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ORIGINAL COMMUNICATION

Thymomatous myasthenia gravis: novel association with HLA DQB1*05:01 and strengthened evidence of high clinical and serological severity

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Abstract

Background The relative prevalence of myasthenia gravis (MG) subtypes is changing, and their differential features and association with HLA class II alleles are not completely understood.

Methods Age at onset, presence/absence of autoantibodies (Ab) and thymoma were retrospectively considered in 230 adult Italian patients. Clinical severity, assessed by MGFA scale, and the highest Ab titer were recorded. Furthermore, we performed low/high resolution typing of HLA-DRB1 and HLA-DQB1 alleles to detect associations of these loci with MG subtypes.

Results There were two peaks of incidence: under 41 years of age, with female preponderance, and over 60 years, with higher male prevalence. The former group decreased and the latter increased significantly when comparing onset period 2008–2015 to 2000–2007. Thymomatous (TMG) patients showed a higher prevalence of severe phenotype and significantly higher anti-AChR Ab titer than non-thymomatous (NTMG) patients. Among the latter, those with onset after 60 years of age (LO-NTMG) displayed significantly higher Ab titers but lower MGFA grade compared to early-onset patients (< 41 years; EO-NTMG). Significant associations were found between HLA DQB1*05:01 and TMG patients and between DQB1*05:02 and DRB1*16 alleles and LO-NTMG with anti-AChR Ab.

Conclusions Two distinct cutoffs (< 41 and > 60 years) conveniently define EO-NTMG and LO-NTMG, with different characteristics. LO-NTMG is the most frequent disease subtype, with an increasing incidence. TMG patients reach higher clinical severity and higher antibody titers than NTMG patients. Moreover, TMG and LO-NTMG with anti-AChR Ab differ in their HLA-DQ association, providing further evidence that these two forms may have different etiologic mechanisms.

Keywords Myasthenia gravis · Disease subgroups · Late-onset · Early onset · Thymoma · HLA association

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Introduction

Myasthenia gravis (MG) is a heterogeneous autoimmune disorder affecting neuromuscular transmission, determined by the production of auto-antibodies against components of the neuromuscular junction. MG is a rare disease, with marked variability in incidence and prevalence rates according to geographical area and the temporal frame considered [1]. However, recent surveys reported high prevalence rates and an increasing incidence of MG in different areas, including Italy [2, 3].

Subtypes of the disease can be identified by age at onset, symptom distribution, autoantibody profile, and associated thymic abnormalities. An exact categorization of the patients according to the above-mentioned characteristics is important for determining the most appropriate therapeutic and management strategies for each patient. Concerning age at onset, a cutoff placed at the age of 50 years to distinguish early from late-onset MG is most often reported in the literature [4]. However, other age cutoffs are taken into account and, in facts, there is no consensus about which age should be considered as the most accurate limit. As for the majority of autoimmune disorders, MG has been considered more prevalent among young women; however, several studies have shown a shift towards a later onset of disease in the recent decades [5]. The factors responsible for the increasing incidence of late-onset MG need to be clarified.

MG has a complex pathogenesis: genetic, immunological and environmental factors are involved in predisposition to and development of the disease. Recently, genome-wide studies reported various associations of MG with single nucleotide polymorphisms next to genes involved in the regulation of the immune response to antigens [6, 7]. With a different approach, other studies have directly investigated the association of HLA genes alleles with MG, reporting heterogeneous results in different populations and disease subgroups [8]. Our preliminary observations suggested correlations of late-onset MG with specific HLA class II alleles [9]. Aims of the present work were to ascertain the relative prevalence of MG subtypes in a larger series of patients and to further explore the immunogenetic features of the largest MG subgroups.

Patients and methods

All adult Italian MG patients ($n = 230$) evaluated between 2006 and 2016 by the neuromuscular units at Policlinico Tor Vergata (PTV) and S. Andrea Hospital, entered in the survey. The study protocol was approved by the independent ethics committee of PTV and all patients signed an informed consent.

The diagnosis of MG was based on clinical evidence of fluctuating weakness of voluntary muscles and a consistent serological and/or electrophysiological pattern [10]. Age at onset, presence of serum anti-acetylcholine receptor (AChR) Ab (normal range = 0–0.2 nmol/l) or anti-muscle-specific kinase (MuSK) antibodies (normal range = < 0.05 nmol/l), presence of thymoma, as assessed by chest CT scan and confirmed by histopathological examination, were additional criteria used to categorize the patients in MG subgroups. The Myasthenia Gravis Foundation of America (MGFA) severity score recorded at clinical nadir in all patients was retrieved.

HLA typing

In 179 patients, we had the opportunity to perform an immunogenetic analysis as previously described [9]. In brief, DNA was obtained from blood samples by a fully automated system (Maxwell, Promega, Milano, Italy). Low-resolution typing for HLA-DRB1 loci was performed using polymerase chain reaction-sequence-specific oligonucleotide (PCR-SSO) (Luminex, One Lambda, Canoga Park, CA, USA); high resolution typing for DQB1 was performed using polymerase chain reaction sequence-specific primers (PCR-SSP) (Olerup, Stockholm, Sweden; Invitrogen, Carlsbad, CA, USA).

Statistical analysis

Differences in anti-AChR Ab titers between MGFA classes or between MG subtypes were assessed using one-way ANOVA and the Student's t test. HLA allele frequencies were estimated by direct counting in patients and in 315 healthy Italian controls. To compare differences between the allele frequencies in the control and MG groups, a 2×2 contingency table analysis was performed using the Fisher exact test. The strength of association between HLA alleles and MG was estimated by odds ratio and 95% confidence intervals. P values were corrected (p_c) for multiple comparisons according to the Bonferroni method. p_c values of < 0.05 and < 0.01 were considered significant and highly significant, respectively.

Results

Demographic, clinical and serological features

Out of the 230 patients recruited, 52% were female, with a mean age at onset of 45 years for females and 61 years for

males. None of the patients reported a family history of MG, although the prevalence of other autoimmune diseases was conspicuous in their first-degree relatives. Among patients, autoimmune disorders were frequently encountered (30%), with thyroiditis being by far the most common comorbidity (58%). Other autoimmune disorders associated with MG included: rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondylitis, Sjogren's syndrome, scleroderma, polymyositis, polymyalgia rheumatica, psoriatic arthritis, inflammatory bowel disease, anti-phospholipid syndrome, autoimmune diabetes, multiple sclerosis, neuromyelitis optica, MGUS-related neuropathy, hemolytic anemia, primary biliary cirrhosis, psoriasis, vitiligo, lichen, and pemphigus. Patients' distribution, according to age at onset and gender, showed two main peaks of incidence: one up to 40 years of age, with an overwhelming female preponderance, the second one over 60 years, with higher male prevalence (Fig. 1).

To detect variations in age at onset among our patients, a comparison between two contiguous 8-year periods: 2000–2007 and 2008–2015, was made. In the latter, patients with onset after 60 years of age resulted significantly more numerous and those with onset under 41 years of age less numerous than in the former period ($p < 0.05$). Moreover, mean age at onset was significantly higher ($p < 0.05$) for period 2008–2015 (mean: 58 ± 1.8 years) as compared to period 2000–2007 (mean: 53 ± 1.8 years). These findings indicate a shift towards a later onset of MG in recent years (Fig. 2).

Serological characterization of MG patients with routine methods showed that 79% carried anti-AChR Ab, 5% carried anti-MuSK Ab and 16% were double seronegative.

On a clinical ground, patients were categorized according to the MGFA score at nadir. 39 patients were classified as MGFA I, 72 as MGFA II, 62 as MGFA III, 33 as MGFA IV and 19 patients as MGFA V. Among subjects

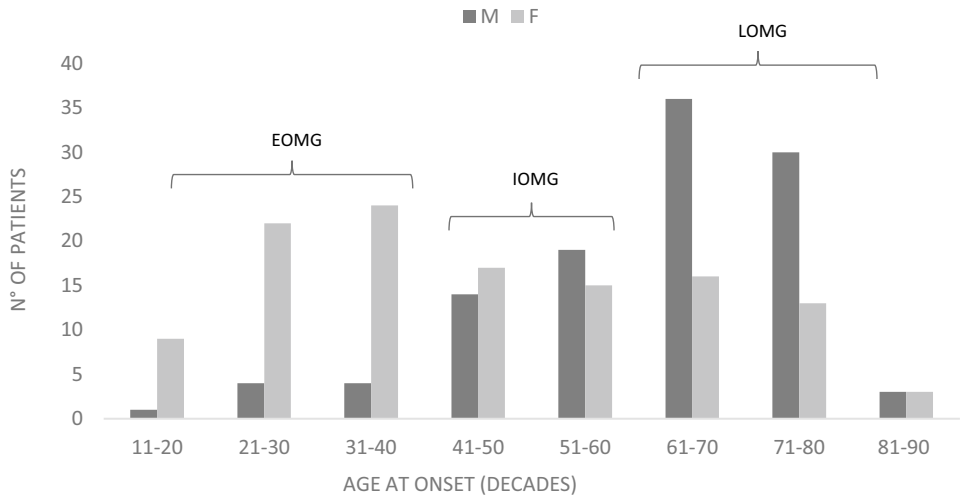

Fig. 1 MG onset distribution per age and sex shows two peaks of incidence: 21–40 years (EOMG), with large female preponderance, and 61–80 years (LOMG), with higher male prevalence. Patients with intermediate age at onset (IOMG) present no gender bias

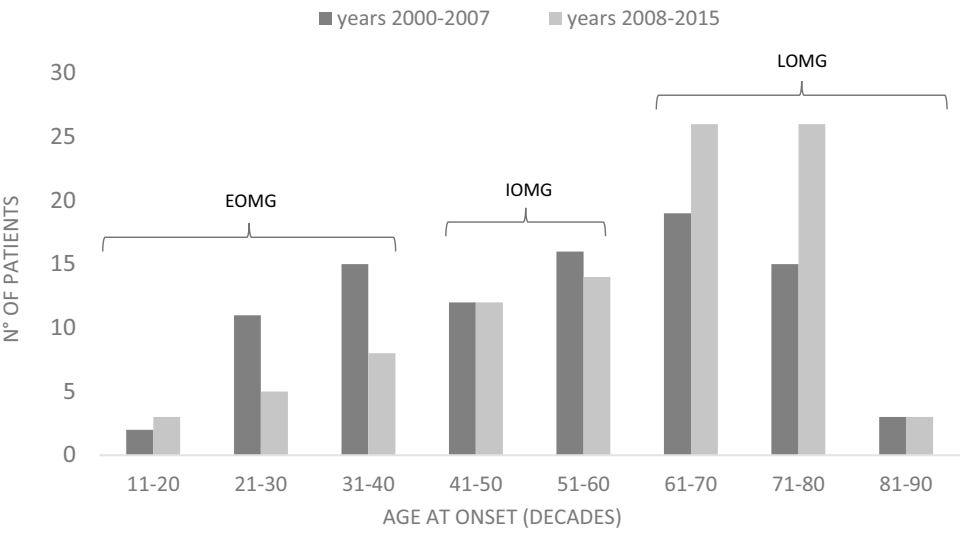

Fig. 2 MG onset distribution with comparison of two consecutive eight years periods shows a marked increase of LO–MG and decrease of EO–MG in the latter as compared to the former eight years ($p < 0.05$)

with anti-AChR Ab, one-way ANOVA demonstrated a significant difference in Ab titers between MGFA classes ($p < 0.05$). A Student's t test showed significantly higher titers ($p < 0.01$) in MGFA V grade ($n = 13/163$) as compared with MGFA I, II and III grade patients ($n = 28$, 54 and 45/163, respectively).

Thymectomy was performed on 91 patients with different surgical approaches. On histopathological examination, findings were: 53 thymoma, 20 follicular lymphoid hyperplasia, 16 thymic atrophy, and 2 thymic cyst. Among patients diagnosed with thymoma, two were identified, according to the WHO classification, as A "medullar", 6 as AB "mixed", 2 as B1 "organoid", 18 as B2 "cortical", 10 as B3 and 4 as C "thymic carcinoma"; for 11 patients, a definition of the histological characteristics of the thymoma was not available.

Comparative analysis of MG subgroups

All patients were stratified, on the basis of age at onset, in three subgroups corresponding to the observed peaks of incidence and to the age range in between. The first two subgroups were defined as Early-Onset (EOMG ≤ 40 years; $n = 64$) and Late-Onset (LOMG > 60 years; $n = 101$). These two groups were characterized by opposite trends in gender prevalence and recent change in incidence (Figs. 1, 2). On the contrary, patients with Intermediate Onset, between 41 and 60 years of age (IOMG; $n = 65$) showed no gender-related bias and no change of incidence in time (Figs. 1, 2). Moreover, within this age range there was a great heterogeneity, with high prevalence of thymoma (37%), MG with anti-MuSK Ab (9%) and double-seronegative MG (21%) cases. Therefore, we considered IOMG patients as "outliers" compared to the first two subgroups (Table 1).

To further characterize MG subtypes, we assigned patients with thymoma (TMG) and anti-MuSK Ab (MuSKMG) to specific subgroups, according to their peculiar pathogenesis. All other non-thymomatous MG (NTMG) patients with or without anti-AChR Ab were stratified on the basis of age at onset, according to our previous observations, as Early Onset NTMG (EO-NTMG), Intermediate Onset NTMG (IO-NTMG) and Late-Onset NTMG (LO-NTMG).

The prevalence of other autoimmune disorders was moderately higher in EO-NTMG (35%) compared to LO-NTMG (26%); however, this difference was non-significant. Moreover, when considering that 86% of EO-NTMG were female, one could hypothesize a gender rather than an age effect on this phenomenon.

When considering clinical severity, TMG patients ($n = 53$) were more often associated with higher MGFA scores than NTMG patients ($n = 177$) and EO-NTMG were more so than LO-NTMG (Fig. 3). Interestingly, mean anti-AChR Ab titer was significantly higher (11.45 nmol/l) in the first group as compared to NTMG (8.45 nmol/l) and in LO-NTMG (9.71 nmol/l) as compared to EO-NTMG (6.22 nmol/l) at Student's t test ($p = 0.01$ and $p < 0.05$, respectively).

Table 1 Characteristics of MG subtypes

EOMG [≤ 40 years] ($n = 64$)	TMG 16 (25%)	AchR	16 (100%)
	NTMG 48 (74%)	AchR	30 (63%)
		MuSK (10%)	5
		DS	13 (27%)
IOMG [41–60 years] ($n = 65$)	TMG 24 (37%)	AchR	24 (100%)
	NTMG 41 (63%)	AchR	21 (51%)
		MuSK (15%)	6
		DS	14 (34%)
LOMG [≥ 61 years] ($n = 101$)	TMG 13 (13%)	AchR (100%)	13
	NTMG 88 (87%)	AchR	77 (88%)
		MuSK (1%)	1
		DS	10 (11%)

EOMG Early onset MG, *IOMG* intermediate MG, *LOMG* late-onset MG, *TMG* thymomatous MG, *NTMG* non-thymomatous MG, *AchR* patients positive for anti-AchR antibodies, *MuSK* patients positive for anti-MuSK antibodies, *DS* double seronegative patients

As regards anti- AChR Ab-negative patients, MuSKMG presented the highest clinical severity, with the largest proportion of MGFA grade IV–V and, at variance with AChR Ab-positive patients, they were partially or totally refractory to first-line treatments, while showing either complete stable, or pharmacologic remission with Rituximab.

On the other hand, double-seronegative MG patients presented the largest prevalence of mild (MGFA I–II) cases (Fig. 3).

Immunogenetic analysis

Out of the 179 patients available for immunogenetic analysis, 48 were TMG, 21 were EO-NTMG with AChR Ab and 49 were LO-NTMG with AChR Ab. In view of these data, we statistically analyzed these more represented subtypes, namely TMG, NTMG and, among the latter, EO-NTMG and LO-NTMG with AChR Ab. The remainder subgroups (MuSKMG, EO-NTMG and LO-NTMG without AChR Ab) were composed only by 11 cases each and, therefore, were not further considered.

After applying Bonferroni's correction, a significant association was confirmed for only three alleles. In particular, statistical analysis showed a positive association between HLA DQB1*05:01 and the TMG subgroup. No

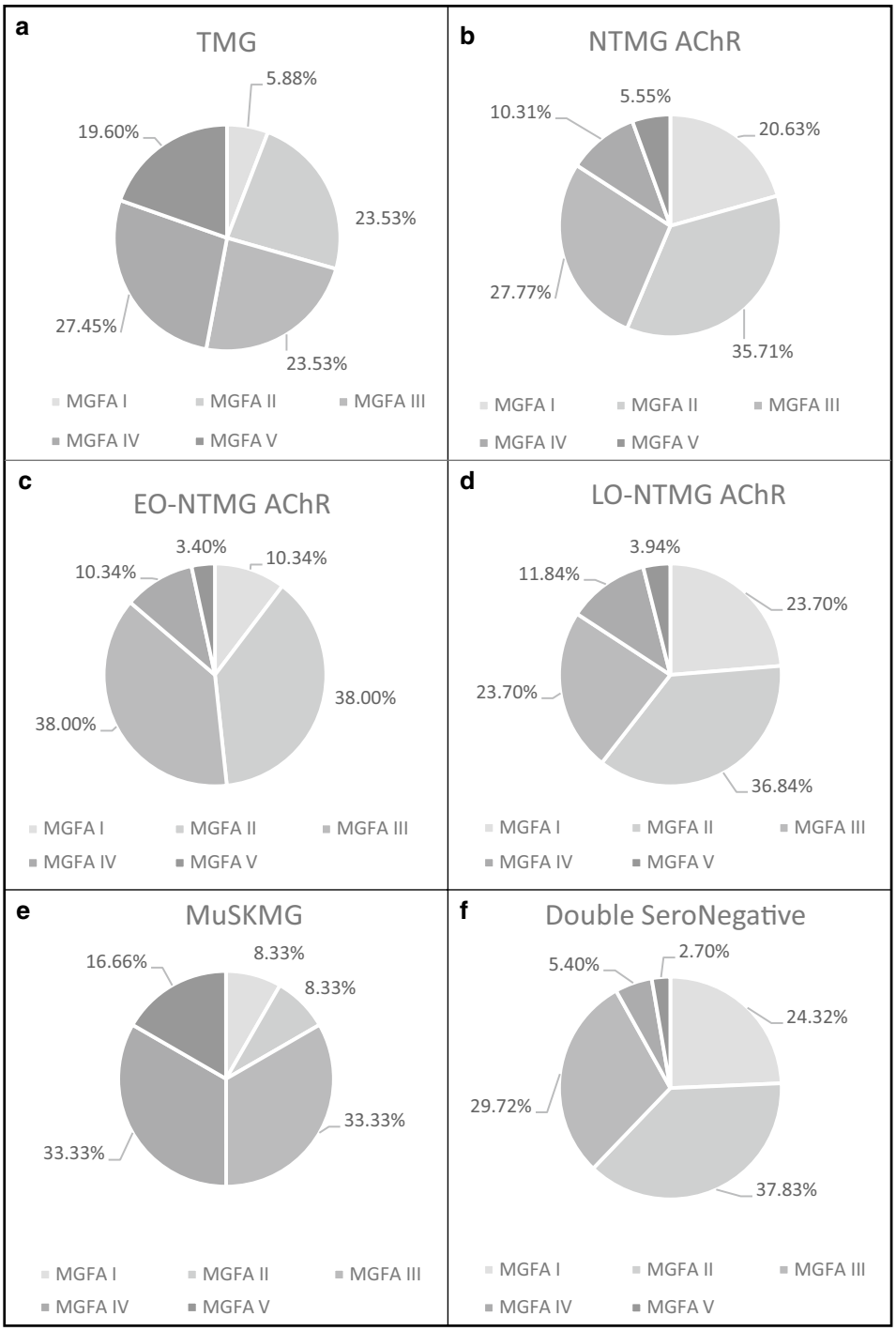

Fig. 3 Disease severity in MG subtypes. TMG shows threefold more prevalent severe (MGFA grade IV + V) forms than NTMG with anti-AChR Ab (**a, b**). Among the latter, those with an early onset have more often a severe form than those with a late onset (**c, d**). Overall, MuSKMG and double seronegative patients show the most and less severe clinical involvement, respectively (**e, f**)

association was detected with NTMG as a whole; however, a significant positive association was observed between the alleles DQB1*05:02 and DRB1*16 and LO-NTMG with AChR Ab. The results of immunogenetic analysis are shown in Table 2.

Discussion

In the present study, we assessed the relative prevalence of different MG subtypes and their association with HLA-II

Table 2 Associations between HLA class II alleles and major MG subtypes

HLA class II gene	DQB1						DRB1		
Allele	05:01			05:02			16		
Frequency	AF	OR	p_c	AF	OR	p_c	AF	OR	p_c
Normal subjects ($n=315$)	0.08	–	–	0.06	–	–	0.05		
TMG ($n=48$)	**0.18**	**2.35**	**0.04**	0.07	1.20	ns	0.04	0.75	ns
NTMG ($n=131$)	0.12	1.51	ns	0.10	1.70	ns	0.10	1.81	ns
EO-NTMG AchR+ ($n=21$)	0.14	1.81	ns	0.05	0.75	ns	0.05	0.86	ns
LO-NTMG AchR+ ($n=49$)	0.11	1.38	ns	**0.16**	**2.95**	**0.01**	**0.15**	**3.10**	**0.02**

Significant results are in bold

AF Allelic frequency, *OR* odds ratio (IC 95%), p_c *p* values corrected according to Bonferroni's, *TMG* thymomatous MG, *ns* non-significant, *NTMG* non-thymomatous MG, *EO-NTMG AchR+* early onset NTMG with AchR antibodies, *LO-NTMG AchR+* late-onset NTMG with AchR antibodies

gene alleles within a large series of consecutive Italian patients. The general and clinical characteristics of our patients are similar, in several aspects, to those previously described in Caucasians. Indeed, frequency of autoantibody subtypes, prevalence of associated thymomas and their most common histotype, and prevalence of other autoimmune disorders were consistent with those reported in other case series [4, 11, 12].

Interestingly, our results indicate that age at onset of MG is increasing, and that LOMG is particularly frequent among male patients, with an expanded incidence in recent years, as opposed to a reduced incidence of EOMG. A partially similar trend has been described in different populations, including Italians, and the causes of such rapid epidemiological modification are still debated [2, 3, 13].

The association we observed between high anti-AChR Ab titers and high MGFA scores indicates that antibody serum concentration can be considered a general marker of severity, although it cannot be regarded as a prognostic indicator in the comparison between single patients [14]. Moreover, the comparative analysis of TMG and NTMG with anti-AChR Ab suggests that the former patients tend to reach a higher clinical severity, in accordance with previous observations. Noteworthy, the higher anti-AChR Ab titers we observed in TMG may concur with other Ab, such as anti-titin Ab, frequently detected in TMG, in determining the higher severity of this disease subtype [14].

At present, there is no consensus about the age threshold that most conveniently separates the early- from the late-onset form of MG; in fact, several cutoffs are used in different studies [15–18]. Our findings suggest that two distinct cutoffs better define MG subgroups according to age at onset. Indeed, EOMG and LOMG, as defined by our age limits of <41 and >60 years, respectively, seem to represent with some consistency different types of the disease, on the basis of demographic, epidemiological and serological peculiarities. The IOMG subgroup probably represents a "grey zone": a miscellanea of outliers from the other two groups, suggested by lack of gender bias and of change in incidence. The composite nature of this group is also confirmed by the high relative prevalence of all disease forms defined by pathogenesis, such as TMG, NTMG with AChR Ab, MuSKMG, double-seronegative MG [1, 4].

Our data also suggest that LO-NTMG is more frequently less severe than EO-NTMG in spite of higher anti-AChR Ab titers. This contrast might reflect the heterogeneity of AChR Ab among different patients' subgroups. It is known, in fact, that anti-AChR Ab with distinct pathogenic properties (binding, blocking and modulating) may coexist in variable concentrations in MG patients, with specific outcomes on clinical severity, independently from Ab titer [19]. Thus, it would be interesting to investigate the prevalence of these distinct Ab categories, also in relation to epitope accessibility, in the above-mentioned MG types [20].

Examining the immunogenetic features of our patients, we report the novel association between TMG and DQB1*05:01. Indeed, no positive associations of HLA alleles with TMG have been described, to our knowledge, when considering data filtered by Bonferroni's correction, whereas a negative (protective) association of TMG with HLA-A*02 was reported [21]. Our observation, if confirmed in larger case series, might represent a genetic marker of TMG.

In parallel, we found a particularly strong association between DQB1*05:02 and LO-NTMG with anti-AChR Ab. The concurrent association with DRB1*16 is likely due to the strong linkage disequilibrium existing between this allele and DQB1*05:02. These data reinforce with higher statistical significance our previous findings obtained in MG patients with anti-AChR Ab and onset after 50 years of age [9].

The DQB1*05:02 allele has been associated, in different populations, with MuSKMG [22, 23] or early-onset MG with undefined serological features [24]. These data, together with our present results, seem to suggest an association of this allele with NTMG as a whole. The small number

of MuSKMG patients in our series does not allow a comparative analysis with other studies.

Therefore, our results show that MG with and without thymoma differ in their HLA class II association, thereby enhancing the existing evidence that these two forms have partially different pathogenic mechanisms. Furthermore, our findings are partially consistent with a recent genome-wide study, which found a significant association of HLA class II regions, next to DRB1, DQB1 and DQA1 genes, and late-onset MG [25].

In conclusion, the meaningfulness of these immunogenetic associations with MG subtypes deserves to be studied in more detail. The HLA region encodes several molecules that play key roles in the immune system, and whose association with autoimmune diseases has been established. Molecules encoded by HLA class II region are involved in exogenous antigen presentation to CD4 + Th cells, indicating the importance of this pathway in autoimmune diseases initiation and maintenance. We could hypothesize that distinct HLA alleles lead to differences in antigen presentation and, subsequently, in antibody production, which could determine variation in age at onset and clinical manifestations of MG. This mechanism could partially explain the lack of correlation between AChR Ab antibody titer and clinical severity in NTMG. Interestingly, current research lines suggest that a detailed characterization of the expression of HLA allelic variants might be useful to design therapeutic strategies aimed at targeting MG-associated HLA epitopes with specific monoclonal Ab [26]. On the other hand, it should be considered that the HLA genes involved might merely be markers of contiguous non-HLA genes responsible for the susceptibility to MG. Further studies might help in elucidating the relevance of the HLA associations in the pathogenesis of MG.

Compliance with ethical standards

Conflicts of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical standards The study protocol was approved by the Independent Ethic Committee of PTV and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Informed consent All participating subjects signed an informed consent.

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