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Myasthenia gravis in the North of Portugal: epidemiological, clinical and serological study and immunopathology of the thymus

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Aos meus pais

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Publications

This work generated seven main scientific articles, that are included in this thesis (four published papers, one manuscript is under the process of review and other two are under submission).

There are other six published articles related to this work that were also cited here.

The original data presented was published in international peer-reviewed journals. The text in the results chapters is exactly as published, but the page formatting was adapted to conform with the layout of the thesis and the reference numbers adjusted to a single bibliography of uniform style. As a consequence, there is some overlap amongst these chapters and between them and the introductory chapter which reviews the literature comprehensively and defines the objectives of each study. The dissertation is completed by general discussion and conclusions.

Publications included in the thesis:

1. **Epidemiology of Myasthenia gravis in Northern Portugal: Frequency estimates and clinical epidemiological distribution of cases.** Ernestina Santos, Ester Coutinho, Isabel Moreira, Ana Martins Silva, Dina Lopes, Henrique Costa, Fernando Silveira, Goreti Nadais, Hugo Morais, Joao Martins, Maria Ceu Branco, Andreia Veiga, Rosa Santos Silva, Augusto Ferreira, Filipa Sousa, Marta Freijo, Ilda Matos, Rui André, Luís Negrão, Carla Fraga, Manuela Santos, Mafalda Sampaio, Carlos Lopes, Maria Isabel Leite, Guilherme Gonçalves. Muscle and Nerve. 2016 Sep;54(3):413-21.

2. **HLA and age of onset in myasthenia gravis.** Ernestina Santos, Andreia Bettencourt, Ana Martins da Silva, Daniela Boleixa, Dina Lopes, Sandra Brás, Paulo Pinho Costa, Carlos Lopes, Guilherme Gonçalves, Maria Isabel Leite, Berta Martins da Silva. Neuromuscular Disorders 2017; 27(7):650-654.

3. **Myasthenia gravis in Pregnancy – experience of a Portuguese Centre.** Antonio Braga, Ernestina Santos, Clara Pinto, Jorge Sousa Braga. Muscle and Nerve 2016;54(4):715-20.

4. **MuSK Myasthenia gravis and Pregnancy.** Ernestina Santos, Denis Gabriel, Antonio Braga, Sara Duarte, Ana Martins da Silva, Ilda Matos, Marta Freijo, Joao Martins, Fernando Silveira, Goreti Nadais, Filipa Sousa, Carla Fraga, Rosa Santos Silva, Carlos Lopes, Guilherme Gonçalves, Clara Pinto, Jorge Sousa Braga, Maria Isabel Leite. Resubmitted after minor revison in Neuromuscular Disorders.

5. **Myasthenia gravis with systemic and neurological polyautoimmunity.** Sara Duarte*, Ernestina Santos*, Joana Martins, Ana Martins Silva, Carlos Lopes, Guilherme Gonçalves, Maria Isabel Leite. Journal of Neurological Sciences. 2017 Oct 15;381: 39-40.

   *the authors contributed equally to the work

6. **Refractory Myasthenia gravis: Clinical, demographic and immunogenetic characteristics in a portuguese cohort.** Ernestina Santos, Andreia Bettencourt, Sara Duarte, Denis Gabriel, Vanessa Oliveira, Ana Martins Silva, Carlos Lopes, Guilherme Gonçalves, Berta Martins Silva, Maria Isabel Leite. *Under submission.*

7. **Thymoma following thymectomy of hyperplastic thymus in young onset Myasthenia gravis: clinical features heralding the thymoma.** Ernestina Santos, Ana Martins Silva, Philipp Stroebel, Antonio Marinho, Nick Wilcox, Guilherme Gonçalves, Carlos Lopes, Alexander Marx, Maria Isabel Leite. *Under submission.*

Publications related with this work and cited in the thesis:

1. **Inflammatory myopathy associated with myasthenia gravis with and without thymic pathology: report of four cases and literature review**. Ernestina Santos, Ester Coutinho, Ana Martins da Silva, António Marinho, Carlos Vasconcelos, Ricardo Taipa, Manuel Melo Pires, Guilherme Gonçalves, Carlos Lopes, Maria Isabel Leite. Autoimmunity Reviews. 2017 Jun;16(6):644-649.

2. **Congenital myasthenic syndrome due to mutation in CHRNE gene with clinical worsening and thymic hyperplasia attributed to association with autoimmune-myasthenia gravis**. Ernestina Santos, Isabel Moreira, Ester Coutinho, Guilherme Gonçalves, Carlos Lopes, Jose Lopes Lima, Maria Isabel Leite. Neuromuscular Disorders. 2015 Dec;25(12):928-31.

3. **Lambert-Eaton myasthenic syndrome and prostatic adenocarcinoma**. Cecília Monteiro, Isabel Moreira, Jose Lopes Lima, Ernestina Santos. Neurological Sciences 2015 Nov;36(11):2145-6.

4. **The protective role of HLA-DRB1 13 in autoimmune diseases**. Andreia Bettencourt, Cláudia Carvalho, Bárbara Leal, Sandra Brás, Dina Lopes, Ana Martins da Silva, Ernestina Santos, Tiago Torres, Isabel Almeida, Fátima Farinha, Paulo Barbosa, António Marinho, Manuela Selores, João Correia, Carlos Vasconcelos, Paulo Pinho Costa, Berta Martins Silva. Journal of Immunology Research 10/2015; 2015:948723. DOI:10.1155/2015/948723.

5. **Myasthenia gravis and neuromyelitis optica spectrum disorder: a multicenter study of 16 patients**. Maria Isabel Leite, Ester Coutinho, Marco Lana-Peixoto, Samira Apostolos, Patrick Waters, Douglas Sato, Luciana Melamud, Monica Marta, Andrew Graham, Jennifer Spillane, Andres Villa, Dagoberto Callegaro, Ernestina Santos, Ana Martins da Silva, Sven Jarius, Robin Howard, Ichiro Nakashima, Gavin Giovannoni, Camilla Buckley, David Hilton-Jones, Angela Vincent, Jacqueline Palace. Neurology. 2012 May 15;78(20):1601-7.

6. **Myasthenia gravis and pregnancy: anaesthetic management - a series of cases.** Carlos Almeida, Ester Coutinho, Daniela Moreira, Ernestina Santos, Jose Aguiar. European Journal of Anaesthesiology. 2010 Nov; 27(11):985-90.

Abbreviations

AChE - acetylcholinesterase
AChR - acetylcholine receptor
AChR Ab- MG – antibody negative acetylcholine receptor myasthenia gravis
AChR-MG – antibody-positive acetylcholine receptor myasthenia gravis
AID- autoimmune disease
AIRE - autoimmune regulatory gene
APECED - autoimmune polyendocrinopathy, chronic mucocutaneous candidiasis and ectodermal dystrophy
ATD - autoimmune thyroid disease
CIDP - chronic inflammatory demyelinating polyneuropathy
ColQ – Collagen Q
CTLA-4 - cytotoxic T-lymphocyte associated protein 4
DSNMG – double seronegative myasthenia gravis
EBV - Epstein-Barr virus
EMG- electromyography
EOMG - early onset (before 50 years old at onset)
GC – germinal centre
GP – general practitioner
GWAS - genome-wide association study
HCDB - hospital clinical databases
HLA - Human Leucocyte Antigens
IFN-gamma- interferon gamma
IL-1b- interleukin 1b
IL-10 – interleukin 10
IVIG - intravenous immunoglobulin
JMG - juvenile myasthenia gravis
LEMS - Lambert-Eaton myasthenic syndrome
LRP4 - receptor related low density lipoprotein-4
MG – myasthenia gravis
MHC – Major histocompatibility complex

MuSK - muscle-specific tyrosine kinase

MuSK-MG – muscle-specific tyrosine kinase myasthenia gravis

NeoMG - neonatal myasthenia gravis

NMJ - neuromuscular junction

NMOSD - neuromyelitis optica spectrum disorder

OAID- other autoimmune disease

PPDB - pyridostigmine prescriptions database

PTPN22 - Protein Tyrosine Phosphatase Non-Receptor Type 22

QMGS- quantitative myasthenia gravis score

RHA - Regional Health Administration

RyR- ryanodine receptor

SCLC-LEMS -small cell lung cancer associated LEMS

SNMG - seronegative myasthenia gravis

TAMG - thymoma associated myasthenia gravis

TCF19- transcription factor 19

TNF-alpha- tumor necrosis factor alpha

Abstract

Myasthenia gravis (MG) is a very well-known autoimmune disorder where there is an antibody mediated attack to the neuromuscular junction, impairing its transmission. It has a constellation of symptoms well characterized. The antibodies that cause the disease are known in more than 95% of the patients with a generalised form of the disease. Yet, there are still a lot to understand regarding some specific issues, namely the changes in the epidemiology trends, its genetic risk factors, effect of the disease on pregnancy and on the newborn of MG mothers, patient susceptibility to polyautoimmunity, refractory disease and also some questions related to thymomatous MG patients and their propensity to immunodeficiency and/or autoimmunity.

The main aim of this research is to increase the knowledge of the epidemiology of Myasthenia gravis in the North of Portugal, and describe more accurately the clinical, serological and thymic immunopathological aspects of the cases identified. The above data will allow (i) to analyse our findings with those of other regions in europe and the rest of the world; (ii) to identify factors that may influence the course of the disease and response to treatments; (iii) to study the effect of the disease on pregnancy; (iv) to register other autoimmune disorders and correlate it with thymus pathology; (v) to study the thymus of the patients with histologically confirmed thymoma, by immunohistochemistry and to correlate the histology of the thymus with the clinical data (MG characteristics, infections, other cancers, other autoimmune disorders).

The epidemiological study involved all the hospitals in the North of Portugal where patients with MG are treated. We used their databases to identify patients. To improve the quality of the source of patients, the method was complemented with identification of the prescriptions of pyridostigmine by the general practitioners in the north of Portugal.

Combining these two sources of information, at 31/12/2013, we estimated a point prevalence for MG of 111.7 patients/10^6 in the north

of Portugal. Prevalence rose with age reaching its maximum in the group over 65 years old, especially in males (288.1/10^6).

During the year of 2013 we estimated an incidence rate of 6.3 per million person/year. Among females, incidence rate was higher in the age group 15-49 (9.1/10^6), while in males, incidence increased with age till the maximum (22.1/10^6) among those aged over 65 years.

This is the first epidemiological study on MG in Portugal. These values are similar to those reported either in studies in southern European countries, or in northern Europe, in studies with similar methods and size of the population studied.

These results are consistent with most of the recent reports that describe higher incidence rates of MG in LOMG group, especially in elderly males. This information has crucial importance for the awareness of clinicians when dealing with this age group, and subsequent improvement in diagnosis and management of MG.

Almost one third of patients (29.4%) had one type of the following comorbidities: other autoimmune diseases (OAID) (organ specific or systemic), tumours or recurrent/serious infections; 2.8% of the patients had ≥2 of those associated problems. The proportion of patients with OAID and infections was identical in EOMG and LOMG groups. Only tumours were more common in LOMG.

Over the years, association of MG with Human Leucocyte Antigens (HLA) has been described in different populations. In European descendent populations HLA-DRB1*03 allele strongly influences MG susceptibility.

In our population, there was also a strong association of HLA-DRB1*03 and HLA-B*08 with MG, confirming that these alleles are important susceptibility factors for this disease, especially in the early onset MG subgroup (EOMG). HLA-DRB1*01 was associated with late-onset subgroup. To the best of our knowledge these results were not reported before and need replication in other populations and in larger cohorts.

MG is not associated with infertility, but it may expose pregnant women to an increased risk of maternal and fetal complications. In our study, 43% of patients experienced an exacerbation of symptoms, especially during

the third trimester and postpartum. This rate of clinical worsening is similar to that found by others. Our study adds to the body literature showing that a more aggressive MG background may increase the risk of clinical exacerbation during pregnancy.

We have also conducted a multicentre study, which included all MuSK-MG in the North of Portugal that underwent through a pregnancy. This is a subgroup of MG patients where the effect of the disease on the pregnancy, the delivery, the foetus and the changes on the MG during this period are much less known as this form of disease is rare. In this series, pregnancy did not seem to precipitate MuSK-MG or influence the MuSK-MG course. Furthermore, there was no apparent negative impact of MG on pregnancy outcomes in those where pregnancy followed the MG onset. One case of neo-natal MG was recorded. All newborns seemed to have normal psychomotor development.

The course of MG is often complicated by concomitant autoimmune disorders. It is important to consider coexistent MG in patients with autoimmune disorders that develop new or aggravated muscular weakness, fatigue or respiratory failure.

On the other hand, while rare, the possibility of neurological autoimmune comorbidity should also be considered in myasthenic patients, especially if there is an unexpected deterioration of muscle weakness or poor response to pyridostigmine (e.g. muscle disease), or if unexpected neurological signs or symptoms arise in MG (e.g. peripheral nervous system involvement in keeping with chronic inflammatory demyelinating polyneuropathy (CIDP); or central nervous system illness such as neuromyelitis optica spectrum disorder (NMOSD); or even rarer manifestations such as autonomic dysfunction). The rapid diagnosis of the second illness will improve management and disease outcomes of both MG and the other autoimmune condition.

A subset of myasthenia gravis patients is refractory to conventional treatments. Identifying their characteristics is important to promptly try to find different therapies effective in this disease subgroup.

In this study, we investigated the clinical features of refractory MG

patients and to investigate a possible association between HLA-DRB1 alleles and refractory MG. 22% of patients were classified as refractory. Compared to the non-refractory patients, the refractory ones were more likely to have a more severe MGFA classification at onset, to have thymomatous MG and to be seropositive, either to anti-AChR or anti-MuSK antibodies. HLA-DRB1*13 allele was less frequent in the non-refractory MG when compared to the refractory group and the control population.

The clinical and demographic characteristics of our refractory patients are similar to those described in other studies. It is important to consider those characteristics from the onset of the disease. HLA-DRB1*13 allele may be a protective allele for the non-refractory group. As far as we were concerned there are no other immunogenetic studies in refractory MG and these results need to be replicated in other populations.

It is extremely rare for early onset myasthenia gravis patients to have thymoma following extended removal of hyperplastic thymus. We described two AChR-MG patients who developed thymoma years after extended transternal thymectomy and highlight the clinical manifestations that heralded the thymoma, consequently of a severe immunodeficiency which resembled APECED (autoimmune polyendocrinopathy, chronic mucocutaneous candidiasis and ectodermal dystrophy) syndrome, with auto-immune regulatory (AIRE) gene defect.

These two cases illustrated the need of searching for a thymoma in MG patients whose disease becomes difficult to treat after removal of a thymus, even if it shows only thymic follicular hyperplasia. In addition, thymoma needs to be considered even more in cases of severe and/or recurrent infections, which could reflect complex paraneoplastic immune-deficiency associated with auto-antibodies to interleukins.

We will continue the study of the thymoma associated MG with immunohistochemistry studies of their thymomas namely with AIRE gene and correlate it with the presence of severe and/or recurrent infections, OAID and tumours.

Resumo

A Miastenia gravis (MG) é uma doença autoimune bem conhecida onde há um ataque à junção neuromuscular prejudicando o seu funcionamento. Caracteriza-se por uma constelação de sintomas bem definidos e os anticorpos que causam a doença são conhecidos em mais de 95% dos doentes com a forma generalizada da doença. No entanto, ainda há muito por entender em relação a algumas questões específicas, nomeadamente as mudanças nas tendências epidemiológicas, os fatores de risco genéticos, o efeito da doença na gravidez e nos recém-nascidos das mães com MG, a suscetibilidade à poliautoimunidade, os doentes refratários e também algumas questões relacionadas com doentes com MG associada ao timoma e a propensão à imunodeficiência e/ou auto-imunidade.

A finalidade deste projeto é aumentar o conhecimento da epidemiologia de Miastenia gravis no Norte de Portugal, com a identificação e descrição dos aspetos clínicos, serológicos e imunopatológicos dos timos. Foram objetivos específicos: comparar a nossa população de doentes com MG com os de outras regiões da Europa e do resto do mundo; identificar fatores que possam influenciar o curso da doença e a resposta aos tratamentos; estudar o efeito da doença na gravidez; registar outras doenças auto-imunes e correlacioná-las com patologia do timo; estudar o timo dos doentes submetidos à timectomia, com timoma, com imuno-histoquímica e correlacionar os estudos de imuno-histoquímica do timo com os dados clínicos (infeções, outras neoplasias e outras doenças auto-imunes).

O estudo epidemiológico envolveu todos os hospitais do Norte de Portugal que tratam doentes com MG. Usámos as bases de dados desses hospitais para identificar os doentes. Para melhorar a qualidade da fonte de doentes, o método foi complementado com a identificação das prescrições de piridostigmina pelos médicos de família no Norte de Portugal. A piridostigimina é um fármaco usado praticamente em todos os doentes com MG e é usada quase exclusivamente na MG.

Combinando estas duas fontes de informação, em 31/12/2013, estimámos uma prevalência pontual para MG de 111,7 doentes/10^6 no Norte de Portugal. A prevalência aumenta com a idade atingindo o seu máximo no grupo com mais de 65 anos, especialmente nos homens (288,1/10^6).

Durante o ano de 2013, estimámos uma taxa de incidência de 6,3 por milhão de pessoas/ano. Entre as mulheres, a taxa de incidência foi maior no grupo etário 15-49 (9,1/10^6), enquanto nos homens a incidência aumentou com a idade até o máximo (22,1/10^6) entre os maiores de 65 anos.

Este é o primeiro estudo epidemiológico em MG em Portugal. E esses números são semelhantes aos reportados em estudos em países do sul da Europa ou no norte da Europa, com métodos e tamanho de amostra populacional similares.

Estes resultados estão de acordo com a maioria das descrições mais recentes que descrevem maiores taxas de incidência de MG no grupo LOMG, especialmente em homens. Esta informação tem importância crucial para a continuação da consciencialização dos clínicos sobre a doença nesta faixa etária, e subsequente melhoria no diagnóstico e tratamento de MG.

Quase um terço dos doentes (29,4%) apresentaram um dos 3 tipos de comorbilidades: outras doenças autoimunes (OAID) (órgão específico ou sistémica), tumores ou infeções recorrentes/graves; 2,8% dos doentes apresentaram ≥ 2 desses problemas associados. A proporção de doentes com OAID e infeções foi idêntica nos grupos EOMG e LOMG. Apenas os tumores foram mais comuns na LOMG.

Ao longo dos anos foi descrita a associação de MG com o HLA em diferentes populações. Nas populações descendentes de europeus, o alelo HLA-DRB1*03 influencia fortemente a suscetibilidade à MG.

Na nossa população, também houve uma forte associação de HLA-DRB1*03 e HLA-B*08 com MG, confirmando que esses alelos são importantes fatores de suscetibilidade para esta doença, especialmente no subgrupo de início precoce. HLA-DRB1*01 foi associado ao subgrupo

de início tardio. Do nosso conhecimento, estes últimos resultados relativos ao subgrupo de início tardio nunca foram publicados anteriormente e precisam ser replicados em outras populações e em coortes maiores.

A MG não está associada à infertilidade, mas expõe as mulheres grávidas a um risco aumentado de complicações maternas e fetais. No nosso estudo, 43% das doentes apresentaram exacerbação de sintomas, especialmente durante o terceiro trimestre e pós-parto. Esta é uma taxa semelhante de agravamento clínico, quando comparamos com outras séries publicadas. O nosso estudo associa-se à literatura mostrando que, na presença de uma MG de base mais agressiva a frequência de exacerbação clínica durante a gravidez pode ser alta.

Realizámos um outro estudo na gravidez, multicêntrico que incluiu todas as doentes com MuSK-MG no Norte de Portugal que tiveram pelo menos uma gravidez. Este é um subgrupo de doentes onde o efeito da doença sobre a gravidez, o parto, o feto e as alterações na MG durante esse período são menos conhecidos. Nesta série, a gravidez não pareceu precipitar MuSK-MG ou influenciar o curso da MuSK-MG, e não houve impacto negativo aparente nos resultados da gravidez naqueles em que a gravidez foi posterior ao início da MG. Registámos um caso de MG neonatal e o peso destes recém-nascidos foi mais baixo, apesar de nenhum cumprir critérios de baixo peso ao nascimento. Todos os recém-nascidos tiveram um desenvolvimento psicomotor normal.

O curso da MG é muitas vezes complicado por outras doenças auto-imunes concomitantes. É importante considerar a MG em doentes com doenças auto-imunes que desenvolvem nova fraqueza muscular, ou agravada, fadiga ou insuficiência respiratória.

Por outro lado, embora seja raro, a possibilidade de comorbilidade auto-imune neurológica também deve ser considerada em doentes com MG, especialmente se houver uma deterioração inesperada da fraqueza muscular ou má resposta à piridostigmina (por exemplo, doença muscular), ou se sinais ou sintomas neurológicos inesperados surgem na MG (por exemplo, envolvimento do sistema nervoso periférico compatível

com uma CIDP, ou doença do sistema nervoso central, como a NMOSD, ou mesmo manifestações mais raras, como a disfunção autonômica). O diagnóstico rápido de uma segunda doença melhorará a resposta ao tratamento da outra doença bem como da MG.

Uma fração de doentes com miastenia gravis é refratária aos tratamentos convencionais. Identificar as suas características é importante para contribuir para tentar encontrar novas formas de tratamento eficaz neste subgrupo. Neste estudo, investigámos os aspetos clínicos dos doentes com MG refratária e uma possível associação entre alelos HLA-DRB1. 22% dos doentes foram classificados como refratários. Em comparação com os pacientes não refratários, os refratários eram mais propensos a ter uma classificação de MGFA mais grave no início da doença, ter MG associada a timoma e ser seropositivo, seja para anticorpos anti-AChR ou anti-MuSK. O alelo HLA-DRB1*13 foi menos frequente na MG não refratária quando comparado ao grupo refratário e à população controlo. As características clínicas e demográficas dos nossos doentes refratários são semelhantes às descritas noutros estudos. É importante considerar essas características desde o início da doença. O alelo HLA-DRB1*13 parece ser um alelo protetor para o grupo das MG não refratárias, à semelhança do que descrito em outras OAID. Do que sabemos, não existem outros estudos imunogenéticos na MG refratária, assim estes resultados precisam ser replicados noutras populações.

É extremamente raro que doentes com miastenia gravis com início precoce tenham timoma anos após a remoção de um timo hiperplásico. Descrevemos dois doentes com AChR-MG que desenvolveram timoma anos após a timectomia transternal extendida e descrevemos as manifestações clínicas que anunciaram o timoma, consequentes a uma imunodeficiência grave que se assemelhava à síndroma APECED (autoimmune polyendocrinopathy, chronic mucocutaneous candidiasis and ectodermal dystrophy) com defeito do gene regulador da autoimunidade (AIRE).

Estes dois casos ilustram a necessidade de procurar um timoma em doentes MG cuja doença se torna difícil de tratar após a remoção de um

timo com hiperplasia folicular tímica. E que o timoma precisa de ser considerado em caso de infeções graves e/ou recorrentes, refletindo uma complexa deficiência imune paraneoplásica, que nos doentes aqui descritos se associou a auto-anticorpos contra interleucinas.

Continuaremos o estudo dos timomas com análise imunohistoquímica, nomeadamente do gene AIRE, e a sua correlação com outras doenças autoimunes, presenças de infeções graves/recorrentes e tumores.

Chapter 1. Introduction

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1.1. Clinical features of MG

1.1.1. Clinical presentations

Myasthenia gravis (MG) was described for the first time by Thomas Willis in 1672.[1] It was the first neurological or neuromuscular disease to be identified as being autoantibody mediated.[2–5]

In around 80% of patients with generalised MG, the disease is caused by autoantibodies to the skeletal muscle nicotinic acetylcholine receptor (AChR) on the postsynaptic membrane of the neuromuscular junction (NMJ); this is called AChR antibody positive MG (AChR-MG).

The AChR antibodies impair neurotransmission by reducing AChR numbers, which is the main consequence of the antibodies and complement mediated damage to the neuromuscular endplate[6,7]; the second consequence is reduction of AChR half-life.[8]

Since the structural and functional integrity of the NMJ is essential for muscle contraction and strength[9–11], and reserves of AChR are limited in humans, AChR loss causes muscle weakness and fatigue, the hallmark of clinical MG. The muscle weakness with fatigue is variable but it involves predominantly certain skeletal muscle groups. The weakness can be focal or generalized, and usually affects ocular, bulbar, neck and proximal extremity muscles. Respiratory muscle weakness develops only rarely, but can be life-threatening. Weakness is typically symmetrical, except in affected external eye muscles, in which the weakness is usually asymmetrical. [12–14]

The evolution of MG is unpredictable, but it is generally characterized by the occurrence of relapses, sometimes subsequent to remissions and a worsening trend in the first months and years. For 85% of MG patients, the maximum severity is reached within less than 3 years.[13,15]

1.1.2. Antibodies in Myasthenia gravis

AChR antibodies

AChR antibodies can be detected by routine assays in 70% of all patients with MG.[16,17] AChR antibodies mostly belong to the IgG_1 and IgG_3 subclasses, which activate the complement cascade, leading to damage of the postsynaptic membrane.[13,16]

Routine screening for AChR antibodies uses radioimmunoprecipitation assay (RIA). It is based on a mixture of solubilized embryonic and adult AChR's, is the most rigorously validated test for AChR antibodies, and is the most reliable among the validated AChR antibody tests. Positive test results have a near 100% specificity for MG in symptomatic individuals. [16,17]

In another 5–10% of patients, AChR antibodies can only be detected with more-sensitive, cell-based assays [18,19], in which AChR molecules are clustered on the membranes of cultured test cells. This technique offers better sensitivity, and enables detection of low-affinity antibodies: they can detect antibodies in 4–66% of patients with ocular or generalized MG in whom antibodies cannot be detected with radioimmunoassay. [18]

The radioimmunoassay is recommended as the first-line test because it has been used successfully in clinical practice; moreover, it not only detects the presence of AChR antibodies but can also quantify their levels. Supplementary cell-based testing should be performed when MG is suspected but the patient is negative for anti-AChR and anti-MuSK antibodies by RIA. [12,20]

Total AChR antibody concentration does not correlate with symptom severity when patients are compared. Although, fluctuations in AChR antibody concentration in an individual patient have been reported to correlate with the severity of muscle weakness and to predict exacerbations. [16,20,21]

MuSK antibodies

In 2001 it was described that up to 40% of them have autoantibodies to the muscle specific tyrosine kinase (MuSK).[22] Subsequent studies confirmed the existence of a subgroup of autoimmune generalised MG patients who lack detectable antibodies to AChR (AChR Ab- MG), but have, instead, MuSK antibodies.

MuSK is another muscle specific protein expressed at the neuromuscular junction and has a role in neuromuscular junction development and in AChR clustering during development.[23,24]

Although both MuSK-MG and AChR Ab- MG clearly improve with plasma exchange or intravenous immunoglobulin (IVIG), patients with MuSK-MG have some particular clinical and laboratory characteristics which differ from the most typical MG.[25]

LRP4 antibodies

More recently, antibodies to receptor related low density lipoprotein-4 (LRP-4) were also found to be associated with some cases of seronegative MG. LRP-4 is a receptor for agrin and for MuSK, which are important in the aggregation of AChR in the neuromuscular junction plate.[26] This subgroup of patients seems to have a milder form of the disease.[12,27]

Other antibodies in MG

Antibodies to agrin, which activates MuSK through Lrp4 binding, and to ColQ (Collagen Q), the collagen tail that anchors acetylcholinesterase to the end-plate basal lamina, were reported in myasthenia gravis patients often in association with other disease-specific antibodies, mostly AChR antibodies. Their pathogenic role has not been proved in animal models.[28]

Other antibodies in thymoma MG and Late Onset MG

Some MG patients have antibodies that bind in a cross-striational pattern to skeletal and heart muscle tissue sections. They are known as "striational antibodies". These autoantibodies recognize epitopes on skeletal muscle proteins, including myosin, actin, actinin, and filamin. [21] Two types of striational antibodies directed against titin, ryanodine receptor (RyR) and cortactin are found in up to 95% of MG patients with thymoma and in 50% of late-onset MG patients.[21,28]

1.1.3. Thymus and Myasthenia gravis

The involvement of the thymus in the immunopathogenic mechanisms of MG is well established. Leopold Laquer, in 1901, was the first to establish a link between the thymus and myasthenia gravis.[1]

In AChR-MG patients with early onset (before 50 years-old at onset, EOMG), the presence of thymic epithelial hyperplasia and multiple lymphoid follicles in lymphocytic infiltrations is very common (>80%).[29-34]

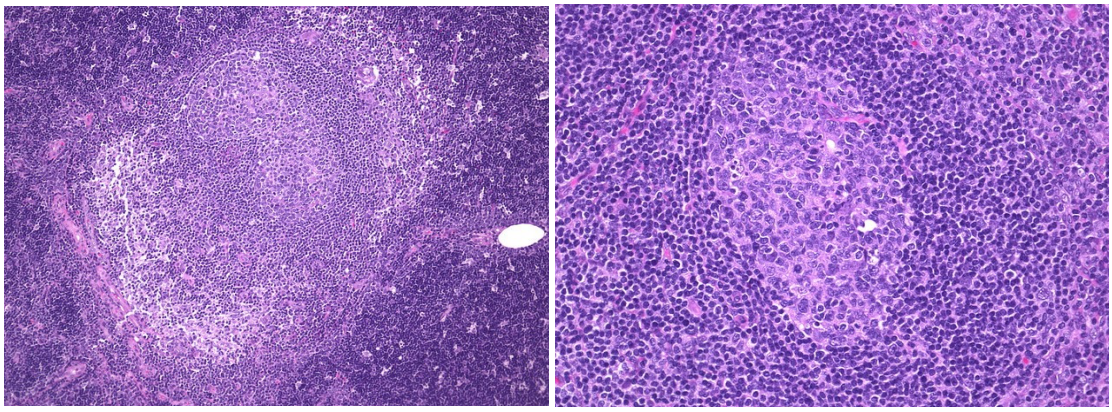

Figure 1.1.3.1. Hyperplastic thymus in a patient with myasthenia gravis. Intermediate view of thymic follicular hyperplasia. The lymphoid follicles are composed of B cells and are of secondary type with formation of germinal centers. Higher power view of a lymphoid follicle.

Moreover, these thymic changes seem to be centred on rare muscle-like cells, thymic myoid cells that express skeletal muscle antigens and are located in the medulla. Their potential roles as a source of AChR for the autoimmunisation and as an early target of the antibody-mediated attack on native AChR, have been suggested through different lines of research.[29,34-37]

The thymic involvement in EOMG may also explain the benefits of thymectomy in these cases, as observed clinically over the years and also in the only clinical trial in this field that was completed and published recently.[38]

By contrast, AChR Ab- thymuses, including those of MuSK-MG cases, are usually reported as histologically normal for age or atrophic, and the clinical response to thymectomy is unclear.[14,39-42]

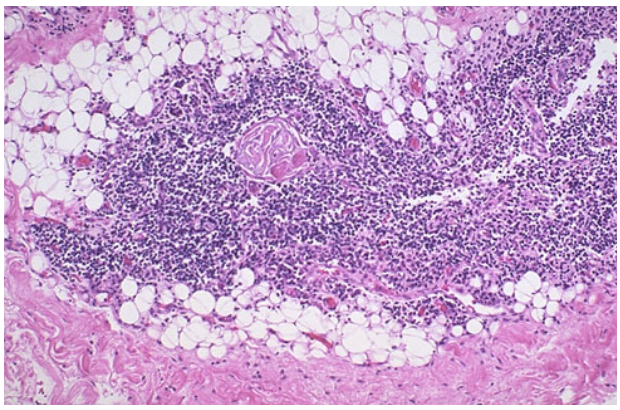

Figure 1.1.3.2. Normal adult thymus is seen at low magnification. Beyond puberty, the thymus continues to atrophy, with decreasing numbers of lymphocytes, so that the adipose tissue becomes more prominent. Occasional Hassall's corpuscles appear in the lymphoid areas.

1.1.4. Diagnosis

The diagnosis of Myasthenia gravis is based on a group on typical symptoms, electrophysiological tests and serological studies. The answer to the therapeutics also help to support the diagnosis.

Typical symptoms

1) The presence of suggestive symptoms, isolated or, more frequently, in variable combination: diplopia, ptosis, without pupillary abnormalities, bulbar manifestations such as dysphonia, dysarthria, dysphagia, difficulty chewing, weakness and fatigue of the limbs, neck muscles and respiratory muscles;

2) A typical variability in the symptoms with exacerbation by exercise;

a) short duration variation during the daytime with worsened symptoms in the evening, during menstruation or during the occurrence of fatigue,

b) relapses corresponding to worsening of the disease over a period of several weeks to several months. Ptosis is particularly helpful in the diagnosis, because it may vary or alternate in a few minutes and worsens after sustained upward gaze. If there is a significant ptosis, the ice test (ice cubes applied on the side of ptosis for 1 min) improves the droopy lid for a few seconds[13,17],

c) the occurrence in a few days of respiratory congestion, shortness of breath, ineffective cough, choking, and rapid motor deterioration predicts a myasthenic crisis that could be life-threatening, requiring immediate hospitalization in intensive care unit. [13,17]

Electrophysiological tests – electromyography

The presence of an EMG decrement is essential and must be investigated in several proximal and distal nerve-muscle pairs and if possible after 12h interruption of acetylcholinesterase (AChE) inhibitors.

The single fiber analysis that reveals an elongated jitter (the time between the potentials of two muscle fibers of the same motor unit) is more sensitive but less specific than the classical EMG and is difficult to perform. It must be limited to difficult cases (a negative EMG, in particular in ocular myasthenia).[13]

Serological tests

Most MG patients have antibodies directed at proteins of the neuromuscular junction. The number of patients with unknown antibodies is less than 5%.

The anti-AChR antibodies are highly specific for MG. If they are negative, it is important to search for the anti-MuSK, LRP4 or clustered AChR. If still negative, reconsider diagnosis.

Response to the treatment

The injection of 0.5 mg neostigmine subcutaneously or intra-muscularly has a significant effect on the deficit signs (ptosis, hypernasal voice, limb weakness) from 15 min and persisting for 2 h. The test of oral cholinesterase over a few weeks is quite justifiable to assess the functional status in daily life and over time.[13]

Thorax CT scan

All patients should perform a thorax CT scan to explore the potential presence of a thymoma. Scan should be reported by experienced radiologist.

Important differential diagnosis

Lambert-Eaton myasthenic syndrome

There is another autoimmune neuromuscular disorder, much rarer than MG, in which the pre-synaptic part of the NMJ is affected, named the Lambert-Eaton myasthenic syndrome (LEMS).[43] It was described for the first time in 1960[44] and are associated to voltage gated calcium channel antibodies.[45]

Up to 50-60% of cases occur as a paraneoplastic disorder (SCLC-LEMS), most commonly in association with small cell lung cancer but can also occur with other types of cancer (prostate, breast, lymphoma).[46–49] The other 40-50% are idiopathic, autoimmune without an identified

tumour.[50] Despite their rarity they are an important differential diagnosis of MG.

Congenital myasthenic syndromes

Another important group that is also a differential diagnosis with MG are the congenital myasthenic syndromes, especially in the cases of double seronegative MG. They are a heterogeneous group caused by genetic mutations of the proteins that interfere with the NMJ transmission.[51] It is very important to differentiate them from autoimmune MG, because the treatment is not based on thymectomy or immunosuppression. Although in very rare cases they can be associated to autoimmune MG.[52]

1.1.5. Treatments

Treatments for Myasthenia gravis can be divided in three major groups:
- symptomatic treatment;
- disease modifying treatment or immunosuppressive treatments;
- and thymectomy.

Symptomatic treatment

The acetylcholinesterase inhibitor pyridostigmine represents the first-choice treatment in all types of autoimmune MG.[17]

All MG subgroups, besides MuSK-MG, usually respond well to this treatment, but individual variation is considerable. In MuSK-MG, is reported a good response in only 50% of patients, and 10% did not respond at all.[19] Some MuSK-MG patients may not tolerate this treatment.

In such patients, 3,4-diaminopyridine, a drug that is more used in Lambert-Eaton myasthenic syndrome, which increases presynaptic release of acetylcholine, may be tried. [53]

Disease modifying treatment or immunosuppressive treatments

Long term immunosuppression

First-line treatments

First-line immunosuppressive drug therapy for MG includes either prednisone, or the combination of prednisone with azathioprine or other immunosuppressive agent. [54–56] According to current recommendations, prednisone alone should be given only as short-term treatment (<1 year). [55]

Long-term prednisolone monotherapy could be considered for the treatment of ocular MG or if is possible to keep a low dose, but for the majority of other patients with MG, combination immunosuppressive treatment is recommended to obtain maximum effect with minimal adverse effects of steroids. [54–56]

Azathioprine (2–3 mg/kg daily) is usually the first line immunosuppressive treatment in MG, but it takes 6–15 months to yield an optimal effect. So, it is used in association to prednisone, which exerts its full effect during the first few weeks and months of treatment. [54–56]

When pharmacological remission or a marked improvement is reached, the immunosuppressive drug should be maintained in the long-term, whereas the steroid dose should be reduced if possible, to avoid adverse effects. Full drug withdrawal may lead to new exacerbations, particularly in MuSK-MG, thymoma MG and late-onset MG. [54–56]

Second-line treatments

There are no controlled studies that compared different drugs in MG.

Mycophenolate mofetil is an option after the failure of first-line therapy. Evidence supports that the use of this drug is strongest in AChR-MG, although more long term studies are required. [57,58]

Rituximab has been increasingly used in MG. It is a monoclonal antibody that binds specifically to the B-lymphocyte surface antigen CD20. For severe MG, and for MuSK-MG in particular, rituximab may be given as first-line immunosuppressive therapy.[59,60] More than 80% of patients with severe or refractory MG responded to rituximab.[60] Most studies to date have used the same induction regimen as for rheumatoid arthritis and then repeated the treatment only if symptoms recurred after many months.[12]

Alternative second-line treatments and third-line treatments

Alternative second-line and third-line treatment options for MG include methotrexate, cyclosporine, tacrolimus and cyclophosphamide.

***Cyclosporin*e** has a proven effect in well-controlled studies, but its use has been limited by a high risk of adverse effects. Several uncontrolled trials suggested that improvement was seen in 65 to 85% of MG patients after 12 to 30 months of treatment.[61–64]

Methotrexate has been used as a second or third line with a steroid sparing effect with reasonable response. Recently there was a study claiming that there is no benefit in the use of methotrexate in MG[65], experience that has not been shared by others. Indeed, despite several limitations, methotrexate has been shown to provide an effective alternative to azathioprine in generalised MG.[61,66–68]

Tacrolimus: there is limited yet promising information to suggest a beneficial role in reducing QMGS and corticosteroid burden in patients with refractory symptoms or new-onset MG.[69] It is similar to cyclosporine, tacrolimus inhibits calcineurin but has the advantage of being less nephrotoxic. Case series have demonstrated its efficacy in MG as a monotherapy or as a corticosteroid sparing agent.[61]

Cyclophosphamide is an alkylating agent that interferes with DNA replication and decreases the production of lymphocytes, monocytes, and

macrophages. According to the literature and expert's opinion, the use of cyclophosphamide has been limited to cases of refractory MG. Remission may be achieved after 12 months, [70] or much earlier as reported in one study (average treatment duration of 3.6 months in half of the patients treated). [71]

Other treatments

Other biologic agents have been used in an even more refractory subpopulation of patients: tocilizumab[72], eculizumab[73,74], belimumab[75] and rescue treatments (autologous hemopoietic stem cell transplantation)[76].

Short term and rapid action immunosuppression

Intravenous immunoglobulins and plasma exchange

Intravenous immunoglobulin (IVIG) is predominantly used as short-term immunoactive therapy in acute situations. IVIG and plasma exchange have similar effects on MG exacerbations. [54,55] Such treatments should always be given for an ongoing or imminent MG crisis, and may be also recommended shortly before situations in which muscle weakness is expected to deteriorate or lead to complications, such as surgery. IVIG or plasma exchange are usually combined with intensified immunosuppressive treatment in patients with severe disease or severe exacerbation. Overall, all MG subgroups — even seronegative MG — respond to IVIG and plasma exchange, although they may respond better to one than to the other treatment. Regarding seronegative patients, the response to those treatment imply that they also have circulating antibodies that are very likely pathogenic.[12,13,16,54,55]

Thymectomy

In thymoma MG, the thymoma of the thymus gland should always be removed to treat the cancer. The response of MG to thymectomy is variable, and improvement of MG symptoms is usually more limited than in early-onset MG.[12]

In early-onset MG, there are a number of controlled studies which showed that thymectomized patients have a more favourable outcome than those who are not.[77,78] Evidence recommends early and complete thymectomy in all patients with early-onset generalized MG with AChR antibodies, particularly those who are not symptom-free on symptomatic drugs alone. The fact that thymic hyperplasia is common in this subgroup supports a therapeutic effect of thymectomy performed early after MG onset. Early thymectomy will prevent export of AChR-specific T cells from the thymus to lymph nodes and peripheral lymphoid tissue. [20,38,77,78] For a good outcome, removal of all thymus tissue is essential.[20,38,77,78]

Late-onset MG is traditionally regarded as less responsive or nonresponsive to thymectomy; however, the evidence regarding thymectomy in patients with late-onset MG is sparse. The fact that the thymus of patients in this subgroup is usually atrophic (an age-normal finding) does not provide any support to the use of thymectomy.[77,78]

In MuSK-MG and LRP4-MG thymectomy is not recommended.[20]

Thymectomy has not shown yet to prevent generalization in ocular MG, or to induce remission. Thymectomy, is therefore, not yet recommended for ocular MG. [55,59], although some experts have been doing thymectomy in selected cases of ocular MG, especially if they have some hint of other muscle involvement in electrophysiology tests.

1.1.6. MG classification and subgroups

Patients with MG are usually classified into subgroups according to clinical presentation and biomarkers.[20] The criteria for these subtypes include clinical symptoms, age of onset, thymic pathology and the autoantibodies.

MG with anti-AChR antibodies

MG with anti-AChR antibodies is divided into early-onset MG (symptom onset before 50 years) and late-onset MG (onset after 50 years).[79] In some studies there is an additional subgroup: the very old late onset MG (onset after 65 years).[80,81]

In AChR-MG, the early-onset and late-onset disease types differ with respect to thymic pathology (Table 1.1.6.1.), HLA genotype and other genetic variables, autoimmune comorbidities, and response to therapy. For example, thymectomy has clear clinical benefits in early-onset MG, but its benefits in late-onset MG are more questionable and possibly non-existent.[20]

MuSK-MG

The presence of anti-MuSK antibodies is usually associated with more-severe and generalized muscle weakness, where bulbar and facial muscles are usually more predominantly involved early in the disease. Sometimes, they also develop muscle atrophy later in the disease course.[53] In MuSK-MG, there seems to be less muscle fluctuation than in other MG subtypes, probably because the severity of the muscle weakness.[42,53,82] Limb weakness is less commonly affected than in AChR-MG, and ocular muscles can be affected in a more symmetric way.[53,82,83] Importantly, respiratory weakness is more likely to affect patients with MuSK-MG than other MG subgroups.[53,82] Therefore, myasthenia crisis is more commonly seen in these patients. MuSK MG is not associated with thymus pathology.

LRP4-MG

MG with LRP4 antibodies is a subgroup more recently described and less well characterized.[27,84,85] It seems that in this subgroup the symptoms are milder, both at initial presentation and over the course of the disease.[86] Myasthenic crisis is very rare. A few patients have been reported to have a combination of anti-AChR and/or anti-MuSK and anti-LRP4 antibodies.[19] Association to thymoma has already been reported, but this is very rarely found.[87]

Seronegative MG

By definition, seronegative MG patients have no anti-AChR, anti-MuSK or anti-LRP4 antibodies. Patients need to have, however, MG symptoms, abnormal neurophysiological tests and favorable response to treatments usually used in typical of MG. They constitute about 10% of generalized MG patients. Is an heterogeneous group, as it includes patients that may have typical antibodies of low affinity or of very low concentration to be detected; it may include also patients with antibodies against relevant antigens that have not yet been identified.[18] Usually the symptoms are milder and the potential benefit of thymectomy has not been confirmed.[20]

Thymoma associated MG

MG associated to thymoma constitutes about 10-15% of all MG cases, and virtually all of the thymoma MG patients have AChR antibodies. Approximately 30% of patients with thymoma may develop MG, especially if they have AChR antibodies.[12,16] MG associated with thymoma usually responds to immunosuppressive treatments, although, when compared to those without thymoma, they may require more and more prolonged immune treatment.[88]

Ocular MG

Approximately 60-80% of patients with MG have ptosis and/or diplopia at onset. At 2 years after onset, 15–20% of patients with initial ocular MG still have purely ocular MG.[16,55] 40%–70% of ocular myasthenia patients have anti-AChR antibodies on the conventional assay.[55,89] Only very few ocular myasthenia cases have antibodies to MuSK.[55,90,91]

Table 1.1.6.1. Myasthenia gravis: classification in subgroups

Subgroup		**Auto-antibodies**	**Age of Onset**	**Frequency in MG**	**Thymus**
AChR-MG	Early-Onset MG	Anti-AChR +	<50y	15-25%	Thymic hyperplasia
	Late-Onset MG	Anti-AChR +	<=50y	35-45%	Atrophic
MuSK-MG		Anti-MuSK +	Any ++Young females	1-10%	Normal
LRP4-MG		Anti-LRP4 +	Any ++Young females	1-5%	Normal
Seronegative		None	Any	10-15%	Normal or thymic hyperplasia
Thymoma MG		Anti-AChR +	Any	10-15%	Thymoma
Ocular		Any Negative in 50%	Any	15%	Variable

1.2. Etiopathogeny

Antibodies targeting the neuromuscular junction cause myasthenia gravis. These antibodies bind to the postsynaptic muscle endplate and attack and destroy postsynaptic molecules. This process leads to impaired signal transduction and, consequently, muscle weakness and fatigability — the hallmark symptoms of MG.[12,16,20]

The aetiology of MG is believed to result from the interaction between genetic and environmental factors.[39]

1.2.1. Immunology – thymus

A key pathogenic finding in EOMG is the occurrence of intrathymic lymphoid follicles and germinal centers described as early as 1901. Once established, germinal centers drive the hypermutation of B cell receptor genes and intrathymic production of high affinity myasthenogenic anti-AChR antibodies.[78]
Initially the thymic myoid cells were assumed to be the initial (triggering) source of AChR for autoimmunization.[29] More recently, new evidence suggests that medullary epithelial cells express unfolded AChR subunits and are under attack by autoantibodies and complement just like adjacent myoid cells. [92–95] In light of this, it has been proposed (REF) that helper T cells are primed by unfolded AChR subunits expressed by MHC class I and II positive hyperplastic medullary epithelial cells. Early antibodies elicited by primed T cells then attack nearby myoid cells (that express whole, native AChRs) and activate complement with subsequent release of AChR/immune complexes. These, in turn, activate professional antigen presenting cells, germinal center formation and autoantibody diversification. [96]

1.2.2. Environment

Environmental factors, such as drugs (i.e., D-penicillamine and IFN-I) and pollutants are also proposed to increase the risk of developing an autoimmune disease. [97]

In EOMG there is strong evidence that a comprehensive autoimmunization against the AChR begins and persists in the thymus. However, the

immunization trigger is unknown. [98] Some suggest that a viral infection may trigger the initiation of this process.[79] Recent candidates are EBV [99,100] and Human Polyomavirus 7 (HPyV7) that were detected in 40% of hyperplastic thymuses (and in thymomas). [101]

Several reports indicate that other viruses, such as cytomegalovirus, human foamy virus, and Nile virus, are associated with MG.[102,103]

1.2.3. Genetics

The association of human leukocyte antigen (HLA) class I and class II genes with MG is clearly established.[39,104,105] The association between HLA-B8 (MHC class I) and -DR3 (MHC class II) and EOMG (and thymic follicular hyperplasia), was confirmed in several studies. [39,104,106] LOMG is associated with HLA-DR2-B7.[39]
Other susceptibility genes have also been studied.[104,107,108] Most of these genes are also susceptibility genes for other autoimmune diseases and include PTPN22, CTLA-4, IL-1b, IL-10, TNF-α, and IFN-γ. [104]
More recently, a genome-wide association study (GWAS) performed in northern European EOMG patients showed an association with the transcription factor TCF19.[109] TCF19 is involved in cell proliferation and differentiation and is up-regulated in human pro-B and pre-B cells. This gene is also highly expressed in GC cells.[110]
Most of the genes associated with MG are involved in regulating the immune system. It is possible that subjects with specific alleles associated with lower immunoregulatory capacity may be more susceptible to autoimmune diseases, particularly to MG.[79]

1.3. Epidemiology of Myasthenia gravis

Myasthenia gravis is a relatively rare neurological disease. Its prevalence is variable, but is around 10–20 per 100.000.[111–115] Some recent reports show a steady increase in MG prevalence, which seems to be due to an increasing incidence of MG in elderly people, particularly in Western countries.[116–120]
Since the fifties, a large number of epidemiology studies on MG have been performed. They have been conducted worldwide describing quite distinct prevalence and incidence rates, according to different methods, classifications of the disease and the inclusion criteria used.[121,122,80,120,123–128]

Two seminal reviews were published on the methodological issues and findings of population-based epidemiological studies of MG.[129,130] Carr et al review covered 55 studies performed between 1950 to 2007 and showed prevalence rates that ranged from 15 to 179 per million persons and incidence rates that ranged from 1.7 to 21.3 per million persons per year.[129] It remains unclear whether this reflects primarily methodological differences or true differences in disease frequencies based on ethnical or other demographic factors.[130]

Some previous studies showed a trend of increasing prevalence with relatively stable incidence, which was interpreted as reflecting the impact of effective treatment and improved diagnostic methods.[112] More recently, prevalence seems quite stable, but in the last two decades several studies report changes on MG incidence, which is mostly due to the group of patients with LOMG.[81,116,117,131]

Differences in MuSK-MG and AChR-MG prevalence and incidence throughout the globe have also been reported. There is clear evidence that MuSK-MG is more common in the southern European countries than in the North of Europe.[82,132,133]

1.4. Other relevant subjects

1.4.1. Polyautoimmunity and Myasthenia gravis

Patients affected by one autoimmune disorder have a higher risk of developing another autoimmune disease. MG patients have an increased risk of other autoimmune disorders compared to the non-MG population. [134–136] The frequency of a second autoimmune disorder is 11–25.7% in MG patients, with most studies showing that this is highest for females and early onset MG (EOMG). [106,112,127,134–136]

In a systematic review, autoimmune thyroid disease (ATD) was the most frequent of 23 associated autoimmune disorders, occurring in 10% of MG patients. [134,137] Other common autoimmune associates with MG are systemic lupus erythematosus (SLE) (1-8%), rheumatoid arthritis (RA) (4%), dermato/polymyositis and neuromyelitis optica. [134,137–140]

Genetic studies on the development of autoimmune disorders reveal common susceptibility genes, most strongly at the human leukocyte antigen (HLA) locus. Genome-wide association studies (GWAS) on EOMG shed light on specific additional genetic hot spots for MG. EOMG is strongly associated with the haplotype HLA-B8-DR3[39], which is also associated with autoimmune thyroid disease, type 1 diabetes mellitus, Sjogren's syndrome, inclusion body myositis, dermato/polymyositis, SLE.[141]

1.4.2. Refractory Myasthenia gravis

Therapeutic options in myasthenia gravis patients include cholinesterase inhibitors, thymectomy, immunosuppressive agents and short-term immunomodulation with plasma-exchange and intravenous

immunoglobulin. [28] Conventional immunosuppressive agents used in MG treatment include azathioprine, mycophenolate mofetil, methotrexate, cyclosporine, tacrolimus and cyclophosphamide.[142] A small proportion of myasthenia gravis patients (10-15%) are classified as refractory due to non-responsiveness to conventional treatments.[142]

Refractory patients were defined as those who could not lower the immunotherapy for MG without clinical relapse, with MG not clinically controlled on their immunotherapy regimen, or who had developed severe adverse effects from immunosuppressive therapy for at least a period of 12 months.[88,142]

Some studies show that these patients are mostly females, have young onset and generalized disease, and are seropositive, predominantly to anti-MuSK antibodies or those with thymoma.[88,142]

This refractory MG population has been the focus of recent publications evaluating the response to biologic agents (rituximab[142,143], tocilizumab[72], eculizumab[73,74], belimumab[75]) and rescue treatments (autologous hemopoietic stem cell transplantation).[76]

1.4.3. Myasthenia gravis in children

Myasthenia that affects children can be classified into the following forms: transient neonatal myasthenia and juvenile myasthenia gravis (JMG). [144] Juvenile myasthenia gravis is a rare autoimmune neuromuscular disorder. It can be divided in two periods: the very early onset MG patients, younger than 8 years old and with a higher proportion of ocular MG (66%), and a puberty onset MG (8-18 years old), that is more similar to the EOMG.[145] JMG is more frequent in Asia than Europe or America. [146][147]
In a French study of 40 patients with JMG, 70% had generalized MG, 52% had positive acetylcholine receptor antibodies, 8% had muscle-specific-kinase antibodies, and 40% were seronegative. [148]

A Chinese study, which included 114 JMG patients, showed that AChR antibodies were present in the majority of JMG patients and were associated with more severe disease, while other antibodies were rare. [145] Genetic analysis revealed that the very early onset JMG had a more prominent genetic predisposition in both an autoantigen gene (CHRNA1) and an immunomodulating gene (CTLA4). Thymus hyperplasia was diagnosed more frequently in those with onset before 8 years. The puberty onset patients (8–18 years) appeared with intermediate characteristics between the very early onset JMG and the young adult patients. [145]. Thymomas were present in 5% of JMG patients.[145]
The pathophysiology of juvenile myasthenia gravis is similar to that of adult myasthenia gravis, though important differences remain regarding presentation and therapeutic options. [149]

One limitation in the treatment of these children is that randomized clinical studies of myasthenia gravis have been carried out primarily in adult populations.[149] Treatment with acetylcholinesterase inhibitors was effective and sufficient in 47% of patients. The 6 patients with generalized JMG treated with rituximab and/or immunoadsorption showed improvement. Thirty percent of the patients required hospitalization in an intensive care unit during follow-up (mean 4.7years). Remission without treatment occurred in 18% of patients.[148]

Thoracoscopic thymectomy is a safe and acceptable treatment for juvenile MG with good disease control. The low morbidity and shorter hospital duration make it an excellent option for consideration.[150]

1.4.4. Late Onset Myasthenia gravis

In myasthenia gravis (MG), clinical manifestations can vary depending on the age of onset. There is no clear consensus on the definition of late-onset myasthenia gravis (LOMG); the cut-off age was originally set at 40 years; then, 50 years of age; this has been more generally used in clinical

practice and in studies.[39,131] However, a cut-off of at 60 years is sometimes used because the current biological status is better, and life expectancy of general population is higher.[151,152]

Studies have shown that the incidence of MG has increased dramatically over the past 20 years, but only among those >65 years of age. [81,153]

Late-onset MG is traditionally regarded as less responsive or nonresponsive to thymectomy however, the evidence regarding thymectomy in patients with late-onset MG is sparse. The fact that the thymus of patients in this subgroup is usually atrophic (an age-normal finding) does not provide any support to the use of thymectomy.

In a study comparing young onset and late onset MG patients, the elderly group, nine (45%) patients had accumulations of lymphocytes, indicating an atrophied thymus with loss of the basic structure. The elderly MG patients with atrophied thymic tissues had higher titres of anti-AChR antibody (59.6+/-81.0 nmol/L) than those with adipose infiltration of the thymus alone (20.1+/-20.9 nmol/L). In immunohistochemical studies, using image analysis, both young patients and elderly patients with atrophied thymic tissues were found to have significantly higher levels of CD20 than age-matched controls ($p < 0.005$).[152]
Thymus remains functional until the sixth decade of life. Atrophy of the thymus and decreased output of T lymphocytes are prominent features in older adults, but they are not the sole changes that occur with age. Increase in marginal zone and B-1 B-cell numbers, a source of autoantibodies, could explain the increased frequency of self-reactive immunoglobulins in older adults.

In patients with MG onset at 50–65 years who have thymic hyperplasia, the response to thymectomy might be expected to be similar to that in early-onset MG, and thymectomy should be considered in selected patients. Biomarkers are needed to establish indications for thymectomy in late-onset MG.

Antibodies against titin and RyR are most common in late-onset MG. Fifty percent of LOMG subgroup patients and almost all MG patients with thymoma have striational antibodies, but it is unknown why these 2 groups have the same antibody profiles.[20,154] It has been suggested that the presence of antibodies to non-AChR muscle antigens, rather than the presence of thymoma or the late age of onset, is responsible for more severe disease in older patients.[155]

Diagnosing MG in older adults can be challenging, weakness can be mistaken for motor neuron disease or a brainstem stroke, and MG is probably underdiagnosed in this age group.[81] Studies from Japan have shown that the ocular form is more common in LOMG.[147]

The diagnosis of patients with LOMG is fundamentally not different from diagnosis in other age groups, although a high index of suspicion should be maintained due to the frequent occurrence of comorbidities in older people that might be confused with MG symptoms.[81]

Treatment is also similar to EOMG, but specific consideration should be given to side effects. One cohort study showed adverse effects of cholinesterase inhibitors in 22 MG patients (age range 30–84 years, 11 of whom were >65 years of age) and found that age correlated with the cholinergic adverse effects.[156]

Unfortunately, they have major side effects to steroids too. They include cataracts, hyperglycemia, diabetes, hypertension, osteoporosis, infections, and weight gain, which have been reported to occur in up to 66.7% of patients; these effects are especially worrisome in older adults.[81]

Some found that LOMG (defined as age 50 years) showed a statistically non-significant trend toward more clinically severe disease than early onset, but patients had a similar treatment response and outcome. [157] However, it was also described a higher pharmacological remission in subjects with an older age at onset.[158]

Considering all described above, MG occurring in older adults seems to be more difficult to manage mainly because of the multiple comorbidities and side effects of medications.[81]

1.4.5. Pregnancy and Myasthenia gravis

MG is not associated with infertility, but it exposes pregnant women to an increased risk of maternal and fetal complications. The clinical course of pregnancy in these patients is unpredictable. Symptoms at the date of conception, the requirement to treat active MG symptoms, as well as the course of previous pregnancies do not seem to correlate with the course of a specific pregnancy in mothers who suffer from MG.[159]

It has been described that about 30% of pregnant women experienced improvement of symptoms while a third face clinical worsening, especially in the first trimester and during postpartum.[159,160]

An increase in the exacerbation rate during pregnancy in the first two - three year after diagnosis was also described.[161] So it is advised to postpone pregnancy one to two - three years after diagnosis.[159,161]

As smooth muscle is not affected in MG, indication for cesarean delivery is usually reserved for obstetric indications. However, it should be emphasise that 3rd stage of labour involves the contraction of voluntary striated muscle and this is where assistence in vaginal delivery is required to reduce the expulsive efforts and then minimize mother's fatigue[159]. Regardless, the incidence of cesarean delivery seems to be increased in these patients.[162,163]

The risk of preterm birth, low neonate birthweight or hypertensive disorders apparently are not increased in MG pregnant women.[163,164]

Neonatal MG (NMG) affects 12-20 % of newborns of such pregnancies. Typical forms characterized by weak cry, swallowing and sucking difficulties usually appear during the first hours of life and disappear in 90% of cases in 2 months. Atypical forms of arthrogryposis multiplex congenita are also described and represent 29% of NMG[165]. Neonatal MG happens in both AChR-MG and MuSK-MG.[166–171]

Chapter 2. Aim and Objectives

Chapter 2. Aim and Objectives

The aim of this research is to increase the knowledge of the epidemiology of Myasthenia gravis in the North of Portugal, and describe the clinical, serological and immunopathological (thymus) aspects of MG cases identified. It is expected that that knowledge will improve the clinical management of the cases of MG in this setting.

The objectives of this study are:

(1) to identify the patients with *Myasthenia gravis* in the North of Portugal;

(2) to study the clinical and demographic characteristics of this population;

(3) to classify the patients according to Myasthenia gravis subgroups;

(4) to calculate the incidence and prevalence of the disease as a whole and its subgroups;

(5) to analise and compare our population of portuguese myasthenic patients with patients from other regions in Europe and the rest of the world;

(6) to identify factors that may influence the course of the disease and response to treatments;

(7) to study the effect of the disease on pregnancy;

(8) to register other autoimmune disorders and correlate it with thymus pathology;

(9) to study the thymus of the patients submitted to thymectomy, with thymoma, with immunohistochemistry;

(10) and to correlate the immunohistochemistry studies of the thymus with the clinical data (infections, other cancers, other autoimmune disorders).

Chapter 3. Results

Chapter 3. Results

3.1. Epidemiological study

Within this research project, an epidemiological study was conducted, in order to estimate the prevalence and incidence of MG in the North of Portugal.

We restricted this study to the North of Portugal and used two different sources: the clinical records from the hospitals and the pyridostigmine prescription register in the study area.

This study included patients from the following hospitals: Hospital São João, Porto, Centro Hospitalar de Vila Nova de Gaia e Espinho, Hospital Pedro Hispano, Matosinhos, Centro Hospitalar do Trás-os-Montes e Alto Douro, Vila Real, Centro Hospitalar do Alto Minho, Viana do Castelo, Centro Hospitalar Entre Douro e Vouga, Feira, Hospital de Braga, Braga, Centro Hospitalar do Nordeste, Mirandela, Hospital de São Teotónio, Viseu, Centro Hospitalar Universitário de Coimbra, Coimbra, Centro Hospitalar do Vale do Sousa, Penafiel and Neuropediatric Departments from Centro Materno Infantil Norte, Centro Hospitalar Porto and Hospital de São João, Porto, Portugal.

The pyridostigmine prescription register in the study area was obtained from Administração Regional de Saúde do Norte.

The serological studies were performed in the clinical Neuroimmunology laboratory at the Nuffield Department of Clinical Neurosciences, Oxford University Hospitals, University of Oxford, United Kingdom, by Dr. Ester Coutinho, under supervision of Prof. Dr. Maria Isabel Leite.

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Epidemiology of Myasthenia Gravis in the North of Portugal: Frequency estimates and clinical epidemiological distribution of cases

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Abstract

We are not aware of previous studies attempting to estimate incidence and/or prevalence of myasthenia gravis (MG), or characterizing the clinical epidemiological patterns of MG in Portugal.

We restricted this study to the North of Portugal and used two different sources: clinical records from the hospitals and pyridostigmine prescription register in the study area.

The objectives were: to estimate the prevalence, incidence and mortality of MG, assessing the availability of data to do those estimates, and to characterise demographic, clinical and laboratory features of the MG cases identified.

At 31/12/2013 we estimated a point prevalence of 111.7 patients/10^6. These figures are similar to those reported either in studies in southern European countries, or in the north of Europe, in studies with similar methods and size of the population studied.

Prevalence rose with age reaching its maximum in the group over 65 years old, especially in males (288.1/10^6).

During the year of 2013 we estimated an incidence rate of 6.3 per million person/year. Our incidence rate was closer to studies from distinct latitudes with similar methods and to others from south of Europe.

Among females, incidence rate is higher in the age group 15-49, while in male's incidence increases with age till the maximum (22.1/10^6) in those aged over 65 years.

The mortality rate attributed to MG was 0.5 per million, which is in the range of what others studies reported. MG features of the patients identified have mostly confirmed common demographic distribution and disease characteristics as published by others.

Introduction

The disease is characterized by fatigable weakness of voluntary muscles and the severity varies from mild ocular symptoms to generalized weakness with bulbar symptoms and respiratory insufficiency. Those phenotype differences led to the classification of the MG in two main subgroups according to the muscle groups involved - ocular and generalised - and also according to their severity.

MG affects people of any age, and this demographic characteristic has allowed additional disease classification in early onset and late onset MG (EOMG and LOMG respectively) with a cut off age that has changed over time from 40 to 50 or even 60 or 65 years at disease onset, depending on the studies and opinions.[39,131]

Clinical, demographic, serological, histological and genetic features of the MG were first analysed together in 1980, where distinct sub-forms of the MG were identified.[39] This study constituted the foundation for a large number of epidemiological, clinical and laboratory research studies ever since performed worldwide.

Epidemiological studies may be one of the ways to investigate predisposing factors for MG such as genetics, climate or eating habits.[126] They are also very important in the understanding autoimmunity at different ages and gender, and to study the impact of the disease in general health care of MG populations.

Since the fifties, a large number of epidemiology studies on MG have been performed. They have been conducted worldwide describing quite distinct prevalence and incidence rates, according to different methods, classifications of the disease and the inclusion criteria used.[121,122,80,120,123–128]

Table 3.1.1. Prevalence and incidence studies on myasthenia gravis

Author, year	Region	Period	Population (n)	Methods of cases identification	Incidence (per million)	Prevalence (per million)
Present study	North of Portugal	2013	3 644 195	Hospital registries and pyridostigmine databases from GP's	6.3	111.7
Aragones, 2014	Osona, Barcelona	2013	155 069	Regional hospitals registries	NA	328.9
Aragones, 2014	Osona, Barcelona	2001-2010	142 337	Regional hospitals registries	28.0	NA
Pedersen, 2013	Denmark	1996-2009	5 511 000	National registry for MG and pyridostigmine databases	9.2	NA
Pallaver, 2011	Trento, Italy	2005-2009	524 826	Hospital and GP registries, AChR serology registries and pyridostigmine databases	14.8	129.6
Montomoli, 2012	Pavia, Italy	1985-2008	493 753	Hospitals registries	NA	240
Anderson, 2014	Norway	1995-2008	4 737 171	Nationwide MG databases and prescription database	8.8-16	131-145
Heldal, 2009	Norway	1985-2008	4 737 131	National registry for AchR test	7.2	115.5
Somnier, 2005	Eastern Denmark	1970-1999	2 300 000	Regional hospital registries	3.5	NA
Poulas, 2001	Greece	1992-1997	10 180 913	Anti-AchR serology registries	7.4	70.6
Guidetti, 1998	Regio-Emilia, Italy	1980-1994	427 493	Multiple sources: registries from different specialities and GP 's	7.8	117.5

MG – myasthenia gravis, GP – general practitioner, AChR – acetylcholine receptor, NA - not applicable

Two seminal reviews were published on the methodological issues and findings of population-based epidemiological studies of MG.[129,130] Carr et al review covered 55 studies performed between 1950 to 2007 and showed prevalence rates that ranged from 15 to 179 per million persons and incidence rates that ranged from 1.7 to 21.3 per million persons per year.[129] It remains unclear whether this reflects primarily methodological differences or true differences in disease frequencies based on ethnical or other demographic factors.[130]

Some previous studies showed a trend of increasing prevalence with relatively stable incidence, which was interpreted as reflecting the impact of effective treatment and improved diagnostic methods.[112]

More recently prevalence seems quite stable, but in the last two decades there have been several reports of a trend on changing the MG incidence, mostly in the group of those with LOMG.[81,116,117,131]

It has been also reported differences in MuSK MG and AChR MG prevalence and incidence throughout the globe. There is clear evidence

that MuSK MG is more common in the southern European countries than in the North of Europe.[82,132,133] It has been also described that AChR MG varies with different latitudes or regions even inside the same country.[172–174]

Recently some authors have addressed promising possibilities of combining different sources of information to identify MG cases in epidemiological studies, using pyridostigmine prescription register combined with databases with clinical records [121,125,175] or based uniquely on pyridostigmine prescription register.[176]

We are not aware of previous studies attempting to estimate incidence and/or prevalence of MG, or characterizing the clinical, demographic and epidemiological patterns of this disease in Portugal. We restricted this study to the North of Portugal and used two different sources: clinical records from the hospitals in the study area and pyridostigmine prescription register, as in the studies we mentioned above.[125,175]

The objectives were: to estimate the prevalence, incidence and mortality of MG, assessing the availability of data to do those estimates and to characterise the identified cases of MG.

Materials and Methods

The study focused on a geographically well-defined area of Portugal – the North region of Portugal, which is officially defined by NUTS II (Nomenclature of territorial units for statistics) and covers an area of 21 278km².[177]

According to the 2011 census 3 689 682 inhabitants lived in the North of Portugal.[177] The estimated population for the same region for 31/12/2013 was 3 644 195 inhabitants.[178]

To calculate de incidence rate for the year of 2013 we could not get the estimated population at the 30th June 2013; instead, we used the estimated population for the 31/12/2013, assuming that for this six

month period there was not a significant change in the size of the population.

In Portugal, the great majority of MG patients are treated in public hospitals. Even when they are diagnosed in a private clinic they are referred to public hospitals for further workup and management. On rare occasions, such as in mild MG or later in the course of the disease, patients may be handed over to private practising neurologists or general practitioners (GP) as per patient choice. In both cases, pyridostigmine prescriptions may be continued by their GP.

MG patients are followed up in one of the eleven public hospitals in this region, where there is at least one neurologist working in a Neurology or Neuropediatrics unit. Three hospitals located in the Centre Region of Portugal have also participated in this study because MG patients living in border areas that are under de RHA of the the North region are often seen there. The names of all hospitals and health units involved in the study are listed under the "affiliations" of the authors working in Portugal.

Data was collected prospectively between 01/01/2013 and 31/12/2013. All patients who had the disease onset and MG diagnosis until the end of this period were included.

Identification of MG patients

Two complementary approaches were used to identify patients: 1) hospital clinical databases and/or clinical records of neurologists of the hospitals participating in the study (HCDB); 2) computorized data base of pyridostigmine prescriptions (PPDB) by the general practitioners (GP) working in the North region.

1) HCBD: neurologists responsible for MG patients in each of the hospitals of catchment area were invited to participate. They were asked to identify, all MG cases currently being treated there. The diagnosis was verified by review of medical records. Information collected included sociodemographic and clinical data; patients that had not been tested yet for AChR or MuSk antibodies or were negative by standard assays

performed locally, were invited to provide a blood sample for antibody tests.

All neurologists were contacted regularly to remind them to register new patients, complete any clinical data still missing and also to collect a blood sample as appropriate.

2) PPDB: The Regional Health Administration (RHA) services have an informatic prescription register that permits access to all the prescriptions made by the GP's. Pyridostigmine bromide (Mestinon®) is used practically in all MG patients with clinically active disease and as first line treatment. At the same time it is used almost exclusively in MG and congenital myasthenic syndromes. Although the initial prescription is usually made by a neurologist it can be continued by a neurologist, a GP or both.

By contacting the RHA services in the North region, all prescriptions for pyridostigmine, made by the general practitioners (GPs) in this region throughout the year 2013 were identified. The name of the doctor who made the prescription, the name of the patient and their national health number were available. We matched the list of patients identified in the HCDB with those identified in the PPDB. For those that were not included in the HCDB we contacted the GP doctor who made the prescription. Depending on their response or not to each form of contact, they were first sent a posted letter, a secure e-mail, and finally called by telephone, requesting the information required (first whether patient had MG or another neurological disease; second, in case of MG, clinical data was collected).

Patients treated with pyridostigmine to other conditions (e.g. congenital myasthenic syndrome, myopathies and other neurological disorders) were excluded from this study.

Combining these two sources of identification of patients was a method considered validated in previous studies in Denmark.[175]

Case definition

Definite MG was considered if the following criteria were fulfilled:

(a) history and examination compatible with myasthenia (fatigable weakness), plus

(b) at least one positive paraclinical test (antibody test, repetitive nerve stimulation, single-fibre electromyogram), and/or

(c) unequivocal improvement with pyridostigmine or immunossupression. [116,179]

If a diagnosis of MG was stated in the medical records, but could not be definitely confirmed or ruled out due to insufficient information on one or more of the aforementioned criteria, cases were classified as *possible MG*. Cases with no identifiable medical records with pertinent information were classified as *non-evaluable*.

Classification of MG subtype

All definite cases were classified in MG subtypes according to antibodies serostatus:

1. AChR MG: Consistent clinical picture, positive AChR antibodies (AChR-Ab);
2. MuSK MG: Consistent clinical picture, positive MuSK antibodies (MuSK-Ab);
3. Seronegative MG: Consistent clinical picture, positive electrophysiological tests and or response to pyridostigmine or immunossupression; seronegative for AChR-Ab and MuSK-Ab.

Definite cases were classified according to the presence/absence of thymoma and age of onset of the disease: before 50 years of age – EOMG and 50 years old or older – LOMG.[117,131,132]

Clinical Data

Clinical and demographic data were collected from the patient's medical records: date of birth, current age, gender, age at MG onset, age at MG diagnosis, neurophysiological test results, antibodies to AChR and MuSK, thymectomy and thymic histology, worst MGFA clinical

score[180], need of artificial ventilation, treatments used during the course of disease and MGFA status after intervention; other autoimmune co-morbidities, tumours and severe/recurrent infections were also recorded.

Serological antibody tests

All patients who had been tested positive for AChR or MuSK antibodies locally were not tested again. Patients whose serological status was negative or unknown provided a blood sample to be tested for AChR and MuSK antibodies (radioimmunoassay and cell-based assays if the first was negative).[18,181] These assays were performed in the Neuroimmunology Laboratory, John Radcliffe Hospital, in Oxford.

Statistical Analysis

The incidence rate (IR) refers to all incident cases in the study area throughout the year 2013. Prevalence date was set as 31/12/2013 for all patients alive and resident in the study area. Prevalence and incidence rate were calculated per million for all MG patients and its subgroups according to sex and age group. SPSS statistics, version 22 was used.

Ethical approval

Ethical approval was obtained from the ethical committees of the intervenient hospitals and from the Portuguese Data Protection Authority (*Comissão Nacional de Proteção de Dados*) to collect demographic and clinical data from all patients and also for blood collection from some patients. Approval was obtained from the Ethical Committee of the Regional Health Administration (North of Portugal) to use the information related to the pyridostigmine prescriptions, to contact the general practitioners and to get patient's information. Informed consent was obtained from each patient to access their clinical data, and for blood collection, if needed.

Results

Patient identification and first disease classification

We identified 371 patients in the HCDB and 259 patients in the PPDB. All records were carefully checked as explained in the methods section, being included or excluded as shown in Figure 3.1.

In the PPDB, we found 499 prescriptions made in the North region by the GPs, corresponding to 259 patients. From these 259 patients, 138 were also identified in the HCDB. Letters were sent to the prescribers GPs of the other 121 patients, requesting information about their patients.

Twenty-one answered by posted letter, 18 by email, and 71 answered only after a phone call to their working place. We could not get proper information regarding 11 patients taking pyridostigmine either because it was not possible to contact the GP (n=5) or because the clinical records did not mention the reason why pyridostigmine was prescribed in first place (n=6). These 11 patients were considered *non-evaluable*.

From the information collected on those 121 patients prescribed with pyridostigmine, 63 patients had other neurological disorders (congenital myasthenic syndrome, mithocondrial myopathies, other myopathies or other neurological conditions); these were rejected from the study.

Forty-seven patients not included in the HCDB were actually MG cases. Overall, 418 MG patients were identified and included in the study; 23/418 were incident cases, with onset in 2013; 11/418 died during 2013 (Figure 3.1.1.).

From the 407 patients alive, at the end of 2013, 398 (97.8%) were classified as *definite* MG, and 9 (2.2%) as *possible* MG. All the *possible* MG cases were identified in the PPDB; in their GP's clinical files they were identified as having MG, but details on the disease could not be found.

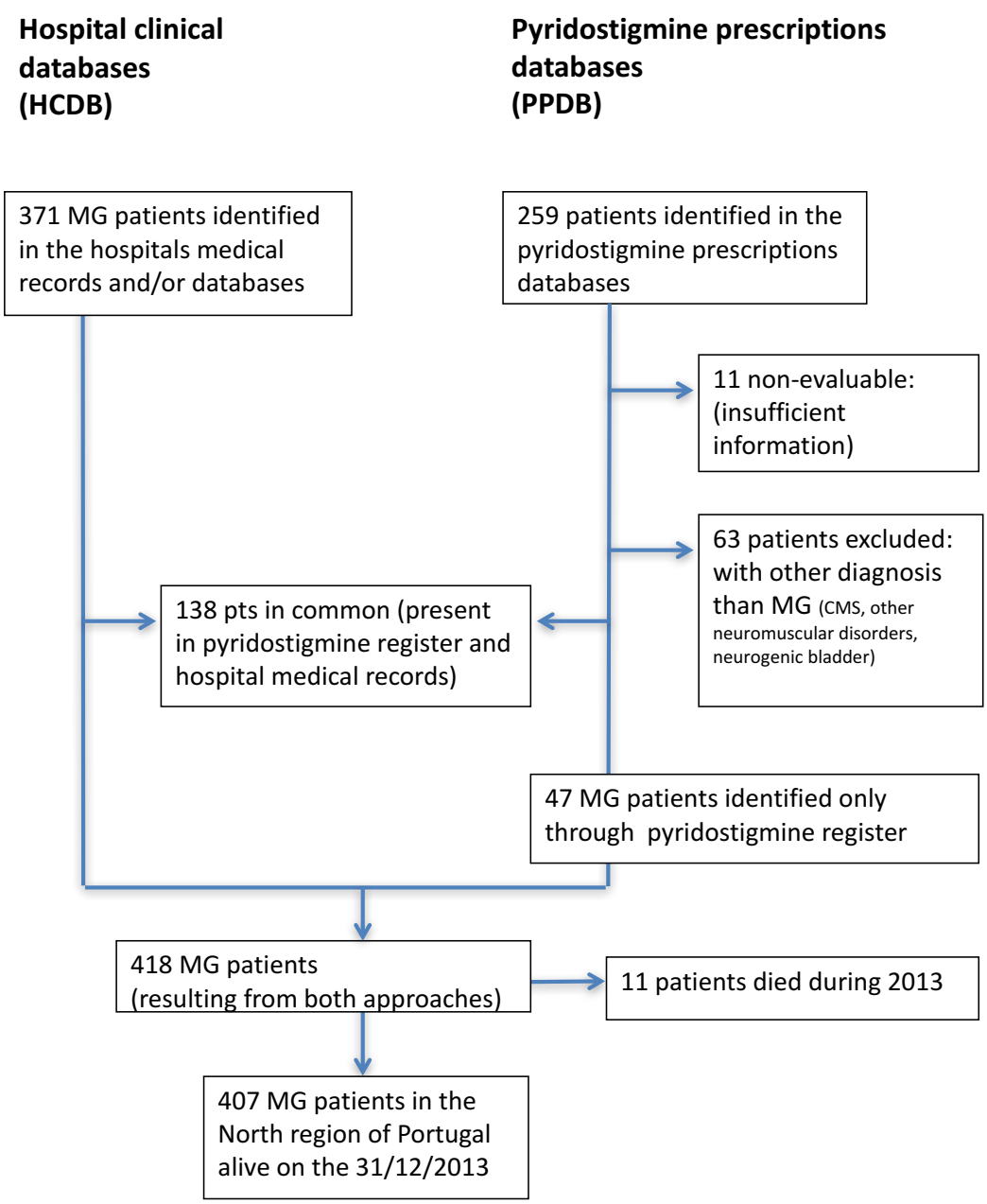

Figure 3.1.1. Patient identification. *Illustration of the sources of identification of the myasthenia gravis patients included in the study. Patients were identified via hospital clinical databases (HCDB) and pyridostigmine prescription databases (PPDB)*

Epidemiological data

At 31/12/2013 we estimated a point prevalence of 111.7 patients per million inhabitants (407 MG patients/3 644 195 inhabitants). During the year of 2013 there were 23 new MG patients. We estimated an incidence rate of 6.3 per million person/year.

We found different prevalence and incidence rates according to the age group and sex (Table 3.1.2. and Figure 3.1.2.). Prevalence rose with age reaching its maximum in the group over 65 years old, especially in males (288.1/10^6) (Table 3.1.2. and Figure 3.1.2A.).

Among females, incidence rate was higher in the age group 15-49 (9.1/10^6), while in males, incidence increased with age till the maximum (22.1/10^6) among those aged over 65 years (Table 3.1.2. and Figure 3.1.2B).

Table 3.1.2. Variation in prevalence and incidence rate with the age

Age Group	Estimated Population of females	n (PR/10^6)	n (IR/10^6)	Estimated Population of males	n (PR/10^6)	N (IR/10^6)	Estimated Total Population	N (PR/10^6)	n (IR/10^6)
0-14	254 838	0 (0)	0 (0)	265 937	1 (3.7)	0 (0)	520 775	1 (1.9)	0 (0)
15-49	877 161	112 (127.6)	8 (9.1)	842 519	40 (47.4)	1 (1.1)	1 719 680	152 (88.3)	9 (5.2)
50-64	393 757	67 (170.1)	1 (2.5)	357 690	52 (145.3)	4 (11.1)	751 447	119 (158.3)	5 (6.6)
≥65	381 601	57 (149.3)	3 (7.8)	270 692	78 (288.1)	6 (22.1)	652 293	135 (206.9)	9 (13.7)

PR/10^6 = Prevalence per million at 31/12/2013. IR/10^6 = Incidence rate per million-persons-year in 2013

Prevalence rates in EOMG and LOMG were similar (106.2/10^6 and 113.2/10^6 respectively) (Table 3.1.3.). However, when the prevalence was analysed in relation to gender, females were predominant in EOMG and males in LOMG group (EOMG prevalence was 144.8/10^6 for females, and 66.7/10^6 for males, whereas LOMG prevalence was 85.1/10^6 for females and 147.9/10^6 for males) (Table 3.1.3. and Figure 3.1.2C.).

Table 3.1.3. Variation in prevalence and incidence rate for EOMG and LOMG

Age of MG onset	Estimated Population for females	n (PR/10^6)	N (IR/10^6)	Estimated Population for males	n (PR/10^6)	n (IR/10^6)	Estimated Total Population	n (PR/10^6)	N (IR/10^6)
EOMG	1 131 999	164 (144.8)	8 (7.0)	1 108 456	74 (66.7)	1 (0.9)	2 240 455	238 (106.2)	9 (4.0)
LOMG	775 358	66 (85.1)	4 (5.1)	628 382	93 (147.9)	10 (15.9)	1 403 740	159 (113.2)	14 (9.9)

EOMG - MG onset between 0-49 y. LOMG – MG onset ≥50 y. PR/10^6 = Prevalence per million at 31/12/2013. IR/10^6 = Incidence rate per million-persons-year in 2013

Incidence rates were 4.0 per million person/year for EOMG and 9.9 for LOMG (Table 3.1.2). When incident rates were compared between genders, EOMG incident cases were predominantly females, whereas LOMG incident cases were predominately males (Table 3.1.2. and Figure 3.1.2D.).

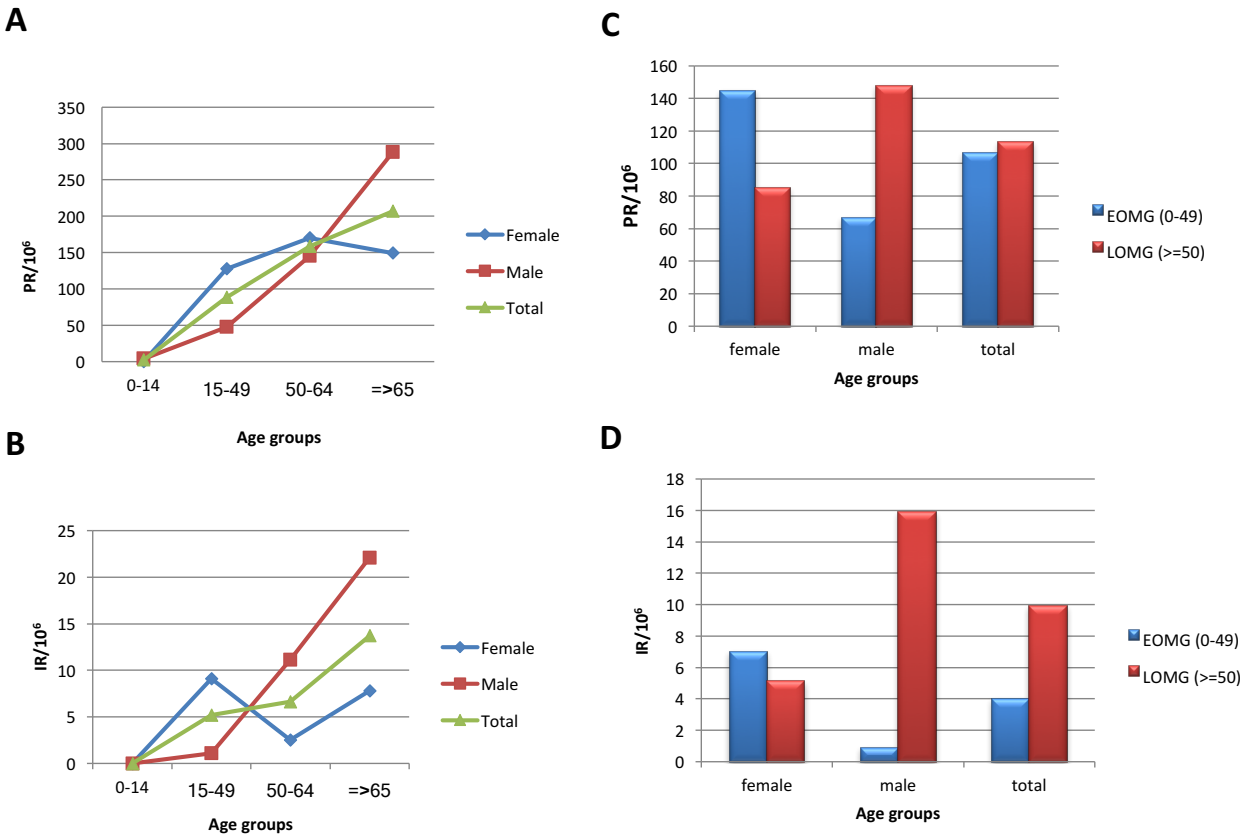

Figure 3.1.2. *Prevalence rate (A and C) and incidence rate (B and D) per million increased overall with age at disease onset, but the striking increase was noticed in the incidence rate in males with late onset MG. PR/106: Prevalence rate per million; IR/106: Incidence rate per million*

Demographical and clinical data of prevalent cases

Characteristics of prevalent cases are summarised in Table 3.1.4.
Most cases were women (58.0%). Age at 31-12-2013 ranged between 7 and 92 years (mean 54.7 years). The age of onset varied between 3 and 87 years (mean 43.7 years), and majority of patient (62.4%) had LOMG.
The ratio female:male in the EOMG group was 2.73:1 and in the LOMG 0.95:1. Ocular forms accounted to 30.1% total cases, and they were predominant among LOMG (57.1%).
The predominant MGFA classification at nadir was class II (57.9%). From 165 patients submitted to thymectomy, majority of them (69,

41.8%) had thymic hyperplasia, whereas (49, 29.7%) had thymoma (40.8% of them occurred in the group 15-49 years and 59.2% after 50 years of age).

Majority of patient (62.8%) were treated with steroids and 33.9% with additional immnunossupression. A relatively high percentage of patients (19%) needed intravenous immunoglobulin (IVIG) or plasma exchange (PEX) at least once during the course of the disease. After a median follow-up period of 7 years (<1 to 42), 41.0% of patients were in remission, either complete (2.6%) or pharmacological (38.4%).
Most of the patients (63%) in remission belonged to the group of EOMG, of which 22.8% had ocular MG and 77.2% had generalised MG.

Almost one third of patients (29.4%) had one type of the following comorbidities: other autoimmune diseases (OAID) (organ specific or systemic), tumours or recurrent/serious infections; 2.8% of the patients had ≥2 of those associated problems.
The proportion of patients with OAID (20.2% vs 16.3%) and infections (4.6% vs 4.4%) was identical in EOMG and LOMG groups. Only tumours were more common in LOMG (12.6% vs 5.9%, p= 0.026).

Table 3.1.4. Demographic and clinical data of the prevalent cases during 2013

Variable	Values		n	%
Sex	F		236	58.0
	M		171	42.0
Age in 31 Dec 2013	0-14		1	0.2
	15-49		152	37.4
	50-64		119	29.2
	>=65		135	33.2
Age of MG onset	0-14		10	2.5
	15-49		228	57.4
	50-64		82	20.7
	>=65		77	19.4
	Missing		10	-
Muscular Involvement (form of disease)	Ocular		120	30.1
	Generalized		279	69.9
	Missing		8	-
Serological status	AChR antibody positive		261	71.5
	MuSK antibody positive		19	5.2
	Seronegative		85	23.3
	Missing		42	-
Thymic histology (thymectomy performed in 165 of the 407 patients (40.5%)	Thymoma		49	34.0
	Thymic hyperplasia		69	47.9
	Normal/Atrophic		25	17.4
	Other (thymic cyst)		1	0.7
	Missing		21	-
MG subtype: According to Ab status and presence of thymoma	AChR MG +		261	71.5
		Thymoma +	46	12.8
		Thymoma -	210	58.3
	MuSK MG +		19	5.3
		Thymoma +	0	0
		Thymoma -	19	5.3
	Seronegative		85	23.6
		Thymoma +	3	0.8
		Thymoma -	82	22.8
	UKN	-	42	-
Worst MGFA Clinical Classification	I		120	30.0
	II		231	58.0
	III		36	9.0
	IV		3	0.8
	V		9	2.2
	Missing		8	-
Non-surgical Treatments	Pyridostigmine		399	98.0
	Steroids		243	61.8
	Imunossupression		134	33.9
	Plasma Exchange		10	2.5
	IV IG		75	19.0
Follow-up (years)	≤ 2		97	24.4
	3-10		169	42.6
	11-20		76	19.1
	>20		55	13.9
	Missing		10	-
MGFA Status Pos-intervention	CR		10	2.6
	PR		150	38.4
	I		91	23.3
	U		131	33.4
	W		9	2.3
	Missing		16	-
Comorbidities (OAID, tumours, or severe infections)	None		279	70.6
	One		105	26.6
	Two or more		11	2.8
	Missing		12	-

F - female, M - male, IVIG – intravenous immunoglobulins, CR - complete remission, PR - pharmacological remission, I - improved, U - unchanged, W - worsened, OAID - other autoimmune disorders.

Serological Data

During the year 2013, 128 blood samples were collected from patients whose serological status had not been completely studied.
Median time between MG onset and the blood collection in that group of patients was 7 years (0 – 42 years, standard deviation 10.7 years).
Of the total 407 patients, we could not have a definite serological status in 42 (10.3%).
Of the 365 patients with complete serological status, 261 had AChR antibodies (71.5%), among which 46 (17.6%) had thymoma. 19 patients had MuSK antibodies (5.2% of the 365 patients tested and 18.2% of the 104 seronegative for AChR); the remaining 85 patients were double seronegative (23.6%) (Table 3.1.4.).

Demographical and clinical data of incident cases

The incident data are summarised in the Table 3.1.5. In the incident cases ratio female:male was similar to the prevalent data; in 60.9%, the age of onset was ≥50 years old, which is also very similar to the ratio registered in the prevalent data (62.4%). 57.1% were AChR positive and 14.3% Musk positive. 56.5% had generalised disease; 17.4% of the patients there was at least one comorbidity.

Table 3.1.5. Demographic and clinical data of the incident cases during 2013

Variables	Values		N (=23)	%
Sex	F		12	52.2
	M		11	48.8
Age in 31 Dec 2013 (= age of onset)	<15		0	0
	15-49		9	39.1
	50-64		5	21.8
	>=65		9	39.1
MG subtype	AChR MG +		12	57.1
		Thymoma +	4	19.0
		Thymoma -	8	38.1
	MuSK MG +		3	14.2
		Thymoma +	0	0
		Thymoma -	3	14.2
	Seronegative		6	28.6
		Thymoma +	0	0
		Thymoma -	6	28.6
	Missing	-	2	-
Thymic Hystology	Thymoma		4	17.4
	Without thymectomy		19	82.6
Muscular Involvement	Ocular		10	43.5
	Generalised		13	56.5
Worst MGFA Clinical Classification	I		10	43.5
	II		10	43.5
	III		3	13.0
	IV		0	0
	V		0	0
Comorbidities (OAID, tumours, or infections)	None		19	82.6
	One		3	13.0
	Two or more		1	4.4

MGFA - Myasthenia Gravis Foundation of America, OAID - other autoimmune disorders

Description of cause of deaths occurred during the year of 2013

The percentage of patients who died during 2013 was 2.6% (11/418) (details in Supplementary Table 3.1.6.). Deaths directly related with MG were 2/418 (0.48%), which represents a mortality rate directly attributed to MG of 0.5 per million (2/3 644 195). These deaths were consequence of severe dysphagia and respiratory failure/infections. They occurred in patients with a recent diagnosis and not yet controlled MG, at, respectively, 85 and 73 years of age. Three patients died with aggressive thymoma related complications; they were 25, 56 and 72 years old (ages of onset had been respectively 25, 41 and 67). In 4 patients (aged between 84 and 89 years at the date of death), the cause of death was not apparently related to MG.

Table 3.1.6. Description of cause of death (n=11) during the year 2013

Number of patients (n)	Cause of Death	Age at disease onset	Age at death
3	Aggressive thymoma	41, 67, 25	56, 72, 25
2	MG related	70, 84	73, 85
3	Acute respiratory tract infection	68, 72, 62	84, 87, 88
1	Malignant haematological disease	88	89
2	UKN	UKN, 50	85, 87

UNK - unknown

Discussion

Epidemiological figures

We found a prevalence of 111.7 MG patients per million, which is similar to those reported in some European studies, e.g. Italy,[122] using similar methods to our study, and in Norway, in studies with similar methods and size of the population studied.[125,126] It is also very close to another previous Italian study in the Regio-Emilia region, with multiple sources of identifying patients but a much smaller size of population studied.[128]

We estimated an incidence rate of 6.3 per million person/year. Our incidence rate is similar to recent studies from different latitudes (Norway and Denmark) with similar methods, where it varied from 7.2 to 9.2 per million persons year,[121,125,126] and also identical to other studies from south of Europe - Greece and Italy,[127,128] where it varied from 7.4 to 7.8 per million person/year. It was, however, much lower than some recently published studies from Spain (Osona, Barcelona) [80] and Australia[176] where they found incidence rates above 20 per million persons year.

Estimated incidence and prevalence rates of MG still vary widely.[129,80,176] A meta-analysis of epidemiological studies reports prevalence rates 15 times greater than incidence rates.[182]

Recent studies report lower prevalence to incidence ratios ranging from 6 to 8.[122,183] In our study this ratio is 17.

When comparing our study with the study in the province of Osona which corresponds geographically to the region closest to the north of Portugal, they presented a much higher prevalence (328.9) and incidence rate (28.0) than ours, but also higher than all other studies. It may be related with the awareness campaign about the disease, mostly in the elderly, that started with the same group in that region in 2003 and also with the small size of the population studied (around 150 000).[80,116,123]

Using two methods of identifying patients (HCDB and PPDB) improves the quality of the data; it was previously described that using a single register source to identify MG cases has a higher sensitivity but a lower positive predictive value than the combined register method applied in this study.[121,175]

In our study, 11 out of the 499 (2.2%) patients of the pyridostigmine prescriptions could not be confirmed to have MG, which is a small proportion of the number of patients studied when compared with other studies where MG diagnosis could not be verified by medical record review in about 27% of the cases.[183]

In some studies that used pyridostigmine prescriptions as the only method to identify patients, the prevalence and especially incidence rate may be overestimated.[176] Reasons may be that pyridostigmine may be also used for other neurological disorders, like congenital myasthenic syndromes, myopathies and neurogenic bladder, and also that one prescription does not correspond necessarily to a new MG patient. In the daily practice sometimes pyridostigmine is used as a trial of treatment in circumstances of diagnostic uncertainty.[176]

To our knowledge, our study was the first to verify with the GP which disease was being treated with pyridostigmine. We found that around one quarter of the prescriptions were for other conditions than MG.

It is possible that, in our study, some patients in complete remission discharged from the hospitals clinics and not taking pyridostigmine

were not captured with our methodology. However, it is common in Portugal for patients in complete remission to continue being followed up yearly by a neurologist.

In this study prevalence rises with age, as reported in many other studies,[121,183] especially in males. The highest value was in males above 65 years, 288.1 per million.

Incidence rates were highest in the female EOMG group and male LOMG group, reaching its peak in males after 65 years (22.1 per million person/year). We do not have information about the evolution of these rates in our population, because there are no previous studies in Portugal. But this result is according to most of the recent reports that describe higher incidence rates of MG in LOMG group, especially in elderly males.[121,122,129,153] This information has crucial importance for the continued awareness of the disease in this age group, and subsequent improvement in diagnosis and management of MG.

Clinical, demographic and serological features

Our prevalent and incident data confirmed that the majority of patients belong to the group of LOMG, confirming others' findings that MG is a disease that affects younger and also elderly people. Overall our clinical, histology and serological data were similar to those reported by other authors. There were, however some discrepancies, which deserve some comments.

Overall a surprisingly high percentage of patients (19%) were treated with IVIG or PEX at least once during the course of the disease, particularly knowing that majority of patients had MGFA I or II at nadir. Majority of patients treated with IVIG or PEX represented a third of the MuSK MG patients and nearly a quarter of the AChR MG patients, which is in keeping with our practice preferring more intense treatment in Ab positive patients with acute bulbar symptoms with or without respiratory manifestations.

The percentage of AChR positive patients in generalised forms of the disease was lower than expected, 76.7% compared with the 85-95% usually reported.[117,181] This might be related with the fact that in 35% (128/365) of the patients completely studied for their serological status, even considering that we used RIA and CBA methods, the blood had been collected at a variable time after the diagnosis (median 7 years). Therefore, it is possible that some of the patients had already become negative as a consequence of treatments.[181] When possible, blood sample collection should be done at the time of the diagnosis and before immune treatments.

Murai et al., in a nationwide survey of MG patients in Japan, reported a prevalence rate of 118 per million, similar to our study and also reported other clinical data similar to ours, including percentage of patients who were AChR-Ab positive at diagnosis. Other similar features included: MGFA I and II accounted for 80% of all MG patients; thymoma was present in 32.0%; and hyperplasia was observed in 38.4%.[147]

Interestingly, in their study, there was 1 important difference compared with ours: They reported a prominent peak in infantile-onset MG (age 0–4 years, 7%), but in our study, there were few infantile MG patients. Our lower number of infantile-onset MG patients (2.5%) is similar to findings from most European and American series where it varied from 1% to 3%.[184]

We found a percentage of 5.3% of MuSk MG in the group of prevalent patients, which is similar to what has been described by other authors in the southern European countries.[185] Interestingly, the percentage in the incident cases is higher, 14.3%. On the other hand, the proportion of AChR-MG in the group of incident cases was lower than expected, possibly because the proportion of ocular MG was relatively high, 43.5%. This may represent variations in incidence of distinct disease subtypes or improvement in diagnosis of rarer and limited forms of MG.

The mortality rate attributed to MG was 0.5 per million, which is in the range of what others studies reported.[122,129] Of note, 3 deaths occurred in relation with aggressive thymomas. Regarding age at death, six occurred between 84 and 89 years, an age that is above the Portuguese life span (82.8 for females and 76.9 for males).[178]

Final Comments

The methods used in this study were robust and allowed us to find a similar prevalence to those described in most of the studies in Europe. The incidence rate was lower to some of recent European studies but we have to consider that our study was, to our knowledge, the first to confront the number of pyridostigmine prescriptions with the pyridostigmine prescriptions for MG and not to other disorders.

The highest prevalence and incidence rates were found in males above 65 years of age, which are very relevant findings for neurologists and GPs daily practice when considering differential diagnosis and disease management in that age group.

3.2. Immunogenetic study

Within this research project, we performed an immunogenetic study to explore the association between HLA-DRB1 susceptibility alleles and age-of-onset in MG, in the cohort of patients followed in the Neuroimmunology Outpatients clinics of Centro Hospitalar do Porto/Hospital Santo António.

The immunogenetic study was performed in Laboratório de Imunogenética, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal, under supervision of Prof. Dr. Berta Martins da Silva.

It was published in ***Neuromuscular Disorders 2017; 27(7):650-654*** and the full text follows.

HLA and age of onset in Myasthenia Gravis

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Abstract

Introduction: Myasthenia gravis (MG) is a classic autoimmune neurological disorder with a range of well-known symptoms and signs. The aetiology of MG is unknown, but both genetic and environmental factors are believed to have important roles. Over the years, association of MG with Human Leucocyte Antigens (HLA) has been described in different populations. In European descendent populations HLA-DRB1*03 allele strongly influences MG susceptibility.

Objective: To investigate the possible association between HLA-DRB1 alleles and age-of-onset in MG.

Patients and Methods: One hundred and fourteen MG patients (82 female and 32 male) and 282 control individuals (CP) were studied. Patients were classified according to the age of onset (early-onset ≤40, n=74 and late-onset ≥ 50, n=20).
Patients with thymoma (n=20) were analysed separately. HLA-DRB1 and HLA-A*08 genotyping was performed using PCR-SSP methodology. A stepwise logistic regression at an allelic level, using forward selection, was applied to identify the HLA-DRB1 genes contributing to MG.

Results: HLA-DRB1*03 allele was overrepresented in the global MG population when compared to the control population (34.5%MG vs. 15.6%CP, p<0.0001, OR=2.85, 95%CI=1.72-4.72). When the early-onset subgroup was considered, this association became even stronger (43.0%MG vs. 15.6%CP, $p=1x10^{-6}$, OR=4.16, 95%CI=2.35-7.22). Regarding the late-onset subgroup, the frequency of HLA-DRB1*01 allele was higher than in the control population (50.0%MG vs. 23.4%CP, p=0.011, OR=3.27, 95%CI=1.31-8.20). For the thymoma subgroup, the HLA-DRB1*10 allele frequency was significantly higher (15.8% MG vs. 3.9% Controls, p=0.029, OR=4.62, 95% CI=1.17-18.23) when compared to the control population. Regarding the HLA-B*08 allele, its frequency was significantly higher in the global MG population (30.6%MG vs. 13.9%PC, p=0.0004, OR=2.72, 95%CI=1.75-4.78).

Conclusions: These results demonstrate a strong association of HLA-DRB1*03 and HLA-B*08 with MG, confirming that these alleles are important susceptibility factors for this disease also in our population. Considering the age of onset, HLA-DRB1*01 was associated with late-onset subgroup. Thymoma MG patients have probably a different genetic (HLA- DRB1*10) background. To the best of our knowledge these results were not reported before and need replication in other populations and in larger cohorts.

Introduction

Myasthenia Gravis is not considered a single entity but a heterogeneous disease with several subgroups. Although these subgroups share a common phenotype they have distinct features based on age of onset, severity of the disease, pathogenic auto-antibodies, thymic abnormalities, response to therapy and different HLA susceptibility alleles.[20,39] So far seven subgroups have been considered: early onset MG (EOMG), late onset MG (LOMG), paraneoplastic MG or thymoma MG, anti-MuSK MG, anti-LRP4 MG, ocular MG and seronegative MG.[20]

The first two subgroups are characterized by anti-acetylcholine receptor antibodies and mainly differ in the age of onset and response to thymectomy. Most authors consider that the age of onset of 50 years separate these two subgroups.[121,122,125,126,183] Another striking feature that divides them is the presence of thymic hyperplasia, which is much more common in the early onset group[186], and the associated HLA susceptibility alleles. Compston was one of the first to describe this difference. In his work the early onset subgroup (up to 40 years of age) had a higher frequency of the HLA B8 DR3 haplotype and the late onset group of the HLA B7 DR2 haplotype.[39] Since then several HLA associations have been described supporting the distinctiveness of these two subgroups.[134,187,188] More recently an association of HLA-DRB1*15:01 and HLA-DQB1*05:02 and DRB1*16 in the late onset group, has been reported.[189,190] Other HLA associations in thymoma MG, MuSK MG and ocular MG have been reported but not so consistently.[187,191]

The main purpose of this study was to evaluate the association of HLA DRB1 alleles and HLA-B*08 with MG and its subgroups in a Portuguese cohort. We want also to highlight the importance of appropriate MG subgrouping according to clinical characteristics and its comparison with similar studies previously made for other ethnic groups.

Patients and Methods

Case Definition

MG was diagnosed if a patient had a typical clinical feature and positive antibodies for anti-acetylcholine receptor and/or positive repetitive nerve stimulation on electromyography. Patients with onset before 50 years of age were considered Early Onset Myasthenia Gravis (EOMG). Late onset Myasthenia Gravis (LOMG) patients were those with onset after 50 years of age. Thymoma MG subgroup were those with histopathological confirmation of the disease. Patients with anti-MuSK antibodies were excluded.

Refractory patients were defined as those that could not scale back their immunotherapy without clinical relapse, were not clinically controlled on an immunotherapy regimen, or developed severe side effects from immunosuppressive therapy.[88]

Polyautoimmunity was defined as the presence of more than one autoimmune disease in a single patient.[192]

HLA Genotyping

Peripheral blood samples were collected in EDTA. Genomic DNA was obtained from proteinase-K–treated peripheral blood leukocytes by using a salting-out procedure.[193] Low-resolution genotyping for HLA–DRB1 locus (i.e., 2-digit HLA nomenclature) was performed using polymerase chain reaction with sequence-specific primers (PCR-SSP), based on methods previously described.[194]

Control population

Patients were compared with a control group consisting of 282 unrelated individuals, from the same geographic origin (north of Portugal).

Statistical Analysis

To identify the HLA-DRB1 alleles contributing to MG, a stepwise logistic regression, using forward selection was applied, which involves starting

with no variables in the model, testing the addition of each variable using a chosen model comparison criterion, adding the variable (if any) that improves the model the most, and repeating this process until none improves the model. It should be noted that odds ratios (ORs) obtained in a multivariable logistic regression analysis are adjusted for all the other genes included in the model and therefore differ from those obtained when a given gene is compared with all other genes. For the HLA-B*08 allele analysis a Chi-square or Fisher's exact test was used, as appropriate. The data were analysed using the IBM SPSS 20 statistical software suite.

Results

Clinical analysis

One hundred and fourteen patients were included in the study. The clinical characteristics of these patients are described on the table 3.2.1.

Table 3.2.1. Clinical characteristics of the myasthenia gravis patients (n=114)

	n=114	Female (%)	Anti-RAch Ab's (%)	Thymectomy/ Thymus histology (%)	G (%)	PolyAI (%)
EOMG	74	63 (84)	61 (82.4)	48 (64.9) 30 Hyperplasia (62.5) 18 Normal (37.5)	69 (93.2)	19 (25.7)
LOMG	20	8 (40)	15 (75)	0/NA	13 (65)	2 (10)
Thymoma MG	20	11 (55)	20 (100)	20 Thymoma	20 (100)	6 (30)

G - Generalized, PolyAI – polyautoimmunity, NA-not applicable.

In the 48 EOMG group, 30 (62.5%) had thymic hyperplasia and performed thymectomy. In the LOMG subgroup all patients had normal thorax CT scan and were not submitted to thymectomy.

Twenty two patients presented criteria for refractory disease [88], 1 in the LOMG (5%), 6 in the thymoma group (30%) and the remaining 15 in the EOMG subgroup (20,3%).

Polyautoimmunity was present in 27/114 (23.7%), and was more common in the EOMG and thymoma subgroups than in the LOMG subgroup (25.7 and 30% vs. 10%).

Immunogenetic analysis

HLA-DRB1*03 allele was overrepresented in the global MG population when compared to the control population (34.5%MG vs. 15.6%CP, p<0.0001, OR=2.85, 95%CI=1.72-4.72), as expected. When the early-onset subgroup was considered, this association became even stronger (43.0%MG vs. 15.6%CP, p=1x10-6, OR=4.16, 95%CI=2.35-7.22).

In the late-onset subgroup, but not in the EOMG subgroup, the frequency of HLA-DRB1*01 allele was higher than in the control population (50.0%MG vs. 23.4%CP, p=0.011, OR=3.27, 95%CI=1.31-8.20).

For the thymoma subgroup, the HLA-DRB1*10 allele frequency was significantly higher (15.8% MG vs. 3.9% Controls, p=0.029, OR=4.62, 95% CI=1.17-18.23) when compared to the control population.

The age of onset was also analysed, disregarding thymic histology, which means thymoma patients were included. In the EOMG group (before 50 years of age) the HLA-DRB1*03 allele frequency was significantly higher compared to controls (39.0% MG vs. 15.6% PC, p<0.0001, OR=3.43, 95%CI=1.99-5.90). The frequency of HLA-DRB1*01 allele remained overrepresented in the MG patients with onset after 50 years vs. CP (42.9% MG vs. 23.4% PC, p=0.024, OR=2.45, 95%CI=1.11-5.45).

Regarding the HLA-B*08 allele, its frequency was significantly higher in the global MG population (30.6% MG vs. 13.9% PC, p=0.0004, OR=2.72, 95%CI=1.75-4.78). This association was only significant in the EOMG subgroup (37.0%MG vs. 13.9% CP, $p=2x10^{-5}$, OR=3.62, 95%CI=1.96-6.71). There was no association of this allele with the LOMG subgroup (20.0% MG vs. 13.9 %CP, p=0.462, OR=1.54, 95%CI=0.48-4.94), nor with the thymoma subgroup (16.7% MG vs. 13.9% CP, p=0.750, OR=1.23, 95%CI=0.34-4.53) were observed.

Table 3.2.2. HLA DRB1 frequencies in myasthenia gravis and control population

	Controls (n=282)	Global MG (n=114)	EOMG (n=74)	LOMG (n=20)	Thymoma MG (n=20)
HLA-DRB1*01	**66 (23.4%)**	29 (25.7%)	16 (21.6%)	**10 (50.0%)**	3 (15.8%)
HLA-DRB1*03	**44 (15.6%)**	**39 (34.5%)**	**32 (43.2%)**	3 (15.0%)	4 (21.1%)
HLA-DRB1*04	69 (24.5%)	30 (26.5%)	18 (24.3%)	7 (35.0%)	5 (26.3%)
HLA-DRB1*07	72 (25.5%)	24 (21.2%)	12 (16.2%)	7 (35.0%)	5 (26.3%)
HLA-DRB1*08	24 (8.5%)	8 (7.1%)	5 (6.8%)	1 (5.0%)	2 (10.5%)
HLA-DRB1*09	14 (5.0%)	3 (2.7%)	3 (4.1%)	0 (0.0%)	0 (0.0%)
HLA-DRB1*10	**11 (3.9%)**	5 (4.4%)	2 (2.7%)	0 (0.0%)	**3 (15.8%)**
HLA-DRB1*11	55 (19.5%)	19 (16.8%)	15 (20.3%)	2 (10.0%)	2 (10.5%)
HLA-DRB1*12	9 (3.2%)	1 (0.9%)	1 (1.4%)	0 (0.0%)	0 (0.0%)
HLA-DRB1*13	84 (29.8%)	21 (18.6%)	11 (14.9%)	4 (20.0%)	6 (31.6%)
HLA-DRB1*14	17 (6.0%)	7 (6.2%)	4 (5.4%)	3 (15.0%)	0 (0.0%)
HLA-DRB1*15	56 (19.9%)	19 (16.8%)	12 (16.2%)	2 (10.0%)	5 (26.3%)
HLA-DRB1*16	13 (4.6%)	10 (8.8%)	8 (10.8%)	1 (5.0%)	1 (5.3%)

The association between HLA genotypes with other MG subgroups (refractory MG patients and the group with polyautoimmunity) was also analysed, but no statistically significant differences were found.

Discussion

Clinical data

Our clinical data shows a predominance of females (84%) in the EOMG subgroup and a predominance of the male gender (60%) in the LOMG subgroup. The frequencies of generalized disease (93.2% vs. 65%) and positivity to antibodies to AChR (82.4 vs. 75%) were higher in the EOMG subgroup than in the LOMG. These findings are similar to other published series.[112,116,124–127,189]

The frequency of polyautoimmunity was high (23.7%) in our cohort. Robertson found a frequency of 27% in a Cambridgeshire (UK) MG population.[112] In the Greek population Poulas described polyautoimmunity in 11.6% of patients.[127] An Italian study registered polyautoimmunity in 14.3% of the cases.[128] In the present study polyautoimmunity was more common in the EOMG (25.7%) and thymoma (30%) subgroups than in the LOMG subgroup (10%). Polyautoimmunity was also more common in the women of the EOMG subgroup in the

Cambridgeshire study (49%).[112] Other series such as Aragones in Barcelona described higher frequency of other autoimmune disorders in the LOMG subgroup than we described in the present study (21.5%).[123] Similar frequencies in the EO and LOMG subgroups[189] in other populations have been reported.

Immunogenetic Data

We registered a positive association with the HLA-DRB1*03 allele in the global MG population, when compared to the control population. This association was even stronger in the EOMG subgroup. This is consistent with other important studies.[39,105]

As reported in other fundamental studies[39,189,195,196] the classical HLA-B*08 allele was associated in this study in the global MG population and in the EOMG subgroup.

Regarding the LOMG subgroup a positive association with the HLA-DRB1*01 allele was found. This association has not been described before. A Tunisian study described an association with HLA DRB1*04 allele[197], a Norwegian study an association with HLADRB1*15[189] and an Italian study with HLA-DQB1*05:02 and DRB1*16 alleles.[190]

Our findings regarding the EOMG and LOMG were still value even when dividing the patients in subgroups just considering the age and not thymic pathology.

For the thymoma MG subgroup we found an association with the HLA-DRB1*10 allele when compared to the control population. The study with Mexican MG mestizo patients showed an association to HLA DRB1*11 in thymomatous patients.[198] In a Chinese study it was found an association of thymomatous MG with HLA-DQA1*0401 and HLA-DQB1*0604 but not with the alleles of HLA DRB1 locus.[199]

Regarding HLA-DRB1*13 allele it was described to be a protective factor for MG in a Norwegian study[189] and to other auto-immune disorders[200], but in our study we could not find the same. It was less frequent in the EOMG subgroup when compared to the CP, but did not

reach a statistically significant difference. This might be related with the sample size.

We could not find any association between HLA genotypes for the group of refractory MG patients or the group with polyautoimmunity. These might be because of the reduced size of the sample.

Taking together these results demonstrate a strong association of HLA-DRB1*03 and HLA-B*08 alleles with MG, confirming that these alleles are important susceptibility factors for this disease also in our population. Considering the age of onset, HLA-DRB1*01 was associated with late-onset subgroup. Thymoma MG patients have probably a different genetic (HLA-DRB1*10) background. To the best of our knowledge these results have not been reported before and warrant replication in other populations and in larger cohorts.

3.3. Pregnancy and Myasthenia gravis

Under this topic, two studies were conducted:

- **Myasthenia gravis in Pregnancy – experience of a Portuguese Centre;**

- **MuSK Myasthenia gravis and Pregnancy.**

3.3.1. Myasthenia gravis in Pregnancy – experience of a Portuguese Centre

Within this research project, we performed a retrospective study on the pregnant women with MG in our cohort in Centro Hospitalar do Porto: Hospital Santo António and Centro Materno-Infantil do Norte Dr. Albino Aroso. We studied both the effect of the disease on pregnancy, delivery and foetus, and the effect of pregnancy on the disease course.

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Myasthenia gravis in Pregnancy – experience of a Portuguese Centre

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Abstract

Objective: Evaluate the clinical course during pregnancy and neonatal outcomes of a cohort of Portuguese patients with myasthenia gravis (MG).

Methods: Retrospective study of pregnancy in a Portuguese cohort of MG patients.

Results: 25 patients with 30 pregnancies were included. The mean maternal age was 32.4±4.1 years. A miscarriage rate of 6.7% was observed, with the delivery of 28 newborns. 43.3% had deterioration of MG symptoms during pregnancy and 46.4% at postpartum. 90% were medicated with pyridostigmine, 43.3% with corticosteroids and 40% needed intravenous immunoglobulin to achieve symptomatic control. There were no maternal or neonatal deaths. Mean gestational time at delivery was 38.2 weeks. No cases of fetal growth restriction, preeclampsia, preterm delivery or fetal demise were observed. Global cesarean rate was 64.3%. Two newborns developed transient neonatal myasthenia.

Conclusions: A high rate clinical worsening of MG status in the mother was observed in this retrospective study, which highlights the importance of a multidisciplinary approach to avoiding maternal adverse outcomes.

Introduction

Myasthenia Gravis (MG) is relatively rare autoimmune disorder of the striate muscle causing neuromuscular transmission failure, clinically characterized by muscle weakness and fatigue.[11]

It is mediated by autoantibodies of the IgG isotype against the striate muscle acetylcholine receptors (AChR).[11] These antibodies reduce the number of AChR present in the end plate of the neuromuscular junction, reducing the efficiency of the neuromuscular synaptic transmission. AChR number is reduced because these antibodies induce complement-mediated lysis of AChR and increasing the rate of AChR internalization by creating AChR-antibody complexes.[11]

AChR antibodies are present in 40-60% of patients with ocular MG and 85% of generalized form.[201] When AChR antibodies are not detected, other humoral factors must be involved in the inactivation of the AChR. Antibodies against the muscle-specific kinase (MuSK), a protein involved in anchoring AChR in the cellular membrane, are detected in up to 40% of these “seronegative” patients, but there is a significant variation in the incidence of MuSK-MG across the world.[202]

More recently antibodies to receptor related low density lipoprotein-4 (LRP-4) were also associated with to some cases of seronegative MG. LRP-4 is a receptor for agrin and for MuSK, which are important in the aggregation of AChR in the neuromuscular junction plate.[26]

Anti-AChR and anti-MuSK antibodies are of IgG isotype, so they have the ability to cross the placental barrier and generate myasthenic symptoms in the fetus in the later, half of pregnancy and transiently in the newborn (transitory neonatal MG).[170,203]

It affects 2 to 3 times more women than men during the second and third decades of life, so association between MG and pregnancy is not improbable with an estimated incidence during pregnancy of 1:20,000.[161]

MG is not associated with infertility, but it exposes pregnant women to an increased risk of maternal and fetal complications.

The clinical course of pregnancy in these patients is unpredictable. Symptoms at the date of conception, the requirement to treat active MG symptoms, as well as the course of previous pregnancies do not seem to correlate with the course of a specific pregnancy in mothers who suffer from MG.[159]

It has been described that about 30% of pregnant women experienced improvement of symptoms while a third face clinical worsening, especially in the first trimester and during postpartum.[159,160] An increase in the exacerbation rate during pregnancy in the first two - three year after diagnosis was also described[161]. So it is advised to postpone pregnancy one to two - three years after diagnosis.[159,161]

As smooth muscle is not affected in MG, indication for cesarean delivery is usually reserved for obstetric indications, however, I would also emphasise that 3rd stage of labour involves the contraction of voluntary striated muscle and this is where assistence in vaginal delivery is required to reduce the expulsive efforts and then minimize mother's fatigue.[159] However, the incidence of cesarean delivery seems to be increased in these patients.[162]

The risk of preterm birth, low neonate birthweight or hypertensive disorders apparently are not increased in MG pregnant women.[164] However, the management of preeclampsia is particularly challenging because the use of magnesium sulfate can exacerbate symptoms of myasthenia and potentially lead to myasthenia crisis and therefore should be used with extreme caution. When eclampsia is present, alternative medication like phenobarbital or anticonvulsant narcotics or sedatives should be used to control seizures.[204]

Neonatal MG (NMG) affects 12-20 % of newborns of such pregnancies. Typical forms characterized by weak cry, swallowing and sucking difficulties usually appear during the first hours of life and disappear in 90% of cases in 2 months. Atypical forms of arthrogryposis multiplex congenita are also described and represent 29% of NMG.[165]

Aim

The aim of this study was to review the data related to our practice with pregnancies in MG patients. We evaluated the clinical course, delivery and neonatal outcome of the pregnancies of these patients.

Methods

Between 2005 and 2013, 25 patients with a total of 30 pregnancies were followed and delivered in the Obstetric Department of Centro Hospitalar do Porto, Oporto, Portugal. The course of pregnancy, delivery and puerperium, as well as the neonatal period of 28 newborns were analyzed retrospectively. Data was collected from patients' medical records, which were all reviewed by the same author. The diagnosis of MG was established by our Neurology department based on typical clinical symptoms, neurophysiological tests, antibodies for the disease and a positive response to acetylcholinesterase inhibitor medication/immunosuppression. This department also followed all patients during pregnancy. NMG was diagnosed on the basis of clinical signs of generalized hypotonia, weak cry, weak reflexes, sucking or respiratory problems.

Myasthenia Gravis Foundation of America post Intervention status classification[180,205] was used to describe the disease severity at the fist obstetric visit, before labor and at post-partum. Clinical improvement or deterioration was defined taking into account the exacerbation or development of new symptoms and changes in the medication dosage (steroids, a significant increase in the dosage of pyridostigmine or introduction of IVIG). Clinical status was characterized by Osserman's classification of Myasthenia Gravis Foundation of America[205]. *Improvement* was represented by patients who had symptoms reduction during pregnancy associated with reduction in medication dosage; *no change* when there were no changes in the symptomatology and no need for medication adjustment; *deterioration* in the patients who required

significant changes of dosages of their ongoing treatments or addition of secondary treatments to control symptoms exacerbation.

Patient's follow-up

All pregnant patients were followed according to our department protocol by a team of obstetricians and neurologists. In the first two trimesters, all patients were seen once a month, then every two weeks between 32nd and 36th weeks, and weekly after 36th week of gestation. All patients had at least one Neurology appointment for trimester, except if there is any clinical exacerbation, where follow-up was adjusted case by case. Foetal ultrasound evaluation was routinely performed in all pregnancies in all trimesters.

Statistical analysis

Descriptive statistics was used to compute the results. Cross tables with Fisher exact test and Pearson X2-test (when applicable) were used to compare clinical exacerbation between cesarean delivery and vaginal delivery group and to compare the group of patients that experienced a deterioration of the symptoms during pregnancy or postpartum with the remaining patients. Mann-Whitney U test was used to compare the interval from the diagnosis of MG to the pregnancy between group of patients that experienced gestational exacerbation and the remaining patients. Two-sided p values less than 0.05 were used to indicate statistical significance.

Results

During the study period, there were 30 pregnancies in 25 patients, with the delivery of 28 newborns. The maternal average age at birth was 32.4±4.1 years (range 27-41). In the first obstetric appointment, and considering the 30 pregnancies individually, 2 patients were in clinical remission without medication, 7 patients in pregnancies were in pharmacological remission, 2 patients had ocular symptomatology (6.7% in Osserman stage I) and 19 patients had mild generalized symptoms

(63.3% in Osserman stage II). 30% were diagnosed in less than two years before pregnancy and 46.7% in the previous five years. One patient was diagnosed during gestation. 12% of our patients had other autoimmune disorders and Hashimoto's thyroiditis was the most frequent association. AChR antibodies were detected in 76% of our patients. Anti-Musk antibodies were present in 2 patients (8%) and seronegative MG was present in 4 patients (16%). Population demographics is given in table 3.3.1.1.

Table 3.3.1.1. Demographic information

Maternal age at delivery (mean)	32.4 ± 4.1 years
Primipara	24 patients
Multipara	6 patients
Time diagnosis before pregnancy (mean-range)	9.3 years (1-20 years)
Thymectomy before pregnancy	18 patients
Thymectomy during pregnancy	1 patient
Thymoma	5 patients
Time between thymectomy and pregnancy (mean - range)	9 years (0 – 16 years)
Association with other autoimmune disorders	3 patients
Hashimoto´s thyroiditis	2 patients
Rheumatoid arthritis	1 patient
Miscarriage	2 pregnancies
Gestational age at delivery (mean)	38.0 ± 1.3 weeks
Vaginal deliveries	10 pregnancies
Cesarean section	18 deliveries
Operative vaginal deliveries	5 deliveries
Elective cesarean section	10 deliveries (55.6% of all cesarean deliveries)

Twenty-four patients were primiparas (80.3%), and only one had a pregnancy before the diagnosis of MG. Two miscarriages occurred in this cohort before the 10th week of gestation (miscarriage rate of 6.7%). In the remaining pregnancies, a full-term delivery was achieved. No multifetal pregnancies were present in this group. 56.7% of pregnant patients experienced a clinical exacerbation during pregnancy or postpartum. Thirteen pregnant patients (43.3%) experienced a deterioration of the symptoms during pregnancy; 1 case was in CSR at beginning of pregnancy, 4 cases were in pharmacologic remission and 1 case had minimal manifestations treated with corticosteroids only. 46.7% of these during the first trimester and 53.3% in the third trimester. No exacerbation was detected in the second trimester. 46.4% of our patients also experienced a symptomatic worsening after delivery (13 patients in 28 full term pregnancies), where 69.2% of these patients also had clinical deterioration during the third trimester of pregnancy. There were no deaths to report in our cohort. One patient experienced a clinical improvement after the first trimester of pregnancy.

Table 3.3.1.2. MGFA Post-intervention status classification and evolution during pregnancy and post-partum

MGFA Post-intervention Status	Before pregnancy	Before Delivery/miscarriage	6 Months after pregnancy/miscarriage
CSR	6.7	3.3	3.3
PR	23.3	10.0	13.3
MM-0	3.3	3.3	0
MM-1	3.3	0	0
MM-2	13.3	6.7	6.7
MM-3	50.0	33.3	33.3
Exacerbation	0	43.3	43.3

Four patients had more than 1 pregnancy in our study. Three patients had 2 pregnancies (one of them with one first trimester miscarriage) and 1 patient had 3 pregnancies (1 first trimester miscarriage). The patient with the 3 pregnancies never experienced any clinical exacerbation in any of her pregnancies, even after the miscarriage. One patient experienced a clinical exacerbation during the first trimester in the first pregnancy, but

the second pregnancy developed without exacerbations. One patient experienced clinical deterioration at the end of the 3rd trimester that was repeated in the subsequent pregnancy. And the patient that had a miscarriage before a full-term pregnancy experienced a clinical exacerbation with worsening of ocular symptoms after the miscarriage as well as during the puerperium of the second pregnancy.

The average gestation age at delivery was 38.2 weeks. There was no need to terminate any pregnancy before term. In our institution, the way of labour is always discussed with the neurologist that follows the pregnant MG patient. In our sample, the global rate of cesarean delivery was 64.3%, with an elective cesarean performed before labor in 46.4% of cases. Elective cesarean for neurologic indication was performed in ten patients and the remaining three elective cesareans were performed for pelvic presentation in primiparas. In 5 cases, an urgent cesarean was made and in all instances secondary to stationary labour. Nine patients delivered vaginally, with the use of the vacuum extractor in five of these cases (55.5%). Assisted vaginal delivery was used to shorten the second stage of labour for maternal fatigue. There were no significant differences in maternal (clinical exacerbation during puerperium) or fetal outcomes (5-minute Apgar index) between the group of patients that delivered vaginally or by cesarean section.

During pregnancy, 90% of our patients were medicated with pyridostigmine and 43.3% with corticosteroids (prednisone). The prednisone dosage varied between 5 to 50mg/day, depending on the severity of the signs and symptoms. 40% of patients were treated with intravenous immunoglobulin (IVIG) during pregnancy (repeated cycles in 75% of cases). Azathioprine was used in 2 pregnancies (75mg/day). It was necessary to control symptomatology before pregnancy and it was kept in association with pyridostigmine and prednisone during all gestation. 72% of these women were submitted to thymectomy before the first gestation. In one case, a patient was submitted to a thymectomy at the 20th week of gestation secondary to a clinical exacerbation refractory to medical treatment (pyridostygmine 420 mg/day; prednisone 20 mg/daily;

IVIG cycle). This patient was diagnosed with a thymoma type A. After surgery, this gestation followed without complications, with the birth of a healthy newborn by an elective cesarean at 37 weeks of gestation. This patient experienced a 2nd pregnancy 4 years later without complications.

In 57.9% a follicular thymic hyperplasia was described. Thymoma was present in 15.8% of cases (all thymoma type A of the WHO classification), and a normal thymic histology was described in 26.5% of these women. All pregnant women kept their medication until labor. During the active phase of the first stage and second stage of labor, neostigmine was used. In all cases, locoregional anesthesia was used.

The average newborn birth weight was 2919.6g (±365.2g). No cases of neonatal asphyxia were diagnosed with all newborns having an Apgar Index above 7 in the 5th minute.

No cases of fetal growth restriction or preeclampsia were diagnosed in this cohort. Seven patients (25%) were diagnosed with gestational diabetes of which 85,7% were treated with corticosteroids to control myasthenic symptoms.

During post-partum, thirteen pregnant patients (46.4%) experienced deterioration of symptomatology. We report one case of myasthenic crisis requiring ventilation support during the postpartum period. This patient was medicated with prednisolone 50mg/day, pyridostigmine 540mg/day and IVIG every four weeks. The clinical aggravation started in the 36th week of gestation. No clinical control was achieved with steroid dose increment and an elective cesarean was performed one week later. Ventilation support was needed during the puerperium. Clinical improvement was achieved with IVIG therapy and plasma exchange.

After the puerperium period, but during the first-year post-partum, three patients worsened the symptoms and in 2 of them, no worsening was detected during the eight weeks of puerperium. In one of these patients neuromyelitis optica (Devic's disease) was diagnosed six months after delivery and 7 years after thymectomy.

Two newborns developed NMG, on the third and fourth day of life. In both cases, the diagnosis was established based on the development of typical

clinical signs of NMG. These infants were floppy and developed feeding difficulties with necessity with feed tube. No ventilator support was needed in any of these patients. They were treated with acetylcholinesterase inhibitors. In both cases, the mother experienced clinical aggravation during pregnancy and was classified as MM-3 according to MGFA post intervention classification at delivery. No major congenital malformations were detected. Two fetuses had pyelectasis grade I and one newborn developed neonatal jaundice. There were no cases of arthrogryposis in our cohort.

Table 3.3.1.3. Osserman classification

Osserman classification	At first obstetric visit	Immunosuppressive medication used at beginning pregnancy	% of patients in each Osserman classification that deteriorate during pregnancy
Classe 0	30%	22.2% without treatment 77.8% treated with: - prednisolone (average dosage 7,5mg/day)	55.5%
Classe I	6.7%	100% under immunosuppressive treatment - prednisolone (average dosage 10 mg/day)	50%
Classe II	63.3%	100% under immunosuppressive treatment - prednisolone (average 25mg/day) - azathioprine (in 2 cases – average dosage 75mg/day) - IVIG (in 12 cases - weigh adjusted dosage)	36.8%

When we compare the group of patients that experienced an aggravation of symptomatology during pregnancy or post-partum with the remaining patients, no statistical differences were detected between groups regarding the interval between diagnosis and pregnancy, parity, medication used, way of delivery or symptomatic state before pregnancy.

Discussion

Pregnancy has a significant effect on MG clinical course with symptomatic exacerbation being reported to be as high as 45% during pregnancy and post-partum. Plauché[206] reported the antenatal exacerbations of 35.4% in the series of 164 pregnancies. Djelmis et al.[207] analyzed their experience of 69 pregnancies in 65 patients with MG. 30.4% showed an exacerbation while 44.9% had no change and 24.6% improved. Schlezinger[208] also described the clinical course of pregnancy in 22 myasthenic women and reported an aggravation in a third of his patients. Téllez-Zentero et al.[165] in a report of 18 pregnancies described an exacerbation rate of 33% and Mitchel & Bebbington[209] in a series of 11 pregnancies reported a clinical aggravation of 45.5% during the third trimester. Batocchi et al.[160] in 64 pregnancies showed clinical exacerbation in 17% of patients without treatment and in 39% of patients under immunosuppressive treatment. In our study, 43% of patients experienced an exacerbation of symptoms, especially during the third trimester and postpartum. This is a similar rate of clinical worsening, when we compare our series to others published. Our patients also needed a higher than usual use of immunosuppressive therapy in association with acetylcholinesterase inhibitors for symptomatic control. In the Djelmis et al.[207] series, 23.2% were free of medication during pregnancy while 33.3% were treated in association with corticosteroids. Téllez-Zentero et al.[165] reported that 61% used pyridostigmine during pregnancy, but only 11% needed corticosteroids or other immunosuppressive medication. In our study, during pregnancy, 90% were treated with acetylcholinesterase inhibitors and 43.3% also required prednisolone to symptomatic control. The corticosteroid dosage was increased or started in 43.3% of our pregnancies and IVIG were started during pregnancy in 6 cases. This probably means that our patients experienced a more aggressive MG, which could justify our rate of clinical worsening and a higher use of corticosteroids during pregnancy. In spite of this, there were no deaths in our cohort and we only experienced one

myasthenic crisis during postpartum and after an aggressive clinical deterioration during the end of the third trimester.

The clinical course of pregnancy in these patients is usually considered unpredictable. Djelmis[207] showed an inverse correlation between the duration of the disease and the frequency of clinical aggravation while Scott described an inverse association between the length of this period and mortality[210]. In our study we didn´t find any association between the clinical exacerbation during pregnancy or in the puerperium and the previous thymectomy, use of corticosteroids or IVIG during pregnancy or the way of delivery. In our cohort, the mean interval between MG diagnosis and pregnancy was smaller in the group of patients with clinical aggravation during pregnancy, but this was not significantly different.

In our group, we describe a case of thymectomy during pregnancy. Usually thymectomy is reserved to patients with thymoma and in young patients with generalized symptoms with the objective to improve long term outcomes[211]. In our study a thymectomy was performed during pregnancy in a patient with a thymoma and with a clinical exacerbation refractory to medical treatment. The objective of the treatment was to keep pregnancy and improve the response to the medical treatment which was achieved.

Our study showed a cesarean delivery rate of 64.3%. During the study period, our institutional cesarean rate was 40%, which means that the frequency of cesarean delivery has higher in MG pregnant women. Some authors[162,207] also showed a higher rate of cesarean delivery in this group of patients. However, this is not a consensual finding[164]. In our institutuion the type of delivery is always discussed with the pregnant women and the neurologist. In the presence of clinical exacerbation in a patient with little motivation to a vaginal delivery, we offer the possibility of a cesarean delivery under locoregional anesthesia. This justifies our high rate of cesarean sections. Despite this, we didn´t find any maternal or fetal outcome difference between these two groups.

We described an NMG rate of 7.1%. In our study, two newborns developed classic signs of NMG during the first 72-96 hours of life, which is little

later than usually described. In the literature, the onset of symptoms occur during the first 24 hours in 80% of cases[212]. They were treated with acetylcholinesterase inhibitors with spontaneous resolution of symptoms during the first two months of life.

In conclusion, the present literature in pregnant patients with MG is still limited. Our study adds to the body literature showing that in the presence of a more aggressive MG background, the frequency of clinical exacerbation during pregnancy could be high. A multidisciplinary approach with neurologists and trained obstetricians allowed a good clinical control, avoiding maternal adverse outcomes. In this group of patients, pregnancy should be planned to avoid the use of foetus toxic medication during pregnancy, as well as, to advise women to become pregnant during a stable phase of the disease[54].

Our study also showed that an elective caesarean to prevent labor in unmotivated patients is safe without an increased rate of clinical exacerbation during puerperium.

3.3.2 MuSK Myasthenia gravis and Pregnancy

Within this research project, we performed a retrospective study on the pregnant women with MuSK-MG in our cohort and other hospitals that also participated in the epidemiological study.
As in the previous study, we analysed the effects of the disease on the pregnancy, the delivery and the foetus, as well as the effects of pregnancy on MuSK-MG.

Part of the results of this study was presented at the XXIII World Conference of Neurology, in Kyoto, in September 2017. The abstract was published in the *Journal of Neurological Sciences*.

The complete study was submitted to Neuromuscular Disorders and is under review after resubmission.

MuSK Myasthenia gravis and Pregnancy

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Abstract

Background: Muscle specific kinase (MuSK) myasthenia gravis (MG, MuSK-MG) is a rare subgroup of MG affecting mainly women during childbearing years.

Objective: To investigate the influence of pregnancy in the course of MuSK-MG and pregnancy outcomes in females with MuSK-MG.

Methods: A multicentric cohort of 17 women with MuSK-MG was identified; 13 of them with ≥1 pregnancy, were studied retrospectively.

Results: MuSK-MG onset age was 35,4 years (15-66); 23,0% had other autoimmune disorder; 46,2% were treatment refractory. Thirteen women experienced 27 pregnancies, either after MG onset (group I) (n=4; maternal age at conception = 29.8 years) or before MG onset (group II) (n=23; maternal age at conception = 26.2 years). In group I pregnancy occurred in average 9.8 years after the MG onset; it occurred in average 17.0 years before MG in group II. In group I, all were on steroids at time of conception, one on azathioprine and another receiving IVIG regularly. There were mild exacerbations that responded to treatment adjustments. There were no relapses in the 12 months following the delivery. There was no preeclampsia, birth defects or stillbirths in either group; 3 miscarriages in group II. One case of neo-natal MG was recorded. All newborns seemed to have normal psychomotor development.

Discussion: In this series, pregnancy did not seem to precipitate MuSK-MG or to influence the MuSK-MG course, and there was no apparent negative impact in pregnancy outcomes in those where pregnancy followed the MG onset.

Introduction

Muscle specific kinase (MuSK) myasthenia gravis (MG, MuSK-MG) is a subgroup of MG described for the first time in 2001.[213] It corresponds to around 5% of the total MG cases[12] and up to 40% of the AChR negative MG patients.[42] MuSK-MG affects mainly oculo-facial-bulbar muscles and may develop myopathic weakness and muscle atrophy. [83] Early respiratory crises are frequent, [214] and traditionally is seen as a more severe and treatment refractory form of the disease. [142,215]

Frequently it affects women during childbearing years. In a study that included two cohorts of 110 MuSK-MG patients 85% were female, with disease onset typically in the fourth decade.[53]

MG, regardless its antibody status, is not associated with infertility, but it may expose pregnant women to an increased risk of maternal and fetal complications. The clinical course of pregnancy in these patients is unpredictable.[159,216] It was described that about 30% of pregnant women experienced improvement of symptoms while a third face clinical worsening, especially in the first trimester and during postpartum. [159,160,217]

An increase in the exacerbation rate during pregnancies taking place in the first two - three years of disease was also described.[160,161] So patients are sometimes advised to postpone pregnancy to two - three years following disease onset.[160] The risk of preterm birth, low neonate birthweight or hypertensive disorders apparently are not increased in MG pregnant women in general.[54]

Neonatal MG (NeoMG) affects 7-20 % of newborns of such pregnancies. Typical forms characterized by weak cry, swallowing and sucking difficulties usually appear during the first hours of life and disappear in 90% of cases in 2 months.[165,216] There have been descriptions of neonatal MG in MuSK-MG. [166,218,219]

Specifically, the effect of pregnancy in MuSK-MG was not described yet. It is important to understand whether pregnancy influences the course of MuSK-MG and vice-versa.

Methods

A multicentric cohort of 17 women with MuSK-MG from 7 hospitals in the North of Portugal was identified. Those with ≥1 pregnancy (n=13) were studied retrospectively.

Data was collected from patients' medical records by their doctor. Informed consent was obtained.

MG clinical information

The diagnosis of MuSK-MG was based on clinical symptoms, neurophysiological tests, and positive anti-MuSK antibodies. Age of MG onset, past medical history, including miscarriage and preeclampsia, polyautoimmunity and treatment at the time of conception were recorded. Refractory MG was defined according predefined criteria.[88] Myasthenia Gravis Foundation of America post Intervention status classification[205] was used to describe the disease severity at the fist obstetric visit, before delivery and post-partum. Clinical improvement or deterioration was defined taking into account the exacerbation, including myasthenia crisis, or development of new symptoms; changes in the medication dosage (steroids, pyridostigmine) or introduction of IVIG were also recorded and analysed in the clinical context. Clinical status was characterized by classification of Myasthenia Gravis Foundation of America.[205]

Pregnancy related information

Age at conception and interval between conception and MG onset were recorded. Information on intrauterine growth retardation, hydramnios, fetal akinesia, if was pre-term/full-term delivery, type of delivery, type of anesthesia, weight of the neonate at birth, Apgar index and presence of neo-natal MG (NeoMG) were collected. NeoMG was diagnosed on the basis of clinical signs of generalized hypotonia, weak cry, weak reflexes, sucking or respiratory problems.

Results

The results are summarized in table 3.3.2.1.

Patients – MG and pregnancy

Thirteen women with MuSK-MG had at least one pregnancy. The age at MG onset was 35,4 years (15-66), 46,2% were refractory[88] and 23,0% had other autoimmune disorder (anti-phospholipid syndrome, sacroiliitis and thyroid autoimmune disorder). These thirteen women experienced 27 pregnancies, either after MG onset (group I) (n=4; maternal age at delivery = 29.8 years) or before MG onset (group II) (n=23; maternal age at delivery = 26.2 years). There was no statistically difference between the age of MG onset in the two groups (p=0.26, Fisher exact test).

In group I pregnancy occurred in average 9.8 (4-14) years after the MG onset and it occurred in average 17.0 (2-39) years before MG in group II (pregnancy before MG). There was no case of maternal MG (onset during pregnancy or in the postpartum period of 6 months).[220]

In group I (pregnancy after MG onset), all four patients were on pyridostigmine and steroids at the time of conception, one was also taking azathioprine and another one was receiving IVIG monthly. Concerning MGFA status, one was at pharmacological remission (PR), one with minimal symptoms (MM), and two were at IIB in the beginning of pregnancy. There were mild exacerbations that responded to a small increase on the dose of pyridostigime, but one patient that worsened from IIB to IIIB and the interval for IVIG was shortened to every 3 weeks. There was no myasthenia crisis during pregnancy. There were no miscarriages. There was no record of intrauterine growth retardation, hydramnios or fetal akinesia. There were two pre-term deliveries (35 w and 36+6w, because of premature rupture of membranes). Two underwent cesarean sections (one obstetric reason and one per choice); all four patients received anesthesia (one general and three epidural).

In group II, there were 3 miscarriages; one of unknown cause and two occurred in the patient with anti-phospholipid syndrome. There was no evidence of intrauterine growth retardation, hydramnios or fetal akinesia.

Neonatal health

There was one record of neo-natal MG; the mother was the patient that had worsened to the stage IIIB and was receiving IVIG. The neonate was full-term, born by cesarean section and under general anesthesia. The Agar Index was 9/10, but had a very weak sucking reflex, weak cry and was difficult to wake up during the two first weeks of life. The mother had papillary carcinoma of the thyroid; baby was also tested for thyroid hormones, which were normal. Anti-MuSK antibodies were not tested in that period. Baby recovered completely by the end of the third week of life. In group I, there were no exacerbations in the 12 months postpartum. All newborns seemed to have normal psychomotor development.

There was no preeclampsia, birth defects or stillbirths in either group. There was lower neonate birthweight in group I when compared with group II (2761g vs 3313g, p=0.008), but none had criteria for low neonate birth weight (<2500g).

Considering the group II, there were 4 cesarean sections and 16 vaginal deliveries. All the cesarean sections were performed for obstetric reasons (ex: pelvic presentation). All newborns had normal development. Three women developed another autoimmune disease years after the pregnancy.

Table 3.3.2.1. MuSK-MG: Summary of clinical data of the mother, pregnancy, delivery and the newborn

	Group I (pregnancy after MG onset)	**Group II** (pregnancy before MG onset)
MuSK-MG patients (n)	4	9
Age at MG onset	19,8 y	44,0 y
Polyautoimmunity at the time of the conception	0	3 1 TAD 1 Sacroiliitis 1 APS
MG severity at Conception	IIB (2) PR (1) MM (1)	NA
Treatments at Conception	Pyridostigmine (4) Azathioprine (1) IV IG (1)	NA
Exacerbations	1 (IIB to IIIB)	NA
Myasthenia crisis	0	NA
Changes in treatments	1 (decreased interval of IV IG)	NA
Pregnancy (n)	4	23
Age at Conception	26,5 y	29,8 y
Interval between MG onset and pregnancy (min-max)	9,8 y (4-14)	-17,0 y (2-39)
Miscarriages	0	3 (1 UKN cause, 2 mother with APS)
Pre-eclampsia	0	0
Hydramnios	0	0
Birth defects	0	0
Fetal akinesia	0	0
Intrauterine growth retardation	0	0
Delivery	4	20
Pre-term/full-term delivery	2/2 (35w, 36w+6d)	0/20
Type of delivery	2 C-section (1 per choice, 1 obstetric) 2 vaginal	4 C-section 16 vaginal
Type of anesthesia	1 general 3 epidural	4 general 2 epidural
Newborn – Infant	4	20
Birth weight	2761	3313
Neonatal MG	1	0
PMD	All normal	All normal

NA- not applicable, TAD – thyroid autoimmune disease, APS- anti-phospholipid syndrome, PMD- Psycho-motor development.

Discussion

In the group that pregnancy occurred after Musk-MG onset, group I, it seems there was not a major effect of the pregnancy on the disease; only one experienced moderate worsening (from IIB to IIIB), probably because the pregnancy occurred many years after the MG onset when the disease was already on treatment, and stable, for a long period of time.

At the same time, it did not seem that MuSK-MG or its treatment had impacted negatively on the pregnancy, attending that it did not occur any complications as miscarriages, pre-eclampsia, birth defects, intrauterine growth retardation, hydramnios, fetal akinesia or stillbirths. And none of the new-born had low birth weight, although the weight in this group was slightly lower than in the group II. Whether this could be related to treatments is difficult to know.

Despite of the rarity reported[166], we registered a case of a newborn with symptoms suggestive of neonatal MG.

Regarding the group II pregnancies occurred many years before the onset of the disease (average of 17.0 years, 2-39 years). Reports point to pregnancy and the postpartum period as a risk period for the start of the disease, [221–223] but, in this cohort of patients (9 women with 23 pregnancies), pregnancy did not seem a precipitating factor for the development of MuSK-MG.

Recently a case-control study using population data from Norway and the Netherlands, studied 246 females with onset of the disease in their reproductive years (15–45 years of age), including AchR-MG and Musk-MG patients, and found 15% of cases of maternal MG. [220] From the 246 MG cases, 6 were MuSK-MG and they found one case of maternal MuSK-MG. There are also reports of other cases of maternal MuSK MG.[167,218,219]

In group II the average age of MuSK-MG onset was 44,0 years, it might be that the risk factors for the development of the disease had less influence of the hormonal changes of the pregnancy [224] but were more similar to those of the late onset MG.[153]

Musk-MG is a rarer form of the disease, it would very important to study

these issues in bigger multicentric cohorts of this subgroup of the disease or case-control studies. Meanwhile, and taking into account our study and those published[166,216,218–220] there seems to be a consensual clinical impression and evidence that women with MuSK-MG may have healthy pregnancies. Furthermore, their MG may remain well-controlled during pregnancy, around delivery time and in the post-partum period. Close monitoring and appropriate management by experienced Obstetricians, Pediatricians and Neurologists will be, however, required.

3.4. Polyautoimmunity and Myasthenia gravis

Within this research project, we performed a retrospective study on the coexistence of other autoimmune disorders and MG in our cohort in Centro Hospitalar do Porto/Hospital de Santo António and analysed the characteristics of this group of patients.

It was published in **Journal of the Neurological Sciences, 2017 Oct 15;381:39-40** and the full text follows:

Myasthenia gravis with systemic and neurological polyautoimmunity

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Introduction

Autoimmune diseases share symptoms and signs, pathophysiological mechanisms and genetic and environmental susceptibilities, facts that support the existence of a common origin [225] though it may be influenced by multiple factors. Polyautoimmunity refers to the presence of more than one autoimmune disease in a single patient and this is well documented in great majority of the spectrum of autoimmune diseases, particularly those mediated by autoantibodies, including myasthenia gravis (MG).[226]

Myasthenia gravis is caused by antibodies directed against the acetylcholine receptor (AChR), muscle-specific kinase (MuSK), or, less frequently, to lipoprotein-related protein 4 (Lrp4) or agrin, all located in the postsynaptic membrane at the neuromuscular junction[12]. Anti-AChR positive MG is traditionally associated with thymus pathology, either thymic hyperplasia or thymoma. The frequency of polyautoimmunity in MG has been reported between 11.6%-32% [112,127,189], which is superior to the general population risk (5%). [141]

Material and methods

We analysed all patients with MG (214) in follow-up between 1992 and 2016 in a Portuguese tertiary centre for the co-occurrence of other autoimmune disorders, with a special focus on the neurological ones. MG and the other diseases were diagnosed based on well-established clinical, electrophysiological, imagiological and serological criteria.

Results

Associated systemic autoimmune disorders in myasthenia gravis

We found other autoimmune disorders in 37 patients with MG (17%). Six patients have a combination of more than one autoimmune disease besides MG (multiple autoimmune syndrome). Autoimmune thyroid disease was the most frequent, followed by rheumatoid arthritis and anti-phospholipid syndrome (table 3.4.1.).

Table 3.4.1. Systemic autoimmune comorbidities

Autoimmune disorders (17%)	**n=37/214**
Autoimmune thyroiditis	14
Rheumatoid arthritis	5
Anti-phospholipid syndrome	5
Psoriasis	4
Sjogren syndrome	3
Systemic lupus erythematosus	2
Pernicious anemia	2
Vitiligo	2
Acquired immunodeficiency (C1q deficit)	1
Alopecia areata	1
Idiopathic thrombocytopenic purpura	1
Lichen planus	1
Primary biliary cirrhosis	1
Reactive arthritis	1
Reactive lymphadenitis	1
Rheumatic fever	1
Seronegative arthritis	1
Total	**46**

As expected, the frequency of a second autoimmune disorder is highest for females (68%) with early onset MG (EOMG) (mean age at onset is found to be 41 years). The majority of these patients have the generalized form of myasthenia (78%) and the anti-AChR antibodies were positive in 78%. Thymectomy was performed in 19 patients (51%), with thymic hyperplasia being the most common histological diagnosis. In most cases, MG preceded the onset of the other autoimmune disease (62%) and immunosuppression didn't seem to affect the development of a second condition (68% had not been exposed to immunosuppressive agents when the other disorder emerged).

Associated neurological autoimmune disorders in myasthenia gravis

Of 214 patients with MG 11 (5%) have another autoimmune neurological disease: three of the central nervous system, seven of the peripheral nervous system and one of the autonomic nervous system (table 3.4.2.).

Table 3.4.2. Neurological autoimmune comorbidities

Pt nr	Gender	Age at MG onset	MG form	Anti-AChR Ab	Thymectomy (Yes or No) Histology	Treatment at the time of the second illness presentation	Time between MG and other AI disease (years)	Other AI neurological disease
1	F	28	G	Pos	Yes TH	0	14	Neuromyelitis optica with AQP4 antibodies
2	F	18	G	Pos	Yes TH	0	9	Neuromyelitis optica with AQP4 antibodies
3	M	39	Oc	Neg	No	0	9	Autoimmune encephalitis with GAD65 antibodies
4	F	27	G	Pos	No	NA	0	Myositis
5	M	32	G	Pos	Yes Thymolipoma	NA	0	Myositis
6	F	39	G	Pos	No	Methotrexate; prednisolone	-6	Myositis
7	M	42	G	Pos	No	IVIG; prednisolone	-4	Myositis
8	M	69	G	Neg	No Thymoma	NA	0	Myositis
9	M	38	G	Pos	Yes Normal	0	21	Parsonage-Turner syndrome
10	F	39	G	Pos	Yes TH	0	2	CIDP
11	F	30	G	Neg	Yes Thymoma	NA	0	Intestinal pseudo-obstruction syndrome with anti-CV2/CRPM5

Pt nr: patient number; **F**: female; **M**: male; **MG**: myasthenia gravis; **G**: generalized, **Oc**: ocular. **AChR**: acetylcholine receptor; **pos**: positive; **neg**: negative; **TH:** Thymic hyperplasia; **AI:** autoimmune; **NA**: not applicable; **IVIG**: Intravenous immunoglobulin; **CIDP**: chronic inflammatory demyelinating polyradiculoneuropathy.

The patients were examined for a second neurological condition only when unusual signs or symptoms arose, when there was an unexpected deterioration or poor response to treatment. The majority (54.5%) of these patients are females with EOMG (average age at MG onset is found to be 36 years), and almost all have generalized MG. Anti-AChR antibodies were positive in 73% (8/11); there were other autoantibodies in relation with other autoimmune disease in six cases. Six patients (55%) were thymectomized. As previously reported [138,140], thymoma seemed to associate more frequently with muscle disease, whereas hyperplasia seemed to be linked to neuromyelitis optica spectrum disease (NMOSD). The MG manifestations preceded or coincided with those of other autoimmune neurological disorder in most cases (9/11; 82%) (average time between onset of symptoms of MG and the other disease was 4 years). The average diagnostic delay of the second neurological disease was 1.5 years. The majority of patients (5/7; 71%) had not yet been exposed to immunosuppression when the second illness emerged.

Discussion and conclusions

The course of MG is often complicated by concomitant autoimmune disorders. Our results are in line with this observation and appear to be relevant in general as well as in specialised neurological clinical practice. It is important to consider coexistent MG in patients with autoimmune disorders that develop new or aggravated muscular weakness, fatigue or respiratory failure.

On the other hand, while rare, the possibility of neurological autoimmune comorbidity should also be considered in myasthenic patients, especially if there is an unexpected deterioration of muscle weakness or poor response to pyridostigmine (e.g. muscle disease), or if unexpected neurological signs or symptoms arise in MG (e.g. peripheral nervous system involvement in keeping with CIDP; or central nervous system illness such as NMOSD; or even rarer manifestations such as of autonomic dysfunction) [2]. The rapid diagnosis of the second illness will improve

management and disease outcomes of both MG and the other autoimmune condition.

3.5. Refractory Myasthenia gravis

Under this topic we studied the clinical, demographic and immunogenetic characteristics of the refractory MG patients in our cohort.

Part of the results were presented in international conferences:

- **Refractory Myasthenia gravis: clinical and demographic data,** presented at the XXIII World Conference of Neurology, in Kyoto, September 2017. The abstract was published in the *Journal of Neurological Sciences.*
- **Refractory Myasthenia gravis and HLA,** presented in the European School of Neuroimmunology, in Venice, June 2017. The abstract was published in the *Journal of Neuroimmunology*.

The manuscript is currently under submission; the full text is as follow.

- **Refractory Myasthenia gravis: Clinical, demographic and immunogenetic characteristics in a portuguese cohort.**

3.5.1. Refractory Myasthenia gravis: Clinical, demographic and immunogenetic characteristics in a portuguese cohort

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Abstract

Background: A subset of myasthenia gravis patients is refractory to conventional treatments. Identifying their clinical and genomic characteristics may be important to contribute for the development of effective strategies in this subgroup.

Objective: The aim of our study is to describe the clinical features of refractory MG patients and to investigate a possible association between HLA-DRB1 alleles and refractory MG.

Methods: A retrospective study of 172 MG patients followed in the Neuroimmunology Outpatients clinics was performed. Patients were classified as refractory or non-refractory based on predefined criteria, and clinical features were compared. Chi-square/Fisher's exact and Mann-Whitney tests were used to investigate associations between clinical and demographics characteristics and response to treatment. 114 MG patients (22 refractory and 92 non-refractory) were genotyped for the HLA-DRB1 locus using PCR-SSP methodology. A group of 282 healthy individuals was used as control population.

Results: Thirty eight out of 172 patients were classified as refractory (22%). Compared to the non-refractory patients, the refractory ones were more likely to have a more severe MGFA classification at onset (p=0.02), to have thymomatous MG (p=0.03) and to be seropositive, either to anti-AChR (p=0.05) or anti-MuSK antibodies (p=0.03). Differences regarding sex, age of onset, presence of other autoimmune diseases, malignancies (other than thymus) or severe/opportunistic infections were not observed. HLA-DRB1*03 was more frequent in the non-refractory vs control population (38.0% vs. 15.6%, $p=3x10^{-6}$). HLA-DRB1*13 allele was less frequent in the non-refractory group when compared to the control population (13.0% vs. 29.8% respectively, p=0.002). And HLA-DRB1*13 allele was more frequent in the refractory MG when compared to the non-refractory group (40.9% vs 13.0%, p=0.003).

Discussion: Refractory MG patients represent a substantial subgroup in the disease. It is important to consider their characteristics from the onset of the disease for a better management of these patients. HLA-DRB1*13 allele appears to have a protective role as it was reported before in other autoimmune disorders.

Introduction

Myasthenia gravis, a rare autoimmune disorder of the neuromuscular transmission, antibody-mediated, characterized by fatigable muscle weakness is increasingly acknowledged as a syndrome more than as a single disease.[28]

In the generalized form of the disease around 85% of patients have antibodies detected against the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction. The remaining patients have antibodies against other components of the postsynaptic muscle endplate, such as muscle specific receptor tyrosine kinase (MuSK), LRP4 or are triple seronegative (unidentified or undetected antibody).[20] Around 10 to 15% of MG cases are associated to thymoma.[20]

Therapeutic options in myasthenia gravis patients include cholinesterase inhibitors, thymectomy, immunosuppressive agents and short-term immunomodulation with plasma-exchange and intravenous immunoglobulin. [28] Conventional immunosuppressive agents used in MG treatment are azathioprine, mycophenolate mofetil, cyclosporine, tacrolimus and cyclophosphamide.[142] A proportion of myasthenia gravis patients are classified as refractory due to non-responsiveness to conventional treatment. It corresponds to a small but important portion of the MG population (10-15%). [142]

Refractory patients were defined as those who could not lower the immunotherapy for MG without clinical relapse, with MG not clinically controlled on their immunotherapy regimen, or who had developed severe adverse effects from immunosuppressive therapy for at least a period of 12 months.[88,142]

Some studies show that these patients are mostly females, have young onset and generalized disease, and are seropositive, predominantly to anti-MuSK antibodies or associated to thymoma.[88,142]

This refractory MG population has been the focus of recent publications evaluating the response to biologic agents (rituximab[142,143], tocilizumab[72], eculizumab[73,74], belimumab[75]) and rescue treatments (autologous hemopoietic stem cell transplantation)[76].

Over the years, association of MG with Human Leucocyte Antigens (HLA) has been described in different populations.[49,105,106,189–191,196] Apparently, their relation with a refractory course was not studied in myasthenia gravis so far. It was studied in other autoimmune disorders, as in rheumatoid arthritis.[227] It was also related to disease severity and progression in rheumatoid arthritis[227–229] and in multiple sclerosis.[230,231]

Identifying refractory patient's characteristics is important to contribute to a better management of treatment approaches and to contribute to the development of therapies effective in this subgroup.

Objective

Our aim was to describe the clinical and demographic features of refractory MG patients and compare them to those with a non-refractory disease. This study also explored the existence of a correlation between the refractory group of patients and HLA-DRB1 locus susceptibility alleles.

Methods

We did a retrospective study of 172 MG patients followed in the Neuroimmunology Outpatients clinics of Centro Hospitalar do Porto/Hospital Santo Antonio. Patients were classified as refractory or non-refractory based on predefined criteria[88]: those who could not lower their immunotherapy without clinical relapse, were not clinically controlled on their immunotherapy regimen, or had severe side effects from immunosuppressive therapy.

Clinical features were compared in the two groups regarding demographic and clinical characteristics: age of onset, sex, generalized versus ocular, MGFA classification, MG antibodies status, thymectomy and thymic pathology, presence of other autoimmune disorders, malignancies (other than thymus) or severe/opportunistic infections. Chi-square/Fisher's exact and Mann-Whitney tests were used to investigate associations between clinical and demographics characteristics and response to treatment.

From the total 172 MG patients mentioned above, 114 MG patients (22 refractory and 92 non-refractory) were genotyped for the HLA-DRB1 locus using PCR-SSP methodology. A group of 282 healthy individuals was used as control population.

HLA Genotyping

Peripheral blood samples were collected in EDTA. Genomic DNA was obtained from proteinase-K–treated peripheral blood leukocytes by using a salting-out procedure.[193] Low-resolution genotyping for HLA–DRB1 locus (i.e., 2-digit HLA nomenclature) was performed using polymerase chain reaction with sequence-specific primers (PCR-SSP), based on methods previously described.[194]

Control population

Patients HLA alleles frequencies were compared with the ones from a control group consisting of 282 unrelated individuals, healthy blood donors, from the same geographic origin (north of Portugal).

Statistical Analysis

Chi-Square/Fisher's exact and Mann-Whitney tests were used to investigate associations between clinical/demographics characteristics, response to treatments and HLA DRB1 alleles frequencies. The data were analyzed using the IBM SPSS 20 statistical software suite.

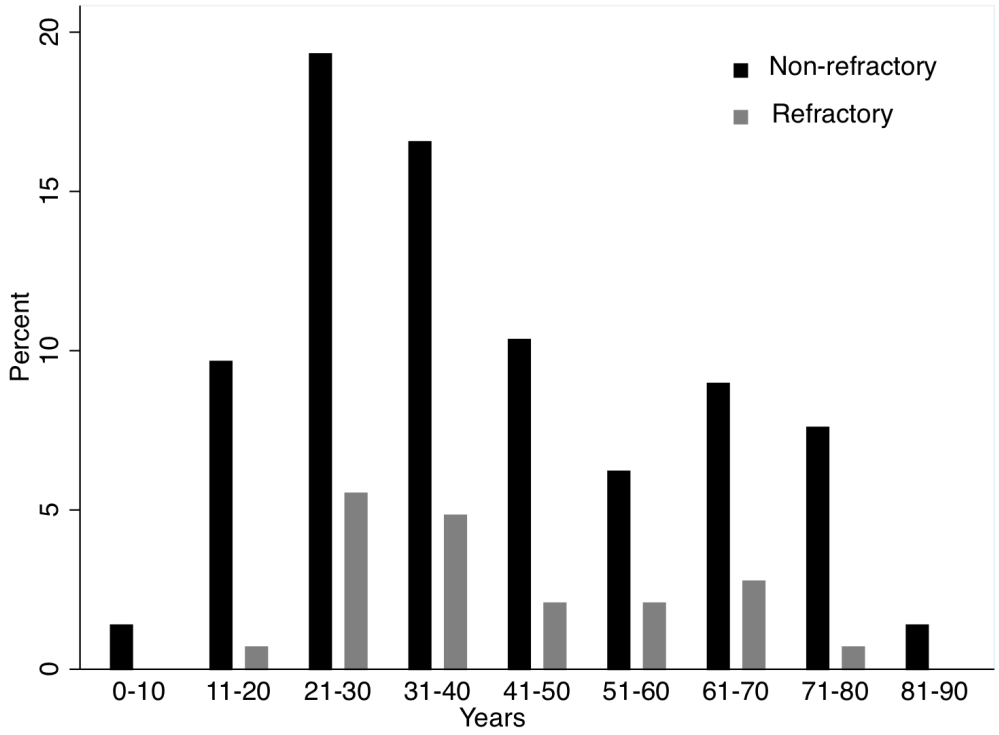
Results

Clinical and demographic data

38 out of 172 patients were classified as refractory (22%).[88] Some of the patients fulfilled more than one of the criteria for refractory MG. Eighteen patients were doing IV IG chronically, thirteen had been under two or more different immunossupressors agents and fourteen were under prednisone = or > than 20 mg per day. The average interval of follow-up was 12 years (2-40).

Average age at onset was 37 years in the non-refractory group versus 33.5

years in the refractory group (p>0.05). The distribution of age of onset in the two groups is illustrated in the Graph 3.5.1.1. There were no differences regarding sex, females were 68% in the non-refractory and 71% in the refractory group(p>0.05). Ocular form was present in 93,1% in the non-refractory versus 6.9% in the refractory group and the generalized form was 78,5% in the non-refractory group vs 21,5% in the refractory group (p>0.05).



Graph 3.5.1.1. Distribution of the MG age of onset in the two groups refractory and non-refractory MG

Compared to the non-refractory patients (Table 3.5.3.1.), the refractory ones were more likely to have a more severe MGFA classification at onset (p=0.02), to have thymomatous MG (p=0.03) and to be seropositive, either to anti-AChR (p=0.05) or anti-MuSK antibodies (p=0.03). Seronegative MG was more common in the non-refractory group 30% vs 7.9% in the refractory (p=0.006).

We could not find any difference regarding presence of other autoimmune diseases, malignancies (other than thymus) or severe/opportunistic infections. Polyautoimmunity was present in 26.9% in the non-refractory group vs. 18.4% in the refractory group (p>0.05).

Table 3.5.1.1. Clinical and demographic characteristics of MG patients

	Non-refractory (n = 134)	Refractory (n = 38)	p-value
Female	92 (68.7%)	27 (71%)	0.78
Age, median (IQR)	57 (44-69)	49.5 (40-63)	0.11
Age at diagnosis	37 (25-59)	33.5 (24-53)	0.58
Clinical manifestation			**0.07**
Ocular	27 (93.1%)	2 (6.9%)	
Generalized	91 (78.5%)	25 (21.5%)	
MGFA classification			**0.02**
I	32 (23.9%)	12 (23.9%)	
IIa	32 (23.9%)	13 (34.2%)	
IIb	10 (7.5%)	3 (7.9%)	
IIIa	0	1 (2.6%)	
IIIb	0	1 (2.6%)	
Iva	0	1 (2.6%)	
IVb	0	1 (2.6%)	
Pharmacologic remission	46 (34.3%)	6 (15.8%)	**0.03**
Complete remission	14 (10.5%)	1 (2.6%)	0.13
Serologic status			
AChR+	87 (65.8%)	31 (81.6%)	**0.05**
MuSK	5 (8.93%)	4 (30.8%)	**0.03**
Seronegative	39 (30%)	3 (7.9%)	**0.006**
Thymectomy	54 (40.3%)	21 (55.3%)	**0.10**
Thymoma	12 (23%)	10 (50%)	**0.03**
Other autoimmune diseases	36 (26.9%)	7 (18.4%)	0.52
Malignancy	9 (16.4%)	2 (15.4%)	0.93
Severe/opportunistic infections	5 (10%)	1 (9%)	0.93

IQR – interquartile range. MGFA – Myasthenia Gravis Foundation of America Clinical Classification. Statistically significant if p-value < 0.05.

Immunogenetic data

We found that HLA-DRB1*03 was more frequent in the non-refractory vs control population (38.0% vs. 15.6%, $p=3x10^{-6}$).

HLA-DRB1*13 allele was less frequent in the non-refractory group when compared to the control population (13.0% vs. 29.8% respectively, p=0.002). At the same time, HLA-DRB1*13 allele was more frequent in the refractory MG when compared to the non-refractory group (40.9% vs 13.0%, p=0.003) (Table 3.5.1.2.).

Table 3.5.1.2. HLA-DRB1 genotyping and Refractory Myasthenia gravis

HLA-	A: Controls (n=282)	B: Refractory MG (n=22)	C: Non-refractory (n=92)	P global	p A vs. B	P A vs. C	P B vs. C
DRB1*01	66 (23.4%)	6 (27.3%)	23 (25.0%)	0.889	0.681	0.716	0.847
DRB1*03	**44 (15.6%)**	**4 (18.2%)**	**35 (38.0%)**	**$2.5x10^{-5}$**	**0.749**	**$3x10^{-6}$**	**0.073**
DRB1*04	69 (24.5%)	7 (31.8%)	23 (25.0%)	0.745	0.443	0.877	0.533
DRB1*07	72 (25.5%)	4 (18.2%)	20 (21.7%)	0.604	0.443	0.494	0.696
DRB1*08	24 (8.5%)	3 (13.6%)	5 (5.4%)	0.396	0.416	0.350	0.182
DRB1*09	14 (5.0%)	1 (4.5%)	2 (2.2%)	0.517	0.930	0.257	0.539
DRB1*10	11 (3.9%)	0 (0.0%)	5 (5.4%)	0.496	0.345	0.514	0.261
DRB1*11	55 (19.5%)	3 (13.6%)	16 (17.4%)	0.743	0.500	0.685	0.657
DRB1*12	9 (3.2%)	1 (4.5%)	0 (0.0%)	0.196	0.732	0.085	0.041
DRB1*13	**84 (29.8%)**	**9 (40.9%)**	**12 (13.0%)**	**0.002**	**0.276**	**0.002**	**0.003**
DRB1*14	17 (6.0%)	0 (0.0%)	7 (7.6%)	0.405	0.236	0.574	0.179
DRB1*15	56 (19.9%)	3 (13.6%)	16 (17.4%)	0.704	0.477	0.632	0.657
DRB1*16	13 (4.6%)	2 (9.1%)	8 (8.7%)	0.276	0.350	0.132	0.965

Discussion

Clinical and demographics

Refractory MG represents a significant subgroup of the disease. In our cohort, the frequency of the refractory MG patients was 22%. This rate is slightly higher than in other populations where it corresponds to 10-15%.[142] Maybe it happens in our cohort because it is a tertiary centre, where more difficult patients are sent to second opinion.
To have a worse MGFA classification at onset, being seropositive either for AChR or anti-MuSK antibodies were the characteristics associated with a refractory course in our cohort.

MuSK-antibody positive MG patients generally respond to conventional immunotherapy, but require higher corticosteroid doses to manage symptoms and have lower remission rates than AChR- antibody positive patients.[82,232] In our study, refractory MuSK-antibody MG patients corresponded to 44,4% of the total MuSK-antibody MG patients.

Nine out of 22 patients (40,9%) with thymoma MG were refractory patients. [233] Akaishi found that patients with MuSK-Ab showed worst severity at the same level as thymoma-associated MG patients, although this result was not statistically significant because of the small number of patients.[234] We also found that another important refractory MG subgroup is thymoma MG.

This study mentioned previously that included 923 japanese MG patients also found that those with ocular MG and those who are seronegative have a better response to treatments.[234] In our study, seropositive patients were more likely to have a generalized disease.

Thymectomy, regardless of the histology, was found in other studies to be associated with a refractory course.[88] In our study we could only find that in those with thymoma, and not thymic hyperplasia, normal histology or atrophic histology.

These characteristics, worse MGFA classification at onset, being seropositive either for AChR or anti-MuSK antibodies associated with a refractory course of the disease should prompt for a more aggressive treatment from the beginning of the disease, more careful weaning of the steroids and earlier start of immnussupressors agents.

Immunogenetics

The functional basis of the association between specific HLA alleles and development of autoimmune disorders is not completely understood. The molecular mimicry hypothesis proposes that certain HLA alleles are more efficient in presenting pathogen epitopes that share structural features with self-peptides to mature T cells. Once the response to the pathogen is initiated the self-antigen is also recognized and disease ensues. The central selection failure theory propose that certain HLA alleles are less efficient at presenting self-peptides to developing T cells in the thymus, so negative selection fails. [235] Another hypothesis postulate that different alleles can modulate the immunologic profile of an individual, through antigen-independent mechanisms, resulting in either promoting a higher autoimmune predisposition or, in opposition, a more efficient immune regulation. [200]

Considering that there is an association of HLA-DRB1 alleles with different autoimmune diseases[200,231] and their course, and also the well-known susceptibility alleles for the different MG subgroups[49,106,189] we explored which HLA-DRB1 alleles could be influencing a refractory course in myasthenia gravis.

The previous immunogenetic analysis of this cohort confirmed the well-known association of MG with HLA-DRB1*03. [106] In this study compelling evidence is provided that this association is driven by non-refractory patients and controls, as no significant difference between refractory patients and controls was observed.

We also uncovered a protective role for HLA-DRB1*13 in the non-refractory group, that was masked in the previous study, as the subgroup division

was by age of onset and not considering the refractory course of the disease.

HLA-DRB1*13 allele has also been pointed has protective factor for several other autoimmune disorders: Systemic Lupus Erythematosus, Psoriasis or Psoriatic Arthritis, Rheumatoid Arthritis, Systemic Sclerosis and Multiple Sclerosis.[200]

This is the first time, to our knowledge that an association of immunogenetic biomarkers and refractory MG was reported.

Final Comments

In conclusion, our results show that refractory MG patients are a subset of MG patients with clinical features that are distinct from those of non-refractory patients. These patients are more likely to be seropositive, either to anti-AChR or to anti-MuSK antibodies, to have a thymoma and to have a more severe MGFA classification at onset.

The distinct characteristics of refractory patients suggest underlying biological differences between the non-refractory and refractory MG groups. We found that the refractory group has a different immunogenetic background, being more similar to the controls than the non-refractory subgroup.

It is important to be aware of refractory traits characteristics from the onset of the disease for a better management of these patients. The identification of biomarkers for refractory disease will be essential for the development of newer therapeutic approaches.

3.6 Infections, neoplasms and other autoimmunity in thymoma associated Myasthenia gravis

This chapter was based in two studies, the first was the study of two case reports and the analysis of their thymus at two moments of the disease:

- **Thymoma following thymectomy of hyperplastic thymus in young onset Myasthenia gravis: clinical features heralding the thymoma;**

And the second is still being conducted. It has the objective to study the occurrence of infections, neoplasms (other than thymus) and systemic autoimmunity in thymoma associated MG with AIRE gene and other immunohistochemistry studies:

- **Infections, neoplasms and other autoimmunity in thymoma MG and AIRE gene.**

3.6.1. Thymoma following thymectomy of hyperplastic thymus in young onset Myasthenia gravis: clinical features heralding the thymoma

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Thymoma following thymectomy of hyperplastic thymus in young onset Myasthenia gravis: clinical features heralding the thymoma

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Abstract:

Background: It is extremely rare for young onset myasthenia gravis (MG) patients to have thymoma following extended removal of hyperplastic thymus. We present two anti-acetylcholine receptor MG (AChR-MG) patients who developed thymoma years after extended transternal thymectomy and highlight the clinical manifestations that heralded the thymoma, consequently of a severe immunodeficiency which resembled APECED (autoimmune polyendocrinology, chronic mucocutaneous candidiasis and ectodermal dystrophy) syndrome, with auto-immune regulatory (AIRE) gene defect.

Case 1: a 20 years-old female with generalized MG received extended transternal thymectomy revealing thymic follicular hyperplasia. After 15 years of stable, mild MG on chronic steroid therapy, she got worse (recurrent severe respiratory infections needing ventilation and plasma exchange). She also developed alopecia totalis, oral candidiasis, recurrent genital Herpes simplex infections and recurrent severe respiratory infections. A WHO B2 type thymoma was removed, but MG and associated manifestations did not improve, and patient died at 52 years-old with a severe pneumonia and respiratory failure. Retrospectively, she was found to have high levels of anti-IL22, IL-17A and IL-17F antibodies.

Case 2: a 27 years-old male with generalized MG was treated with immunosuppression and extended transternal thymectomy revealing thymic follicular hyperplasia. MG was initially difficult to treat needing plasma exchanges, but became stable with steroids and cyclosporine. Seven years after MG onset, he presented extensive skin lesions and worsened myasthenic symptoms with generalized severe bulbar and respiratory involvement. Imaging and histology revealed thymoma type B3 with pleural dissemination. Skin lesions were diagnosed as paraneoplastic exfoliative erythoderma. He had severe immunodeficiency (CD4 lymphocytes= 99/µL) with several nosocomial infections (*P. aeruginosa*, *Klebsiella*, CMV and became HBV DNA positive), chronic

diarrhoea and a chronic hepatitis with unknown cause (infeccious vs autoimmune). Thoracic radiotherapy (dose 30 Gy) was followed by chemotherapy, but the patient died of massive pulmonary thromboembolism. He was found to have high levels of anti-IL22, IL-17A and IL-17F antibodies.

Discussion: These two cases illustrate the need of searching for a thymoma in MG patients whose MG becomes difficult to treat after removal of a thymus with thymic follicular hyperplasia. Thymoma needs to be considered even more in case of severe and/or recurrent infections, which could reflect complex paraneoplastic immune-deficiency that in the here described patients were associated with auto-antibodies to interleukins.

Thymoma following thymectomy of hyperplastic thymus in young onset Myasthenia gravis: clinical features heralding the thymoma

Background:

Thymomas are rare thymic epithelial tumors that are often associated with autoimmune disorders among which myasthenia gravis (MG) is the commonest.[227,228]. In addition, autoimmune and other mechanisms underlie thymoma-associated humoral and cellular immune defects (e.g. hypogammoglobulinemia) that commonly entail life-threatening infections.[227,228]

Thymectomy is an accepted therapeutic option for myasthenia gravis[229,230]. Extended thymectomy aims to dissect the macroscopically recognizable thymus together with the anterior mediastinal fat, taking into account that mediastinal adipose tissue may harbor substantial thymic extensions on microscopic examination [229]. There are several reports on recurrences of MG because of incomplete removal or ectopy of thymic tissue, while reports on thymomas arising from remnant or ectopic thymic tissue following transternal thymectomy are sparse.[231–235] Associated diseases, particularly MG, are often the presenting manifestation of thymomas, but others occur after thymectomy and may sometimes herald thymoma recurrence. This also applies to thymoma-associated immunodeficiencies and subsequent severe and/or chronic infections such as chronic mucocutaneous candidiasis (CMC), cryptococcal meningitis and Kaposi sarcoma.[228]

APECED is the eponym of a genetic syndrome that typically comprises autoimmune polyendocrinology, chronic mucocutaneous candidiasis and ectodermal dystrophy. The endocrine and infectious findings have an immune basis and result from mutations of *AIRE* (*autoimmune regulator*), which is a crucial gene in the induction of thymic self-tolerance[236]: AIRE protein mediates expression of thousands of tissue-specific self-antigens by medullary thymic epithelial cells, while this expression is missing in AIRE deficiency states, in which multi-organ autoimmunity develops because of faulty negative selection of auto-reactive T cells.[237]

Some of the APECED syndrome features are also found in patients with thymoma regardless the development of MG.[236] There is some evidence that the clinical features of APECED syndrome correlate with the absence/reduction of specific pro-inflammatory interleukins (IL17 A, IL 17F, IL 22) and the presence of neutralizing antibodies to these interleukins. Neutralizing antibodies to IL17 were also found in some thymoma patients with CMC.[237] In those patients it was hypothesized that production of autoantibodies against these TH17-produced cytokines was related to thymocyte development with defective negative selection in thymomas with lack of AIRE expression.[237]

Objective:

To report two patients with early onset MG, submitted to extended transternal thymectomy, with thymic hyperplasia, that later in life developed clinical manifestations heralding a thymoma.

Case Reports:

Case 1:

A 20 years old woman started to suffer from occasional unilateral eyelid ptosis. Few years later, after delivery, she had diplopia, unilateral eyelid ptosis and mild dysphagia. Electromyography confirmed decrement of compound muscle action potential (CMAP) amplitude on repetitive stimulation. MG was diagnosed. She improved with pyridostigmine, but mild ptosis persisted. In 1987, at 35 years, she worsened, experiencing also limb weakness, mild dysphagia, dysphonia. It was decided to start treatment with steroids. After this she rapidly worsened, needing admission to intensive care unit because of respiratory failure. She was submitted to extended transternal thymectomy and histology revealed thymic follicular hyperplasia.

She improved and remained stable (MGFA IIA) for more than 10 years, although steroids could never be completely discontinued.

In 1997, at age 45, she had recurrent respiratory tract infections, some of which with admission to the intensive care unit for ventilation support and worsening of myasthenic symptoms requiring treatment with plasma exchange. Anti-AChR antibodies and CT scan was not performed at this time. She presented with chronic oral and genital candidiasis and severe onychomycosis. She also had recurrent herpes simplex genital ulcers that were painful, difficult to treat and became chronic. She developed *alopecia totalis*, because of that and the recurrent severe infections thymoma was suspected. Thorax MRI confirmed the presence of a mediastinal mass. She was submitted to thymoma removal. Histological analysis revealed a WHO B2 type thymoma. After the surgery, she was free of myasthenic symptoms and required no treatment but soon she developed neck weakness requiring treatment with pyridostigmine and a mild dose of steroids. Antibodies to AChR were positive, 8.0 nmol/L.

Seven years after the surgery, myasthenic symptoms got worse that were largely refractory to plasma exchange and intravenous immunoglobulins, developed severe recurrent respiratory tract infections, requiring repeated admissions to the intensive care unit. Genital herpes simplex ulcers and onychomycosis also persisted, being refractory to treatment. She died of a severe pneumonia at 52 years of age.

Retrospective study of her serum showed high levels of neutralizing antibodies to the proinflammatory interleukins IFN-α, IFN-ω, IL-17A, IL-17F and IL-22 by the time the thymoma was diagnosed. Immunohistochemistry of archival paraffin material revealed loss of AIRE expression in the thymoma but not in the hyperplastic thymus removed during the first surgery, although it had a weak expression.

Figures

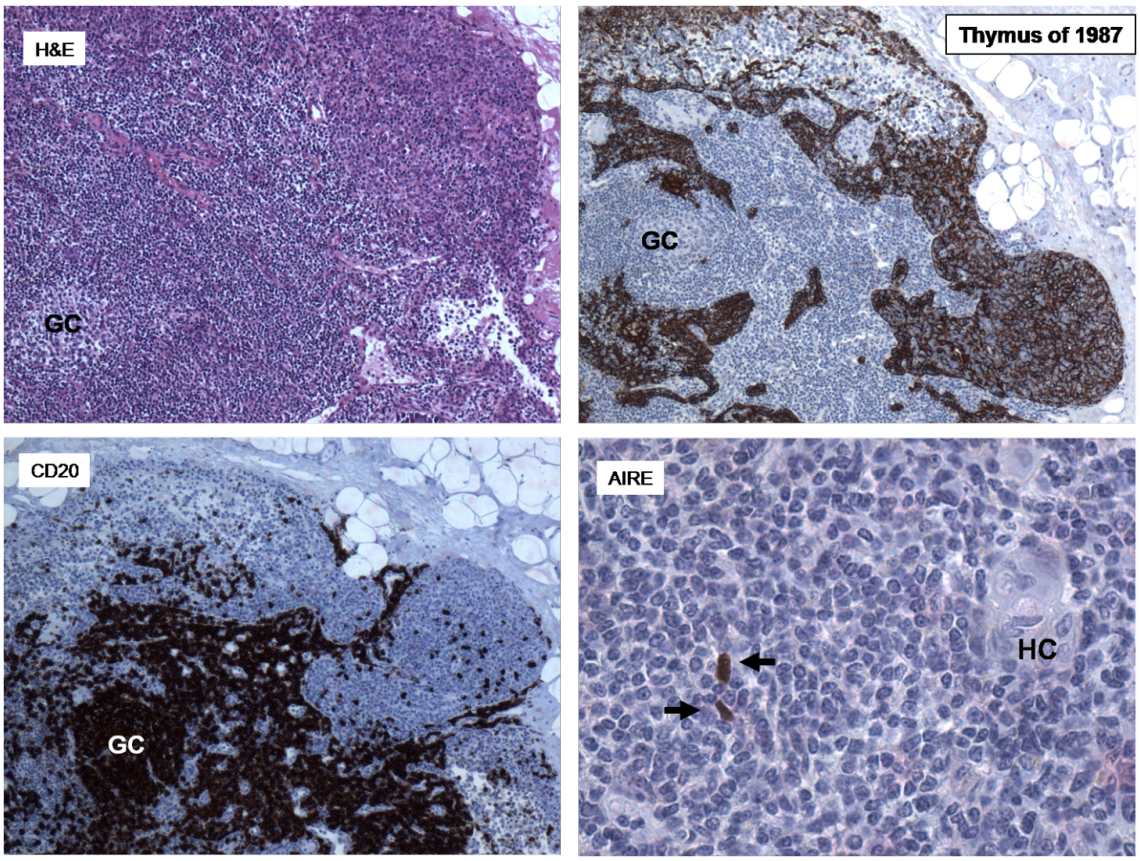

Fig. 3.6.1.1. Thymus as resected in 1987, following corticosteroid treatment. **a.** Non-neoplastic thymic tissue with moderate involution of the cortex (C) and lymphoid follicular hyperplasia as revealed by a germinal centre (GC) in the medulla (M) [H&E, x50]. **b.** Cytokeratin 19 staining highlights epithelial cells in the cortex (C) and medullary epithelial bands (MEB). **c.** High number of CD20-positive B cells with clear-cut follicle formation in the thymic medulla. **d.** Staining for AIRE revealed an inadequate paucity of AIRE-positive cells (arrows), likely due to corticosteroid treatment. Hassall's corpuscle (HC). [Immunoperoxidase in b. – d., x50 in b. and c., x200 in d.]

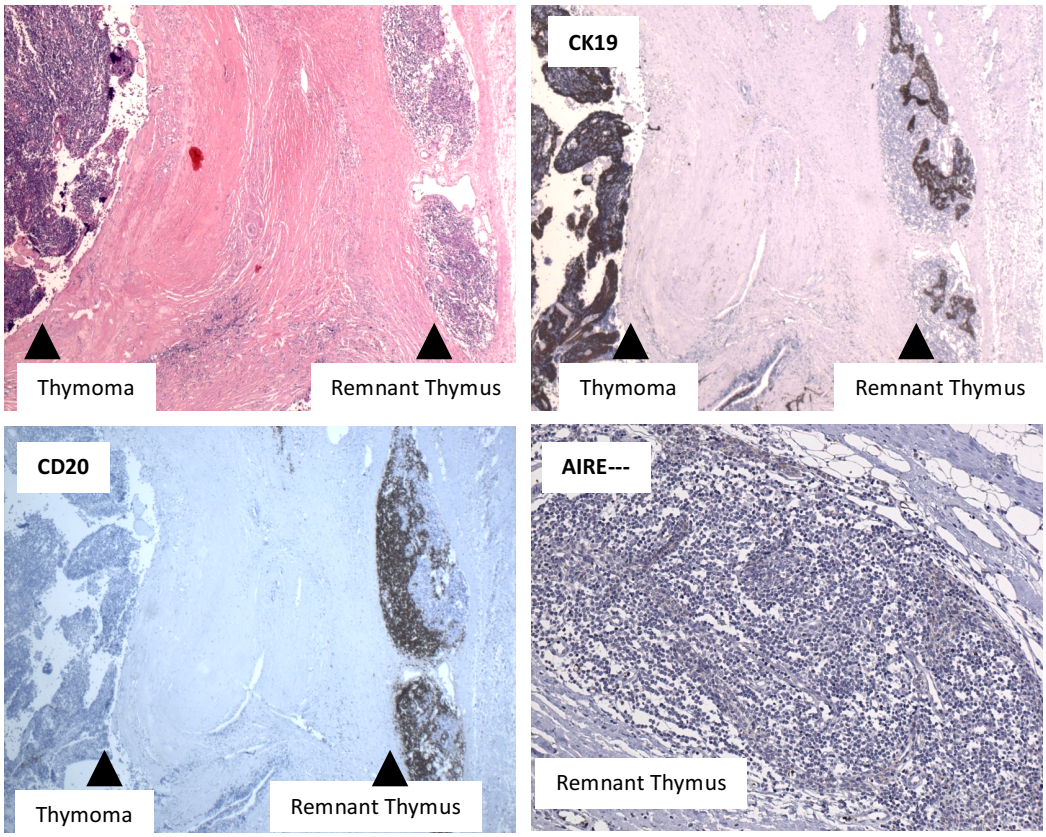

Fig. 3.6.1. 2. Overview of the resection specimen of 1997. **a.** Adjacent to the capsule surrounding the thymoma (left) there is atrophic non-neoplastic thymic tissue (right) with massive cortical involution and paucity of Hassall's corpuscles [H&E, x50]. **b.** Cytokeratin 19 staining highlights epithelial cells in the thymoma and the remnant thymic tissue. **c.** CD20-positive B cells (without follicle formation) are conspicuous only in the thymus. **d.** Staining of the thymus for AIRE revealed a lack of AIRE-positive cells (again after antecedent immunosuppressive treatment with corticosteroids). [Immunoperoxidase in b. – d., x50 in b. and c.; x200 in d.]

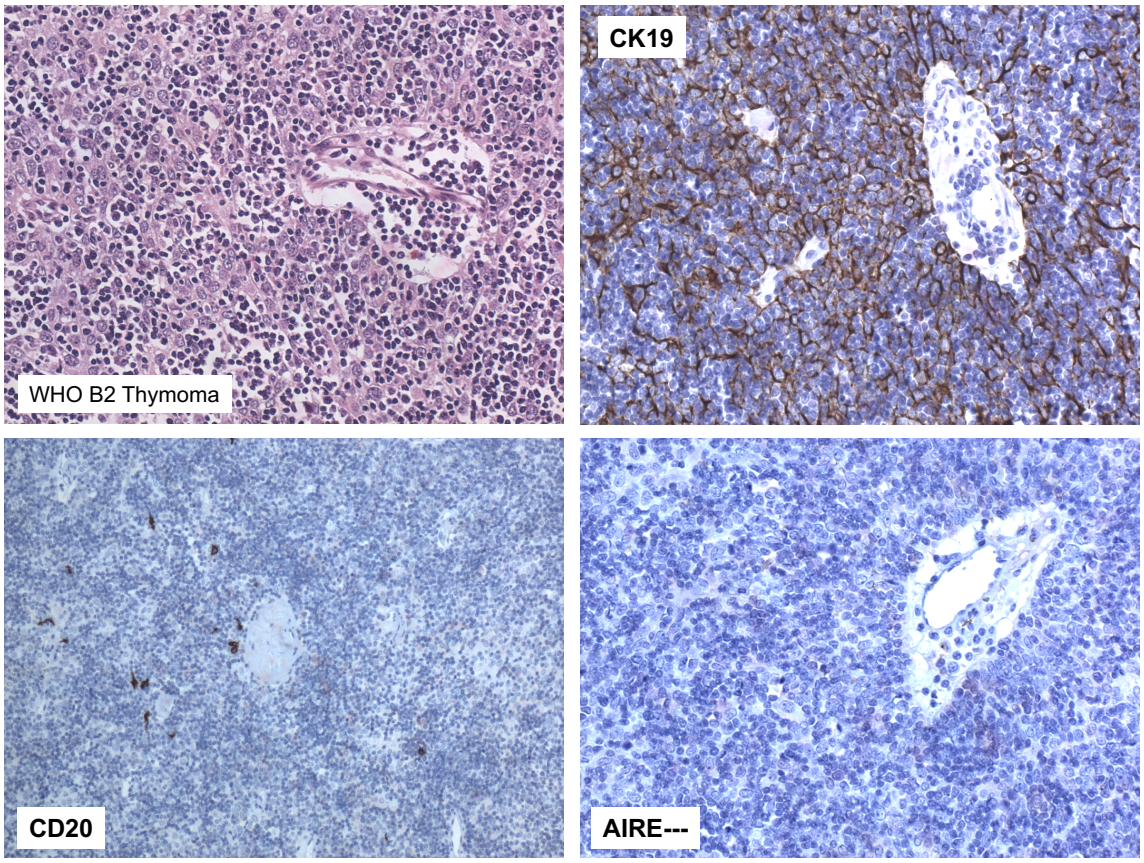

Fig. 3.6.1.3. **a.** WHO type B2 thymoma with a conspicuous perivascular space [H&E, x200]. **b)** Cytokeratin 19 staining highlights the epithelial-free perivascular space with vague surrounding "palisade" of epithelial cells. **c.** Typical paucity of CD20-positive B cells in the thymoma (compare to Fig 1c.). **d.** Lack of AIRE expression in B2 thymoma as is typical for >95% of thymomas, including apparently all B2 thymomas, irrespective of the presence or absence of mucocutaneous candidiasis (Ströbel, 2007; Kisand, 2010). [Immunoperoxidase in b. – d.; x50 in c.; x200 in b. and d.]

Case 2:

In June 2003, a 27 years old man started generalized weakness, difficulties in playing football with falls, difficulty in chewing, dysphonia, diplopia and unilateral ptosis. Electromyography showed decrement of CMAP's in the repetitive nerve stimulation of the facial and cubital nerves and a high titre of AChR antibodies. Generalized MG was diagnosed and he started pyridostigmine, 300 mg/day with partial improvement. Thorax CT scan showed an enlargement of the thymic tissue. In August 2003, he had a thoracic trauma after a fall. He worsened his weakness of the neck and the limbs and started dysphagia. He was treated with prednisolone 90 mg/day and pyridostimine 420 mg/day 20 days later. One month later he was readmitted for extended transternal thymectomy. Histology showed a thymic follicular hyperplasia. Six days after surgery he developed severe dysphagia and herpes zoster infection at the level of the T8 dermatome. He was successfully treated with IVIG and acyclovir. Five days later he worsened again of the dysphagia and neck muscles weakness he was treated with plasma exchange. But soon he developed respiratory weakness, requiring mechanical ventilation. At that time, *S. aureus* sepsis related to the plasma exchange catheter was diagnosed. Because of the bulbar symptoms IVIG was repeated and started cyclosporin A progressively up to 4 mg/kg/day. During the following year, the steroids were tapered slowly and he was stable.

He was stable under cyclosporine 200mg/day and pyridostigmine 420 mg/day until July 2010. At this time, he developed an erythematous rash spreading from the thorax to the upper limbs and simultaneously ocular symptoms, dysphonia and dysphagia reappeared. Steroids were reintroduced without significant improvement. Skin biopsies were performed and it was diagnosed a pityriasis rosea in the first and a liquen planus in the second. In November 2010, he was readmitted because of bulbar symptoms, proximal tetraparesis and dyspnoea. Thorax CT scan showed a mediastinal mass around the supra-aortic trunk with contrast enhancement and multiple lesions in the pleura. A mediastinal biopsy showed a thymoma type B3. PET scan suggested that the disease was

locally invasive. Skin lesions were later considered to be paraneoplastic erytrodherma. He was treated with plasma exchange, but soon worsened again needing mechanical ventilation. Plasma exchange was repeated for 23 sessions. He continued to deteriorate, always dependent on mechanical ventilation and a feeding tube. The tumour was considered unresectable. He had several infections: *Pseudomonas aeruginosa* pneumonia and then became persistently present in the respiratory secretions, Klebsiella p. infection, CMV systemic infection, positive HBV DNA), chronic hepatitis with uncertain cause (CMV, HBV, toxic or autoimmune) and persistent diarrhoea, with malabsorption, causing severe nutritional deficits, and which cause was not found. He had a severe immunodeficiency (CD4+=99/μL). Since the patient's condition precluded chemotherapy, radiotherapy (dose 30 Gy) to the mediastinum was performed. His nutritional state slowly improved and in February 2011 he started chemotherapy with (cyclophosphamide, doxorubicin and cisplatin). After the first session of chemotherapy his condition deteriorated again needing invasive ventilation. Thoracic CT scan showed a massive pulmonary thromboembolism. Despite the treatment with intravenous heparin he died 48 hours later.

Retrospective analysis of patient's serum from 2010, when his thymoma was diagnosed, revealed showed high levels of neutralizing antibodies to IFN-α, IFN-ω, IL-17A, IL-17F and IL-22.

Discussion:

We report here on two patients with early onset AChR MG who showed exacerbated MG and unusual other paraneoplastic phenomena in conjunction with thymoma development many years after extended transternal thymectomy. To the best of our knowledge, only five thymomas developing after thymectomy for non-thymomatous MG have been described so far, [231–235] however without reporting on anti-cytokine autoantibodies that in one of the patients was associated with features of APECED.

Our review of sections from the original thymectomy specimens confirmed the diagnosis of TFH and provided no evidence that a thymoma had been overlooked. Therefore, it is most likely that the thymomas in our patients arose from thymic remnants that escaped removal during the first surgery in spite of 'extended transsternal thymectomy'. This approach aims to remove all extensions and heterotopic foci of thymic tissue in the mediastinal and part of the cervical fat in addition to the main body of the thymus. However, about 5% of thymic tissue is left behind as compared to the abandoned "maximal thymectomy" approach that requires combined transsternal and transcervical surgery without apparently improving the control of MG symptoms[238]. Microscopic extensions of thymic tissue may escape surgical removal particularly around and between large vessels in the vicinity of the aortic arch[239]. This possibility is particularly obvious in our 2nd patient, because his thymoma occurred in an unusual location, "around the supra-aortic trunk" (see above). The alternative scenario that a late metastasis of an overlooked thymoma was the source of the tumors appears less probable. However, a caveat concerns the paucity of slides that were available for our review of the historic thymectomy material, reflecting the lack of guidelines about how many paraffin blocks should be prepared from non-neoplastic thymectomy specimens. "Microthymomas" in particular may escape macroscopic detection and limited sampling[240,241]. In fact, on extensive sampling as required in the recent MGTX study on non-thymomatous AChR-MG one unexpected thymoma was detected among 60 thymectomy specimens[38]. In any case, the admittedly low risk of malignant transformation of remnant thymic tissue may be considered in the debate on whether extensive or limited approaches are preferable in conventional or robotic thymectomy for non-thymomatous MG.[38,229,242,243]

An unusual finding in our two patients concerns their immunological features in addition to exacerbated MG in connection with thymoma development: both patients showed identical profiles of anti-cytokine autoantibodies, including those that are typical of thymoma patients in

general (IFN-α, IFN-ω), but also rare neutralizing autoantibodies to TH-17 cytokines, IL-17A, IL-17F and IL-22 that are typical of APECED syndrome patients, i.e. patients with germline mutations of the 'autoimmune regulator gene', *AIRE*. By contrast, almost all thymomas (including the tumor of patient 1) have shown deficient AIRE protein expression in their neoplastic thymic epithelial cells despite a wild-type *AIRE* gene [244], and it is unknown, why APECED features are so uncommon[244] and why autoantibodies to TH-17 cytokines are confined to a minority of thymoma patients[237]. On the other hand, since the autoantibody-mediated functional defect of TH17 cytokines underlies the innate immunodeficiency that leads to chronic candidiasis[245], only the rare thymoma patients with these autoantibodies show chronic candidiasis[237] or even a broader spectrum of APECED symptoms (ref. [236] and our patient 1). Therefore, it is not too surprising that patient 1 showed APECED features, while it is a new and unexpected observation that patient 2 did not have such features - despite the presence of anti-TH17 cytokine. The reason for this discrepancy between the two patients is currently unknown.

Instead of APECED-like symptoms, patient 2 showed more 'unspecific' serious infections (including Pseudomona aeruginosa and other bacterial infections and CMV infection) in connection with acquired severe deficiency of CD4+ T cells that is known to occur in variable association with autoimmune B lymphopenia in a subset of thymoma patients. [246–249]

In any case, it is most remarkable that the two thymoma patients presented here had a coincidence of two findings: One that is rare among thymoma patients, namely severe immunodeficiency against a background of anti-TH17 autoimmunity, and another one that is even much rarer, namely thymoma development years after subtotal thymectomy for non-thymomatous AChR MG. More such cases need in depth immunological investigation to get a hint whether this coincidence is more than fortuitous.

Conclusion:

These cases are extraordinarily informative, enabling us to study in each patient the clinical, serological and thymic features of MG as both autoimmune and paraneoplastic disease. Not only are the alopecia and severe and recurrent infections, particularly the mucocutaneous candidiasis, known complications of thymoma: they are also signs of a complex underlying immunodeficiency that apparently has an autoimmune basis, as in APECED patients.

In cases of autoimmune MG submitted to extended transternal thymectomy that become refractory to treatments a thymoma should be searched.

Acknowledgment: Prof. Willcox and Meager for anti-interleukin antibody detection.

3.6.2 Infections, neoplasms and other autoimmunity in thymoma MG and AIRE gene

This study is still being performed.

The clinical and demographic data has been collected. We analysed the frequency of infections (severe, recurrent or opportunistic), neoplasms and other autoimmunity in patients with thymoma MG of our cohort. Some of this data was already presented in international meetings, as described below.

The histopathological studies are currently being performed.

The objective of the study is to correlate the clinical data with the histopathological findings.

Works presented in international meetings:

- "***Clinical review of thymoma in Myasthenia gravis patients. Viral and fungal infections may indicate poor prognosis***", in the International Conference of Neuromuscular Disorders, in Naples July 2010 and the abstract was published in Journal of Neuromuscular Diseases;

- "***Extra-thymic malignancies in myasthenia gravis patients***", in the 12th International Conference on Myasthenia Gravis and Related Disorders, in New York, May 2012, organized by Myasthenia Gravis Foundation of America and the New York Academy of Sciences;

- ***"Polyautoimmunity, tumours and infections in myasthenia gravis",*** in the 3rd International Congress on Controversies in Rheumatology & Autoimmunity, in Sorrento, March 2014.

Infections, neoplasms and other autoimmunity in thymoma MG and AIRE gene

Introduction

The thymus, a lymphoid organ with a lobular structure, is important for the development of T cells. Specifically, thymocytes (T cell precursors) are subjected to both negative and positive selection in the thymus. [97,98]

Each lobule of the thymus has a cortex that contains densely packed CD4 and CD8 double-positive thymocytes and a medulla that contains sparser CD4 or CD8 single-positive thymocytes.[97,98]

Mainly in the cortex, thymocytes are subjected to positive selection, in which precursors with low reactivity to the MHC complex are deleted/eliminated. Subsequently, the thymocytes are subjected to negative selection in the medulla, a process that deletes/eliminates cells that have reactivity against self-antigens.

Thymic epithelial cells (TECs) and thymic dendritic cells (tDCs) are considered to be responsible for the positive and negative selection of thymocytes.

In most cases of thymoma associated MG (TAMG) the source of the autoreactive T cells is almost certainly the highly abnormal but thymopoietically active thymoma microenvironment, as also suggested by unusual antigen specificities of CD4+ T cells in thymoma patients.[77,98]

The role of CD8+ T cells in the pathogenesis of AChR-MG is less clear but likely of high relevance at least in EOMG and LOMG considering their association with MHC class I risk alleles.[78]

TAMG typically occurs after 50 years of age but children may rarely be affected. In contrast to EOMG, there is no major gender bias and no strong HLA association. Immunodeficiency states of which some have an autoimmune basis, are also quite frequent. [237,246] Apart from rare

exceptions that showed anti-MuSK antibodies[250,251] or were seronegative. [252,253]

TAMG is more commonly than EOMG accompanied by additional autoimmune diseases: the spectrum of them is broader but partially overlapping with the spectrum in EOMG [254–256]. Immunodeficiency states are also quite frequent [237,246].

Another pathogenic feature, although it is shared by almost all MG(+) and MG(-) thymomas, is defective expression of the autoimmune regulator AIRE. [244]

In thymoma-associated Myasthenia gravis the focus is on the role of abnormal intratumorous T cell selection and activation, lack of intratumorous myoid cells and regulatory T cells as well as deficient expression of the autoimmune regulatory gene (AIRE) by neoplastic thymic epithelial cells. [98]

The failure of peripheral tolerance mechanisms in such tumor patients is by all likelihood also a facet of the tolerance breakdown observed in thymomas. [254] However, the unusually high frequency (>50%) of thymoma-associated autoimmune phenomena compared to their low frequency (<5%) in other tumor types suggests that additional mechanism are likely operative in TAMG [246,256].

Taking into account that autoimmune diseases are more prevalent in patients with thymopoietically active thymomas than in thymopoietically inactive thymomas, and that MG is virtually never encountered in patients with thymic carcinoma (that are generally devoid of thymopoietic activity) [254,256,257], a pathogenetic role of central self-tolerance failure in TAMG seems to be important.

The Thymus and AIRE gene

The thymus selects T cells, thus ensuring T cell tolerance. Thymoma can be associated with immune dysregulation manifesting as autoimmunity and/or immunodeficiency. A particular challenge to physicians treating patients with thymoma is the possible occurrence of both autoimmunity—requiring immunosuppression—and immunodeficiency. [78,246]

Major laboratory findings of this syndrome are hypogammaglobulinemia, few or absent circulating B and T cells, and an abnormal CD4:CD8 T cell ratio.[98,246,249]

Immune dysregulation in thymoma patients has only been described in case reports and small case series. To date, no clear link between histologic type of thymoma and immunologic manifestations can be drawn. [246]

The pathogenesis of immunodeficiency in patients with thymoma has long remained a mystery, yet recently functionally relevant anticytokine autoantibodies have been linked to the clinical phenotype observed in these patients. [237,245,254,255,258,259]

Autoantibodies neutralizing key components of the immune system can thus lead to an increased risk of infections.[237]

Given the high incidence of immunodeficiency/infections in these patients, thymoma should probably be excluded in adults presenting with recurrent (severe) infections and suspicion of primary immunodeficiency. [227,249,260]

Knowing the fact that autoimmune regulator mutations are associated with autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, lack of autoimmune regulator expression in a subset of thymomas may partially explain the loss of tolerance and the development of autoimmunity seen in these patients. [237,244]

In the absence of the AIRE protein, many tissue-specific self-antigens are not expressed in the thymus, and multi-organ autoimmunity develops because of faulty negative selection of autoreactive T cells. [236]

Autoimmune polyendocrinopathy syndrome type 1 (APS1), also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, is caused by defects in a single gene. Patients with this rare disease have mutations in both copies of *AIRE* gene (for autoimmune regulator). [259]

By definition, the patients have at least two of the "APS1 triad"—hypoparathyroidism (HP), Addison disease (AD), and chronic mucocutaneous candidiasis (CMC). However, the clinical phenotype is highly variable. Characteristically, patients present with CMC and/or skin disorders, usually early in childhood; these symptoms are followed (sometimes 1–3 decades later) by autoimmune endocrine disorders, which may also target the gonads and/or endocrine cells in the gut, pancreatic islets, and thyroid gland. [254,259]

In addition, some had alopecia, keratoconjunctivitis, vitiligo, and vasculitis, and few were growth-hormone-deficient. [259]

Starting in infants or young children, the typical diagnostic triad of APS-I comprises hypoparathyroidism (HP), autoimmune adrenal insufficiency (AI) and chronic mucocutaneous candidiasis (CMC). Many patients develop other autoimmune manifestations, e.g. premature ovarian insufficiency (POI), vitiligo, alopecia, autoimmune hepatitis, keratitis, enamel dysplasia, and/or intestinal malabsorption. Phenotypes vary widely, even within families; some patients are first recognized in adulthood. [254]

Generation of the T cell repertoire in the thymus involves selection of those T cells that recognize only foreign substances. T cells that can react against self-antigens are eliminated, and the *AIRE* gene is thought to be involved in this "education process." Like those with APS1, patients with thymomas make autoantibodies not only against target organs (especially muscle in their case), but also against interferon alpha (IFN-α) and

interferon omega (IFN-ω), two secreted immune regulators.

The most obvious link between these disparate syndromes is that, in nearly all thymomas, the neoplastic TECs fail to express AIRE detectably, implying reduced expression of AChR, insulin and GAD65. [254]

Material and Methods

Clinical and Demographic Data

Here we retrospectively analysed the frequency and clinical presentation of autoimmune manifestations, infections and neoplasms (other than thymus) in patients with MG and a histologically confirmed thymoma diagnosed in a multicentric study which included 5 hospitals in the north of Portugal: Centro Hospitalar do Porto, Centro Hospitalar de Vila Nova de Gaia, Hospital de São João, Instituto Português de Oncologia e Hospital de Pedro Hispano.

Histopathological studies of the thymomas cases

Currently, we are studying the samples of thymomas of TAMG cases from the mentioned above hospitals.

The histopathological studies of the thymus are being performed at Serviço de Anatomia Patológica, Centro Hospitalar do Porto, under supervision of Dr. Jose Ramon Vizcaíno and Prof. Doutor Carlos Lopes.

We are performing the studies necessary to classify thymomas according to WHO classification, and also additional immunohistochemistry studies.

These includes the study of:

- expression of AIRE gene;
- expression of CD8+CD45RA+ and CD103 in order to understand whether the cases with opportunistic infections might be associated with a poor CD8+CD45RA+ export capacity.

- expression of CD20*, CD79a, PAX5, CD23 and CD138. Since anti-cytokine antibodies are said to be produced within the thymoma, we want to study whether there is a correlation between autoantibody-positivity in the sera and the occurrence of B cells (CD20*, CD79a, PAX5), lymphoid follicles (CD23) and plasma cells (CD138, kappa/lambda) inside the respective thymomas (not only in the adjacent remnant thymus).

Objective

The objective of the study is to correlate the clinical data, the frequency and clinical presentation of autoimmune manifestations, infections and neoplasms (other than thymus) in patients with TAMG with AIRE gene expression, poor CD8+CD45RA+ export capacity and/or the presence of B cells and lymphoid follicles inside the thymoma.

Results

This is an ongoing study.

Chapter 4. Summary and Discussion

Chapter 4. Summary and Discussion

Epidemiological study

We estimated a point prevalence for MG of 111.7 patients/10^6 in the North of Portugal (a 31/12/2013). Our method of estimation is valid, as explained in the methods section and in the published paper. This estimate is similar to those reported either in studies in southern European countries, or in the north of Europe, in studies with similar methods and size of the population studied. [122,125,126,128]

Prevalence rose with age reaching its maximum in the group over 65 years old, especially in males (288.1/10^6), as reported in many other studies.[121,183]

During the year of 2013 we estimated an incidence rate of 6.3 per million person/year. Our incidence rate was similar to those reported in studies from distinct latitudes with similar methods and to others from south of Europe. [121,125–128]

Among females, incidence rate was higher in the age group 15-49 (9.1/10^6), while in males, incidence increased with age till the maximum (22.1/10^6) among those aged over 65 years. We do not have information on changes in incidence over time, because these is the first epidemiological study on MG in Portugal. But this result is in agreement with most of the recent reports that describe higher incidence rates of MG in LOMG group, especially in elderly males.[121,122,129,153] This information has crucial importance for the awareness of clinicians when dealing with this age group, and subsequent improvement in diagnosis and management of MG.

The mortality rate attributed to MG was 0.5 per million, which is in the range of what others studies reported.[122,129]

Of note, 3 deaths occurred in relation with aggressive thymomas. Regarding age at death, six occurred between 84 and 89 years, an age that is above the Portuguese life span (82.8 for females and 76.9 for males).

Clinical, demographic and serological features

Our prevalent and incident data confirmed that the majority of patients belong to the group of LOMG, confirming others' findings that MG is a disease that affects younger and also elderly people. Overall our clinical, histology and serological data were similar to those reported by other authors. There were, however some discrepancies, which deserve some comments.

Overall a surprisingly high percentage of patients (19%) were treated with IVIG or PEX at least once during the course of the disease, particularly knowing that majority of patients had MGFA I or II at nadir. Majority of patients treated with IVIG or PEX represented a third of the MuSK MG patients and nearly a quarter of the AChR MG patients, which is in keeping with our practice preferring more intense treatment in Ab positive patients with acute bulbar symptoms with or without respiratory manifestations.

Almost one third of patients (29.4%) had one type of the following comorbidities: other autoimmune diseases (OAID) (organ specific or systemic), tumours or recurrent/serious infections; 2.8% of the patients had ≥2 of those associated problems.

The proportion of patients with OAID (20.2% vs 16.3%) and infections (4.6% vs 4.4%) was identical in EOMG and LOMG groups. Only tumours were more common in LOMG (12.6% vs 5.9%, p= 0.026), which is in keeping with what is expected in general population.

Our lower number of infantile-onset MG patients (2.5%) is similar to findings from most European and American series where it varied from 1% to 3%. [184]

We found a percentage of 5.3% of MuSK MG in the group of prevalent patients, which is similar to what has been described by other authors in the southern European countries. [185] Interestingly, the percentage in the incident cases is higher, 14.3%. On the other hand, the proportion of AChR-MG in the group of incident cases was lower than expected, possibly because the proportion of ocular MG was relatively

high, 43.5%. This may represent variations in incidence of distinct disease subtypes or improvement in diagnosis of rarer and limited forms of MG.

Immunogenetic study

Over the years, association of MG with Human Leucocyte Antigens (HLA) has been described in different populations. In European descendent populations HLA-DRB1*03 allele strongly influences MG susceptibility.[39,134,187,188]

This study investigated the possible association between HLA-DRB1 alleles and age-of-onset in MG.

The results of the study demonstrated a strong association of HLA-DRB1*03 and HLA-B*08 with MG, confirming that these alleles constitute important susceptibility factors for this disease also in our population. Considering the age of onset, HLA-DRB1*01 was associated with late-onset subgroup. Thymoma MG patients have probably a different genetic (HLA- DRB1*10) background.

To the best of our knowledge these results were not reported before and need replication in other populations and in larger cohorts.

Pregnancy and Myasthenia gravis

It is well recognised that MG does not affect fertility, but it may expose pregnant women to an increased risk of maternal and fetal complications. The clinical course of pregnancy in these patients is not well known.

This study evaluated the clinical course, delivery and neonatal outcome of the pregnancies of our MG patients.

Regarding the influence of the pregnancy on the MG, 43% of patients experienced an exacerbation of symptoms, especially during the third trimester and postpartum. This is a similar rate of clinical worsening as in other studies published.[209] 90% were treated with acetylcholinesterase inhibitors and 43.3% also required prednisolone to symptomatic control.

There were no deaths in our cohort and we only experienced one myasthenic crisis during postpartum, following an aggressive clinical deterioration that occurred by the end of the third trimester.
We described an NMG rate of 7.1%. Two newborns developed classic signs of NMG during the first 72-96 hours of life, which is little later than usually described. They were treated with acetylcholinesterase inhibitors with complete resolution of symptoms during the first two months of life.
Our study adds to the body literature showing that a more aggressive MG background is a predictive factor for clinical exacerbation during pregnancy. A multidisciplinary approach with neurologists and trained obstetricians allowed a good clinical control, avoiding maternal adverse outcomes. In this group of patients, pregnancy should be planned to avoid the use of foetus toxic medication during pregnancy, as well as, to advise women to become pregnant during a stable phase of the disease.

The other study on pregnancy and MG was a multicentric study that included all MuSK-MG in the North of Portugal that underwent through a pregnancy. This is a subgroup of MG patients where the effect of the disease on the pregnancy, the delivery, the foetus and the changes on the MG during that period are less known. As far we are concerned there are no other series on MuSK-MG and pregnancy.
In our series, pregnancy did not seem to precipitate MuSK-MG or to influence the MuSK-MG course, and there was no apparent negative impact in pregnancy outcomes in those where pregnancy followed the MG onset. These results should be regarded with caution because is a small series.

Polyautoimmunity in Myasthenia gravis

Polyautoimmunity refers to the presence of more than one autoimmune disease in a single patient and this is well documented in great majority of the spectrum of autoimmune diseases, particularly those mediated by autoantibodies, including myasthenia gravis.

We found other autoimmune disorders in 37 patients with MG (17%). Six patients have a combination of more than one autoimmune disease besides MG (multiple autoimmune syndrome).

As expected, the frequency of a second autoimmune disorder is highest for females (68%) with early onset MG (EOMG) (mean age at onset is found to be 41 years). The majority of these patients have the generalized form of myasthenia (78%) and the anti-AChR antibodies were positive in 78%. Thymectomy was performed in 19 patients (51%), with thymic hyperplasia being the most common histological diagnosis. In most cases, MG preceded the onset of the other autoimmune disease (62%) and immunosuppression didn't seem to affect the development of a second condition (68% had not been exposed to immunosuppressive agents when the other disorder emerged).

Of 214 patients with MG 11 (5%) have another autoimmune neurological disease: three of the central nervous system, seven of the peripheral nervous system and one of the autonomic nervous system.

The course of MG is often complicated by concomitant autoimmune disorders. It is important to consider coexistent MG in patients with autoimmune disorders that develop new or aggravated muscular weakness, fatigue or respiratory failure.

On the other hand, while rare, the possibility of neurological autoimmune comorbidity should also be considered in myasthenic patients, especially if there is an unexpected deterioration of muscle weakness or poor response to pyridostigmine (e.g. muscle disease), or if unexpected neurological signs or symptoms arise in MG (e.g. peripheral nervous system involvement in keeping with CIDP; or central nervous system illness such as NMOSD; or even rarer manifestations such as of autonomic dysfunction). The rapid diagnosis of the second illness will improve management and disease outcomes of both MG and the other autoimmune condition.

Refractory Myasthenia gravis

A subset of myasthenia gravis patients is refractory to conventional treatments. Identifying their characteristics is important to contribute to try to find different therapies effective in this subgroup.

38 out of 172 patients were classified as refractory (22%). This rate is slightly higher than in other populations where it corresponds to 10-15%. [142] Maybe it happens in our cohort because it belongs to a tertiary centre where more difficult patients are referred for treatment.
Compared to the non-refractory patients, the refractory ones were more likely to have a more severe MGFA classification at onset, to have thymomatous MG and to be seropositive, either to anti-AChR or anti-MuSK antibodies.
We could not find any difference regarding sex, age of onset, presence of other autoimmune diseases, malignancies (other than thymus) or severe/opportunistic infections.
HLA-DRB1*13 allele was less frequent in the non-refractory MG when compared to the refractory group and the control population.
The clinical and demographic characteristics of our refractory patients are similar to those described in other studies. It is important to consider those characteristics from the onset of the disease. HLA-DRB1*13 allele was less common in the non-refractory group. HLA-DRB1*13 allele may be a protective allele for the non-refractory MG patients. As far as we were concerned there are no other immunogenetic studies in refractory MG and these results need to be reproduced in other populations.

Infections, neoplasms and other autoimmunity in thymoma associated Myasthenia gravis

It is extremely rare for young onset myasthenia gravis (MG) patients to have thymoma following extended removal of hyperplastic thymus. We described two anti-acetylcholine receptor MG (AChR-MG) patients who

developed thymoma years after extended transternal thymectomy and highlight the clinical manifestations that heralded the thymoma, consequently of a severe immunodeficiency which resembled APECED (autoimmune polyendocrinology, chronic mucocutaneous candidiasis and ectodermal dystrophy) syndrome, with auto-immune regulatory (AIRE) gene defect.

These two cases illustrated the need to search for a thymoma in MG patients where MG becomes difficult to treat years after removal of a thymus with thymic follicular hyperplasia. And that thymoma needs to be considered even more in case of severe and/or recurrent infections, which could reflect complex paraneoplastic immune-deficiency associated with auto-antibodies to interleukins, as found in genetic conditions associated with AIRE mutations.

This knowledge led to another study that is still going on.
We studied the frequency and clinical presentation of autoimmune manifestations, infections and neoplasms (other than thymus) in patients with MG and a histologically confirmed thymoma diagnosed in a multicentric study which included 5 hospitals in the north of Portugal. The aim is to correlate it with AIRE gene expression, poor CD8+CD45RA+ export capacity and/or the presence of B cells and lymphoid follicles inside the thymoma in the immunohistochemistry analysis of the thymoma samples.

5. Conclusions and Final Comments

Chapter 5. Conclusions and Final Comments

This epidemiological work enabled to Know the prevalence of the disease in the North of Portugal, as well as to know the prevalence for the different MG subgroups. More importantly, it allowed us to recognize that MG prevalence is higher in the elderly.
Furthermore, the study allowed us to calculate the incidence of MG in the same region. Like in other populations in different parts of the world, the MG incidence in the north or Portugal has been rising mostly in the elderly men.
This is a very important message to communicate to other neurologists, internal medicine doctors and general practitioners once awareness for such a treatable, but potentially serious, condition in older age groups will permit earlier intervention and thus a better outcome.

Being a model of antibody-mediated autoimmune disease, MG has been well recognised as having immunological, genetic and environmental influences. It was therefore, reassuring that our immunogenetic study confirmed the HLA susceptibility alleles as previously described in many different EOMG populations. On the other, we were able to find that LOMG has different immunogenetic characteristics, and found a different HLA susceptibility allele for this subgroup, which may explain some distinct characteristics of these patients in comparison to the EOMG, the most relevant of which is the gender bias (males in LOMG). Whether the LOMG HLA susceptibility alleles are indeed protective against an early onset of disease is a question that would be interesting to look into in future.

The study of the pregnant MG women identified an important proportion of patients that worsens during pregnancy and deserve special attention and care. There were no important complications in their children. We also focused in the MuSK-MG pregnant women analysis and it showed that pregnancy did not seem to precipitate MuSK-MG or to influence the MuSK-MG course, and there was no apparent negative impact in pregnancy

outcomes in those where pregnancy followed the MG onset. Pregnancy in MG is in general welcome and any MG patient at childbearing age should be offered expert counselling on this matter. MG services should have a robust support from obstetrics to provide helpful advice, and good quality and safe service to pregnant MG patients at any stage of their pregnancy, delivery and post-partum.

Polyautoimmunity in MG is a subject of great interest and relevance. We found that polyautoimmunity is common, either with other organ-specific, systemic or neurological autoimmune disorders. Is very important to diagnose them early and treat them promptly to improve the outcome of both MG and other diseases.

Recognizing and, if possible, predicting, the characteristics of MG patient with refractory disease is crucial. It allows making clear plans for monitoring the disease course and treating the disease more intensively from as early stages as possible to prevent major complications. Immunogenetic studies in larger disease cohorts may help to identify factors of poor prognosis and poor response to conventional treatments in MG.

Finally, we identified that some of the thymoma MG patients were more susceptible to severe/recurrent infections, endocrine or autoimmune disorders. We are performing additional studies to try to understand the mechanism why it happens.

Future studies in MG should concentrate on understanding why MG is becoming more common in older populations and also define strategies to identify such patients promptly and treat them early.
Given the difficulty in understanding the reason for such increase in prevalence and incidence of LOMG, it would be helpful to identify biological markers and environmental factors potentially associated with the risk of the disease.

It would be worth identifying new treatments for MG patients, in particular for those over the age of 50 or 60 years, where side effects of steroids and other chronic immunosuppressive treatments cause serious complications. Comorbidities are very frequent and interaction of poly-medications can also contribute to increased morbidity in MG.

We are confident that the studies conducted and presented in this thesis will have a positive impact in clinical practice in MG not only in the hospital where the studies were performed, but in the hospitals where the patients were recruited from. Patients' diagnosis and management will continue improving regardless of their age or gender, their social background or where they live.

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