



Autoimmune associations and autoantibody screening show focused recognition in patient subgroups with generalized myasthenia gravis [☆]



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ABSTRACT

Autoimmune associations in myasthenia gravis (MG)-patients and their relatives have not been re-assessed since their separation into early- or late-onset MG (EOMG, LOMG), or thymoma-associated MG.

Here, we analysed 226 EOMG-, 97 LOMG-, and 150 thymoma-patients for autoimmune disorders in themselves and their relatives. From 283 of them sera were tested for different organ- and non-organ-specific autoantibodies (autoAbs) by immunofluorescence test (IFT) and ELISA; genotyping was performed in 213 patients.

Relatives with autoimmune disorders were reported by more patients with EOMG (40% of 210) than LOMG (20% of 89; $p < 0.01$) than thymomas (8% of 150; $p < 0.001$). In 150 genotyped EOMG-females, the known risk allele of the immuno-regulatory *PTPN22* 2 (R620W) appeared commoner in those with second autoimmune diseases ($p \sim 0.06$), or with autoimmune relatives ($p \sim 0.03$), than in those without. Organ-specific autoAbs were found in $\sim 30\%$ of all MG-patients, autoAbs to striated muscle only in patients with thymoma-MG (62%) or LOMG (61%). Titers against adrenal cortex were lower in LOMG-patients. Disease-associated autoAbs against systemic targets or 'natural autoAbs' – except of autoAbs to nuclei – were uncommon in all groups ($\leq 13\%$).

Thus-with rare exceptions in EOMG and LOMG-we found minimal support for the notion that autoimmune patients have wide-ranging autoreactivity that causes disease only if it targets such Achilles' heels as the muscle acetylcholine receptor; even in thymoma-patients the autoAbs are sharply focused on a restricted range of muscle, cytokine and endocrine targets.

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1. Introduction

Autoimmune diseases tend to occur together in the same patient or family [1]; sometimes these include myasthenia gravis (MG), as first highlighted by Simpson [2,3]. MG is a classic autoan-

tibody (autoAb)-/complement-mediated disorder, where attack on muscle acetylcholine receptors (AChRs) reduces their numbers [4,5]. The resulting fatigable weakness often first targets eye/eyelid movements, and usually 'generalizes' within 2–3 years to affect the face, throat and even breathing [5,6]. Anti-AChR autoAbs are

Abbreviations: AChR, acetylcholine receptor; AIRE, auto-immune regulator; ANA, anti-nuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; APS-I, Autoimmune Polyendocrine Syndrome type I; cANCA, ANCA with a cytoplasmic fluorescence pattern; autoAb, autoantibody; autoAg, autoantigen; CMC, chronic mucocutaneous candidiasis; dsDNA, double-stranded deoxyribonucleic acid; ELISA, enzyme-linked immuno-sorbent assay; EOMG, early-onset myasthenia gravis; GAD, glutamic acid decarboxylase 65; GPC, gastric parietal cells; GWAS, genome-wide association study; IDDM, insulin-dependent diabetes mellitus; IFN, interferon; IFT, immunofluorescence test; IL, interleukin; ITP, idiopathic thrombocytopenic purpura; LOMG, late-onset MG; MG, myasthenia gravis; MS, multiple sclerosis; NMO, neuromyelitis optica; NMT, neuro-myotonia; NS, not significant; PA, pernicious anemia; pANCA, ANCA with a perinuclear fluorescence pattern; PBC, primary biliary cirrhosis; PMR, polymyalgia rheumatica; PTPN22, protein tyrosine phosphatase, non-receptor type 22; RA, rheumatoid arthritis; SD, standard deviation; SLE, systemic lupus erythematosus; TG, thyroglobulin; TNIP1, tumor necrosis factor α -induced protein 3-interacting protein 1; TPO, thyroid peroxidase; WHO, World Health Organization.

[☆] We dedicate this paper in affectionate gratitude to the memory of the late Prof. John Newsom-Davis, who collected so many of the present patients and recorded their details so systematically.

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almost MG-specific, and are found in ~85% of the patients with generalized MG [7,8]. In the West, as first highlighted in the 1970s [5,6,9], these fall into three main subgroups with: associated thymomas (~10%) or early- or late-onset MG (EOMG or LOMG; onset before or after age 45). Those with LOMG are also heterogeneous; as in thymoma patients, they show only weak sex and HLA biases [9–12], so we have few etiological clues. By contrast, EOMG is particularly well-defined, with very frequent follicular 'hyperplasia' in the thymic medulla, onset between puberty and the menopause, and strong female and HLA-DR3 and -B8 biases [10–14] as well as modest associations with the immunomodulatory tyrosine phosphatase, non-receptor type 22 (*PTPN22*) and TNF- α -induced protein 3-interacting protein 1 (*TNIP1*) [14].

Thymomas are neoplasms of thymic epithelial cells, and frequently generate and export large numbers of T-cells [15–17]. The neoplastic epithelial cells often lose HLA-class II antigens [16–19], and rarely express the autoimmune regulator (*AIRE*) [20], and presumably, therefore, the autoantigens (autoAgs) it normally up-regulates [20,21]. Thus, one might predict defects in self-tolerance induction in the T-cells developing in thymomas – by analogy with current concepts of pathogenesis in *AIRE*-mutant patients with the Autoimmune Polyendocrine Syndrome type I (APS-I) [20–23]. If these defects alone cause the autoimmunity, it should target many "standard" autoAgs commonly recognized in other autoimmune disorders. In fact, in APS-I, it most frequently focuses on Th17/Th22 cells [24,25], the parathyroids and the adrenal cortex [22,23] – which are very unusual targets in sporadic autoimmunity. Likewise, among all thymoma patients, at least 30% develop MG, and their other autoimmune associations, such as bone marrow aplasias, are unusual too [26].

Many patients with thymomas have additional autoAbs against certain striated muscle Ags, notably titin in >90% [27], and others neutralizing type I interferons (IFNs; ~70%) and/ or IL-12 (~50%), but not the many other cytokines tested [28]. These additional autoAbs are all also found in LOMG, though less frequently, but almost never in EOMG, other autoimmune disorders or healthy controls, so they are again largely thymoma-/LOMG-specific [27,28].

In MG overall, autoimmune associations include thyroid and Addison's diseases, insulin-dependent diabetes mellitus (IDDM), pernicious anemia (PA), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), as noted initially by Simpson [3,6], and later in case reports and small to modest screens for the autoAbs known in the 1970s [29,30]. Simpson had, therefore, suggested that MG was one component of a multi-system autoimmune syndrome analogous to SLE [3], and recently Burn et al. [31] hypothesized that "*PTNP22 variants might predispose to some general autoimmune diathesis*". Likewise, Vrolix et al. [32] suggested that autoimmune patients tend to respond to multiple different autoAgs: only if these responses happen to target crucial Achilles' heels like the AChR do they cause the corresponding disease(s).

We have tested these hypotheses in a large horizontal screen of thymoma, LOMG and EOMG patient sera for additional autoAbs characteristic of the most relevant autoimmune disorders, whether: (i) organ-specific – against endocrine or gastro-intestinal targets; (ii) non-organ-specific autoAgs (in nuclei or neutrophils); (iii) 'naturally occurring' autoAbs known to be induced by infections [33,34], e.g., against sarcolemma, endothelial cells or further nuclear antigens.

2. Materials and methods

2.1. Patients

With informed consent and local Ethics Committee approval, we sampled patients at our Referral Centres for Neurology/thy-

mectomy (London or Oxford, UK) or for Pathology/thymectomy (Mannheim, Germany) (Table 1, Appendix A, Table A.1). The UK patients were from two partially overlapping series of each MG subgroup (>90% assessed by the late Prof. J. Newsom-Davis), one tested genetically, and one serologically (see Table 2 and Appendix A). The disease controls were patients assessed similarly by Prof. J. N-D for (non-autoimmune) inherited myasthenic syndromes (Appendixes A and B). We scanned hospital notes for evidence of other autoimmune disorders in the patients themselves and their blood relatives (self-reported; Table 1a). We have previously reported most of the UK patients with EOMG, LOMG or thymomas [12,28,35], and the German patients [20]. The thymomas were confirmed histologically (in 148 of 150 patients) and radiologically in two who were too frail for surgery.

Diagnosis of generalized MG was based on clinical and electromyographic features plus elevated serum anti-AChR autoAbs in every case. Taken between 1981 and 2004, the samples represent a wide range of MG onset-ages, durations and follow-up periods (Table 1a).

The healthy controls were 30 adult blood donors aged 25–70 years (sera kindly provided by Prof. D. Wernet, Department of Transfusion Medicine, University of Tübingen, Germany).

2.2. Assays for autoAbs

2.2.1. Immunofluorescence tests (IFTs) on

- (A) Cryostat sections from rat kidney, liver, heart, stomach, and from human pancreas and adrenal cortex [36,37]; this method can detect the most relevant 'standard' autoAbs in one assay, namely against nuclear, mitochondrial or microsomal antigens; also against striated muscle or sarcolemma (in heart), and smooth muscle or endothelial cells (in blood vessels), and against islet and adrenal cortex cells.
- (B) Chang liver cell cultures, the better to distinguish autoAbs to nuclei, centromeres, nuclear dots (sp100) or nucleoli [38]; this method is more sensitive for anti-nuclear autoAbs (ANAs), but less discriminating than the IFT on cryostat sections because it also detects 'natural' autoantibodies (Section 4.3). A and B are valuable for diagnosis of connective tissue, autoimmune liver and organ specific autoimmune disorders.
- (C) Human neutrophils, for autoAbs against neutrophil cytoplasm (cANCA) or perinuclear regions (pANCA) [39]; while both are important for diagnosing different forms of vasculitis, pANCA can also occur in patients with ulcerative colitis and primary sclerosing cholangitis.

Patients' sera were diluted 1:10 for the demonstration of autoAbs in the IFT on cryostat sections, cell cultures and neutrophils with the exception of antibodies to adrenal glands and islet cells (undiluted; [30,37,40]). In the IFT on cryostat sections titers above 1:40 (for autoAbs to adrenal glands and islet cells 1:2), in the IFT on cell cultures and neutrophils titers above 1:80 were considered positive.

In the IFT on cryostat sections a polyvalent fluorescence-conjugated anti-human IgG-, IgA- and IgM-antibody was used (DAKO, Hamburg, Germany; dilution 1:200), with the exception for the detection of antibodies to islet cells and adrenal glands where a monovalent fluorescence-conjugated anti-human IgG antibody was used (DAKO, Hamburg, Germany; dilution 1:200). Also for the IFT on cell cultures and neutrophils a monovalent fluorescence-conjugated anti-human IgG antibody was applied (DIANOVA, Hamburg, Germany; dilution 1:1.000).

Table 1

Other autoimmune disorders in the MG patient probands or their blood relatives.

(a) Patients' characteristics								
Patient groups	EOMG		LOMG		Thymoma		Disease controls	
	Probands	Relatives	Probands	Relatives	Probands	Relatives	Probands	Relatives
Number of subjects	226	210	97	89	150	150	113	118
F:M	5:1		1:2		1:1		1.1:1	
<i>Myasthenia gravis</i>								
Onset-age:range (years)	2–45	–	51–84	–	17–80	–	0–29	
Duration	3 mo–19 y	–	5 mo–8 y	–	3 mo–33 y	–		
Probands with second autoimmune disorders themselves or in >1 blood relatives number (%)	41 (18) ^a	85 (40)^{b, c, d}	13 (13)	19 (20)	28 (19) ^a	13 (8)^d	9 (8)	17 (14)
Total disorders (number)	49^b	98^e	15	19	32 ^b	14	9	22
(b) Prevalence of autoimmune disorders								
Autoimmune disorders	EOMG		LOMG		Thymoma		Disease controls	
	Probands <i>n</i> = 226	Relatives <i>n</i> = 210	Probands <i>n</i> = 97	Relatives <i>n</i> = 89	Probands <i>n</i> = 150	Relatives <i>n</i> = 150	Probands <i>n</i> = 113	Relatives <i>n</i> = 118
Number (%)								
Organ-specific								
Addison's disease	0		0		1 (1)		0	
Thyroiditis	15 (7)	34 (16)^b	5 (5) ^f		8 (5)		7 (5)	3 (3)
Ovarian failure	3 (1)	0			1 (1)		0	
Diabetes mellitus type I	4 (2)	13 (12)	0		2 (1)		6 (4)	4 (3)
Pernicious anemia	2 (1)	4 (2)	4 (4) ^f		0		1 (1)	0
Celiac disease	0	2 (1)	0		0		0	
Nephrotic syndrome	0		0		1 'nephritis'	2 (1)	0	
Hematologic								
Thrombocytopenic purpura	3 (1)	1	0		0		0	
Pure red cell aplasia	0	0	0		2 (1)		0	
Hypogammaglobulinemia	0		0		2 (1)		0	
Mucocutaneous candidiasis	0		1 ^f		3 (2)		0	
Rheumatologic								
Rheumatoid arthritis	3 (1)	21 (10)^c	1	0	1	1	0	5 (4)
Ankylosing spondylitis	0	1	0	1 (1)	0	0	0	
SLE	8 (4)	2 (1)	1 (1)	0	3 (2)	1	0	1 (1)
Neurologic								
Myasthenia gravis	226 (100)^{e, g}	9 (4)	97 (100)^{e, g}	5 (6)	130 (87) ^{e, g}	1	1 (1) ^h	1 (1)
Multiple sclerosis	1	2 (1)	0		1	3 (2)	1 (1)	1 (1)
Neuromyelitis optica	2 (1)		0		0		0	
Neuromyotonia	0	1	2 (2) ⁱ		8 (5) ⁱ		0	
Ectodermal								
Vitiligo, alopecia, urticaria	2, 0, 1	–, –, –	1 ^f , 0, 0	1, –, –	3, 4, 2	–, 1, –	1, 0, 0	
Others (one or two of each)	Crohn's, PBC, uveitis	Crohn's, PMR	PBC, PMR, pulmonary fibrosis ^f , COPD	PMR	Crohn's, Behcet's, ulcerative colitis, systemic sclerosis, COPD		Crohn's, psoriasis, PMR	
Not specified	10 (2)		7 (5)	1	2 (1)			

Values remaining significantly different after Bonferroni-correction are printed in bold.

COPD, chronic obstructive pulmonary disease; PBC, primary biliary cirrhosis; PMR, polymyalgia rheumatica; SLE, systemic lupus erythematosus.

^a Significant as compared to disease controls *p* < 0.05.^b Significant as compared to disease controls *p* < 0.01.^c Significant as compared to thymoma patients *p* < 0.001.^d Significant as compared to LOMG *p* < 0.01.^e Significant as compared to disease controls *p* < 0.001.^f The same LOMG patient had all of these features.^g High prevalence of MG because it was a selection criterion.^h This patient had mutations in the AChR ε-subunit, and developed anti-AChR-positive autoimmune MG aged 34 [68].ⁱ These reflect a referral bias because of the UK Centre's known interest in neuromyotonia.

2.2.2. Enzyme-linked immunosorbent assays (ELISAs)

We used ELISA kits to detect antibodies to double-stranded (ds) DNA, thyroperoxidase (TPO) and thyroglobulin (TG) (all from Vita Diagnostics, Freiburg, Germany). These kits detect only IgG-antibodies.

Antibodies to mitochondrial proteins and microsomes were measured by published in-house assays [33,41,42]. We used a recently recommended citrullinated peptide (cfc6) to detect antibod-

ies to CCP [43] synthesized by Biotrend (Cologne, Germany). Patients' sera were diluted 1:500–1:4,000 depending upon the antigen used [33,41,42]. Bound antibodies were detected with peroxidase-conjugated human IgG-specific second layer antibodies (DIANOVA, Hamburg, Germany; dilution 1:2000). All tests were performed in triplicate together with high-, medium-, and low-titer and negative standard sera. Normal values against the different antigens were determined in sera from 30 healthy controls; the

Table 2

Autoantibody prevalences (%) in different MG patient groups and controls.

AutoAbs against	Method for detection	EOMG <i>n</i> = 114	LOMG <i>n</i> = 44	Thymoma		Healthy Controls <i>n</i> = 30
				With MG <i>n</i> = 110	Without MG <i>n</i> = 15	
Striated muscle	IFT (cryostat sections)	4	6^{a,b}	75^a	40^{a,c}	0
Organ-specific						
Gastric parietal cells	IFT (cryostat sections)	7	14	5	0	3
Adrenal cortex	IFT (cryostat sections)	13 ^d	7	15	13	0
Thyroid (TG and/or TPO)	ELISA	16	9 ^b	17	29	7
Islet cells	IFT (cryostat sections)	3	0	4	0	0
Total organ-specific		35^e	27	27 ^d	43	10
Non-organ-specific						
Disease-specific						
Nuclei (ANA)	IFT (cryostat sections)	11	23 ^b	8	26	7
Nucleoli	IFT (cell cultures)	7	7	13	20	3
Nuclear dots	IFT (cell cultures)	2	0	3	13	0
Centromeres	IFT (cell cultures)	0	0	0	0	0
Mitochondria	IFT (cryostat sections)	6	5	1	0	0
Microsomes	IFT (cryostat sections)	0	0	1	0	0
Actin	IFT (cryostat sections)	2	5	0	0	0
Neutrophils: cANCA	IFT (human neutrophils)	1	9	2	0	0
Neutrophils: pANCA	IFT (human neutrophils)	2	2	2	0	0
CCP	ELISA	2	0	5	8	0
Natural antibodies (not disease-specific)						
Nuclei (ANA)	IFT (cell cultures)	23 ^h	25 ^b	66^{d,f}	67 ^g	40
Sarcolemmal antigens	IFT (cryostat sections)	5 ^h	9	1 ^e	0	17
Endothelial cells	IFT (cryostat sections)	6	5	0	0	7

Values remaining significantly different after Bonferroni-correction are printed in bold.

^a Significant as compared to healthy controls and EOMG: *p* < 0.001.^b The same LOMG patient as in Table 1 had all of these autoAbs.^c Significant as compared to thymoma with MG: *p* < 0.05.^d Significant as compared to healthy controls: *p* < 0.05.^e Significant as compared to healthy controls: *p* < 0.01.^f Significant as compared to EOMG and LOMG: *p* < 0.001.^g Significant as compared to EOMG and LOMG: *p* < 0.01.^h Apparently lower than in healthy controls: *p* = 0.06.

means of their absorbances ($\times 1000$) + 3 SD were taken as the upper limits of the normal range.

2.2.3. PTPN22 and TNIP1

PTPN22 and TNIP1 genotyping (on 649 European EOMG-patients and 2596 controls) was described recently [14].

2.3. Statistics

We used Fisher's exact test followed by Bonferroni correction for comparing prevalences; also ANOVA and Kruskal–Wallis analyses for comparing antibody levels followed by post-hoc tests or Mann–Whitney *U* tests for unpaired data in some instances. We give *P* values for all the significant differences; they are printed bold if they remain significant after correction for multiple comparisons by post-hoc tests. For completeness, we also mention several trends that do not achieve significance because of small numbers.

Our study design was as practical as possible in a busy referral Centre, but inevitably has the limitations of any horizontal retrospective analysis of rare subgroups with self-reported family histories. Nevertheless, the UK patients were assessed similarly, >90% of them by the late Prof. J. Newsom-Davis, systematically using a standard protocol. Most of the autoimmune blood relatives were siblings, parents or grandparents, but there were some aunts, uncles and cousins; if there were >1 per proband, they were still counted as a single instance.

3. Results

Anti-AChR autoAbs were a key inclusion criterion, so all the MG patients were positive for them.

3.1. Early-onset MG patients (EOMG)

3.1.1. Associations with other autoimmune disorders

3.1.1.1. In the UK patients themselves. Other autoimmune disorders were more prevalent in EOMG patients (18%) than in the non-immune disease controls (8%; *p* = 0.02; Table 1a, b), as expected. In this characteristically young-/female-biased EOMG series (>4:1), thyroid disorders and SLE were the commonest, as already reported [6], followed by various others, mostly with onset before the MG. Two patients developed neuromyelitis optica (NMO) 12–22 years after onset of MG/thymectomy [44].

3.1.1.2. In blood relatives. Among 210 EOMG patients 85 (40%) reported blood relatives with at least one autoimmune disorder (total *n* = 98; Table 1a), which is significantly more than the 14% in the disease controls (*p* < 0.01). In those 85, EOMG onset-ages were only marginally younger (22.0 vs 23.5; not significant [NS]), and second autoimmune disorders slightly commoner (25.7% vs 17.0%; NS), than in the remaining 125 (not shown). Thyroid disorders were again the commonest among the relatives (*p* < 0.01 vs controls), but RA and IDDM also appear more prevalent than in the disease controls (NS: *p* = 0.09 and 0.31), unlike SLE. Only 4% had relatives with MG. Some striking examples are listed in Appendix B.

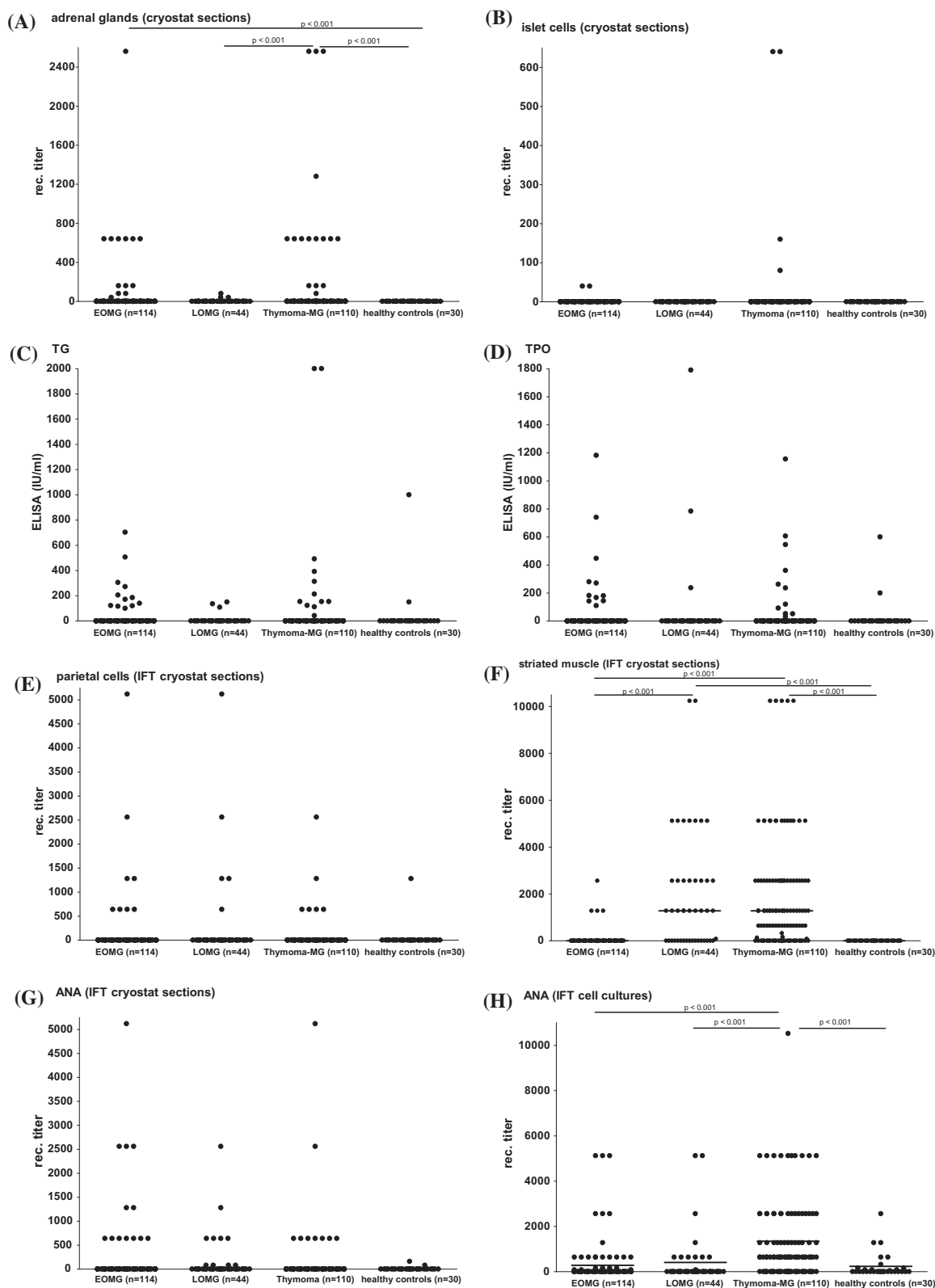


Fig. 1. Antibody reactivity against adrenal cortex (A), islet cells (B), thyroglobulin (TG; C) and thyroperoxidase (TPO; D), gastric parietal cells (E), striated muscle (F), and nuclear antigens (G, H), as demonstrated by immunofluorescence tests (IFT) on cryostat sections (A, B, E, F, G), cell cultures (H) or ELISA (C, D). Individual values (●) and medians (–) are given. (A) ANOVA and Kruskal–Wallis analyses revealed significant heterogeneity among the MG groups. In post-hoc tests, the indicated differences remain significant after correction for multiple comparisons. (B) There was no significant heterogeneity among the MG groups and also no difference from the healthy controls, by ANOVA and Kruskal–Wallis analyses. (C–E) There was no significant heterogeneity among the MG groups, or differences from the controls, by ANOVA and Kruskal–Wallis analyses. (F) There was significant heterogeneity among the groups as determined by ANOVA and Kruskal–Wallis analyses ($p < 0.001$). Post-hoc tests confirmed the expected differences, as indicated. (G) There was no significant heterogeneity among the MG groups, nor differences from the controls, whether using ANOVA- or Kruskal–Wallis analyses or Mann–Whitney U -tests. (H) There was significant heterogeneity among the MG groups and the healthy controls by ANOVA and Kruskal–Wallis analyses ($p < 0.001$). In post-hoc tests, the thymoma patients differ significantly from the other MG groups and from the controls, even after correction for multiple comparisons.

3.1.2. Additional autoantibodies

Organ-specific autoAbs were found more frequently in the EOMG patients than in the controls ($p < 0.01$) (Table 2), especially against adrenal cortex (13% vs none, $p < 0.05$) and TPO and/or TG (16% vs 7%; $p = 0.24$). Antibody reactivity to adrenal cortex was significantly higher in EOMG patients than in healthy controls but did not differ from the other MG-groups (Fig. 1A). There was no significant difference in antibody reactivity to islet cells, thyroid (TG, TPO), or gastric parietal cells within the MG-groups (Fig. 1B–E). Only four patients (4%) tested positive against striated muscle, as expected, and titers were significantly lower than in the patients with LOMG or thymoma-MG (Fig. 1F).

Non-organ-specific autoAbs were detected in up to 11% of patients, mainly against nuclear antigens (ANAs) (Table 2, Fig. 1G). The three patients with SLE had anti-nuclear Abs (ANAs) plus autoAbs to dsDNA (Table 3). Although no patients had evident liver disease, 6% had anti-mitochondrial autoAbs, two of them recognizing the PBC-specific M2-antigen (α -ketoacid dehydrogenase complex; not shown). Two patients had anti-CCP autoAbs, but without RA; conversely, the two with RA were anti-CCP negative (and non-smokers, whereas smoking associates strongly with anti-CCP-positive RA [45]).

'Natural' autoAbs seem less prevalent in EOMG than in controls, especially against nuclear antigens in the IFT on cell cultures (23% vs 40%; $p = 0.06$; Table 2); also against sarcolemma in IFTs on cryostat sections (5% vs 17%; $p = 0.06$; Table 2). Indeed, both, these and endothelial autoAbs were infrequent in all three MG subgroups.

In Table 3, we collate the additional autoAbs with the corresponding autoimmune disorders. In general, the autoAbs were more prevalent than the disorders, and they co-occurred surprisingly rarely in the same patient in any of the MG subgroups. Adrenal autoAbs were found in one of the three EOMG patients tested with ovarian failure (Table 1), but she had no evidence of Addison's disease.

Overall, 41% of the patients had none of the autoAbs tested or only one of them. Even the few extreme cases recognized autoAbs from four different groups at most (Appendix B). The >7:1 excess of females makes it hard to assess any gender biases in the autoAbs; they showed no obvious correlations with MG onset-ages or durations.

3.1.3. Immunogenetic correlations

In our recent GWAS, the HLA association in EOMG proved to be with HLA-B8 rather than any linked alleles, and was substantially stronger in the females [14]. There were much weaker associations at *TNIP1*, and with the known R620W risk allele at *PTPN22* (found in 31% of 649 EOMG patients vs 20% of 2596 healthy controls [14]). Here, in the ~150 genotyped females, it appeared even more prevalent in the 30 with additional autoimmune disorders (especially thyroid) than in those without (47% vs 27%; $p = 0.065$); also in the 57 with autoimmune relatives (42% vs 31% of 187; $p = 0.04$; especially RA). We found no such bias at *TNIP1*; nor for HLA-B8, probably because of its very high prevalence (78%) in the 157 HLA-typed patients. In the 64 patients tested both serologically and genetically, we saw no clear correlation between any one of the autoAbs tested and the risk alleles. Those with >3 additional autoAbs showed a weak bias towards the R620W risk allele of *PTPN22* (NS).

3.2. Late-onset MG patients (LOMG)

3.2.1. Associations with other autoimmune disorders

3.2.1.1. In the UK patients themselves. Of the 97 LOMG patients, 13 had at least one other autoimmune disorder (total $n = 15$; not different from the disease controls); they include 5 patients with thyroiditis, 4 with PA, and 2 others with NMT [46] (Table 1).

3.2.1.2. In blood relatives. Blood relatives with other autoimmune disorders were reported by at least 19 LOMG patients (Table 1), i.e. significantly fewer than in EOMG ($p < 0.001$) and more than in the thymoma patients ($p = 0.01$). They again included PA and thyroid disorders, as well as MG in 6%.

3.2.2. Additional autoantibodies

Organ-specific autoAbs were detected in 27% of the 44 LOMG patients (Table 2); especially levels of autoAbs to adrenal cortex were lower than in patients with EOMG or thymoma-MG (Fig. 1A). In contrast, more LOMG patients were positive against gastric parietal cells than thymoma patients or healthy controls, but differences in prevalence and reactivity were not statistically significant (Table 2, Fig. 1E). As expected, 61% of the LOMG patients had striated muscle autoAbs, and titres were significantly higher than in EOMG (Fig. 1F).

Non-organ-specific autoAbs were found in up to 23% of patients, especially ANAs in cryostat sections (Table 2, Fig. 1G). Interestingly, 5 (11%) were positive for p- or cANCA ($p = 0.08$ vs healthy controls), and 5% against mitochondria (though negative against M2; not shown) or actin.

Only 25% had 'natural' autoAbs against nuclear antigens in IFT on cultured cells (Table 2, Fig. 1H), even fewer against sarcolemma or endothelium, as in EOMG and controls.

Again, autoAbs did not correlate clearly with MG onset-ages or durations or gender (HLA types were available for only 21 UK patients) or with the corresponding disorders (Table 3).

Overall, ~65% of the LOMG patients had none or only one autoAb from the 'standard' groups, and 33% had just two; none had more. In one very unusual man, they recognized only nuclei (in both assays), striated muscle and both thyroid Ags, even though he had vitiligo, PA, hypothyroidism, CMC and pulmonary fibrosis (Tables 1 and 2 and Appendix B).

3.3. Thymoma patients

3.3.1. Associations with other autoimmune disorders

3.3.1.1. In the UK patients themselves. Of the 150 thymoma patient probands, 130 (87%) had MG. Twenty-eight (21%) had 32 instances of other autoimmune disorders than MG (Table 1; $p < 0.01$ vs the 8% in the disease controls).

Fifteen of the 20 thymoma patients without MG are detailed in Appendix C.

3.3.1.2. In blood relatives. Just 13 patients (9%) reported blood relatives with autoimmune disorders (Table 1) – apparently much fewer than in EOMG ($p < 0.001$). They include thyroid disease and IDDM but, interestingly, multiple sclerosis (MS) in 3 cases, vs only one with MG.

3.3.2. Thymoma histology and additional autoantibodies

Of the 100 histologically typed MG-thymomas, 8 were WHO-types A; 75 were types B2 or B3, as expected in such an MG-biased series [17,20].

Of the 130 thymoma-MG patients, 110 were tested for autoAbs. *Organ-specific autoAbs* were found in 27% of them, especially those against adrenal cortex, TPO and/or TG (17%; including 4 of the 8 with thyroid disorders) (Table 2, Fig. 1A, C and D). Furthermore, titers against adrenals were higher than in LOMG or controls ($p < 0.001$; Fig. 1A). By contrast, prevalences of striated muscle autoAbs were 75% (vs 40% in the thymoma patients without MG), and marginally lower in the few patients with type A than type B thymomas (37% vs 56%; NS). As expected, their titers were much higher in the thymoma-MG patients than in the EOMG or control subjects (Fig. 1F).

Table 3

Co-occurrence of autoantibodies with the corresponding autoimmune diseases [number (%)].

Disease	autoAb to:	EOMG (n = 91)	LOMG (n = 25)	Thymoma + MG (n = 94)
PA	Gastric parietal cells			
+	+	0	0	0
+	—	0	1 (4) ^a	0
—	+	8 (9)	4 (16)	4 (4) ^b
Adrenal/ovarian failure	Adrenal cortex			
+	+	1 (1) ^c	0	0
+	—	0	0	0
—	+	9 (19)	2 (8)	14 (15) ^b
Thyroiditis	TPO/TG			
+	+	3 (3)	1 (4) ^a	3 (3) ^b
+	—	0	1 (4)	4 (4) ^b
—	+	10 (11)	1 (4)	13 (14)
IDDM	Islet cells			
+	+	0	0	0
+	—	3 (3)	0	2 (2) ^b
—	+	1 (1)	0	3 (3) ^b
SLE	ANA (cryostat sections)			
+	+	3 (3)	0	2
+	—	1 (1) ^d	0	0
—	+	11 (12)	5 (20)	5 (5)
RA	CCP			
+	+	0	0	1 (1)
+	—	1 (1) ^e	0	0
—	+	1 (1)	0	1 (1)
Vasculitis	p- or cANCA			
+	+	0	1	0
+	—	0	0	0
—	+	1 (1)	3 (12)	1 (1)

None of the patients with autoAbs to mitochondria, nuclear dots, microsomes or actin (see Table 2) had evident autoimmune liver disease.

None of the patients with autoAbs to nucleoli (see Table 2) had evident systemic sclerosis or myositis.

The 4 patients with NMT (LOMG n = 1, thymoma n = 3) all had antibodies to VGKC complexes (not shown).

The 2 EOMG patients who later developed NMO also had antibodies to aquaporin 4 (not shown [44]).

^a The same individual LOMG patient had several autoAbs and/or disorders.^b The same individual thymoma patient had several autoAbs and/or disorders.^c This patient had ovarian failure but not Addison's disease.^d One of these patients patient had lupus anti-coagulant syndrome plus anti-phospholipid antibodies.^e One of these patients had juvenile rheumatoid arthritis.

Non-organ-specific autoAbs were remarkably infrequent, apart from those against nuclei in cryostat sections (8%, including two patients with SLE).

Of the 'natural' autoAbs, ANAs on cultured cells were more prevalent in these MG-thymoma patients than in the controls ($p < 0.05$) or the EOMG or LOMG patients ($p < 0.001$), and their titers were also significantly higher (Fig. 1H); other natural autoAbs were almost absent (Table 2).

Overall, 20% of the patients had ≤ 1 of the additional autoAbs against 'standard' targets, and only 14% against >3 autoAg groups (not shown). The various autoAbs showed no clear correlations with MG onset-ages, gender, or thymoma histology (not shown), or with the corresponding autoimmune disorders (Table 3).

3.3.3. Immunogenetic correlations

Of the 17 genotyped patients with a second autoimmune disorder, 35% carried the same risk allele of *PTPN22* as in EOMG (vs 19% in the remaining 42; NS). Of the 10 patients with thyroid autoAbs, 7 carried HLA-DR7 versus 14 of the 49 patients without them (29%; $p = 0.026$). We saw no clear HLA associations with anti-striated muscle or other autoAbs.

4. Discussion

This large horizontal screen highlights a remarkable 'tunnel vision' of autoAbs in the three main groups with generalized MG, even though broad autoreactivity might be expected in patients

with: (a) EOMG, where 18% had a second autoimmune disorder and 40% had an autoimmune relative – especially those with the R620W risk allele *PTPN22*; (b) older patients (i.e. LOMG), where autoimmunity is generally commoner [47–49]; (c) thymomas ($>90\%$ AIRE-deficient), a subgroup notorious for its variety of unusual autoimmune associations [17,26]. Whereas 13–19% of probands in the three MG subgroups had additional autoimmune disorders, these affected relatives more often in EOMG (40%) than LOMG (20%; $p < 0.01$) or thymoma-associated MG (8%; $p < 0.001$). Moreover, non-organ-specific and natural autoAbs were surprisingly uncommon in EOMG and LOMG; even thymoma-MG patients mostly had only 2–3 of them. These findings have diverse implications for etiology and management in these patient groups, as we briefly discuss below.

4.1. EOMG and LOMG

The very high prevalences of HLA-DR3 and especially -B8 in our EOMG patients (78%) make it hard to detect significant correlations between their other autoimmune diseases and their HLA alleles. Interestingly, we noted a modest excess of the R620W risk allele of *PTPN22* – rather than of *TNIP1* or *HLA* – in the EOMG females with second autoimmune diseases or autoimmune relatives, as in some other disorders [50]. That requires confirmation with even larger numbers; it also implies that other predisposing genes must be segregating in other families. The modest associations with SLE/ANAs seen here and previously [50,51] also recall the similar *PTPN22*- and young female- biases in these two autoAb-mediated

disorders [31]. Thus, any novel therapies directed at this pathway could be relevant in EOMG as well as SLE. Although it is debatable [47], young females (e.g., with EOMG), may give better family histories than in the other subgroups [52], where the focus may be more on their (younger) offspring than on (older) forbears. While we may have underestimated autoimmune disorders in probands and their blood relatives in all three subgroups (because of incomplete reporting), both seem commoner in EOMG than in either the disease controls or the respective 4–5% and 15–25% in the general European population [1,47,53].

The apparently stronger familial autoimmune tendencies and immunogenetic associations in patients with EOMG than LOMG might reflect greater heterogeneity among the latter, who include a mixture of ‘delayed-onset EOMG’ females and truly later-onset males [9–11,54]; analyzing those separately in larger numbers might well be more informative. Despite the stronger associations in EOMG, multiplex MG families seem surprisingly rare, and show no obvious EOMG bias; in the only such family we know, onset was after age 55 (1 of the 3 affected sibs is included here; unpublished). In striking contrast in MS, 10–20% of patients have relatives with MS, even though their HLA associations are considerably weaker than in EOMG [55,56].

The autoAbs did not clearly associate with the EOMG risk alleles genotyped, probably because of modest numbers. Also, nearly all our patients were tested at single time-points, so we might well have missed autoAbs that are only found either earlier or later in their disease evolution.

Interestingly, autoAbs against gastric parietal cells seemed slightly commoner in LOMG than EOMG (14% vs 7%; NS), although PA was infrequent in both the probands and their relatives. AutoAbs to adrenal cortex, thyroid and/or islet cells had similar prevalences in all three MG groups, but titers of autoAbs to adrenal cortex were significantly lower in LOMG than in thymoma-MG.

Since the additional autoAbs against IFN- α s and IL-12 [28,35], and especially striated muscle [27], appear very similar in some patients with LOMG and many with thymomas, they might reflect analogous thymic changes in these groups [54]. Otherwise, this almost unique coincidence is very hard to explain. Nevertheless, the sharp focus on these particular targets argues strongly that LOMG is not just a result of random autoreactivity in the elderly. In striking contrast, these additional autoantibodies are all rare in EOMG [28,35,57], despite the expression of striated muscle Ags in the muscle-like thymic myoid cells that are foci of immune attack and germinal center formation in EOMG [19]. Our multi-step hypothesis for EOMG pathogenesis postulates initial priming of helper T-cells by isolated AChR subunits expressed by thymic epithelial cells; that focuses the resulting ‘early’ autoAb response and/or T-cell attack sharply onto the myoid cells’ AChR. The evidence for and against it has already been discussed [19].

4.2. Distinctive features in thymoma patients

Only 8% of patients with thymomas reported autoimmune relatives – a figure close to that in the non-autoimmune disease controls and the general population [1,53]; that cannot be a mere artefact of their ages – which range lower than in LOMG. We propose that thymomas predispose so strongly to MG (see below) that they override modest familial susceptibility factors to autoimmunity. Together with the frequent loss of HLA-class II expression by the neoplastic epithelial cells, that might explain why HLA associations have not been obvious. In addition to their frequent autoAbs against type I IFNs and IL-12 [28,35], and striated muscle [27] (Table 2), many thymoma patients had ‘natural’ ANAs (67%). These are known to increase after intercurrent infections (see Section 4.3) [33], which are common in this group [58], and may well be exaggerated by the frequent use of immunosuppressive drugs. Alternately,

they might be evoked by debris from dying cells [59], which must be abundant in thymomas. However, the highly disease-specific nuclear dot or centromeric staining patterns were rare in all groups, and only three thymoma patients had autoAbs to dsDNA – and showed the reported association with SLE [26,60].

Notably, ‘standard’ disease-related but non-organ-specific autoAbs were seen only sporadically, whether tested by ‘catch-all’ staining on tissue sections or cell cultures or by more focused ELISAs. In general, few patients showed the broad autoreactivity that generation of T-cells in AIRE-deficient thymomas might cause. We favor alternative explanations [61]; since thymomas clearly express epitopes from muscle autoAgs, notably AChR, titin and ryanodine receptor [57], we suggest that these bias selection or actively autoimmunize T-cells – whether those developing *in situ* or others infiltrating from the periphery [17,61]. Analogous processes might lead to the rare autoimmune attack on Th17/Th22 cells/cytokines, and thus to CMC [24] (Table 1). Since some thymomas express GAD65 and 21-hydroxylase [20], we hypothesized analogous autoimmunization against endocrine targets too [17,61], possibly explaining the endocrine autoAbs – notably against adrenal cortex – that we now find in 27% of our thymoma patients.

To us, the unusual focus of autoAbs in patients with thymomas – on endocrine cells and certain cytokines – is explained more neatly by locally expressed autoAgs biasing selection – or even actively autoimmunizing – in their AIRE-deficient micro-environments, whereas pure failure to delete potentially autoaggressive T-cells there should cause much less focused/ more delayed autoimmunity [61]. Hence, we propose that the spectrum of autoAgs targeted in thymoma patients reflects those available to bias T-cell repertoires generated in the absence of AIRE. Conceivably, these repertoires may be further restricted by pre-existing regulatory T-cells; possibly also by AIRE-expressing stromal cells in peripheral lymph nodes [62] whose roles remain to be established, especially in humans [17,20,61,62].

4.3. Pathogenic mechanisms

‘Natural autoAbs’ showed similar or even lower prevalences in EOMG and LOMG than in the controls. They were originally thought to be mainly IgMs, but it is now clear that some are IgGs [34,63]. Here we focused on antibodies to sarcolemma and endothelium – without differentiating between IgGs and IgMs – although they are clearly part of a much larger spectrum [33,34]. In our experience, their production is decreased in autoimmune conditions (as seen here in EOMG; Table 2) but substantially increased during infections [33,40,64]. So are antibodies to nuclei detected by IFT on cell cultures – even of the IgG isotype – e.g., in hepatitis B or C [65]. Typically, these ANA give homogeneous or speckled patterns; however, even when their titers are high, they give no staining in IFTs on cryostat sections. Although they may thus mimic some hallmarks of autoimmunity superficially, they have no clear clinical or pathogenetic relevance [40,65].

In sharp contrast, it is generally agreed that autoAbs against surface receptors or ion/ water channels are pathogenic, e.g., against thyroid stimulating hormone receptor in Graves’ disease, AChR in MG, aquaporin 4 in NMO [44] and potassium channel complexes in NMT [46]. Those against TPO and/or TG were found in some of the patients with autoimmune thyroiditis, but the other organ-specific autoAbs correlated poorly with the corresponding disorders, as is well known [66]. Although they may cause some sub-clinical organ damage, it is widely accepted that the disorders are mediated mainly by T-cells, and that many autoAbs are indirect markers rather than pathogenic effectors. Possibly, disease without autoAbs might result from pathogenic T-cell responses against other targets in the same tissues. Conversely, autoAbs clearly can occur several years before disease, as is well known with islet cell

autoAbs and IDDM [67]. Thus, those against adrenal cortex might serve as an early warning that some thymoma patients are at risk of unexpected Addisonian crises [37].

In conclusion, the present results strongly imply that the autoimmunity in most MG patients is sharply focused rather than random. Indeed, the broad autoreactivity previously predicted [3,31,32] was evident – at most – in only a small minority of the patients we tested here. Hence there should still be scope for selective therapies as well as broad-spectrum immuno-suppressants.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.humimm.2013.06.020>.

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Appendix A

Table A.1 Numbers of patients from the different centers tested for genotypes or autoantibodies

	Analyzed for	EOMG	thymoma	LOMG
German	Autoantibodies	20	22	20
UK	Genotypes	152	48	
	Autoantibodies	30	64*	24
	both	64	39	

*for 2 patients, no family history was recorded

The disease controls were 119 patients assessed in parallel by the late Prof John Newsom-Davis for possible inherited ‘congenital’ myasthenic syndromes. Causative mutations have already been identified in 77 of them. When checking for autoimmune diseases, we excluded patients younger than 11 years; therefore, their ages at last follow-up spanned those in the autoimmune MG subgroups (Table 1). We also counted any pair (or trio) of siblings and/or parents as a single family.

Appendix B

Illustrative patients with:-

EOMG

Single patients/ families were sometimes afflicted by multiple autoimmune disorders, e.g.:-

- a boy whose maternal uncle had RA, and other relatives had thyroid disease, IDDM or PA. Even though he developed juvenile RA at age 7, IDDM at 13 and MG at 15, he had none of the additional autoAbs tested. HLA-DR3, -DR4: -B8, -B15: -A1, -A32.

- a man whose grandmother and mother had hypothyroidism, and whose sister has Graves’ disease. He developed thyroid disease aged 31, and MG at 33. He had autoAbs only to TPO. HLA-DR2, --DR11: -B7: -A2, -A11 but neither *PTNP22* nor *TNIP1* risk alleles.

- a woman whose mother had PA, RA and Crohn's disease (CD). Her brother also had CD, which she herself contracted at age 20 before MG at 24. HLA-DR1, -DR9; -B8, -B62: -A1, -A2, plus the *PTPN22* risk allele.

Multiple autoAbs – even in extreme cases – were found to 4 different autoAg groups (at most) in:-

- a girl of 11.5 (with a hyperthyroid grandmother) with autoAbs to mitochondria (positive by all three methods), striated muscle, gastric parietal cells (GPC) and citrullinated peptide;

- a female aged ~23 (whose grandmother had IDDM) with autoAbs against nuclei (both assays), TPO and TG (and also Aquaporin 4 [not shown], consistent with the NMO she contracted at age 35; [1]). HLA-DR16 and -DR3, -B8 and -B18, -A2 and -A3; homozygous for the risk allele of *PTPN22*.

LOMG

The only extreme case is marked ^f, ^b, and ^a in Tables 1, 2 and 3 respectively (main paper). His father had been hypothyroid. The patient developed vitiligo aged 60, PA at 70, hypothyroidism and CMC at 74 (plus autoAbs to IL-17 and IL-22), LOMG at 75 and pulmonary fibrosis soon afterwards. At that stage, no thymoma was evident on scanning, but he was then lost to follow-up. Nevertheless, he had autoAbs only against TPO and TG, striated muscle and nuclei (in sections and cultures). HLA-DR15, -DR4; -B7, -B44; -A2, -A3. Further genotyping is awaited.

Thymomas

The most extreme cases (Wolff et al; in prep) include two females:-

- MG-onset aged 33, hypothyroidism aged 41; she had autoAbs against striated muscle, islet cells, adrenal cortex, TPO, and nuclei in cultured cells; also to additional endocrine targets (Wolff et al., in preparation). HLA-DR4, -DR7: -B8, -B12: -A1, -A2; she also had the risk alleles at both *PTPN22* and *TNIP1* [2]

- MG onset aged 63, and she also had a papillary thyroid carcinoma. She had autoAbs to GPC, TG and TPO, adrenal cortex, striated muscle, nuclei and nucleoli in cultured cells. Genotyping is awaited.

Appendix C

The thymoma patients without MG included 5 from the UK (also without anti-AChR autoAbs); two presented with myopathy, and one each with Addison's disease plus hypogammaglobulinemia, COPD (who also had adrenal autoAbs, and ANAs on sections), NMT, or systemic sclerosis. None reported any

autoimmune relatives; they all had ANAs on cultured cells. The 10 Germans without MG included one with hyper-thyroidism, and another with pemphigus foliaceus; both had antibodies to TG and TPO and ANAs on cultured cells.

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