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Abbreviations: AChR: acetylcholine receptor, EOMG: early-onset myasthenia gravis, GC: germinal center, HEV: high endothelial venule, IL: Interleukin, IFN: Interferon, MG: myasthenia gravis, MGT: thymoma-associated MG, MuSK: muscle-specific tyrosine kinase, sCD40L: soluble CD40 ligand, Tfh: T follicular helper, TOMA: thymoma without MG.

ABSTRACT

Thymomas are associated with a very high risk of developing Myasthenia Gravis (MG). Our objectives were to identify histological and biological parameters to allow early diagnosis of thymoma patients susceptible to developing MG.

We conducted a detailed retrospective analysis from a patient database, searching for differences between patients with thymoma-associated MG (MGT, n=409) and thymoma without MG (TOMA, n=111) in comparison with nonthymomatous MG patients (MG, n=1246). We also performed multiplex and single molecule arrays to measure the serum levels of cytokines in these groups of patients and controls (n=14-22).

We identified a set of parameters associated with MG development in thymoma patients: 1) detection of anti-acetylcholine receptor (AChR) antibodies, 2) development of B1 or B2 thymoma subtypes, 3) presence of ectopic thymic germinal centers (GCs), 4) local invasiveness of thymoma, and 5) being a woman under 50 years old. Among these parameters, 58.8% of MGT patients displayed GCs with a positive correlation between the number of GCs and anti-AChR titers. By immunohistochemistry, we found thymic GCs in the adjacent tissues of thymomas encircled by high endothelial venules (HEVs) that could favor peripheral cell recruitment. We also clearly associated MG symptoms with higher IFN-γ, IL-1β and sCD40L serum levels, specifically in MGT patients compared to TOMA patients.

Altogether, these analyses allowed the clear identification of histological, in particular the presence of GCs, and biological parameters that would facilitate the evaluation of the probability of the MG outcome postoperatively in thymoma patients.

Key words: Autoimmune diseases, Myasthenia Gravis, Paraneoplastic syndrome, Thymus, Tertiary lymphoid structures, Cytokines

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction characterized by weakness and fatigability of skeletal muscles. MG is mediated by autoantibodies against proteins of the neuromuscular junction, mainly the acetylcholine receptor (AChR). Several classifications of MG patients are made based on 1) the autoantibody target, 2) the symptoms (ocular versus generalized presentation) and their severity, 3) the age at onset (early onset form (EOMG) before 45-50 years old or late onset development after 50 years old), and 4) the association with a thymic pathology [1]. Indeed, thymic abnormalities are observed in AChR-MG patients [2]. Different thymic abnormalities are described in AChR-MG patients: 1) 85% of the patients, mainly females, have thymic follicular hyperplasia, which is characterized by B-cell infiltration leading to ectopic germinal center (GC) development [1]; and 2) 10-15% of MG patients have thymoma, predominantly developing after 50 years old without sex predominance [3].

Thymomas correspond to thymic epithelial cell neoplasms and are among the most frequent anterior mediastinal tumors in adults. Several paraneoplastic syndromes are associated with thymoma. The most common is MG, but others are also described, such as systemic lupus erythematosus, rheumatoid arthritis, neuromyotonia, vitiligo, or pemphigus [4] [5]. For patients with a thymoma, MG incidence occurs in approximately 30% of cases, but this rate is highly variable from one study to another [6]. Symptoms in thymoma-associated MG (MGT) patients are usually more severe with a higher frequency of generalized disease with bulbar and respiratory symptoms, and patients need more immunosuppressive treatments relative to MG patients without thymoma [7]. MG symptoms allow earlier detection of a thymoma and favor thymoma patient's survival [8]. However, MG can develop after thymectomy. Here, we conducted a detailed retrospective analysis from our patients' database, searching for differences between MGT and thymoma without MG (TOMA) patients, in comparison with MG patients without thymoma. We also measured the serum levels of diverse cytokines in these different groups of patients to potentially identify predictive biomarkers for MG. This study aimed to identify risk factors for thymoma patients developing MG symptoms after thymectomy.

2. Materials & Methods

2.1. Patients' Database

This study is a retrospective analysis from a computerized and anonymized French database established by Dr. Sonia Berrih-Aknin in collaboration with the Marie Lannelongue Hospital (Le Plessis-Robinson, France), Cochin Hospital (Paris, France), Institut Mutualiste Montsouris (Paris, France), Raymond Poincaré Hospital (Garches, France), Pitié-Salpêtrière Hospital (Paris, France) and Hautepierre Hospital (Strasbourg, France). From 1990 to 2018, 1766 patients were registered in this database, and 3 groups of patients have been identified (Fig. 1): 1) thymoma patients without MG at the time of surgery (TOMA, n=111), 2) thymoma-associated MG patients, (MGT; n=409) and 3) MG patients without thymoma, (MG; n=1246) (Fig. 1). Patients with a diagnosis of thymic carcinoma were excluded. Clinical and biological data included biographic data, thymic histology, anti-AChR antibody titer (serum titer measured from 90 days before and up to 8 days after thymectomy and considered positive ($AChR^+$) when above 0.5 nmol/L), and immunosuppressive treatments. The diagnosis of MG was based on clinical, pharmacological and electrophysiological criteria (Table 1).

2.2. Immunohistochemistry on thymic sections

Thymic biopsies from MGT patients, either the thymoma (n=7) or the adjacent thymic tissue (n=7), were analyzed and compared to those from MG patients. Frozen thymic sections (7 µm) were fixed in ice-cold acetone for 20 min. Immunofluorescence staining was performed to label B cells and follicular dendritic cells with a FITC mouse antihuman CD21 (clone BL13, Beckman Coulter) and the epithelial network with an eFluor 615 mouse antihuman cytokeratin (clone AE1/AE3, eBioscience). High endothelial venules (HEVs) were labelled successively with a rat antimouse PNAd (peripheral node addressin) carbohydrate epitope (MECA 79, BD Pharmingen), a biotin mouse antirat IgM (clone G53-238, BD Pharmingen) and Alexa Fluor 350 streptavidin (Invitrogen). Images were acquired with a Zeiss Axio Observer Z1 Inverted Microscope.

2.3. Human serum samples

Blood was collected from TOMA (n=15-20; mean age 59.2±2.4 [age range 37-78]), MGT (n=16-19; mean age 53.6±2.2 [age range 41-74]), early-onset MG patients (EOMG, n=14-15; mean age 28.2±2.3 [age range 17-45]) and non-MG controls (n=20-22; mean age 43.6±2.4 [age range

17-69]). Blood was collected before thymectomy from MG, MGT and TOMA patients that were not treated with immunosuppressors. Blood from control healthy donors was from the French Blood Establishment (EFS). Blood was collected under a vacuum container in a dried tube with separating gel (BD Vacutainer SST™ Advance Ref 367953) for coagulation and centrifugation. Serum samples were stored at -80°C until use. Studies on blood samples were approved by local ethics committees [RCB 2010-A00250-39].

2.4. Multiplex assays

Multiplex cytokine analyses were performed with the Bio-Plex Pro™ Human Th17 Cytokine Panel 15-plex (IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IFN-γ, sCD40L, TNF-α) (BioRad, Marnes-La-Coquette, France) on an immunomonitoring platform (IMRB, VRI, INSERM U955 - UPEC, France). The samples were diluted 1:4 in the kit diluent and incubated overnight at room temperature before analysis. Differences in serum levels were analyzed using the fluorescence signals (minus the fluorescence background) as the serum levels were low and could not be determined precisely from the cytokine standard curves.

2.5. IFN-α measurement with Simoa assay

Serum levels of all interferon (IFN)-α subtypes were quantified using a single molecule array (Simoa) from Quanterix [9]. Each serum sample was diluted 1:3, and the limit of detection was calculated as the mean value + 2SD of reactivity from all blank runs and found to be 0.04 fg/ml.

2.6. Statistical analyses

GraphPad 5.0 software was used to perform statistical analyses and generate graphic representations. The results are expressed as the means and standard error of the mean (SEM) for the different experiments. We used Student’s t-test or the Mann-Whitney test for 2-by-2 comparisons, or the one way ANOVA with Bonferroni's Multiple Comparison Tests. Fisher’s exact test was used for 2-by-2 table analyses and was performed using the online tool GraphPad QuickCalcs. The tests used are specified in the figures or table legends, and p-values are indicated on graphs when <0.05.

3. Results

3.1. Main characteristics of the patient cohort

We reviewed 1766 patients registered in the database with a clinical diagnosis of nonthymomatous MG (MG, n=1246), or thymoma (n=520) either associated with MG (MGT, n=409) or without MG (TOMA, n=111) (Fig. 1). From this database, 78.7% of thymoma patients had MG. The female/male sex ratios were 2.7 for MG, 1.4 for MGT and 1.0 for TOMA patients (Table 1). The proportion of female patients was significantly higher among MG patients relative to MGT and TOMA patients, and the proportion of female patients was also slightly higher among MGT than TOMA patients. As expected, nonthymomatous MG patients who develop symptoms at an early age were thymectomized at a younger age than patients with a thymoma (TOMA or MGT patients) (Fig. 2A). However, we also showed that MGT patients were thymectomized at a younger age than TOMA patients (Fig. 2A). In addition, MGT patients were thymectomized more rapidly after symptom onset than MG patients (Fig. 2B), probably because thymectomy must be processed rapidly once the thymoma is diagnosed [7].

The histological classification used is the one established by the World Health Organization (WHO), with type A, B1, B2, B3 and AB thymomas. Type A is often considered a medullary thymoma, types B1 and B2 are cortical thymomas, type AB is a mixed thymoma (involving both cortical and medullary epithelial cells) and type B3 is atypical thymomas. Here, more than 80% of the MGT patients displayed a B2 (54.8%) or B1 (25.5%) subtype. In the TOMA group, the proportions of the A, AB, B1 and B2 subtypes were more homogenous (25.0%, 16.2%, 22.1% and 34.8%, respectively). Comparing TOMA and MGT patients, significant differences were observed for the B2 subtype, which was highly represented in MGT patients, and the A subtype was more highly represented in TOMA patients (Table 1). Local invasiveness of the thymoma outside of the thymic capsule was significantly more frequent for the MGT group than the TOMA group (Table 1). As MGT patients were also treated with immunosuppressive drugs significantly more often (Table 1), we analyzed local invasiveness in treated and untreated patients. We observed that patients with immunosuppressive treatment more frequently have invasive tumors when patients were analyzed altogether (TOMA and MGT patients) or in MGT patients (data not shown). For TOMA patients, it was difficult to draw conclusions as only a very small number were treated with immunosuppressive drugs (data not shown).

We analyzed the anti-AChR antibody titer for all patients when measured before or at the time of thymectomy. A total of 76.6% of nonthymomatous MG patients had anti-AChR antibodies (Table 1). In 23.4% of seronegative patients, a few may have low-affinity anti-AChR antibodies that were not yet measurable with the appropriate cell-based assay in clinical laboratories in France, and the others could be positive for anti-muscle-specific tyrosine kinase (MuSK) or anti-LRP4 antibodies. In the MGT group, almost all patients (98.5%) were positive for anti-AChR antibodies (Table 1). This was not surprising as MGT is not associated with anti-MuSK or anti-LRP4 antibodies. In the TOMA group, 37 patients were analyzed for anti-AChR antibodies, and 51.3% were positive at thymectomy, suggesting that these patients were at high risk to develop MG symptoms after surgery. For all AChR$^+$ patients, comparing the anti-AChR levels in the three groups of patients, we showed that MG patients had higher levels than MGT and TOMA patients, and MGT patients might also have higher levels than TOMA patients (Fig. 2C). Additionally, we analyzed the anti-AChR antibody titers in MGT patients according to different parameters. We did not observe significant differences according to the delay between MG onset and thymectomy (Fig. 2D) or between males and females (Fig. 2E). However, MGT patients with immunosuppressive treatments had higher AChR titers (Fig. 2F). We also observed a tendency for higher titers of anti-AChR antibodies for the B1, B2 and B3 thymoma subtypes relative to the A and AB thymoma subtypes (Fig. 2G).

3.2. Focus on germinal centers (GCs)

In nonthymomatous MG, the thymus is frequently characterized by B-cell infiltration and ectopic GC development [1]. In our database, 64.5% of the nonthymomatous MG patients displayed ectopic GCs. The percentage of GCs reached 78.8% in nonthymomatous AChR$^+$ MG patients under 45 years old (data not shown). The presence of GCs was investigated in nonthymomatous MG by pathologists, while this was not always the case for MGT or TOMA patients. Nevertheless, in our database, for some patients with a thymoma, the presence of GCs was screened in the adjacent tissue of thymoma biopsies. Surprisingly, we observed that 58.8% of the MGT patients displayed ectopic GCs. In contrast, only 15.6% of TOMA patients were characterized by the presence of ectopic GCs (Table 1 and Fig. 3A). Knowing the impact of corticosteroids on GCs [10], data were analyzed by excluding patients with immunosuppressive treatments, and the same observations were made (Fig. 3B). In MG patients, females under 45 years old have many more thymic GCs than males, but this

difference disappears for patients over 45 years old [10]. Here, among the 274 MGT patients for which the presence of GCs has been analyzed (average age of 48.8±0.7 years old), there was no significant difference between female and male MGT patients, with 60.3% and 56.7% of displaying GCs, respectively. GCs were observed in MGT or TOMA patients, regardless of the thymoma subtype (data not shown). Interestingly, we observed that MGT patients with thymic GCs developed symptoms at a younger age than patients without GCs (Figs. 3C-D), and the degree of thymic hyperplasia was higher in younger patients (Figs. 3E-F). In addition, anti-AChR antibody levels were available for 179 MGT patients (146 MGT patients without IS treatments) in which the presence of GCs was evaluated. We clearly showed that the levels of autoantibodies were significantly higher in MGT patients with numerous thymic GCs (Figs. 3G-H). All of these observations were made in all MGT patients or in MGT patients without IS treatments (Figs. 3B, D, F and H).

We investigated the presence of GCs by immunohistochemistry in the adjacent thymic tissue or the thymomas of MGT and TOMA patients relative to the thymus of nonthymomatous MG patients. We observed the presence of GCs in 2 out of 7 adjacent thymic tissues, but none in the thymomas examined. As for MG patients, we observed the presence of HEVs around GCs of MGT patients (Figs. 4A-B). The development of these specific endothelial vessels suggests the abnormal recruitment of peripheral cells in the thymus of MGT, as observed for MG patients.

3.3. Multiplex analyses for cytokines

Multiplex cytokine analyses in the sera were performed for 15 cytokines and analyzed by fluorescence intensity. Among them, we selected those that were upregulated in MGT relative to TOMA patients or to controls, namely IFN-γ, IL1-β, Interleukin (IL)-10 and sCD40L.

IFN-γ levels were increased in all MG, EOMG and MGT subgroups relative to control donors and TOMA patients (Fig. 5A). IL-10 levels were increased in all MG, EOMG and MGT patients relative to control donors. However, no difference was observed between MGT and TOMA patients (Fig. 5B), but a significant difference was observed between TOMA and EOMG patients. IL-1β and sCD40L were only overexpressed in MGT patients relative to control donors or TOMA patients. No overexpression was observed in EOMG patients (Figs. 5C-D). These analyses showed that higher serum levels for IL-10 and IFN-γ were clearly associated with MG

symptoms, while higher serum levels for IL-1β and sCD40L were clearly specific for MGT patients. ROC curves were computed to evaluate the sensitivity and specificity of these cytokines in discriminating MGT and TOMA patients. The results showed that only IFN-γ, IL-1β and sCD40L (Figs. 5 F-H), but not IL-10 (data not shown), might have potential clinical significance.

3.4. Simoa analysis for IFN-α

The ultrasensitive assays performed with Simoa technology allow the detection of all IFN-α subtypes. Here, we observed that IFN-α levels were between 0.04 (limit of detection, LOD) and 14.17 fg/ml for healthy controls, similar to the range previously reported by Rodero et al. [9]. We did not observe significant differences in MG, EOMG, MGT and TOMA patients relative to controls and between patient subgroups (Fig. 5E). IFN-α levels were above the LOD for 95% and 75% of the heathy controls and EOMG patients, respectively. However, many of the thymoma patient samples were below the LOD. Indeed, IFN-α was only detectable in 63% of MGT patients and 50% of TOMA patients.

4. Discussion

The well-known association between MG and thymoma prompts clinicians to search for a thymoma at MG diagnosis. Inversely, when the thymoma is diagnosed first, how can we predict the emergence of MG afterwards?

4.1. Global cohort analysis

Our database gathered a large number of nonthymomatous MG (MG) or thymomatous MG (MGT) patients relative to thymoma without MG (TOMA). The high incidence (78.7%) of MG among our cohort of thymoma patients was because our research team is mainly dedicated to the study of MG. The incidence of MG in thymoma patients is considered to be approximately 30% but is highly variable from one study to another and can range from 17 to 67% [11].

We analyzed in detail our cohort of patients, searching for distinct characteristics between TOMA and MGT patients. As already described in the literature, more than 80% of MGT patients present mainly a B2 or a B1 subtype [7]. In addition, mean age at thymectomy was lower for MGT than TOMA patients. This is because MG symptoms favor an earlier diagnosis of thymoma. Among thymoma patients, MGT patients more often present a local invasion of the tumor compared to TOMA. This link between invasiveness and MGT was already observed in other studies [7] [12]. A recent study even suggests that tumor progression is associated with B-cell infiltration [13]. This could be linked to the fact that MGT patients have GCs, as discussed below. Regarding anti-AChR antibodies, almost all MGT patients were positive at the time of surgery. For TOMA patients, from 24%-37% (in the literature) [14] [15] [16] [17] to 49% (in our cohort) have AChR antibodies, and these patients are clearly at risk of developing MG symptoms later on. Only a few studies have published data on post-thymectomy development of MG, and only on a small group of patients [14] [15] [18] [17] [19]. Among the TOMA patients in our database, we had information on only 8 patients who developed MG symptoms postoperatively. This group was not representative of the incidence of MG, as the follow-up of TOMA patients was not recorded in our database. However, we could clearly identify that 6 patients were females, and 5 out of 6 had a B1 or B2 thymoma subtype.

4.2. Ectopic germinal centers are associated with MG, even in thymoma patients

GCs constitute sites of antibody affinity maturation through processes of clonal proliferation, somatic mutation, and selection and are essentially observed in secondary lymphoid organs. GCs can sometimes be observed in inflammatory organs named tertiary lymphoid organs, as in the thymus of $AChR^+$ EOMG [20]. Here, in our database, we highlight that 58.8% of the MGT patients displayed thymic GCs. In TOMA patients, 15.6% also have GCs. Knowing that TOMA patients are at risk of developing MG symptoms after thymectomy, we suspect that the presence of CGs in these patients could be a predictive factor. We also showed a clear association between the degree of thymic follicular hyperplasia and the age, with the youngest MGT patients having the highest degree, as observed in EOMG [10]. However, we did not observe a significant gender distinction for thymic GCs in MGT patients.

In 1966, Watanabe was the first to comment on the presence of GCs in the adjacent thymic tissue of thymoma [21]. Next, in 1984, Monden et al. observed in a larger cohort of patients that GCs were present in 91.1% of MGT patients and 64.3% of nonthymomatous MG patients [22]. GCs are usually described in the adjacent thymic tissue of the thymoma, but a few studies observed the presence of B cells and GCs in the neoplastic epithelial tissue [18] [23] [24]. After these publications from the seventies to the nineties, GCs in thymomas were hardly studied and seemed to have been forgotten. Only recently, Song et al. analyzed the localization of T follicular helper (Tfh) cells in the adjacent thymic tissue of thymomas in MGT and TOMA patients. These cells are known to favor GC development by interacting with GC-B cells. They demonstrated that MGT patients have a higher percentage of Tfh cells than TOMA patients or controls [25]. In addition, Cavalcante et al. demonstrated an increased proportion of B cells and of the B-cell chemokine CXCL13 in the thymuses of MGT patients relative to TOMA patients or controls [26]. Here, by immunohistochemistry, we confirmed the presence of GCs in the adjacent thymic tissue but not in the thymomas of MGT patients. In particular, we showed the development of HEVs all around these GCs, exactly as in the thymus of EOMG patients [27]. In secondary lymphoid organs and in chronically inflamed tissues, such as the MG thymus, lymphocyte homing is directed through HEVs, a specialized endothelium bearing on its luminal surface diverse chemokines and expressing high levels of PNAd carbohydrate ligands [27] [28]. In MG patients, thymic HEVs expressed the chemokines CXCL12 [27] and CXCL17 [29]. It would be of interest to analyze chemokines that are expressed on HEVs in MGT thymuses. Regardless, the development of these specific endothelial vessels suggests the

abnormal recruitment of peripheral cells in the thymuses of MGT patients, as observed for EOMG patients.

Ectopic GCs often develop at sites of inflammation where they influence the course of infection, autoimmune disease, transplant rejection and cancer. What is the exact role of thymic GCs in the thymuses of MGT patients?

In some cancers, as for colorectal carcinoma, the presence of GCs is associated with better patient survival, as GCs are involved in local and systemic antitumor responses [30]. An elevated number of GCs also correlates with the long-term survival of patients with non-small-cell lung cancer [31] [32]. Consequently, thymic GCs could be protective against the tumor itself and favor thymoma patient survival. However, it is difficult to draw conclusions, as thymectomy is part of the treatment, and it is not possible to relate patient survival with the presence of thymic GCs in the long term.

Ectopic GCs in various tumors can participate in the immune response against tumor-associated antigens or self-antigens. Here, we observed that the number of GCs is correlated with higher titers of anti-AChR antibodies, as also observed for EOMG [33]. In addition, MGT patients are known to display various autoantibodies associated with other autoimmune diseases but also autoantibodies against antistriated muscle antigens or cytokines [34] [35]. Consequently, thymic GCs could have a negative role, favoring autoimmunity in particular.

4.3. Elevated cytokine levels associated with MG development in thymoma

We searched for blood biomarkers that could characterize MGT patients and define TOMA patients at risk of developing MG. As high levels of IFN-I subtypes are detected in the thymuses of MGT and not of TOMA patients [36], we used ultrasensitive Simoa technology to measure all IFN-α subtypes [9]. However, we did not detect any increased levels of IFN-α in MGT or TOMA patients, and we even observed that serum samples from MGT and TOMA patients were frequently below the LOD relative to controls and EOMG patients. MGT patients as well as TOMA patients can display anti-IFN-α autoantibodies [34], which may potentially explain our results and requires further study. As for IFN-β, which plays a central role in thymic changes in EOMG [37], we also analyzed serum levels by ELISA in controls and MGT patients but did not observe any variation (data not shown). Similarly, using Bio-Plex technology, Uzawa et al. did not observe serum variation for IFN-β in controls, EOMG, late-onset MG and MGT patients [38].

With a multiplex approach, we found 4 cytokines at higher levels in the sera of MGT patients. IFN-γ and IL-10 were upregulated in all MG patients, both MGT and EOMG patients, as specific markers for MG. IL-1β and sCD40L were only detected significantly at higher levels in sera of the MGT group. Data analysis using ROC curves demonstrated that only IFN-γ, IL-1β and sCD40L could discriminate MGT from TOMA patients.

Fang et al. also observed increased serum levels of IFN-γ in MG patients [39]. IFN-γ has pleiotropic effects, and a role in mediating autoimmune diseases has been previously suggested [40]. IFN-γ is important in inducing B-cell proliferation and differentiation, consequently favoring anti-AChR antibody production. IFN-γ is also required to induce experimental MG [41]. Elevated serum levels of IFN-γ could thus be a good predictive biomarker in thymoma patients susceptible to developing MG symptoms.

In 2014, Uzawa et al. measured the expression of 27 cytokines/chemokines in MGT and MG patients. They also described the significant upregulation of IL-1β in MGT patients relative to controls [42]. IL-1β is a proinflammatory cytokine that drives Th1 and Th17 cell differentiation [43] and favors autoimmunity. IL1-β knockout mice are also resistant to the induction of an experimental autoimmune MG model [44]. The role of higher levels of IL-1β in MGT is difficult to decipher as this cytokine can also to be involved in tumor induction [45].

Of interest, our study is the first to show elevated levels of sCD40L in MGT patients. CD40 and CD40L are expressed on a wide variety of cells. In the circulation, sCD40L mainly derives from activated T cells and platelets. It corresponds to a cleaved form of the membrane CD40 protein but retains considerable biological activity and the ability to bind to the CD40 receptor. Elevated sCD40L levels are observed in pathological conditions, such as inflammation, neoplasia and autoimmune diseases [46]. It was also demonstrated that sCD40L increases serum IgG levels and GC formation in mice [47]. In an experimental model of MG, the use of anti-CD40L has been shown to suppress ongoing chronic MG [48]. The role of sCD40L thus seems complex and difficult to understand in MGT patients.

Altogether, these observations showed elevated levels of IFN-γ, IL-1β and sCD40L in MGT patients. The levels of these cytokines should be analyzed prospectively in TOMA patients to correlate these cytokines with the emergence of MG symptoms.

5. Conclusion

Our retrospective analysis of patients from our database, together with a review of the literature, lead us to propose a list of clinical parameters for thymoma patients at risk of developing MG symptoms after thymectomy. We clearly demonstrated that ectopic thymic GCs were associated with MG in thymoma patients and should be systematically searched by pathologists. From our analyses, a risk score could be established with the following parameters: 1) detection of anti-AChR antibodies before surgery, 2) B2 or B1 thymoma subtypes, 3) presence of ectopic thymic GCs, 4) local invasiveness of thymoma, 5) women before 50 years old, and 6) high serum levels of IFN-γ, IL-1β and sCD40L. Now, a prospective study would be of interest to confirm the validity of these parameters. Early identification of patients at risk of developing MG is very important for specific follow-up after surgery. In addition, the benefits of early immunosuppressive treatment of these patients to prevent MG symptoms and/or to decrease their intensity should be discussed, knowing that this form of MG is more severe with bulbar and respiratory symptoms leading to prolonged treatment despite thymectomy [49].

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Author Contributions

CL, CP, O-MF and SM performed and analyzed experiments, FT collected samples and managed patient information in the database, VB and DD performed Simoa experiments, VM, M-RG, AM-L, MA and PV provided histopathological reports after thymectomy. EF, BE and AB provided blood samples. EF and DG provided thymic samples. SB-A established the database. CL, CP, AB and SB-A read and revised the manuscript. CL and RLP designed the study, analyzed the experiments and wrote the manuscript.

References

[1] S. Berrih-Aknin, R. Le Panse. Myasthenia gravis: A comprehensive review of immune dysregulation and etiological mechanisms. J Autoimmun, 2014;52:90-100.

[2] M. Raica, A. M. Cimpean, D. Ribatti. Myasthenia gravis and the thymus gland. A historical review. Clin Exp Med, 2008;8:61-4.

[3] Y. Fujii. Thymus, thymoma and myasthenia gravis. Surg Today, 2013;43:461-6.

[4] S. Shelly, N. Agmon-Levin, A. Altman, Y. Shoenfeld. Thymoma and autoimmunity. Cell Mol Immunol, 2011;8:199-202.

[5] A. Evoli, G. M. Minicuci, R. Vitaliani, A. Battaglia, G. Della Marca, L. Lauriola *et al.* Paraneoplastic diseases associated with thymoma. J Neurol, 2007;254:756-62.

[6] S. K. Padda, X. Yao, A. Antonicelli, J. W. Riess, Y. Shang, J. B. Shrager *et al.* Paraneoplastic Syndromes and Thymic Malignancies: An Examination of the International Thymic Malignancy Interest Group Retrospective Database. J Thorac Oncol, 2018;13:436-46.

[7] A. Evoli, C. Minisci, C. Di Schino, F. Marsili, C. Punzi, A. P. Batocchi *et al.* Thymoma in patients with MG: characteristics and long-term outcome. Neurology, 2002;59:1844-50.

[8] S. Margaritora, A. Cesario, G. Cusumano, E. Meacci, R. D'Angelillo, S. Bonassi *et al.* Thirty-five-year follow-up analysis of clinical and pathologic outcomes of thymoma surgery. Ann Thorac Surg, 2010;89:245-52; discussion 52.

[9] M. P. Rodero, J. Decalf, V. Bondet, D. Hunt, G. I. Rice, S. Werneke *et al.* Detection of interferon alpha protein reveals differential levels and cellular sources in disease. J Exp Med, 2017;214:1547-55.

[10] F. Truffault, V. de Montpreville, B. Eymard, T. Sharshar, R. Le Panse, S. Berrih-Aknin. Thymic Germinal Centers and Corticosteroids in Myasthenia Gravis: an Immunopathological Study in 1035 Cases and a Critical Review. Clin Rev Allergy Immunol, 2017;52:108-24.

[11] G. Maggi, C. Casadio, A. Cavallo, R. Cianci, M. Molinatti, E. Ruffini. Thymoma: results of 241 operated cases. Ann Thorac Surg, 1991;51:152-6.

[12] L. Maggi, F. Andreetta, C. Antozzi, F. Baggi, P. Bernasconi, P. Cavalcante *et al.* Thymoma-associated myasthenia gravis: outcome, clinical and pathological correlations in 197 patients on a 20-year experience. J Neuroimmunol, 2008;201-202:237-44.

[13] Y. Lu, F. Meng, Y. Yang, L. Li, D. Wang, Y. Cui *et al.* Significance of B10 cell in patients with thymoma complicated with myasthenia gravis. Oncotarget, 2017;8:73774-86.

[14] J. Nakajima, T. Murakawa, T. Fukami, A. Sano, S. Takamoto, H. Ohtsu. Postthymectomy myasthenia gravis: relationship with thymoma and antiacetylcholine receptor antibody. Ann Thorac Surg, 2008;86:941-5.

[15] Y. Yamada, S. Yoshida, T. Iwata, H. Suzuki, T. Tagawa, T. Mizobuchi *et al.* Risk factors for developing postthymectomy myasthenia gravis in thymoma patients. Ann Thorac Surg, 2015;99:1013-9.

[16] J. Nakajima, M. Okumura, M. Yano, H. Date, T. Onuki, M. Haniuda *et al.* Myasthenia gravis with thymic epithelial tumour: a retrospective analysis of a Japanese database. Eur J Cardiothorac Surg, 2016;49:1510-5.

[17] M. Ohta, M. Itoh, H. Hara, N. Itoh, H. Nishitani, K. Hayashi *et al.* Anti-skeletal muscle and anti-acetylcholine receptor antibodies in patients with thymoma without myasthenia gravis: relation to the onset of myasthenia gravis. Clin Chim Acta, 1991;201:201-5.

[18] T. Namba, N. G. Brunner, D. Grob. Myasthenia gravis in patients with thymoma, with particular reference to onset after thymectomy. Medicine (Baltimore), 1978;57:411-33.

[19] K. Kondo, Y. Monden. Myasthenia gravis appearing after thymectomy for thymoma. Eur J Cardiothorac Surg, 2005;28:22-5.

[20] M. A. Cron, S. Maillard, J. Villegas, F. Truffault, M. Sudres, N. Dragin *et al.* Thymus involvement in early-onset myasthenia gravis. Ann N Y Acad Sci, 2017.

[21] H. Watanabe. Thymoma and thymic germinal center. Keio J Med, 1971;20:69-75.

[22] Y. Monden, K. Nakahara, K. Kagotani, Y. Fujii, A. Masaoka, Y. Kawashima. Myasthenia gravis with thymoma: analysis of and postoperative prognosis for 65 patients with thymomatous myasthenia gravis. Ann Thorac Surg, 1984;38:46-52.
[23] F. Fend, T. Kirchner, A. Marx, H. K. Muller-Hermelink. B-cells in thymic epithelial tumours. An immunohistochemical analysis of intra- and extraepithelial B-cell compartments. Virchows Arch B Cell Pathol Incl Mol Pathol, 1993;63:241-7.
[24] M. J. Kornstein, S. Kay. B cells in thymomas. Mod Pathol, 1990;3:61-3.
[25] Y. Song, L. Zhou, F. Miao, G. Chen, Y. Zhu, X. Gao *et al.* Increased frequency of thymic T follicular helper cells in myasthenia gravis patients with thymoma. J Thorac Dis, 2016;8:314-22.
[26] P. Cavalcante, S. Marcuzzo, S. Franzi, B. Galbardi, L. Maggi, T. Motta *et al.* Epstein-Barr virus in tumor-infiltrating B cells of myasthenia gravis thymoma: an innocent bystander or an autoimmunity mediator? Oncotarget, 2017;8:95432-49.
[27] J. M. Weiss, P. Cufi, J. Bismuth, B. Eymard, E. Fadel, S. Berrih-Aknin *et al.* SDF-1/CXCL12 recruits B cells and antigen-presenting cells to the thymus of autoimmune myasthenia gravis patients. Immunobiology, 2013;218:373-81.
[28] S. Berrih-Aknin, N. Ruhlmann, J. Bismuth, G. Cizeron-Clairac, E. Zelman, I. Shachar *et al.* CCL21 overexpressed on lymphatic vessels drives thymic hyperplasia in myasthenia. Ann Neurol, 2009;66:521-31.
[29] C. Cordiglieri, R. Marolda, S. Franzi, C. Cappelletti, C. Giardina, T. Motta *et al.* Innate immunity in myasthenia gravis thymus: pathogenic effects of Toll-like receptor 4 signaling on autoimmunity. J Autoimmun, 2014;52:74-89.
[30] D. Coppola, M. Nebozhyn, F. Khalil, H. Dai, T. Yeatman, A. Loboda *et al.* Unique ectopic lymph node-like structures present in human primary colorectal carcinoma are identified by immune gene array profiling. Am J Pathol, 2011;179:37-45.
[31] C. Germain, S. Gnjatic, F. Tamzalit, S. Knockaert, R. Remark, J. Goc *et al.* Presence of B cells in tertiary lymphoid structures is associated with a protective immunity in patients with lung cancer. Am J Respir Crit Care Med, 2014;189:832-44.
[32] E. B. Gottlin, R. C. Bentley, M. J. Campa, D. S. Pisetsky, J. E. Herndon, 2nd, E. F. Patz, Jr. The Association of Intratumoral Germinal Centers with early-stage non-small cell lung cancer. J Thorac Oncol, 2011;6:1687-90.
[33] S. Berrih-Aknin, E. Morel, F. Raimond, D. Safar, C. Gaud, J. Binet *et al.* The role of the thymus in myasthenia gravis: immunohistological and immunological studies in 115 cases. Annals of the New York Academy of Sciences, 1987;505:50-70.
[34] A. Meager, M. Wadhwa, P. Dilger, C. Bird, R. Thorpe, J. Newsom-Davis *et al.* Anti-cytokine autoantibodies in autoimmunity: preponderance of neutralizing autoantibodies against interferon-alpha, interferon-omega and interleukin-12 in patients with thymoma and/or myasthenia gravis. Clin Exp Immunol, 2003;132:128-36.
[35] F. E. Somnier, P. J. Engel. The occurrence of anti-titin antibodies and thymomas: a population survey of MG 1970-1999. Neurology, 2002;59:92-8.
[36] P. Cufi, P. Soussan, F. Truffault, R. Fetouchi, M. Robinet, E. Fadel *et al.* Thymoma-associated myasthenia gravis: On the search for a pathogen signature. J Autoimmun, 2014;52:29-35.
[37] P. Cufi, N. Dragin, N. Ruhlmann, J. M. Weiss, E. Fadel, A. Serraf *et al.* Central role of interferon-beta in thymic events leading to myasthenia gravis. J Autoimmun, 2014;52:44-52.
[38] A. Uzawa, T. Kanai, N. Kawaguchi, F. Oda, K. Himuro, S. Kuwabara. Changes in inflammatory cytokine networks in myasthenia gravis. Sci Rep, 2016;6:25886.
[39] T. K. Fang, C. J. Yan, J. Du. CTLA-4 methylation regulates the pathogenesis of myasthenia gravis and the expression of related cytokines. Medicine (Baltimore), 2018;97:e0620.
[40] J. R. Lees. Interferon gamma in autoimmunity: A complicated player on a complex stage. Cytokine, 2015;74:18-26.
[41] G. X. Zhang, B. G. Xiao, X. F. Bai, P. H. van der Meide, A. Orn, H. Link. Mice with IFN-gamma receptor deficiency are less susceptible to experimental autoimmune myasthenia gravis. J Immunol, 1999;162:3775-81.

[42] A. Uzawa, N. Kawaguchi, K. Himuro, T. Kanai, S. Kuwabara. Serum cytokine and chemokine profiles in patients with myasthenia gravis. Clin Exp Immunol, 2014;176:232-7.
[43] G. Lopalco, L. Cantarini, A. Vitale, F. Iannone, M. G. Anelli, L. Andreozzi *et al.* Interleukin-1 as a common denominator from autoinflammatory to autoimmune disorders: premises, perils, and perspectives. Mediators Inflamm, 2015;2015:194864.
[44] D. Huang, F. D. Shi, R. Giscombe, Y. Zhou, H. G. Ljunggren, A. K. Lefvert. Disruption of the IL-1beta gene diminishes acetylcholine receptor-induced immune responses in a murine model of myasthenia gravis. Eur J Immunol, 2001;31:225-32.
[45] R. Bent, L. Moll, S. Grabbe, M. Bros. Interleukin-1 Beta-A Friend or Foe in Malignancies? Int J Mol Sci, 2018;19.
[46] C. van Kooten, J. Banchereau. CD40-CD40 ligand. J Leukoc Biol, 2000;67:2-17.
[47] B. D. Elzey, J. F. Grant, H. W. Sinn, B. Nieswandt, T. J. Waldschmidt, T. L. Ratliff. Cooperation between platelet-derived CD154 and CD4+ T cells for enhanced germinal center formation. J Leukoc Biol, 2005;78:80-4.
[48] S. H. Im, D. Barchan, P. K. Maiti, S. Fuchs, M. C. Souroujon. Blockade of CD40 ligand suppresses chronic experimental myasthenia gravis by down-regulation of Th1 differentiation and up-regulation of CTLA-4. J Immunol, 2001;166:6893-8.
[49] G. O. Skeie, F. Romi. Paraneoplastic myasthenia gravis: immunological and clinical aspects. Eur J Neurol, 2008;15:1029-33.

Figures legends

Figure 1: Description of the parameters analyzed from the database

Figure 2: Analyses of biological parameters in TOMA, MGT and MG patients

(A) Age of patients at the time of thymectomy for 105 TOMA, 405 MGT, and 1125 MG patients. (B) Delay between the onset of MG symptoms and thymectomy for 353 MGT, and 1017 MG. (C) Anti-AChR titer (>0.5nmol/L) for 19 TOMA, 261 MGT, and 541 MG patients. (D) Anti-AChR titers in $AChR^+$ MGT patients according to delay between MG onset and thymectomy. (E) Anti-AChR titers in 165 females and 96 males in $AChR^+$ MGT patients. (F) Anti-AChR titers in 208 untreated- and 58 treated-$AChR^+$ MGT patients. (G) Anti-AChR titers in $AChR^+$ MGT patients in different thymoma subtypes. p-values were assessed using the one-way ANOVA with Bonferroni post hoc tests (A, C, D, G) or the student t-test (B, E, F). p-values are indicated if $p<0.05$ as follows: (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

Figure 3: Analyses of thymic GCs

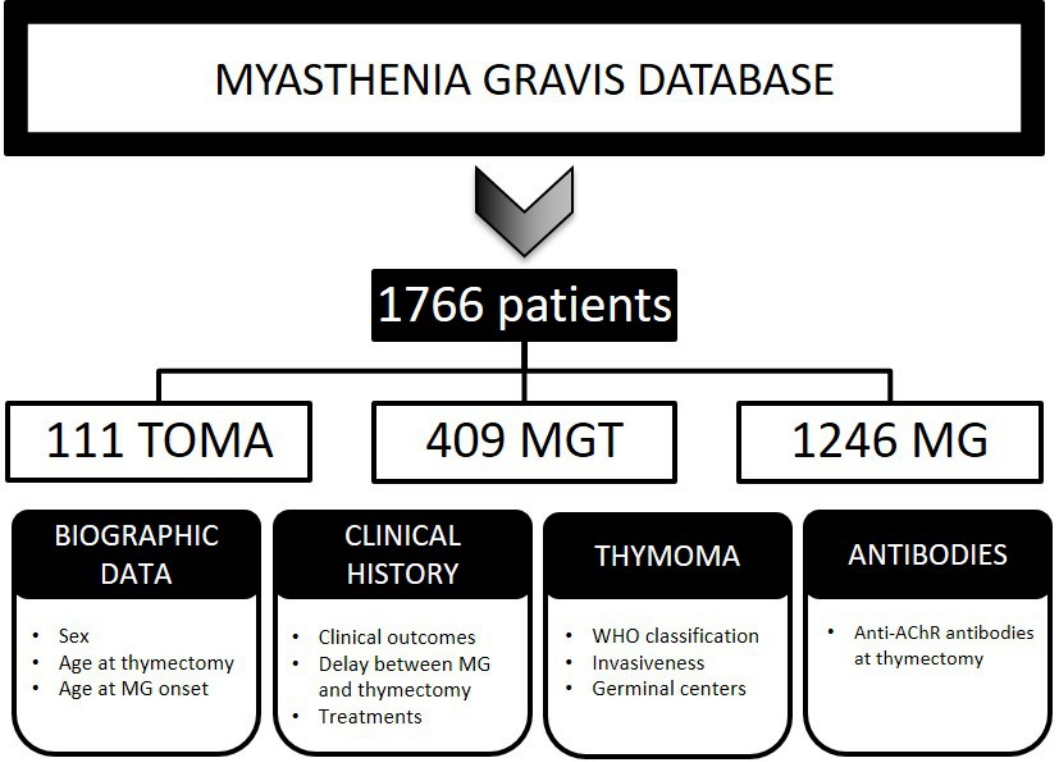
(A-B) Percentages of patients with or without thymic GCs. (C-D) Age at MG onset according to the presence or not of thymic GCs in all MGT patients. (E-F) MGT patients were divided in four subgroups according to the age ≤40, 40-50, 50-60 and ≥60 to analyze the degree of thymic hyperplasia that was graded as follows: no GC=0; few GCs = 1; many GCs = 2; numerous GCs = 3. The data correspond to mean values ± SEM. (G-H) Anti-AChR titers in 179 MGT patients (E) and 144 MGT patients without immunosuppressive treatments (F) according to the proportion of thymic GCs. (A, C, E, F) All patients and (B, D, F, G) patients without immunosuppressive treatments. p-values were assessed using the one-way ANOVA with Bonferroni post hoc tests (C-H) and p-values are indicated if $p<0.05$ as follows: (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

Figure 4: Immunohistochemistry for thymic GCs in EOMG and MGT patients

(A-B) Immunofluorescence staining of representative thymic sections from EOMG (A) and MGT (B) patients with an anti-CD21 antibody to detect GCs (in green), an anti-PNAd for HEVs (in bleu) and an anti-cytokeratin for thymic epithelial cells (in red). Images were acquired with a Zeiss Axio Observer Z1 Inverted Microscope. (C) Negative control of the staining on a serial thymic section of the EOMG patients showed in A. Images are composed of six mosaic pictures. Scale bars = 100 µm

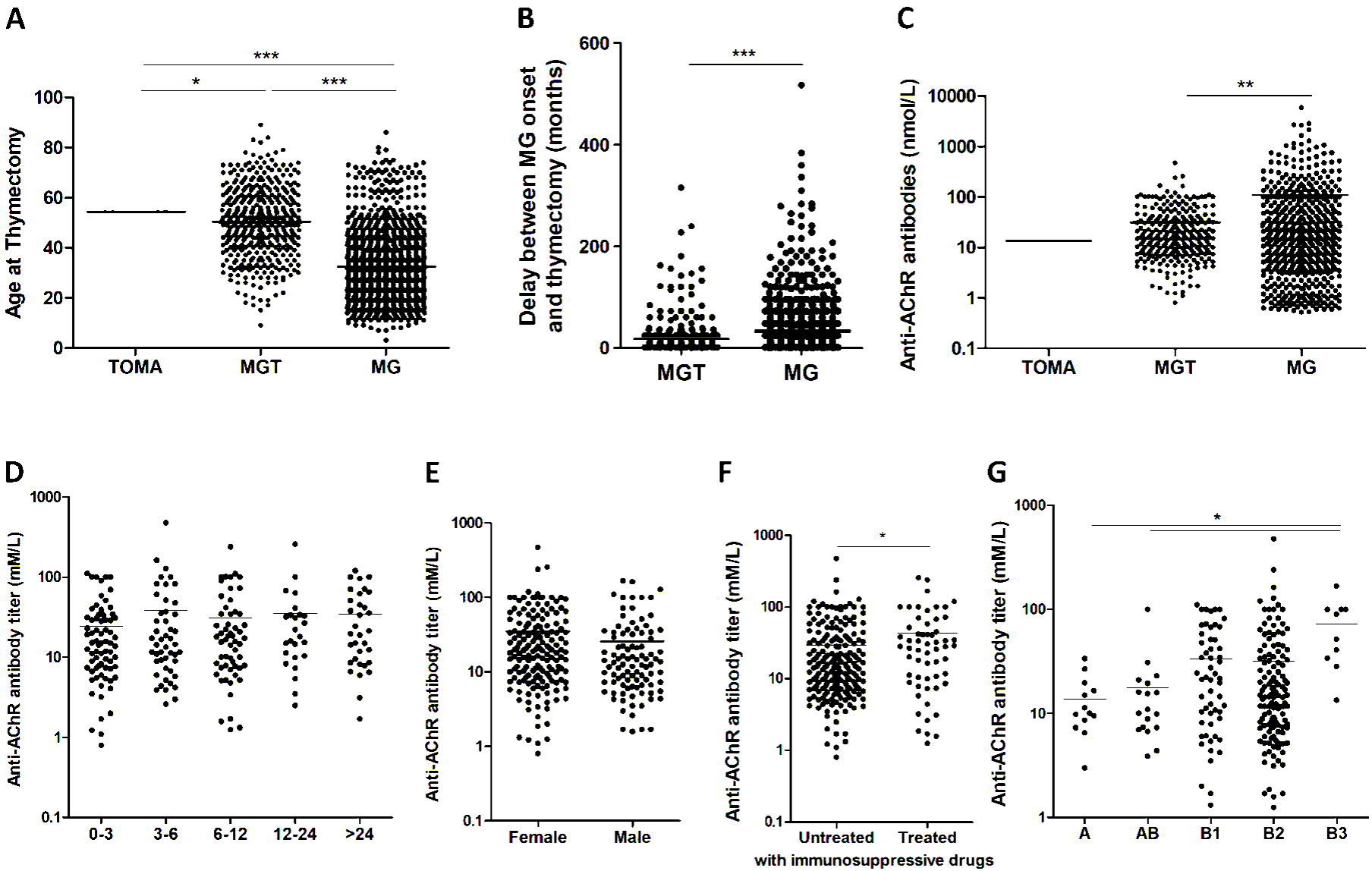
Figure 5: Serum levels of cytokines in different groups of patients.

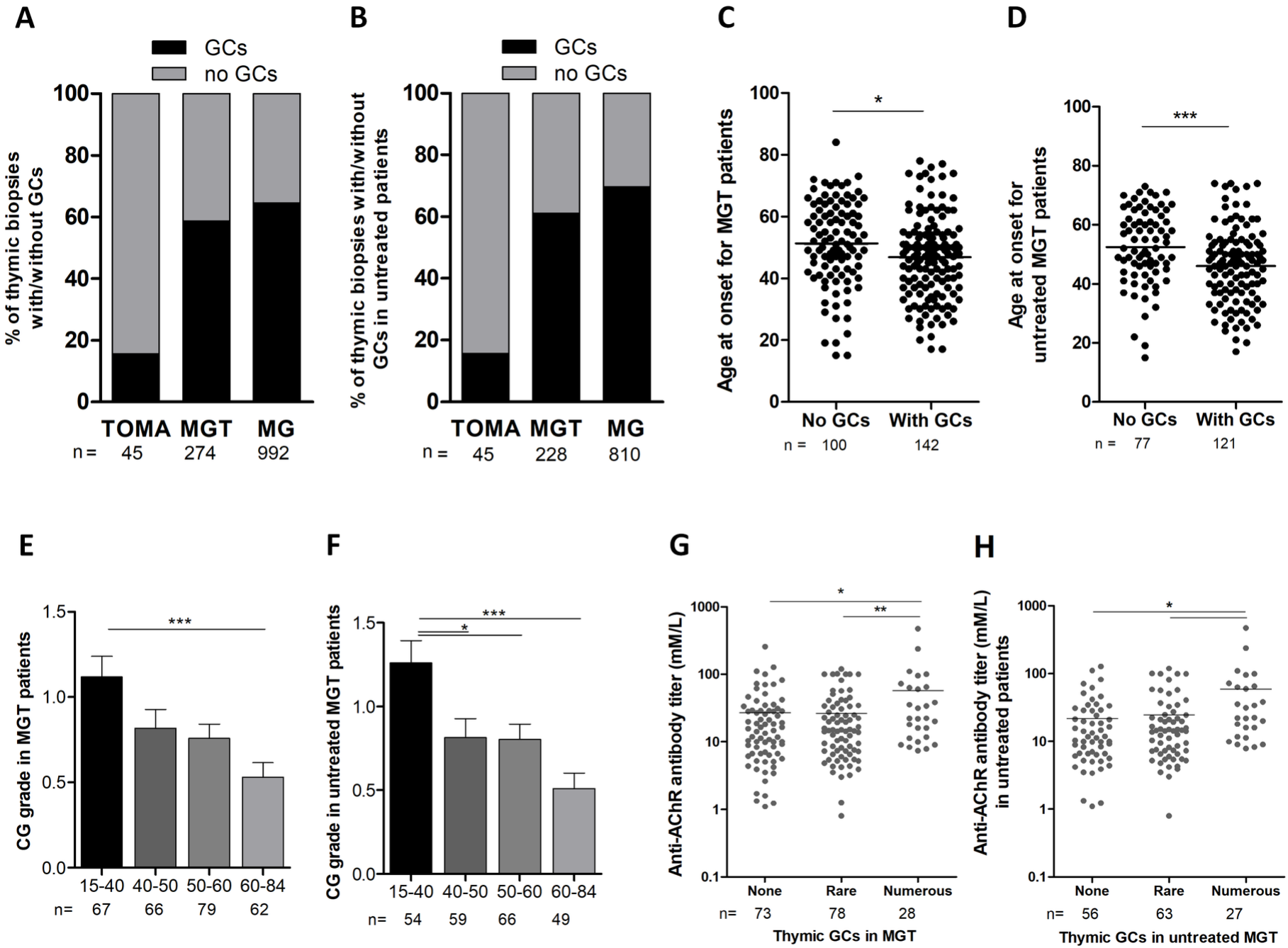
(A-D) Multiplex analyses for IFN-γ (A), IL-10 (B), IL-1b (C) and sCD40L (D) in control (CT, n=22), EOMG (n=14), MGT (n=16) and TOMA (n=15) donors. The "all MG" subgroup (n=30) corresponds to EOMG and MGT patients altogether. Cytokine expression levels were analyzed using the fluorescence signal (minus the fluorescence background). (E) Simoa analysis for IFN-α subtypes in control (CT, n=20), EOMG (n=15), MGT (n=19) and TOMA (n=20) donors. The "all MG" subgroup (n=34) corresponds to EOMG and MGT patients altogether. (A-E) p-values were assessed using a Kruskal-Wallis test with Dunn's multiple comparisons and p-values are indicated as follows: (#) $p<0.1$, (*) $p<0.05$, (**) $p<0.01$. (F-H) Analyses of the sensitivity and specificity of IFN-γ (F), IL-1β (G) and sCD40L (H) as markers to discriminate MGT from TOMA patients. The ROC curves were computed using the expression level of the cytokines in MGT and TOMA patients.

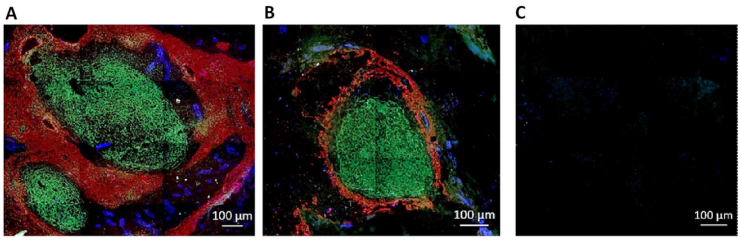


Abbreviations:

TOMA: thymoma without myasthenia gravis at time of thymectomy
MGT: myasthenia gravis diagnosed before thymoma
MG: myasthenia gravis without histological diagnostic of thymoma
WHO: World Health Organization
AChR: acetylcholine receptor


A
Age at Thymectomy
100
80
60
40
20
0
***
*
***
TOMA
MGT
MG
B
Delay between MG onset and thymectomy (months)
600
400
200
0
***
MGT
MG
C
Anti-AChR antibodies (nmol/L)
10000
1000
100
10
1
0.1
**
TOMA
MGT
MG
D
Anti-AChR antibody titer (mM/L)
1000
100
10
1
0.1
0-3
3-6
6-12
12-24
>24
E
Anti-AChR antibody titer (mM/L)
1000
100
10
1
0.1
Female
Male
F
Anti-AChR antibody titer (mM/L)
1000
100
10
1
0.1
*
Untreated
Treated
with immunosuppressive drugs
G
Anti-AChR antibody titer (mM/L)
1000
100
10
1
*
A
AB
B1
B2
B3


A
GCs
no GCs
% of thymic biopsies with/without GCs
100
80
60
40
20
0
TOMA MGT MG
n = 45 274 992
B
GCs
no GCs
% of thymic biopsies with/without GCs in untreated patients
100
80
60
40
20
0
TOMA MGT MG
n = 45 228 810
C
Age at onset for MGT patients
*
100
80
60
40
20
0
No GCs With GCs
n = 100 142
D
Age at onset for untreated MGT patients
***
100
80
60
40
20
0
No GCs With GCs
n = 77 121
E
CG grade in MGT patients
***
1.5
1.0
0.5
0.0
15-40 40-50 50-60 60-84
n= 67 66 79 62
F
CG grade in untreated MGT patients
***
*
1.5
1.0
0.5
0.0
15-40 40-50 50-60 60-84
n= 54 59 66 49
G
Anti-AChR antibody titer (mM/L)
*
**
1000
100
10
1
0.1
None Rare Numerous
n= 73 78 28
Thymic GCs in MGT
H
Anti-AChR antibody titer (mM/L) in untreated patients
*
1000
100
10
1
0.1
None Rare Numerous
n= 56 63 27
Thymic GCs in untreated MGT


A
100 µm
B
100 µm
C
100 µm

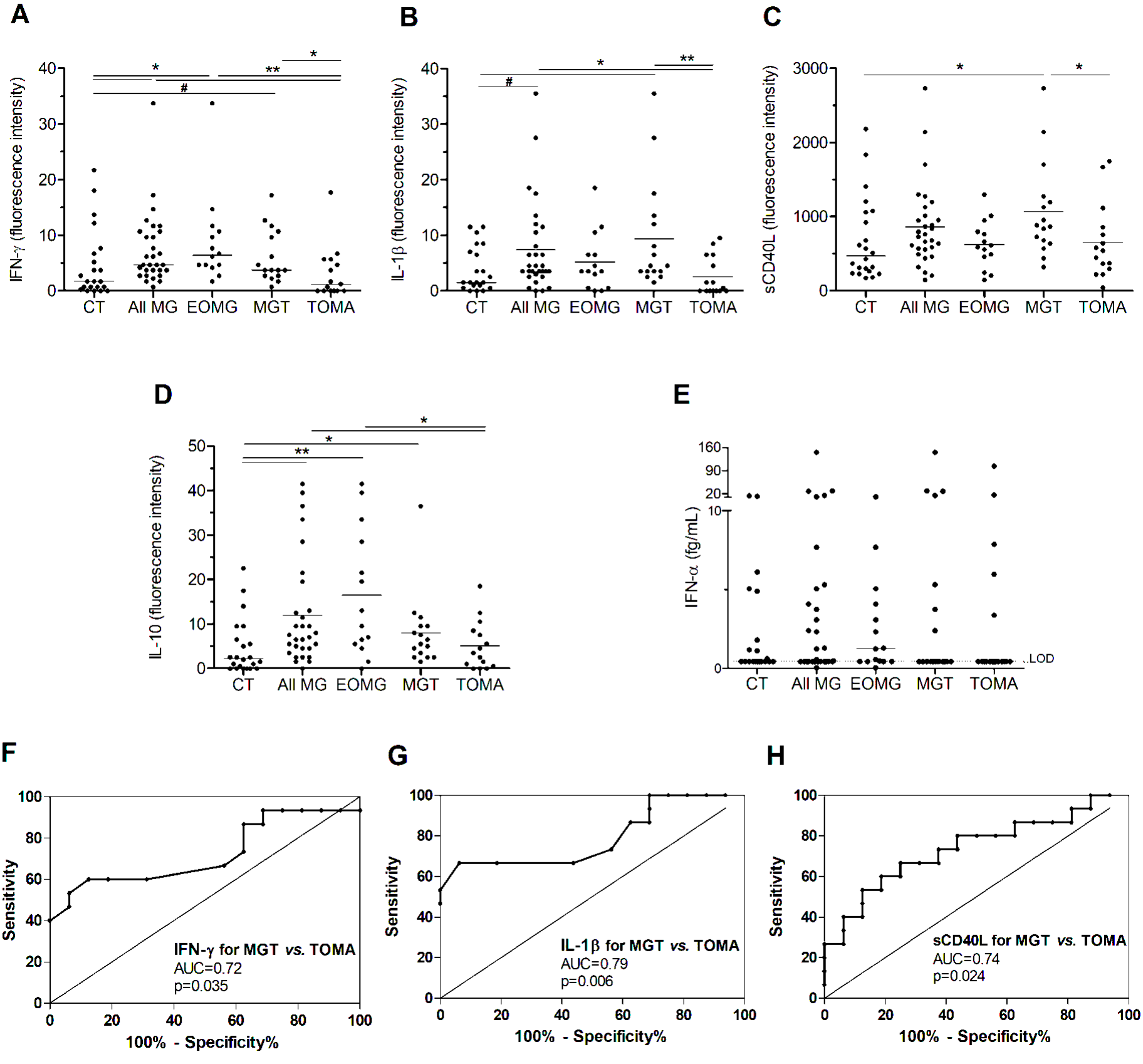

A
IFN-γ (fluorescence intensity)
CT
All MG
EOMG
MGT
TOMA
B
IL-1β (fluorescence intensity)
C
sCD40L (fluorescence intensity)
D
IL-10 (fluorescence intensity)
E
IFN-α (fg/mL)
LOD
F
IFN-γ for MGT vs. TOMA
AUC=0.72
p=0.035
G
IL-1β for MGT vs. TOMA
AUC=0.79
p=0.006
H
sCD40L for MGT vs. TOMA
AUC=0.74
p=0.024
Sensitivity
100% - Specificity%

Table 1: Characteristics of the patients included in the database

		TOMA	MGT	MG
Patients (number of patients)		111	409	1246
Gender: number of female patients (%)[a]		55 (49.5%)	237 (57.9%)	913 (73.3%)
Age at MG onset in years (mean ±SEM)[b]		-	48.8 ± 0.7 (n=356)	31.6 ± 0.4 (n=1115)
Age at thymectomy in years (mean ±SEM)[c] Age range		54.4 ± 1.3 (n=105) 17-89 years old	50.2 ± 0.7 (n=405) 9-89 years old	32.3 ± 0.4 (n=1125) 12-62 years old
Delay between MG onset and thymectomy in months (mean ±SEM)[d]		-	18.4 ± 1.9 (n=353)	33.4 ± 1.6 (n=1017)
Thymoma classification: number of patients (% for known patients)[e]	A	17 (25.0%)	17 (5.1%)	-
	AB	11 (16.2%)	31 (9.3%)	-
	B1	15 (22.1%)	85 (25.5%)	-
	B2	24 (34.8%)	182 (54.8%)	-
	B3	2 (2.9%)	17 (5.1%)	-
	Unknown	42	77	-
Invasive thymoma : number of patients (% for known patients)[f]	Yes	40 (53.3%)	230 (70.6%)	-
	No	35 (46.7%)	96 (29.4%)	-
	Unknown	36	83	-
Immunosuppressive treatments: number of patients (% for all patients)[g]	Yes	4 (3.6%)	83 (20.3%)	235 (18.9%)
	None	107	326	1011
AChR antibodies: number of patients (% for known patients)[h] Serum titer were measured from 90 days before and up to 8 days after thymectomy	Positive at thymectomy	19 (51.3%)	261 (98.5%)	541 (76.6%)
	Negative at thymectomy	18 (48.7%)	4 (1.5%)	165 (23.4%)
	Unknown	74	144	540
Presence of germinal centers: number of patients (% for known patients)[i]	Yes	7 (15.6%)	161 (58.8%)	640 (64.5%)
	None	38 (84.4%)	113 (41.2%)	352 (35.5%)
	Unknown	66	135	254

[a] Gender: TOMA vs MG $p<0.0001$; MGT vs MG $p<0.0001$ (Fisher's exact test).

[b] Age at MG onset: MGT vs MG $p<0.0001$ (Student t test).

[c] Age at thymectomy: TOMA vs MGT ($p<*$), TOMA vs MG ($p<***$), MGT vs MG ($p<***$) (one way anova test Bonferroni post hoc tests) - Figure 2A.

[d] Delay between MG onset and thymectomy : MGT vs MG $p<0.0001$ (Student t test) - Figure 2B.

[e] Thymoma WHO classification for TOMA vs MGT (comparison of each subtype versus all others for known patients): type A $p=0.0001$ and type B2 $p=0.002$ (Fisher's exact test).

[f] Invasive thymoma for known patients independently from histological type: TOMA vs MGT $p=0.0060$ (Fisher's exact test).

[g] Immunosuppressive treatment (corticosteroids, azathioprine, mycophenolate mofetil, methotrexate, cyclophosphamide, rituximab): TOMA vs MGT or MG, p*** (Fisher's exact test).

[h] Presence of anti-AChR antibodies: TOMA vs MGT or MG $p<0.0001$, MGT vs MG $p<0.0001$ (Fisher's exact test) - Figures 3 for further investigations.

[i] Germinal centers for known patients: TOMA vs MGT or MG $p<0.0001$ (Fisher's exact test).