, only test for AChR ab and MuSK ab are commercially available.

2.4.5 MG with thymoma

Although paraneoplastic thymoma associated MG accounts for 10% of all MG cases (60), it is rare in children compared to adults. Thymomas are neoplasms of thymic epithelial cells and constitute approximately 4% of paediatric mediastinal tumours (61). The peak incidence is seen in the fourth and fifth decades of life (62). MG occurs in 30-50% of adults with thymoma (62), but is only reported in 15% with paediatric thymomas (61).

2.4.6 Ocular MG (OMG)

OMG implies that the weakness is limited to the extra ocular muscles for 2 years without becoming generalized (63). The incidence of pure ocular JMG varies widely in retrospective studies. It is more frequent in Asian populations where it occurs in 50-83% (31, 64, 65), while in European populations, OMG is reported in 10-20% (35, 66, 67). Children with African ancestry also show a higher risk for OMG compared with those of European ancestry (39), and OMG seems more frequent among prepubertal than postpubertal onset cases (35, 67).

2.4.7 Neonatal myasthenia gravis

Children born to females with MG may have a transient neonatal myasthenia due to placental transfer of maternal antibodies. This occurs in approximately 10 – 15 % of births in females with MG (68, 69). Symptoms like hypotonia, feeding difficulties, weak cry or respiratory distress, manifest within the first 3 days of life and last 2-4 weeks in general (29, 70). Treatment is symptomatic with acetylcholinesterase inhibitor, assisted ventilation or IVIg, however, rarely needed. The severity in maternal symptoms does not necessarily correlate with neonatal symptoms.

2.5 Diagnostics

JMG is diagnosed based on history, repeated physical examination and supplementary tests (16, 71, 72). Standardized tests to evaluate muscle strength and additional testing procedures are the same as used in adults, but may be

difficult to perform in infants and the youngest children, thus challenging the diagnosis.

2.5.1 Clinical evaluation

Clinical examination should show typical findings with fluctuating muscle weakness and fatigability without other neurological deficits. It is important to assess strength repetitively during maximum effort and after rest. Asymmetric ptosis and weakness of several muscles in both eyes, not characteristic of lesion of one nerve, is typical. Pupillary responses are normal. The facial muscles are weak giving the classical myasthenic snarl. The voice may be nasal, especially after prolonged talking. Any trunk or limb muscle may be weak. However, neck flexors are usually weaker than extensors; deltoids, triceps and extensors of the wrist and fingers, and ankle dorsiflexors are frequently weaker than other muscles (1, 16, 73).

2.5.2 Serum antibodies

The presence of elevated AChR ab or MuSK ab in serum confirms the MG diagnosis. AChR ab can be tested using a radio immunoprecipitation assay (RIPA) or an ELISA technique. The specificity of the RIPA test is 100% in all MG types (74). The ELISA has the advantage of no radioactive isotopes to contend with, however sensitivity is lower and there are also described 5% false positive results (74). MuSK ab are measured by immunofluorescence on transfected cells or by RIPA technique.

A substantial fraction of JMG patients reveal no detectable AChR or MuSK ab, using the standard clinical immunoprecipitation assays. Sensitive cell-based assays (CBAs) have recently shown that many of these seronegative patients do indeed possess autoantibodies (55, 75). It is possible that some children may have clustered AChR ab, but the diagnostic technique to detect this is routinely not available.

Other studies have found that some double seronegative MG patients possessed LRP4 ab (mainly IgG1 and IgG2) (15). LRP4 antibodies are also found in juvenile MG. Antibodies against titin are mainly detected in late onset adult MG and in thymoma-associated MG (76, 77). Other detected autoantibodies in MG are agrin ab, Collagen Q ab and cortactin ab; however their clinical and pathogenic significance requires further investigations.

2.5.3 Neurophysiological testing

Repetitive nerve stimulation (RNS) and single-fiber EMG (SFEMG) can demonstrate abnormal neuromuscular transmission, but require patient cooperation and are difficult in children under age 8-10 years without sedation. RNS is the most specific and most commonly used technique to evaluate the neuromuscular transmission. A decremental response of more than 10% is very suggestive of transmission defect. This is best seen with slow repetitive depolarization of the nerve at 3 Hz. If progressively fewer muscle fibers respond to nerve stimulation, producing a decrementing pattern, the result is abnormal (78). See Figure 4. Due to the clinical pattern of weakness in JMG, the RNS will more likely be positive when proximal or cephalic muscles are tested (78).

Figure 4

A prototype decrementing response to repetitive nerve stimulation in myasthenia gravis. The amplitude of the initial response is normal, and the decrement is maximal in the fourth response after which the responses may increase slightly, giving an envelope shape to the train of responses. (79) *Ann Indian Acad Neurol.* 2013;16(1):34-41. With permission.

AChE inhibitors must be withheld for 24 hours to prevent false negative results (32). A normal test will not exclude MG, since RNS is relatively insensitive (1, 16, 32). Amandusson et al showed that RNS had a 54% sensitivity in ocular MG, 77% sensitivity when predominantly bulbar symptoms and 83% sensitivity when predominantly limb weakness (80).

SFEMG is more sensitive with increased jitter greatest in the weak muscles in MG reported in 90-99% (32). Padua et al showed a 100% sensitivity of SFEMG on orbicularis oris in ocular MG, while RNS had 15% sensitivity (81). However, SFEMG is less specific and other disorders affecting nerve and muscle may show increased jitter and must be excluded (79). See Figure 5. SFEMG is technically more difficult and demanding, involves patient cooperation and not easily available in children. The SFEMG technique records muscle fiber action potentials from one single motor unit (32). The variation in latency from stimulus to response is the neuromuscular jitter, and an increased jitter or blocking are signs of neuromuscular transmission defect (32).

Figure 5

In the diagram on the left of the figure, SFEMG electrode can record electrical activity from two muscle fibers of the same motor unit. In the figure on right two muscle fiber action potentials, and variability in the interval between potentials is seen as a variable position of the second potential with increased jitter and occasional blockings (arrows) in myasthenic muscle (79).

Ann Indian Acad Neurol. 2013;16(1):34-41. With permission.

RNS and SFEMG cannot differentiate JMG and congenital myasthenic syndromes (CMS), however, normal jitter in a weak muscle excludes JMG as the cause (1, 32, 79).

2.5.4 Pharmacological testing (Tensilon test)

Intravenous application of the rapid-acting cholinesterase inhibitor edrophonium is used as a diagnostic test (16, 72). Easy monitored symptoms like ptosis or dysarthria must be present, and a positive test consists of transient resolution. Due to potential cholinergic side effects, especially bradycardia and hypotension, the test must be conducted in a monitored setting with atropine, an anticholinergic drug, available. Positive test may also be seen in CMS, amyotrophic lateral sclerosis and Lambert Eaton syndrome among others (1).

2.5.5 "Ice pack" test

Muscle cooling improves myasthenic weakness. Cool ptotic eyelid for 2 min and then assess improvement in ptosis (1).

2.5.6 Chest image

Computed tomography (CT) of the chest, or preferentially magnetic resonance imaging (MRI) in children to reduce radiation exposure, is done to check for thymus hyperplasia or thymoma (16).

2.5.7 MicroRNAs

Circulating microRNAs have recently been described as a potential biomarker in MG, also to monitor disease course and therapeutic response. Elevated miR-150-5p and miR-21-5p have been found in MG patients, and levels of miR-150-5p were lower in immunosuppressed patients and in patients with clinical improvement following thymectomy (82).

When characteristic myasthenic symptoms are present and AChR ab or MuSK ab are elevated, the diagnosis is virtually assured and further testing is often not necessary (1).

2.6 Differential diagnosis

2.6.1 Congenital myasthenic syndrome (CMS)

In the paediatric age group it is most important to distinguish between seronegative autoimmune MG and a CMS (83). CMS is a heterogeneous group of disorders all due to a mutation in genes coding for a protein important to the neuromuscular transmission. The common clinical characteristic is fatigable muscle weakness, and at least 24 genes are identified (84). See Figure 6.

Figure 6

Localization of the proteins encoded by the genes mutated in congenital myasthenic syndromes (CMSs). CHRNE is the gene most often mutated (marked +++), followed by COLQ, RAPSN, and DOK7 (marked ++). The other genes are only rarely identified. Glycosylation enzymes (GFPT1, DPAGT1, ALG2, and ALG14) are ubiquitously expressed and have acetylcholine receptor (AChR) as one of their targets. AChE, acetylcholinesterase.(85) *Curr Opin Neurol.* 2013;26(5):561-8. With permission

The mutations are mainly autosomal recessive. They are classified to whether they involve presynaptic, synaptic or postsynaptic proteins. Postsynaptic mutations are most common. The incidence of genetically verified CMS among

children < 18 years of age was 9.2 per million in the UK (21). In future years the detected prevalence of genetically confirmed CMS will continue to increase as new genetic subtypes are identified and clinicians become better at identifying the phenotype of children with suspected CMS.

2.6.2 Other differential diagnoses to have in mind are:

1. Congenital myopathy may have the same distribution of weakness but less fluctuation and diagnosis will be helped by increased CK, findings on muscle biopsy and genetics.
2. Mitochondrial myopathy may have further organ manifestation and typical findings on muscle biopsy and metabolic workup in blood/urine.
3. Myotonic dystrophy has characteristic EMG pattern and can be confirmed by genetics
4. Lambert-Eaton myasthenic syndrome is rare in children and may present additional autonomic symptoms and abnormal muscle stretch reflexes. Antibodies against voltage gated calcium channels may be detected.
5. Chronic progressive bulbar paralysis (Fazio-Londe disease) will involve additional cranial nerves and confirmed by genetics
6. Botulism will often present with additional vegetative symptoms.
7. Guillain-Barré syndrome will present with diminished reflexes and typical findings on electrophysiological studies and in cerebrospinal fluid.
8. Brainstem tumour will often have other associated neurological symptoms and are verified by MRI

9. Möbius syndrome with cranial nerve VI and VII palsy will be present from birth

2.7 The immune system

The immune system is the body's defence system against disease. It is divided into innate (humoral) immunity and adaptive (cell-mediated) immunity, and consists of five major kinds of cells, all derived from the pluripotent hematopoietic stem cells in the bone marrow.

1. B-lymphocytes are responsible for antibody production and mediate the humoral immune response. Checkpoints are in the bone marrow, spleen and lymph nodes. Important surface proteins are B cell specific immunoglobulins (IgG, IgA, IgM, IgD, IgE) which act as receptors for specific antigens.
2. T lymphocytes are responsible for the cellular immune response and move from the bone marrow to the thymus to mature. Immature cortical thymocytes differentiate into CD4+ (helper T cells, T regulatory cells) or CD8+ (cytotoxic cells) T cells as they move from cortex to medulla in the thymus. Both B and T cells have specific immunity due to DNA rearrangement in immunoglobulins or T cell receptor (TCR) genes. As opposed to B cells, T cells cannot recognize antigens in their free state. The protein antigen must be broken down and presented by major histocompatibility complex (MHC) molecules on self-cells (like APC, virus-infected cells, cancer cells) to be recognized by the T cells. MHC, called human leucocyte antigens (HLA) in humans, are cell-surface

glycoproteins presenting antigenic peptides to T cells. The MHC/HLA gene complex resides on chromosome 6 and exhibits extensive genetic polymorphism with a wide variability between individuals, necessary to cope with the ever-increasing range of pathogens. See Figure 7.

Endogenous antigens are presented to CD8⁺ T cells by MHC class I antigens, while exogenous antigens are presented to CD4⁺ T cells by MHC class II antigens. The MHC class III region encodes proteins involved in the complement system (86).

- a. Helper T cells (CD4⁺) enhance B cell antibody production, macrophage activation and cytotoxic T cell activation.
 - b. Cytotoxic T cells (CD8⁺) kill virus-infected cells, transplanted tissue and cancer cells.
 - c. Memory T cells are more easily activated and recruit helper T cells providing a more rapid immune response.
 - d. Regulatory T cells key role is immunoregulation by dampening down immune responses, like maintaining peripheral self-tolerance.
3. NK cells kill virus-infected cells and cancer cells. They have no antigen specific receptors and are not affected by antigens, but are activated by cytokines derived from dendritic cells.
4. Macrophages are not antigen specific. They phagocytise and exhibit peptide on MHC II molecules as APC, have secretory functions regulating inflammatory response and cytotoxic effect.

5. Dendritic cells are APC (antigen presenting cells) signalling through cytokine production.

Figure 7

The extended HLA complex on the short arm of chromosome 6. Only very few gene loci are shown. Those encoding the “classical” HLA class I or II molecules are shown as green squares, and the number of known alleles (by late 2004) are indicated.⁽⁸⁷⁾ *Transpl Immunol. 2005;14(3-4):175-82. With permission.*

In addition to immune cells, the immune system consists of the lymphoid organs responsible for maturation, concentration, interaction and deployment of lymphocytes.

1. **The bone marrow:** All lymphoid cells originate in the bone marrow and it is the site for differentiation of B cells and NK cells
2. **The thymus** is the site for T cell differentiation. Here, anti-self specificities are eliminated and T cell repertoires to protect against infection are selected. “The thymus selects the useful, neglects the useless and destroys the harmful” (88). The gland lies behind the breastbone, resting on the pericardium above the heart. The thymus lobes are divided into lobules consisting of cortex and medulla. It has the fastest growth

during the first 2 years of life, but continues to grow until puberty where it is at its biggest, 40 g. Then it gradually involutes. The thymus never disappears, however the thymic T cell output is reduced. The T cell differentiation is performed through two steps; positive selection in the cortex where only T cells that recognize self- MHC are matured, then negative selection in the medulla where self-reactive T cells are deleted by apoptosis.

The thymus has a central role in MG and pathological alterations of the thymus is often observed; typically hyperplasia in JMG and EOMG, and thymoma in LOMG (89). The precise nature of this relationship between the thymus and MG is still not clear. See Figure 8.

Figure 8

Hypothetical role of the thymus in myasthenia gravis patients without a thymoma. Thymectomy terminates antiacetylcholine receptor (*anti-AchR*) antibody (*Ab*) production by the thymus and the provision of high affinity anti-AchR antibody-producing cells to peripheral organs (90) *Surg Today*. 2010;40(2):102-7. With permission.

In the hyperplastic MG thymus there are an increased number of B cells organized in germinal centres. These B cells are active and produce AChRab shown in immunodeficient mice grafted with thymic biopsies from MG patients (91). The thymus contains all components necessary for the immunopatogenesis of MG (89): myoid cells that express AChR antigen, antigen-presenting, anti-AChR auto reactive T cells, and B cells producing AChRabs (26).

3. **The lymph nodes** contain antigens, antigen-bearing dendritic cells and macrophages. It is a site for phagocytosis and antibody production, act as a junction between the lymphatic and the circulatory systems, support induction, proliferation and differentiation of lymphocytes and allow recirculation of lymphocytes.
4. **The spleen** responds to antigen in the blood and contains 25% of the mature lymphocytes, both B and T cells. Here blood-born antigens processed by dendritic cells are presented to T cells, which in turn activate B cells.
5. **Mucosa-associated lymphoid tissue** (including adenoid, tonsils).

2.8 Autoimmunity

Immunologic tolerance is the acquired ability not to respond to a self-antigen. All immune cells must recognize self before they can react to non-self. At least half of the lymphocyte antigen receptors made by random recombination react to self-antigen. Central tolerance is developed in the primary lymphoid organs, B cells in the bone marrow and T cells in the thymus. Here, B- and T-cells with

receptors binding self-antigens are deleted if they fail to edit their receptors to less reactive ones.

As a backup, the peripheral tolerance makes self-reactive lymphocytes anergic through absence of co-stimulatory signals, suppression by T reg cells or deletion by apoptosis, when they encounter peripheral self-antigens. AIRE (autoimmune regulator gene) controls the development of central tolerance in the thymus.

Autoimmunity occurs when these two strategies fail and self-reactive lymphocytes are present and activated by auto antigens, leading to a humoral or cell-mediated attack on self. Autoimmunity has a multifactorial aetiology, including both genetics and environmental factors (86).

Possible contributing factors in autoimmune disease are:

1. Alteration of self-antigens by chemicals or viruses
2. Cross-reactive antibody production
3. Exposure to sequestered self-antigens
4. Decrease in regulatory T-cell number or function
5. Overactive or absence of TH cell function (polyclonal B-cell activation)
6. Inappropriate MHC class II molecule expression on APCs
7. Thymic defects
8. Polyclonal activation
9. Genetic factors
10. Hormonal factors

Autoimmune disorders are either organ specific like in MG, idiopathic thrombocytopenic purpura (ITP) and Addison's disease, or systemic like in

systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and ankylosing spondylitis).

2.9 Pathogenesis of juvenile MG

2.9.1 The neuromuscular junction

A neuromuscular junction is the relay between a motor neuron and a muscle fiber, and has a complex structure. See Figure 9. All components of the neuromuscular junction have to function and cooperate to achieve a muscle contraction. The signal from a motor neuron is transmitted across the synaptic cleft through a chemical transmitter, acetylcholine (ACh). ACh binds to ACh receptors (AChRs) on the cellular membrane of the muscle fibers. This binding induces an influx of sodium, which depolarizes the muscle membrane. If strong enough, the signal will initiate muscle contraction. Diverse disorders may affect this process causing defects of the neuromuscular transmission. The defects can affect presynaptic, synaptic or postsynaptic structures and well-known pathogenesis are genetic like in CMS, toxic as in botulism or autoimmune like in JMG and Lambert-Eaton myasthenic syndrome.

2.9.2 Immunopathogenesis

Juvenile MG is an autoimmune disease in which antibodies bind to AChRs, or to other functionally related proteins in the postsynaptic membrane at the neuromuscular junction, and through loss of functional AChR the neuromuscular transmission is impaired. The aetiology, as in other autoimmune diseases, is not clearly established but assumed to be multifactorial, involving genetic, epigenetic

and environmental factors (92). AChR ab are the most frequent ab in juvenile MG patients, and there is ample evidence that these ab are pathogenic.

Figure 9

Structure of the NMJ. As it enters the muscle and approaches its target fibers, each α -motor neuron axon divides into branches that innervate many individual muscle fibers. Each branch loses its myelin sheath and further subdivides into many presynaptic boutons, which contain ACh-loaded synaptic vesicles and face the surface of the muscle fiber at the NMJ. The synaptic bouton and the muscle surface are separated by the synaptic cleft, which contains AChE and proteins and proteoglycans involved in stabilizing the NMJ structure. The NMJ postsynaptic membrane has characteristic deep folds, and the AChR is densely packed at the fold top. When the nerve action potential reaches the synaptic bouton, ACh is released into the synaptic cleft, where it diffuses to reach and bind the AChR. ACh binding triggers the AChR ion channel opening,

permitting influx of Na^+ into the muscle fiber. The resulting EPP activates voltage-gated Na^+ channels at the bottom of the folds, leading to further Na^+ influx and spreading of the action potential along the muscle fiber. Other proteins, including Rapsyn, MuSK, and agrin, which are involved in AChR clustering, are also present on the muscle membrane in close proximity to the AChR. MASC, myotube-associated specificity component; RATL, rapsyn-associated transmembrane linker. (5) *J Clin Invest.* 2006;116(11):2843-54. With permission.

Figure 10

Effector mechanisms of anti-AChR Abs. (A) Ab binding to the AChR activates the complement cascade, resulting in the formation of membrane attack complex (MAC) and localized destruction of the postsynaptic NMJ membrane. This ultimately leads to a simplified, altered morphology of the postsynaptic membrane of the NMJ of MG patients, which lacks the normal deep folds and has a relatively flat surface. (B) Abs cross-link AChR molecules on the NMJ postsynaptic membrane, causing endocytosis of the cross-linked AChR molecules and their degradation (antigenic modulation). This ultimately leads to a reduced number of AChR molecules on the postsynaptic membrane. (C) Ab binding the ACh-binding sites of the AChR causes functional block of the AChR by interfering with binding of ACh released at the NMJ. This results in failure of neuromuscular transmission. (5) *J Clin Invest.* 2006;116(11):2843-54. With permission.

AChR ab are mainly of the immunoglobulin IgG1 and IgG3 subtypes. Binding of these antibodies to AChR leads to complement-mediated destruction of the postsynaptic membrane. In addition, divalent bindings to adjacent AChRs increase the rate of internalization and lysozymal destruction of AChRs. AChR blocking effect has been shown, although most of the AChR ab do not cause direct block of the AChR (93). See Figure 10.

In MuSK MG the endplate damage does not rely upon the same immunopathology. MuSK ab are mainly of IgG4 subtype which lack complement activating properties and are functionally monovalent (93). MuSK ab bind to IgG-like regions on MuSK and directly block the binding of LRP4 and thereby the assembly and activation of the agrin-LRP4-MuSK complex with dispersal of AChR clustering and loss of AChR (94).

In both AChRab MG and MuSKab MG, the effect is a reduction in functional AChRs with impaired neuromuscular transmission leading to fatigable muscle weakness.

The pathogenic significance of LRP4 ab are not yet fully established. LRP4 ab are of IgG1 subtype and able of complement activation. Inhibition of agrin-induced aggregation of AChRs has also been implicated as a pathogenic mechanism (93, 95).

2.9.3 Genetics aspects in JMG

MG is not transmitted by a classical Mendelian inheritance, however, involvement of genetic factors in disease development is evident from twin studies showing higher concordance rate of MG among monozygotic twins compared to dizygotic twins (96, 97). Although familial autoimmune MG is very rare (98), familial autoimmunity (the co-occurrence of other autoimmune diseases in family members) is relatively common among MG patients, suggesting some common genetic predisposition (99).

The most studied and most important genetic factor involved in MG, is the human leukocyte antigen complex (HLA) alleles. The HLA gene products are involved in the immune system's differentiation between self and non-self. The HLA genotypes associated with MG vary with age of onset, ethnic origin, clinical symptoms and thymic histology. The association of HLA A1-B8-DR3-DQ2 (8.1) haplotype with EOMG in Caucasians has been reproduced by several groups (100-102). The 8.1 haplotype has also been shown associated with several other autoimmune disorders like RA, autoimmune thyroid disease and SLE (103, 104), supporting the hypothesis of some common genetic risk factors. Further studies refining the association of the 8.1 haplotype with MG, have showed that the predominant association is with the HLA-B8 allele in Caucasian EOMG (102, 105, 106). In LOMG the strongest association was with the HLA-DRB1*15:01 allele in a Norwegian population (102), while with HLA-DQB1*05:02 and DRB1*16 in an Italian (107). For MuSK MG being a separate entity, a strong association with HLA-DQ5 has been shown in both a Dutch and an Italian cohort (108, 109). HLA associations in juvenile MG have been investigated in Asian populations, where onset before 15 years of age were associated with DQ3, and onset before 3

years of age with DR9 and DR13 in Japanese MG patients (110). HLA-B*4601-DRB1*0901 has been shown to be positively associated with juvenile MG in Chinese (111), and especially with juvenile ocular disease in a Southern Chinese Han population (112). Shinomoya et al suggest that latent generalized juvenile MG is a specific clinical subtype associated with HLA DRB1*1302/DQA1*0102/DQB1*0604 in Japanese (113).

Other non-HLA susceptibility genes have also been described in MG:

- In a Chinese JMG population, a genetic predisposition in CHRNA1 (cholinergic receptor nicotinic alpha 1 subunit) and CTLA4 (cytotoxic T lymphocyte-associated 4) genes were found (114). The CHRNA1 gene regulates expression of the α -subunit of AChR, the main immune region for the AChR ab in MG. CTLA4 encodes a T cell membrane receptor acting as a negative regulator for their activation, thus helping to maintain self-tolerance (100).
- PTPN22 (Protein Tyrosine Phosphatase non-receptor type 22) is involved in the TCR signalling pathway, leading to inhibition of T cell activation (115). The R620W mutation in the PTPN22 gene has been shown to be associated with multiple autoimmune disorders including MG, by several groups (100, 105, 115).
- TNIP1 (TNF- α -induced protein 3 interacting protein 1) inhibits signal transduction and is required for termination of TLR responses. A GWAS in European EOMG identified an association with a change the TNIP1 loci associated with an exacerbated inflammatory state (105, 116).

2.9.4 Epigenetic mechanisms in JMG

Epigenetic mechanisms link environment and genetics and include, among others, micro RNA, DNA methylation and histone acetylation. These epigenetic changes may explain the high occurrence of disease-discordant pairs in monozygotic twins (116).

- MicroRNAs are small non-coding RNAs that mediate post-transcriptional regulation of target genes. Dysregulation of microRNAs has been described in several autoimmune diseases (116). MiR-155 and miR-146a seem to be related to inflammation, and in MG miR-150-5p levels have been shown to have the highest association and were also significantly reduced after thymectomy (82).

2.9.5 Environmental aspects in JMG

Environmental factors are thought to contribute to the onset of autoimmune disease, including MG. The incomplete concordance of MG in monozygotic twins indicates this influence of environmental factors (96). Possible environmental factors include drugs, pollutants, pathogens, diet, vitamin D, microbiota, smoking, occupation and hormones (117-120). We still have limited knowledge about the direct influence of these factors in MG, and there are no publications directly addressing this aspect in juvenile MG.

2.9.6 Infections

A hypothesis of infectious agents being involved in the development of autoimmune diseases has existed for a long time, but is still only well-

documented in a few cases, like Guillain-Barré syndrome and rheumatic fever (92). Suggested mechanisms involving infectious agents are molecular mimicry and local inflammation leading to increased immunogenicity of self-antigens (92). Epstein –Barr virus (EBV) has been associated with several autoimmune diseases including MG, and EBV-infected B cells have been detected in hyperplastic MG thymus (89). However, EBV is very common and whether the association represents causation is still open; studies are contradictory (116).

2.9.7 Pharmacological drugs

Several drugs have been reported to induce autoantibodies and autoimmune disease. The prototype disease is SLE, where drug-induced SLE accounts for 10% of all cases in the United States (121). Drug-induced MG has also been described. MG is induced in 1-7% of patients on D-penicillamine, likely via modulation of the immune system, and reversible upon drug withdrawal (116). Cases of MG have also been described after INF- α treatment and anti-TNF drugs (116, 122). Other drugs may interfere with the neuromuscular transmission and exacerbate myasthenic symptoms, the aminoglycosides, macrolides and fluoroquinolones as well as neuromuscular blocking drugs, magnesium, statins and sedatives; and they should be avoided (34). If there is a clinical deterioration after starting a new drug in JMG patients, the drug should be withdrawn. When in remission or mild symptoms, MG patient often tolerate the drugs but they should be used with caution (34).

2.9.8 Hormones

The majority of patients diagnosed with an autoimmune disease are females, 78% (123, 124). This gender bias is thought to be due mainly to sex hormones. Oestrogens, androgens and prolactin have been shown to modulate the immune system, both the innate and adaptive system (125). Women generally have a stronger humoral and cellular immune response compared to men, with higher CD4:CD8 ratio and higher level of circulating antibodies (126). In addition to the female preponderance in early onset MG, the exacerbation of MG symptoms during pregnancy and by menstruation, might be attributed to influence of sex-hormones (119, 127).

2.10 Treatment

Treatment in juvenile MG is mainly based on case series and experience from adult MG patients where there are a few randomized controlled studies (RCT) (71, 128). Due to MG's rarity and good response to existing treatment, it is challenging to do new trials. Recently an International consensus was published to guide clinicians managing MG, and some aspects on treatment in juvenile MG were included (129). The treatment goal is Minimal Manifestation Status (PIS) or better, with only mild medication side effects.

Available treatment can be divided into four categories:

2.10.1 Symptomatic

Acetyl cholinesterase (AChE) inhibitors are symptomatic treatment and first line therapy in juvenile MG. Most commonly used is Pyridostigmine (Mestinon). AChE

inhibitors reduce the ACh breakdown and prolong the ACh activity in the synaptic cleft. Ocular JMG may be managed by AChE inhibitors alone, but usually generalized juvenile MG needs additional treatment (71, 129). Concerning ocular symptoms, AChE inhibitors are more effective on ptosis than ophthalmoplegia (130). Progressive loss of efficacy due to compensatory overproduction of acetylcholine esterase isoforms may occur after prolonged use (71). In MuSK MG AChE inhibitors are less effective and more frequently induce side effects (50). Side effects are due to cholinergic stimulation in the autonomic nervous system and including diarrhea, nausea, sweating and bradycardia.

2.10.2 Immunotherapies

Immunosuppressive drug treatment includes glucocorticosteroids and other immunosuppressants. The mainstay of therapy is oral steroids and good clinical response is shown in both adults and children in retrospective studies with improvement in 80.2 % and 61% respectively (67, 131). However there is a lack of good quality RCTs. An initial deterioration of myasthenic symptoms may occur within the first 3 weeks after starting steroid treatment, and especially when initiated at high doses (71, 132). Long-term steroid treatment have numerous, significant and potential chronic side effects in children, like growth retardation and poor bone health in addition to weight gain, hyperglycaemia and mood alteration(133).

If oral steroids fail to control symptoms satisfactory, or if high doses of steroids are required for symptom control, other immunosuppressants are usually added.

Most frequently used in children as in adults, is azathioprine. There are only paediatric case studies on effect, but azathioprine has been shown effective in adult MG (134). Other immunosuppressants used in MG are cyclosporine A, mycophenolate mofetil, methotrexate, tacrolimus, rituximab and eculizumab; however, experience in children is limited. Adverse effects and increased risk of neoplasia related to protracted immunotherapy, pose challenges for long-term management (135).

In the most severe cases, immunomodulating intervention therapy with PLEX or intravenous immunoglobulins (IVIg) is necessary before initiating steroids, as well as in myasthenic crisis. IVIg has been used in several paediatric neurological disorders and has been shown well tolerated and safe (136, 137). IVIg have immunomodulating effect through several mechanisms mediated through their large quantity of Fab- or Fc-fragments of the polyclonal healthy donor immunoglobulins, including neutralizing autoantibodies, suppression of cytokines and complement, blockade of leucocyte-adhesion-molecule binding, among others (76) . PLEX is a quick way to eliminate autoantibodies and other pathogenic humoral factors from the bloodstream. Liew et al showed that PLEX might be superior and have a more consistent response in JMG (138).

2.10.3 Thymectomy

An involvement of the thymus in MG pathogenesis is supported by several findings including the positive association with thymus pathology and the intrathymic auto sensitization of T cells and production of AChR antibodies (89).

So far no randomized controlled studies on thymectomy in JMG have been conducted. However, the benefit of thymectomy has been shown in several retrospective studies with higher remission rates in children after thymectomy than the rate of spontaneous remission (139-143). A recent systematic review on thymectomy in juvenile MG (144) concludes that thymectomy appears to be safe, and that the majority show improvement after thymectomy, but the quality of data is poor and prospective studies are warranted.

Controversy regarding timing of thymectomy in children exists, especially due to the role of the thymus in thymocyte differentiation and maturation, and there is concerns that thymectomy in young age may lead to premature immunosenescence (145). However, studies indicate that thymectomy can be safe for JMG as early as age 17 months (72). Some data suggest that early thymectomy within 12 months of onset, is more effective (146). The effect of thymectomy is gradual and observed up to 2 years postoperatively (36, 71), thus it is an elective procedure and should be performed on a stable patient (129). All thymus tissue has to be removed, either through transsternal approach, video-assisted thoracoscopic or robotic-assisted methods. Thymectomy is mandatory if thymoma is present. In peripubertal and postpubertal children with AChR ab positive moderate to severe JMG, thymectomy is indicated (71, 129). In prepubertal children, seronegative JMG, pure ocular JMG and MuSK JMG, the indication is still debated (71, 129).

2.10.4 Supportive therapy

- In addition to medical treatment, MG patients may need and have benefit from supportive therapy as follows:
- Respiratory therapy during myasthenic crisis or chronic nocturnal respiratory support in patients with poor response to medical therapy
- Speech therapy if dysarthria/dysphagia
- Dietary intervention if dysphagia and weight loss

2.11 Course of disease

The mortality for MG has decreased from 23-30% in the mid-1950s to 1.2-2.2% in recent years (89). Juvenile MG is a chronic disease like adult onset MG. The rate of spontaneous remission, however, tends to be higher among children with MG than adults (40), and especially high in those with prepubertal onset (35). Evoli et al (35) found a good therapeutic result in 79-83% in the Italian JMG population, and a good outcome with 83% showing clinical improvement was also reported by Ashraf et al (147). However, studies vary and in a Chinese juvenile MG population improvement were only seen in 16.7% (31) and in a Jamaican study only 12 % showed improvement (148). The inclusion criteria, follow-up length and treatment vary between the studies and might explain some of the difference, however, there are several findings suggesting that juvenile MG is influenced by race (39).

2.11 1 Comorbidity

Juvenile MG patient with lack of therapeutic response, unexpected deterioration or new symptoms should be examined for comorbidity. The most frequently reported comorbidity in juvenile MG is an additional autoimmune disease occurring in 6-17% and most common is thyroid disorders (31, 39, 141).

Autoimmune diseases are also frequent in adult MG, reported in 13-22%, and more frequent in EOMG (99, 149). Other comorbidities reported in juvenile MG are epilepsy (30, 31, 141), migraine (148), and asthma (30, 141, 148).

Infections can lead to MG exacerbation and should be treated early and vigorously (1).

Several drugs may interfere with the neuromuscular transmission and exacerbate myasthenic symptoms as described in paragraph 2.9.7.

3 AIMS OF THE THESIS

The overall aim of the work presented in this thesis was to identify factors contributing to clinical characteristics, treatment response and outcome of JMG in Norway. This knowledge can help us to a better understanding of the disease pathogenesis, increase the awareness of the disease and thereby improve future diagnostics and treatment of JMG patients.

The specific aims

- To determine the incidence and prevalence of JMG in Norway. (Paper I)
- To characterize the JMG population in Norway including clinical characteristics, immunological status, treatment and long-term outcome especially focusing on potentially differences between prepubertal and postpubertal onset. We hypothesized that JMG in Norway presents with similar characteristics as in other Western population and that there are some differences between prepubertal and postpubertal onset. (Paper II)
- To identify predisposing HLA alleles in the Norwegian JMG population. We hypothesized that postpubertal onset JMG was associated with the same HLA alleles as the Norwegian EOMG population. While in the prepubertal onset JMG, being described with some different clinical characteristics, we hypothesize that there are potential differences in gene expression. (Paper III)
- To study the long-term effects of thymectomy on the immune system when performed in JMG patients, including potential changes in immunosenescence. (Paper IV)

4 METHODS

4.1 Study design

The results in Paper II and IV are based on data from a population based retrospective cohort study. Our aim was to identify all patients with juvenile MG in Norway, and all four papers are based on the same patient material. Paper I was a population based incidence and prevalence study. Paper III was constructed as a case-control study.

- Population based study: the cases are collected from a geographically defined population.
- Retrospective cohort: the cohort is assembled by going back into the past, like from medical records, and then followed as a group forward to the present.
- Case-control study: Two samples are collected, patients with the disease and otherwise similar people who do not have the disease.

4.2 Study population

Norway is a country situated in Northern Europe, and stretches from latitude 57° N in the south to 71° N in the north. The Norwegian population constituted 5.1 mill people on 31 December 2013, 50.3% males and 49.7% females (Statistics Norway, www.ssb.no).

We aimed to identify all juvenile MG patients in Norway with onset at age ≤ 18 years and used three different strategies for the identification:

- I) A computer search on the hospitals' electronic coding system, including the 15 main hospitals in Norway with either a neurological or paediatric department, or both, were done in collaboration with a clinician at respective hospitals. The search was based on the International Classification for Disease (ICD) coding for MG, and patients < 19 years of age registered with the code 358 (ICD-9) or G 70 (ICD-10) in the period from 1989 through 2013, were identified. Juvenile MG diagnosis according to inclusion criteria was verified by the in-house clinician or by the main investigator.
- II) A search in the AChR ab database at Haukeland University Hospital for positive test on patients ≤ 18 years of age was made. In this laboratory database from 1983 to 2012 (150), antibodies were detected by a commercial kit (IBL International GmbH, Hamburg, Germany) and concentration ≥ 0.4 nmol/L was regarded as positive.
- III) We extracted registered patients reporting MG onset ≤ 18 years of age from the nationwide population-based MG database from the Department of Neurology at Oslo University Hospital (118, 151).

The diagnostic criteria for study inclusion were clinical juvenile MG and two out of the following three: 1) elevated AChR ab or MuSK ab 2) electrophysiological findings showing impaired neuromuscular transmission 3) Reversal of weakness after administration of acetyl cholinesterase inhibitor and/or immunomodulation therapy.

In the nationwide MG database, some patients reported onset before the age of 18, but no medical evaluation or documentation of early symptoms existed.

These cases were not included. Patients with verified or suspected congenital myasthenic syndrome (symptoms from birth; positive family history; no antibody detection and no response to immunomodulation) were not included.

The juvenile MG patients were divided into prepubertal onset cases or postpubertal onset cases according to age at first MG symptom; before age 12 years or at age 12-18 years respectively.

All the juvenile MG patients identified were contacted by mail with an information letter, or asked by treating physician, to participate in the studies. They were asked to 1) permit clinical data to be obtained from their medical journal, 2) to donate blood for DNA preparation and TREC-/ T-cell subset analysis, 3) meet at Oslo University Hospital for an updated clinical evaluation.

Paper I

In this paper we included all patients who fulfilled the inclusion criteria and had disease onset <18 years of age. The incidence rates are based on population numbers for each year in the study period, 1989 to 2013, (Statistics Norway, www.ssb.no). The number of Norwegian inhabitants under the age of 18 was used to calculate the incidence rate. Prevalence rates were also estimated based on the Norwegian juvenile population under the age of 18.

Figure 11

Flow diagram showing the identification of **JMG patients in Norway** and the **selection of JMG patients included in the different papers**. Three different sources were used for patient identification: hospital records (ICD code), AChR ab database at Haukeland University Hospital and the national adult MG database.

Paper II

In this part of the study we included all identified patients with MG onset ≤ 18 years, who fulfilled the given inclusion criteria and who gave their written consent.

Paper III

The results in paper III are based on the same study population as in paper II. However, since haplotype and genotype frequencies may vary between different populations, all non-ethnic Norwegians were excluded to reduce the effect of population stratification. The healthy controls were randomly selected through the Norwegian Bone Marrow Donor Registry (NBMDR,). In the NBMDR Norwegian HLA-typed volunteer bone marrow donors are registered. These controls have also been used in previous studies (152, 153). The controls had a different gender distribution with 30% females and 70% males, however, no difference in the HLA allelic distribution was seen between female and male controls.

Paper IV

47 patients from Paper II gave blood samples for TREC and T cell subset analysis and were included in Paper IV. Among these, 32 were thymectomized, while 15 were not.

4.3 Data collection

Clinical data were obtained from 1) medical journals, 2) clinical evaluation with standardized interview.

4.4 Medical records

Standardized clinical variables such as age at onset, phenotype, antibody status, results from neurophysiological examination, history of thymectomy, thymus histology, treatment and comorbidity, were collected from the patients' medical journals by the main investigator. To access the medical journals, the main investigator visited all four University Hospitals that had evaluated and treated the patients. We found that all juvenile MG patients had been evaluated at a Norwegian University Hospital. For supplementary and/or updated clinical data, medical charts from the treating hospitals were also obtained.

4.5 Clinical evaluation with standardized interview

Information about the long-term outcome with an updated clinical examination, as well as clinical variables including on-going treatment, was obtained from the patients who met for a clinical evaluation at Oslo University Hospital during 2015. The 12 patients, who for different reasons could not come to our clinic for an updated clinical evaluation, were scored based on information from their last evaluation by their local neurologist at a primary hospital.

4.6 MGFA clinical classification

MG symptoms at diagnosis and last follow up were categorized according to the Myasthenia Gravis Foundation of America (MGFA) clinical classification Scale (154).

Class I: pure ocular weakness

Class II: mild generalized weakness

Class III: moderate generalized weakness

Class IV: severe generalized weakness

Class V: intubation

Class II, III and IV are designated “a” if predominantly limb weakness, and “b” if predominately oropharyngeal.

4.7 MGFA Postintervention Status

Response to therapy was classified according to the MGFA Post intervention Status Scale (PIS) (154).

Complete stable remission (CSR): no symptoms and no MG therapy for at least one year

Pharmacological remission (PR): as CSR but in need of some MG therapy except AChE inhibitor

Minimal manifestation 0-3 (MM0-3): No symptoms or functional limitations, but some weakness on examination

MM0: no treatment for at least one year

MM1: on immunosuppression, but no AChE inhibitor

MM2: only low dose AChE inhibitor for at least one year

MM3: AChE inhibitor and immunosuppression during the past year

Improved (I): clinical improvement or substantial reduction in MG medication

Unchanged (U)

Worse (W)

Died of MG (D)

4.8 Blood samples and laboratory measures

Peripheral blood samples were collected by venepuncture from all patients who met for a clinical evaluation at Oslo University Hospital. In addition, two patients had blood samples collected at their local hospital (Haukeland University Hospital and Innlandet Hospital) and sent to Oslo University Hospital.

4.9 HLA genotyping (Paper III)

DNA sequencing methods have evolved substantially since the first DNA sequences were obtained in the early 1970s. The next generation sequencing (NGS) techniques, also called high-throughput sequencing, are a number of different sequencing technologies allowing a much quicker and cheaper DNA/RNA sequencing (155). The HLA genotyping in this study was done by a NGS technique, the Illumina sequencing by synthesis technology. In this method, surface bound, clonally amplified short DNA fragments are sequenced in cycles by a synthesis process in which a blocked fluorescent base is incorporated and then imaged. After the block is removed the next base can be incorporated and a new cycle starts. The images are analysed and the coloured spots are translated into a base sequence.

The NGSgo kit from GenDx (Utrecht, The Netherlands) was used to sequence the HLA-A, -B, -C, -DRB1 and -DQB1 genes with MiSeq Reagent Kit v2 on an Illumina

MiSeq (Illumina, San Diego, USA). The sequencing was performed at the Norwegian Sequencing Centre (NSC, www.sequencing.uio.no), University of Oslo, Norway. The platform-independent NGSengine v2.1 analysis software (GenDx) for the high-resolution identification of HLA alleles by NGS was used to analyse the sequencing results and obtain the HLA-genotypes. All patients included in the study were successfully genotyped for the five HLA loci.

4.10 Immunophenotyping (Paper IV)

Analysis of the different cell populations, the immunophenotyping, was done using flow cytometry, and was performed at the Department of Immunology, Oslo University Hospital, Rikshospitalet.

Monoclonal antibodies identify specific proteins (antigens) on the cell surface, known markers termed clusters of differentiation, CD (156). A CD-marker may be expressed on more than one cell type and therefore combinations of CD-markers are used, e.g. CD3 are expressed on all T cells, CD31 are expressed on RTE (recent thymic emigrants) and CD 19 are expressed on B cells.

In Paper IV, the following subpopulations were determined according to IPID (Immune phenotyping in Immunodeficiency), European Society of Immunodeficiencies.

T-cells were gated as CD3+ and further as

- Naive CD4+ (CD4+ CD45RA+)
- Recent thymic emigrants (CD4+ CD45RA+ CD31+)
- CD4+ memory (CD4+ CD45RO+)
- Follicular like CD4+ (CD4+ CD45RO+ CCR5+)

- Regulatory T-cells (CD4+ CD25++ CD127-)
- Naive CD8+ (CD8+ CD27+ CD28+)
- CD8+ early effector memory (CD8+ CD27+ CD28-)
- CD8+ late effector memory (CD8+ CD27- CD28-)

EDTA-blood was incubated with optimally titrated antibodies for 15 min at room temperature, followed by erythrocyte lysis (BD FACS Lysing Solution, Becton Dickinson, San Jose, CA, USA). Data acquisition was performed on a Canto II flow cytometer (Becton Dickinson) and 100 000 cells was acquired when possible.

4.11 TREC analysis (Paper IV)

T cell receptor rearrangement excision circles (TRECs) are DNA fragments generated during rearrangement of the T cell receptor gene when the T cells mature in the thymus. TREC quantification can be useful as a measure of thymic output, and it has shown a negative correlation with age, which has been attributed to the correlation with senescence of the immune system (157, 158). The TREC analysis was done as described in Paper IV. The BLOOD DNA kit (Omega-Biotek, USA) was used for extraction of genomic DNA from 200µl whole blood in 100µl elution solution. The extracts were analysed by PCR as previously described in detail in a previous publication (159). The only modification was using Via7 for analyses. The standard curve, ranging from 40000 to 10 copies, was made using a TREC plasmid generated by Douek, provided by the Medical College of Wisconsin. TREC plasmid concentration was measured using Nanodrop (Spectrophotometer ND-1000). All qPCR assays fulfilled the quality requirements of similar slopes and with R^2 values > 0.99. To assure adequate

DNA extraction Betaactin was used as housekeeping gene to assure adequate DNA extraction for PCR.

4.12 Ethical aspects

The Regional Committee for Medical and Health Research Ethics South East (REK South East) approved all studies included in this thesis. All the patients, or their parents when under 16 years of age, gave their written informed consent before participating in the study. All data were treated confidentially, de-identified and stored in accordance with the guidelines from REK. The biological data, blood and DNA, were preserved in a research bio bank at Oslo University Hospital.

4.13 Statistical considerations

Statistics were performed using STATA 13 (Stata Corp, College Station, TX, USA) (Paper I), OpenEpi.com (Paper I + II), EpiInfo version 3.5.3 (Centers for Disease Control and Prevention, Atlanta, GA) (Paper II), IBM SPSS Statistics for Windows, version 23 (Armonk, NY, USA: IBM Corp) (Paper IV) and UNPHASED v.3.0.10 (Paper III).

Detailed description of the different statistical analysis and methods are given in the original papers. In general, Chi Square test or Fisher exact test when appropriate, were used for comparison of categorical variables between groups.

4.13.1 Type I and Type II error

In statistical hypothesis testing, two situations may lead to a false conclusion. We can reject the null hypothesis when it is in fact true, called Type I error (false-positive) (160). The likelihood of making a Type I error is expressed by the P-value. The P-value equals the probability of the occurrence of a result showing a difference between groups happening by chance, when it in fact is no difference between the groups. We used a significance threshold of $p < 0.05$ in order to reject the null hypothesis.

The other false conclusion we can make is to fail to reject the null hypothesis when it is false, called Type II error (false-negative). The probability that we do not make a Type II error equals the power of the test. When the power is high, the probability of Type II error is low. Increasing the sample size or the precision of the measurements can increase the power. Juvenile MG is rare and therefore challenges the sample size and our ability to show difference between subgroups like prepubertal and postpubertal onset, thus may have led to Type II error.

4.13.2 Confounding

Before attributing a difference in outcome to the exposure, it is important to look for other factors that differ between the groups and which also affect the outcome, called confounding factors. Failure to control for these can lead to confounding bias. The problem of confounding bias during data analysis can be solved by stratification where the comparisons are restricted to individuals who have the same value of the confounding factor, or to use regression models. In Paper IV we used linear regression analysis was performed to investigate the

relationships between thymectomized patients and non-thymectomized patients and the total counts of T cell subsets, and to adjust for the possible confounding effect of chronological age.

4.13.3 Internal and external validity

The internal validity refers to the degree to which the results are correct for the sample of patients being studied, and is determined by how well the design, data collection and analyses are done. The internal validity is threatened by selection bias, measurement bias, confounding bias and random variation. External validity on the other hand, is the degree to which the results of an observation are generalizable to other settings and patients. The best strategy obtain generalizability, is to ensure good internal validity (161).

In Paper I and II we aimed at identifying all juvenile MG cases in Norway, and used three different strategies that all yielded unique cases. Nevertheless, we might have missed some cases. We did not include private and general physicians, but juvenile MG is a diagnosis that usually requires specialized knowledge. Patient with mild and/or transient symptoms may not have been recognized or misdiagnosed. Finally, we did not include the MG patients who reported juvenile onset, but had medical evaluation and documentation of symptoms completed many years later. We tried to avoid misclassification and all patients were checked diagnose verification according to the inclusion criteria, and we excluded cases with suspected congenital myasthenic syndrome. The participation rate in Paper II was high, 84%, also ensuring the external validity.

4.13.4 HLA association analyses

Linkage is the tendency of DNA sequences or a group of genes to be inherited together due to their closeness on a chromosome. Linkage disequilibrium (LD) occurs when there is a non-random association of alleles at different loci in a population; the combination of alleles or genotypes are found in a higher or lower frequency than what would be expected if they were independent and associated randomly. HLA genes are closely located on the MHC region and in close LD (100, 162). We used the haplotype method (163) and the Svejgaard method (164) to assess which alleles and loci showed the primary association and which appeared to be secondary due to linkage disequilibrium (LD). When testing several variables the problem of multiple testing occurs. When doing twenty comparisons we would expect one to be statistically significant by chance at the 5% level, giving a false positive result. We corrected for multiple testing by adjusting the p-values after the variables that was tested at each locus ($n=8$ for HLA-A, $n=9$ for HLA-B, $n=9$ for HLA-C, $n=10$ for HLA-DRB1, and $n=10$ for HLA-DQB1) (165). We did not correct for the total number of alleles tested, as the alleles at HLA loci do not fully represent independent tests due to the strong LD.

5 SUMMARY OF RESULTS

5.1 Paper I

Juvenile myasthenia gravis in Norway: A nationwide epidemiological study

The reported incidence and prevalence of MG, both with juvenile and adult onset, vary in studies and can be explained by geographical as well as methodological differences. The objective in this retrospective study was to assess the incidence rate and prevalence of juvenile MG in Norway in the study period from 1989 to 2013. Cases were collected at hospitals nationwide by ICD-codes as well as from the AChR ab database at Haukeland University Hospital and the clinical MG database at Oslo University Hospital. Diagnosis was verified through medical records. Inclusion criteria were clinical MG with onset before 18 years of age and antibodies against AChR or MuSK. If seronegative, supportive electrophysiological findings and improvement after treatment with acetylcholinesterase inhibitor or immunomodulating therapy were needed. Sixty-three juvenile MG patients were identified. Using the Norwegian population under 18 years of age as reference, we found a stable average annual incidence rate of juvenile MG of 1.6 (95% CI, 1.2-2.1) per million person years in Norway in 1989-2013. The incidence rate was higher in the postpubertal onset group compared to the prepubertal onset group, 3.1 (95% CI, 2.1-4.4) vs. 0.9 (95% CI 0.5-1.4), and in females compared to males 2.8 (95% CI, 2.0-3.8) vs. 0.4 (95% CI, 0.2-0.9). Prevalence of juvenile MG was 3.6-13.8 per million.

5.2 Paper II

Juvenile myasthenia gravis in Norway: Clinical characteristics, treatment, and long-term outcome in a nationwide population based cohort

Studies on juvenile MG have reported some demographic and clinical differences. In this study we aimed to characterize the clinical aspects, treatment and long-term course of juvenile MG in a Norwegian cohort, comparing prepubertal and postpubertal onset. Among the 75 juvenile MG patients identified with onset ≤ 18 years, 63 gave their consent and were included, 1/3 with prepubertal onset and 2/3 with postpubertal onset. There was all over a female preponderance and in the majority of patients, 91.5%, symptoms were generalized. Median time from onset to diagnosis was 6 months and AChR antibodies were present in 75 %. Myasthenic crisis was more frequent when prepubertal onset, and within the first year after diagnosis. All patients were treated with pyridostigmine. There were no difference in the use of oral prednisolone (29% vs. 48%); however, other immunosuppressive agents were more often used in the postpubertal group 36% vs. 10%, $p=0.03$) which could indicate a more severe disease. Thymectomy was performed in 50 (79%) patients. Outcome was overall favourable, with clinical improvement in 94%; 51 % were asymptomatic at last follow up and the frequency of complete remission was especially high in the thymectomized prepubertal onset group. The most prominent comorbidity was a co-occurring autoimmune disease present in one-third of the patients. Typically evolving after the JMG diagnosis and most frequent were thyroid disease and psoriasis. We also found a high occurrence of tonsillectomy and/or adenotomy.

5.3 Paper III

Juvenile myasthenia gravis in Norway: HLA-DRB1*04:04 is positively associated with prepubertal onset

In this case-control study, next generation sequencing of five HLA loci (HLA-A, -B, -C, -DRB1 and -DQB1) were performed in 43 Norwegian juvenile MG patients. Corresponding data from 368 healthy controls were used. In the cases, a positive association was seen with HLA-B*08 and HLA-DRB1*04:04. HLA-B*08 is known to be associated with early onset adult MG. Our focus was especially on genetic risk factors in the prepubertal onset subgroup, and we found that HLA-DRB1*04:04 was only positively associated with this subgroup, while HLA-B*08 was most frequent in cases with postpubertal onset.

This study provides novel information about HLA susceptibility alleles in Norwegian juvenile MG where HLA-DRB1*04:04 was associated with prepubertal onset.

5.4 Paper IV

Thymectomy in juvenile myasthenia gravis- any long term effect on immunosenescence markers?

Thymectomy is an established treatment in adult MG and also recommended for the treatment of postpubertal onset juvenile MG. Whether the youngest children should be thymectomized is still debated. Signs of premature aging of the immune system have been shown in studies on early perioperative thymectomy in children with congenital heart defects (145).

In this study we measured and compared T cell subsets and TRECs in peripheral blood in 32 thymectomized and 15 non-thymectomized juvenile MG patients.

We found a significant lower number of naïve helper T cells, an increased proportion of memory helper T cells and a significant lower number of naïve cytotoxic T cells in the thymectomized patients, as well as a significant reduction in the number of TRECs and proportion of recent thymic emigrants. There was no correlation with age at thymectomy.

No increased frequency of malignancies or infection was found in either group.

Autoimmune disorders were frequent in both groups, but without significant difference. These finding indicate a possible premature immunosenescence in the T cell compartment in juvenile MG patients, however, no clinical consequences in our cohort so far.

6 GENERAL DISCUSSION

Juvenile MG is a rare disorder and that makes research on this patient group challenging. Geographical differences as well as the absence of a uniform age cut off for the juvenile onset MG vs. EOMG in different studies; also challenge the interpretation and comparison of data. In this work we have tried to collect all Norwegian MG patients with juvenile onset to get extended knowledge about this group of patients. In this section I will discuss the interpretation of our main findings.

6.1 Incidence of juvenile MG in Norway

As already stated, juvenile MG is a rare disease and our findings confirm it's rarity in Norway with an overall annual incidence rate of 1.6 per million. Our data show that prepubertal onset is even more rare with an annual incidence rate of 0.9 per million. These results are in accordance with other epidemiological studies on juvenile MG in western countries. Parr et al found an incidence of 1.5 per million in the UK and VanderPluym et al 2.0 per million in Canada (20, 21). A lower incidence in the prepubertal age group has also been shown in Denmark where the incidence rate was 0.3 per million in the 0-9 years age group vs. 2.2 among those 10-19 years of age (166). On the contrary, a study from Taiwan found the highest incidence rate in the youngest age group 0-4 years with 8.9 per million. The overall annual incidence of juvenile MG is also reported higher in Taiwan with 3.7 -8.9 per million as well as and in South Korea with 7.0 per million, and also in South Africa with 4.3 per million (23, 24, 167).

This may reflect real geographical and racial differences in risk, but the studies differ in methodology and make direct comparison complicated.

Are our incidence numbers correct for the Norwegian JMG population? Our method of multiple case finding strategies and a long study period helped completing the study population. When we compare our numbers with neighbouring countries like Denmark and the UK and a recent review (17, 21, 166), we have similar results. We cannot exclude having missed some cases thus having a somewhat underestimated number. However, based on the overall incidence of MG in Norway shown to be 7.2-16 per million in the same timespan (26, 150), JMG constitute 10-22%. Thus we argue that our findings are representative for our study population.

We found no significant change in the incidence of juvenile MG over time in the study period from 1989 to 2013. There has been shown an overall increase in the MG incidence from the mid-1980s, but sub analyses have showed that the increase is in the LOMG group with a stable occurrence of juvenile MG (18, 22). Our figures corroborate this.

We observed a female preponderance among the juvenile MG patients. This was anticipated based on earlier studies on JMG (39, 67, 114, 141), and females have been shown to have an overall increased risk to acquire an autoimmune disease (124). Several mechanisms have been proposed to explain this. The peak of MG incidence in females in the 2nd to 3rd decades and have been attributed to the influence of sex hormones (127). An equal incidence of JMG among the sexes in

the prepubertal age, but with a female preponderance in postpubertal age shown in some studies, has been argued to support this assumption. However, our data in accordance with several others, point towards a female preponderance also in the prepubertal age supporting the influence of additional gender specific properties like X-linked genes. Females are otherwise also shown to have a stronger immune response compared to men (125).

6.2 Clinical characteristics and outcome

In Paper II we present clinical characteristics describing the Norwegian JMG cohort. With a high participation rate of 84%, we assume that our data are representative for the whole study population.

6.2.1 What is the typical Norwegian JMG patient?

The majority of Norwegian JMG patients are females and the disease typically starts with ocular symptoms, most frequently ptosis. However, in most of the patients symptoms generalize and in only 9.5 % they remain pure ocular. This differs from what has been reported from other JMG cohorts. It is known that Asian population have more OMG (30, 31, 114), but also compared to numbers from USA, South Africa and Italy, we have a high proportion of generalized symptoms, 90.5% vs. 66-74% (35, 39, 168). This may indicate genuine differences in JMG subtypes within Europe, or might be explained by a bias due to missed cases, or missed diagnosis of cases with slight ocular symptoms only.

Interestingly, a recent study on adult MG also showed lower occurrence of OMG in Norway (14%) compared to the Netherlands (36%) (151).

In accordance with other studies on JMG (40, 42, 67, 114), the share of AChR ab positive cases were somewhat lower in the Norwegian JMG cohort compared to what is reported in adult MG, and this was especially prominent in the prepubertal onset group where only 62% were positive for AChR ab or MuSK ab. Another difference between the two subgroups was the higher rate of myasthenic crisis in those with prepubertal onset. This has also been shown in an Italian cohort. Based on the limited data, it is difficult to say if this is a true difference indicating a more aggressive disease among the prepubertals or if it is due to delayed diagnosis or treatment in the youngest.

6.2.2 What is the prognosis?

The overall outcome was good with the majority of patients improving after diagnosis and 57% being asymptomatic at last follow up. All though we could not show statistical significance, the proportion of CSR was highest among those with prepubertal onset and especially among those who had been thymectomized. A relatively benign course of JMG, especially when prepubertal onset, has also been described previously (40). A total of 51% achieving CSR was quite high compared with other studies showing remission in 12-45% of patients (31, 39, 148, 168). These studies differ in methodology and have a shorter follow-up, which might explain some of the difference.

The postpubertal onset group was more often treated with immunosuppressive medication, and among these fewer achieved CSR, which could indicate a more

severe disease, a poor response to thymectomy or prednisolone therapy, or biological differences between the postpubertal and prepubertal MG subtypes.

6.2.3 What about comorbidity?

We confirm a high frequency of autoimmune disorders among JMG patients. This is the most frequent comorbidity occurring in one-third of the patients, equal in the prepubertal and postpubertal onset group. In all but one patient JMG was diagnosed before the additional autoimmune disease. See Figure 12.

Autoimmune comorbidity has been reported in 6-17% in earlier studies on JMG, and most common is thyroid disorders in accordance with our data (31, 39, 141). Immunosuppressive treatment was the only factor having a significant influence on the risk of developing a second autoimmune disorder, and showed a protective effect. This was also observed in an Italian adult MG cohort (169).

Epileptic seizures occurred in five patients (7.9%). A 5-fold increased risk of epilepsy in patients with MG was shown in an American population-based study (170), but reported frequency of epileptic seizures in JMG varies. A heightened risk of epilepsy has also been shown in several other autoimmune diseases, and especially pronounced in children (170). The relationship between MG and epileptic seizures is not yet clear. Epilepsy is not a single disease entity, but a symptom of an underlying brain dysfunction. The specific antibodies may not be directly pathogenic but the susceptibility of epilepsy in autoimmune diseases could be attributable to the general inflammatory components in MG and other autoimmune diseases (170).

Figure 12

Time, in years, from JMGO onset to thymectomy and to onset of an additional autoimmune disorder (AAD). Five patients (#15-19) were not thymectomized.
 Black diamond: thymectomy
 Grey square: AAD

Interestingly, we found a high occurrence of tonsillectomy and/or adenotomy. A large Swedish study also found that tonsillectomy was associated with an increased risk of autoimmune disease, and the majority of tonsillectomies were performed due to adenotonsillar hypertrophy (171). Studies have shown evidence for extrathymic T cell development in the tonsils. However, if and to what extent tonsillectomy affects the immune system, is still a matter of debate (172, 173).

6.3 Thymectomy in juvenile MG

The majority of our JMG patients were thymectomized. Thymectomy has recently been shown beneficial in a randomized controlled trial among 126 adult MG patients. The study, however, did not disclose safety and efficacy findings in patients below the age of 18 years since these were excluded (10). In the latest international consensus guidelines for the management of MG, thymectomy is recommended for the treatment of postpubertal onset juvenile MG (129).

Although there are no randomised controlled studies on thymectomy in juvenile MG, several prospective studies have shown positive effects (139, 174). It is still debated whether the youngest children should be thymectomized, due among other things to a higher proportion of spontaneous remission and to potential long-term side effects.

In our JMG cohort, the remission rate was equal in the thymectomized group compared to the non-thymectomized group. When stratified by age of onset, however, CSR was most frequent in the prepubertal thymectomized group. This could support the use of thymectomy also in the treatment in prepubertal onset MG. Nevertheless, we are in need of a prospective study on thymectomy in JMG to get more knowledge about both efficacy and safety.

Some earlier studies have indicated that thymectomy within one year from JMG onset is more beneficial (139, 146), however our data did not corroborate this.

Per/postoperative complications occurred in five patients. In two, major complications required surgical treatment, but no mortality and no long-term sequela that we were aware of occurred.

In paper IV we focused more in detail on the potential long-term immunological consequences of thymectomy, and especially whether there was any association with age at thymectomy. We showed changes in the T cell compartment that resemble findings characterizing the normal aging of the immune system (175). The thymectomized JMG patients had significant alterations in the peripheral T cell subsets, especially in the CD4⁺ subset with a decrease in CD4⁺CD45RA⁺ naïve helper T cell with a relative increase in CD4⁺CD45RO⁺ memory helper T cells, and a decrease in CD8⁺CD27⁺CD28⁺ naïve cytotoxic T cells. We found no correlation of the T cell subsets with age at thymectomy. The same changes in the immunosenescence markers have been shown in studies on children thymectomized while undergoing cardiac surgery (145, 176, 177). In an adult MG population neither lower total T cells, nor lower naïve T cells were found (178).

TRECs and RTE were both lower in the Tx patients compared to the non-Tx patients, reflecting the expected reduced thymic activity after thymectomy. No effect on the T reg cells was found when comparing the Tx and non-Tx juvenile MG patients.

An increased susceptibility to viral infections and autoimmune disease are features of Down syndrome and DiGeorge syndrome (22q11.2 deletion syndrome), both syndromes comprise thymus hypoplasia (179, 180). The incidence of autoimmune disorders also increase with general aging (181). Autoimmune comorbidity is also a known feature in patients with JMG and was frequently occurring in our cohort; however, we could not show an association with thymectomy. No increased tendency to infections in the thymectomized JMG patients was found. Other potential clinical implications of early

immunosenescence are reduced antibody response to vaccines and increased occurrence of cancer (175, 182). Malignancies were not frequently occurring in our cohort. Our study was not designed to look at response to vaccines.

All though indications for a premature immunosenescence in the T cell compartment in thymectomized juvenile MG patients, we could not show any clinical consequences in our population at last follow up. The change in immunosenescence markers was not related to age at thymectomy. Thus, our study could not confirm any increased risk of thymectomy in prepubertal JMG compared to postpubertal JMG. The retrospective methodology, small sample size and variability within the study population however, are limitations of the study.

Interestingly, an analysis of immunosenescent markers in patients with different autoimmune disorders showed signs of accelerated aging also before clinical disease onset (183).

Additional prospective studies including healthy controls are necessary to elucidate the effect of thymectomy in JMG further.

6.4 HLA associations in Norwegian juvenile MG – a novel finding

In paper III we provide novel information about HLA associations in Norwegian juvenile MG, where HLA-DRB1*04:04 was associated with prepubertal onset while in postpubertal onset juvenile MG, the HLA association was with HLA-B*08. The association of specific HLA alleles with MG has been known for decades (184). The associated HLA alleles vary with different MG subgroups and

with racial origin. This is the first study on genetics in Norwegian JMG patients and to our best knowledge the first on HLA associations in European juvenile MG patients. The HLA alleles reported in Asian juvenile MG populations, was not detected (HLA-B*46:01) or present at a low frequency (HLA-DRB1*09:01 and HLA-DRB1*13:02) among our juvenile MG population (111-113).

The HLA-B*08 association is well established in European EOMG (105) and has also been described in Norwegian EOMG (102). The association with HLA-DRB1*04:04 is a new finding not earlier described in MG patients.

Sub analysis showed that the HLA associations differed according to the age at JMG onset. The HLA-DRB1*04:04 was only positively associated with prepubertal onset. However, HLA-B*08 was associated with both prepubertal and postpubertal onset. Clinical studies on JMG in Western populations have also showed some differences in disease characteristics between prepubertal and postpubertal onset groups, with higher frequency of seronegativity and pure ocular symptoms in the prepubertal group (16, 29, 35, 40). The main difference between the HLA-DRB1*04:04 and HLA-B*08 prepubertal cases, was the age at MG onset. DRB1*04:04 was associated with an earlier onset than B*08. On the other hand, the HLA-B*08 positive juvenile MG cases showed the same characteristics as adult EOMG with female preponderance, AChR ab positivity and thymus hyperplasia. This could support adult EOMG being a continuum of postpubertal onset juvenile MG. The similarity of postpubertal onset MG to adult EOMG has been addressed earlier (29). It has also been hypothesized earlier that prepubertal onset JMG is a distinctive subset of the disorder (110) and our findings can corroborate this.

Just as a comment and interesting observation, the JMG patient with earliest onset at age 1 year, had both HLA-DRB1*04:04 and HLA-B*08 (Paper III, Table 4). This patient had AChR ab and thymus hyperplasia when thymectomized at age 2 years. It is intriguing to hypothesize that there could be an additive effect of having both associated risk HLA alleles causing this early onset of JMG.

The definition of prepubertal onset is somewhat difficult, especially in a retrospective study. Age 12 years is often used as an arbitrary cut of defining prepubertal onset in clinical publications on juvenile MG. We registered age of menarche in all the patients. Only those with MG onset before menarche were labelled prepubertal, and this was coherent with the cut off at age 12 years.

Age of sexual maturity vary interindividually and also with ethnicity and geographical latitude. Based on this and our findings, one could ask whether a cut-off at age 12 for the prepubertal and postpubertal sub classification is meaningful.

However, our sample size was too small to show significant differences between the subgroups, and further studies are needed to elaborate on this and to confirm the DRB1*04:04 association with prepubertal JMG.

7 CONCLUSION

In this thesis we have provided new knowledge about juvenile MG in Norway:

- We found that the incidence rate of juvenile MG in the Norwegian population has been stable over the last 25 years, and is consistent with the incidence in comparable countries like Denmark and UK. The incidence rate is higher among females and higher in the postpubertal age group.
- The symptoms were most often generalized, and the generalization occurred typically within 2 years from onset.
- The overall outcome was good, and seemed even more favourable among the prepubertal cases.
- The most frequent comorbidity to keep in mind is co-occurring autoimmune diseases.
- The HLA class II allele DRB1*04:04 showed to be the strongest risk allele in prepubertal onset juvenile MG.
- The HLA class I allele B*08 known to be a major risk allele in EOMG, also showed to be a risk allele in juvenile MG, especially in the postpubertal onset group.
- We found indications for a premature immunosenescence in the T cell compartment in thymectomized juvenile MG patients; however, no certain clinical consequences were seen.

8 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVE

We have shown that JMG is a rare disease, but with a significant female preponderance in both prepubertal and postpubertal age. Further, this study has increased our knowledge about JMG in Norway, which is helpful both during the diagnostic process and in the follow up and treatment of patients, for instance the higher risk of myasthenic crisis in the prepubertal group the first year from onset. Interestingly, we have a very high proportion with generalized disease compared to other Western populations. However, more research is needed to establish if there is a real difference between the JMG cohorts and why; for instance if it could be due to different treatment strategies, thus having implication for our future handling of JMG patients. It could for instance be of interesting to look into children evaluated at the Dept. of Ophthalmology due to diplopia or ptosis, to see if there are cases of ocular JMG missed in this population.

It is important to be aware of the autoimmune comorbidity frequently occurring in these patients. When lack of therapeutic response, unexpected deterioration or new symptoms we should keep in mind the possibility of autoimmune comorbidity since it has therapeutic implication and typically develops after the JMG diagnosis. A lower occurrence of autoimmune comorbidity in the JMG patients treated with immunosuppressants was an interesting finding and should be looked more into in future studies.

Concerning the debate on thymectomy and its indication in JMG, our study contributes with valuable data both on effect on outcome, and potential long-term complications. Due to its retrospective methodology, it is necessary to conduct prospective studies and also follow the JMG cohort further for potential complications later in life.

We have provided evidence for different genetic risk factors according to age at onset in the JMG cohort. This may have implication for the subgrouping and treatment of JMG patient. The definition of juvenile MG with prepubertal and postpubertal sub classification should be looked further into, and it will be necessary to get more knowledge from larger study populations considering clinical and genetic differences. Due to the rarity of JMG in Western populations, international collaboration will be imperative to achieve this.

It would be of interest to look more into the positive association between JMG and tonsillectomy, both etiological and immunological aspects.

9 REFERENCES

1. Sanders DB, Massey JM. Clinical features of myasthenia gravis. *Handb Clin Neurol*. 2008;91:229-52.
2. Afifi AK. Myasthenia gravis from Thomas Willis to the present. *Neurosciences (Riyadh)*. 2005;10(1):3-13.
3. Hughes T. The early history of myasthenia gravis. *Neuromuscul Disord*. 2005;15(12):878-86.
4. Millichap JG, Dodge PR. Diagnosis and treatment of myasthenia gravis in infancy, childhood, and adolescence: a study of 51 patients. *Neurology*. 1960;10:1007-14.
5. Conti-Fine BM, Milani M, Kaminski HJ. Myasthenia gravis: past, present, and future. *J Clin Invest*. 2006;116(11):2843-54.
6. Osserman KE, Teng P. Studies in myasthenia gravis: neonatal and juvenile types. *J Mt Sinai Hosp N Y*. 1956;23(5):711-27.
7. Levin PM. Congenital myasthenia in siblings. *Arch Neurol Psychiatry*. 1949;62(6):745-58, illust.
8. Buzzard EF. THE CLINICAL HISTORY AND POST-MORTEM EXAMINATION OF FIVE CASES OF MYASTHENIA GRAVIS. *Brain*. 1906;28(3-4):438-83.
9. Blalock A, Mason MF, Morgan HJ, Riven SS. MYASTHENIA GRAVIS AND TUMORS OF THE THYMIC REGION: REPORT OF A CASE IN WHICH THE TUMOR WAS REMOVED. *Ann Surg*. 1939;110(4):544-61.
10. Wolfe GI, Kaminski HJ, Aban IB, Minisman G, Kuo HC, Marx A, et al. Randomized Trial of Thymectomy in Myasthenia Gravis. *N Engl J Med*. 2016;375(6):511-22.
11. Pearce JM. Mary Broadfoot Walker (1888-1974): a historic discovery in myasthenia gravis. *Eur Neurol*. 2005;53(1):51-3.
12. Patrick J, Lindstrom J. Autoimmune response to acetylcholine receptor. *Science*. 1973;180(4088):871-2.
13. Lindstrom JM, Seybold ME, Lennon VA, Whittingham S, Duane DD. Antibody to acetylcholine receptor in myasthenia gravis. Prevalence, clinical correlates, and diagnostic value. *Neurology*. 1976;26(11):1054-9.
14. Hoch W, McConville J, Helms S, Newsom-Davis J, Melms A, Vincent A. Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. *Nat Med*. 2001;7(3):365-8.
15. Higuchi O, Hamuro J, Motomura M, Yamanashi Y. Autoantibodies to low-density lipoprotein receptor-related protein 4 in myasthenia gravis. *Ann Neurol*. 2011;69(2):418-22.
16. Della Marina A, Trippe H, Lutz S, Schara U. Juvenile myasthenia gravis: recommendations for diagnostic approaches and treatment. *Neuropediatrics*. 2014;45(2):75-83.
17. McGrogan A, Sneddon S, de Vries CS. The incidence of myasthenia gravis: a systematic literature review. *Neuroepidemiology*. 2010;34(3):171-83.

18. Cetin H, Fulop G, Zach H, Auff E, Zimprich F. Epidemiology of myasthenia gravis in Austria: rising prevalence in an ageing society. *Wiener klinische Wochenschrift*. 2012;124(21-22):763-8.
19. Gattellari M, Goumas C, Worthington JM. A national epidemiological study of Myasthenia Gravis in Australia. *Eur J Neurol*. 2012;19(11):1413-20.
20. VanderPluym J, Vajsar J, Jacob FD, Mah JK, Grenier D, Kolski H. Clinical characteristics of pediatric myasthenia: a surveillance study. *Pediatrics*. 2013;132(4):e939-44.
21. Parr JR, Andrew MJ, Finnis M, Beeson D, Vincent A, Jayawant S. How common is childhood myasthenia? The UK incidence and prevalence of autoimmune and congenital myasthenia. *Arch Dis Child*. 2014;99(6):539-42.
22. Carr AS, Cardwell CR, McCarron PO, McConville J. A systematic review of population based epidemiological studies in Myasthenia Gravis. *BMC Neurol*. 2010;10:46.
23. Mombaur B, Lesosky MR, Liebenberg L, Vreede H, Heckmann JM. Incidence of acetylcholine receptor-antibody-positive myasthenia gravis in South Africa. *Muscle Nerve*. 2014.
24. Lai CH, Tseng HF. Nationwide population-based epidemiological study of myasthenia gravis in taiwan. *Neuroepidemiology*. 2010;35(1):66-71.
25. Heldal AT, Owe JF, Gilhus NE, Romi F. Seropositive myasthenia gravis: a nationwide epidemiologic study. *Neurology*. 2009;73(2):150-1.
26. Andersen JB, Heldal AT, Engeland A, Gilhus NE. Myasthenia gravis epidemiology in a national cohort; combining multiple disease registries. *Acta Neurol Scand Suppl*. 2014(198):26-31.
27. Yu YL, Hawkins BR, Ip MS, Wong V, Woo E. Myasthenia gravis in Hong Kong Chinese. 1. Epidemiology and adult disease. *Acta Neurol Scand*. 1992;86(2):113-9.
28. Zhang X, Yang M, Xu J, Zhang M, Lang B, Wang W, et al. Clinical and serological study of myasthenia gravis in HuBei Province, China. *J Neurol Neurosurg Psychiatry*. 2007;78(4):386-90.
29. Evoli A. Acquired myasthenia gravis in childhood. *Curr Opin Neurol*. 2010;23(5):536-40.
30. Wong V, Hawkins BR, Yu YL. Myasthenia gravis in Hong Kong Chinese. 2. Paediatric disease. *Acta Neurol Scand*. 1992;86(1):68-72.
31. Gui M, Luo X, Lin J, Li Y, Zhang M, Zhang X, et al. Long-term outcome of 424 childhood-onset myasthenia gravis patients. *J Neurol*. 2015;262(4):823-30.
32. Kaminski HJ. *Current Clinical Neurology : Myasthenia Gravis and Related Disorders* (2). Totowa: Totowa, NJ, US: Humana Press; 2009.
33. Kirzinger L, Khomenko A, Schulte-Mattler W, Backhaus R, Platen S, Schalke B. Myopathy in Childhood Muscle-Specific Kinase Myasthenia Gravis. *Pediatr Neurol*. 2016;65:90-2.
34. Elsaïs A, Popperud TH, Melien O, Kerty E. [Drugs that may trigger or exacerbate myasthenia gravis]. *Tidsskr Nor Laegeforen*. 2013;133(3):296-9.
35. Evoli A, Batocchi AP, Bartoccioni E, Lino MM, Minisci C, Tonali P. Juvenile myasthenia gravis with prepubertal onset. *Neuromuscul Disord*. 1998;8(8):561-7.

36. Gilhus NE, Verschuuren JJ. Myasthenia gravis: subgroup classification and therapeutic strategies. *Lancet Neurol.* 2015;14(10):1023-36.
37. Li HF, Xie Y, Yue YX. Myasthenia gravis: subgroup classifications. *Lancet Neurol.* 2016;15(4):355-6.
38. Gilhus NE, Verschuuren JJ. Myasthenia gravis: subgroup classifications - Authors' reply. *Lancet Neurol.* 2016;15(4):357-8.
39. Heckmann JM, Hansen P, Van Toorn R, Lubbe E, Janse van Rensburg E, Wilmshurst JM. The characteristics of juvenile myasthenia gravis among South Africans. *S Afr Med J.* 2012;102(6):532-6.
40. Chiang LM, Darras BT, Kang PB. Juvenile myasthenia gravis. *Muscle Nerve.* 2009;39(4):423-31.
41. Berrih-Aknin S, Frenkian-Cuvelier M, Eymard B. Diagnostic and clinical classification of autoimmune myasthenia gravis. *J Autoimmun.* 2014;48-49:143-8.
42. Andrews PI, Massey JM, Sanders DB. Acetylcholine receptor antibodies in juvenile myasthenia gravis. *Neurology.* 1993;43(5):977-82.
43. Pineles SL, Avery RA, Moss HE, Finkel R, Blinman T, Kaiser L, et al. Visual and systemic outcomes in pediatric ocular myasthenia gravis. *Am J Ophthalmol.* 2010;150(4):453-9 e3.
44. Murai H, Noda T, Himeno E, Kawano Y, Ohyagi Y, Shiraishi H, et al. Infantile onset myasthenia gravis with MuSK antibodies. *Neurology.* 2006;67(1):174.
45. Evoli A, Padua L. Diagnosis and therapy of myasthenia gravis with antibodies to muscle-specific kinase. *Autoimmunity reviews.* 2013;12(9):931-5.
46. Romi F, Aarli JA, Gilhus NE. Seronegative myasthenia gravis: disease severity and prognosis. *Eur J Neurol.* 2005;12(6):413-8.
47. Evoli A, Tonali PA, Padua L, Monaco ML, Scuderi F, Batocchi AP, et al. Clinical correlates with anti-MuSK antibodies in generalized seronegative myasthenia gravis. *Brain.* 2003;126(Pt 10):2304-11.
48. Pasnoor M, Wolfe GI, Nations S, Trivedi J, Barohn RJ, Herbelin L, et al. Clinical findings in MuSK-antibody positive myasthenia gravis: a U.S. experience. *Muscle Nerve.* 2010;41(3):370-4.
49. Tsiamalos P, Kordas G, Kokla A, Poulas K, Tzartos SJ. Epidemiological and immunological profile of muscle-specific kinase myasthenia gravis in Greece. *Eur J Neurol.* 2009;16(8):925-30.
50. Guptill JT, Sanders DB, Evoli A. Anti-MuSK antibody myasthenia gravis: clinical findings and response to treatment in two large cohorts. *Muscle Nerve.* 2011;44(1):36-40.
51. Verschuuren J, Strijbos E, Vincent A. Neuromuscular junction disorders. *Handb Clin Neurol.* 2016;133:447-66.
52. Zisimopoulou P, Evangelakou P, Tzartos J, Lazaridis K, Zouvelou V, Mantegazza R, et al. A comprehensive analysis of the epidemiology and clinical characteristics of anti-LRP4 in myasthenia gravis. *J Autoimmun.* 2014;52:139-45.
53. Zhang B, Tzartos JS, Belimezi M, Ragheb S, Bealmear B, Lewis RA, et al. Autoantibodies to lipoprotein-related protein 4 in patients with double-seronegative myasthenia gravis. *Arch Neurol.* 2012;69(4):445-51.

54. Kaminski HJ. Seronegative Myasthenia Gravis-A Vanishing Disorder? *JAMA neurology*. 2016;73(9):1055-6.
55. Rodriguez Cruz PM, Huda S, Lopez-Ruiz P, Vincent A. Use of cell-based assays in myasthenia gravis and other antibody-mediated diseases. *Exp Neurol*. 2015;270:66-71.
56. Rodriguez Cruz PM, Al-Hajjar M, Huda S, Jacobson L, Woodhall M, Jayawant S, et al. Clinical Features and Diagnostic Usefulness of Antibodies to Clustered Acetylcholine Receptors in the Diagnosis of Seronegative Myasthenia Gravis. *JAMA neurology*. 2015;72(6):642-9.
57. Gasperi C, Melms A, Schoser B, Zhang Y, Meltoranta J, Risson V, et al. Anti-agrin autoantibodies in myasthenia gravis. *Neurology*. 2014;82(22):1976-83.
58. Gallardo E, Martinez-Hernandez E, Titulaer MJ, Huijbers MG, Martinez MA, Ramos A, et al. Cortactin autoantibodies in myasthenia gravis. *Autoimmunity reviews*. 2014;13(10):1003-7.
59. Cortes-Vicente E, Gallardo E, Martinez MA, Diaz-Manera J, Querol L, Rojas-Garcia R, et al. Clinical Characteristics of Patients With Double-Seronegative Myasthenia Gravis and Antibodies to Cortactin. *JAMA neurology*. 2016;73(9):1099-104.
60. Marx A, Pfister F, Schalke B, Saruhan-Direskeneli G, Melms A, Strobel P. The different roles of the thymus in the pathogenesis of the various myasthenia gravis subtypes. *Autoimmunity reviews*. 2013;12(9):875-84.
61. Fonseca AL, Ozgediz DE, Christison-Lagay ER, Detterbeck FC, Caty MG. Pediatric thymomas: report of two cases and comprehensive review of the literature. *Pediatr Surg Int*. 2014;30(3):275-86.
62. Kumar R. Myasthenia gravis and thymic neoplasms: A brief review. *World journal of clinical cases*. 2015;3(12):980-3.
63. Luchanok U, Kaminski HJ. Ocular myasthenia: diagnostic and treatment recommendations and the evidence base. *Curr Opin Neurol*. 2008;21(1):8-15.
64. Murai H, Yamashita N, Watanabe M, Nomura Y, Motomura M, Yoshikawa H, et al. Characteristics of myasthenia gravis according to onset-age: Japanese nationwide survey. *J Neurol Sci*. 2011;305(1-2):97-102.
65. Lee HN, Kang HC, Lee JS, Kim HD, Shin HY, Kim SM, et al. Juvenile Myasthenia Gravis in Korea: Subgroup Analysis According to Sex and Onset Age. *J Child Neurol*. 2016;31(14):1561-8.
66. Lindner A, Schalke B, Toyka KV. Outcome in juvenile-onset myasthenia gravis: a retrospective study with long-term follow-up of 79 patients. *J Neurol*. 1997;244(8):515-20.
67. Batocchi AP, Evoli A, Palmisani MT, Lo Monaco M, Bartoccioni M, Tonali P. Early-onset myasthenia gravis: clinical characteristics and response to therapy. *Eur J Pediatr*. 1990;150(1):66-8.
68. Tellez-Zenteno JF, Hernandez-Ronquillo L, Salinas V, Estanol B, da Silva O. Myasthenia gravis and pregnancy: clinical implications and neonatal outcome. *BMC Musculoskelet Disord*. 2004;5:42.
69. Oosterhuis HJ. The natural course of myasthenia gravis: a long term follow up study. *J Neurol Neurosurg Psychiatry*. 1989;52(10):1121-7.

70. Jovandaric MZ, Despotovic DJ, Jesic MM, Jesic MD. Neonatal Outcome in Pregnancies with Autoimmune Myasthenia Gravis. *Fetal Pediatr Pathol.* 2016;35(3):167-72.
71. Ionita CM, Acsadi G. Management of juvenile myasthenia gravis. *Pediatr Neurol.* 2013;48(2):95-104.
72. Liew WK, Kang PB. Update on juvenile myasthenia gravis. *Curr Opin Pediatr.* 2013;25(6):694-700.
73. Parr JR, Jayawant S. Childhood myasthenia: clinical subtypes and practical management. *Dev Med Child Neurol.* 2007;49(8):629-35.
74. Oger J, Frykman H. An update on laboratory diagnosis in myasthenia gravis. *Clin Chim Acta.* 2015;444:126-31.
75. Devic P, Petiot P, Simonet T, Stojkovic T, Delmont E, Franques J, et al. Antibodies to clustered acetylcholine receptor: expanding the phenotype. *Eur J Neurol.* 2014;21(1):130-4.
76. Lee JI, Jander S. Myasthenia gravis: recent advances in immunopathology and therapy. *Expert Rev Neurother.* 2017;17(3):287-99.
77. Gautel M, Lakey A, Barlow DP, Holmes Z, Scales S, Leonard K, et al. Titin antibodies in myasthenia gravis: identification of a major immunogenic region of titin. *Neurology.* 1993;43(8):1581-5.
78. Pitt M. Neurophysiological strategies for the diagnosis of disorders of the neuromuscular junction in children. *Dev Med Child Neurol.* 2008;50(5):328-33.
79. Cherian A, Baheti NN, Iype T. Electrophysiological study in neuromuscular junction disorders. *Ann Indian Acad Neurol.* 2013;16(1):34-41.
80. Amandusson A, Elf K, Grindlund ME, Punga AR. Diagnostic Utility of Repetitive Nerve Stimulation in a Large Cohort of Patients With Myasthenia Gravis. *J Clin Neurophysiol.* 2017;34(5):400-7.
81. Padua L, Stalberg E, LoMonaco M, Evoli A, Batocchi A, Tonali P. SFEMG in ocular myasthenia gravis diagnosis. *Clin Neurophysiol.* 2000;111(7):1203-7.
82. Punga AR, Punga T. Circulating microRNAs as potential biomarkers in myasthenia gravis patients. *Ann N Y Acad Sci.* 2018;1412(1):33-40.
83. Keeseey JC. Clinical evaluation and management of myasthenia gravis. *Muscle Nerve.* 2004;29(4):484-505.
84. Beeson D. Congenital myasthenic syndromes: recent advances. *Curr Opin Neurol.* 2016;29(5):565-71.
85. Hantai D, Nicole S, Eymard B. Congenital myasthenic syndromes: an update. *Curr Opin Neurol.* 2013;26(5):561-8.
86. Elgert KD. *Immunology : understanding the immune system.* 2nd ed. ed. Hoboken, N.J: Wiley-Blackwell; 2009.
87. Thorsby E, Lie BA. HLA associated genetic predisposition to autoimmune diseases: Genes involved and possible mechanisms. *Transpl Immunol.* 2005;14(3-4):175-82.
88. von Boehmer H, Teh HS, Kisielow P. The thymus selects the useful, neglects the useless and destroys the harmful. *Immunol Today.* 1989;10(2):57-61.
89. Cavalcante P, Le Panse R, Berrih-Aknin S, Maggi L, Antozzi C, Baggi F, et al. The thymus in myasthenia gravis: Site of "innate autoimmunity"? *Muscle Nerve.* 2011;44(4):467-84.

90. Okumura M, Inoue M, Kadota Y, Hayashi A, Tokunaga T, Kusu T, et al. Biological implications of thymectomy for myasthenia gravis. *Surg Today*. 2010;40(2):102-7.
91. Cron MA, Maillard S, Villegas J, Truffault F, Sudres M, Dragin N, et al. Thymus involvement in early-onset myasthenia gravis. *Ann N Y Acad Sci*. 2018;1412(1):137-45.
92. Bach JF. The etiology of autoimmune diseases: the case of myasthenia gravis. *Ann N Y Acad Sci*. 2012;1274:33-9.
93. Phillips WD, Vincent A. Pathogenesis of myasthenia gravis: update on disease types, models, and mechanisms. *F1000Research*. 2016;5.
94. Huijbers MG, Zhang W, Klooster R, Niks EH, Friesse MB, Straasheijm KR, et al. MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. *Proc Natl Acad Sci U S A*. 2013;110(51):20783-8.
95. Yan M, Xing GL, Xiong WC, Mei L. Agrin and LRP4 antibodies as new biomarkers of myasthenia gravis. *Ann N Y Acad Sci*. 2018;1413(1):126-35.
96. Bogdanos DP, Smyk DS, Rigopoulou EI, Mytilinaiou MG, Heneghan MA, Selmi C, et al. Twin studies in autoimmune disease: genetics, gender and environment. *J Autoimmun*. 2012;38(2-3):J156-69.
97. Ramanujam R, Pirskanen R, Ramanujam S, Hammarstrom L. Utilizing twins concordance rates to infer the predisposition to myasthenia gravis. *Twin research and human genetics : the official journal of the International Society for Twin Studies*. 2011;14(2):129-36.
98. Salvado M, Canela M, Ponseti JM, Lorenzo L, Garcia C, Cazorla S, et al. Study of the prevalence of familial autoimmune myasthenia gravis in a Spanish cohort. *J Neurol Sci*. 2016;360:110-4.
99. Mao ZF, Yang LX, Mo XA, Qin C, Lai YR, He NY, et al. Frequency of autoimmune diseases in myasthenia gravis: a systematic review. *Int J Neurosci*. 2011;121(3):121-9.
100. Giraud M, Vandiedonck C, Garchon HJ. Genetic factors in autoimmune myasthenia gravis. *Ann N Y Acad Sci*. 2008;1132:180-92.
101. Vandiedonck C, Beaurain G, Giraud M, Hue-Beauvais C, Eymard B, Tranchant C, et al. Pleiotropic effects of the 8.1 HLA haplotype in patients with autoimmune myasthenia gravis and thymus hyperplasia. *Proc Natl Acad Sci U S A*. 2004;101(43):15464-9.
102. Maniaol AH, Elsais A, Lorentzen AR, Owe JF, Viken MK, Saether H, et al. Late onset myasthenia gravis is associated with HLA DRB1*15:01 in the Norwegian population. *PLoS One*. 2012;7(5):e36603.
103. Avidan N, Le Panse R, Berrih-Aknin S, Miller A. Genetic basis of myasthenia gravis - a comprehensive review. *J Autoimmun*. 2014;52:146-53.
104. Price P, Witt C, Allcock R, Sayer D, Garlepp M, Kok CC, et al. The genetic basis for the association of the 8.1 ancestral haplotype (A1, B8, DR3) with multiple immunopathological diseases. *Immunol Rev*. 1999;167:257-74.
105. Gregersen PK, Kosoy R, Lee AT, Lamb J, Sussman J, McKee D, et al. Risk for myasthenia gravis maps to a (151) Pro-->Ala change in TNIP1 and to human leukocyte antigen-B*08. *Ann Neurol*. 2012;72(6):927-35.

106. Janer M, Cowland A, Picard J, Campbell D, Pontarotti P, Newsom-Davis J, et al. A susceptibility region for myasthenia gravis extending into the HLA-class I sector telomeric to HLA-C. *Hum Immunol.* 1999;60(9):909-17.
107. Testi M, Terracciano C, Guagnano A, Testa G, Marfia GA, Pompeo E, et al. Association of HLA-DQB1 *05:02 and DRB1 *16 Alleles with Late-Onset, Nonthymomatous, AChR-Ab-Positive Myasthenia Gravis. *Autoimmune Dis.* 2012;2012:541760.
108. Niks EH, Kuks JB, Roep BO, Haasnoot GW, Verduijn W, Ballieux BE, et al. Strong association of MuSK antibody-positive myasthenia gravis and HLA-DR14-DQ5. *Neurology.* 2006;66(11):1772-4.
109. Bartoccioni E, Scuderi F, Augugliaro A, Chiatamone Ranieri S, Sauchelli D, Alboino P, et al. HLA class II allele analysis in MuSK-positive myasthenia gravis suggests a role for DQ5. *Neurology.* 2009;72(2):195-7.
110. Matsuki K, Juji T, Tokunaga K, Takamizawa M, Maeda H, Soda M, et al. HLA antigens in Japanese patients with myasthenia gravis. *J Clin Invest.* 1990;86(2):392-9.
111. Chen WH, Chiu HC, Hseih RP. Association of HLA-Bw46DR9 combination with juvenile myasthenia gravis in Chinese. *J Neurol Neurosurg Psychiatry.* 1993;56(4):382-5.
112. Feng HY, Yang LX, Liu WB, Huang X, Qiu L, Li Y. The HLA-B*4601-DRB1*0901 haplotype is positively correlated with juvenile ocular myasthenia gravis in a southern Chinese Han population. *Neurol Sci.* 2015;36(7):1135-40.
113. Shinomiya N, Nomura Y, Segawa M. A variant of childhood-onset myasthenia gravis: HLA typing and clinical characteristics in Japan. *Clin Immunol.* 2004;110(2):154-8.
114. Hong Y, Skeie GO, Zisimopoulou P, Karagiorgou K, Tzartos SJ, Gao X, et al. Juvenile-onset myasthenia gravis: autoantibody status, clinical characteristics and genetic polymorphisms. *J Neurol.* 2017;264(5):955-62.
115. Zagoriti Z, Kambouris ME, Patrinos GP, Tzartos SJ, Poulas K. Recent advances in genetic predisposition of myasthenia gravis. *BioMed research international.* 2013;2013:404053.
116. Berrih-Aknin S. Myasthenia Gravis: paradox versus paradigm in autoimmunity. *J Autoimmun.* 2014;52:1-28.
117. Berrih-Aknin S, Le Panse R. Myasthenia gravis: a comprehensive review of immune dysregulation and etiological mechanisms. *J Autoimmun.* 2014;52:90-100.
118. Maniaol AH, Boldingh M, Brunborg C, Harbo HF, Tallaksen CM. Smoking and socio-economic status may affect myasthenia gravis. *Eur J Neurol.* 2013;20(3):453-60.
119. Wang Z, Yan Y. Immunopathogenesis in Myasthenia Gravis and Neuromyelitis Optica. *Front Immunol.* 2017;8:1785.
120. Cavalcante P, Cufi P, Mantegazza R, Berrih-Aknin S, Bernasconi P, Le Panse R. Etiology of myasthenia gravis: innate immunity signature in pathological thymus. *Autoimmunity reviews.* 2013;12(9):863-74.
121. Chang C, Gershwin ME. Drugs and autoimmunity--a contemporary review and mechanistic approach. *J Autoimmun.* 2010;34(3):J266-75.

122. Fee DB, Kasarskis EJ. Myasthenia gravis associated with etanercept therapy. *Muscle Nerve*. 2009;39(6):866-70.
123. Fairweather D, Frisancho-Kiss S, Rose NR. Sex differences in autoimmune disease from a pathological perspective. *Am J Pathol*. 2008;173(3):600-9.
124. Jacobson DL, Gange SJ, Rose NR, Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin Immunol Immunopathol*. 1997;84(3):223-43.
125. Quintero OL, Amador-Patarroyo MJ, Montoya-Ortiz G, Rojas-Villarraga A, Anaya JM. Autoimmune disease and gender: plausible mechanisms for the female predominance of autoimmunity. *J Autoimmun*. 2012;38(2-3):J109-19.
126. Amadori A, Zamarchi R, De Silvestro G, Forza G, Cavatton G, Danieli GA, et al. Genetic control of the CD4/CD8 T-cell ratio in humans. *Nat Med*. 1995;1(12):1279-83.
127. Nussinovitch U, Shoenfeld Y. The role of gender and organ specific autoimmunity. *Autoimmunity reviews*. 2012;11(6-7):A377-85.
128. Sussman J, Farrugia ME, Maddison P, Hill M, Leite MI, Hilton-Jones D. Myasthenia gravis: Association of British Neurologists' management guidelines. *Pract Neurol*. 2015;15(3):199-206.
129. Sanders DB, Wolfe GI, Benatar M, Evoli A, Gilhus NE, Illa I, et al. International consensus guidance for management of myasthenia gravis: Executive summary. *Neurology*. 2016;87(4):419-25.
130. Kerty E, Elsais A, Argov Z, Evoli A, Gilhus NE. EFNS/ENS Guidelines for the treatment of ocular myasthenia. *Eur J Neurol*. 2014;21(5):687-93.
131. Schneider-Gold C, Gajdos P, Toyka KV, Hohlfeld RR. Corticosteroids for myasthenia gravis. *The Cochrane database of systematic reviews*. 2005(2):Cd002828.
132. Skeie GO, Apostolski S, Evoli A, Gilhus NE, Illa I, Harms L, et al. Guidelines for treatment of autoimmune neuromuscular transmission disorders. *Eur J Neurol*. 2010;17(7):893-902.
133. Pascuzzi RM, Coslett HB, Johns TR. Long-term corticosteroid treatment of myasthenia gravis: report of 116 patients. *Ann Neurol*. 1984;15(3):291-8.
134. Palace J, Newsom-Davis J, Lecky B. A randomized double-blind trial of prednisolone alone or with azathioprine in myasthenia gravis. Myasthenia Gravis Study Group. *Neurology*. 1998;50(6):1778-83.
135. Engel AG, Engel A. Myasthenia gravis and myasthenic disorders. New York: Oxford University Press; 2012.
136. Jolles S, Sewell WA, Misbah SA. Clinical uses of intravenous immunoglobulin. *Clin Exp Immunol*. 2005;142(1):1-11.
137. Nosadini M, Mohammad SS, Suppiej A, Sartori S, Dale RC. Intravenous immunoglobulin in paediatric neurology: safety, adherence to guidelines, and long-term outcome. *Dev Med Child Neurol*. 2016;58(11):1180-92.
138. Liew WK, Powell CA, Sloan SR, Shamberger RC, Weldon CB, Darras BT, et al. Comparison of plasmapheresis and intravenous immunoglobulin as maintenance therapies for juvenile myasthenia gravis. *JAMA neurology*. 2014;71(5):575-80.
139. Tracy MM, McRae W, Millichap JG. Graded response to thymectomy in children with myasthenia gravis. *J Child Neurol*. 2009;24(4):454-9.

140. Adams C, Theodorescu D, Murphy EG, Shandling B. Thymectomy in juvenile myasthenia gravis. *J Child Neurol.* 1990;5(3):215-8.
141. Rodriguez M, Gomez MR, Howard FM, Jr., Taylor WF. Myasthenia gravis in children: long-term follow-up. *Ann Neurol.* 1983;13(5):504-10.
142. Ware TL, Ryan MM, Kornberg AJ. Autoimmune myasthenia gravis, immunotherapy and thymectomy in children. *Neuromuscul Disord.* 2012;22(2):118-21.
143. Andrews PI, Massey JM, Howard JF, Jr., Sanders DB. Race, sex, and puberty influence onset, severity, and outcome in juvenile myasthenia gravis. *Neurology.* 1994;44(7):1208-14.
144. Madenci AL, Li GZ, Weil BR, Zurakowski D, Kang PB, Weldon CB. The role of thymectomy in the treatment of juvenile myasthenia gravis: a systematic review. *Pediatr Surg Int.* 2017.
145. Sauce D, Larsen M, Fastenackels S, Duperrier A, Keller M, Grubeck-Loebenstien B, et al. Evidence of premature immune aging in patients thymectomized during early childhood. *J Clin Invest.* 2009;119(10):3070-8.
146. Seybold ME. Thymectomy in childhood myasthenia gravis. *Ann N Y Acad Sci.* 1998;841:731-41.
147. Ashraf VV, Taly AB, Veerendrakumar M, Rao S. Myasthenia gravis in children: a longitudinal study. *Acta Neurol Scand.* 2006;114(2):119-23.
148. Melbourne Chambers R, Forrester S, Gray R, Tapper J, Trotman H. Myasthenia gravis in Jamaican children: a 12-year institutional review. *Paediatrics and international child health.* 2012;32(1):47-50.
149. Fang F, Sveinsson O, Thormar G, Granqvist M, Askling J, Lundberg IE, et al. The autoimmune spectrum of myasthenia gravis: a Swedish population-based study. *J Intern Med.* 2015;277(5):594-604.
150. Heldal AT, Eide GE, Gilhus NE, Romi F. Geographical distribution of a seropositive myasthenia gravis population. *Muscle Nerve.* 2012;45(6):815-9.
151. Boldingh MI, Maniaol AH, Brunborg C, Dekker L, Heldal AT, Lipka AF, et al. Geographical Distribution of Myasthenia Gravis in Northern Europe--Results from a Population-Based Study from Two Countries. *Neuroepidemiology.* 2015;44(4):221-31.
152. Spurkland A, Saarinen S, Boberg KM, Mitchell S, Broome U, Caballeria L, et al. HLA class II haplotypes in primary sclerosing cholangitis patients from five European populations. *Tissue Antigens.* 1999;53(5):459-69.
153. Lorentzen AR, Karlsen TH, Olsson M, Smestad C, Mero IL, Woldseth B, et al. Killer immunoglobulin-like receptor ligand HLA-Bw4 protects against multiple sclerosis. *Ann Neurol.* 2009;65(6):658-66.
154. Jaretzki A, 3rd, Barohn RJ, Ernstoff RM, Kaminski HJ, Keesey JC, Penn AS, et al. Myasthenia gravis: recommendations for clinical research standards. Task Force of the Medical Scientific Advisory Board of the Myasthenia Gravis Foundation of America. *Ann Thorac Surg.* 2000;70(1):327-34.
155. Mardis ER. A decade's perspective on DNA sequencing technology. *Nature.* 2011;470(7333):198-203.
156. Chan JK, Ng CS, Hui PK. A simple guide to the terminology and application of leucocyte monoclonal antibodies. *Histopathology.* 1988;12(5):461-80.

157. Strawa K, Markowska A, Miskiewicz P, Kus A, Ambroziak U, Szymanski K, et al. Increased concentration of T-cell receptor rearrangement excision circles (TREC) in peripheral blood in Graves' disease. *Clin Endocrinol (Oxf)*. 2014;81(5):769-74.
158. Hazenberg MD, Verschuren MC, Hamann D, Miedema F, van Dongen JJ. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. *J Mol Med (Berl)*. 2001;79(11):631-40.
159. Gul KA, Overland T, Osnes L, Baumbusch LO, Pettersen RD, Lima K, et al. Neonatal Levels of T-cell Receptor Excision Circles (TREC) in Patients with 22q11.2 Deletion Syndrome and Later Disease Features. *J Clin Immunol*. 2015;35(4):408-15.
160. Kirkwood BR, Sterne JAC. *Essential medical statistics*. 2nd ed. ed. Malden: Blackwell; 2003.
161. Fletcher RH, Fletcher SW. *Clinical epidemiology : the essentials*. 4th ed. ed. Baltimore: Lippincott Williams & Wilkins; 2005.
162. Ahmad T, Neville M, Marshall SE, Armuzzi A, Mulcahy-Hawes K, Crawshaw J, et al. Haplotype-specific linkage disequilibrium patterns define the genetic topography of the human MHC. *Hum Mol Genet*. 2003;12(6):647-56.
163. Valdes AM, Thomson G. Detecting disease-predisposing variants: the haplotype method. *Am J Hum Genet*. 1997;60(3):703-16.
164. Svejgaard A, Ryder LP. HLA and disease associations: detecting the strongest association. *Tissue Antigens*. 1994;43(1):18-27.
165. Rice TK, Schork NJ, Rao DC. Methods for handling multiple testing. *Adv Genet*. 2008;60:293-308.
166. Pedersen EG, Hallas J, Hansen K, Jensen PE, Gaist D. Identifying patients with myasthenia for epidemiological research by linkage of automated registers. *Neuroepidemiology*. 2011;37(2):120-8.
167. Park SY, Lee JY, Lim NG, Hong YH. Incidence and Prevalence of Myasthenia Gravis in Korea: A Population-Based Study Using the National Health Insurance Claims Database. *J Clin Neurol*. 2016;12(3):340-4.
168. Castro D, Derisavifard S, Anderson M, Greene M, Iannaccone S. Juvenile myasthenia gravis: a twenty-year experience. *J Clin Neuromuscul Dis*. 2013;14(3):95-102.
169. Evoli A, Caliandro P, Iorio R, Alboini PE, Damato V, LaTorre G, et al. Poly-autoimmunity in patients with myasthenia gravis: A single-center experience. *Autoimmunity*. 2015;48(6):412-7.
170. Ong MS, Kohane IS, Cai T, Gorman MP, Mandl KD. Population-level evidence for an autoimmune etiology of epilepsy. *JAMA neurology*. 2014;71(5):569-74.
171. Ji J, Sundquist J, Sundquist K. Tonsillectomy associated with an increased risk of autoimmune diseases: A national cohort study. *J Autoimmun*. 2016;72:1-7.
172. Brandtzaeg P. Immunology of tonsils and adenoids: everything the ENT surgeon needs to know. *Int J Pediatr Otorhinolaryngol*. 2003;67 Suppl 1:S69-76.

173. Pidelaserra Marti G, Isdahl Mohn KG, Cox RJ, Brokstad KA. The influence of tonsillectomy on total serum antibody levels. *Scand J Immunol.* 2014;80(5):377-9.
174. Hennessey IA, Long AM, Hughes I, Humphrey G. Thymectomy for inducing remission in juvenile myasthenia gravis. *Pediatr Surg Int.* 2011;27(6):591-4.
175. Zlamy M, Prelog M. Thymectomy in early childhood: a model for premature T cell immunosenescence? *Rejuvenation research.* 2009;12(4):249-58.
176. Prelog M, Keller M, Geiger R, Brandstatter A, Wurzner R, Schweigmann U, et al. Thymectomy in early childhood: significant alterations of the CD4(+)CD45RA(+)CD62L(+) T cell compartment in later life. *Clin Immunol.* 2009;130(2):123-32.
177. Gudmundsdottir J, Oskarsdottir S, Skogberg G, Lindgren S, Lundberg V, Berglund M, et al. Early thymectomy leads to premature immunologic ageing: An 18-year follow-up. *J Allergy Clin Immunol.* 2016;138(5):1439-43.e10.
178. Sempowski G, Thomasch J, Gooding M, Hale L, Edwards L, Ciafaloni E, et al. Effect of thymectomy on human peripheral blood T cell pools in myasthenia gravis. *J Immunol.* 2001;166(4):2808-17.
179. Lima K, Folling I, Eiklid KL, Natvig S, Abrahamsen TG. Age-dependent clinical problems in a Norwegian national survey of patients with the 22q11.2 deletion syndrome. *Eur J Pediatr.* 2010;169(8):983-9.
180. Sauce D, Appay V. Altered thymic activity in early life: how does it affect the immune system in young adults? *Curr Opin Immunol.* 2011;23(4):543-8.
181. Prelog M. Aging of the immune system: a risk factor for autoimmunity? *Autoimmunity reviews.* 2006;5(2):136-9.
182. Prelog M, Wilk C, Keller M, Karall T, Orth D, Geiger R, et al. Diminished response to tick-borne encephalitis vaccination in thymectomized children. *Vaccine.* 2008;26(5):595-600.
183. Thewissen M, Somers V, Venken K, Linsen L, van Paassen P, Geusens P, et al. Analyses of immunosenescent markers in patients with autoimmune disease. *Clin Immunol.* 2007;123(2):209-18.
184. Pirskanen R. Genetic aspects in myasthenia gravis. A family study of 264 Finnish patients. *Acta Neurol Scand.* 1977;56(5):365-88.

ELSEVIER

Official Journal of the European Paediatric Neurology Society

Original article

Juvenile myasthenia gravis in Norway: A nationwide epidemiological study

T.H. Popperud ^{a,b,*}, M.I. Boldingh ^a, C. Brunborg ^c, K.W. Faiz ^d,
A.T. Heldal ^e, A.H. Maniaol ^a, K.I. Müller ^{f,g}, M. Rasmussen ^{h,i}, K. Øymar ^{e,j},
E. Kerty ^{a,b}

^a Department of Neurology, Oslo University Hospital, Oslo, Norway

^b Institute of Clinical Medicine, University of Oslo, Oslo, Norway

^c Oslo Centre for Biostatistics and Epidemiology, Research Support Services, Oslo University Hospital, Oslo, Norway

^d Department of Neurology, Akershus University Hospital, Lørenskog, Norway

^e Department of Clinical Medicine, University of Bergen, Bergen, Norway

^f Department of Neurology and National Neuromuscular Centre, University Hospital of North Norway, Tromsø, Norway

^g Department of Clinical Medicine, UiT – The Arctic University of Norway, Tromsø, Norway

^h Department of Clinical Neuroscience for Children, Section for Child Neurology, Oslo University Hospital, Oslo, Norway

ⁱ Unit for Hereditary and Inborn Neuromuscular Disorders, Department of Neurology, Oslo University Hospital, Oslo, Norway

^j Department of Pediatrics, Stavanger University Hospital, Stavanger, Norway

ARTICLE INFO

Article history:

Received 30 June 2016

Received in revised form

17 August 2016

Accepted 3 September 2016

Keywords:

Juvenile myasthenia gravis

Incidence

Prevalence

Frequency

ABSTRACT

Background: The aim of this study was to assess the incidence rate and prevalence of autoimmune myasthenia gravis (MG) among children in Norway.

Methods: This retrospective population-based study was performed in Norway from January 2012 to December 2013. Cases of juvenile MG (JMG) with onset < 18 years were identified through searches in coding systems of electronic patient records at the 15 main hospitals in Norway from 1989 to 2013. In addition, the acetylcholine receptor antibody database at Haukeland University Hospital and the clinical nationwide MG database at Oslo University Hospital were searched for cases of JMG. Diagnosis and age at onset were verified through medical records. Incidence and prevalence rates were calculated using the Norwegian population as reference.

Results: In total 63 unique JMG cases were identified. This corresponds to an average annual incidence rate of 1.6 per million. Incidence rate was stable over the study period. Prevalence of JMG was 3.6–13.8 per million. Females constituted the majority of JMG cases (55 vs

* Corresponding author. Department of Neurology, Oslo University Hospital, Rikshospitalet, PB 4950 Nydalen, 0424 Oslo, Norway. Fax: +47 23074961.

E-mail address: t.h.popperud@medisin.uio.no (T.H. Popperud).

<http://dx.doi.org/10.1016/j.ejpn.2016.09.001>

1090-3798/© 2016 European Paediatric Neurology Society. Published by Elsevier Ltd. All rights reserved.

8 males). The risk of JMG was higher among females both in the postpubertal and prepubertal group ($p < 0.001$ and $p = 0.02$, respectively).

Conclusion: This study confirms the rarity of JMG in Norway, especially among males, and shows a stable incidence rate over the last 25 years.

© 2016 European Paediatric Neurology Society. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disorder caused by autoantibodies directed against components of the neuromuscular junction, most commonly the acetylcholine receptors (AChR). Other targets are the protein muscle specific kinase (MuSK) and the agrin receptor low-density lipoprotein receptor-related protein 4 (LRP4).¹ A subgroup of MG patients has no detectable autoantibodies using commercially available methods. The distribution of MG patients with no detectable autoantibodies is greater amongst children.² MG patients experience fluctuating muscle weakness and fatigability. All skeletal muscles may be affected, including respiratory muscles. However, the ocular, bulbar and proximal extremity muscles are most typically involved. The disease may cause significant disability and is potentially fatal if not treated. Since the disability can influence normal physical and visual functioning, it is important to prevent delays in diagnosis and treatment in children.

MG with onset in childhood or adolescence is termed juvenile myasthenia gravis (JMG). The upper age limit for JMG varies, but is often set at age 19 years.³ The age span represents different phases of immune maturation, and the group is usually divided into prepubertal and pubertal/postpubertal onset with a cut off at age 12.⁴

MG in adults varies widely with prevalence 5–317 per million^{5,6} and incidence rate of 4.1–30 per million per year.⁷ The same applies to JMG with studies reporting prevalence 2.2–6.2 per million^{8,9} and incidence rate of 1.5–4.3 per million.^{10,11} Geographical and also methodological differences may explain the variation. It is estimated that approximately 10–15% of MG cases in Caucasian populations have juvenile onset.³ In Asian populations childhood onset has been reported more frequently, representing up to 50% of MG cases.¹²

Postpubertal JMG has a higher incidence among females.^{11,13,14} In prepubertal JMG many studies report no gender differences,^{11,14,15} but some have shown female predominance^{10,13,16} and other male predominance.⁹

The aim of this nationwide study was to assess the incidence and prevalence of MG with juvenile onset in Norway.

2. Materials and methods

2.1. Study area and population

This cross-sectional study is based on the Norwegian population, which constituted 5.1 mill people (50.3% males and

49.7% females) on 31 December 2013 (Statistics Norway, www.ssb.no).

Norway is situated in Northern Europe, latitude 57°–71° N. The country has a public healthcare system providing free healthcare for children under the age of 16 years. Norway is divided into four health regions (North, Central, West and South-East). All regions have neurologic and pediatric expertise.

All Norwegian residents have a unique national ID number enabling identification of individual cases.

2.2. Case identification and confirmation

Case identification commenced in January 2012 and was completed in December 2013. Multiple sources were used to identify JMG cases (Figs. 1 and 2) from the whole of Norway.

At Norwegian hospitals all in- and out-patient discharges are coded according to the Norwegian version of the International Classification of Disease (ICD). Codes are registered in hospitals' information and patient systems, either using the

Fig. 1 – Flow diagram showing identification of juvenile myasthenia gravis (JMG) cases in Norway. Three different sources were used: hospital records (ICD code), AChR ab database at Haukeland University Hospital and the national adult MG database. Cases identified through more than one source were only counted once (excluded as duplicate or triplicate). AChR ab = acetylcholine receptor antibodies. MG = myasthenia gravis.

Fig. 2 – Venn diagram demonstrating sources overlap.
AChR ab = acetylcholine receptor antibody.
MG = myasthenia gravis.

Distributed Information- and Patient system for hospitals (DIPS ASA, Norway) or the DocuLive electronic system (Siemens, Norway). Potential cases were identified through ICD codes (ICD-9: 358, ICD-10: G 70) registered in the period from 1989 through 2013 at the 15 main hospitals in Norway that have either a pediatric or a neurological department or both. This was done in collaboration with in-house physicians.

An additional search was made in the AChR-antibody database at Haukeland University Hospital, a laboratory database from 1983 to 2012.¹⁷ Antibodies were detected by a commercial kit (IBL International GmbH, Hamburg, Germany) and concentration ≥ 0.4 nmol/L was regarded as positive.¹⁷

A clinical nationwide population-based MG database from the Department of Neurology at Oslo University Hospital included living Norwegian MG patients age 16 years or older on prevalent cases day, 1 November 2009.^{18,19} We extracted registered patients reporting MG onset before the age of 18 from this database.

2.3. Inclusion and exclusion criteria

Diagnostic criteria for inclusion were symptoms typical for MG (fatigable ocular, bulbar and/or extremity weakness) with onset before age 18 years, and two out of the following three: 1) Antibodies against AChR or MuSK. 2) Supportive electrophysiological findings. Repetitive nerve stimulation showing impaired neuromuscular transmission and/or single-fiber electromyography revealing increased jitter. 3) Reversal of weakness after administration of acetylcholinesterase inhibitor (edrophonium or pyridostigmine) and/or immunomodulating therapy (steroids, intravenous immunoglobulin, immunosuppressive agent, thymectomy).

JMG diagnosis and inclusion criteria were verified through patients' medical charts. This was done either by the in-hospital physicians or by the authors (THP, KWF, KIM).

Some patients in the nationwide MG database reported onset before the age of 18, but medical evaluation and documentation of symptoms were completed many years later. These cases were not included. Cases were excluded if symptoms started at birth and/or with positive family history, and if there was no antibody detection and no response to immunomodulation, as congenital myasthenic syndrome was suspected.

The Norwegian National Registry was consulted to verify that the patients were alive on the prevalence day.

2.4. Definitions

JMG was defined as MG with onset of clinical symptoms <18 years. Prepubertal JMG was defined as clinical onset <12 years, and postpubertal JMG ≥ 12 years.

2.5. Data collection

In addition to the clinical variables given as inclusion criteria, the following variables were noted from the medical charts: time from onset to diagnosis, which month of the year symptoms started, geographic place of residence at time of diagnosis, if the patient had been evaluated at a University Hospital.

2.6. Statistical analysis

Average annual incidence rate was estimated for five-year periods and for the 25 years long study period, 1989–2013. An estimation of population at risk was calculated as follows: the mean value of the juvenile population (<18 years) in Norway at the beginning of each year in the whole study period, or the five-year periods (Statistics Norway, www.ssb.no/tabell/07459). Incidence rates, including age- and sex-specific rates, were calculated based on new JMG cases occurring in the 25 years long study period or the five-year periods, divided by the estimated population at risk, and divided by 25 or 5 respectively to get the average annual rate. The results are presented as incidence rates (new cases per million person years) with 95% Confidence Intervals (CI).

The point prevalence rates were estimated based on prevalent cases alive on five specific dates, December 31, 1993, 1998, 2003, 2008 and 2013. Denominator was the juvenile population (<18 years) in Norway on respective date (Statistics Norway, www.ssb.no/tabell/07459).

The 95% CIs were estimated using the Mid-P exact test on openepi.com. Chi Square test was used to test for linear trend and to compare differences in ratios. These statistical analyses were done by STATA 13 (Stata Corp, College Station, TX, USA).

2.7. Ethics

The study was approved by the Regional Committee for Medical and Health Research Ethics, and data was registered in accordance with Norwegian guidelines.

Table 1 – Age and gender specific annual average incidence of juvenile myasthenia gravis in Norway 1989–2013.

Age, yrs	Incidence females				Incidence males				Incidence females and males			
	N per 25 yrs	Person yrs $\times 10^6$	Cases per mill/yr	(95% CI)	N per 25 yrs	Person yrs $\times 10^6$	Cases per mill/yr	(95% CI)	N per 25 yrs	Person yrs $\times 10^6$	Cases per mill/yr	(95% CI)
1–11	12	0.34	1.4	(0.8–2.4)	3	0.36	0.3	(0.1–0.9)	15	0.7	0.9	(0.5–1.4)
12–17	24	0.17	5.7	(3.8–8.3)	3	0.18	0.7	(0.2–1.8)	27	0.35	3.1	(2.1–4.4)
1–17	36	0.51	2.8	(2.0–0.9)	6	0.54	0.4	(0.2–0.9)	42	1.05	1.6	(1.2–2.1)

Incident cases per million person-years with 95% confidence interval (CI).
N = number of incident cases.
Yr = year.

3. Results

3.1. Incidence

We identified a total of 234 potential JMG cases. After inclusion criteria verification, our study population constituted 63 unique JMG patients (Fig. 1). 42 of the 63 identified JMG cases were incident cases in the period 1989–2013. Estimated average crude incidence rate of JMG in this period was 1.6 (95% CI, 1.2–2.1) per million person years. The postpubertal group had a significantly higher incidence rate of 3.1 (95% CI, 2.1–4.4) compared to the prepubertal group with incidence rate of 0.9 (95% CI, 0.5–1.4), $p < 0.001$ (see Table 1).

Females constituted 86% of the incident cases. Gender specific incidences are shown in Table 1. The risk of being diagnosed with JMG in the study period was highest among females, both in the prepubertal and postpubertal group with OR 8.6 (95% CI, 2.6–28.5), $p < 0.001$, and 4.2 (95% CI, 1.2–14.8), $p = 0.02$, respectively. We compared age-specific incidence in five 5-year periods (see Table 2). No change in incidence was found in the study period, neither in the prepubertal or postpubertal group, $p = 0.07$ and $p = 0.08$ respectively.

Incidence in the different health regions is shown in Table 3. The western region had the highest incidence rate of 2.7 per million, but there were no statistically significant differences among the regions.

3.2. Prevalence

Point prevalence was estimated on five specific dates in the study period, December 31 1993, 1998, 2003, 2008 and

2013 (Numbers shown in the [supplementary material](#)). Prevalence was highest on December 31, 2003 and lowest on December 31, 2013, 13.8 per mill and 3.6 per mill, respectively. This difference was not statistically significant.

3.3. Characteristics of JMG cases

The gender distribution was 55 (87%) females and 8 (13%) males, corresponding to a female-to-male odds ratio (OR) of JMG of 7.0 (95% CI, 3.3–14.6), $p < 0.001$. Autoantibodies (ab) were detected in 48 patients (AChR ab in 47 and MuSK ab in one). Nine patients were negative for both AChR ab and MuSK ab. Information about MuSK ab was missing in seven. Impaired neuromuscular transmission was verified by electrophysiological examination (repetitive nerve stimulation and/or single fiber EMG) in 43 patients. Improved muscle strength after administration of acetylcholinesterase inhibitor was documented in 54 patients.

Median age of onset was 13.0 years (range 1–17, IQR 9.0–16.0), females 13.0 (IQR 10.0–16.0) and males 11.0 (IQR 6.0–16.5). A total of 23 (37%) patients had prepubertal onset, of which 18 (78%) were female. Also among the 40 patients constituting the postpubertal group the majority, 37 (93%) patients, were female (Fig. 3). The commonest initial clinical presentation was ocular, presented in 69%, most often ptosis. Median time from onset until diagnosis was 6 months, range 0–120, without any significant difference between the prepubertal and postpubertal onset groups. We have information about month of JMG onset in all but nine patients. Onset was spread all through the year with no seasonal difference in incidence. All patients had at least one evaluation at a University Hospital.

Table 2 – Age specific annual average incidence of juvenile myasthenia gravis (JMG) in Norway 1989–2013 stratified in 5-year periods.

Annual incidence	JMG < 18 yrs				JMG < 12 yrs				JMG 12–17 yrs			
	N	Person-yrs $\times 10^6$	Cases per mill/yr	(95% CI)	N	Person-yrs $\times 10^6$	Cases per mill/yr	(95% CI)	N	Person-yrs $\times 10^6$	Cases per mill/yr	(95% CI)
1989–93	7	0.98	1.4	(0.6–2.8)	5	0.64	1.5	(0.6–3.4)	2	0.34	1.2	(0.2–3.9)
1994–98	7	1.02	1.4	(0.6–2.7)	4	0.70	1.1	(0.4–2.8)	3	0.32	1.9	(0.5–5.2)
1999–03	10	1.06	1.9	(1.0–3.4)	3	0.72	0.8	(0.2–2.2)	7	0.34	4.2	(1.9–8.4)
2004–08	9	1.1	1.6	(0.8–3.0)	1	0.72	0.3	(0.01–1.3)	8	0.38	4.3	(2.0–8.2)
2009–13	9	1.12	1.6	(0.8–3.0)	2	0.74	0.5	(0.09–1.8)	7	0.38	3.7	(1.6–7.2)

Incidence per million person years with 95% confidence interval (CI).

Table 3 – Average annual incidence of juvenile myasthenia gravis in 1989–2013 in the four different health regions in Norway.

Health region	Incident cases 1989–2013	Population at risk $\times 10^6$	Incidence per mill person years	(95% CI)
South-East	21	0.56	1.5	(1.0–2.3)
West	16	0.23	2.7	(1.3–4.3)
Central	1	0.15	0.3	(0.0–1.3)
North	4	0.11	1.5	(0.5–3.5)

Fig. 3 – Distribution of age at onset of juvenile myasthenia gravis (JMG) in males and females in Norway in 1989–2013.

4. Discussion

This is the first comprehensive epidemiological study of JMG in Norway. The study confirms the rarity of JMG, with an incidence rate of 1.6 per million per year in the study period 1989–2013. The results are comparable to earlier reports from western countries. In the UK, [JMG incidence rate was 1.5 per million in 2003–2007], and in a Danish population estimated JMG incidence rate was 0.3 in the 0–9 years age group and 2.2 in the age range 10–19 years.²⁰ In Canada the incidence rate of JMG was 2.0 and 0.9 in 2010 and 2011, respectively.¹³ Higher incidence rate was found in South Africa with 4.3 per million¹¹ and also in Taiwan with JMG incidence of 3.7–8.9 per million, highest in the age group 0–4 years with 8.9 per million.²¹ The studies differ in methodology, exclusion criteria and age limits, and in addition, potential geographical and ethnic differences in risk complicate direct comparisons. Incidence rates both from the UK and Denmark are in accordance with the incidence rate in Norway. However, JMG is reported more frequently in Taiwan and in South Africa, reflecting geographical variations in risk.³ We found that the incidence rate was lowest in the prepubertal group, which agrees with data from other western countries.^{11,20}

Data regarding adult onset MG show an increased incidence of MG in the mid-1980s, especially late onset MG.⁵ This increase is explained by some by the introduction of AChR ab assays and also improved epidemiological methodology.⁵ In our 25-years data we found no statistically significant

evidence for an increase in JMG incidence since 1989, a finding supported by an Austrian study.⁸

Autoimmune disease mechanisms are complex, influenced among others by genetic predispositions, environment and hormonal factors. Autoimmune diseases have been shown to be more frequent among females,^{22,23} and the influence of sex hormones on development of autoimmune disease has been discussed.²⁴ A higher incidence among females also applies to MG,^{20,25} especially early adult onset,⁹ and to JMG with postpubertal onset.^{10,11,26} In JMG with prepubertal onset, an equal gender distribution is shown in several studies,^{8,11,14,15} supporting the significance of sex hormones and puberty in disease pathogenesis. However, there are divergent results in the prepubertal group. Some studies show female preponderance^{10,13,16} but male predominance has also been described.⁹ Due to differences in methodology, exclusion criteria and age limits, direct comparisons of the studies are difficult. In addition, age at pubertal onset varies geographically and is shown to be higher in the north European countries compared to Italy and to both African-Americans and Caucasians in the USA.²⁷ In the Norwegian JMG group the majority of those studied was female. The risk of being diagnosed with JMG was significantly higher among females both in the postpubertal and prepubertal group. Our study population is small, but also suggests an increased risk among females in the prepubertal group, which could argue against the influence of sex hormones and pubertal shift on JMG onset, and instead point towards the influence of other gender-specific properties such as X-linked genes.²⁴

Geographical differences in frequencies, such as a north-south gradient, have been described in several autoimmune disorders.²⁸ However, we found no significant difference in JMG incidence among different geographical regions within Norway. We do have a small study population, but the result is in accordance with earlier studies on adult MG in Norway where no latitude gradient has been found.^{17,18}

We found that all JMG patients in Norway had been evaluated at least once at a University Hospital.

On one hand, centralizing the clinical management of rare diseases may optimize patients' outcome, but the JMG patients are spread out over a far-flung country. Access to and availability of specialists is challenging in many remote areas. The importance of primary physicians' knowledge to provide referrals for proper diagnosis and treatment should not be underestimated. In our population the median time until diagnosis was 6 months, but in some cases up to 120 months. Rapid diagnosis and treatment is important in children, since JMG may have a negative impact on their motor function or lead to permanent visual disturbances such as amblyopia due to ptosis and ophthalmoplegia.

A strength of this study is that, in addition to the long 25-year study period, multiple strategies to identify JMG cases were used. This gave a more complete study population since the three different sources each yielded unique cases (Fig. 2). We chose to do the diagnostic search only at hospitals and not include general practitioners or private practitioners, since neurology and pediatric services are well covered in all regions in Norway, and JMG is a rare disease, which in our experience is always evaluated by an in-hospital specialist. On

the other hand, undiagnosed cases with mild symptoms could have been missed due to diagnostic failure. We might also have missed some JMG cases in the ICD searches due to incomplete diagnostic registrations in the earliest years. Other potentially missed JMG cases might have been diagnosed before 1989, with complete remission and no medical follow-up after 1989.

This study confirms the rarity of JMG in Norway, especially among males. We find that the incidence has been stable over the last 25 years.

Conflict of interest

The authors report no conflict of interest.

Acknowledgements

We are grateful to all the participating neurological and pediatric departments in Norway for their contributions.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejpn.2016.09.001>.

REFERENCES

- Berrih-Aknin S, Frenkian-Cuvelier M, Eymard B. Diagnostic and clinical classification of autoimmune myasthenia gravis. *J Autoimmun* 2014;**48**:49–143–8.
- Chiang LM, Darras BT, Kang PB. Juvenile myasthenia gravis. *Muscle Nerve* 2009;**39**:423–31.
- Evoli A. Acquired myasthenia gravis in childhood. *Curr Opin Neurol* 2010;**23**:536–40.
- Della Marina A, Trippe H, Lutz S, Schara U. Juvenile myasthenia gravis: recommendations for diagnostic approaches and treatment. *Neuropediatrics* 2014;**45**:75–83.
- Carr AS, Cardwell CR, McCarron PO, McConville J. A systematic review of population based epidemiological studies in Myasthenia Gravis. *BMC Neurol* 2010;**10**:46.
- Lavrnjic D, Basta I, Rakocevic-Stojanovic V, et al. Epidemiological study of adult-onset myasthenia gravis in the area of Belgrade (Serbia) in the period 1979–2008. *Neuroepidemiology* 2013;**40**:190–4.
- McGrogan A, Sneddon S, de Vries CS. The incidence of myasthenia gravis: a systematic literature review. *Neuroepidemiology* 2010;**34**:171–83.
- Cetin H, Fulop G, Zach H, Auff E, Zimprich F. Epidemiology of myasthenia gravis in Austria: rising prevalence in an ageing society. *Wien Klin Wochenschr* 2012;**124**:763–8.
- Gattellari M, Goumas C, Worthington JM. A national epidemiological study of myasthenia gravis in Australia. *Eur J Neurol* 2012;**19**:1413–20.
- Parr JR, Andrew MJ, Finnis M, Beeson D, Vincent A, Jayawant S. How common is childhood myasthenia? The UK incidence and prevalence of autoimmune and congenital myasthenia. *Arch Dis Child* 2014;**99**:539–42.
- Mombaur B, Lesosky MR, Liebenberg L, Vreede H, Heckmann JM. Incidence of acetylcholine receptor-antibody-positive myasthenia gravis in South Africa. *Muscle Nerve* 2015;**51**:533–7.
- Zhang X, Yang M, Xu J, et al. Clinical and serological study of myasthenia gravis in HuBei Province, China. *J Neurol Neurosurg Psychiatry* 2007;**78**:386–90.
- VanderPluym J, Vajsar J, Jacob FD, Mah JK, Grenier D, Kolski H. Clinical characteristics of pediatric myasthenia: a surveillance study. *Pediatrics* 2013;**132**:e939–44.
- Haliloglu G, Anlar B, Aysun S, et al. Gender prevalence in childhood multiple sclerosis and myasthenia gravis. *J Child Neurol* 2002;**17**:390–2.
- Evoli A, Batocchi AP, Bartocconi E, Lino MM, Minisci C, Tonali P. Juvenile myasthenia gravis with prepubertal onset. *Neuromuscul Disord* 1998;**8**:561–7.
- Castro D, Derisavifard S, Anderson M, Greene M, Iannaccone S. Juvenile myasthenia gravis: a twenty-year experience. *J Clin Neuromuscul Dis* 2013;**14**:95–102.
- Heldal AT, Eide GE, Gilhus NE, Romi F. Geographical distribution of a seropositive myasthenia gravis population. *Muscle Nerve* 2012;**45**:815–9.
- Boldingh MI, Maniaol AH, Brunborg C, et al. Geographical distribution of myasthenia gravis in Northern Europe—results from a population-based study from two countries. *Neuroepidemiology* 2015;**44**:221–31.
- Maniaol AH, Boldingh M, Brunborg C, Harbo HF, Tallaksen CM. Smoking and socio-economic status may affect myasthenia gravis. *Eur J Neurol* 2013;**20**:453–60.
- Pedersen EG, Hallas J, Hansen K, Jensen PE, Gaist D. Late-onset myasthenia not on the increase: a nationwide register study in Denmark, 1996–2009. *Eur J Neurol* 2013;**20**:309–14.
- Lai CH, Tseng HF. Nationwide population-based epidemiological study of myasthenia gravis in taiwan. *Neuroepidemiology* 2010;**35**:66–71.
- Anaya JM. Common mechanisms of autoimmune diseases (the autoimmune tautology). *Autoimmun Rev* 2012;**11**:781–4.
- Jacobson DL, Gange SJ, Rose NR, Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin Immunol Immunopathol* 1997;**84**:223–43.
- Rubtsov AV, Rubtsova K, Kappler JW, Marrack P. Genetic and hormonal factors in female-biased autoimmunity. *Autoimmun Rev* 2010;**9**:494–8.
- Cavalcante P, Cufi P, Mantegazza R, Berrih-Aknin S, Bernasconi P, Le Panse R. Etiology of myasthenia gravis: innate immunity signature in pathological thymus. *Autoimmun Rev* 2013;**12**:863–74.
- Rodriguez M, Gomez MR, Howard Jr FM, Taylor WF. Myasthenia gravis in children: long-term follow-up. *Ann Neurol* 1983;**13**:504–10.
- Juul A, Teilmann G, Scheike T, et al. Pubertal development in Danish children: comparison of recent European and US data. *Int J Androl* 2006;**29**:247–55. discussion 286–290.
- Ellis JA, Kemp AS, Ponsonby AL. Gene-environment interaction in autoimmune disease. *Expert Rev Mol Med* 2014;**16**:e4.

ELSEVIER

Official Journal of the European Paediatric Neurology Society

Original article

Juvenile myasthenia gravis in Norway: Clinical characteristics, treatment, and long-term outcome in a nationwide population-based cohort

T.H. Popperud ^{a,b,c,*}, M.I. Boldingh ^a, M. Rasmussen ^{c,d}, E. Kerty ^{a,b}^a Department of Neurology, Oslo University Hospital, Oslo, Norway^b Institute of Clinical Medicine, University of Oslo, Oslo, Norway^c Unit for Hereditary and Inborn Neuromuscular Disorders, Department of Neurology, Oslo University Hospital, Oslo, Norway^d Department of Clinical Neuroscience for Children, Section for Child Neurology, Oslo University Hospital, Oslo, Norway

ARTICLE INFO

Article history:

Received 30 December 2016

Received in revised form

29 March 2017

Accepted 10 April 2017

Keywords:

Juvenile myasthenia gravis

Treatment

Thymectomy

Outcome

Comorbidity

ABSTRACT

Background: This study aimed to characterize juvenile myasthenia gravis in a national population-based cohort in Norway, and to evaluate long-term outcome and potential differences correlated with prepubertal versus postpubertal disease onset.

Patients and methods: Patients with onset of myasthenia gravis aged ≤ 18 years were identified through multiple strategies. Retrospective clinical data were collected by means of medical charts. All patients had an updated clinical examination. Cases were divided into prepubertal and postpubertal onset using age 12 years as the cut off.

Results: In total, 75 patients were identified of whom 63 were included in the study: 21 in the prepubertal and 42 in the postpubertal onset group. There was a female preponderance in both groups. In total, 59% presented with ocular symptoms, but the great majority of patients in both groups generalized during the two first years of the disease. Myasthenic crisis was more frequent in the prepubertal onset group. All patients were initially treated with pyridostigmine, 26 with steroids, and 17 with other immunosuppressive treatment. The postpubertal cases were more often treated with immunosuppressive therapy. Fifty patients (79%) underwent thymectomy. The general outcome was favourable: 57% became asymptomatic and only four subjects failed to attain clinical improvement. One-third had at least one additional autoimmune disease.

Conclusion: Despite frequent symptom generalization and a subgroup of prepubertal onset with severe disease, the long-term outcome was good, especially in the thymectomized prepubertal onset group. Polyautoimmunity occurred in both groups in one-third.

© 2017 European Paediatric Neurology Society. Published by Elsevier Ltd. All rights reserved.

* Corresponding author. Department of Neurology, Oslo University Hospital, Rikshospitalet, pb 4950 Nydalen, 0424 Oslo, Norway. Fax: +47 23074961.

E-mail address: t.h.popperud@medisin.uio.no (T.H. Popperud).

<http://dx.doi.org/10.1016/j.ejpn.2017.04.003>

1090-3798/© 2017 European Paediatric Neurology Society. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Myasthenia gravis (MG) is a neuromuscular disorder characterized by fatigability and fluctuating muscle weakness. MG can be localized purely to the ocular muscles, which is termed ocular MG (OMG), or affect other voluntary muscles, which is termed generalized MG (GMG). The pathogenesis is autoimmune, and mediated by autoantibodies against components of the neuromuscular endplate that lead to impaired synaptic transmission. In the majority of cases, antibodies (ab) are directed against acetylcholine receptors (AChR), but other targets including muscle specific kinase (MuSK) and the receptor related low density lipoprotein-4 (LRP4) have been described.¹ In western countries, 10–15% of cases have onset in childhood, which is termed juvenile myasthenia gravis (JMG).² Differences in populations with early onset and late onset JMG have been reported for some demographic and disease aspects (e.g. sex prevalence and severity of disease)³; racial variations have also been described.^{4–7}

Even though spontaneous remission is reported to be high in JMG, most studies suggest a higher remission rate after thymectomy.^{8–11} Thymectomy has recently been shown to be beneficial in a randomized controlled trial among 126 adult MG patients, but the study did not disclose safety and efficacy findings in patients below the age of 18 since these were excluded.¹²

There are several recent studies, some quite large, that describe clinical characteristics and outcome of JMG in Asian populations^{6,13,14}; and other studies from the USA and South Africa, which comprised mixed racial groups.^{7,15} However, there are few population-based reports on JMG and few studies conducted with European cohorts. Our study aimed to address the clinical aspects, benefits of thymectomy, and long-term course of prepubertal and postpubertal juvenile MG in a nationwide population-based cohort in Norway.

2. Material and methods

2.1. Case identification and confirmation

Patient identification was conducted from January 2012 to April 2016 using multiple strategies. Systematic searches for the code for MG were made on ICD (International Statistical Classification of Diseases) across the 15 main hospitals in Norway for patients with onset at age ≤ 18 years from 1989 through 2013. In addition, the AChR ab database at Haukeland University Hospital and the national adult MG database at Oslo University Hospital were checked.

MG diagnosis was verified through patients' medical charts. Diagnosis was based on typical MG symptoms with fatigable muscle weakness in ocular and/or other skeletal muscles, positive response to cholinesterase inhibitor, and the presence of AChR or MuSK antibodies. If no antibodies to AChR or MuSK were identified, typical neurophysiological findings with pathologic decrement and/or jitter and positive response to immunotherapy supportive of MG diagnosis were needed for inclusion. However, if onset was before age 1 year and/or there were additional findings like scoliosis,

contractures, and apnoea, cases were excluded due to suspicion of congenital myasthenic syndrome.^{16,17} Full details about the study population and inclusion criteria are detailed in a previous publication.¹⁸ Cases of transient neonatal MG and congenital myasthenic syndromes were not included. The patients were divided into prepubertal onset group when MG onset was <12 years and postpubertal onset group when MG onset was 12–18 years.¹⁶

2.2. Data collection

Retrospective clinical data were collected by means of medical charts. In addition, 51 patients went through an updated clinical evaluation at our clinic during 2015. MG symptoms at diagnosis and last follow up were categorized according to the Myasthenia Gravis Foundation of America (MGFA) clinical classification Scale¹⁹: Class I, pure ocular weakness; Class II, mild generalized weakness; Class III, moderate generalized weakness; Class IV, severe generalized weakness; and Class V, intubation. Class II, III and IV are designated “a” if predominantly limb weakness, and “b” if predominately oropharyngeal. Response to therapy was classified according to the MGFA Post intervention Status Scale (PIS): complete stable remission (CSR), pharmacological remission (PR), minimal manifestation 0–3 (MM0–3), improved (I), unchanged (U), worse (W), or died of MG (D).¹⁹ The 12 patients, who for different reasons could not come to our clinic for an updated clinical evaluation, were scored based on information from their last evaluation by their local neurologist at a primary hospital.

2.3. Statistical analysis

Descriptive analysis, including median and range, were performed using EpiInfo version 3.5.3 (Centers for Disease Control and Prevention, Atlanta, GA). To evaluate differences between groups, chi-square and mid-P exact tests with 2-tailed p value on openepi.com were used. A P-value < 0.05 was considered statistically significant.

2.4. Ethics

The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics, South East Office. All patients, or their parents when under age, gave written informed consent. Data was collected and registered in accordance with Norwegian guidelines.

3. Results

3.1. Demographic and clinical characteristics

In total, 75 patients with MG onset ≤ 18 years were identified. Sixty-three patients, who all fulfilled the diagnostic criteria, gave their written consent and were included in this study. Patients were followed up for a median of 14.8 years (IQR: 7.3–35.5). MG onset was prepubertal in 1/3 of the patients and postpubertal in 2/3. There was a female preponderance both in the prepubertal and postpubertal onset group of 71% and

90%, respectively. Clinical characteristics stratified by age at onset are summarized in Table 1. In 59% of patients, the reported presenting symptoms were pure ocular, most frequently ptosis. However, only 29% were pure ocular at diagnosis (Fig. 1), and disease in the majority of patients evolved into generalized MG. See Table 1. Altogether, 6 cases (9.5%) remained pure ocular. Six patients experienced myasthenic crisis with need for respiratory assistance during the disease course; five out of these cases occurred during the first year after MG onset.

3.2. Diagnostics

MGFA clinical classification scores at diagnosis are shown in Fig. 1. The median time from onset until diagnosis was 6 months (IQR 2–11). Diagnosis was based on typical symptoms and positive AChR antibodies in 47 (75%) patients, and positive MuSK antibodies in one. In the majority of patients, 77% of prepubertal and 86% of postpubertal onset cases, abnormal antibodies were found in relation to time of diagnosis, and in the rest at later retesting. Five of the AChR antibody negative

Fig. 1 – MGFA score at diagnosis and at last follow-up (median 14.8 years (IQR 7.3–35.5) from diagnosis), stratified by age of onset. Data are presented as proportion (percentage) of patients in each category.

Table 1 – Comparison of clinical characteristics and treatment in prepubertal and postpubertal onset juvenile MG.

	Prepubertal, N = 21	Postpubertal, N = 42
Gender, F:M	2.5:1	9.5:1
Onset age in years, median (Range)	7.0 (1–11)	16.0 (12–18)
Follow up in years, median (IQR)	27 (11–39)	13 (7–34)
AChR ab positive, N (%)	12 (57%)	35 (83%)
Clinical classification		
Ocular MG at onset, N (%)	13 (62%)	24 (57%)
• Generalization first 6 months	6 (29%)	8 (19%)
• Generalization within 2 years	8 (38%)	19 (45%)
• Generalization after 2 years	2 (9.5%)	2 (5%)
Generalized MG at onset, N (%)	8 (38%)	18 (43%)
Myasthenic crisis with respiratory assistance	4 (19%)	2 (5%)
Medical treatment during disease course		
Pyridostigmine po, N (%)	21 (100%)	42 (100%)
Prednisolone po, N (%)	6 (29%)	20 (48%)
Aza/Cic/MyM, N (%)	2 (10%)	15 (36%)
IVIg, N (%)	5 (24%)	15 (36%)
Plex, N (%)	9 (43%)	9 (21%)
Surgical treatment		
Chest imaging (UL, CT, MRI)	21	42
• hyperplasia/thymoma	1	12
Thymectomy, N (%)	13 (62%)	37 (88%)
IVIg/Plex prior to surgery	7 (54%)	18 (43%)
Thymus histology		
• Hyperplasia, N (%)	7 (54%)	23 (62%)
• Normal, N (%)	3 (23%)	9 (25%)
• Thymoma, N (%)	0	0
• Unknown, N (%)	3 (23%)	5 (14%)
Median time from onset to thymectomy in years (IQR)	2 (1.2–3.7)	1.3 (0.8–2.2)
Abbreviations: Aza = azathioprine; Cic = cyclosporine; MyM = mycophenolate mofetil; IVIg = intravenous immunoglobulin; Plex = plasma exchange, UL = ultra sound; CT = computed tomography; MRI = magnetic resonance imaging.		

patients were diagnosed before the AChR ab test was commercially available, and were negative when tested later after treatment was initiated. Eleven of the 15 AChR ab negative patients were negative for MuSK ab as well. Results were missing for four patients. Two of the AChR ab negative patients had pure ocular symptoms. See Fig. 2 for results from diagnostic investigations.

3.3. Possible triggers

Ten (16%) patients were born by Caesarean section, and there were no significant differences in birth method between the prepubertal and postpubertal onset groups. Among the prepubertal JMG cases, six (29%) were born prematurely (before completion of 37 weeks of pregnancy), while only four cases (9.5%) were premature in the postpubertal group. A special triggering event at disease onset, such as infection, vaccination, or surgery, was reported by 17 patients (27%). Familial MG occurred in 4.8% of patients, one pair of siblings and one mother-daughter. All were AChR ab positive.

3.4. Treatment

All patients were initially treated with pyridostigmine. Additional treatments during the disease course are shown in Table 1. There were no significant differences in the use of oral prednisolone between the prepubertal and postpubertal onset groups ($p = 0.16$). Use of other immunosuppressive agents were significantly higher in the postpubertal onset group ($p = 0.03$). Spontaneous remission occurred in five patients, three (14%) with prepubertal onset and two (4.8%) with postpubertal onset. All five had generalized MG, and three were AChR ab positive.

Fig. 2 – All 63 JMG patients included had typical MG symptoms. The diagnostic workup included: serology, testing for antibodies against AChR; electrophysiology, repetitive stimulation, and in some cases additional SF-EMG; pharmacological testing with intravenous edrophonium. The figure shows the number of patients with positive results for each diagnostic test and how the different diagnostic test results overlap.

3.5. Thymectomy and safety

Fifty patients (79%) underwent thymectomy, with a higher rate in the postpubertal onset group, $p = 0.02$ (Table 1). Four thymectomized patients had pure ocular symptoms and 43 (86%) were AChR ab positive. The median time from onset until thymectomy was 18 months (IQR 9–31). The surgical method was transsternal in 42 and thoracoscopic in eight. Thymus hyperplasia was pathologically verified in 30 patients, none had thymoma. Complications related to thymectomy occurred in five (7.9%) cases, all of whom underwent the transsternal approach. Major complications occurred in two cases: bleeding due to aorta injury during surgery in one case and mediastinitis/abscess in the other. Both incidents necessitated special surgical treatment. In three cases, post-operative infection occurred, which was treated with antibiotics in each. There was no mortality or long-term sequelae that we were aware of.

3.6. Outcome at last visit

Outcome data was available for all 63 patients. MGFA scores at last follow up are shown in Fig. 1 and treatment status in Fig. 3. Altogether, 59 patients (94%) achieved clinical improvement. At last follow-up, 36 patients (57%) were asymptomatic. Thirty-two (51%) patients attained complete stable remission (CSR) or pharmacological remission (PR).

Only three patients were clinically unchanged with the patients in worse condition as compared to symptoms at diagnosis. Due to the small number of patients in both groups, it is difficult to do statistical analysis. However, when comparing the prepubertal vs. postpubertal onset groups at last follow up, the MGFA clinical score, the need of medical treatment, and the PIS score were lower in the prepubertal group (Figs. 1 and 3). When stratifying the patients by age at onset and thymectomy status, the outcome seemed especially good in the prepubertal onset thymectomized group in which 69.2% of patients achieved CSR (Fig. 3). Somewhat unexpectedly, we found that the rate of CSR was higher among those thymectomized 12 months or later after JMG onset, compared to those with early thymectomy, 57% vs 25% respectively ($p = 0.03$). Neither sex, age at onset, AChR ab status, thymus histology, MGFA at diagnosis, nor additional autoimmune disorder were associated with the likelihood of achieving CSR.

3.7. Comorbidity

Associated disease was most frequently a second autoimmune disorder, affecting twenty patients (32%). All but one evolved after MG diagnosis. The most frequent comorbidities were thyroid disorders and psoriasis. There was no difference in occurrence of autoimmune disorders in the postpubertal compared to the prepubertal onset group ($p = 0.85$). Likewise, autoimmune disorders did not occur more frequently in the

Fig. 3 – Clinical outcome at last follow up in prepubertal and postpubertal onset groups stratified by thymectomy status. Data are presented as proportion of patients (percentage) in each MGFA Post intervention Status (PIS) category: complete stable remission (CSR), pharmacological remission (PR), minimal manifestation 0-3 (MM0-3), improved (I), unchanged (U).

thymectomized group ($p = 0.57$). However, we found that a second autoimmune disorder was more frequent in JMG patients not treated with a glucocorticoid sparing immunosuppressive agent ($p = 0.04$). For information on other associated diseases, see [Table 2](#).

4. Discussion

This is the first study describing features of a nationwide JMG cohort in Norway with a long follow up of median 14.8 years. JMG is an uncommon disease in Norway as in other western countries, with an incidence of 1.6 per million person years, and even more rare in prepuberty.¹⁸ We found a female preponderance in both the prepubertal and postpubertal onset group, in accordance with other studies.^{15,20,21} The majority of our JMG cases started with pure ocular symptoms, but most of them evolved generalized MG within 2 years from onset, similar to adult MG.²² There were no significant differences between the prepubertal and postpubertal onset groups concerning primary symptoms ([Table 1](#)), symptoms at diagnosis ([Fig. 1](#)), and generalized disease. However, in the prepubertal onset group the symptoms seemed to generalize somewhat earlier than in the postpubertal group and myasthenic crisis was more frequent ([Table 1](#)) as also reported from an Italian population.³ It could be more difficult to examine the youngest children and pick up signs of an impending crisis. We might also be more reluctant to initiate treatment with immunosuppressive agents in the youngest. The higher rate of myasthenic crisis among the prepubertal onset cases may indicate a subgroup with more severe disease in need of more aggressively immunosuppressive treatment.

In our cohort, only 9.5% of JMG cases remained as pure OMG over the disease course, which is in contrast to other studies, which describe a greater incidence of OMG in the juvenile group, especially in the youngest patients.^{3,5,16,23} It has been shown that Chinese, Japanese and South Korean JMG populations have a high proportion of pure OMG.^{6,13,14,24} More

notably, our group had fewer pure ocular JMG cases than cohorts from USA, South Africa, and Italy.^{7,15} Lindner et al. reported more comparable numbers of 10% pure ocular cases in a German cohort.²⁵ However, this study only included patients with JMG onset from age 12 to 18, excluding the prepubertal onset cases where OMG typically have been reported more frequent and not directly comparable with our study population. Our results may indicate genuine differences in JMG subtypes within Europe, or might be explained by a bias due to missed cases or missed diagnosis of cases with slight ocular symptoms only.

Table 2 – Comparison of comorbidities in prepubertal and postpubertal onset juvenile MG.

Comorbidity	Prepubertal, N = 21	Postpubertal, N = 42
Autoimmune disorder, N (%)	7 (33%)	13 (31%)
Thyroid	4 (19%)	3 (7.1%)
Psoriasis	1 (4.8%)	3 (7.1%)
Mb Crohns/UC	1 (4.8%)	2 (4.8%)
AS		2 (4.8%)
RA	1 (4.8%)	1 (2.4%)
SLE		1 (2.4%)
Sarcoidosis		1 (2.4%)
Scleroderma		1 (2.4%)
Celiac disease		1 (2.4%)
ITP		1 (2.4%)
DM1	1 (4.8%)	
Epileptic seizure	1 (4.8%)	4 (9.5%)
Neoplasia (skin)		1 (2.4%)
Heart: WPW		1 (2.4%)
HUS		1 (2.4%)
Tonsillectomy	2 (9.5%)	5 (11.9%)
Adenotomy	2 (9.5%)	2 (4.8%)

Abbreviations: UC = ulcerative colitis; AS = ankylosing spondylitis; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; ITP = idiopathic thrombocytopenic purpura; DM1 = diabetes mellitus type 1; WPW = Wolff Parkinson white; HUS = haemolytic uremic syndrome.

The share of AChR ab positive cases are often lower in JMG compared to adult MG.⁵ This was the case for the Norwegian JMG cohort, especially in the prepubertal onset group where only 62% were positive for AChR ab or MuSK ab. Pure ocular MG has a higher incidence of AChR ab negativity than generalized MG cases.²⁶ However, in our cohort OMG was quite rare, and not explainable by a high number of seronegative individuals. Congenital myasthenic syndromes (CMS) are important differential diagnoses when no AChR ab or MuSK ab is found, especially given that these conditions do not benefit from immunomodulating treatment but are susceptible to potential side effects. We took measures to exclude potential CMS cases as described in the methodology section, but such misdiagnoses cannot be completely ruled out in autoantibody negative cases.

Even though a high share of patients exhibited symptom generalization, the JMG cohort showed a good overall outcome. The relatively benign course of JMG, especially with prepubertal onset, has been previously described.⁵ The majority of JMG cases (94%) in this study, improved after diagnosis and 57% were asymptomatic at last follow-up. A total of 51% achieved CSR, which is quite high compared with other studies showing remission in 12–45% of patients.^{6,7,15,27} These studies however have a shorter follow-up which might explain some of the difference.

Corticosteroids and azathioprine are the most used immunosuppressive drugs for JMG worldwide,¹⁶ as in our cohort. The postpubertal onset group was more often treated with immunosuppressive medication (Table 1). This could be due to a more reluctant use among the youngest children. However, among the cases treated with immunosuppressive agents, fewer achieved CSR, which could indicate a more severe disease and a possible biological difference between the postpubertal and prepubertal MG subtypes.

In our JMG cohort, 79% were thymectomized, which is considerably higher than similar studies where the proportion of thymectomized cases ranged from 32 to 57%.^{7,15,28} There was not a significantly higher remission rate in the thymectomized group compared to the non-thymectomized group. However, when stratified by age of onset, CSR was especially frequent in the prepubertal thymectomized group (Fig. 3). This could support the indication for thymectomy in prepubertal onset MG. It is important to emphasize that these are retrospective observational data, and a proper randomized controlled trial in juvenile MG is necessary to disclose safety and efficacy of thymectomy.

Some studies indicate that early thymectomy within the first year after onset of the disease is more beneficial.^{10,29,30} We found there was not a benefit of thymectomy shortly after symptom onset, rather the opposite was observed with a higher proportion of CSR in the later thymectomy group (>12 months from onset). This may support an indication for thymectomy also later in the disease course. Despite findings on chest imaging indicating enlarged thymus and possible thymoma in 13 patients, there were no cases of thymoma, further confirming its rarity in JMG. In accordance with other studies, thymectomy in JMG seems safe with no mortality or long-term sequel in our population.

The prepubertal group had a higher proportion of prematurity than the postpubertal group. Moreover, using Medical

Birth Registry of Norway data indicating a 6.3% rate of prematurity as a reference for the general population (Medical Birth Registry of Norway, statistikkbank.fhi.no/mfr/), the incidence of prematurity was significantly higher in the prepubertal JMG group ($p = 0.002$) but not in the postpubertal group ($p = 0.4$). Evans et al. described an association between prematurity and early-onset MG, and discuss the possibility that an immature immune system, abundance of the foetal Ach receptor, and medical complications from prematurity may together increase the risk of MG development.³¹

One-third of the patients had a second autoimmune disorder, which is higher than in the general population, and higher than has been found in adult MG^{32,33} except in a South African triple seronegative adult MG cohort where interestingly 39% had concomitant autoimmune disorder.³⁴ A Swedish population based study found that 22% of adult MG patients had at least one other autoimmune disease, with an odds ratio of 2.8 compared to control subjects.³⁵ In a Chinese JMG cohort, autoimmune comorbidity was present in 19% of cases.⁶ The high frequency of polyautoimmunity applied both to the prepubertal and postpubertal onset cases in our cohort. Most frequent were thyroid disorders (Table 2), which is in accordance with other studies both on JMG and adult MG.^{6,24,33} When looking for potential factors influencing the risk of developing a second autoimmune disorder, immunosuppressive treatment was the only factor having a significant protective effect. This was also observed in an Italian adult MG cohort.³³

Epileptic seizures occurred in five patients (7.9%), see Table 2. There is an ongoing discussion whether MG affects the central nervous system, and one of the issues is the rate of epileptic seizures. Frequency of epileptic seizures in JMG vary, and are reported up to 12.5%.³⁶ A 5-fold increased risk of epilepsy in patients with MG was shown in an American population-based study.³⁷ However, the relationship between MG and epileptic seizures is not yet clear.

Interestingly, our JMG cohort had a high occurrence of tonsillectomy and/or adenotomy (Table 2). If and to what extent tonsillectomy affects the immune system, is still a matter of debate.^{38,39} All but one of the tonsillectomies/adenotomies was done before JMG onset, raising the question about causation. Recent studies show evidence for extra-thymic T cell development in the tonsils.⁴⁰ A large Swedish study found that tonsillectomy was associated with an increased risk of autoimmune disease, although there was not statistical significance for MG specifically.⁴¹

This study is retrospective and is thus subject to associated limitations in data collection and recall bias. Our dataset is not complete due to 12 patients not consenting, and we may have missed cases in the identification process. These would probably not include severe and/or complicated cases, but rather those with more modest symptoms and spontaneous remission, which did not necessitate contact with the health care system.

5. Conclusion

We found that symptoms frequently generalized in our JMG cohort; however the long-term outcome was good. Outcome seemed more favourable among the prepubertal onset cases,

especially among those thymectomized. Efficacy and safety of thymectomy in JMG must still be further addressed in prospective studies. The postpubertal cases were more often given immunosuppressive treatment, indicating a potentially more severe disease. However, myasthenic crisis were more frequent among the prepubertal cases, and it is important to be aware of this subgroup with potentially severe disease. Management should keep in mind the high occurrence of polyautoimmunity among JMG patients.

Disclosure statement

The authors report no conflict of interest.

Disclaimer

This study has used data from the Medical Birth Registry of Norway. The interpretation and reporting of these data is the sole responsibility of the authors, and no endorsement by the Medical Birth Registry of Norway is intended nor should be inferred.

REFERENCES

- Oger J, Frykman H. An update on laboratory diagnosis in myasthenia gravis. *Clin Chim Acta* 2015;**444**:126–31.
- Phillips 2nd LH, Torner JC, Anderson MS, Cox GM. The epidemiology of myasthenia gravis in central and western Virginia. *Neurology* 1992;**42**:1888–93.
- Evoli A, Batocchi AP, Bartoccioni E, Lino MM, Minisci C, Tonali P. Juvenile myasthenia gravis with prepubertal onset. *Neuromuscul Disord* 1998;**8**:561–7.
- Zhang X, Yang M, Xu J, et al. Clinical and serological study of myasthenia gravis in HuBei Province, China. *J Neurol Neurosurg Psychiatry* 2007;**78**:386–90.
- Chiang LM, Darras BT, Kang PB. Juvenile myasthenia gravis. *Muscle Nerve* 2009;**39**:423–31.
- Gui M, Luo X, Lin J, et al. Long-term outcome of 424 childhood-onset myasthenia gravis patients. *J Neurol* 2015;**262**:823–30.
- Heckmann JM, Hansen P, Van Toorn R, Lubbe E, Janse van Rensburg E, Wilmshurst JM. The characteristics of juvenile myasthenia gravis among South Africans. *S Afr Med J* 2012;**102**:532–6.
- Tracy MM, McRae W, Millichap JG. Graded response to thymectomy in children with myasthenia gravis. *J Child Neurol* 2009;**24**:454–9.
- Hennessey IA, Long AM, Hughes I, Humphrey G. Thymectomy for inducing remission in juvenile myasthenia gravis. *Pediatr Surg Int* 2011;**27**:591–4.
- Andrews PI, Massey JM, Howard Jr JF, Sanders DB. Race, sex, and puberty influence onset, severity, and outcome in juvenile myasthenia gravis. *Neurology* 1994;**44**:1208–14.
- Heng HS, Lim M, Absoud M, et al. Outcome of children with acetylcholine receptor (AChR) antibody positive juvenile myasthenia gravis following thymectomy. *Neuromuscul Disord* 2014;**24**:25–30.
- Wolfe GI, Kaminski HJ, Aban IB, et al. Randomized trial of thymectomy in myasthenia gravis. *N Engl J Med* 2016;**375**:511–22.
- Murai H, Yamashita N, Watanabe M, et al. Characteristics of myasthenia gravis according to onset-age: Japanese nationwide survey. *J Neurol Sci* 2011;**305**:97–102.
- Lee HN, Kang HC, Lee JS, et al. Juvenile myasthenia gravis in Korea: subgroup analysis according to sex and onset age. *J Child Neurol* 2016;**31**:1561–8.
- Castro D, Derisavifard S, Anderson M, Greene M, Iannaccone S. Juvenile myasthenia gravis: a twenty-year experience. *J Clin Neuromuscul Dis* 2013;**14**:95–102.
- Della Marina A, Trippe H, Lutz S, Schara U. Juvenile myasthenia gravis: recommendations for diagnostic approaches and treatment. *Neuropediatrics* 2014;**45**:75–83.
- Schara U, Della Marina A, Abicht A. Congenital myasthenic syndromes: current diagnostic and therapeutic approaches. *Neuropediatrics* 2012;**43**:184–93.
- Popperud TH, Boldingh MI, Brunborg C, et al. Juvenile myasthenia gravis in Norway: a nationwide epidemiological study. *Eur J Paediatr Neurol* 2017;**21**:312–7.
- Jaretzki 3rd A, Barohn RJ, Ernstoff RM, et al. Myasthenia gravis: recommendations for clinical research standards. Task force of the medical scientific advisory board of the myasthenia gravis foundation of America. *Ann Thorac Surg* 2000;**70**:327–34.
- VanderPluym J, Vajsaar J, Jacob FD, Mah JK, Grenier D, Kolski H. Clinical characteristics of pediatric myasthenia: a surveillance study. *Pediatrics* 2013;**132**:e939–44.
- Parr JR, Andrew MJ, Finnis M, Beeson D, Vincent A, Jayawant S. How common is childhood myasthenia? The UK incidence and prevalence of autoimmune and congenital myasthenia. *Arch Dis Child* 2014;**99**:539–42.
- Evoli A, Tonali P, Bartoccioni E, Lo Monaco M. Ocular myasthenia: diagnostic and therapeutic problems. *Acta Neurol Scand* 1988;**77**:31–5.
- Batocchi AP, Evoli A, Palmisani MT, Lo Monaco M, Bartoccioni M, Tonali P. Early-onset myasthenia gravis: clinical characteristics and response to therapy. *Eur J Pediatr* 1990;**150**:66–8.
- Wong V, Hawkins BR, Yu YL. Myasthenia gravis in Hong Kong Chinese. 2. Paediatric disease. *Acta Neurol Scand* 1992;**86**:68–72.
- Lindner A, Schalke B, Toyka KV. Outcome in juvenile-onset myasthenia gravis: a retrospective study with long-term follow-up of 79 patients. *J Neurol* 1997;**244**:515–20.
- Evoli A. Acquired myasthenia gravis in childhood. *Curr Opin Neurol* 2010;**23**:536–40.
- Melbourne Chambers R, Forrester S, Gray R, Tapper J, Trotman H. Myasthenia gravis in Jamaican children: a 12-year institutional review. *Paediatr Int Child Health* 2012;**32**:47–50.
- Rodriguez M, Gomez MR, Howard Jr FM, Taylor WF. Myasthenia gravis in children: long-term follow-up. *Ann Neurol* 1983;**13**:504–10.
- Seybold ME. Thymectomy in childhood myasthenia gravis. *Ann N Y Acad Sci* 1998;**841**:731–41.
- Andrews PI. Autoimmune myasthenia gravis in childhood. *Semin Neurol* 2004;**24**:101–10.
- Evans OB, Vig V, Parker CC. Prematurity and early-onset juvenile myasthenia gravis. *Pediatr Neurol* 1992;**8**:51–3.
- Mao ZF, Yang LX, Mo XA, et al. Frequency of autoimmune diseases in myasthenia gravis: a systematic review. *Int J Neurosci* 2011;**121**:121–9.
- Evoli A, Caliendo P, Iorio R, et al. Poly-autoimmunity in patients with myasthenia gravis: a single-center experience. *Autoimmunity* 2015;**48**:412–7.
- Huda S, Woodhall MR, Vincent A, Heckmann JM. Characteristics of acetylcholine-receptor-antibody-negative myasthenia gravis in a South African cohort. *Muscle Nerve* 2016;**54**:1023–9.

35. Fang F, Sveinsson O, Thormar G, et al. The autoimmune spectrum of myasthenia gravis: a Swedish population-based study. *J Intern Med* 2015;277:594–604.
36. Snead 3rd OC, Benton JW, Dwyer D, et al. Juvenile myasthenia gravis. *Neurology* 1980;30:732–9.
37. Ong MS, Kohane IS, Cai T, Gorman MP, Mandl KD. Population-level evidence for an autoimmune etiology of epilepsy. *JAMA Neurol* 2014;71:569–74.
38. Brandtzaeg P. Immunology of tonsils and adenoids: everything the ENT surgeon needs to know. *Int J Pediatr Otorhinolaryngol* 2003;67(Suppl 1):S69–76.
39. Pidelaserra Marti G, Isdahl Mohn KG, Cox RJ, Brokstad KA. The influence of tonsillectomy on total serum antibody levels. *Scand J Immunol* 2014;80:377–9.
40. McClory S, Hughes T, Freud AG, et al. Evidence for a stepwise program of extrathymic T cell development within the human tonsil. *J Clin Invest* 2012;122:1403–15.
41. Ji J, Sundquist J, Sundquist K. Tonsillectomy associated with an increased risk of autoimmune diseases: a national cohort study. *J Autoimmun* 2016;72:1–7.

RESEARCH ARTICLE

Juvenile myasthenia gravis in Norway: HLA-DRB1*04:04 is positively associated with prepubertal onset

T. H. Popperud^{1,2,3*}, M. K. Viken⁴, E. Kerty^{1,2}, B. A. Lie^{4,5}

1 Department of Neurology, Oslo University Hospital, Oslo, Norway, **2** Institute of Clinical Medicine, University of Oslo, Oslo, Norway, **3** Unit for hereditary and inborn neuromuscular disorders, Department of Neurology, Oslo University Hospital, Oslo, Norway, **4** Department of Immunology, University of Oslo and Oslo University Hospital, Oslo, Norway, **5** Department of Medical Genetics, University of Oslo and Oslo University Hospital, Oslo, Norway

* t.h.popperud@medisin.uio.no

Abstract

Background

Juvenile myasthenia gravis (MG) is a rare autoantibody mediated autoimmune disorder targeting the neuromuscular endplate. The clinical hallmark is muscle weakness and fatigability. Disease aetiology is complex, including both genetic and environmental factors. The involvement of genes in the human leukocyte antigen (HLA) is well established in adult MG. However, HLA associations in European juvenile MG have not been studied. This case-control study aimed to investigate and characterize genetic risk factors in prepubertal and postpubertal onset juvenile MG.

Methodology/Principal findings

A population based Norwegian cohort of 43 juvenile MG patients (17 with prepubertal onset, 26 with postpubertal onset) and 368 controls were included. Next generation sequencing of five HLA loci (HLA-A, -B, -C, -DRB1 and -DQB1) was performed, and a positive association was seen with HLA-B*08 (OR (95% CI) = 3.27 (2.00–5.36), $P_c = 0.00003$) and HLA-DRB1*04:04 (OR (95% CI) = 2.65 (1.57–4.24), $P_c = 0.03$). Stratified in postpubertal and prepubertal onset, HLA-DRB1*04:04 was only positively associated with the latter ($P = 0.01$). The HLA-B*08 allele (12.9% in the controls), previously described associated with early onset adult MG, was most frequently observed in postpubertal onset MG (40.4%, $P = 0.0002$) but also increased among prepubertal onset MG (23.5%, $P = 0.05$).

Conclusion

This study provides novel information about HLA susceptibility alleles in Norwegian juvenile MG where HLA-DRB1*04:04 was associated with prepubertal onset.

OPEN ACCESS

Citation: Popperud TH, Viken MK, Kerty E, Lie BA (2017) Juvenile myasthenia gravis in Norway: HLA-DRB1*04:04 is positively associated with prepubertal onset. PLoS ONE 12(10): e0186383. <https://doi.org/10.1371/journal.pone.0186383>

Editor: Christos Papadelis, Boston Children's Hospital / Harvard Medical School, UNITED STATES

Received: May 3, 2017

Accepted: September 30, 2017

Published: October 16, 2017

Copyright: © 2017 Popperud et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the University of Oslo. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Myasthenia gravis (MG) is a rare autoimmune disorder affecting the neuromuscular endplate. The disease can occur at any age, and when onset in childhood it is termed juvenile MG. The upper age-cut off for juvenile MG varies between studies, but is often set at age ≤ 18 years [1]. Due to its heterogeneous nature, MG is in addition to age at onset, sub classified according to clinical presentation (ocular MG vs generalised MG), thymus histopathology (thymoma, thymus hyperplasia) and autoantibody (ab) profile (acetylcholine receptor (AChR) ab, muscle-specific kinase (MuSK) ab, lipoprotein receptor-related protein 4 (Lrp4) ab) [2]. In the majority of cases autoantibodies are directed towards the AChRs [3, 4]. The immune response, which is B cell mediated, T cell dependent and also involving complement factors, leads to impaired neuromuscular transmission, and the MG patients experience intermittent and fatigable weakness of skeletal muscles [5].

The disease aetiology in MG is complex and multifactorial involving both genetic and environmental factors [6]. MG does not show a strong heritability, and the frequency of familial MG cases is reported to be low, from 3–7% [7–9]. However, co-occurrence of autoimmune diseases among family members of MG patients [10, 11], the fact that clinical manifestations of MG differ between racial groups [12] and a higher concordance rate of MG among monozygotic compared to dizygotic twins [13], suggests an influence by genetic factors in disease pathogenesis. The strongest genetic determinant is the involvement of genes in the human leukocyte antigen (HLA) complex located on the short arm of chromosome 6 [14, 15]. The associated HLA alleles vary with the different MG subgroups and with racial origin. In European populations, an association with HLA-B*08 in early onset MG (EOMG, onset < 40 years) have been shown [14, 16], while the strongest HLA risk allele in late onset MG (LOMG, onset > 60 years) is DRB1*15:01 allele [16, 17]. None of the European studies have focused on juvenile MG. However, in East-Asian populations where juvenile MG is more frequent, HLA genes have been studied in this subgroup. A positive association has been found with the HLA-B*46 -DRB1*09 haplotype and ocular juvenile MG in a Chinese population [18], and with the HLA-DRB1*1302 haplotype and latent generalized juvenile MG in a Japanese population [19].

Studies on clinical presentation suggest that juvenile MG, and especially when prepubertal onset, differ from adult MG in some aspects such as autoantibody status and disease severity [1, 20].

This study aimed to investigate and characterize genetic risk factors in a cohort of Norwegian juvenile MG patients through comprehensive genotyping of HLA class I and II loci, and in particular, whether there were specific HLA risk alleles in the prepubertal onset subgroup.

Methods

Patients

In this population-based study, juvenile MG cases were identified from Jan 2012 to Apr 2016, through multiple strategies: i) through neurological and/or paediatric departments at the 15 main hospitals in Norway, ii) through the national AChR ab database at Haukeland University Hospital and iii) through the national adult MG database at Oslo University Hospital. Inclusion criteria were acquired MG with typical clinical symptoms and onset ≤ 18 years of age, in addition to AChR antibody positivity and/or neurophysiologic findings consistent with MG (pathological decrement after repetitive nerve stimulation and/or increased jitter on single-fibre electromyogram) [21].

A total of 75 juvenile MG patients were identified, and 53 (71%) gave consent to participate and donate a blood sample. Inclusion criteria were verified through patients' medical records

and the following additional clinical information were registered: gender, age at onset, thymectomy status, thymus histology and co-occurring autoimmune disorders. A more detailed description of the clinical characteristics in the Norwegian JMG cohort is given in a previous publication [22].

To avoid possible population stratification, only ethnically Norwegian cases were included. Hence, eight non-ethnic Norwegians were excluded, as well as one MuSK positive patient and one out of a pair of siblings. No thymoma patients were found.

All together 43 unrelated ethnic Norwegian juvenile MG patients were included in the study. The patients were divided into prepubertal onset group when MG onset age < 12 years, and postpubertal onset group when MG onset age 12–18 years. In addition we registered age at menarche in all female patients, and all cases defined as prepubertal onset had MG onset before menarche.

Controls

Previously genotyped HLA-data for 368 healthy controls, randomly selected among Norwegian bone marrow donors recruited through the Norwegian Bone Marrow Donor Registry (<http://www.nordonor.org/>), were utilized [23, 24]. 30% were female and 70% were male, however, no difference in the HLA allelic distribution was seen between female and male controls.

HLA genotyping

Genomic DNA was isolated from blood samples with Gentra Autopure LS (Qiagen, Hilden, Germany). For HLA-genotyping, the NGSgo kit from GenDx (Utrecht, The Netherlands) was used to sequence the HLA-A, -B, -C, -DRB1 and -DQB1 genes with MiSeq Reagent Kit v2 (300-cycles) and 2 x 150 paired-end sequencing on an Illumina MiSeq (Illumina, San Diego, USA). The sequencing was performed at the Norwegian Sequencing Centre (NSC, www.sequencing.uio.no), University of Oslo, Norway. The sequencing results were analysed and HLA-genotypes obtained by using the NGSengine v2.1 analysis software (GenDx).

Statistics

Statistical analysis of genetic associations was performed using UNPHASED v.3.0.10 [25]. Rare alleles ($n < 2$ in both patients and controls) were excluded. The expectation maximization algorithm was used to estimate maximum likelihood haplotype frequencies. The haplotype method [26] and the Svejgaard method [27] were used to assess which alleles and loci showed the primary association and which appeared to be secondary due to linkage disequilibrium (LD). Values for D' and r^2 were calculated for allele combinations. Odds ratios (OR) and 95% confidence intervals (CI) were calculated with Woolf's formula comprising Haldane's correction. P_c values < 0.05 after correction for number of comparisons in the initial global locus tests ($n = 5$) were considered significant. For allelic associations, we present both uncorrected P values (P_{nc}) and P -values corrected (P_c) for the number of tested alleles at each locus ($n = 8$ for HLA-A, $n = 9$ for HLA-B, $n = 9$ for HLA-C, $n = 10$ for HLA-DRB1, and $n = 10$ for HLA-DQB1). We did not correct for the total number of alleles tested, as the alleles at HLA loci do not fully represent independent tests due to the strong LD.

Ethics

The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics, South East Office. All patients, or their parents when underage, gave written

informed consent. Data was collected and registered in accordance with Norwegian guidelines.

Results

The main clinical characteristics of the 43 unrelated ethnic Norwegian juvenile MG patients included in the study are listed in [Table 1](#). Age at onset ranged from 1 year to 18 years, 17 had prepubertal onset and 26 postpubertal onset.

All patients included in the study were successfully genotyped for the five HLA loci (HLA-A, B, C, DRB1 and DQB1). Genotype success rate among controls were above 95% for all HLA loci. At locus level, two of the HLA genes were significantly associated with juvenile MG; i.e. HLA-B ($P_c = 0.004$) and HLA-DRB1 ($P_c = 0.00003$). The frequencies of HLA alleles significantly increased or decreased among the juvenile MG patients compared to the controls are shown in [Table 2](#).

A positive association was observed between juvenile MG and alleles on the well-established ancestral haplotype 8.1 (AH8.1; A*01-B*08-C*07-DRB1*03:01-HLA-DQB1*02:01). The strongest positive association was seen with HLA-B*08 (OR (95% CI) = 3.27 (2.00–5.36), $P_c = 0.00003$), and only B*08 together with A*01 and DRB1*03:01 were significant on AH8.1 after correction for multiple testing. These three alleles occurred on a susceptibility haplotype A*01-B*08-DRB1*03:01 present in 19.8% of cases compared to 8.0% of controls ($P = 0.0004$). Conditional haplotype analysis showed that HLA-B*08 conferred the strongest association ($p < 0.02$) while neither HLA-DRB1*03 nor A*01 were associated in the absence of HLA-B*08 ($p > 0.3$).

In addition, a positive association between juvenile MG and HLA-DRB1*04:04 was observed (OR (95% CI) = 2.65 (1.57–4.24), $P_c = 0.03$). Several alleles were also found to be negatively associated; however, none of these remained significant after correction for multiple testing.

When stratifying the juvenile MG patients into prepubertal onset and postpubertal onset groups, we found that only prepubertal onset juvenile MG was associated with the HLA-DRB1*04:04 allele ($P = 0.01$), where it occurred in 26% compared to 6.4% among controls. HLA-DRB1*04:04 was observed in 7.7% among the postpubertal onset MG, not increased compared to the controls ($P = 0.3$) and significantly less frequent than in the prepubertal onset MG ($P = 0.01$). HLA-B*08 was associated with both groups, but most pronounced with the postpubertal onset juvenile MG cases ($P = 0.0002$) where it was present in 40.4% compared to 23.5% in the prepubertal onset cases, and 12.9% in the controls.

Next, we compared the clinical characteristics of all the juvenile MG patients carrying the two risk alleles, HLA-B*08 and HLA-DRB1*04:04. Among the HLA-B*08+ cases, AChR

Table 1. Clinical characteristic of the Norwegian juvenile myasthenia gravis cohort stratified by age at onset.

	PREPUBERTAL ONSET (n = 17), n (%)	POSTPUBERTAL ONSET (n = 26), n (%)
Female (n = 35)	13 (76%)	22 (85%)
GMG (n = 40)	16 (94%)	24 (92%)
AChR ab + (n = 31)	9 (53%)	22(85%)*
HP/TX (n = 20/32)	5/9 (56%)	15/23 (65%)
CAD (n = 12)	5 (29%)	7 (27%)

* Compared with prepubertal onset, $P < 0.05$.

CAD = co-occurring autoimmune disorder other than myasthenia gravis. GMG = Generalised myasthenia gravis. AChR ab = acetylcholine receptor antibodies. HP = Thymus hyperplasia. TX = thymectomy

<https://doi.org/10.1371/journal.pone.0186383.t001>

Table 2. HLA alleles showing association ($P_{nc} < 0.05$) with juvenile myasthenia gravis (JMG).

HLA ALLELE	JMG (n = 43) n (%)	CONTROLS (n = 368) n (%)	P_{nc}	OR (95% CI)	P_c
A*01	27 (31.4%)	119 (16.2%)	0.0005	2.38 (1.46–3.88)	0.005
A*02	12 (19.8%)	240 (32.7%)	0.02	0.52 (0.30–0.89)	ns
B*08	28 (32.6%)	94 (12.9%)	0.000003	3.27 (2.00–5.36)	0.00003
B*40	17 (19.8%)	75 (10.3%)	0.007	2.18 (1.23–3.85)	ns
C*07	42 (48.8%)	246 (34.8%)	0.01	1.78 (1.14–2.79)	ns
DRB1*03:01	26 (30.2%)	106 (14.5%)	0.0002	2.56 (1.57–4.24)	0.002
DRB1*04:01	4 (4.7%)	92 (12.6%)	0.04	0.38 (0.15–0.96)	ns
DRB1*04:04	13 (15.1%)	47 (6.4%)	0.003	2.65 (1.40–5.04)	0.03
DQB1*02:01	26 (30.2%)	137 (19.3%)	0.02	1.83 (1.12–2.98)	ns
DQB1*03:01	4 (4.7%)	117 (16.5%)	0.006	0.28 (0.11–0.69)	ns

Odds Ratio (OR) and 95% confidence interval (CI) are shown for uncorrected P values (P_{nc}). P-values corrected for the number of tested alleles at each locus (P_c)

<https://doi.org/10.1371/journal.pone.0186383.t002>

antibodies and thymus hyperplasia were more frequent and age of onset higher, compared to the HLA-B*08- cases (Table 3). When comparing the clinical characteristics in the prepubertal onset group sub classified according to risk allele association, the main difference was age at onset (Table 4). In the HLA-DRB1*04:04+/HLA-B*08- cases, the median age at onset was 5 years compared to 9 years among the HLA-DRB1*04:04-/HLA-B*08+ cases.

The HLA-B*46:01 allele reported in Asian juvenile MG populations was not detected in our juvenile MG cohort, and the HLA-DRB1*09:01 was present at a low frequency both in patients and controls, 1.2% and 0.8% respectively. The DRB1*13:02 allele reported to be associated with latent generalized juvenile MG in Japan, was insignificantly ($P_{nc} = 0.1$) increased among our juvenile MG population (10.5%) vs controls (5.3%).

Discussion

The association of specific HLA alleles with MG has been known for decades [9], but this is to our best knowledge the first study on HLA associations in European juvenile MG patients. We investigated HLA class I and II alleles in a nationwide Norwegian cohort of MG patients with juvenile disease onset, exploring potential risk alleles in this MG subgroup and identified a positive association with HLA-B*08 and HLA-DRB1*04:04. The HLA-B*08 association is well established in European EOMG[14], while the association with HLA-DRB1*04:04 is a new finding not earlier described in MG patients. However, in other autoimmune disorders like rheumatoid arthritis and autoimmune Addison's disease, especially in early onset cases, an association with HLA-DRB1*04:04 has been shown[28–30].

Interestingly, we found that the HLA associations were differed according to the age at MG onset. The HLA-B*08 was most frequent in the cases with postpubertal onset, while the HLA-DRB1*04:04 was only positively associated with prepubertal onset.

In the prepubertal onset group, the main difference between the HLA-DRB1*04:04 and HLA-B*08 cases, was the age at MG onset. DRB1*04:04 was associated with an earlier onset than B*08 (Table 4). Since an arbitrary cut of at age <12 years were used to define the groups, this support an association with true prepubertal onset. AChR ab positivity and thymus hyperplasia seemed more frequent among the HLA-B*08 cases; however, the sample size was too small to establish a true difference between the two groups of prepubertal onset.

Table 3. Clinical characteristics of the juvenile myasthenia gravis cohort stratified by HLA-B*08 association.

		HLA-B*08+ (n = 27)	HLA-B*08- (n = 16)
Median age at onset in years		14	8.5
Age at onset	<12 years	8 (30%)	9 (56%)
	≥ 12 years	19	7
AChR ab status	Positive	23 (85%)*	8 (50%)
	Negative	4	8
Gender	Female	24 (89%)	11 (69%)
	Male	3	5
Thymectomy	Yes	24 (89%)**	8 (50%)
	No	3	8
Thymus histology	Yes	18 (75%***)	2 (13%)
	No	4	4
	Unknown	2	2
CSR	Yes	13 (48%)	8 (50%)
	No	14	8
CAD		9 (33%)	3 (19%)
GMG		25 (93%)	15 (93%)

* Compared with HLA-B08-, p = 0.02.

** Compared with HLA-B*08, p = 0.008.

*** Compared with HLA-B*08, p = 0.005.

CAD = co-occurring autoimmune disorder other than myasthenia gravis. AChR ab = acetylcholine receptor antibodies. CSR = complete stable remission. GMG = generalised myasthenia gravis

<https://doi.org/10.1371/journal.pone.0186383.t003>

The HLA-B*08 positive juvenile MG cases in our material showed the characteristics typical for adult EOMG; female preponderance, AChR ab positivity and thymus hyperplasia (Table 3). The similarity of postpubertal onset MG to adult EOMG has been addressed earlier [31], and our findings concerning clinical picture and HLA association could support postpubertal onset juvenile MG being a continuum of adult EOMG. Prepubertal onset MG on the other hand, could comprise a distinctive subset of the disorder as hypothesized by Matsuki et al [32], and further supported by the differences we observed in the HLA associations.

Several studies have described the clinical presentation of juvenile MG in Western populations [20, 31, 33–36]. A challenge when comparing these studies, is their heterogeneity due to discrepancy on upper age cut off for the designation juvenile MG. However, several studies

Table 4. Clinical characteristics of prepubertal onset myasthenia gravis stratified by HLA association.

	DRB1*04:04+/B*08-, n(%)	DRB1*04:04-/B*08+, n(%)	DRB1*04:04+/B*08+, n(%)	DRB1*04:04-/B*08-, n(%)
Total (n = 17)	7	7	1	2
Median age at onset in years	5	9	1	2
Female	5 (72%)	6 (86%)	1 (100%)	1 (50%)
AChR ab	3 (43%)	5 (72%)	1 (100%)	0
HP/TX	1/3	3/5	1/1	0/0
CAD	2 (29%)	2 (29%)	1	0
CSR	4 (71%)	4 (71%)	1 (100%)	1 (50%)

AChR ab = acetylcholine receptor antibodies. HP = Thymus hyperplasia. TX = Thymectomy. CAD = co-occurring autoimmune disorder other than myasthenia gravis. CSR = complete stable remission

<https://doi.org/10.1371/journal.pone.0186383.t004>

have differentiated between prepubertal onset and postpubertal onset, and show differences in disease characteristics between the two groups. The prepubertal onset cases are associated with higher frequency of seronegativity, higher frequency of ocular MG but also some with severe disease although with good prognosis [1, 20, 31, 37]. Although subtle, the clinical differences together with the current HLA findings, could suggest that prepubertal onset MG constitute a distinctive subset of the disorder.

The main strength of this study is the comprehensive HLA genotyping done on a population based study cohort with extensive clinical mapping. However, the sample size is small due to the rarity of juvenile MG, and this limits the study. Further research including larger number of juvenile MG cases, especially those with prepubertal onset, is necessary to confirm the DRB1*04:04 association and to better describe the differences between the clinical subgroups.

In conclusion, this study provides novel information about HLA associations in European juvenile MG, where HLA-DRB1*04:04 is associated with prepubertal onset. In postpubertal onset juvenile MG, the HLA association is with HLA-B*08 like in EOMG.

Supporting information

S1 Table. Distribution of HLA alleles observed in the juvenile MG patients compared to controls.
(DOCX)

Acknowledgments

We would like to thank colleagues at the Departments of Neurology and Paediatrics around Norway for help in including MG patients to this study, and Siri T. Flåm for technical assistance. We would like to thank the MG patients for participating, and also the Norwegian Bone Marrow Donor Registry for supplying the control samples.

Author Contributions

Conceptualization: T. H. Popperud, E. Kerty, B. A. Lie.

Formal analysis: T. H. Popperud, M. K. Viken, B. A. Lie.

Investigation: T. H. Popperud, M. K. Viken.

Supervision: E. Kerty.

Writing – original draft: T. H. Popperud.

Writing – review & editing: T. H. Popperud, M. K. Viken, E. Kerty, B. A. Lie.

References

1. Della Marina A, Trippe H, Lutz S, Schara U. Juvenile myasthenia gravis: recommendations for diagnostic approaches and treatment. *Neuropediatrics*. 2014; 45(2):75–83. <https://doi.org/10.1055/s-0033-1364181> PMID: 24470240.
2. Berrih-Aknin S, Frenkian-Cuvelier M, Eymard B. Diagnostic and clinical classification of autoimmune myasthenia gravis. *J Autoimmun*. 2014; 48–49:143–8. <https://doi.org/10.1016/j.jaut.2014.01.003> PMID: 24530233.
3. Lindstrom JM, Seybold ME, Lennon VA, Whittingham S, Duane DD. Antibody to acetylcholine receptor in myasthenia gravis. Prevalence, clinical correlates, and diagnostic value. *Neurology*. 1976; 26(11):1054–9. Epub 1976/11/01. PMID: 988512.
4. Leite MI, Waters P, Vincent A. Diagnostic use of autoantibodies in myasthenia gravis. *Autoimmunity*. 2010; 43(5–6):371–9. <https://doi.org/10.3109/08916930903541208> PMID: 20380582.

5. Vincent A. Unravelling the pathogenesis of myasthenia gravis. *Nat Rev Immunol*. 2002; 2(10):797–804. Epub 2002/10/03. <https://doi.org/10.1038/nri916> PMID: [12360217](#).
6. Berrih-Aknin S, Le Panse R. Myasthenia gravis: A comprehensive review of immune dysregulation and etiological mechanisms. *J Autoimmun*. 2014; 52:90–100. <https://doi.org/10.1016/j.jaut.2013.12.011> PMID: [24389034](#)
7. Namba T, Brunner NG, Brown SB, Muguruma M, Grob D. Familial myasthenia gravis. Report of 27 patients in 12 families and review of 164 patients in 73 families. *Arch Neurol*. 1971; 25(1):49–60. Epub 1971/07/01. PMID: [5146412](#).
8. Salvado M, Canela M, Ponseti JM, Lorenzo L, Garcia C, Cazorla S, et al. Study of the prevalence of familial autoimmune myasthenia gravis in a Spanish cohort. *J Neurol Sci*. 2016; 360:110–4. Epub 2016/01/03. <https://doi.org/10.1016/j.jns.2015.11.049> PMID: [26723985](#).
9. Pirskanen R. Genetic aspects in myasthenia gravis. A family study of 264 Finnish patients. *Acta Neurol Scand*. 1977; 56(5):365–88. Epub 1977/11/01. PMID: [596124](#).
10. Fang F, Sveinsson O, Thormar G, Granqvist M, Askling J, Lundberg IE, et al. The autoimmune spectrum of myasthenia gravis: a Swedish population-based study. *J Intern Med*. 2015; 277(5):594–604. Epub 2014/09/25. <https://doi.org/10.1111/joim.12310> PMID: [25251578](#).
11. Evoli A, Caliendo P, Iorio R, Alboini PE, Damato V, LaTorre G, et al. Poly-autoimmunity in patients with myasthenia gravis: A single-center experience. *Autoimmunity*. 2015; 48(6):412–7. Epub 2015/04/15. <https://doi.org/10.3109/08916934.2015.1031890> PMID: [25868386](#).
12. Andrews PI, Massey JM, Howard JF Jr., Sanders DB. Race, sex, and puberty influence onset, severity, and outcome in juvenile myasthenia gravis. *Neurology*. 1994; 44(7):1208–14. PMID: [8035917](#).
13. Bogdanos DP, Smyk DS, Rigopoulou EI, Mytilinaiou MG, Heneghan MA, Selmi C, et al. Twin studies in autoimmune disease: genetics, gender and environment. *J Autoimmun*. 2012; 38(2–3):J156–69. Epub 2011/12/20. <https://doi.org/10.1016/j.jaut.2011.11.003> PMID: [22177232](#).
14. Gregersen PK, Kosoy R, Lee AT, Lamb J, Sussman J, McKee D, et al. Risk for myasthenia gravis maps to a (151) Pro→Ala change in TNIP1 and to human leukocyte antigen-B*08. *Ann Neurol*. 2012; 72(6):927–35. Epub 2012/10/12. <https://doi.org/10.1002/ana.23691> PMID: [23055271](#); PubMed Central PMCID: PMC3535539.
15. Seldin MF, Alkhairy OK, Lee AT, Lamb JA, Sussman J, Pirskanen-Matell R, et al. Genome-wide Association Study of Late-Onset Myasthenia Gravis: Confirmation of TNFRSF11A, and Identification of ZBTB10 and Three Distinct HLA Associations. *Mol Med*. 2015. Epub 2015/11/13. <https://doi.org/10.2119/molmed.2015.00232> PMID: [26562150](#); PubMed Central PMCID: PMC4749491.
16. Maniaol AH, Elsaïs A, Lorentzen AR, Owe JF, Viken MK, Saether H, et al. Late onset myasthenia gravis is associated with HLA DRB1*15:01 in the Norwegian population. *PLoS One*. 2012; 7(5):e36603. <https://doi.org/10.1371/journal.pone.0036603> PMID: [22590574](#); PubMed Central PMCID: PMC3348874.
17. Testi M, Terracciano C, Guagnano A, Testa G, Marfia GA, Pompeo E, et al. Association of HLA-DQB1*05:02 and DRB1*16 Alleles with Late-Onset, Nonthymomatous, AChR-Ab-Positive Myasthenia Gravis. *Autoimmune Dis*. 2012; 2012:541760. Epub 2012/10/24. <https://doi.org/10.1155/2012/541760> PMID: [23091703](#); PubMed Central PMCID: PMC3474207.
18. Feng HY, Yang LX, Liu WB, Huang X, Qiu L, Li Y. The HLA-B*4601-DRB1*0901 haplotype is positively correlated with juvenile ocular myasthenia gravis in a southern Chinese Han population. *Neurol Sci*. 2015; 36(7):1135–40. Epub 2015/05/09. <https://doi.org/10.1007/s10072-015-2235-9> PMID: [25953150](#).
19. Shinomiya N, Nomura Y, Segawa M. A variant of childhood-onset myasthenia gravis: HLA typing and clinical characteristics in Japan. *Clin Immunol*. 2004; 110(2):154–8. <https://doi.org/10.1016/j.clim.2003.10.004> PMID: [15003812](#).
20. Evoli A, Batocchi AP, Bartoccioni E, Lino MM, Minisci C, Tonali P. Juvenile myasthenia gravis with prepubertal onset. *Neuromuscul Disord*. 1998; 8(8):561–7. PMID: [10093062](#).
21. Popperud TH, Boldingh MI, Brunborg C, Faiz KW, Heldal AT, Maniaol AH, et al. Juvenile myasthenia gravis in Norway: A nationwide epidemiological study. *Eur J Paediatr Neurol*. 2017; 21(2):312–7. Epub 2016/09/27. <https://doi.org/10.1016/j.ejpn.2016.09.001> PMID: [27666466](#).
22. Popperud TH, Boldingh MI, Rasmussen M, Kerty E. Juvenile myasthenia gravis in Norway: Clinical characteristics, treatment, and long-term outcome in a nationwide population-based cohort. *Eur J Paediatr Neurol*. 2017; 21(5):707–14. Epub 2017/05/02. <https://doi.org/10.1016/j.ejpn.2017.04.003> PMID: [28457757](#).
23. Lorentzen AR, Karlsen TH, Olsson M, Smestad C, Mero IL, Woldseth B, et al. Killer immunoglobulin-like receptor ligand HLA-Bw4 protects against multiple sclerosis. *Ann Neurol*. 2009; 65(6):658–66. Epub 2009/07/25. <https://doi.org/10.1002/ana.21695> PMID: [19630074](#).

24. Spurkland A, Saarinen S, Boberg KM, Mitchell S, Broome U, Caballeria L, et al. HLA class II haplotypes in primary sclerosing cholangitis patients from five European populations. *Tissue Antigens*. 1999; 53(5):459–69. Epub 1999/06/18. PMID: [10372541](#).
25. Dudbridge F. Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol*. 2003; 25(2):115–21. Epub 2003/08/14. <https://doi.org/10.1002/gepi.10252> PMID: [12916020](#).
26. Valdes AM, Thomson G. Detecting disease-predisposing variants: the haplotype method. *Am J Hum Genet*. 1997; 60(3):703–16. Epub 1997/03/01. PMID: [9042931](#); PubMed Central PMCID: PMCPMC1712525.
27. Svejgaard A, Ryder LP. HLA and disease associations: detecting the strongest association. *Tissue Antigens*. 1994; 43(1):18–27. Epub 1994/01/01. PMID: [8023317](#).
28. Erichsen MM, Lovas K, Skinningsrud B, Wolff AB, Undlien DE, Svartberg J, et al. Clinical, immunological, and genetic features of autoimmune primary adrenal insufficiency: observations from a Norwegian registry. *J Clin Endocrinol Metab*. 2009; 94(12):4882–90. Epub 2009/10/28. <https://doi.org/10.1210/jc.2009-1368> PMID: [19858318](#).
29. Prahalad S, Thompson SD, Conneely KN, Jiang Y, Leong T, Prozonic J, et al. Hierarchy of risk of childhood-onset rheumatoid arthritis conferred by HLA-DRB1 alleles encoding the shared epitope. *Arthritis Rheum*. 2012; 64(3):925–30. Epub 2011/09/29. <https://doi.org/10.1002/art.33376> PMID: [21953520](#); PubMed Central PMCID: PMCPMC3276774.
30. Myhre AG, Undlien DE, Lovas K, Uhlving S, Nedrebo BG, Fougner KJ, et al. Autoimmune adrenocortical failure in Norway autoantibodies and human leukocyte antigen class II associations related to clinical features. *J Clin Endocrinol Metab*. 2002; 87(2):618–23. Epub 2002/02/12. <https://doi.org/10.1210/jcem.87.2.8192> PMID: [11836294](#).
31. Evoli A. Acquired myasthenia gravis in childhood. *Curr Opin Neurol*. 2010; 23(5):536–40. <https://doi.org/10.1097/WCO.0b013e32833c32af> PMID: [20581680](#).
32. Matsuki K, Juji T, Tokunaga K, Takamizawa M, Maeda H, Soda M, et al. HLA antigens in Japanese patients with myasthenia gravis. *J Clin Invest*. 1990; 86(2):392–9. Epub 1990/08/01. <https://doi.org/10.1172/JCI114724> PMID: [1974553](#); PubMed Central PMCID: PMCPMC296740.
33. Batocchi AP, Evoli A, Palmisani MT, Lo Monaco M, Bartoccioni M, Tonali P. Early-onset myasthenia gravis: clinical characteristics and response to therapy. *Eur J Pediatr*. 1990; 150(1):66–8. PMID: [2079081](#).
34. Castro D, Derisavifard S, Anderson M, Greene M, Iannaccone S. Juvenile myasthenia gravis: a twenty-year experience. *J Clin Neuromuscul Dis*. 2013; 14(3):95–102. <https://doi.org/10.1097/CND.0b013e318253a48e> PMID: [23492460](#).
35. Rodriguez M, Gomez MR, Howard FM Jr., Taylor WF. Myasthenia gravis in children: long-term follow-up. *Ann Neurol*. 1983; 13(5):504–10. <https://doi.org/10.1002/ana.410130506> PMID: [6870202](#).
36. Heckmann JM, Hansen P, Van Toorn R, Lubbe E, Janse van Rensburg E, Wilmshurst JM. The characteristics of juvenile myasthenia gravis among South Africans. *S Afr Med J*. 2012; 102(6):532–6. PMID: [22668957](#).
37. Chiang LM, Darras BT, Kang PB. Juvenile myasthenia gravis. *Muscle Nerve*. 2009; 39(4):423–31. <https://doi.org/10.1002/mus.21195> PMID: [19229875](#).