Glycobiology, 2021, vol. 31, no. 9, 1230–1238
https://doi.org/10.1093/glycob/cwab053
Advance Access Publication Date: 14 June 2021
Original Article

Translational Glycobiology

Analysis of site-specific glycan profiles of serum proteins in patients with multiple sclerosis or neuromyelitis optica spectrum disorder—a pilot study

Peng Peng Ip[2,†], Qiongyu Li[4,†], Wei-Han Lin[2,†], Chien-Ching Chang[2], Cathy Shen-Jang Fann[2], Huan-Yuan Chen[2], Fu-Tong Liu[2], Carlito B Lebrilla[1,4], Chih-Chao Yang[1,3], and Fang Liao[1,2]

[2]Institute of Biomedical Sciences, Academia Sinica, No.128, Sec. 2, Academia Road, Taipei 115, Taiwan,
[3]Department of Neurology, National Taiwan University Hospital, No. 7, Chung-Shan S. Road, Taipei 100, Taiwan, and
[4]Department of Chemistry, University of California Davis, One Shields Avenue, Davis, CA 95616, USA

[1]To whom correspondence should be addressed: Tel: +886 226523905; Fax: +886 227829224; e-mail: fl9z@ibms.sinica.edu.tw (F, Liao); Tel: +886 905938950; Fax: +886 227042816; e-mail: jess6627@gmail.com (C.-C., Yang); Tel: +1 530-752-6364; Fax: +1 530-754-5609; e-mail: cblebrilla@ucdavis.edu (C.B., Lebrilla).

[†]These authors contributed equally to this work.

Received 12 October 2020; Revised 7 June 2021; Accepted 8 June 2021

Abstract

Glycosylation is important for biological functions of proteins and greatly affected by diseases. Exploring the glycosylation profile of the protein-specific glycosylation and/or the site-specific glycosylation may help understand disease etiology, differentiate diseases and ultimately develop therapeutics. Patients with multiple sclerosis (MS) and patients with neuromyelitis optica spectrum disorder (NMOSD) are sometimes difficult to differentiate due to the similarity in their clinical symptoms. The disease-related glycosylation profiles of MS and NMOSD have not yet been well studied. Here, we analyzed site-specific glycan profiles of serum proteins of these patients by using a recently developed mass spectrometry technique. A total of 286 glycopeptides from 49 serum glycoproteins were quantified and compared between healthy controls ($n = 6$), remitting MS ($n = 45$) and remitting NMOSD ($n = 23$) patients. Significant differences in the levels of site-specific *N*-glycans on inflammation-associated components [IgM, IgG1, IgG2, complement components 8b (CO8B) and attractin], central nerve system-damage-related serum proteins [apolipoprotein D (APOD), alpha-1-antitrypsin, plasma kallikrein and ADAMTS-like protein 3] were observed among three study groups. We furthered demonstrated that site-specific *N*-glycans on APOD on site 98, CO8B on sites 243 and 553 are potential markers to differentiate MS from NMOSD with an area under receiver operating curve value > 0.75. All these observations indicate that remitting MS or NMOSD patients possess a unique disease-associated glyco-signature in their serum proteins. We conclude that monitoring one's serum protein glycan profile using this high-throughput analysis may provide an additional diagnostic criterion for differentiating diseases, monitoring disease status and estimating response-to-treatment effect.

Key words: multiple sclerosis, neuroinflammation, neuromyelitis optica, serum glycan proteins, site-specific glycan profiles

Introduction

Glycosylation is the most common post-translational modification of proteins, and the decoration of the protein with a variety of glycans leads to the diversity of protein structures. Unlike protein and nucleic acid biosynthesis, glycan synthesis is not template-driven, making the complexity of glycans and leading to difficulty and challenge for glycan analysis (Cummings et al. 2014). Studying the impacts of glycans on biological/physiological functions has been hampered due to the limitation of the technology. Recently, advanced technologies have been developed and applied to the analysis of glycosylation of proteins (Li et al. 2020) and led to understanding the diversity and difference of glycans in certain protein, suggesting site-specific site of glycosylation of the protein plays important roles in physiological and pathological functions (Reily et al. 2019). Given that glycans regulate the structure/conformation, stability and functionality of proteins, glycans are thought to play important roles in the regulation of various biological functions, such as protein–protein interactions (Cummings 2019), leukocyte trafficking (Regal-McDonald et al. 2020), tissue homeostasis (Ohtsubo et al. 2006), pathogen recognition (Greco et al. 2006; Ohtsubo et al. 2006; Thompson et al. 2019), immune cell regulation (Calarese et al. 2003; Nitschke 2014; Dias et al. 2018), cancer cell migration and invasion (Julien et al. 2011; Glavey et al. 2014; Stanczak et al. 2018) and so on. Alterations of glycosylation have been observed in multiple diseases, such as cancers (Pinho et al. 2015; Mereiter et al. 2019; Rodrigues et al. 2021), autoimmune diseases (Lauc et al. 2013; Maverakis et al. 2015; Pagan et al. 2018; Martin et al. 2020) and congenital glycosylation disorders (Medina-Cano et al. 2018; Ng et al. 2018). Thus, understanding the glycosylation profile of a disease status will provide information on the understanding of the disease etiology, the identification of diagnostic and prognostic markers and the development of glycotherapeutics.

Multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD) are relapsing-remitting neuro-inflammatory autoimmune disorders with distinct etiology. MS is the most common demyelinating disease of central nervous system (CNS) caused by autoreactive T cells specific to myelin destroying the myelin sheath of nerve cells (Olsson et al. 1990), whereas NMOSD is a female-predominant disease mostly caused by the autoreactive immunoglobulin (Ig) against aquaporin 4 (AQP-4) expressing on astrocytes (Lennon et al. 2004). Although diagnosis criteria for MS and NMOSD had been established (Wingerchuk et al. 2006; Polman et al. 2011; Lalan et al. 2012), differentiating these diseases might sometimes be problematic as these patients present very similar clinical manifestations and magnetic resonance imaging (MRI) findings. Supportive diagnoses for NMOSD include MRI evidence of longitudinally extensive spinal cord lesion and the presence of serum anti-AQP-4 antibody (Kim et al. 2017). Nevertheless, differential diagnosis for patients with AQP-4 antibody seronegative is quite challenging. Glycan profiles of serum/plasma proteins have been shown to be altered in various diseases, for example, gastric cancer (Kodar et al. 2012), lung cancer (Vasseur et al. 2012), colorectal cancer (Doherty et al. 2018), ulcerative colitis (Miyahara et al. 2013), Type 2 diabetes (Dotz et al. 2018), and inflammatory bowel diseases (Clerc et al. 2018; Dotz et al. 2019). It has been observed that the glycosylation patterns of IgG in the cerebrospinal fluid (CSF) in patients with MS are altered as compared with healthy volunteers and the levels of alteration of glycosylation is occurred shortly after relapse (Wuhrer et al. 2015). However, a comprehensive comparison of glycan profiles of serum proteins between these two diseases has not been studied. As MS and NMOSD with different etiology may display different glycan profiles, we believed that studying the glycosylation in serum proteins may provide novel information to differentiate these two diseases. In this study, we are interested in identifying differential markers from blood sample, which is easy to access, to improve the diagnostic accuracy for MS and NMOSD.

The majority of earlier studies on analyzing the glycosylation of serum proteins were all focused on the analysis of total plasma *N*-glycome (TPNG), in which the profile of total glycans released from plasma proteins was analyzed. The drawback of TPNG analysis is that it fails to reveal the site-specific glycan profile on a given serum/-plasma protein. Of note, the site-specific glycosylation profile on a given serum/plasma protein would be more informative compared with TPNG. To this end, we took advantage of a recently developed dynamic multiple reaction monitoring (dMRM) mass spectrometry technique (Li et al. 2019) to analyze the site-specific glycan profiles of serum glycoproteins from MS and NMOSD patients. To do this, the measurement was performed at defined retention time (RT) of each glycopeptide instead of monitoring throughout the chromatographic run. Moreover, this measurement requires no sample enrichment that may affect the quality of quantification (Li et al. 2019). In this study, we quantified and compared the site-specific glycosylation on serum proteins from healthy controls, patients with remitting MS and patients with remitting NMOSD.

Results

Measurement of protein and glycopeptide quantities with dMRM

The abundance (ion counts) of 286 glycopeptides on 49 serum glycoproteins was measured and analyzed (Supplementary Table SI). A complete list of transition of each glycopeptide and normalizing peptide has been previously published (Supplementary File 1; Li et al. 2019). A list of fragment areas of each glycopeptide and normalizing peptides of each participant and some representative extracted-ion chromatogram spectra are provided in Supplementary File 2 and Supplementary Figure S1, respectively. The protein name, the site of asparagine conjugated with glycan and the glycan composition on that site in each protein are designated as "protein name_glycosylation site_glycan composition" (Supplementary File 2). The composition of a glycan is referred according to $Hexose_AHexNAc_BFucose_CNeu5Ac_D$, and ABCD indicates the number of different monosaccharides (Li et al. 2019). To study the abundance of the glycopeptide on each specific site of a given protein independent of the level of that protein, the abundance of the glycopeptide was normalized to the abundance of the quantitated peptide of that protein (response normalized to peptide, RNP). For the sake of statistical analysis, only those glycopeptides, which were detected in >80% of participants in each group with signal-to-noise (S/N) ratio > 3 (limit of detection, LOD), were statistically analyzed. As a result, a total of 135 glycopeptides (47% of total quantified glycopeptides) were compared among participating groups.

Glycan profiles of participants

The 135 measured glycopeptides, whose ion counts were above the LOD in >80% of participants in each group, were subjected to statistical analyses. Given that the differences of both age and gender of the three study groups (MS vs. NMOSD vs. Healthy controls) are at borderline significances (Table I) and that levels of some

Table I. Participants characteristics

	No. of subjects	Age (median years)	Gender (% female)
Healthy	6	34.5	50
MS	45	33	68.89
MNOSD	23	39	91.3
P-value	–	0.044*	0.049$

*Kruskal–Wallis test. $Chi-Square test.

glycans on serum glycoproteins has been shown affected by age and gender (Merleev et al. 2020), we thus analyzed levels of glycopeptides between three study groups using analysis of covariance (ANCOVA) after controlling age and gender as covariates (Figure 1, Supplementary Table SII). The glycopeptides shown significant differences (*P*-value < 0.05) among three study groups were further analyzed by ANCOVA adjusted with age and gender to determine which two study groups were significantly different (Figure 1, Supplementary Table SII). NMOSD and MS patients shared similar levels of the 5411, 5412 and 5501 glycan on N46 of IgM; however, those glycan levels were significantly lower in healthy donors. These results may suggest that the increased levels of site-specific *N*-glycans of IgM might be associated with neuro-inflammation. Interestingly, the levels of the 4311 glycan on N46 of IgM (IgM_46_4311) in MS and NMOSD patients were not only significantly altered as compared with healthy controls, but also significantly differed between NMOSD and MS patients (NMOSD > MS > Healthy). We speculate that high levels of IgM_46_4311 might be associated with CNS demyelinating autoimmune diseases, which might be more profound in NMOSD patients as compared with MS patients. Furthermore, we observed that site-specific glycans including the 5402 glycan on N271 of Alpha-1-antitrypsin (A1AT_271_5402), the 6610 glycan on N243 of complement component 8B (CO8B_243_6610), the 5400 glycan on N180 of IgG1 (IgG1_180_5400) and the 5401 glycan on 127 of plasma kallikrein (KLKB1_127_5401) were significantly differed only between NMOSD and MS patients but not between either MS patients and healthy donors or NMOSD patients and healthy donors. These results may suggest that these specific glycans on those proteins might potentially differentiate NMOSD and MS.

Prediction models for differentiating MS and NMOSD patients

Because we observed the dissimilarities of glycan levels between NMOSD and MS, we further tested if these altered glycans could be used as potential biomarkers for differentiating NMOSD from MS. To this end, age, gender and eight variables which have a *P*-value below 0.05 in both ANCOVA analysis between three study groups and ANCOVA analysis between MS and NMOSD were selected for logistic regression analysis (Table II). Area under curve (AUC) of the receiver operating characteristic (ROC) analysis (MS vs. NMOSD) of each variable could be increased when age and/or gender were included in ROC analysis (Supplementary Table SIII). The best prediction model with an AUC of 0.913 was constructed using stepwise logistic regression analysis (Table III, Figure 2). This prediction model (model 1) contains APOD_98_6520, IgG1_180_5400 and IgG2_176_4510 with a sensitivity of 88.2% and a specificity of 80.6% (Table III and Figure 2). Of note, variables with the highest AUC in univariate analysis (CO8B_243_6610 and CO8B_553_5402; Table II) were excluded in this model as these variables were significantly correlated with IgG1_180_5400 (Supplementary Table SIV). Therefore, we further performed an age- and gender-adjusted multivariate logistic regression analysis with forced-in variables, which have an AUC values > 0.75 (APOD_98_6520, CO8B_243_6610 and CO8B_553_5402). As CO8B_243_6610 and CO8B_553_5402 were significantly correlated (Supplementary Table SIV), they were allocated into two prediction models (model 2 and model 3; Table IV, Figure 3). As these site-specific glycans showed a high AUC in ROC analyses, we speculate that these site-specific glycans in those serum glycoproteins may be considered as potential indicators for differentiating NMOSD from MS.

Discussion

In this study, by utilizing the dMRM mass spectrometry methods, we quantified the site-specific glycan levels on serum proteins of healthy controls, remitting MS and NMOSD patients. To our best knowledge, this is the first report comparing the site-specific serum glycan profiles of MS and NMOSD patients. Despite the fact that both MS and NMOSD patients were at the remission phase of diseases, we still observed alternations of multiple glycans between any two study groups. Given that glycans play a role in leukocyte migration and cell adhesion which are important for regulating inflammatory autoimmune diseases, site-specific glycan profiles may provide one more layer of information in addition to protein quantities for serving as biomarkers to differentiate these two neuro-inflammatory autoimmune diseases for diagnosis purpose. However, the functions of these altered glycans in the context of neuro-inflammatory autoimmune diseases await further investigation. Of note, factors other than the disease itself, such as gender and age (Knezevic et al. 2010; Catera et al. 2016), ongoing immuno-modulatory treatments (Saldova et al. 2012) and additional diseases presented in these patients may as well contribute to these glycan alternations (Gudelj et al. 2018). Indeed, Merleev et al., has recently published that gender and age may influence the glycan levels of some serum glycopeptides (Merleev et al. 2020). Thus, we compared the levels of serum glycopeptides between groups in consideration of age and gender as covariates. In this pilot study, among serum proteins analyzed for site-specific glycans, we found that site-specific glycan levels were altered in inflammation-inducing determinants (IgG1, IgG2, IgM and CO8B, attractin), and in pathology-related molecules (APOD, A1AT, plasma kallikrein and ADAMTS-like protein 3), suggesting that the site-specific glycan profiles on serum proteins may be potentially used for differentiating NMOSD and MS. Hereby, we speculate that glycans at CO8B (N243_6610 and N553_5402) and APOD (N98_6520), which have a AUC value > 0.75 in univariate logistic regression analysis, and variables in our three prediction models may be considered as potential differential diagnostic markers for distinguishing NMOSD and MS patients.

Previously, studying glycosylations of plasma proteins mainly analyzed TPNG, in which glycans released from all plasma proteins were analyzed and quantified. Although TPNG representing the global levels of glycans released from all plasma proteins may be informative, the analysis of TPNG cannot provide the information on the site-specific glycans of any specific plasma protein. Thus, in order to gain a protein-specific glycan profile, we studied the glycan levels changed at specific sites of any given serum protein using a recently developed dMRM mass spectrometry technique (Li et al. 2019). This may ultimately assist the investigation of the glycan functions of any given specific protein. The advantages of this approach include followings: (1) only a small volume of serum without enrichment is

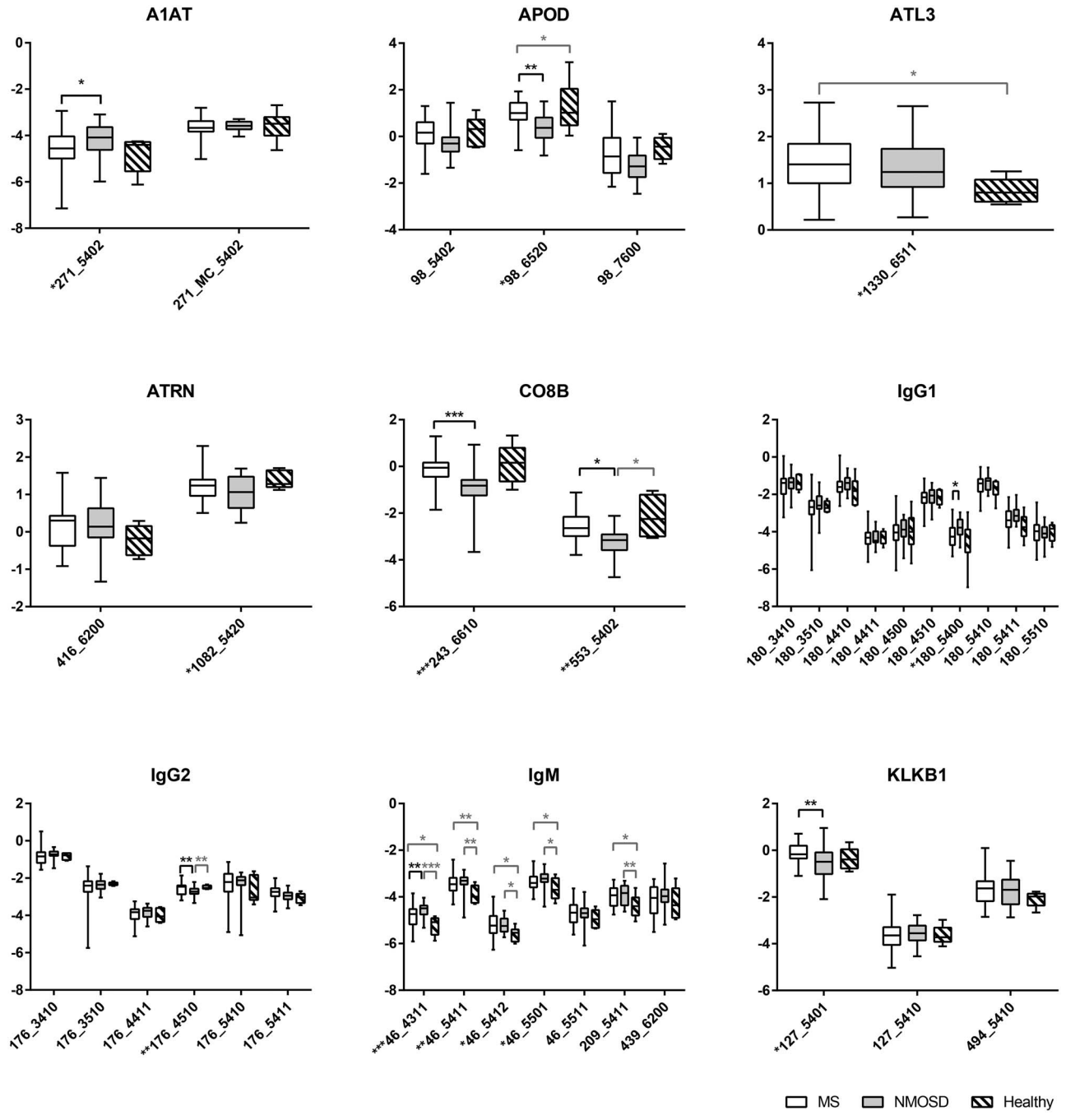


Fig. 1. Glycosylation profiles of serum proteins with significant site-specific glycan alterations between groups. *X*-axis shows the glycosylation site and glycan structure of the serum protein (denoted as glycosylation site_glycan composition). Glycan composition is denoted as the number of different monosaccharides (ABCD) according to $Hexose_AHexNAc_BFucose_CNeu5Ac_D$. *Y*-axis shows the natural log-transformed value of the RNP. Only serum proteins with glycan levels above the limit of detection (S/N > 3) in 80% of samples of each study group are shown. Differences between three groups and differences between any two study-groups were analyzed with ANCOVA test adjusted with age and gender. The glycosylation site labeled with asterisks in the *X*-axis indicates statistical differences among three study groups by ANCOVA test. The asterisk shown in the box-and-whisker plot indicates statistical differences between any two study groups by ANCOVA test. ∗, $P < 0.05$; ∗∗, $P < 0.01$ and ∗∗∗, $P < 0.001$. ANCOVA, analysis of covariance; RNP, response normalized to peptide.

required (2 µl of serum for each run); (2) samples are analyzed with a high throughput method and (3) standardized analytic methods can be used easily for clinical applications. However, this technique is still at its preliminary stage and requires further optimizations, such as methods to increase the detect sensitivity in order to detect relatively low abundant glycopeptides and to obtain better signal-to-noise ratio. Furthermore, it would be more informative if both TPNG and site-specific glycosylation of serum samples are analyzed simultaneously, in which glycan alterations in a global and site-specific manner will be revealed, providing more information on the identification of disease-specific glycan signatures of any given specific serum proteins.

Table II. Logistic regression with variables which have significantly differences among three study groups and between MS and NMOSD patients

Variable	OR (95% CI)	*Z*-value	*P*-value	AUC of ROC
Age	1.077 (1.019–1.146)	2.5051	0.0122	0.686
Gender	4.74 (1.163–32.216)	1.9281	0.0538	0.612
A1AT_271_5402	2.263 (1.098–5.391)	2.0321	0.0422	0.677
APOD_98_6520	0.229 (0.087–0.528)	−3.2350	0.0012	0.763
CO8B_243_6610	0.169 (0.054–0.427)	−3.3973	0.0007	0.805
CO8B_553_5402	0.144 (0.036–0.422)	−3.1401	0.0017	0.785
IgG1_180_5400	4.723 (1.837–14.284)	3.0035	0.0027	0.737
IgG2_176_4510	0.151 (0.024–0.787)	−2.1445	0.0320	0.66
IgM_46_4311	7.55 (1.686–46.945)	2.4173	0.0156	0.702
KLKB1_127_5401	0.193 (0.053–0.566)	−2.7641	0.0057	0.724

Abbreviations: AUC, area under curve; CI, confidence interval; OR, odd ratio; ROC, receiver operating characteristic.

Table III. The best prediction model identified with stepwise logistic regression

Model 1							
	OR (95% CI)	*Z*-value	*P*-value	AUC of ROC	Optimal probability level	Sensitivity	Specificity
APOD_98_6520	0.061 (0.006–0.302)	−2.8426	0.0045	0.9134	0.3045	88.2%	80.6%
IgG1_180_5400	17.187 (3.202–231.97)	2.7356	0.0062				
IgG2_176_4510	<0.001 (<0.001–0.084)	−2.4906	0.0128				

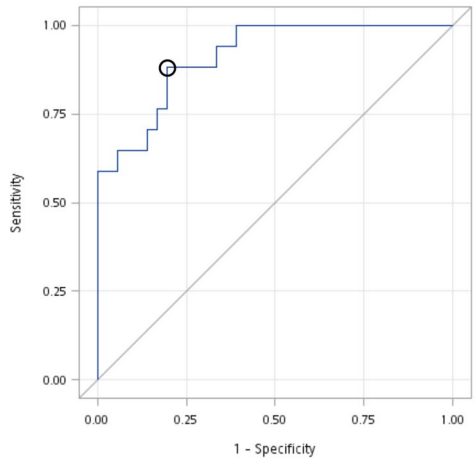
Abbreviations: AUC, area under curve; CI, confidence interval; OR, odd ratio; ROC, receiver operating characteristic.

MS and NMOSD are CNS autoimmune diseases, in which immunoglobulins/antibodies and complements play important roles. Despite that patients were at the remission phase of the disease, we still observed changes in glycan levels on antibodies (IgM, IgG1 and IgG2) and component of the complement system (CO8B) between any two study groups. The Fab portion of antibodies recognizes specific antigens while the Fc portion of antibodies determines the effector functions of antibodies via interaction with specific Fc receptors. It has been reported that glycans covalently attached to Fc of antibodies significantly affect the conformation/structure of antibodies interacting with Fc receptors or complements, thus affecting effector functions of antibodies (Yamaguchi et al. 2020). There are five N-glycosylation sites (N46, N209, N272, N279 and N439) on IgM; glycans at N46, N209 and N272 are complex glycan while N279 and N439 are high-mannose glycans (Chandler et al. 2019). We found that, of interest, the site-specific *N*-glycan of IgM that showed a significant difference between NMOSD and MS is located at N46, a site with complex glycans. A study showed that the sialylation of IgM μ chain results in suppression of inflammation by inhibiting T-cell activation (Colucci et al. 2015). The increased glycan levels at N46 of IgM (IgM_46_4311, 5411, 5412 and 5501) in NMOSD and MS patients compared with healthy controls, were all sialylated, suggesting that patients may be under immuno-suppressive responses, which may be partly due to the effect of the immuno-modulatory treatments.

The treatment of NMOSD patients focuses on suppressing antibody responses as NMOSD is caused by self-reactive anti-AQP-4 antibody (Kimbrough et al. 2012). The majority of pathogenic anti-AQP-4 antibodies belongs to the IgG1 subtype (Reiber 1998; Hinson et al. 2007; Kalluri et al. 2010). IgG1 carries only one glycosylation site at N180 in the Fc region and the *N*-glycan of IgG1 is structurally important for its stability and effector functions. For example, fucosylation at the IgG glycan core structure reduces the IgG binding affinity to FcγRIIIA and FcγRIIIB, which results in reduced antibody-dependent cellular cytotoxicity (ADCC) (Shinkawa et al. 2003; Nimmerjahn et al. 2008); increased sialylation of IgG promotes anti-inflammatory effect due to the reduced binding affinity of sialylated IgG to activating FcγRs (Kaneko et al. 2006; Seeling et al. 2017); the agalactosylated IgG activates the complement system and, as expected, an increase of agalactosylated IgG glycans has been associated with inflammatory diseases (Gudelj et al. 2018). Intriguingly, we observed an alteration in the levels of proinflammatory nonsialylated glycans (5400) on IgG1 of NMOSD patients. It is plausible that the exact function of the IgG1-linked glycans may depend on the overall glycan structures and the specificity of the antigen recognition. To understand the potential contribution of these IgG1 glycan alterations to NMOSD, it would be worth studying the glycan functions and the antigen-specificity of those IgG1.

The activation of the complement system has been shown to play a role in the pathology of MS and NMOSD (Tatomir et al. 2017). In NMOSD patients, it has also been reported that the destruction of AQP-4-expressing astrocytes by AQP-4-IgG1 is a complement-dependent response (Liu et al. 2017). Several complement proteins such as clusterin have been shown to be increased in MS plaques and may be potential biomarkers (Ingram et al. 2014). Here, we observed that the levels of site-specific glycans on CO8B showed differences between MS and NMOSD patients. CO8B is one of the components of the membrane attack complex (MAC) and glycosylation of CO8 may play a role in MAC formation (Franc et al. 2018). The effect of site-specific glycans on complement components in MS and NMOSD warrants further investigation.

Both MS and NMOSD patients exhibit neuronal damages and the critical repair may take place after the relapse phase of the diseases. The increased oxidative stress and serum lipid peroxidation, which may damage oligodendrocytes, have been observed in NMOSD patients (Penton-Rol et al. 2009; Shu et al. 2017). The



Model 1
Variables: APOD_98_6520, IgG1_180_5400 and IgG2_176_4510

AUC	0.9134
Sensitivity	88.2%
Specificity	80.6%
Optimal probability level	0.3045

Fig. 2. Prediction diagnostic model for differentiating NMOSD and MS patients constructed with stepwise logistic regression (model 1). Eight variables showing significant differences between three study groups and between NMOSD and MS patients, age and gender were included in a stepwise logistic regression analysis. The resulting best model contains APOD_98_6520, IgG1_180_5400 and IgG2_176_4510. Circle on the plot shows the optimal cut-off point of the ROC curve, which was selected based on Youden index analysis. AUC, area under curve; MS, multiple sclerosis; NMOSD, neuromyelitis optic spectrum disorder; ROC, receiver operating characteristic. (This figure is available in black and white in print and in color at Glycobiol).

serum protein, APOD, which physically associates with high-density lipoprotein (HDL) fractions (Rassart et al. 2000), is also expressed in the central and peripheral nervous systems to protect brains from lipid peroxidation (Provost et al. 1991; Seguin et al. 1995). Of note, APOD located in the brain is less glycosylated compared with serum APOD (Li et al. 2016). We found that NMOSD patients have decreased glycosylation levels in APOD compared with MS patients as well as healthy controls. There are two N-glycosylation sites on APOD at N65 and N98 (Schindler et al. 1995). The altered glycan at N98 of APOD may have some physiological impacts on NMOSD, likely acting on brain lesions to reduce oxidative stress.

In this pilot study, using an advanced mass spectrometry technique, we have investigated the site-specific glycan profiles of serum proteins in MS, NMOSD and healthy controls. We have demonstrated that site-specific glycan alterations are found in many serum proteins in MS and NMOSD patients compared with healthy controls; however, the biological functions of the altered site-specific glycans on serum proteins have been rarely studied and is worthy of further investigation. Our study has also shown the differences of site-specific glycans on some serum proteins between MS and NMOSD. The differential glycan profiles might serve as a building block for new diagnostic biomarkers in MS and NMOSD. In the future, the site-specific glycan profiles from larger study cohorts will be required for building up a glycan library of these diseases.

Materials and methods

Participants

Serum samples from a previously described study cohort, which contained 45 MS patients and 23 NMOSD patients, were analyzed in this study (Ip et al. 2018). MS and NMOSD were identified in accordance with McDonald's criteria (Polman et al. 2005) and Wingerchuk's criteria (Wingerchuk et al. 2006), respectively. In brief, all patient participants were at the remission phase of diseases and most of them were under immuno-modulating therapies. The general characteristics of the participating patients were summarized in the previous study (Ip et al. 2018). Serum samples from six healthy adults were served as controls. All participants had agreed and signed a written informed consent form prior to participation. This study protocol was approved by the Ethic Committee of the National Taiwan University Hospital, Taipei, Taiwan (201308022RINB) and Academia Sinica, Taipei, Taiwan (AS-IRB-BM13070).

Mass spectrometry and data analysis

The detail procedures for glycoproteomics and glycopeptides quantitation were previously described (Li et al. 2019). In brief, a 2-μL aliquot of each sample was transferred to a 96-well plate. Samples were firstly reduced with 2 μL of 450 mM dithiothreitol (DTT) at 60°C for 50 min followed by the alkylation with 2 μL of 550 mM iodoacetamide (IAA) at room temperature in the dark for 30 min. Another 2 μL of DTT were added and mixed with samples for 1 min to quench the alkylation. The reduced serum samples then were subjected to trypsin digestion by adding 4 μg of trypsin was added to each sample and incubated in a water bath at 37°C for 18 h. Digested samples were acidified with formic acid (FA) to a final concentration of 1% and then subjected to liquid chromatography–mass spectrometry (LC–MS) for peptide separation and quantitation. Tryptic digested serum samples were separated with an Agilent 1290 infinity ultra-high-pressure liquid chromatography (UHPLC) system, using an Agilent Eclipse plus C18 (RRHD 1.8 μm, 2.1 mm × 150 mm) column coupled to an Agilent Eclipse plus C18 (RRHD 1.8 μm, 2.1 mm × 5 mm) guard column. A 70-min binary gradient was used for separation, with solvent A of water containing 3% of acetonitrile (ACN), 0.1% of FA and solvent B containing 90% of ACN and 0.1% of FA. Mass spectrometry analysis was conducted on an Agilent 6495 triple quadrupole (QqQ) mass spectrometer and the scan mode of the instrument used was dMRM. The delta retention time (ΔR) of peptides were 1 min and that of glycopeptides varied from 1.5 to 2 min. The cycle time is 500 ms and 15 points were collected for each transition. The resolution of the Agilent QqQ system was set as 0.7 u at full width half maximum (FWHM). RT of each glycopeptide was predicted based on the hydrophobicities of its peptide backbone and mapped by analyzing the RTs of synthesized peptide standards. The dMRM data were analyzed using Agilent MassHunter Quantitative Analysis B.5.0 software. The representative MS/MS spectra of Hemopexin glycopeptides (Hemo_187_5402 and Hemo_187_5421) and IGg1 glycopeptides (IgG1_3410 and IgG1_4410) identified with Thermo Q Exactive-Orbitrap Mass spectrometer are provided (Supplementary Figures S2 and S3). The RT on the LC, fragmentation and the annotated fragments are shown on the spectra.

Statistical analysis

Data were natural log transformed to remove skewness and the normality of each variable was examined by the use of Kolmogorov–Smirnov test. Only normally distributed variables were tested with

Table IV. Prediction models with forced-in variables

Model 2							
	OR (95% CI)	*Z*-value	*P*-value	AUC of ROC	Optimal probability level	Sensitivity	Specificity
APOD_98_6520	0.346 (0.105–1.011)	−1.8668	0.0619	0.8667	0.5022	69.6%	93.3%
CO8B_243_6610	0.163 (0.041–0.49)	−2.8480	0.0044				
Age	1.034 (0.966–1.114)	0.9326	0.3505				
Gender	3.609 (0.477–41.875)	1.1602	0.2460				
Model 3							
	OR (95% CI)	*Z*-value	*P*-value	AUC of ROC	Optimal probability level	Sensitivity	Specificity
APOD_98_6520	0.348 (0.104–1.019)	−1.8432	0.0653	0.8500	0.4527	80.0%	90.2%
CO8B_553_5402	0.233 (0.053–0.742)	−2.2170	0.0266				
Age	1.044 (0.971–1.131)	1.1440	0.2524				
Gender	2.572 (0.279–58.712)	0.7548	0.4504				

Abbreviations: AUC, area under curve; CI, confidence interval; OR, odd ratio; ROC, receiver operating characteristic.

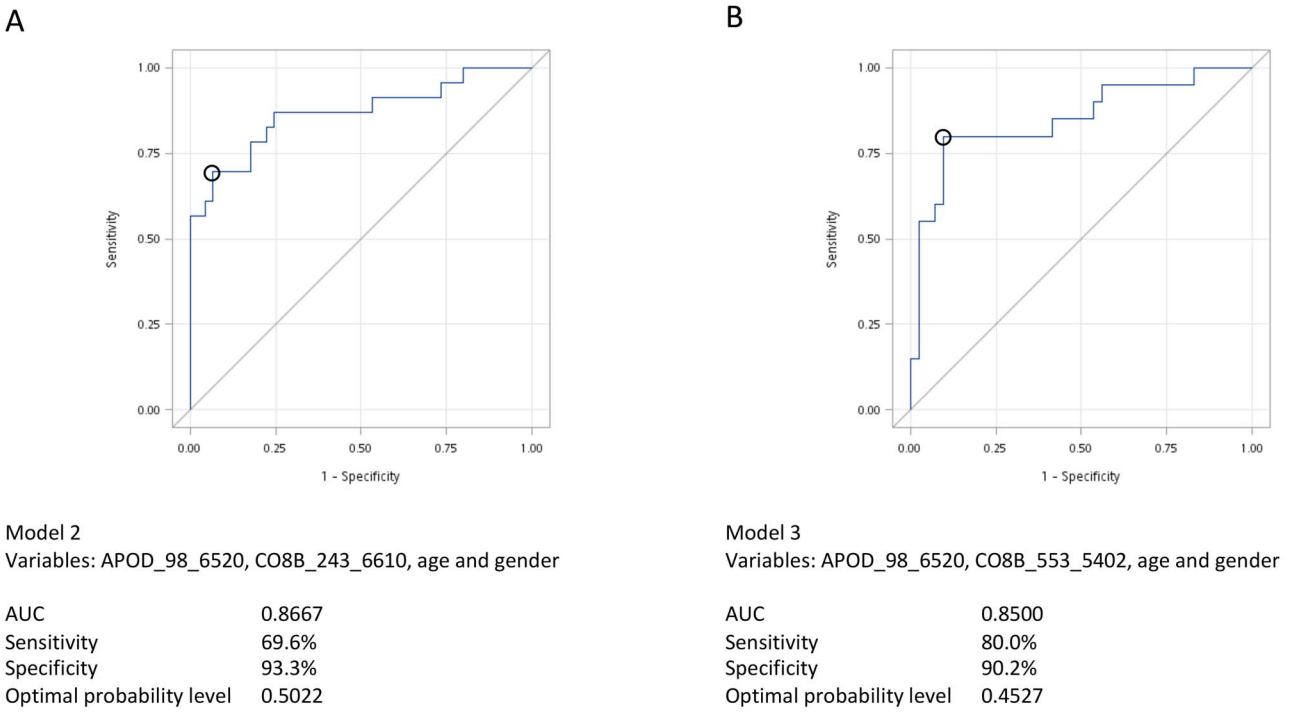


Fig. 3. Prediction diagnostic models for differentiating NMOSD and MS patients constructed with forced-in variables. **A** Variables in model 2 are APOD_98_6520, CO8B_243_6610, age and gender. **B** Variables in model 3 are APOD_98_6520, CO8B_553_5402, age and gender. Circle on the plot shows the optimal threshold point of the ROC curve selected based on Youden index analysis. AUC, area under curve; MS, multiple sclerosis; NMOSD, neuromyelitis optic spectrum disorder; ROC, receiver operating characteristic. (This figure is available in black and white in print and in color at Glycobiol).

ANCOVA. ANCOVA controlled with age and gender as covariates was used to measure differences among three study groups (MS vs. NMOSD vs. healthy controls) and differences between two study groups (MS vs. NMOSD, MS vs. healthy controls and NMOSD vs. healthy controls). Correlations between variables were measured by Pearson correlation coefficient. Disease predicted value of differentially expressed glycans was tested with logistic regression and receiver operating curve (ROC). Prediction models were calculated with stepwise logistic regression or with forced-in multivariate logistic regression. The cut-off value having the largest Youden index (sensitivity-specificity-1) was selected as the optimal cut-off of the ROC analysis. Statistical analysis was performed using SAS software (v.9.4) and Graphpad Prism software. The *P*-value <0.05 was considered significant different.

Supplementary data

Supplementary data are available at *Glycobiology* online.

Acknowledgements

We thank Mr. Shang-Jung Wu (Institute of Biomedical Sciences, Academia Sinica, Taiwan) for statistical advices. Ms. Yu-Fang Lee (Department of

Neurology, National Taiwan University Hospital, Taiwan) and Dr. Chen-Yen Chung (Institute of Biomedical Sciences, Academia Sinica, Taiwan) for sample collections and preparations.

Funding

This work was supported by Institute of Biomedical Sciences at Academia Sinica, Taiwan (grant number IBMS-CRC101-P01).

Conflict of interest statement

None declared.

References

Calarese DA, Scanlan CN, Zwick MB, Deechongkit S, Mimura Y, Kunert R, Zhu P, Wormald MR, Stanfield RL, Roux KH, et al. 2003. Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. *Science*. 300:2065–2071.

Catera M, Borelli V, Malagolini N, Chiricolo M, Venturi G, Reis CA, Osorio H, Abruzzo PM, Capri M, Monti D, et al. 2016. Identification of novel plasma glycosylation-associated markers of aging. *Oncotarget*. 7:7455–7468.

Chandler KB, Mehta N, Leon DR, Suscovich TJ, Alter G, Costello CE. 2019. Multi-isotype glycoproteomic characterization of serum antibody heavy chains reveals isotype- and subclass-specific N-glycosylation profiles. *Mol Cell Proteomics*. 18:686–703.

Clerc F, Novokmet M, Dotz V, Reiding KR, de Haan N, Kammeijer GSM, Dalebout H, Bladergroen MR, Vukovic F, Rapp E, et al. 2018. Plasma N-glycan signatures are associated with features of inflammatory bowel diseases. *Gastroenterology*. 155:829–843.

Colucci M, Stockmann H, Butera A, Masotti A, Baldassarre A, Giorda E, Petrini S, Rudd PM, Sitia R, Emma F, et al. 2015. Sialylation of N-linked glycans influences the immunomodulatory effects of IgM on T cells. *J Immunol*. 194:151–157.

Cummings RD. 2019. Stuck on sugars – how carbohydrates regulate cell adhesion, recognition, and signaling. *Glycoconj J*. 36:241–257.

Cummings RD, Pierce JM. 2014. The challenge and promise of glycomics. *Chem Biol*. 21:1–15.

Dias AM, Correia A, Pereira MS, Almeida CR, Alves I, Pinto V, Catarino TA, Mendes N, Leander M, Oliva-Teles MT, et al. 2018. Metabolic control of T cell immune response through glycans in inflammatory bowel disease. *Proc Natl Acad Sci U S A*. 115:E4651–E4660.

Doherty M, Theodoratou E, Walsh I, Adamczyk B, Stockmann H, Agakov F, Timofeeva M, Trbojevic-Akmacic I, Vuckovic F, Duffy F, et al. 2018. Plasma N-glycans in colorectal cancer risk. *Sci Rep*. 8:8655.

Dotz V, Lemmers RFH, Reiding KR, Hipgrave Ederveen AL, Lieverse AG, Mulder MT, Sijbrands EJG, Wuhrer M, van Hoek M. 2018. Plasma protein N-glycan signatures of type 2 diabetes. *Biochim Biophys Acta Gen Subj*. 1862:2613–2622.

Dotz V, Wuhrer M. 2019. N-glycome signatures in human plasma: Associations with physiology and major diseases. *FEBS Lett*. 593:2966–2976.

Franc V, Zhu J, Heck AJR. 2018. Comprehensive proteoform characterization of plasma complement component c8alphabetagamma by hybrid mass spectrometry approaches. *J Am Soc Mass Spectrom*. 29:1099–1110.

Glavey SV, Manier S, Natoni A, Sacco A, Moschetta M, Reagan MR, Murillo LS, Sahin I, Wu P, Mishima Y, et al. 2014. The sialyltransferase ST3GAL6 influences homing and survival in multiple myeloma. *Blood*. 124:1765–1776.

Greco A, Ho JG, Lin SJ, Palcic MM, Rupnik M, Ng KK. 2006. Carbohydrate recognition by *Clostridium difficile* toxin A. *Nat Struct Mol Biol*. 13:460–461.

Gudelj I, Lauc G, Pezer M. 2018. Immunoglobulin G glycosylation in aging and diseases. *Cell Immunol*. 333:65–79.

Hinson SR, Pittock SJ, Lucchinetti CF, Roemer SF, Fryer JP, Kryzer TJ, Lennon VA. 2007. Pathogenic potential of IgG binding to water channel extracellular domain in neuromyelitis optica. *Neurology*. 69:2221–2231.

Ingram G, Loveless S, Howell OW, Hakobyan S, Dancey B, Harris CL, Robertson NP, Neal JW, Morgan BP. 2014. Complement activation in multiple sclerosis plaques: An immunohistochemical analysis. *Acta Neuropathol Commun*. 2:53.

Ip PP, Chung CY, Chang CC, Lee YF, Wang HM, Lian IB, Fann CS, Yang CC, Liao F. 2018. Differentiation of remitting neuromyelitis optica spectrum disorders from multiple sclerosis by integrating parameters from serum proteins and lymphocyte subsets. *J Neuroimmunol*. 318:45–52.

Julien S, Ivetic A, Grigoriadis A, QiZe D, Burford B, Sproviero D, Picco G, Gillett C, Papp SL, Schaffer L, et al. 2011. Selectin ligand sialyl-Lewis x antigen drives metastasis of hormone-dependent breast cancers. *Cancer Res*. 71:7683–7693.

Kalluri SR, Illes Z, Srivastava R, Cree B, Menge T, Bennett JL, Berthele A, Hemmer B. 2010. Quantification and functional characterization of antibodies to native aquaporin 4 in neuromyelitis optica. *Arch Neurol*. 67:1201–1208.

Kaneko Y, Nimmerjahn F, Ravetch JV. 2006. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science*. 313:670–673.

Kim SM, Kim SJ, Lee HJ, Kuroda H, Palace J, Fujihara K. 2017. Differential diagnosis of neuromyelitis optica spectrum disorders. *Ther Adv Neurol Disord*. 10:265–289.

Kimbrough DJ, Fujihara K, Jacob A, Lana-Peixoto MA, Leite MI, Levy M, Marignier R, Nakashima I, Palace J, de Seze J, et al. 2012. Treatment of neuromyelitis optica: Review and recommendations. *Mult Scler Relat Disord*. 1:180–187.

Knezevic A, Gornik O, Polasek O, Pucic M, Redzic I, Novokmet M, Rudd PM, Wright AF, Campbell H, Rudan I, et al. 2010. Effects of aging, body mass index, plasma lipid profiles, and smoking on human plasma N-glycans. *Glycobiology*. 20:959–969.

Kodar K, Stadlmann J, Klaamas K, Sergeyev B, Kurtenkov O. 2012. Immunoglobulin G Fc N-glycan profiling in patients with gastric cancer by LC-ESI-MS: Relation to tumor progression and survival. *Glycoconj J*. 29:57–66.

Lalan S, Khan M, Schlakman B, Penman A, Gatlin J, Herndon R. 2012. Differentiation of neuromyelitis optica from multiple sclerosis on spinal magnetic resonance imaging. *Int J MS Care*. 14:209–214.

Lauc G, Huffman JE, Pucic M, Zgaga L, Adamczyk B, Muzinic A, Novokmet M, Polasek O, Gornik O, Kristic J, et al. 2013. Loci associated with N-glycosylation of human immunoglobulin G show pleiotropy with autoimmune diseases and haematological cancers. *PLoS Genet*. 9:e1003225.

Lennon VA, Wingerchuk DM, Kryzer TJ, Pittock SJ, Lucchinetti CF, Fujihara K, Nakashima I, Weinshenker BG. 2004. A serum autoantibody marker of neuromyelitis optica: Distinction from multiple sclerosis. *Lancet*. 364:2106–2112.

Li H, Ruberu K, Karl T, Garner B. 2016. Cerebral apolipoprotein-D is hypoglycosylated compared to peripheral tissues and is variably expressed in mouse and human brain regions. *PLoS One*. 11:e0148238.

Li Q, Kailemia MJ, Merleev AA, Xu G, Serie D, Danan LM, Haj FG, Maverakis E, Lebrilla CB. 2019. Site-specific glycosylation quantitation of 50 serum glycoproteins enhanced by predictive glycopeptidomics for improved disease biomarker discovery. *Anal Chem*. 91: 5433–5445.

Li Q, Xie Y, Wong M, Barboza M, Lebrilla CB. 2020. Comprehensive structural glycomic characterization of the glycocalyxes of cells and tissues. *Nat Protoc*. 15:2668–2704.

Liu Y, Given KS, Harlow DE, Matschulat AM, Macklin WB, Bennett JL, Owens GP. 2017. Myelin-specific multiple sclerosis antibodies cause complement-dependent oligodendrocyte loss and demyelination. *Acta Neuropathol Commun*. 5:25.

Martin TC, Simurina M, Zabczynska M, Martinic Kavur M, Rydlewska M, Pezer M, Kozlowska K, Burri A, Vilaj M, Turek-Jabrocka R, et al. 2020. Decreased immunoglobulin G core fucosylation, a player in antibody-dependent cell-mediated cytotoxicity, is associated with autoimmune thyroid diseases. *Mol Cell Proteomics*. 19:774–792.

Maverakis E, Kim K, Shimoda M, Gershwin ME, Patel F, Wilken R, Raychaudhuri S, Ruhaak LR, Lebrilla CB. 2015. Glycans in the immune system and the altered glycan theory of autoimmunity: A critical review. *J Autoimmun*. 57:1–13.

Medina-Cano D, Ucuncu E, Nguyen LS, Nicouleau M, Lipecka J, Bizot JC, Thiel C, Foulquier F, Lefort N, Faivre-Sarrailh C, et al. 2018. High N-glycan multiplicity is critical for neuronal adhesion and sensitizes the developing cerebellum to N-glycosylation defect. *Elife*. 7:e38309.

Mereiter S, Balmana M, Campos D, Gomes J, Reis CA. 2019. Glycosylation in the era of cancer-targeted therapy: Where are we heading? *Cancer Cell*. 36:6–16.

Merleev AA, Park D, Xie Y, Kailemia MJ, Xu G, Ruhaak LR, Kim K, Hong Q, Li Q, Patel F, et al. 2020. A site-specific map of the human plasma glycome and its age and gender-associated alterations. *Sci Rep*. 10:17505.

Miyahara K, Nouso K, Saito S, Hiraoka S, Harada K, Takahashi S, Morimoto Y, Kobayashi S, Ikeda F, Miyake Y, et al. 2013. Serum glycan markers for evaluation of disease activity and prediction of clinical course in patients with ulcerative colitis. *PLoS One*. 8:e74861.

Ng BG, Freeze HH. 2018. Perspectives on glycosylation and its congenital disorders. *Trends Genet*. 34:466–476.

Nimmerjahn F, Ravetch JV. 2008. Anti-inflammatory actions of intravenous immunoglobulin. *Annu Rev Immunol*. 26:513–533.

Nitschke L. 2014. CD22 and Siglec-G regulate inhibition of B-cell signaling by sialic acid ligand binding and control B-cell tolerance. *Glycobiology*. 24:807–817.

Ohtsubo K, Marth JD. 2006. Glycosylation in cellular mechanisms of health and disease. *Cell*. 126:855–867.

Olsson T, Zhi WW, Hojeberg B, Kostulas V, Jiang YP, Anderson G, Ekre HP, Link H. 1990. Autoreactive T lymphocytes in multiple sclerosis determined by antigen-induced secretion of interferon-gamma. *J Clin Invest*. 86:981–985.

Pagan JD, Kitaoka M, Anthony RM. 2018. Engineered sialylation of pathogenic antibodies in vivo attenuates autoimmune disease. *Cell*. 172:564, e513–577.

Penton-Rol G, Cervantes-Llanos M, Martinez-Sanchez G, Cabrera-Gomez JA, Valenzuela-Silva CM, Ramirez-Nunez O, Casanova-Orta M, Robinson-Agramonte MA, Lopategui-Cabezas I, Lopez-Saura PA. 2009. TNF-alpha and IL-10 downregulation and marked oxidative stress in Neuromyelitis Optica. *J Inflamm (Lond)*. 6:18.

Pinho SS, Reis CA. 2015. Glycosylation in cancer: Mechanisms and clinical implications. *Nat Rev Cancer*. 15:540–555.

Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, Fujihara K, Havrdova E, Hutchinson M, Kappos L, et al. 2011. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol*. 69:292–302.

Polman CH, Reingold SC, Edan G, Filippi M, Hartung HP, Kappos L, Lublin FD, Metz LM, McFarland HF, O'Connor PW, et al. 2005. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol*. 58:840–846.

Provost PR, Villeneuve L, Weech PK, Milne RW, Marcel YL, Rassart E. 1991. Localization of the major sites of rabbit apolipoprotein D gene transcription by in situ hybridization. *J Lipid Res*. 32:1959–1970.

Rassart E, Bedirian A, Do Carmo S, Guinard O, Sirois J, Terrisse L, Milne R. 2000. Apolipoprotein D. *Biochim Biophys Acta*. 1482:185–198.

Regal-McDonald K, Patel RP. 2020. Selective recruitment of monocyte subsets by endothelial N-glycans. *Am J Pathol*. 190:947–957.

Reiber H. 1998. Cerebrospinal fluid–physiology, analysis and interpretation of protein patterns for diagnosis of neurological diseases. *Mult Scler*. 4:99–107.

Reily C, Stewart TJ, Renfrow MB, Novak J. 2019. Glycosylation in health and disease. *Nat Rev Nephrol*. 15:346–366.

Rodrigues JG, Duarte HO, Reis CA, Gomes J. 2021. Aberrant protein glycosylation in cancer: Implications in targeted therapy. *Biochem Soc Trans*. 49:843–854.

Saldova R, Huffman JE, Adamczyk B, Muzinic A, Kattla JJ, Pucic M, Novokmet M, Abrahams JL, Hayward C, Rudan I, et al. 2012. Association of medication with the human plasma N-glycome. *J Proteome Res*. 11:1821–1831.

Schindler PA, Settineri CA, Collet X, Fielding CJ, Burlingame AL. 1995. Site-specific detection and structural characterization of the glycosylation of human plasma proteins lecithin:cholesterol acyltransferase and apolipoprotein D using HPLC/electrospray mass spectrometry and sequential glycosidase digestion. *Protein Sci*. 4:791–803.

Seeling M, Bruckner C, Nimmerjahn F. 2017. Differential antibody glycosylation in autoimmunity: Sweet biomarker or modulator of disease activity? *Nat Rev Rheumatol*. 13:621–630.

Seguin D, Desforges M, Rassart E. 1995. Molecular characterization and differential mRNA tissue distribution of mouse apolipoprotein D. *Brain Res Mol Brain Res*. 30:242–250.

Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, Sakurada M, Uchida K, Anazawa H, Satoh M, Yamasaki M, et al. 2003. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem*. 278:3466–3473.

Shu Y, Li R, Qiu W, Chang Y, Sun X, Fang L, Chen C, Yang Y, Lu Z, Hu X, et al. 2017. Association of serum gamma-glutamyltransferase and C-reactive proteins with neuromyelitis optica and multiple sclerosis. *Mult Scler Relat Disord*. 18:65–70.

Stanczak MA, Siddiqui SS, Trefny MP, Thommen DS, Boligan KF, von Gunten S, Tzankov A, Tietze L, Lardinois D, Heinzelmann-Schwarz V, et al. 2018. Self-associated molecular patterns mediate cancer immune evasion by engaging Siglecs on T cells. *J Clin Invest*. 128:4912–4923.

Tatomir A, Talpos-Caia A, Anselmo F, Kruszewski AM, Boodhoo D, Rus V, Rus H. 2017. The complement system as a biomarker of disease activity and response to treatment in multiple sclerosis. *Immunol Res*. 65:1103–1109.

Thompson AJ, de Vries RP, Paulson JC. 2019. Virus recognition of glycan receptors. *Curr Opin Virol*. 34:117–129.

Vasseur JA, Goetz JA, Alley WR Jr, Novotny MV. 2012. Smoking and lung cancer-induced changes in N-glycosylation of blood serum proteins. *Glycobiology*. 22:1684–1708.

Wingerchuk DM, Lennon VA, Pittock SJ, Lucchinetti CF, Weinshenker BG. 2006. Revised diagnostic criteria for neuromyelitis optica. *Neurology*. 66:1485–1489.

Wuhrer M, Selman MH, McDonnell LA, Kumpfel T, Derfuss T, Khademi M, Olsson T, Hohlfeld R, Meinl E, Krumbholz M. 2015. Pro-inflammatory pattern of IgG1 Fc glycosylation in multiple sclerosis cerebrospinal fluid. *J Neuroinflammation*. 12:235.

Yamaguchi Y, Barb AW. 2020. A synopsis of recent developments defining how N-glycosylation impacts immunoglobulin G structure and function. *Glycobiology*. 30:214–225.