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Mass Spectrometric Determination of IgG Subclass-Specific Glycosylation Profiles in Siblings Discordant for Myositis Syndromes

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Abstract

Many autoimmune conditions are believed to result from chronic inflammation as a consequence of the interaction of genetic and environmental factors in susceptible individuals. One common feature in some autoimmune diseases is the decrease in terminal galactosylation of the constant region *N*-glycan of the total plasma immunoglobulin. To determine whether a similar pattern is characteristic for the autoimmune disorder myositis, we analyzed the antibody subclass specific glycosylation in patients with myositis, their asymptomatic siblings, and healthy unrelated age- and sex-matched controls. The antibody subclass specific glycosylation was determined from the LC-MS analyses of the IgG glycopeptides generated by trypsin digestion of the antibody heavy chain. The glycosylation profiles of the IgG subclasses were determined relative to the abundance of the monogalactosylated core-fucosylated glycoform, G_1F. We found elevated amounts of glycoforms lacking terminal galactose in myositis patients. Pairwise statistical analyses reveals that galactosylation is statistically different between the myositis patients and control groups. Furthermore, the trend analysis for glycosylation indicates a pattern of decreasing galactosylation in the order: controls>siblings>myositis patients, suggesting the existence of a genetic, immune-related predisposition in the group of asymptomatic siblings that can be detected before the onset of clinical symptoms at the level of plasma proteins.

Keywords

myositis; plasma; twin-sibling clinical study; antibody glycosylation; glycopeptide; mass spectrometry

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Supporting Information Available
Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Introduction

The idiopathic inflammatory myopathies (IIM), collectively referred to as the myositis syndromes, represent a rare group of systemic autoimmune disorders characterized by progressive muscle weakness and infiltration of multiple muscles and other tissues by immune cells. Within the group of IIM, with an overall prevalence of 0.001%, the best recognized disease subsets are dermatomyositis (DM), polymyositis (PM) and inclusion body myositis (IBM) [1, 2]. While some myositis autoantigens, such as histidyl-tRNA synthetase (Jo-1) [3, 4], signal recognition particle [5] and the nuclear protein Mi-2, have been identified, the mechanisms leading to the breach in immune tolerance and disease pathogenesis in IIM remain largely unknown [3–8]. The symptoms of myositis are non-specific, often resembling those of other diseases, and phenotypes may differ considerably among individuals. Although the diagnosis of myositis syndromes may be challenging, several helpful tests include muscle biopsy and measurements of autoantibody titers and muscle enzymes in serum[7, 9–11]. Growing evidence suggests that both genetic and environmental factors contribute to the development of myositis [12].

Despite the clinical heterogeneity among autoimmune diseases, substantial evidence indicates the existence of a common genetic component associated with alleles of the major histocompatibility complex region [13–15]. Furthermore, gene expression analyses of family members discordant for autoimmune disorders revealed increased susceptibility of unaffected siblings and twins compared to the unrelated population. These studies found a distinct gene expression signature in the unaffected family members, which resembled, to a considerable extent, the pattern found in autoimmune patients, and to a lesser extent the pattern found in non-related controls [14, 16, 17].

At the protein level, the effector molecules of the immune system – the antibodies –undergo structural changes in autoimmune rheumatic disorders such as rheumatoid arthritis (RA) [18, 19], Sjøgrens's syndrome [20], psoriatic arthritis, and systemic lupus erythematosus (SLE) [21]. These changes are mainly related to the glycoform distribution of the complex type *N*-linked glycan attached at Asn 297, the conserved glycosylation site on the antibody constant region. The shared feature among all these diseases is the elevation of the glycoforms lacking terminal galactose – collectively referred to as IgG Gal-0 – in patients compared to healthy controls.

The molecular mechanisms of the pro- and anti-inflammatory properties of serum IgG are largely modulated by its constant region glycosylation [22, 23]. Until recently, it was believed that the Gal-0 portion of the glycoforms may trigger pro-inflammatory responses by activating the complement pathway through interaction of the unmasked GlcNAc residue with the mannan-binding lectin [24, 25]. More recent studies, however, suggest that IgG-Gal 0 antibodies engage the classical Fcγ receptors pathways [26], and that the sialylated portion of the IgG is required and sufficient to suppress inflammation [27–29]. In RA, the dynamic levels of IgG-Gal 0 correlate with disease progression, disease activity, and spontaneous remission of the disease in pregnancy and post-partum flares of disease [19, 30, 31]. Although the pathogenic role of the IgG-Gal 0 glycoforms is not fully understood, the sugar signature of serum IgG was shown to be an useful indicator to differentiate between early RA and RA [32].

As part of a twin-sibling study aimed at identifying genetic and environmental risk factors of systemic rheumatic disorders [33], the subclass specific IgG glycosylation in fourteen patients with myositis, ten asymptomatic siblings of ten of the patients, and twelve healthy unrelated age-matched controls was characterized. Aims of the study were to determine: *(i)* whether a similar pattern of elevated IgG Gal-0, as seen in RA, is characteristic for myositis patients, and *(ii)* whether susceptibility can be defined at the protein level, from the phenotypes of

IgG glycosylation in patients, siblings and controls. The methodology employed to address these questions combines Protein G affinity capture of plasma IgG, proteolytic degradation and high performance liquid chromatography – mass spectrometry (LC-MS) at the glycopeptide level [18, 34, 35]. The main advantages of characterizing antibody glycopeptides by mass spectrometry are: *(i)* subclass specific changes in IgG glycosylation can be identified, and *(ii)* Fc and Fab glycans can be distinguished [34]. The abundance values relative to the core fucosylated monogalactosyl glycoform in each subclass were determined for the eleven most abundant glycoforms observed by LC-MS. The results were statistically analyzed with regard to galactosylation, sialylation, bisecting GlcNAc, and lack of core fucosylation.

Experimental section

Materials

Dithiothreitol, ammonium bicarbonate, and 96% formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Sequencing grade-modified porcine trypsin was obtained from Promega (Madison, WI). The Protein G-agarose kit was obtained from KPL (Washington DC). NuPage 4 – 12 % Bis-Tris pre-cast gels, sample loading and running buffers and Coomasie SimplyBlue were purchased from Invitrogen (Carlsbad, CA). Acetonitrile was purchased from Caledon Laboratories, Ltd. (Georgetown, Ontario). Purified water (17.8 MΩ) was obtained from an in-house Hydro Picopure 2 system. All chemicals were used without further purification unless otherwise specified.

Study Population

The present study is part of the clinical study NCT00055055, aimed at identifying genetic and environmental risk factors in families with twins or siblings discordant for rheumatic disorders, including rheumatoid arthritis, systemic lupus erythematosus and myositis [33]. The participants in this study were selected as follows: cases – adults or children with one of the above autoimmune conditions, who have a healthy twin or sibling of the same sex within 5 years of age; cases' unaffected twins or siblings, and unrelated controls – normal, age- and sex-matched volunteers. Blood samples were collected at a single time point. Out of these, plasma samples from myositis patients (M, n = 14), asymptomatic twins/siblings (S, n = 10) and unrelated age-matched controls (C, n = 12) were selected for the study of IgG glycosylation. All patients met the criteria for probable or definite PM/DM, as defined by Bohan and Peter [36] and modified by the International Myositis Assessment and Clinical Study Group (IMACS) [37]. Physician global disease activity was assessed by a 100 mm visual analogue scale [38]. The characteristics of the study population, including the disease activity assessed by the physician and medication at the time point of blood collection, are presented in Supplemental Table 1. The subjects in this study were followed with annual mailings of questionnaires asking about new diseases or medications for 3–4 years and none developed new autoimmune diseases. None of the subjects showed clinical or laboratory signs of other inflammatory diseases.

Protein G-affinity Purification of the IgG Isolation

The isolation of plasma IgG was carried out in 0.5 mL compact reaction columns (CRCs), packed with agarose-bound Protein G, which binds all four human IgG subclasses. Washing/binding and elution buffers were provided in the Protein G-agarose kit and were used as suggested by the manufacturer. For each plasma sample, a 0.5 mL column was packed with ~ 200 μL drained agarose, as follows: 400 μL of slurry were mixed with 400 μL washing/binding buffer, transferred to the column, and allowed to flow by gravity. The packed affinity resin was equilibrated with 5 mL washing/binding buffer. Plasma samples (20 μL) were diluted to 100 μL with washing/binding buffer and applied on the resin. A volume of

200–300 μL of washing/binding buffer was subsequently added, in order to fill the remaining dead volume. A Nutator was used to mix the content of the column in a three dimensional, gentle rocking motion, for 45 minutes at room temperature. The non-bound protein fraction was removed by washing the resin with 5 mL washing/binding buffer, followed by 5 mL deionized water. Elution of the IgG was performed by adding 0.5 mL elution buffer and incubation for 15 minutes at room temperature. The eluted IgG was brought to physiologic pH by the addition of 150 μL of 5X washing/binding buffer.

SDS-PAGE and In-gel Digestion

Following elution and neutralization to pH 7.4, 30 μL of the solution containing the isolated IgG were mixed with 10 μL sample loading buffer containing 100 mM DTT. Reduction was carried out at 95°C for 10 minutes, followed by SDS-PAGE and automated in-gel digestion with trypsin of the heavy chain band, as described previously [35]. To ensure complete trypsinization of the heavy chain, an additional digestion step was employed as follows: to each lyophilized sample, 25 μL ammonium bicarbonate (pH 7.4) containing 0.83 μg trypsin were added. The digestion was performed overnight at 37°C. The samples were stored at −80°C until further use.

Mass Spectrometry

The IgG glycopeptides were analyzed by LC/MS on a Waters Q-Tof Premier mass spectrometer equipped with a nanoAcquity UPLC system (Waters, Milford, MA). Analyses were performed on a 3 μm, 100 μm × 100 mm, Atlantis dC18 column (Waters, nanoAcquity), using a flow rate of 300 nL/min. A C18 trapping column (180 μm × 20 mm) with 5 μm particle size (Waters, nanoAcquity) was positioned in-line of the analytical column and upstream of a micro-tee union used both as a vent for trapping and as a liquid junction. Trapping was performed for 3 min at 5 μL/min flow rate, using the initial solvent composition. A 4 μL aliquot of the digest sample was injected onto the column. Peptides were eluted by using a linear gradient from 98% solvent A (0.1% formic acid in water (v/v)) and 2% solvent B (0.1% formic acid in acetonitrile (v/v)) to 40% solvent B over 90 minutes. Mass spectrometer settings for the MS analysis were: capillary voltage of 3.2 kV, cone voltage of 20 V, collision energy of 5.0 V and source temperature of 80°C. Mass spectra were acquired over the mass range 200 – 2000 Da. For subclass specific glycosylation analysis, triplicate analyses were acquired in the MS only mode. For calibration, an orthogonal reference spray (LockSpray) of a solution of Glu^1-Fibrinopeptide B (500 fmol/μL) in water/acetonitrile 80:20 (v/v) and 0.1% formic acid, having a reference mass of 785.8496 (2+) was used.

Data Interpretation

The LC-MS data were analyzed using MassLynx 4.1 software (Waters, Milford, MA). The monosaccharide and glycoform annotations used in the following sections are as follows: G – galactose, F – fucose, B – bisecting *N*-acetyl glucosamine, SA – sialic acid. For example, the annotation G_1FSA refers to the core fucosylated, complex biantennary glycan, bearing one galactose and one terminal sialic acid residue. Because no species containing two sialic acid or two fucose moieties were observed, the presence/absence of SA or F in the annotation will indicate the presence of one/zero residues of that kind. The obtained LC-MS/MS data do not provide unambiguous linkage information to differentiate between isobaric structures, such as triantennary glycans and those containing bisecting GlcNAc. Because of the considerable number of previous studies of human IgG glycosylation[18,19,34], we assigned those structures containing an extra GlcNAc moiety in addition to the two antennas, as glycans with bisecting GlcNAc. The IgG subclass specific glycosylation profiles were determined by averaging the MS scans over the chromatographic retention time in which glycopeptides from a specific subclass eluted. The IgG tryptic glycopeptides

ions were observed as the +2 and +3 charge states. For each of the eleven most abundant glycoforms (shown in Figure 1B) the abundances of each species were summed and normalized to the abundance of the fucosyl monogalactosyl glycoform (G_1F) within each IgG subclass. From these analyses, it was not possible to differentiate between the IgG_2 and IgG_3 subclasses, because the glycopeptides have identical peptide backbones.

Statistical Analysis

Statistical analyses were performed using the statistical software package SAS (version 9.1). All variables analyzed are defined as ratios of their total ion abundance to the sum of the abundances of the eleven most abundant glycoforms observed in the LC-MS analyses. The data were normalized to the sum of all glycoforms so that statistical analyses of each individual variable can be performed. Pairwise comparisons among patients (M), asymptomatic siblings (S) and unrelated controls (C) in the IgG_1 and $IgG_{2–3}$ subclasses were performed for each of the glycoforms as well as the following summed variables: Gal-0 (agalactosylation) defined as the sum ($G_0F + G_0FB + G_0$); bisecting GlcNAc (B) defined as the sum ($G_0FB + G_1FB + G_2FB$); sialic acid (SA) defined as the sum ($G_1FSA + G_2FSA$); and Fuc-0 (afucosylation) defined as ($G_0 + G_1 + G_2$). All pairwise comparisons were performed by treating matched groups and subjects nested within groups as random effects to account for any correlations among the data within subject and within matched groups. We performed standard mixed effects analysis of variance in SAS 9.1 using the SAS procedure called PROC MIXED. A comparison of variables was determined to be statistically significant at a $P<0.05$. Since the patients, their asymptomatic siblings, and the unrelated controls may be viewed as ordered groups, we performed a trend analysis, using ORIOGEN methodology[39,40], to investigate the changes in variable over these ordered categories. The expectation is that there is a continuum from patients to asymptomatic siblings to unrelated controls with the asymptomatic siblings serving as the middle group. Because the IgG_4 glycopeptides were observed with low relative abundance, and in some samples they were not detected above the noise level, no statistical analyses of the IgG_4 glycosylation were performed.

Results and discussion

Subclass specific glycosylation profiling by LC/MS

Human IgG contains four distinct IgG subclasses – IgG_1, IgG_2, IgG_3 and IgG_4 – sharing more than 95% sequence homology. All four subclasses can be isolated from serum using Protein G affinity capture [18]. Their concentration in human plasma may vary considerably among individuals, with IgG_1 (10–12 mg/mL) and IgG_2 (2–6 mg/mL) having the highest concentrations, followed by IgG_3 (0.5–1 mg/mL) and IgG_4 (0.2–1 mg/mL) [41]. Structural differences are found in the hinge region, disulfide bridges, and in the CH2 domain of the heavy chain, around the consensus site for *N*-glycosylation, Asn 297. The minimal constant region tryptic peptide containing the *N*-linked site has the sequence EEQ*X*^{297}NST*X*R, where X represents characteristic residues for each IgG subclass, as follows: X = both Y for IgG_1, both F for $IgG_{2–3}$, and simultaneously F296/Y300 for IgG_4. As a result of these amino acid variations, glycopeptides from each subclass exhibit differences in their hydrophobic character, a feature which was used in the present study to selectively determine the glycoform distribution within each subclass. Glycopeptides derived from IgG_2 and IgG_3 are identical and could not be characterized separately. However, because the concentration of IgG_2 is typically higher than that of IgG_3, it is reasonable to assume that the major glycoform contribution for the EEQF^{297}NSTFR glycopeptides is derived from IgG_2.

Because the complex type glycan moieties attached to peptides are potentially labile under ESI conditions, the extracted ion current (EIC) for the protonated *N*-acetyl glucosamine

($GlcNAc^+$, *m/z* 204.1) [35, 42–46] facilitated identification of glycopeptides in LC-MS without enrichment of the sugar containing species (as shown in Figure 1A for one patient sample). In our experience, glycopeptides always show some level of in-source decomposition, which can be roughly estimated from the abundance of sugar oxonium ions, such as *m/z* 204.1, 366.1, relative to that of non-glycopeptide ions. For each IgG subclass, glycopeptide ions containing partial glycan structures, arising from in source decomposition, can be detected in the high *m/z* region (see the discussion of Figure 1B). Their abundance relative to that of non-glycopeptide ions is also indicative of the extent of in-source decay. For the semi-quantitative determination of the IgGs, it is desirable to minimize the extent of in-source decay, and this can be achieved by careful adjustment of the mass spectrometer settings. The cone voltage, defined in the Waters QT of Premier configuration as the potential offset between capillary voltage and the counter electrode, was found to have a dramatic effect on both in-source decomposition and sensitivity. Therefore, its value was adjusted such that good sensitivity for the detection of all glycoforms, and low in-source decomposition were achieved. Although the low abundance of oxonium ions resulted in higher noise in the EIC of *m/z* 204.1, this did not impact negatively the detection of glycopeptides.

Glycopeptides derived from IgG_1 were found to elute first, followed by those derived from IgG_4 and lastly $IgG_{2–3}$ (Figure 1A). Within each subclass, neutral glycopeptides elute earlier than those containing terminal sialic acid. In order to obtain the picture of glycoform distribution for each subclass, the MS scans were averaged over the retention time window in which the glycopeptide ions characteristic of each specific subclass were detected. As an example, Figure 1B shows the averaged MS data obtained for the IgG_1 glycopeptides with the sequence $EEQY^{297}NSTYR$. From these data, the most abundant glycoforms observed are $G_{0–2}F$, followed by those containing bisecting GlcNAc and terminal sialic acid. The glycans lacking the core fucose were detected with lowest abundance. A species arising from in-source decomposition of IgG_1 glycopeptides, assigned to the partial glycan $GlcNAc_3Man_3Fuc$, was detected as the ion *m/z* 1216.063. Ions corresponding to this glycan structure were detected for all IgG subclasses. As described above for the ion of *m/z* 204.1, the abundance of this ion can be minimized through adjustment of source parameters. For the quantitative analyses, the abundances of the observed charge states for each glycoform (+2 and +3 charge state) were summed to determine the total abundance of each glycoform. The relative abundances of each glycoform was then determined relative to the total abundance of all the glycoforms. In several instances the IgG_4 glycopeptides were of low abundance and poor S/N ratio, therefore, their relative glycoform distributions are not presented and further statistical analyses were not performed.

To more easily visualize these data, a bar graph representing the relative abundance of each glycoform of the IgG_1 subclass observed in one plasma sample set is shown in Figure 1C. The bars represent the mean of triplicate LC-MS analyses and the error bars are the result of three technical replicates of each individual sample. Of the most abundant glycoforms observed (G_0F, G_1F, and G_2F), a considerable increase in the relative abundance of the G_0F glycoform in the IgG_1 subclass was observed for the myositis patient compared to either the asymptomatic sibling or the unrelated control. Interestingly, the observed abundance for the G_0F glycoform for the sibling was intermediate between the myositis patient and the unrelated control. A similar trend was observed for the G_1F glycoform, however, the differences in abundance of this glycoform where less among the three samples. For the G_2F glycoform, the asymptomatic sibling had the highest level compared to the patient or control.

To evaluate the entire data set, the relative abundances for each glycoform from the IgG_1 and $IgG_{2–3}$ subclasses were averaged across each sample group (patient, sibling, control). The resulting average for each glycoform in the IgG_1 subclass and $IgG_{2–3}$ subclass is shown

in Figure 2 and Supplemental Table 2. In Figure 2, the bar graphs correspond to the average glycoform relative abundance and the error bars represent the standard deviation across all samples within that specific group (myositis, sibling, or control). The triplicate LC-MS analyses of the relative abundances showed very good reproducibility with an average standard deviation of 0.004 for all the variables analyzed (Supplemental Table 2). From these graphs, one can determine that the glycan profile in patients suffering from myositis tend to lack terminal galactose residues. The relative abundance of the G_0F glycoform in both the IgG_1 and $IgG_{2–3}$ subclasses is elevated relative to the control. This observation correlates with findings reported for the glycan profile of rheumatoid arthritis patients[21]. Furthermore, the asymptomatic siblings exhibit G_0F glycan levels intermediate between the myositis patients and the controls in both subclasses. To ascertain whether the measured differences in the relative abundances of the glycoforms are significant, a statistical evaluation was applied to all variables from the LC/MS analyses.

Statistical analyses of glycosylation

Because decreased levels of galactosylation were first noted in autoimmune patients suffering from rheumatoid arthritis[19], the galactosylation levels (G_0F) in myositis patients was of particular interest. In addition to changes in the individual glycoforms in the IgG_1 and $IgG_{2–3}$ subclasses, changes in specific glycan features were also evaluated. It is known, for example, that fucose and sialic acid are sugars involved in various pathological processes[47,48,49]. To determine whether changes in a specific glycan feature could be observed in myositis patients, the individual glycoforms representing a specific glycan feature were added together. These features were evaluated to account for the total agalactosylation (Gal-0), total bisecting N-acetylglucosamine (GlcNAc), total sialic acid (SA), and total afucosylation (Fuc-0). These summed variables are defined as Gal-0=(G_0F + G_0FB + G_0), bisecting GlcNAc=(G_0FB + G_1FB + G_2FB), SA=(G_1FSA + G_2FSA), and Fuc-0=(G_0 + G_1 + G_2). The relative abundances for each glycoform in the IgG_1 and $IgG_{2–3}$ subclasses as well as the summed glycoforms are shown in Supplemental Table 2.

Statistical analyses of agalactosylation

To determine the statistical significance of the glycosylation changes, pairwise comparisons among the three sample groups (patients, siblings, and controls) were performed using standard mixed effects analysis of variance in SAS 9.1 using the SAS procedure called PROC MIXED. Of the different individual and summed glycoforms analyzed, the variables representing agalactosylation showed significant differences ($P < 0.05$) among the sample groups in the pairwise comparisons (Table 1). In general, higher levels of G_0F were found in myositis patients compared to either siblings or controls (Figure 3). For the G_0F values, highly statistically significant differences ($P < 0.0001$) were observed between the groups of myositis patients and controls for both IgG_1 and $IgG_{2–3}$. The groups of myositis patients and their asymptomatic siblings were also found statistically different with regard to G_0F in the IgG_1 subclass ($P = 0.0098$), whereas the statistical significance for G_0F between these groups in the $IgG_{2–3}$ subclass was borderline significant ($P = 0.0504$). No significant difference ($P > 0.05$) was observed for the IgG_1 G_0F variable between the groups of siblings and unrelated controls, however, the $IgG_{2–3}$ G_0F variable was observed as statistically significant between these two groups ($P = 0.0175$).

To account for the presence of all galactose deficient sugars, the variable Gal-0 = (G_0F + G_0FB + G_0) among the three groups was evaluated. Similarly, the two extreme groups of myositis patients (M) and controls (C) were highly statistically different ($P < 0.0001$) with regard to Gal-0 in both the IgG_1 and the $IgG_{2–3}$ subclasses. Additionally, Gal-0 was found statistically different between the groups of myositis patients (M) and their asymptomatic siblings (S) in both the IgG_1 subclass ($P = 0.0105$) and the $IgG_{2–3}$ subclass ($P = 0.0221$).

Similar to what was observed for G_0F between the siblings (S) and control (C) groups, no significant difference ($P > 0.05$) was observed for Gal-0 in the IgG_1 subclass, but this variable was significantly different in the $IgG_{2–3}$ subclass ($P = 0.0071$). In addition to G_0F, the G_0FB and G_0 variables may contribute to the statistical significance of Gal-0. Of these two variables in either IgG subclass which showed pairwise statistical relevance was the G_0FB variable in IgG_1 subclass (Table 1). These results indicate that the major component of the Gal-0 sum is most likely represented by the G_0F variable.

As an extension of the statistical analyses, because the siblings in this clinical study were asymptomatic, we grouped the siblings and the controls into one “healthy” group and performed a comparison of this group with the patient group. From this analysis, the agalactosylation variables that were observed as significant between either the patients vs siblings or the patients vs controls were found to be highly statistically different between the patient group vs the combined healthy group for both the IgG_1 and $IgG_{2–3}$ subclasses (Table 1).

A previous study demonstrated that IgG Gal-0 can be an useful prognostic indicator to differentiate between early RA and RA [32]. Furthermore, genetic data suggested that unaffected siblings of autoimmune individuals have gene expression signatures that share more similarities with the affected family members than with unrelated controls [16]. As noted earlier in the Experimental Section, the study groups were ordered with the asymptomatic siblings serving as the intermediate group. Given this order, it is reasonable to expect the responses to exhibit a trend over the groups (increasing or decreasing). Hence, we employed statistical trend analysis to determine whether there was a pattern of decreasing IgG Gal-0 among the patient, siblings, and control groups. Trend tests have been employed over the past several decades in areas ranging from toxicology to clinical trials[50–55]. In situations where the explanatory variable is ordinal, as is the present situation with the three groups, trend tests might provide useful interpretation of the data. Often there is a tendency for the mean response to increase (or decrease) gradually over ordered categories and classical pairwise tests cannot always detect such gradual systematic changes. The variables that were determined to be statistically significant among the three sets (Table 1) were interrogated for the existence of a trend using the ORIOGEN methodology described previously[39,40]. The G_2F variable (galactose-containing glycan) was also included in the trend analyses as this variable would be complementary to the agalactosylation variables. The results of the trend analysis are shown in Table 2 and suggest a significant decreasing pattern ($P < 0.001$) of the mean G_0F and Gal-0 responses in the order myositis>sibling>control in both IgG_1 and $IgG_{2–3}$, which remained significant even after adjusting for multiple testing using the Bonferroni correction ($P < 0.002$). A bar graph representation of the trend analysis results of the G_0F variable for IgG_1 and $IgG_{2–3}$ are shown in Figure 3. Although it appears that Gal-0 is largely dominated by G_0F, the second component of Gal-0, G_0FB, also displayed a significant decreasing trend ($P < 0.001$) in the same direction in the IgG_1 data (Table 2). This suggests that, despite the fact that IgG_1 Gal-0 is dominated by G_0F, the term G_0FB contributes significantly to the sum as well. Similar to the pairwise comparisons, the trend analysis of the G_0FB variable was not statistically significant in the $IgG_{2–3}$ subclass data set ($P > 0.05$ without adjustment for multiple testing). The decreasing trends determined for G_0F and Gal-0 in the order myositis>sibling>control are validated by the increasing trend of G_2F in the opposite direction in both IgG_1 and $IgG_{2–3}$ data. The existence of the trend towards increasing agalactosylation of serum IgG in the order control<sibling<myositis is further evidence that IgG Gal-0 may be a useful predictor to assess the susceptibility of an individual to develop an autoimmune condition before the onset of clinical symptoms. Although the healthy siblings have not been monitored to the point when they showed disease symptoms, this finding is consistent with previous studies of SLE and diabetes in

patients and their asymptomatic siblings, showing that the development of autoantibodies in the last group preceded clinical manifestations of the disease.

Statistical analyses of sialylation, bisecting GlcNAc and fucosylation

As described for agalactosylation, similar statistical pairwise and trend analyses were performed for the glycoforms containing terminal sialic acid (SA), bisecting GlcNAc, and for those lacking the core fucose (Fuc-0). In each case, all glycoforms containing the distinctive monosaccharide were analyzed together, regardless of the number of galactose residues. In the statistical analyses, no significant changes were found for any of the above variables in either IgG_1 or $IgG_{2–3}$ subclass.

Statistical analyses of other variables

It is known that physiological variations, such as age and gender, can influence plasma IgG glycosylation[56,57,58]. Additionally, a correlation between the use of prednisone and the levels of IgG_2 galactosylation was found recently for RA patients[19]. Therefore, we performed multiple regression analysis to evaluate the effect of various factors, namely age, diagnosis (DM/PM), medication (Yes/No), and disease activity, on each variable in the IgG_1 and $IgG_{2–3}$ subclasses and found none of the factors to be significant at $P < 0.05$ for any of the variables in the two data sets. Because the statistical power of these analyses is limited by the number of cases in the study, however, these analyses could be more meaningful for a larger number of patients.

The unique design of this study also provides an opportunity to investigate the potential role that genes and the environment have on the glycosylation profile of IgGs. Because one sample set was obtained from monozygotic twins discordant for myositis, the IgG glycosylation pattern of genetically identical individuals could be interrogated. One can thus assume that the discordance for myositis is due to an environmental influence. Although only one sample set was available from monozygotic twins, the results are interesting and, thus, will be mentioned briefly. From the relative abundance of the IgG_1 glycoforms from this sample set, it was determined that the IgG_1 G_0F abundance was higher in the patient vs their asymptomatic sibling (Supplemental Figure 1). These data reflect the same trend observed for the averages of all patients and all siblings (Figure 2A). Although only one sample set from monozygotic twins was available, these IgG glycosylation data may indicate that different serologic phenotypes may be associated with discordance of monozygotic twins. A larger cohort of monozygotic twins, however, would be necessary. This hypothesis is also supported by previous studies of monozygotic twins which revealed a link between the phenotypic differences observed in identical twins discordant for SLE, RA, and DM, and environmental epigenetic events at the level of DNA methylation affecting expression of genes associated with the immune function [14, 59, 60].

Conclusions

Since the initial observation that galactosylation of serum IgG antibodies is decreased in RA patients [21], there has been a great amount of effort devoted to improve the understanding of the implications of the conserved immunoglobulin glycosylation in physiologic and pathologic states. Although the altered IgG glycosylation was initially regarded as a feature related only to RA, it became clear that the decrease in terminal galactosylation is associated not only with RA, but it may represent a shared feature among several autoimmune and non-autoimmune diseases[32].

The present study represents the first investigation of the myositis syndromes with regard to glycosylation of serum IgG, from the perspective of a twin/sibling clinical study.

Characterization of the IgG glycopeptides by LC-MS showed that myositis patients have statistically significant elevated amounts of glycoforms lacking galactose compared to their healthy siblings and unrelated controls, which is consistent with the previous findings involving RA. Statistical significant differences in the glycosylation between patients with dermatomyositis and those with polymyositis were not observed, nor could we find any correlation with disease activity. A potential limitation of this study is the small number of patients analyzed and their heterogeneity within the group, derived from the differential diagnosis and the use of medication. The novelty, however, comes from the group of asymptomatic siblings for which a galactosylation phenotype intermediate between the patients and controls was found. The overall incidence of myositis (0.001% incidence of all syndromes) and the qualification criteria for candidates of this clinical study (i.e. patients must have a healthy twin or sibling of the same sex within 5 years of age) render selection of possible subjects difficult. Despite this fact, statistically significant differences were observed between patients and controls as well as the patients and their asymptomatic siblings for the abundances of the G_0F and Gal-0 glycoforms. In addition, trend analysis indicates an increasing pattern in IgG Gal-0 in the order controls<siblings<patients in both IgG_1 and $IgG_{2–3}$. This result is validated by the significantly decreasing trend of the digalactosylated glycoform G_2F in the reverse order. This observation is consistent with results from previous genetic studies which revealed similarities in the gene expression profiles between autoimmune patients and first degree relatives [16]. Hence, in addition to genome, susceptibility can be defined at the proteome level as well. Because IgG Gal-0 was found elevated in several autoimmune disorders, we believe it represents a more general indicator for the presence of an inflammatory condition rather than a disease-specific marker. The usefulness of IgG Gal-0 as a potential prediction tool of an autoimmune condition needs to be further explored in order to define intervals characteristic for disease, susceptibility and normal levels. This would imply the analysis of a larger cohort of autoimmune patients and their siblings in different age brackets. In the emerging era of individualized medicine, such a test measuring the IgG galactosylation could be routinely employed not only as a complimentary diagnostic tool, but also in preventive care. With the knowledge of a certain serum IgG glycosylation pattern together with the individual's family history, prophylactic strategies can be adopted to prevent onset of clinical symptoms in susceptible individuals. While autoantibodies are usually heterogeneous and characteristic only for a limited number of individuals, determining serum IgG galactosylation may compliment the diagnosis of autoimmune diseases. In conclusion, this study indicates that myositis patients have increased levels of core-fucosylated agalactosyl glycans and that susceptibility in asymptomatic siblings can be identified at the level of serum proteins in the pattern of IgG glycosylation. We believe that the present study of myositis, although performed on a small cohort, sets an important foundation for future studies of this and other autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IIM	idiopathic inflammatory myopathies
DM	dermatomyositis
PM	polymyositis
LC-MS/MS	liquid chromatography tandem mass spectrometry

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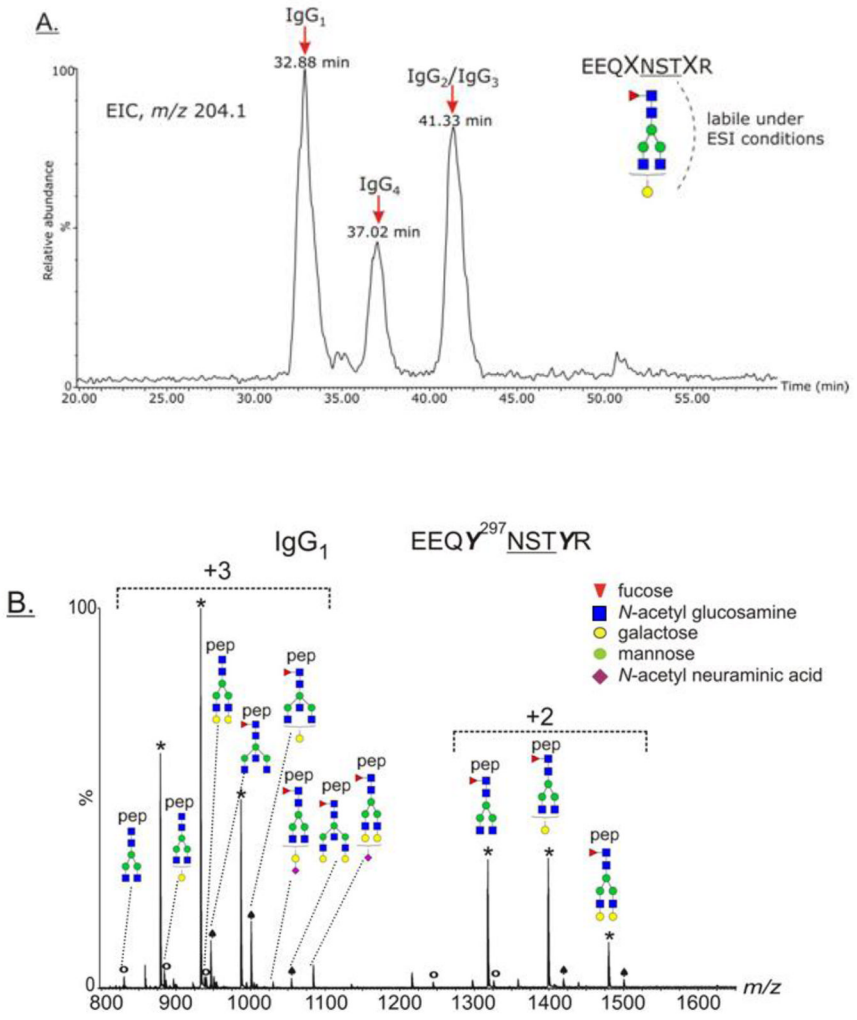

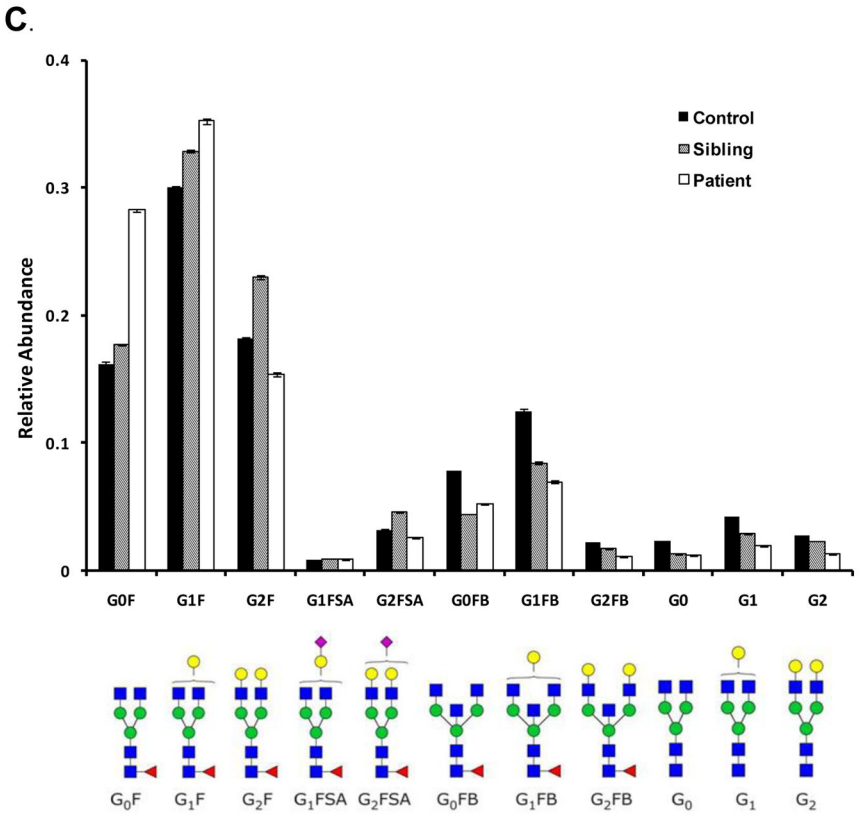

Figure 1.

A) Representative extracted ion current (EIC) of the ion of *m/z* 204.1 over the chromatographic time window 20–60 minutes from the LC-MS analysis of the total IgG heavy chain digest from one myositis patient. These data show the characteristic chromatographic profile of the subclass specific IgG glycopeptides. B) Typical mass spectrum of IgG_1 glycopeptides showing the 2+ and 3+ charge states of the eleven most abundant glycoforms analyzed in the present study. Different charge states of the glycopeptides $G_{0–2}F$ are indicated with a star (*), those of $G_{0–2}FB$ are indicated with a spade (♠) and those of $G_{0–2}$ are indicated with an empty circle (○) The monosaccharide color code is shown at the top right. C) Relative abundances of the IgG_1 glycans from one sample set (Sample Set 5). Solid black bars - controls, hatched bars - siblings, solid white bars - myositis patients. The eleven glycoforms analyzed are indicated at the bottom of the figure. The abundance of each glycoform was determined relative to the total abundance of all glycoforms. The bars represent the mean of triplicate LC-MS analyses and the error bars are the result of three technical replicates of each individual sample.

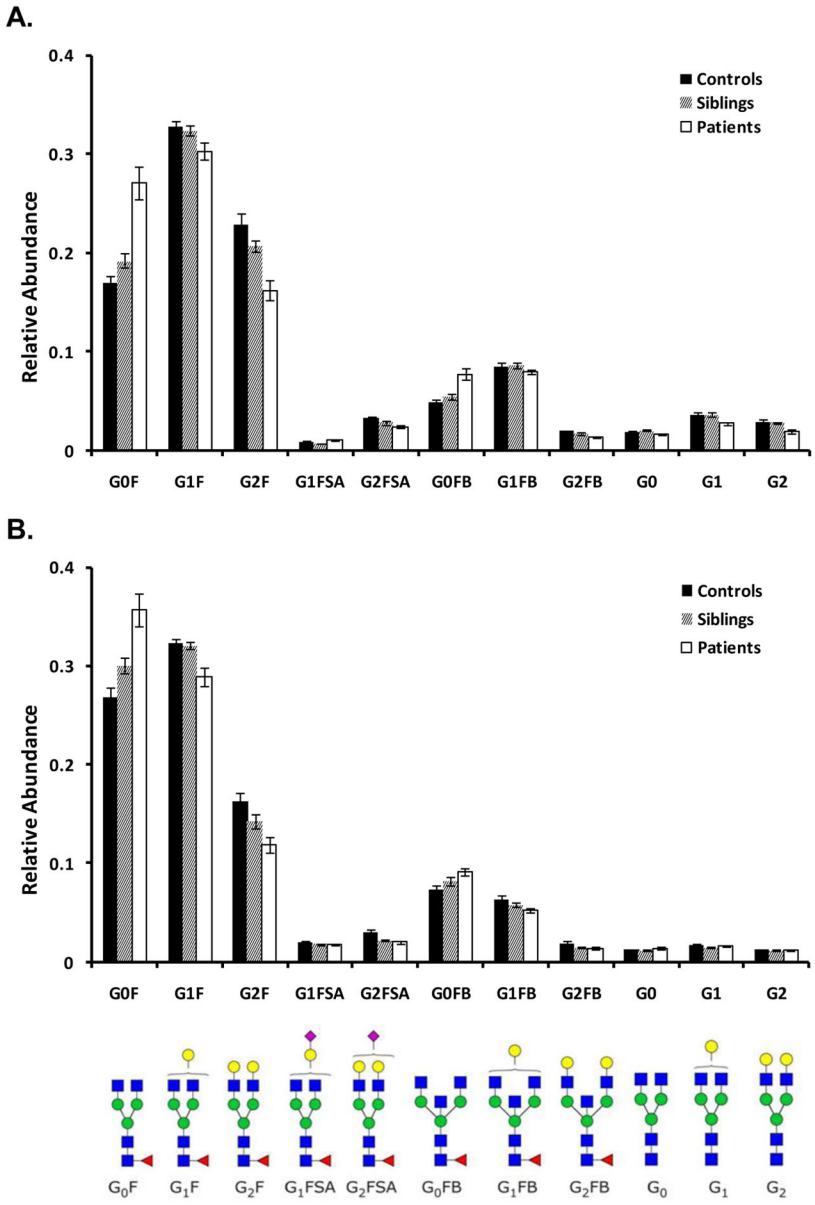

Figure 2.

Mean relative abundance of subclass specific IgG glycosylation profiles. A) IgG_1 subclass and B) IgG_{2-3} subclass. Solid black bars – controls, hatched bars – siblings, solid white bars – myositis patients. The eleven glycoforms analyzed are indicated at the bottom of the figure. The abundance of each glycoform was determined relative to the total abundance of all glycoforms. The bars represent the mean of each sample set (myositis patients, asymptomatic siblings, and controls) and the error bars are the standard deviations of each mean.

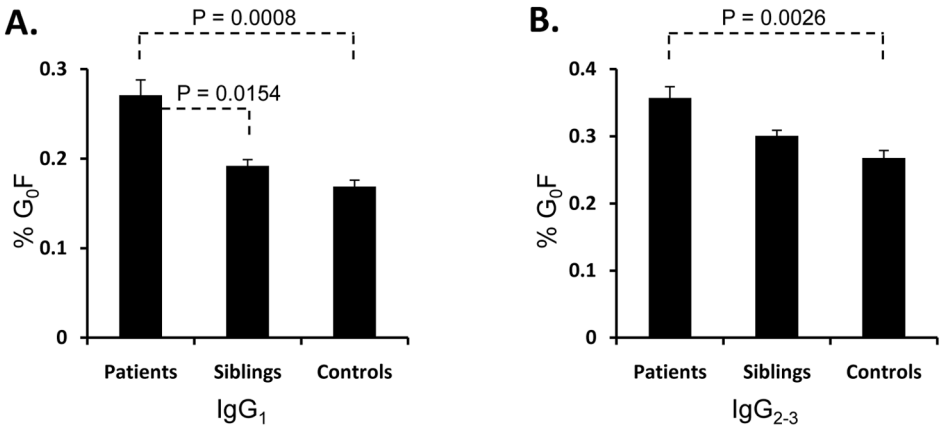

Figure 3.

Mean relative abundance of the percentage of G_0F relative to the total glycoform abundance in the antibody subclasses among the groups of myositis patients, asymptomatic siblings, and unrelated age-matched controls. A) IgG_1 subclass and B) $IgG_{2–3}$ subclass. The P values obtained from the pairwise comparisons of the different groups that were considered statistically significant ($P<0.05$) are indicated at the top of each bar graph.

Table 1

Pairwise comparison of IgG glycosylation between myositis patients, asymptomatic siblings, and unrelated age-matched controls. Statistically significant results are highlighted.

		P value[a]			
Subclass	**Variable[b]**	**Patient vs Control**	**Patient vs Sibling**	**Sibling vs Control**	**Patients vs Controls plus Siblings**
IgG_1	Gal-0	<0.0001	0.0105	0.1241	<0.0001
	G_0F	<0.0001	0.0098	0.1663	<0.0001
	G_0FB	0.0008	0.0256	0.324	0.0034
IgG_{2-3}	Gal-0	<0.0001	0.0221	0.0071	<0.0001
	G_0F	<0.0001	0.0504	0.0175	0.0002

[a] P<0.05 is considered statistically significant

[b] Gal-0=G_0F+G_0FB+G_0

Table 2

Trend analyses of IgG glycosylation between myositis patients, asymptomatic siblings, and unrelated, age-matched controls.

					Mean Relative Abundance		
Subclass	**Variable[a]**	**Trend[b]**	**P value[c]**	**Bonferonni[d] P value**	**Patient**	**Sibling**	**Control**
IgG_1	G_0F	↓	0.0001	0.0016	0.271 ± 0.017	0.192 ± 0.007	0.169 ± 0.007
	Gal-0	↓	0.0001	0.0016	0.364 ± 0.021	0.266 ± 0.009	0.236 ± 0.01
	G_2F	↑	0.0001	0.0016	0.162 ± 0.01	0.207 ± 0.006	0.229 ± 0.011
	G_0FB	↓	0.0001	0.0016	0.077 ± 0.006	0.054 ± 0.003	0.048 ± 0.003
IgG_{2-3}	G_0F	↓	0.0001	0.0016	0.357 ± 0.017	0.301 ± 0.008	0.268 ± 0.011
	Gal-0	↓	0.0001	0.0016	0.462 ± 0.019	0.395 ± 0.011	0.353 ± 0.012
	G_2F	↑	0.0003	0.0048	0.119 ± 0.008	0.143 ± 0.007	0.163 ± 0.008

[a] Gal-0=$G_0F+G_0FB+G_0$

[b] ↓ Decreasing trend in the order Patient>Sibling>Control; ↑ Increasing trend in the order Patient<Sibling<Control

[c] P<0.05 is considered statistically significant

[d] Bonferonni correction factor of 16 was used to adjust for multiple testing