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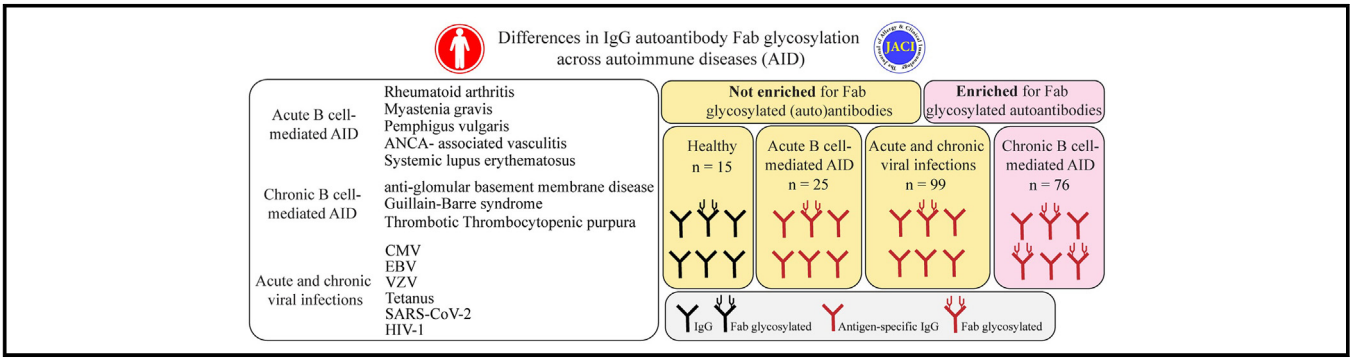
Amsterdam, Leiden, Groningen, Bilthoven, and Rotterdam, The Netherlands; and Paris, France

GRAPHICAL ABSTRACT



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Disclosure of potential conflict of interest: M.G. Huijbers and J.J.G.M. Verschuuren are coinventors on patent applications based on muscle-specific tyrosine kinase –related research. The Leiden University Medical Center, M.G. Huijbers, and J.J.G.M. Verschuuren receive royalties from these patents. Leiden University Medical Center receives royalties for a muscle-specific tyrosine kinase ELISA. N.A.G. Graça is an inventor of a patent application regarding autoantibody-resistant ADAMTS13 variants. The rest of the authors declare that they have no relevant conflicts of interest.

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Background: Increased prevalence of autoantibody Fab glycosylation has been demonstrated for several autoimmune diseases.

Objectives: To study whether elevated Fab glycosylation is a common feature of autoimmunity, this study investigated Fab glycosylation levels on serum IgG and its subclasses for autoantibodies associated with a range of different B cell–mediated autoimmune diseases, including rheumatoid arthritis, myasthenia gravis subtypes, pemphigus vulgaris, antineutrophil cytoplasmic antibody–associated vasculitis, systemic lupus erythematosus, anti–glomerular basement membrane glomerulonephritis, thrombotic thrombocytopenic purpura, and Guillain-Barré syndrome.

Methods: The level of Fab glycosylated IgG antibodies was assessed by lectin affinity chromatography and autoantigen-specific immunoassays.

Results: In 6 of 10 autoantibody responses, in 5 of 8 diseases, the investigators found increased levels of Fab glycosylation on IgG autoantibodies that varied from 86% in rheumatoid arthritis to 26% in systemic lupus erythematosus. Elevated autoantibody Fab glycosylation was not restricted to IgG_4, which is known to be prone to Fab glycosylation, but was also present in IgG_1. When autoimmune diseases with a chronic disease course were compared with more acute autoimmune illnesses, increased Fab glycosylation was restricted to the chronic diseases. As a proxy for chronic autoantigen exposure, the investigators determined Fab glycosylation levels on antibodies to common latent herpes viruses, as well as to glycoprotein 120 in individuals who are chronically HIV-1–infected. Immunity to these viral antigens was not associated with increased Fab glycosylation levels, indicating that chronic antigen-stimulation as such does not lead to increased Fab glycosylation levels.

Conclusions: These data indicate that in chronic but not acute B cell–mediated autoimmune diseases, disease-specific autoantibodies are enriched for Fab glycans. (J Allergy Clin Immunol 2023;151:1646-54.)

Key words: *Autoimmune diseases, autoantibodies, IgG, Fab glycosylation*

Abbreviations used

AAV: ANCA-associated vasculitis
AChR: Acetylcholine receptor
ACPA: Anti-citrullinated protein antibodies
BCR: B-cell receptor
CarP: Carbamylated protein
CCP: Cyclic citrullinated protein
CMV: Cytomegalovirus
dsDNA: Double-stranded DNA
GBM: Glomerular basement membrane
GBS: Guillain-Barré syndrome
IQR: Interquartile range
MG: Myasthenia gravis
MuSK: Muscle-specific tyrosine kinase
PR3: Proteinase 3
PV: Pemphigus vulgaris
RA: Rheumatoid arthritis
SLE: Systemic lupus erythematosus
SNA: *Sambucus nigra* agglutinin
TT: Tetanus toxoid
TTP: Thrombotic thrombocytopenic purpura
VZV: Varicella-zoster virus

A central role of the immune system is to protect the host from invading pathogens, while maintaining tolerance to self. Failure to distinguish self from nonself is at the basis of autoimmunity, and if improperly regulated this can lead to pathology and disease.[1] To date nearly 100 distinct autoimmune diseases are described that collectively affect 3%-5% of the general population, with ever rising incidence. Autoimmune diseases are highly diverse and diseases differ in severity, affected tissue(s), and effector mechanism that cause damage. Although incompletely understood, autoimmunity is thought to result from a combination of loss of tolerance mechanisms, genetic susceptibility, and environmental factors.[2]

The presence of autoantibodies is a common feature of many autoimmune diseases, and for some diseases these can be useful for diagnosis and classification and for others may correlate with the disease status or predict further clinical evolution of the disease.[3] Autoantibodies can be directed against a variety of molecules, such as nucleic acids, lipids, or proteins and can mediate both systemic inflammation and tissue damage.[4] For several IgG autoantibody responses, an increased prevalence of antibody variable region (Fab) glycosylation has been observed,[5] such as anti-cyclic citrullinated protein (CCP) in rheumatoid arthritis (RA)[6,7]and anti-myeloperoxidase in antineutrophil cytoplasmic antibody–associated vasculitis (AAV).[8,9] Fab glycans are attached to consensus *N*-glycosylation sites (N-X-S/T) that are mainly introduced via the process of somatic hypermutation during antigen-specific immune responses, as they are largely absent in the naive B-cell repertoire.[10] In healthy individuals, about 10%-14% of serum IgG is Fab glycosylated[11-15] with IgG_4 antibodies showing higher levels of Fab glycosylation (44%) compared to the other IgG subclasses (IgG_1: 12%, IgG_2: 11%, IgG_3: 15%).[15] Furthermore, mass spectrometry glycan analysis revealed that most Fab glycans have a complex-type biantennary structure with high levels (>90%) of terminal sialic acid residues.[13,16,17]

The role of Fab glycans in autoimmunity, as for immunity in general, is poorly understood. Fab glycans can affect antigen binding.[18-20] Therefore, it has been postulated that Fab glycans may reduce autoimmunity by masking the autoantigen binding sites of autoantibodies.[21] Likewise, Fab glycans expressed by autoreactive B-cell receptors (BCRs) have been shown to enhance BCR signaling and to prolong its expression on the cell surface after antigenic triggering.[22] In certain B-cell lymphomas, such as follicular or diffuse large B-cell lymphoma, the introduction of Fab glycans on the BCR might allow for interaction with lectins in the germinal center and thereby provide survival signals to sustain tumor growth.[23,24] In addition, Fab glycans may also arise on chronic antigen exposure because elevated Fab glycosylation levels are found on IgG_4 and IgE antibodies, which are associated with repeated or chronic antigen exposure.[10,15,25,26] Furthermore, anti-hinge autoantibodies in both patients with RA and healthy individuals were extensively Fab glycosylated, suggesting that elevated Fab glycosylation may develop in

TABLE I. Description of included autoimmune diseases for determination of autoantibody Fab glycosylation levels

Disease	Autoantigen(s)	Autoantibody subclass(es)	Organ(s) affected	Patients included, n
RA	CCP2, CarP	IgG_1- IgG_4	Joints, lungs, heart, skin, eyes and others	12
MG	MuSK, AChR	IgG_1, IgG_4	Muscle	24
PV	Dsg3	IgG_1, IgG_4	Oral mucosa, skin	9
AAV	PR3	IgG_1, IgG_3	Blood vessel walls	21
SLE	dsDNA	IgG_1, IgG_3	Skin, joints, kidneys, lungs, heart, others	10
Anti-GBM disease	α3(VI) NC1	IgG_1, IgG_4	Kidneys and lungs	8
GBS	Gangliosides	IgG_1, IgG_3, IgG_4	Peripheral nervous system	8
TTP	ADAMTS13	IgG_1 (IgG_4)	Central nervous system, kidneys, and others	9

response to an inflammatory microenvironment that is not per se restricted to autoimmunity.[27]

Although several IgG autoantibody responses have been characterized with increased levels of Fab glycans, it is not known whether this is a general characteristic acquired by autoantibodies that develop in the context of autoimmunity. Therefore, characterization of Fab glycosylation levels on a broad spectrum of autoantibody responses is important because it may provide a more detailed understanding of the role of Fab glycans in pathological conditions.

METHODS

Patients and healthy controls were included by the various collaborating teams at the University Medical Centers in Amsterdam, Leiden, Rotterdam, Groningen, and Paris according to the approved study protocols and with written consent of the patients according to the Declaration of Helsinki. In this study cross-sectional samples were included prior to (B-cell–targeted) therapy or more than 6 months after immunosuppressive treatment. For samples, lectin (*Sambucus nigra* agglutinin [SNA]) affinity chromatography, total and specific IgG immunoassays, and gel filtration chromatography were used; see details in File E1 in this article's Online Repository (available at www.jacionline.org).

Statistical analysis

Differences between 2 groups were analyzed using a paired or unpaired *t*-test and between multiple groups using a Kruskal-Wallis ANOVA and a Dunn posttest for multiple comparisons. Nonparametric correlations were analyzed with a Spearman rank correlation test. A P value <.05 was considered significant. The statistical analyses were carried out using GraphPad Prism 9.1.1 (GraphPad Software, San Diego, Calif).

RESULTS

Variable levels of autoantibody Fab glycosylation across multiple autoimmune diseases

To investigate whether elevated levels of Fab glycosylation are a general characteristic acquired by antibodies that develop in the context of autoimmunity, we analyzed the level of Fab glycosylation for 10 autoimmune disease–associated IgG autoantibody responses in cross-sectional serum samples taken before B-cell–targeted therapy across 8 different autoimmune diseases (Table I). To do so, we fractionated sera of patients with autoimmune disease (n = 101) and healthy controls (n = 15) using SNA (sialic acid binding lectin) affinity chromatography (Fig 1, *A*). SNA affinity chromatography of serum results in an SNA+ fraction (enriched for sialylated antibodies) and an SNA− fraction (devoid of sialylated antibodies). Total and specific IgG is measured in the initial serum and in SNA+ and SNA− fractions by quantitative ELISA, RIA, Luminex, fluoro enzyme immunoassay, or multiplex immunoassay (see Fig E1 in this article's Online Repository at www.jacionline.org). The percentage of Fab sialylated antibodies is calculated by dividing the amount of (antigen-specific) IgG detected in the SNA+ fraction by the combined amount of (antigen-specific) IgG detected in the SNA+ and SNA− fractions (amount refers to arbitrary units measured in each fraction) (see Table E1 in this article's Online Repository at www.jacionline.org). This technique allows for the enrichment of Fab sialylated antibodies, but not for Fc glycans, and provides a good estimate for the level of Fab glycosylation because >90% of Fab glycans carry terminal sialic acid residues.[12,13,28]

In line with previous studies,[6,15,29] for anti-citrullinated protein antibodies (ACPA) in RA, we found high levels of Fab glycosylation (anti-CCP2: 86%; interquartile range [IQR]: 71-90) that were significantly elevated compared with those of total IgG (14%; IQR: 12-16; P <.0001) (Fig 1, *B*). For the subset of samples with quantifiable anti-carbamylated protein (CarP) antibody levels, we also found high levels of Fab glycosylation for anti-CarP antibodies (51%; IQR: 42-77), but significantly lower than for anti-CCP2 antibodies (P = .05). Significantly increased levels of autoantibody Fab glycosylation were also observed for anti-Dsg3 antibodies found in patients with pemphigus vulgaris (PV) (49%; IQR: 37-55; P <.0001), anti-proteinase 3 (PR3) antibodies found in patients with AAV (31%; IQR: 15-40; P <.0001) and anti–double-stranded DNA (dsDNA) (26%; IQR: 19-34; P = .02) antibodies found in patients with systemic lupus erythematosus (SLE) when compared to Fab glycosylation levels on their total IgG (Fig 1, *C*). In contrast, Fab glycosylation levels on autoantibody responses found in patients with anti-glomerular basement membrane (GBM) glomerulonephritis, Guillain-Barré syndrome (GBS), and thrombotic thrombocytopenic purpura (TTP), anti-alpha3(IV) noncollagenase domain 1 antibodies (10%; IQR: 2-23), anti-gangliosides antibodies (<3%; IQR: 2-4), and anti-ADAMTS13 antibodies (3%; IQR: 1-10), respectively, were found to be similar or decreased compared with that of their total IgG (GBM: 14%; P = .07; GBS: 11%; P < .0001; and TTP: 12%; P = .05) (Fig 1, *D*). Remarkably, in patients with myasthenia gravis (MG), we found high levels of Fab glycosylation for anti-muscle-specific tyrosine kinase (MuSK) antibodies (46%; IQR: 29-64; P = .0001) but levels comparable to those of total IgG (11%; IQR: 11-17) for anti-acetylcholine receptor (AChR) antibodies (15%; IQR: 1-20; P = .63). Contrary to anti-CCP2 and anti-CarP antibodies in RA, anti-MuSK and anti-AChR antibodies in MG were not measured in the same individuals because these rarely coexist. Because anti-dsDNA autoantibodies in patients with SLE revealed elevated Fab glycosylation, we additionally analyzed 2 other autoantibody responses in the same patients. For anti-Smith antibodies, which are specifically associated with SLE, Fab glycosylation levels were

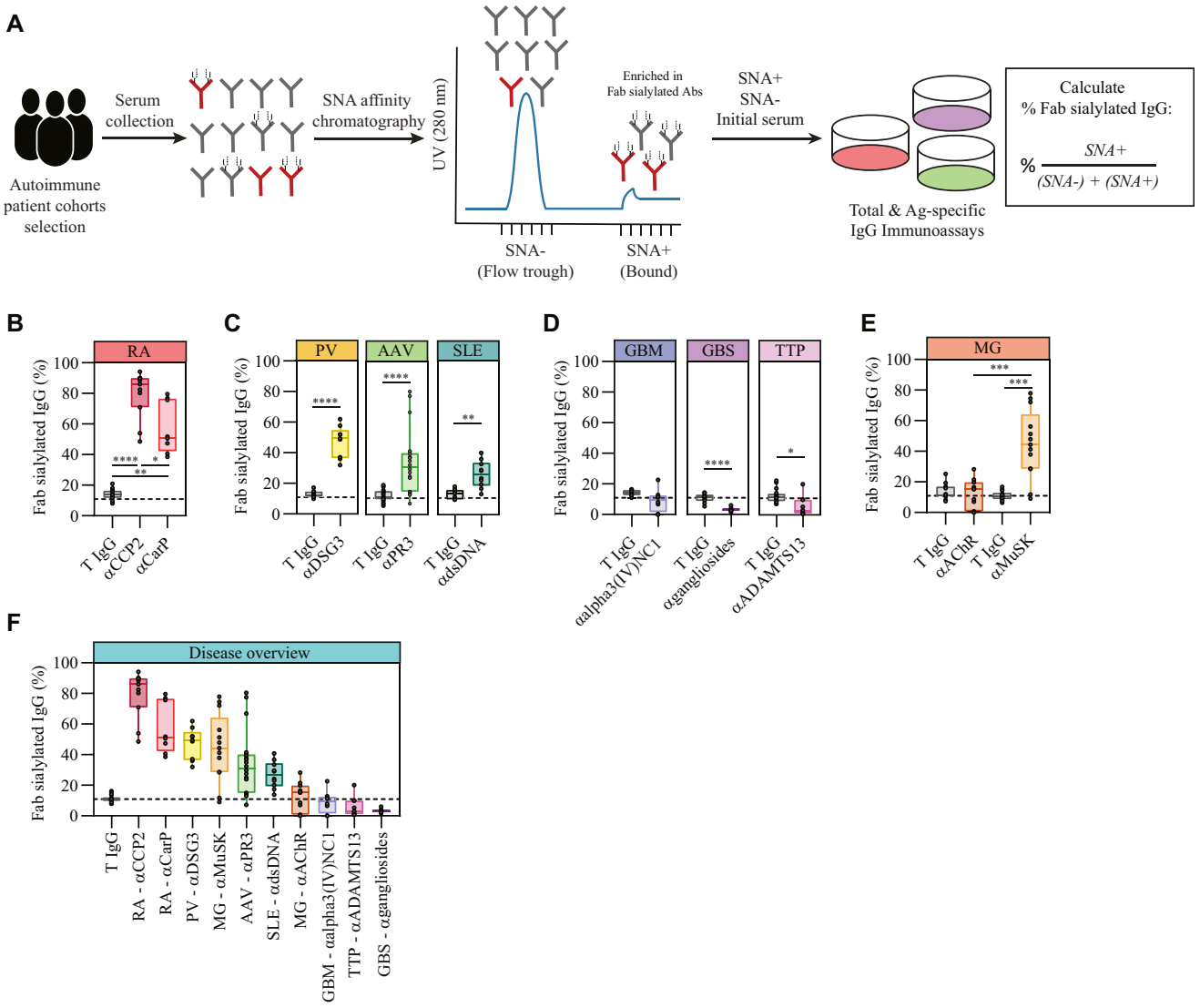

FIG 1. Prevalence of IgG autoantibody Fab glycosylation across multiple autoimmune diseases. **(A)** Schematic overview of methodology. Cross-sectional serum samples taken before B-cell–targeted therapy or >6 months after immunosuppressive treatment from 8 different autoimmune patient cohorts were fractionated using SNA affinity chromatography generating a sialic acid enriched (SNA+) and depleted (SNA−) pool of serum proteins. Total (*T*) and antigen (*Ag*)-specific IgG is determined for both fractions. The percentage of Fab glycosylated antibodies was calculated by dividing the amount of IgG in SNA+ by the amount of IgG in the combined SNA+ and SNA− fractions (ie, arbitrary units measured in each fraction). Percentage of Fab sialylated T IgG, **(B)** anti-CCP2 (n = 11), and anti-CarP (n = 8) in RA; **(C)** anti-Dsg3 (n = 9) in PV; anti-PR3 (n = 21) in AAV; anti-dsDNA (n = 10) in SLE; **(D)** anti-alpha3(IV) noncollagenase domain 1 (*NC1*) (n = 8) in anti-GBM glomerulonephritis; anti-gangliosides (n = 8) in GBS; anti-ADAMTS13 (n = 9) in TTP; and **(E)** anti-MuSK (n = 13) and anti-AChR (n = 11) in MG. **(F)** Overview of Fab glycosylation levels of T IgG in healthy controls and 10 IgG autoantibody responses across 8 different autoimmune diseases. *Dashed lines* represent the median for Fab glycosylation of T IgG in healthy donor sera (11%; IQR: 11-14%; n = 18). *Box plots* show median and IQR. Statistical differences were determined using a paired or unpaired *t*-test or Kruskal-Wallis ANOVA and Dunn's multiple comparison test. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001.

elevated (n = 6; 23%; IQR: 15-31; *P* = .04), contrary to the less disease-specific anti-Ro52 antibodies (n = 7; 14%; IQR: 12-19; *P* = .38) (see Fig E2, *A* in this article's Online Repository at www.jacionline.org). Fig 1, *F* provides an overview of Fab glycosylation levels on total IgG in healthy individuals and disease-associated autoantibody responses ordered by decreasing median Fab glycosylation levels. SNA+/SNA− antigen detection values of quantitative immunoassays are reported in the supporting information. Size-exclusion chromatography was performed to confirm the presence of Fab glycans on antigen-specific autoantibodies (larger hydrodynamic volume) for anti-PR3 and anti-MuSK antibodies (Fig E2, *B*), as previously described for anti-CCP2 and anti-hinge antibodies in RA.[6,27] For anti-MuSK IgG_4 antibodies, the size shift was less pronounced probably due to the fact that most IgG_4 molecules carry only a single Fab glycosylated Fab arm due to half-molecule exchange.[30] Autoantibody levels did not correlate with total IgG levels (Fig E2, *B*) nor with Fab glycosylation levels (see Fig E3 in this article's Online

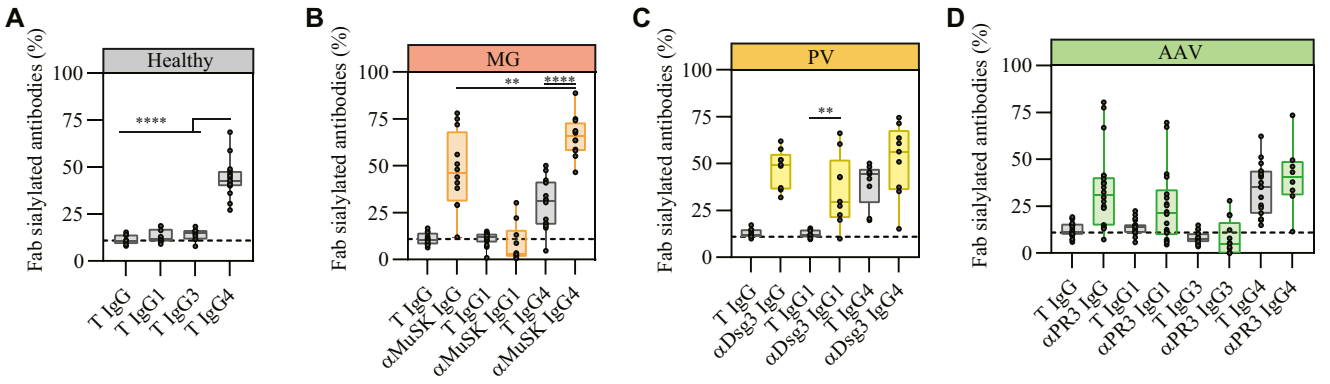

FIG 2. Fab glycosylation levels of IgG autoantibody subclasses. **(A)** Percentage of sialylated antibodies for T IgG and IgG_1, IgG_3, and IgG_4 in healthy donor sera (n = 18). **(B)** Fab sialylated antibody levels for total and specific anti-MuSK IgG (n = 12), IgG_1 (n = 10), and IgG_4 (n = 12) in patients with MG. **(C)** Fab sialylated antibody levels for total and specific anti-Dsg3 IgG, IgG_1, and IgG_4 (n = 9) in patients with PV. **(D)** Fab glycosylation levels for total and specific anti-PR3 IgG (n = 22), IgG_1 (n = 18), IgG_3 (n = 15), and IgG_4 (n = 11) in patients with AAV. *Box plots* show median and IQR. Statistical differences were determined using a Kruskal-Wallis ANOVA and Dunn's multiple comparison test. $^{**}P < .01$, $^{****}P < .0001$.

Repository at www.jacionline.org) for any of the diseases, which suggests that high or low level of Fab glycosylation is not the result of the level of antibodies produced, in line with earlier studies.[27,31]

IgG autoantibody subclass distribution and Fab glycosylation levels

Next, we determined Fab glycosylation levels of IgG subclasses in autoantibody responses that showed elevated levels of Fab glycosylation. In healthy individuals IgG_4 Fab glycosylation levels are increased (43%; IQR: 40-48) compared with that of other IgG subclasses (IgG_1: 12%; IQR: 11-17; IgG_3: 15%; IQR 12-16), and of total IgG (11%; IQR 9-14) (Fig 2, *A*), of which IgG_4 antibodies are only a minor fraction.[10,15] Within anti-MuSK and anti-Dsg3 autoantibody responses, a large fraction is of the IgG_4 subclass.[32,33] Therefore, we investigated whether the increased Fab glycosylation levels observed for these responses could be explained by a high proportion of IgG_4 antibodies, which have elevated levels of Fab glycans in general. For MuSK MG we found that levels of Fab glycosylation of anti-MuSK IgG_4 antibodies (66%; IQR: 58-73) were significantly higher than that of total IgG_4 (31%; IQR: 19-41; $P < .0001$) and anti-MuSK IgG (46%; IQR: 29-64; $P = .005$), whereas Fab glycosylation levels of anti-MuSK IgG_1 antibodies (3%; IQR: 2-15) were not elevated compared to total IgG (11%; IQR: 8-14; $P = .99$) or IgG_1 (12%; IQR: 9-14; $P = .99$). This indicates a subclass-specific increased selection for Fab glycosylation of anti-MuSK antibodies, restricted to the IgG_4 subclass (Fig 2, *B*). Fab glycosylation for PV-associated anti-Dsg3 followed a different pattern (Fig 2, *C*). Fab glycosylation levels on anti-Dsg3 IgG_1 antibodies (30%; IQR: 21-52) were significantly higher than total IgG_1 (12%; IQR 11-15; $P = .003$) but not different from anti-Dsg3 IgG (49%; IQR: 37-55; $P = .39$). Anti-Dsg3 IgG_4 antibodies (56%; IQR: 36-68) were not different from total IgG_4 (45%; IQR: 29-47; $P = .32$) nor from anti-Dsg3 IgG (49%; IQR 37-55; $P = .98$). Anti-Dsg3 IgG_3 antibodies were only detectable in a small fraction of patients (n = 3) and presented variable levels of Fab glycosylation levels with high interpatient variation (26%; IQR: 11-56) (see Fig E4, *A* in this article's Online Repository at www.jacionline.org) not significantly different from total IgG_3 (10%; IQR: 8-12; $P = .25$). Fab glycosylation levels for anti-PR3 antibodies in AAV, a response dominant in IgG_1 and IgG_3, were elevated for anti-PR3 IgG_1 (21%; IQR: 10-34) and anti-PR3 IgG_4 antibodies (40%; IQR: 31-49), and low for anti-PR3 IgG_3 antibodies (5%; IQR: 0-16) (Fig 2, *D*). Fab glycosylation of total IgG_4 (35%; IQR: 21-44) and anti-PR3 IgG_4 antibodies (40%; IQR: 31-49; $P = .93$) were not significantly different. Here, although not significant overall, some individuals showed a remarkable increase in anti-PR3 IgG_1 Fab glycosylation compared to total IgG_1 (14%; IQR: 11-15; $P = .14$). Six patients with AAV were included at first onset of disease and 15 patients during relapse. Interestingly, the median Fab glycosylation level of anti-PR3 IgG, and thus IgG subclasses, was significantly lower in patients at first onset of disease (14%; IQR: 12-27; $P = .009$) than in those in relapse (36%; IQR: 26-41) and not different from total IgG (12%; IQR: 10-17; $P = .91$) (Fig E4, *B*). Anti-Dsg3 and anti-PR3 IgG_1 Fab glycosylation levels were significantly higher than those of anti-MuSK IgG_1, whereas Fab glycosylation levels for anti-MuSK IgG_4 were higher compared to anti-PR3 IgG_4 but not anti-Dsg3 IgG_4 (Fig E4, *C*). For RA, no reliable data were obtained for IgG_2 to IgG_4. This is in line with the observation that the ACPA subclass composition is dominated by IgG_1, with a minor contribution of other subclasses, including IgG_4, which is estimated to contribute, on average, 5% to the overall ACPA IgG composition.[34,35] Assays for reliable measurements of anti-dsDNA IgG subclasses were lacking and therefore not included in this study.

Chronic viral antigen stimulation or repeated tetanus toxoid immunization does not lead to increased levels of antigen-specific IgG Fab glycosylation

To investigate whether elevated levels of Fab glycosylation are characteristic of situations of chronic antigen exposure, we analyzed Fab glycosylation levels on antibodies targeting several different herpes viruses in the same patient groups and in healthy controls. Infection with a single or multiple of these herpes viruses is common in the general adult population. Once infected,

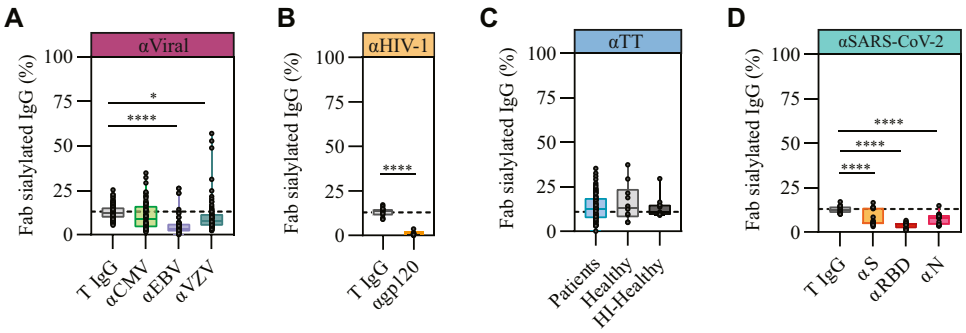

FIG 3. IgG Fab glycosylation levels after chronic and acute viral antigen exposure. **(A)** Percentage of Fab sialylated antibodies for T IgG, anti-CMV, anti-EBV, and anti-VZV in serum of patients with autoimmune disease (including RA, PV, AAV, SLE, TTP, and GBS; CMV: n = 41; EBV: n = 44; VZV: n = 53) and healthy controls (CMV: n = 6; EBV: n = 9; VZV: n = 13). **(B)** Percentage of Fab sialylated antibodies for T IgG and anti-glycoprotein 120 antibodies in serum of individuals with chronic HIV-1 infection (n = 14). **(C)** Percentage of Fab sialylated IgG for anti-TT in patients with autoimmune disease (including RA, MG, PV, AAV, SLE, TTP, and GBS; n = 110), healthy controls (n = 10), and TT hyperimmunized (n = 11). **(D)** Fab sialylated antibodies for T IgG, anti–spike protein (*S*), anti–receptor-binding domain (*RBD*) and anti–nucleocapsid protein (*N*) IgG in healthy individuals previously infected with severe acute respiratory syndrome coronavirus 2 (*SARS-CoV-2*) (S: n = 19; RBD: n = 19; N: n = 18). *Box plots* show median and IQR. Statistical differences were determined using a paired *t*-test or Kruskal-Wallis ANOVA and Dunn's multiple comparison test. $*P < .05$, $****P < .0001$.

individuals establish a lifelong latency with repeated periods of viral reactivation and exposure.[36] Fab glycosylation levels were determined on IgG antibodies specific for human cytomegalovirus (CMV), EBV, and Varicella-zoster virus (VZV) in patients with autoimmune disease (n = 69) and healthy controls (n = 15) that tested seropositive for ≥1of these viruses. The prevalence of CMV/EBV/VZV infections among the included patients with autoimmune disease and healthy controls were fairly similar (see Fig E5, *A* in this article's Online Repository at www.jacionline.org). Fab glycosylation levels for IgG antibodies against CMV (9%; IQR: 4-16) in patients and healthy controls were comparable to total IgG levels (12%; IQR: 10-15; $P = 0.18$) (Fig 3, *A*). Interestingly, Fab glycosylation levels on anti-EBV (3%; IQR: 2-5) and anti-VZV antibodies (8%; IQR: 5-11) were significantly lower compared to total IgG (12%; EBV: $P < .0001$; VZV: $P = .02$). Furthermore, Fab glycosylation levels were also evaluated for anti-glycoprotein 120 antibodies in individuals with treatment-naive chronic HIV-1 infection. Altered Fc glycosylation levels as well as specific Fab glycans on broadly neutralizing antibodies were previously reported.[37,38] However, also in this case, we observed that Fab glycosylation levels were lower rather than elevated (0.5%; IQR: 0.2-1.1) compared to total IgG (13%; IQR: 9-17; $P = .0001$) (Fig 3, *B*). Fab glycosylation levels for antibodies to tetanus toxoid (TT) (12%; IQR: 8-18), a typical vaccine antigen that mainly induces IgG_1, were similar to those of total IgG (12%; IQR 10-15; $P = .84$) across all autoimmune diseases and comparable to those of healthy individuals (13%; IQR: 8-23; $P = .5$) and to those hyperimmunized with TT (HI-healthy: 11%; IQR: 10-15; $P = .99$) (Fig 3, *C*). There were no significant differences in Fab glycosylation levels of CMV/EBV/VZV/TT antibody responses when separated per disease (Fig E5, *B*). IgG Fab glycosylation levels were also studied in individuals that recently underwent an acute primary viral infection. Fab glycosylation levels on anti-spike (5%; IQR: 5-14), anti-spike protein receptor-binding domain (4%; IQR: 2-5), and anti-nucleocapsid protein (N) (8%; IQR: 4-10) IgG antibodies in individuals with severe acute respiratory syndrome coronavirus 2 infection were found to be significantly lower compared to those of total IgG (13%; IQR: 11-14; $P < .0001$) (Fig 3, *D*). Taken together, antibodies developed during both chronic and acute viral antigen exposure show normal to low levels of Fab glycosylation.

DISCUSSION

In this disease-overarching study, we compared IgG autoantibody Fab glycosylation levels among 10 different disease-associated IgG autoantibody responses across 8 different autoimmune diseases. We observed elevated levels of autoantibody Fab glycosylation in a number of chronic B-cell–mediated autoimmune diseases, including, for the first time, anti-Dsg3, anti-MuSK, anti-PR3, and anti-dsDNA IgG autoantibody responses, but not for autoantibody responses found in acute B-cell–mediated autoimmune diseases. Hence, chronic B-cell–mediated autoimmune diseases may share a common pathophysiological mechanism of immune dysregulation hallmarked by elevated Fab glycosylation levels. Furthermore, within autoantibody responses we observed subclass-specific increases of Fab glycosylation and no enhanced Fab glycosylation levels were found on antibodies directed against viral antigens, including antigens from common latent herpes viruses, indicating that chronic or repeated antigen exposure in itself does not necessarily lead to increased antibody Fab glycan levels and is context-dependent.

In recent years it has become increasingly clear that Fab glycans play a role in antibody function as well as in immune function. Besides diversification of the antibody repertoire, several functional attributes have been demonstrated to involve Fab glycans, including impact on antigen binding,[15,18-20,39] antibody half-life and stability,[40-44] and engagement of (endogenous) lectins,[23,45] such as SIGLEC CD22.[46] The level of Fab glycosylation for any given IgG autoantibody response is the result of the subclass distribution and their individual level of Fab glycosylation. Functional characteristics of the different IgG subclasses are major determinants in the differences between IgG_1-/IgG_3- and IgG_4-dominant autoimmune diseases in the way they contribute to inflammation and damage.[47-49] The level of Fab

glycosylation on anti-MuSK IgG_4 antibodies significantly exceeded levels of total IgG_4, whereas Fab glycosylation on anti-MuSK IgG_1 antibodies was low. This indicates a subclass-specific increased selection for Fab glycosylation of anti-MuSK, being in this case restricted to the IgG_4 subclass. In PV, another archetypical IgG_4-dominated autoimmune disease, Fab glycosylation levels of anti-Dsg3 IgG_4 antibodies were high, but not significantly elevated compared to total IgG_4. In both these diseases, an association between high levels of Fab glycans and the presence of pathogenic IgG_4 autoantibodies is observed. The presence of Fab glycans may create an additional layer that can contribute to the pathogenicity of these autoantibodies. Of note, altered *N*-glycosylation of anti-PLA2R1 IgG_4 in patients with membranous nephropathy has been reported to result in local activation of complement via the lectin pathway, thereby contributing to pathogenicity.[50] Fab glycosylation of anti-PLA2R1 IgG_4 was not assessed specifically in this study. Different from MuSK MG, though, the Fab glycosylation levels of anti-Dsg3 IgG_1 antibodies in PV were significantly increased compared to total IgG_1. Hence, high levels of autoantibody Fab glycosylation in PV are not exclusive to IgG_4 when determined in the same individuals. IgG_1 Fab glycosylation levels were also elevated for anti-PR3 antibodies in a fraction of patients with AAV and was previously observed for total IgG_1 in patients with IgG_4-related disease.[51] Fab glycosylation levels on antigen-specific IgG_3 was generally low. However for anti-Dsg3 IgG_3, we observed several cases with elevated levels of Fab glycosylation, whereas anti-PR3 IgG_3 antibody Fab glycosylation levels were low. Autoantibody responses thus widely differ in the preferred subclass expression and the level of Fab glycosylation on these antibodies. Subclass-specific enrichment of Fab glycans may occur under conditions where Fab glycans are functionally relevant.

A commonality among several autoantibody responses is a skewing toward the IgG_4 isotype, a subclass that has elevated levels of Fab glycans in general and is elicited on T_H2-type responses, associated with chronic antigen exposure and sometimes tolerance build-up. Chronic or relapsed viral infections are not associated with an IgG_4 skewing. Hence, chronic antigen exposure, within specific contexts, could still result in elevated Fab glycosylation.

For patients with AAV, at first onset of disease Fab glycosylation levels were significantly lower than those who suffered from a relapse, suggesting that Fab glycans are positively selected during the course of the disease. In RA, selection in favor of Fab glycans was also observed in a longitudinal study into ACPA response development.3[1,52] Accumulation of Fab glycans as a natural by-product of ongoing B-cell responses is unlikely. For ACPA, the number of variable region mutations did not correlate with the frequency of *N*-glycosylation sites.[29] Furthermore, both IgG_4 and IgE antibodies have elevated levels of Fab glycans despite having similar or even fewer variable region mutation levels as other isotypes.[10,25,26] It remains unclear how selection for Fab glycans takes place. There might be a role for the antigen or the context of the antigen to drive Fab glycosylation. However, binding of antibodies or BCRs to antigens is not consistently enhanced or decreased by Fab glycans.[18-20,22,39,40] Alternatively, Fab glycans on BCRs may interact with lectins as indicated for B-cell lymphomas,[23,24] and thereby acquire a survival advantage compared to non-Fab glycosylated BCRs. Further evidence is needed to support this hypothesis.

Autoantibodies in chronic progressive autoimmune diseases all, except anti-AChR, displayed elevated levels of Fab glycosylation. Autoantibodies in TTP, anti-GBM glomerulonephritis, and GBS had normal or even decreased levels of Fab glycosylation. These diseases generally run a relapsing-remitting or acute monophasic disease course instead of being chronic. Possible discrepancies between monophasic and chronic disease states are prolonged exposure to antigen, ongoing inflammation, evolving B-cell responses (see above), and epitope spreading. As a proxy for chronic antigen exposure, we examined antibodies to common latent herpes viruses and HIV. Fab glycosylation levels were not elevated, indicating that chronic antigen stimulation as such does not lead to increased Fab glycosylation levels. By contrast, antibodies formed against therapeutic proteins, another setting with prolonged antigen exposure, were previously found to display elevated Fab glycosylation levels.[15,27] Possibly, antibodies against microbes may evolve in a microbe-specific context in which Fab glycans are not favorable. Because most enveloped viruses have an overall negative charge due to the phospholipids on the cell surface,[53,54] the potentially hampered antibody binding due to charge repulsion by antibodies carrying negatively charged sialylated Fab glycans[12,13] may result in negative selection for the introduction of these glycans, even on repeated exposure. In line with this hypothesis, IgG autoantibodies against rhesus D, present on the negatively charged surface of red blood cells, were also characterized by low level of Fab glycans.[15] The AChR antigen has also been described to have a negative surface charge[55] different from that of the MuSK antigen, which is largely positively charged,[56] potentially explaining the nonelevated levels of Fab glycans for anti-AChR antibodies in MG. Moreover, the majority of patients with GBS, TTP, or anti-GBM glomerulonephritis report a viral or bacterial infection before disease onset. The immune system generates antibodies to fight infection, that coincidently trigger autoimmunity in genetically susceptible individuals due to cross-reactivity with self-antigens.[57-59] The low levels of Fab glycans observed on autoantibodies in GBS, TTP, and anti-GBM glomerulonephritis might stem from these antibodies originating from cross-reactive anti-microbe immune responses. In line, recent studies further strengthen the link between EBV infection and multiple sclerosis etiology.[60] It will be interesting to study whether autoantibodies in multiple sclerosis originate from cross-reactive EBV antibodies and display low levels of Fab glycans.

Although the included number of patients per disease is limited, the strength of the current study lies in the determination of autoantibody Fab glycosylation levels on a broad spectrum of autoimmune diseases. To determine the clinical prognostic value of Fab glycans, it will be of interest to study longitudinal Fab glycosylation levels and profiles on disease-associated autoantibody responses in more individuals and correlate these with clinically relevant parameters such as disease severity, remission versus active disease, or treatment status. Whether alterations in autoantibody Fab glycosylation levels will affect the course of the disease or change on immunosuppressive treatment needs to be determined. For RA, Fab glycans are described to predict progression to RA and thereafter stabilizes once disease is established.[31,52]

Thus, considering the importance of the autoantibody subclass and glycosylation status for the pathogenic potential of a

specific-autoantibody response, it might be helpful to include these parameters for diagnostic purposes. Taken together, the variable emerging autoantibody Fab glycosylation levels indicates that Fab glycosylation on autoantibodies is not a random process but is, rather, subject to context-dependent selection mechanisms during autoimmune responses.

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Key messages

- **Levels of autoantibody Fab glycosylation levels are variable across autoimmune diseases and therefore not a general hallmark of autoimmunity.**
- **Chronic but not acute B-cell–mediated autoimmune diseases associate with (pathogenic) autoantibodies enriched for Fab glycans.**
- **Chronic (auto)antigen exposure in itself does not necessarily lead to increased levels of Fab glycosylation and was context-dependent.**
- **Autoantibody responses display IgG subclass-specific enrichment of Fab glycosylation.**

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