Short Communication

Enrichment of serum IgG4 in MuSK myasthenia gravis patients

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A B S T R A C T

Muscle-specific kinase (MuSK) myasthenia gravis (MG) is a neuromuscular autoimmune disease belonging to a growing group of IgG4 autoimmune diseases (IgG4-AIDs), in which the majority of pathogenic autoantibodies are of the IgG4 subclass. The more prevalent form of MG with acetylcholine receptor (ACHr) antibodies is caused by IgG1-3 autoantibodies. A dominant role for IgG4 in autoimmune disease is intriguing due to its anti-inflammatory characteristics. It is unclear why MuSK autoantibodies are predominantly IgG4. We hypothesized that MuSK MG patients have a general predisposition to generate IgG4 responses, therefore resulting in high levels of circulating IgG4. To investigate this, we quantified serum Ig isotypes and IgG subclasses using nephelometric and turbidimetric assays in MuSK MG and AChR MG patients not under influence of immunosuppressive treatment. Absolute serum IgG1 was increased in both MuSK and AChR MG patients compared to healthy donors. In addition, only MuSK MG patients on average had significantly increased and enriched serum IgG4. Although more MuSK MG patients had elevated serum IgG4, for most the IgG4 serum levels fell within the normal range. Correlation analyses suggest MuSK-specific antibodies do not solely explain the variation in IgG4 levels. In conclusion, although serum IgG4 levels are slightly increased, the levels do not support ubiquitous IgG4 responses in MuSK MG patients as the underlying cause of dominant IgG4 MuSK antibodies.

1. Introduction

Human immunoglobulin G (IgG) is divided into four subclasses depending on small variations in the constant domain (Fc). These variations dictate their functionalities, such as their ability to activate complement, bind to Fc receptors on immune cells or undergo Fab-arm exchange (Vidarsson et al., 2014). In a healthy adult population, the following reference ranges have been determined as normal by the national Dutch blood bank: IgG1 (4.9–11.4 g/L), IgG2 (1.5–6.4 g/L), IgG3 (0.11–0.85 g/L) and IgG4 (0.03–2.0 g/L) (Meulenbroek, 2008). IgG subclass levels can be abnormal due to a deficiency of IgG subclasses (hypogammaglobulinemia) or due to abnormal ratios of subclasses (dysgammaglobulinemia). Hypogammaglobulinemia is observed in diseases affecting class switching, such as CD40 ligand or activation-induced cytokine deaminase (cofactor) deficiencies, or in immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome (Durandy and Kracker, 2012; Weemaes et al., 2013). Dysgammaglobulinemia can be observed in for example selective IgG subclass deficiencies, monoclonal gammopathies or IgG4-related diseases (The International Myeloma Working Group, 2003; Lighaam and Rispens, 2006).
Antibody-mediated autoimmune diseases are caused by antigen-specific autoantibodies, often predominantly of a specific IgG subclass. Muscle-specific kinase (MuSK) myasthenia gravis (MG) is part of a growing group of IgG4 autoimmune diseases (IgG4-AIDs), in which the majority of pathogenic autoantibodies are of the IgG4 subclass (Huijbers et al., 2015; Koneczny, 2020). Since IgG4 has anti-inflammatory characteristics, its role in autoimmune disease was surprising at first sight. In the past decade, several studies have confirmed that IgG4 is indeed responsible for the pathology observed in these IgG4-AIDs. The predominant mechanism appears to be functional blocking of its antigen rather than antigenic modulation or complement activation common to IgG1–3 autoimmune diseases (Huijbers et al., 2015; Koneczny, 2020). However, why these diseases are dominated by IgG4 autoantibody responses is unknown. Altered levels of serum IgG subclasses have been found in several autoimmune diseases, such as primary Sjögren syndrome, systemic lupus erythematosus, rheumatoid arthritis, AChR MG and pemphigus (Liu et al., 2011; Funakoshi et al., 2012; Chen et al., 2014; Zhang et al., 2015). Therefore, we hypothesized that patients with IgG4-AID may have a general predisposition to generate IgG4 responses.

To assess possible dysregulation in the immunoglobulin response in MuSK MG, we measured IgG subclasses, Ig isotypes and antigen-specific titers in patients not under the influence of immunosuppressive treatments.

2. Materials and methods

2.1. Subjects

MG patients were included from databases and biobanks at the Leiden University Medical Centre (LUMC, Leiden, the Netherlands), Policlinico Gemelli Università Cattolica del Sacro Cuore (Rome, Italy), Hospital Sant Pau UAB (Barcelona, Spain), Uppsala University (Uppsala, Sweden) and University of California Davis (Davis, CA, USA). The included serum samples from patients fulfilled the following criteria: 1) positive for either AChR or MuSK antibodies, and 2) immunosuppression naïve or no immunosuppressive treatment for at least one year prior to the sample date. Patients who had received anti-CD20 treatment in their medical history were excluded from the study. Seventeen samples were excluded because the patients were receiving immunosuppression (IS) at time of sampling. These were used in a supplementary analysis excluding because the patients were receiving IS at time of sampling. These were used in a supplementary analysis. The included serum samples from patients fulfilled the following criteria 1) positive for either AChR or MuSK antibodies, and 2) immunosuppression naïve or no immunosuppressive treatment for at least one year prior to the sample date. Patients who had received anti-CD20 treatment in their medical history were excluded from the study. Seventeen samples were excluded because the patients were receiving immunosuppression (IS) at time of sampling. These were used in a supplementary analysis examining IgG levels in IS vs non-IS treated patients. Samples and retrospective data were obtained from biobanks, in compliance with the institute's medical ethics committee guidelines. Demographics and clinical parameters were retrospectively collected from the medical history and gathered in an anonymized database. Sera were stored at −80 °C until analysis. Data from healthy adult donors were derived from the stem cell donor database of the Willem-Alexander Children's Hospital (LUMC, Leiden, the Netherlands).

2.2. Laboratory tests

All measurements were performed by the clinical chemistry laboratory at the LUMC under standard operating procedures based on manufacturer's instruction. IgG subclasses, total IgG, total IgA and IgM were determined using nephelometric methods on BN-prospek (Siemens; IgG subclasses, total IgG, total IgA and IgM) or the turbidimetric assay using the Cobas8000 (Roche; total IgG, IgA and IgM). Total IgE was determined using the FEIA technique on ImmunoCAP250 (Thermo Fisher Diagnostics). AChR antibody titers were determined using radioimmunoprecipitation assay (RIA, RSR) and MuSK antibody titers with either RIA (RSR) or Enzyme-linked ImmunoSorbent Assay (ELISA, IBL).

2.3. Statistical analysis

Statistical analyses were performed using SPSS statistics v25 (IBM). Statistical analyses were performed using SPSS statistics v25 (IBM). Demographics were analyzed with a Chi-square test (sex) or Kruskal-Wallis test (age). Absolute immunoglobulin levels were log-transformed before analysis. For group comparisons, ANCOVA with posthoc comparisons was used and included age and sex as covariates. Elevated IgG4 proportions were tested with Fisher's Exact test.

To be able to combine MuSK antibody titers measured with the RIA and the ELISA, the values were ranked per variable for each assay separately. These sets of ranks were each converted to a scale from zero to one to normalize for the difference in the number of individuals analyzed between the two assays. The datasets for the MuSK RIA and MuSK ELISA were then combined and analyzed with a Spearman correlation.

Due to limited sample volume for some patient samples, we could not perform all predefined measurements. The group sizes available for individual measurements due to missing data are provided in the figure legends.

3. Results

3.1. Donor demographics

Sera from five medical centers were included in this study. The MuSK MG group contained relatively more female patients, while AChR MG patients were generally older at time of sampling (Table S1). These demographic characteristics match the known epidemiological patterns for MuSK MG (female predominance) and AChR MG (bimodal age of disease onset, Table S1) (Verschuuren et al., 2010). Since the groups were significantly different in sex (Chi-square test, p = 0.024) and age (Kruskal-Wallis, p < 0.001) (Table S1), these were included as covariates in the statistical analyses. This is important because serum IgG4 levels can depend on both (Aucouturier et al., 1984; Harkness et al., 2020). Clinical information of the MG patient groups can also be found in Table S1.

3.2. Elevated and enriched serum IgG4 in MuSK MG patients

IgG subclasses were measured in MuSK MG patients and compared with AChR MG patients (IgG1-AID) and healthy donors. MuSK MG patients had increased IgG1, similar to AChR MG patients (Fig. 1A). In contrast, MuSK MG patients on average had higher serum IgG4 compared to both AChR MG patients and healthy donors, while no differences between groups were detected for IgG2 and IgG3 (Fig. 1B-D). In line with previous findings, males had higher IgG4 levels than females (p < 0.01) and this holds true for all groups (Fig. S1) (Harkness et al., 2020; Endmayer et al., 2022). Elevated IgG4 is defined as >1.35 g/L in IgG4-related disease (Palazzo et al., 2014). In the MuSK MG group, 22% of patients had elevated serum IgG4 versus 3% in healthy donors and 10% in AChR MG patients, despite the female predominance in MuSK MG (Fig. 1D, Fisher exact test, p < 0.01).

To assess whether the subclass distribution differs between groups, we calculated the percentage of each subclass versus the sum of all IgG subclasses. In AChR MG patients, the relative proportion of IgG1 was higher, while IgG2 was relatively lower (Fig. 1E-F). For MuSK MG patients, the relative proportion of IgG4 was significantly higher compared to the other subclasses (Fig. 1H). The IgG subclass that is increased in each MG subtype is the subclass which dominates the antigen-specific autoantibodies. Ig isotypes were measured to investigate whether an overall dysregulation of the immunoglobulin response is present. For a subset of patients, a separate measurement of total IgG revealed increased IgG4 for AChR MG and a similar trend for MuSK MG (Fig. 2A). The average serum IgM level in MuSK MG patients did not significantly differ from healthy donors and AChR MG (Fig. 2B). A trend towards elevated serum IgA was present in both MG groups (Fig. 2C). 43% of the MuSK MG patients had elevated IgE (>99 IU/mL) versus 31% of the AChR MG patients, this was not significantly different (Fisher's exact test, p = 0.415). In the general
Fig. 1. Increased IgG4 in MuSK MG patients. Absolute values of IgG1 (A), IgG2 (B), IgG3 (C) and IgG4 (D). Dotted line at 1.35 g/L represents cutoff for elevated IgG4 in IgG4-RD (Palazzo et al., 2014). IgG1 (E), IgG2 (F), IgG3 (G) and IgG4 (H) as a percentage of the sum of the IgG subclasses. Data are presented as geometric mean and geometric 95% confidence interval (CI) in A-D and as mean and 95% CI for E-H. * p < 0.05, ** p < 0.01, *** p < 0.001. HD: n = 77, MuSK MG: n = 27, AChR MG: n = 50. HD = healthy donors.

Fig. 2. Ig Isotypes. Absolute values of IgG (A), IgM (B), IgA (C) and IgE (D). Data is presented as geometric mean and geometric 95% CI. * p < 0.05. HD: n = 75, MuSK MG: n = 24 (n = 20 for IgG, n = 21 for IgE), AChR MG: n = 50 (n = 44 for IgG, n = 48 for IgE). HD = healthy donors.
adult population 32% have elevated IgE (Kerkhof et al., 2003).

3.3. MuSK-specific titers correlate with serum IgG4

To assess whether MuSK-specific antibodies contribute to the elevated serum IgG subclass levels, the relationship between MuSK antibody titers and absolute serum IgG1 or IgG4 was investigated, as these IgG subclasses showed elevated absolute levels in MuSK MG. A spearman correlation of ranks was performed to assess this relationship, because the data is non-parametric in nature. MuSK Ab titers significantly correlated with serum IgG4 (spearman’s rho = 0.511, p < 0.05, n = 22), but not with IgG1 (spearman’s rho = 0.156, ns, n = 22). MuSK MG patients with higher serum IgG4 thus also more often have higher MuSK Ab titers.

4. Discussion

IgG4 MuSK antibodies cause MuSK MG and correlate with disease severity both between and within patients (Niks et al., 2008; Klooster et al., 2012; Huijbers et al., 2016). In this study, we show that total serum IgG4 is increased in MuSK MG patients not under the influence of immunosuppressive treatment. MuSK MG patients with relatively high serum IgG4 also more often have relatively high MuSK titers, suggesting similar processes may underlie both. However, the observed individual variation suggests it is unlikely that the increased IgG4 levels are only due to MuSK-specific antibodies, indicating that MuSK MG patients experience an overall mild increase in IgG4 production. Absolute IgG1 levels were also elevated in both MuSK and AChR MG patients, resulting in an higher proportion of IgG1 vs all IgG subclasses in AChR MG patients only. Taken together, MuSK MG patients appear to have a tendency to mild increase in serum immunoglobulins, suggesting the antibody response in general may be more active. However, since circulating IgG4 levels fall within the normal range for most MuSK MG patients, it is unlikely that a predisposition to ubiquitously generate IgG4 responses causes the dominance of IgG4 antibodies targeting MuSK.

Total serum IgG4 is also selectively enriched in other IgG4-AIDs pemphigus vulgaris and pemphigus foliaceus (Funakoshi et al., 2012). In contrast, several recent studies did not find elevated serum IgG4 in various neurological IgG4-AIDs, including MuSK MG (Basile et al., 2018; Basile et al., 2021; Endmayr et al., 2022). This may partly be explained by the low numbers of patient samples in these studies. Secondly, although samples were sometimes taken early in the disease course, a proportion of patients was receiving immunosuppression, which can reduce serum IgG4 levels and thus influence how the immunoglobulin response relates to disease activity (Fig. S2, (Tabata et al., 2011; Iseda et al., 2014)). To further support the observation that increased circulating IgG4 levels are a general phenomenon in IgG4-AIDs, sera of other IgG4-AID patient groups need to be investigated prior to starting immunosuppressive therapies.

IgG4-related diseases (IgG4-RD) are hallmarked by IgG4 producing B-cell infiltrates in organs, causing swelling and dysfunction (Palazzo et al., 2014; Bledsoe et al., 2018). Over 50% of these patients have elevated serum IgG4 levels with some reaching an order of magnitude higher levels than normal (Osemi et al., 2011; Endmayr et al., 2022). It has been hypothesized that IgG4-RD and IgG4-AID are similar disease entities (Rabagkar et al., 2017). However, our results and previous studies in pemphigus (Funakoshi et al., 2012) found that serum IgG4 elevations are modest and less prevalent in IgG4-AIDs. Immune cell infiltrates have been found in pemphigus skin lesions, but it is unknown whether these are IgG4-producing B-cells (Yuan et al., 2017). Further, B-cell infiltrates were not observed at the neuromuscular junctions of intercostal muscles in MuSK MG patients (Jennekens et al., 1992; Selcen et al., 2004; Niks et al., 2010). Finally, IgG4-AIDs have a clear causative antigen, while this is not yet established for IgG4-RD (Hubers et al., 2018). Thus far, the overlap between these two disease classes is therefore limited.

The IgG4-response is associated with T helper 2 (Th2) cytokines interleukin (IL)-4, IL-13 and IL-10, regulatory T-cells and chronic antigen exposure (Lighaam and Rispens, 2016). Class-switching to IgE and IgG4 are linked by their dependency on IL-4 or IL-13, while IL-10 appears to direct a class switch to IgG4 over IgE (Lighaam and Rispens, 2016). The observed serum IgE levels in MuSK MG patients would be in line with a heightened Th2 response; although this was not statistically significant, likely in part because this analysis was underpowered. Surprisingly, in vitro activation of T-cells from MuSK MG patients suggests a higher Th1/17 versus Th2 response compared to healthy controls, although no differences could be found in plasma cytokine levels (Yi et al., 2014; Yilmaz et al., 2015a). Furthermore, less IL-10-producing regulatory B-cells were found in vivo, and in vitro CD40-stimulated B-cells produced less IL-10 (Sun et al., 2014; Yilmaz et al., 2015b). These results suggest that enhanced Th2 activation may not be the main driver of the IgG4 response in MuSK MG. However, the vast majority of the MuSK MG patients in these studies were on immunosuppressive medication, introducing a potential bias. An active immunization model of MuSK MG in mice did result in a predominant IgG1 (IgG4 equivalent in mouse) response accompanied by increases in IL-4 and IL-10, suggesting MuSK immunization can induce a Th2-like response (Ulusoy et al., 2014). In summary, it is yet unclear which immunological mechanisms cause the selective increase in IgG4 production or dominance in

Fig. 3. MuSK antibody titers correlate with serum IgG4. Scatter plot of MuSK antibodies (Abs) versus IgG1 (A) or IgG4 (B). MuSK Abs detected by ELISA in black and by radioimmunoprecipitation assay (RIA) in grey.

\[ \text{spearman's rho} = 0.156, \text{ ns} \]

\[ \text{spearman's rho} = 0.511, \text{ p<0.05} \]
autoantibody subclass in IgG4-AIDs. In our experience it was very challenging to compile a set of samples fitting the immunosuppression naïve criteria, in spite of reaching out to 20 different MG expertise centers. The relatively low numbers studied here are a direct consequence of this and limit the study power. To further investigate the etiology of IgG4-AIDs it will be of importance to study immunosuppressive naïve serum and immune cells, as the exposure to such treatments may significantly limit our view on the immunological characteristics of these diseases.

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CRediT authorship contribution statement
Dana L.E. Vergoossen: Conceptualization, Formal analysis, Visualization, Project administration, Writing – original draft. Annabel M. Ruitert: Investigation, Data curation. Kevin R. Keene: Data curation. Erik H. Niks: Conceptualization, Resources. Martijn R. Tannemaat: Resources, Writing – review & editing. Ellen Strijbos: Data curation. Alexander F. Lipka: Data curation, Writing – review & editing. Els C. Jol van der Zijde: Resources, Data curation. Maarten J.D. van Tol: Resources, Data curation, Writing – review & editing. Jaap A. Bakker: Methodology, Writing – review & editing. Brigitte A. Wevers: Methodology. Elisabet Westerberg: Resources, Writing – review & editing. Lúcia S. Borges: Resources. Olivia C. Tong: Resources. David P. Richman: Resources. Isabel Illa: Resources, Writing – review & editing. Anna Rosstedt Punga: Resources, Writing – review & editing. Amelia Evoli: Resources, Writing – review & editing. Silvere M. van der Maarel: Conceptualization, Supervision, Writing – review & editing. Jan J. Verschueren: Conceptualization, Resources, Supervision, Writing – review & editing. Maartje G. Huijbers: Conceptualization, Formal analysis, Supervision, Writing – review & editing.

Declaration of Competing Interest
OT, LB and DR receive research support from NIH and Cibaletta Biopharma. JV, SM, MH are co-investors on MusK-related patents. LUMC and JV, SM and MH receive royalties from these patents. LUMC receives royalties on a MusK ELISA. JV is consultant for Argexen, Alexion, NMD Pharma. ARP is consultant for Argexen. MH receives financial support from the LUMC (OIO 2017, Gisela Their Fellowship 2021), Top Sector Life Sciences & Health to Samenwerkende Gezondheidsfonds (LSHM18055-SGF and LSHM19130), Prinses Beatrix Spierfonds (W.OR-19.13). The remaining authors declare no interests. The LUMC is part of the European Reference Network for Rare Neuromuscular Diseases [ERN EURO-NMD] and the Netherlands Neuromuscular Center.

Data availability
Data will be made available on request.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2022.577978.
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