GlYcoLISA: antigen-specific and subclass-specific IgG Fc glycosylation analysis based on an immunosorbent assay with an LC-MS readout

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Protocol

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GlYcoLISA: antigen-specific and subclass-specific IgG Fc glycosylation analysis based on an immunosorbent assay with an LC–MS readout

David Falck & Manfred Wuhrer

Abstract

Immunoglobulin G (IgG) fragment crystallizable (Fc) glycosylation modulates effector functions such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Consequently, assessing IgG Fc glycosylation is important for understanding the role of antibodies in infectious, alloimmune and autoimmune diseases. GlYcoLISA determines the Fc glycosylation of antigen-specific IgG by an immunosorbent assay with a liquid chromatography–mass spectrometry (LC–MS) readout. Detection of antigen-specific IgG glycosylation in a subclass- and site-specific manner is realized by LC–MS-based glycopeptide analysis after proteolytic cleavage. GlYcoLISA addresses challenges related to the low abundance of specific IgG and the high background of total IgG by using well-established immunosorbent assays for purifying antibodies of the desired specificity using immobilized antigen. Alternative methods with sufficient glycan resolution lack these important specificities. GlYcoLISA is performed in a 96-well plate format, and the analysis of 160 samples takes ~5 d, with 1 d for sample preparation, 2 d of LC–MS measurement and 2 d for partially automated data processing. GlYcoLISA requires expertise in LC–MS operation and data processing.

Key points

- This protocol describes a method for profiling fragment crystallizable glycosylation of antigen-specific antibodies isolated from clinical samples.
- By characterizing IgG specific for an antigen of interest and at a high molecular resolution, the technique allows more confident and functionally relevant characterization of glycosylation alterations in disease than could be achieved with less-specific methods.

Key references

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Introduction

Antibody glycosylation

Antibodies are key players in the host defense against pathogens and are initiators of various alloimmune and autoimmune responses. Immunoglobulin G (IgG) is the most abundant antibody in circulation and is generally considered the most relevant antibody in the defense against viral and bacterial infection, initiating the majority of cytotoxic and phagocytic responses—with the exception of mucosal tissue responses, which are dominated by IgA. IgG is a homodimer with four peptide chains, two heavy chains and two light chains, and is divided into two domains, the fragment crystallizable (Fc) and the antigen-binding fragment (Fab)[1,2]. IgG will form immune complexes in the presence of antigen, thereby activating cytotoxic, phagocytic and other inflammatory pathways.

The Fc domain contains one conserved glycosylation site on each heavy chain (Fig. 1). Glycosylation occurs at over 98% prevalence, and glycan structures make up 1% of the molecular weight of IgG, making IgG glycosylation the most abundant and structurally impactful post-translational modification (PTM) next to disulfide bridges. Furthermore, glycosylation modifications are directly involved in and regulate key protein–protein interactions of IgG, including those involved in the activation of Fcγ receptors (FcγRs) and complement[3,4] (Table 1). For example, IgG1 lacking the core fucose has stronger interactions with FcγRIIIa and FcγRIIIb than fucosylated IgG1 has[5], which leads to increased antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells and macrophages, and to antibody-dependent cellular phagocytosis by neutrophils ex vivo[6–8]. Terminal galactosylation seems to be the dominant glycosylation feature influencing complement activation by IgG[9], an effect that has recently been attributed to changes in the hexamerization potential of IgG[4]. Furthermore, in 15–25% of circulating IgG, the Fab domain contains additional glycosylation sites in the variable domain that can affect antigen binding and have been suggested to affect B cell maturation[1,10].

IgG glycosylation is tightly controlled and highly stable in homeostasis due to its strong functional impact[11]. However, it is also very sensitive to natural changes such as pregnancy and pathologies such as infection or the development of autoimmunity[12–14]. Consequently, it is often involved in pathogenic mechanisms and is therefore an excellent target for diagnosis and therapy[2].

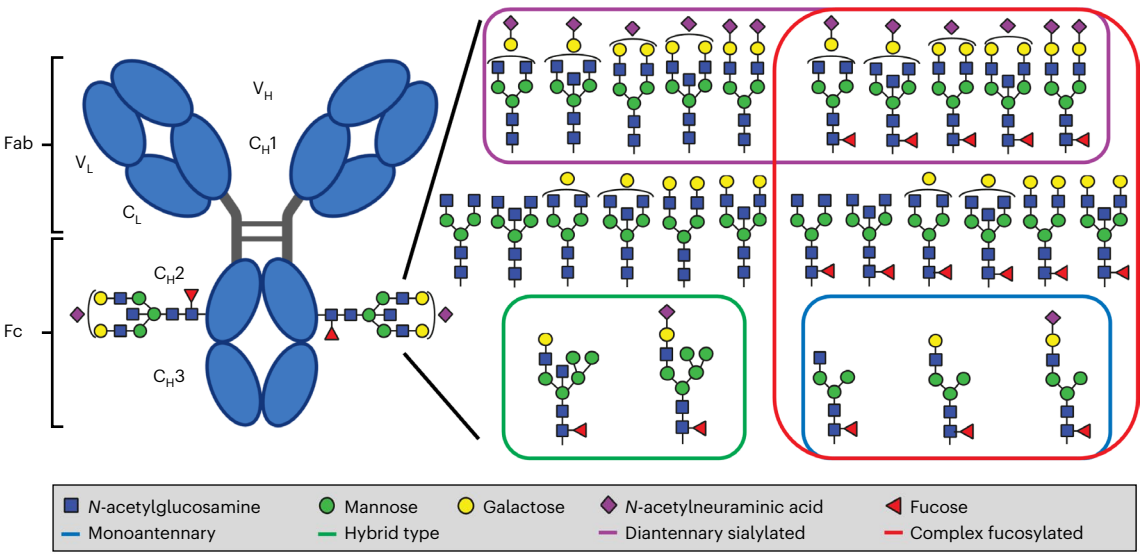

Fig. 1 | IgG structure and expected glycosylation structures. The C_H2 glycosylation of IgG is conserved in all subclasses. For potential glycosylation structures, see also Extended Data Table 1.

Table 1 | Examples of the impact of IgG Fc glycosylation on effector functions

Glycosylation trait	Function affected	Via interaction	Correlation	Cell type affected
Fucosylation[5]	ADCC	FcγRIIIa	Negative	NK cells and macrophages
	ADCP	FcγRIIIb	Negative	Neutrophils
Galactosylation[4,9]	ADCC	FcγRIII	Positive	NK cells
	CDC	Hexamerization, C1q	Positive	Autolysis
Sialylation[61,62]	ADCC	FcγRIIb	Negative	Macrophages
	Upregulation of FcγRIIb	Unknown	Negative	Macrophages

ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity.

IgG Fc glycosylation is dynamically and differentially regulated for each antigen

Recent studies have shown convincing evidence that antibody glycosylation is separately regulated for different antigens, either by the temporal or spatial separation of antibody-producing cells[15–17]. Consequently, co-existing antibodies against different antigens differ in glycosylation; for example, vast differences have been observed in the relative abundance of IgG1 core fucosylation depending on the nature of the antigen context[13,18,19]. Low fucosylation is imprinted in an as-yet-unknown manner and persists for years in the absence of novel stimuli[14]. By contrast, antigen-specific IgG glycosylation, including fucosylation, changes strongly in the presence of stimuli, making it highly dynamic in both acute and chronic scenarios. The dynamics in acute responses are apparent in the context of infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), in which anti-spike (anti-S) IgG1 shows low fucosylation, galactosylation and sialylation at the initial challenge[13,20]. These glycosylation traits increase over time as inflammation resolves until they reach a long-term equilibrium. However, for example in the context of rheumatic diseases, it has been demonstrated that glycosylation of antigen-specific antibodies (ASAs) may shift, for example, when a chronic disease re-activates[21]. Furthermore, repeated challenges change the imprinting in such a way that the equilibrium state differs from that of the initial challenge[18].

The regulation of IgG glycosylation is controlled by the microenvironment of the antibody-producing cells. Residence in certain organizational structures can affect IgG glycolylation; for example, germinal center residence of antibody-producing B cells has been shown to suppress antibody sialylation in animal models[15]. In humans, afucosylated IgG responses are notably reduced in the absence of a functional spleen both under healthy homeostatic and chronic inflammatory conditions[16]. Furthermore, temporal changes in IgG glycosylation can be affected via cytokines or hormones[17,22,23].

As the glycosylation of IgG directed against different antigens is differentially regulated, analysis of the glycosylation status of the total IgG pool will produce a result that reflects neither the equilibrium nor the dynamics of the IgG directed against antigens of interest in a given setting. On the contrary, the absence of antigens for the vast majority of total IgG constituents results in neutrality or even a reduction in inflammatory responses due to competition with the immune complexes of the antigen-specific IgG of interest. Nonetheless, glycosylation analysis of IgG from circulation or other biofluids is largely performed on the level of total IgG, in a way that is indifferent to antigen specificity. Studies in cases of autoantibody-associated vasculitis and intravenous IgG show that total IgG glycosylation can be important as a clinical marker in its own right[21,24], although insufficient differentiation between antigen-specific and total IgG effects complicates the interpretation of such studies.

Due to the structural, regulatory and functional differences between the glycosylation of antigen-specific IgG antibodies and total IgG glycosylation, antigen-specific IgG glycosylation analysis yields more relevant and straightforward results and avoids the pitfalls of overinterpreting or misinterpreting the impact of the glycosylation of total IgG. By providing a protocol for the analysis of the glycosylation of antigen-specific IgG antibodies, we hope that users will be able to resolve the many existing questions and contradictions in the field of IgG glycosylation.

Development of the protocol

GlYcoLISA (Glyco: glycosylation analysis; Y: antibody; L: LC–MS; ISA: immunosorbent assay) combines over a decade of high-sensitivity, throughput-optimized antibody glycosylation analysis by liquid chromatography–mass spectrometry (LC–MS) with enzyme-linked immunosorbent assay (ELISA). It allows the assessment of the Fc glycosylation of low-abundance ASAs in large clinical cohorts, relying on 96-well plate-based purification of antibodies from serum or plasma (Fig. 2). Additionally, it can be used for antibody glycosylation analysis of total IgG, preclinical, in vitro or biotechnological samples, and saliva or synovial fluid.

ELISA is used extensively to (semi)quantitatively assess ASAs, combining the affinity purification of ASAs with a biochemical signal conversion and a simple spectrophotometric readout. Importantly, ELISA is highly amenable to parallelization and allows 96-well (or higher) formats. However, it does not provide proteoform resolution, preventing the assessment of antibody glycosylation. GlYcoLISA addresses this shortcoming by replacing the spectrophotometric readout of ELISA with an LC–MS-based characterization of IgG Fc glycosylation. As sample preparation and measurements of the LC–MS method are throughput optimized, compatibility with the 96-well affinity purification is maintained[25].

The following choices and developments in sample preparation and LC–MS analysis[25,26] were instrumental in allowing the characterization of the glycosylation of low-abundance ASAs in large clinical cohorts[25,26]. Reduction of the LC dimensions and the consequential reduction of the flow rate to a nanoliter flow regime allows very efficient nano electrospray ionization, partially remedying the ionization preference of peptides over glycopeptides under common reversed-phase conditions[27]. Furthermore, glycopeptide ionization was increased by implementing a sheathless nano-electrospray ionization (nano-ESI) interface, together with dopant-enriched nitrogen gas[25,28]. A straightforward 96-well-based sample preparation, an on-line desalting and preconcentration step, and a relatively short LC–MS measurement time[27,29] allow the processing of 1,000s of samples in a matter of weeks[30]. The resulting large amounts of complex data are processed using a number of (pre)processing steps, a workflow that was developed in synergy with the physical method[29,31]. Continuous improvements in sensitivity now allow the LC–MS-based glycoprofiling of even low-nanogram amounts of IgG[32].

Integration of the 96-well-plate-based affinity purification of ASAs and their LC–MS glycoprofiling presented a challenge due to the limited compatibility of LC–MS for involatile buffers. This challenge was first overcome in 2009 in the earliest version of GlYcoLISA[33].

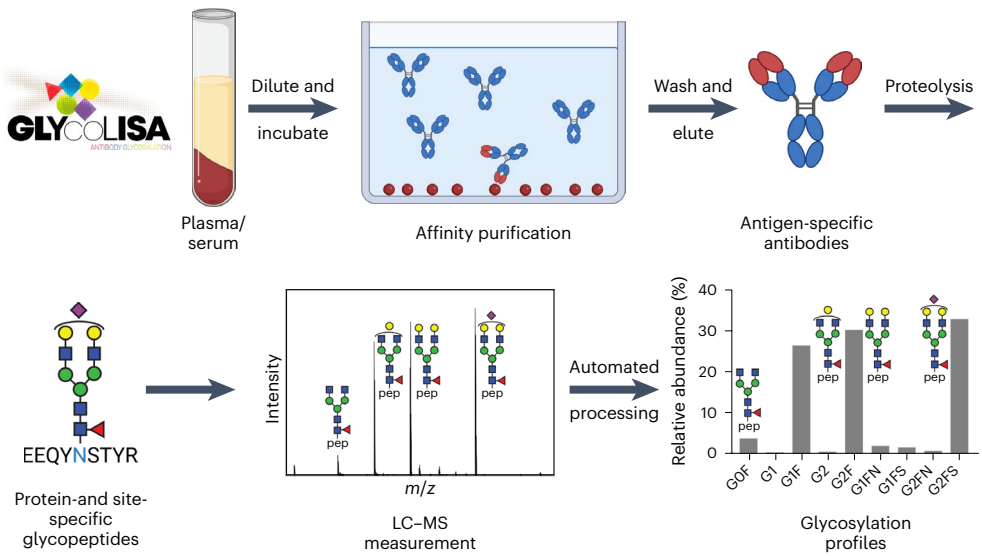

Fig. 2 | GlYcoLISA workflow. GlYcoLISA combines a classic 96-well-based immunosorbent assay with LC–MS profiling of IgG Fc *N*-glycosylation in a high-sensitivity, throughput-optimized manner. Automated data-processing strategies are essential to GlYcoLISA. Pep, peptide; depends on IgG subclass and allotype. Created with BioRender.com. GlYcoLISA logo by Marco Ceschi.

Applications of the method

An overview of previous applications of GlYcoLISA can be found in Table 2. GlYcoLISA lends itself primarily to studying the glycosylation of disease-specific antibodies, but can also be applied to studying antibody glycosylation in the context of vaccine responses, or changes in the glycoform composition of therapeutic antibodies in preclinical pharmacokinetics models. Differences in antibody glycosylation between different diseases can provide important clues to disease mechanisms and improve the understanding of basic immunology. Differences and similarities in antibody glycosylation between natural infections and vaccination-induced responses could also give hints to the efficacy and safety of vaccines.

GlYcoLISA was first employed to study autoantibodies in rheumatoid arthritis[12,34]. This was extended to other autoimmune diseases, such as autoantibody-associated vasculitis and autoimmune hemolytic anemia[21,35,36], and then to alloimmune pathologies, including fetal or

Table 2 | Overview applications of GlYcoLISA

Disease or intervention	Study[a]	Antigen	Main findings[b]
Rheumatoid arthritis	Scherer[33]	Citrullinated peptides	Anti-citrullinated protein autoantibodies (ACPAs) exhibit reduced Gal and Sia compared with total IgG1
	Rombouts[34]	Citrullinated peptides	ACPAs exhibit high Fuc and acquires low Gal in the transition from arthralgia to arthritis[c]
	Bondt[12]	Citrullinated peptides	Pregnancy-induced amelioration of Rheumatoid Arthritis associates with high Gal of ACPA[c]
Autoantibody-associated vasculitis	Wuhrer[35]	Proteinase 3 (PR3)	Low Gal, Sia and Bisec of anti-PR3 antibodies compared with total IgG of patients and healthy volunteers
	Kemna[21]	Proteinase 3	Low Gal and Sia of total IgG1, but not anti-PR3 antibodies, predicts disease reactivation[c]
Autoimmune hemolytic anemia	Sonneveld 2017 (*Scientific Reports*)[36]	Red blood cells (RBCs)	Patients show low Gal and Sia of anti-RBC antibodies compared to healthy volunteers. In patients, anti-RBC antibodies show low Gal and high Sia compared to total IgG1
Fetal or neonatal alloimmune thrombocytopenia	Kapur 2014 (*Blood*)[14]	Human platelet antigen 1a (HPA1a)	Low Fuc of anti-HPA1a antibodies correlates with disease severity
Hemolytic disease of the fetus and newborn	Kapur 2014 (*British Journal of Haematology*)[37]	Blood antigen D	Very low Fuc of anti-D antibodies correlates with ADCC activation and disease severity
	Sonneveld 2017 (*British Journal of Haematology*)[38]	Blood antigen c, E and K	High Gal and Sia for anti-c antibodies and low Fuc for anti-K antibodies correlates with disease severity
	Sonneveld[39]	Blood antigen K	Low Fuc, low Bisec and high Gal of anti-k antibodies associated with disease severity
Malaria	Larsen[18]	*Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1)	Low Fuc anti-PfEMP1 antibodies result from natural infection and not from vaccination[c]
COVID-19	Larsen[13]	SARS-CoV-2 S and nuclear protein	Low Fuc characterizes early anti-S responses and correlates with disease severity[c]
	Pongracz[20]	SARS-CoV-2 S protein	Early low Gal, low Sia and high Bisec of anti-S antibodies correlate with disease severity and inflammatory markers[c]
	Siekman[40]	SARS-CoV-2 S protein	Hospitalized patients with COVID-19 show low Fuc and Bisec of anti-S antibodies compared to outpatients[c]
	Sustic[63]	SARS-CoV-2 S protein	Comparable anti-S antibody Fuc levels were measured by GlYcoLISA and a Fucose-sensitive ELISA for antigen-specific IgG (FEASI)
SARS-CoV-2 vaccination	Van Coillie[43]	SARS-CoV-2 S protein	The BNT162b2 mRNA SARS-CoV-2 vaccine induces transient low Fuc anti-S antibodies
	Buhre[19]	SARS-CoV-2 S protein	The long-term anti-S glycosylation profiles induced by mRNA and vector vaccines against SARS-CoV-2 are comparable[c]
Platelet refractoriness	Van Osch[44]	Class I HLA	Anti-HLA antibodies are characterized by high Gal and Sia. These glycosylation features associate with disease severity
Antibody-mediated kidney rejection	Bharadwaj[45]	HLA A2	Afucosylated anti-HLA A2 antibodies might drive rejection through more efficacious FcγRIIIa binding, leading to increased ADCC

[a]First author surname and reference number, with year of publication and journal name provided where ambiguous. [b]All statements relate to IgG1 throughout. [c]Longitudinal study. Bisec, bisection; Fuc, fucosylation; Gal, galactosylation; Sia, sialylation.

neonatal alloimmune thrombocytopenia[14] and hemolytic disease of the fetus and newborn[37–39]. Associations of IgG glycosylation with disease severity and treatment effects are frequently observed. In principal, the technique could be used to conduct research on the involvement of IgG glycosylation in any autoimmune or alloimmune disease with a prominent autoantibody response of the IgG class.

Recent studies have demonstrated the utility of GlYcoLISA in studying infectious diseases, including malaria[18] and coronavirus disease 2019 (COVID-19)[13,20,40]. In malaria infections, the low fucosylation of antiparasite antibodies was observed[18]. These potentially protective responses were specific to membrane-bound antigens formed in the red blood cell stages of the parasite in natural infections and were not found in total IgG, specific IgG directed against antigens of the merozoite stage, or in the IgG of individuals vaccinated with a subunit vaccine. By contrast, in SARS-CoV-2 infections that led to COVID-19, the low fucosylation of anti-S protein antibodies (compared with total IgG) positively correlated with the development of acute respiratory distress syndrome, and interleukin-6 and C-reactive protein levels[13]. Consistent with that, low fucosylation of anti-S antibodies boosted interleukin-6 production by macrophages in vitro[13]. Anti-human immunodeficiency virus antibody glycosylation has received recent attention and could potentially benefit from implementation of the GlYcoLISA approach[41]. Furthermore, antibody glycosylation in response to tuberculosis has been studied using alternative methods that could be exchanged for or supplemented with GlYcoLISA[42]. Interestingly, skewed antipathogen antibody glycosylation has been implicated in both beneficial and detrimental immune activation[13,41].

In addition to its use in studying pathogens, GlYcoLISA has been applied to the investigation of antibody responses elicited by therapeutic interventions, including those induced in vaccinations[18,19,43], or as side effects in platelet transfusion[44] and kidney transplantation[45].

GlYcoLISA is optimized for investigating IgG responses, but there are subsets of diseases in which antibody isotypes other than IgG play a prominent role. For example, aberrant IgA responses have been implicated in rheumatoid arthritis and IgA nephropathy[46,47], and IgA glycosylation has been recognized as being potentially important in these diseases[47,48]. Throughput-optimized methods for the site-specific and protein-specific glycosylation analysis of IgA, compatible with the GlYcoLISA strategy, have now been published[49]. Multi-isotype methods would be most advantageous in this context, as they avoid the selective enrichment of isotypes in addition to antigen-based enrichment[49,50]. Although investigation of IgA glycosylation is not possible using the current protocol, we expect that the antigen-specific analysis of IgA glycosylation following the GlYcoLISA principle will be feasible in the coming years.

Comparison with other methods

Affinity purification

Instead of immobilizing the antibody target directly to the 96-well plate, the target may be immobilized on beads, for example, agarose or magnetic beads[41]. Using 96-well filter plates for agarose beads or magnetic retention, such formats can be easily used in the same workflow[25]. However, for many applications, plates for direct immobilization or protocols for their preparation already exist from ELISAs. Thus, it is most efficient to use these by implementing LC–MS-compatible wash and elution conditions.

There are many other formats for affinity purification, such as columns and cartridges. However, these are largely inconvenient for multiplexing. Furthermore, large elution volumes will increase the time consumption for the eluate drying (Step 7). Nonetheless, where these formats are sufficiently miniaturized and multiplexed, they should offer suitable alternatives.

A limitation shared with most ELISA formats is that the immobilization of soluble antigens may affect the ability to target conformational or quaternary epitopes[51]. In ELISA, this can be solved by first forming the antigen–antibody complex in solution and then capturing the first antibody with an immobilized second one, albeit at the expense of creating a more complex assay. Whether a similar approach would be possible for GlYcoLISA is unknown and will probably depend on the antigen and how much interference its tryptic peptides would create during the LC–MS measurements.

Other detection methods

LC–MS offers multiple dimensions of high selectivity that are often needed to obtain high-quality data for ASA glycosylation. By contrast, more convenient detection methods, such as lectin microarrays[52] or panels of anticarbohydrate antibodies, suffer from low glycan specificity and lack of protein specificity[2]. Proteolytic cleavage steps (Steps 9 and 10) used in this protocol, which produce tryptic glycopeptides of the conserved Fc-glycosylation site, provide protein specificity and site specificity in the LC–MS analysis. IgG1, IgG3 and IgG4 produce tryptic glycopeptides with unique amino acid sequences, which allows their chromatographic separation and differential glycosylation profiling[25]. If present, IgG2 produces tryptic peptides with the same amino acid sequence as IgG3 or IgG4 depending on the allotypes, the former being dominant in white populations and the latter being dominant in studies of Chinese citizens, for example. Therefore, for most white donors, IgG2 and IgG3 glycosylation cannot be distinguished if both responses occur. Protein specificity is key to robust performance and easy adaptation of existing immunosorbent assays, as it is also provided in ELISAs by the secondary (detection) antibody.

Released glycan-based detection methods, such as capillary electrophoresis with laser induced fluorescence detection have been used to study antigen-specific IgG glycosylation but lack protein specificity[2,42,53]. With these methods, nonspecific binding in the immunosorbent step needs to be much more tightly controlled than with a protein-specific method. This is especially true when profiling ASAs due to their low concentration compared with the total antibody pool. Importantly, only a protein-specific method can provide an isotype-resolved and subclass-resolved glycosylation analysis, as the immunosorbent step indiscriminately enriches all isotypes and subclasses with the correct antigen specificity.

Expertise needed to implement the protocol

This protocol requires specific training in protein analysis by LC–MS. Such expertise is typically found in proteomics research groups or proteomics core facilities. As the latter are established in most larger companies and research institutes with an interest in antibodies, relevant researchers should be able to access this expertise. All such facilities should have sufficient bioinformatics expertise to implement the data processing although the data processing approaches necessarily differ markedly from standard proteomics workflows; facilities without experience in glycoproteomics should be aware of time investments for their implementation.

Limitations

The LC–MS and data processing expertise needed for GlYcoLISA is a hurdle for widespread implementation. However, many MS core facilities are beginning to implement glycoproteomics in their portfolio or have experience with other PTMs. Consequently, and due to the comparably low complexity of performing glycoproteomics on a single protein with a single glycosylation site, the implementation of GlYcoLISA seems timely and feasible. All MS core facilities offering proteomics services will have experience with automated data processing and great advances have been made in glycoproteomics data processing, encompassing commercial and open-source tools. For laboratories already specialized in LC–MS glycoproteomics, the off-the-shelf approach to affinity purification used by GlYcoLISA will greatly facilitate implementation and implementation at MS core facilities will give many groups at the respective institution access to the method. Despite limitations regarding widespread implementation, we thus expect the method to become available to many researchers over time.

ELISAs are omnipresent in laboratories and readily accessible, and a vast number of protein targets are available as commercial ELISAs or present in laboratories already studying an antigen of interest. Furthermore, protein that can be recombinantly produced can be implemented in an ELISA in a relatively straightforward way. While GlYcoLISA is currently limited to IgG, its extension to other isotypes will greatly expand the application range.

Due to the high sensitivity of GlYcoLISA, sample volume requirements are often much lower than for other biomolecular analyses and absolute sensitivities in the low nanomole range have been achieved. However, there are some scenarios where this might be insufficient, such as if analyzing samples from presymptomatic patients or those with immunodeficiency.

As we discussed in the 'Comparison with other methods' section, released glycan-based methods have important drawbacks in specificity, sensitivity and throughput. However, they provide some additional information regarding important isomeric glycan structures, such as distinguishing monogalactosylation of the α1,3-arm versus the α1,6-arm, which affects FcγRIIIa binding[54]. Such structural details are not resolved in GlYcoLISA owing to the focus on throughput.

Maybe the most important limitation of GlYcoLISA is the fact that a relative quantitation is obtained. Alhough this has advantages in terms of precision and robustness, the ultimate functional impact of an antibody is determined by a combination of its glycoprofile and its concentration. Both properties can be obtained separately by GlYcoLISA and quantitative ELISA, and integrated post hoc. However, a direct absolute quantitation of antibody glycoforms using the method would be an important step forward. We have obtained very promising preliminary results in this respect, but choose to omit such considerations here before their strengths have been sufficiently demonstrated in applications.

Experimental design

Choice of sample

The protocol is optimized to be performed on plasma or serum from patients positive for IgG directed against the antigen of interest. However, other biofluids could be considered[55]. Often, antigen positivity has been measured for diagnosis or can be reliably deduced from it. When antigen positivity is unclear, a simple assay, such as ELISA, should be performed before selecting samples for GlYcoLISA. Importantly, plasma or serum from healthy volunteers or patients that have not been exposed to the antigen of interest or do not make antibodies against it for other reasons should be included as negative controls. As positive controls and technical replicates, a pool of patient plasma or sera with confirmed antigen positivity will be generated. Twenty microliters of plasma or serum is required per patient, but this may depend on expected concentrations of specific IgG. A small amount of extra material is needed for the pools and for the total IgG glycoanalysis (where indicated). Antibody glycosylation is very stable upon storage of serum or plasma[56,57]; therefore, the protocol can be performed on residual sample from legacy cohorts to gain new insights, provided sufficient coverage of consent and ethical clearance exists.

Randomization and treatment of confounders

Antigen-specific and total IgG samples should be kept on separate plates. This measure will limit the effect of any cross-contamination during sample preparation and carryover in consecutive LC–MS runs. For cross-sectional comparisons, samples should be randomized. However, a randomization that equally distributes sample groups between batches (typically pairs of 96-well plates) is preferable to a completely random setup as it avoids artifacts if batch correction is needed. In contrast, for longitudinal studies, samples from one patient or volunteer should be keep closely together because they will be compared with each other. This avoids the introduction of batch effects. Age and sex are well known confounders of IgG glycosylation, which need to be addressed by age matching and sex matching of the clinical groups or by the application of a statistical model capable of estimating and correcting for confounders, such as treatment with immunosuppressant drugs.

When subjects are treated with therapeutic monoclonal or polyclonal antibodies, these will be cocaptured in the total IgG (or in the specific IgG purifications if polyclonal antibodies are derived from antigen-experienced donors). Therefore, sampling should be performed with sufficient delay after treatment so that the therapeutic antibodies have been cleared to an extent that their contribution to antibody glycosylation profiles is minimal. Hormone therapy, especially estrogen, and altered physiological states, such as pregnancy, may likewise affect antibody glycosylation.

Immunosorbent assay

The capacity of the capturing material should be considered when planning the amount of serum/plasma to be used. High-affinity antibodies might outcompete those with low affinity if this capacity is exceeded, which could bias the analysis toward high-affinity antibodies.

Separate readouts for the glycosylation of IgG2 and IgG3 can be achieved through an additional affinity separation using Protein A[26]. However, this approach doubles the amount of data acquisition and the additional sample preparation decreases sensitivity. In addition, many immune responses already have a subclass preference, limiting the utility of this option to specific scenarios[39].

We have provided an example of the antigen immobilization and immunosorbent assay in the procedure. However, some details will depend on the specific ELISA from which the desired GlYcoLISA derives. For example, the optimal protein concentration should be taken from the ELISA as it may differ between antigens. Furthermore, the balance between average antibody affinity and propensity for nonspecific binding depends on the physicochemical properties of the antigen and will sometimes require optimization of the washing steps (Step 3).

LC–MS setup

An essential part of assuring high-quality GlYcoLISA data is to monitor cross-contamination introduced during sample preparation and carryover from one LC–MS run to the next. This is typically done by checking for analyte signals in blanks that underwent all sample preparation steps. For efficient troubleshooting, system blanks that do not undergo any sample preparation should be used to distinguish between cross-contamination and carryover, where positive signal in the system blanks indicates carryover. Due to the huge enrichment challenge, the high sensitivity and the asymptotic concentration-response behavior of LC–MS, blanks are rarely entirely devoid of analyte signals, although such signals should be much lower than those observed for the samples (see also, troubleshooting). To avoid a large impact of minor carryover due to large concentration differences, follow the suggestions in Step 12.

For an accurate and robust quantitation, especially of oligomannose-containing and sialic acid-containing glycopeptides, postsource decay must be carefully controlled. With the described setup, postsource decay can be limited to less than 0.5%, although ion-transfer settings may need to be optimized for the specific instrument. Successful minimization can be checked using human IgG from healthy donors; such IgG should contain only a few permille of monoantennary fucosylated glycans. However, as postsource decay generates the corresponding masses/structures from the dominant diantennary fucosylated glycans, a combined relative abundance of monoantennary glycans of 1% or more indicates excessive postsource decay.

Data processing

The quality of samples and LC–MS measurements will largely dictate choices during data processing, especially if analyte signals are low due to low antibody concentrations. A first pass spectral curation is provided by the alignment and calibration settings, excluding analyses failing in these steps. Furthermore, LaCyTools provides—for every glycopeptide in every sample—the mass error after calibration (ppm), the signal-to-noise ratio (S/N) and a measure for how well the isotopic pattern is matching the theoretical expectation for the given elemental composition (IPQ). Analyte and spectral curation is based on minimum requirements for reliable quantitation. The following settings can generally be applied, given a comparable instrumental setup, as they present a good compromise between excluding false positives and avoiding false negatives: $-20 < \text{ppm} > 20$, $S/N \geq 9$ and $IPQ \leq 0.2$. The cutoff percentage can be used for fine-tuning the average quality of the included analytes; a high ratio (≥80%) effectively excludes analytes quantified with low accuracy and precision while a low ratio (25–50%) may be needed at low data quality to be able to include a sensible number of analytes and spectra for sufficient statistical power. All analytes passing curation (consensus list) are quantified in all spectra. Spectral curation is ideally based on comparison to negative controls. In addition, a small percentile (2–5%) of the lowest-quality spectra may be excluded to improve the overall quality of the dataset.

Glycosylation traits represent common biosynthetic events and often represent epitopes determining biological function, for example, via the interaction with lectins[58]. Consequently, the functional, biological and clinical impact of glycosylation traits is often easier to interpret than that of individual glycans. Furthermore, glycosylation traits have important advantages for statistical treatment, such as the reduction of interdependency and the number of features.

Vendor data files (raw data) as well as initial and aligned mzXML are typically between 100 and 200 MB per sample. For data management, we therefore advise to store only the raw

data and delete the mzXML files after processing is finished. The other files generated by LaCyTools are comparably small, but very informative regarding processing and should thus be retained.

Materials

Samples and controls

- IgG samples: the protocol is optimized for use with plasma or serum from patients positive for IgG directed against the antigen of interest
- Control sample: controls can include plasma or serum from healthy controls, patients that have not been exposed to the antigen of interest or patients who do not make antibodies against it for other reasons
- Positive control: a pool of aliquots from the antigen-positive patients

▲ **CRITICAL** Due to the use of patient material and data (such as age, sex and medication use), ethical approval and participants' informed consent must be obtained before conducting the study. Our study was approved by the Medical Ethics Committee Leiden-Den Haag-Delft (NL73740.058.20). The trial was registered in the Dutch Trial Registry (NL8589). The study complied with the latest version of the Declaration of Helsinki.

Reagents

Reagents for affinity purification and proteolytic cleavage

- Recombinant protein antigen

▲ **CRITICAL** It is preferable to use antigen sources and batches that have already been successfully used in ELISAs. Proteins are dissolved in PBS, although other buffers might be tolerated at high stock concentrations around 1 mg/ml or higher. The antigen should not contain other proteins as additives or contaminants; IgG contamination may produce false signals, while other proteins may compromise antigen specificity.

- Deionized and filtered water. We use water deionized and filtered generated with a Purelab Ultra system (Veolia Water Technologies Netherlands B.V.) set at ≤18.2 Ω
- Disodium hydrogenphosphate (Sigma-Aldrich, cat. no. 71643)
- Potassium dihydrogenphosphate (Merck, cat. no. 1.04873)
- Sodium chloride (Sigma-Aldrich, cat. no. 31434)
- Trypsin (Promega, cat. no. V5111)
- Nunc immunoplate Maxisorp 96-well plate (Thermo Fisher Scientific, cat. no. 442404)
- Adhesive plate seal (Westburg, cat. no. AB-0580)
- Tween 20 (Sigma-Aldrich, cat. no. P9416)
- Ammonium bicarbonate (ABC; Merck, cat. no. 09830)
- Formic acid (VWR, cat. no. 84865.180)

▲ **CAUTION** Concentrated formic acid is corrosive. Handle in fume hood and with personal protective equipment.

- Polystyrene V-bottom 96 well Microplate (Greiner Bio-One, cat. no. 651101)
- Trypsin cleavage mix ('Reagent setup')
- Glacial acetic acid (Merck, cat. no. 1.00063)
- Special indicator paper pH 5.5–9.0 (Macherey-Nagel, cat. no. 90209)

Reagents for LC–MS

- 10 ml vials. 46×22.5 mm (Merck, cat. no. 854180-U) with 20 × 0.75 mm polytetrafluoroethylene/silicone septa (Merck, cat. no. 27539) and 20 × 9.5 mm open center aluminum crimp seals (Merck, cat. no. 27230-U)
- 2 ml clear glass vials 12 × 32 × 6 mm (outer diameter (OD) × height (H) × inner diameter (ID)) (Merck, cat. no. 27265)
- Polypropylene screw caps with hole and 1.5 mm polytetrafluoroethylene/silicone septum (Merck, cat. no. 27273)

- Acetonitrile (ACN), HPLC grade (Biosolve, cat. no. 01203502)
- Water, LC–MS grade (Biosolve, cat. no. 0023214102BS)
- Isopropanol, LC–MS grade (Biosolve, cat. no. 16267802)
- Trifluoroacetic acid (TFA), LC–MS grade (Thermo Fisher Scientific, cat. no. 85183)

Equipment

- 1,200 µl, 200 µl and 50 µl multichannel pipets (Mettler Toledo; Rainin L12-1200 XLS cat. no 17014497, Rainin L12-200 XLS cat. no 17013810 and Rainin L12-50 XLS cat. no 17013809, respectively)
- 20, 200 and 1,000 µl pipette tips (Mettler Toledo, cat. nos. 17005091, 17005093/30389299 and 30389292, respectively)
- 20 µl filter pipet tips (Mettler Toledo, cat. no 30389274)
- Rainin AutoRep-E repetition pipet equipped with Rainin 0.5 ml repetition tip (cat no. ENC-500)
- Refrigerator (Liebherr)
- Plate shaker (Heidolph, Titramax 100 or 101, cat. no. 544-11200-00 or 544-11300-0**0**)
- Incubator (Termaks, cat. no. B9051)
- Centrifugal vacuum dryer (Speedvac; Christ, RVC 2-33 CDplus)
- Vortex mixer (Velp Scientifica, ZX3)
- Centrifuge (Beck Coulter, Avanti J-15R)
- Nano-liquid chromatograph-electrospray–MS instrument (Thermo Fisher Scientific, Dionex Ultimate 3000)
  ▲ **CRITICAL** The nano-liquid chromatography instrumentation consists of an autosampler (WPS-3000TFC Analytical), nanoflow pumps with integrated loading pump and column oven (NCS3200RS nano) and a solvent degasser (SRD-3400). The nano-ESI–MS instrumentation consists of an impact HD quadrupole–time-of-flight mass spectrometer (Bruker Daltonics); a CaptiveSpray nano-ESI source with nanoBooster option (Bruker Daltonics); titanium union (Bruker Daltonics, cat. no. 1823008); a CaptiveSpray emitter, tapered tip 20 µm ID (Bruker Daltonics, cat. no. 1822990) and a glass capillary, Glass-D6,48-D0,5-L180-R1GOHM (Bruker Daltonics, cat. no. 873044). See 'Equipment setup' for settings.
- Analytical column, reversed-phase, NanoEase M/Z peptide BEH C18, 1.7 µm particles, 130 Å pores, 75 µm × 100 mm (Waters, cat. no. 186008792)
- Trap column, reversed-phase, PepMap Neo C18, 5 µm particles, 100 Å pores, 0.3 × 5 mm (Thermo Fisher Scientific, cat. nr. 174500)
- 350–250 µm Teflon sleeve (Thermo Fisher Scientific, cat. no. 160489)
- Fused silica capillary, 280 µm OD 20 µm ID (Thermo Fisher Scientific, cat. no. 160475)
- Peek tubing, 300 µm ID (Thermo Fisher Scientific, cat. no. 160492)
- NanoViper tubing, 30 µm ID × 10 cm (Thermo Fisher Scientific, cat. no. 164648)
- Peek tubing, 400 µm ID IDEX sleeve orange 0.062" × 0.16" (SciPro, cat. no. F230)
- TSP fused silica tubing 30.5 cm 100 µm ID 363 µm OD, total volume 2.4 µl (BGB Analytik Benelux BV, cat. no. TSP-100375)
- Modern Windows server for data processing: we operate the software using a 3.10 GHz Intel Xeon Gold 6345 (two processors per user) with 512 GB random-access memory and no dedicated graphics processing unit. The estimations under timing are based on these specification, but they do not constitute minimum requirements
- Software
  - MSConvertGUI of ProteoWizard (version 3.0.8708; https://proteowizard.sourceforge.io/)
  - MZmine (version 2.53; https://mzmine.github.io/)
  - LaCyTools (V2.1.0; GitLab (https://git.lumc.nl/cpm/) or GitHub (https://github.com/Tarskin/LaCyTools))

Reagent setup

PBS

Mix 142.5 g disodium hydrogen phosphate, 212.5 g sodium chloride and 11.9 g potassium dihydrogen phosphate. Add this powder mix in portions of ~50 g to 4 L of water under magnetic stirring allowing for a waiting time of 5 min per portion. Use 1 L water to rinse powder containers

and add to the rest of the solution. A 5× concentrated stock solution is obtained, which has a pH of 7.3 and can be stored for up to 12 months at room temperature (18–23 °C). 200 ml of this 5× stock should be added to 800 ml water before use, resulting in a solution of 32 mM disodiumhydrogenphosphate, 3.5 mM potassiumdihydrogenphosphate and 145 mM sodium chloride with pH 7.6 that can be stored for up to 6 months at 4 °C.

PBS-T (PBS with 0.05% (vol/vol) Tween 20)
Add 50 µl Tween 20 (Sigma-Aldrich, cat. no. P9416) to 100 ml PBS.

50 mM ABC
Dissolve 395.2 mg ABC powder in 100 mL water.

Trypsin cleavage mix
Prepare 20 mM acetic acid by adding 57.2 µl glacial acetic acid to 50 ml water and cool the acetic acid on ice. Reconstitute 20 µg lyophilized sequencing grade trypsin in 100 µl of the ice-cold 20 mM acetic acid. The 20 mM acetic acid can be stored for 6 months at 4 °C. The acidic trypsin solution should be prepared fresh, but can be stored for 1 month at −80 °C given a maximum of three freeze/thaw cycles.

LC–MS eluents
Prepare the LC–MS eluents as follows.
Eluent A, 0.02% (vol/vol) TFA: add 200 µl TFA to 1 L bottled water, sonicate before use.
Eluent B, 95% (vol/vol) ACN: add 25 mL bottled water to 475 ml ACN, sonicate before use.
Seal wash solution, 5% (vol/vol) isopropanol: add 50 mL isopropanol to 950 ml bottled water.
Syringe wash solution, 3% (vol/vol) ACN: Add 30 mL ACN to 970 mL bottled water.
Trap column wash solution: Mix 500 ml isopropanol with 500 ml bottled water and 200 µl TFA
▲ **CRITICAL** Regularly check the solutions for particulate matter or an 'oily' sheen, which indicate fouling. We recommend exchanging these solutions at least every 3 months to avoid extensive shifts in the pH or water/organics ratio.

Equipment setup: LC–MS settings

LC settings

- Loading pump flow 25 µl/min of eluent A
- Column oven (containing trap and analytical column), 45 °C
- Gradient pump flow 600 nL/min of a mix of eluent A and B
- Two-step linear gradient:
  - 0 min: 3% eluent B
  - 4.5 min: 21.7% eluent B
  - 5.5 min: 50% eluent B
  - 8.0 min: 50% eluent B
  - 9.0 min: 3% eluent B
  - 11.5 min: 3% eluent B

Valve settings

- 0 min: injection of sample
- 0–1 min: loading pump via autosampler loop to trap column
- 1–6 min: gradient pump via trap column (reverse direction) to analytical column
- 6–11.5 min: gradient pump directly to analytical column
- 6–11.5 min: loading pump to trap column; three injections of 2.5 µl of trap column wash solution

Ionization and transfer settings

- 500 ml/min Nitrogen gas doped with can supplied via the nanoBooster setup
- Ion source settings:
  - End plate offset 500 V
  - Capillary voltage 1,200 V

  - nanoBooster pressure 0.2 bar
  - Dry gas 3.0 L/min
  - Dry temperature 180 °C
- Transfer settings:
  - Funnel 1 RF 400.0 Vpp
  - Funnel 2 RF 600.0 Vpp
  - isCIS Energy 0 eV
  - Hexapole RF 300.0 Vpp
- Quadrupole settings:
  - Ion energy 2.0 eV
  - Low mass *m/z* 300.00
- Collision cell settings:
  - Collision energy 5.0 eV
  - Collision RF 2,100.0 Vpp
  - Transfer time 110.0 µs
  - Prepulse storage 21.0 µs

MS data acquisition settings

- Timing and duration: 60 s after injection for 300 s
- Ion mode: positive
- Range: *m/z* 550–1,800
- Rate: 1 Hz

Procedure

Antigen immobilization

● **TIMING (per two 96-well plates) 20 min + overnight incubation + an additional 10 min**

1. Dilute the recombinant protein antigen to 1 µg/ml in PBS. 26 ml is enough for one 96-well plate.
   ▲ **CRITICAL STEP** One 96-well plate will accommodate approximately 90 samples and relevant standards and controls.
2. Pipette 250 µl of diluted antigen into the wells of a 96-well Maxisorp plate. Seal the plate and incubate at 4 °C overnight to coat the plate with antigen.
3. Remove protein solution and wash the wells three times with 250 µl PBS-T. Solutions should be removed as completely as possible in each (of these four) instance(s) by firmly tapping the plate upside-down on a stack of tissues. Immediately continue with Step 4.

Immunosorbent assay

● **TIMING (per two 96-well plates) 30 min + 1 h incubation + 45 min + 2–3 h drying**

4. Add 180 µl PBS-T then 20 µl serum to the coated Maxisorp plate. Incubate for 1 h at 37 °C under shaking to capture IgG.
   ▲ **CRITICAL STEP** Shaking should be firm enough to induce movement in the solution, but mild enough to avoid cross-contamination between the wells by spill. We use 300 rpm on the plate shaker.
5. Empty the wells with a multichannel pipette, using a fresh tip for each sample. Wash each well three times with 250 µl PBS-T, then two times with 250 µl PBS and two times with 250 µl 50 mM ABC. Wash liquids can be removed by inversion of the plate (for effective removal, see Step 3).
6. Elute the IgG by adding 200 µl of 100 mM formic acid to each well using a multichannel pipette and incubating for 5 min at room temperature under shaking (see instructions in Step 4 regarding shaking speed). Transfer the eluate into a V-bottom plate.
7. Dry the eluate in a speedvac for 2–3 h at 50–60 °C.
   ▲ **CRITICAL STEP** Check thoroughly that no visible liquid remains in the samples. Insufficiently dried IgG will be exposed to concentrated acid, resulting in loss of sialic acid.

To a lesser extent, sialic acid might be lost upon elongated exposure to the drying process. Operation with infrared heating will speed-up drying but is not essential.

■ **PAUSE POINT** Properly dried samples can be stored for at least 1 month at −20 °C. Alternatively, eluates can be stored for up to a week at −20 °C before drying.

Proteolytic cleavage

● **TIMING (per two 96-well plates) 30 min + overnight incubation**

8. Redissolve the dried IgG in 20 µl 50mM ABC for 5 min under shaking at 450 rpm and room temperature. For improved efficiency, the reader may use 20 µl of a mixture of 85% 50mM ABC and 15% of ACN instead, although avoiding the use of the harmful and highly flammable ACN in this step is slightly preferred for work safety considerations.
9. Dilute acidic trypsin solution in ice cold water 1:20 (vol/vol) to a concentration of 10 ng/µl to obtain trypsin cleavage mix. Add 20 µl of the trypsin cleavage mix to each well and shake for 5 min at 450 rpm and room temperature.

   ▲ **CRITICAL STEP** The trypsin cleavage mix must be freshly prepared. Check the pH for three random samples per 96-well plate by dropping 0.5 µl of sample on the indicator paper. The pH should be between 7 and 8. Residual acid may interfere with cleavage efficiency; if below pH 7, add 1 µl of 1 M ABC, check the pH again and repeat if necessary.
10. Seal the plate with an adhesive plate seal and incubate overnight at 37 °C.

    ■ **PAUSE POINT** After overnight incubation and equilibration to room temperature, samples may be stored at −20 °C (ref. 57). During LC–MS measurements (below), it is possible to keep the samples for several days at 4 °C in the autosampler.

    ◆ **TROUBLESHOOTING**

LC–MS measurements

● **TIMING preparation 45 min (two plates) + measurements 11.5 min per sample**

11. If necessary, thaw samples under shaking at 450 rpm. Centrifuge the plate for 10 min at 780*g* at room temperature. Keep samples in the thermostat autosampler of the LC–MS instrument at 4 °C until injection.

    ▲ **CRITICAL STEP** Homogenization of the sample after freezing is essential to inject a representative portion of the sample and thus obtain reliable results. Centrifugation sediments particles that might otherwise clog and damage the LC–MS instrument.
12. Check the settings of the LC–MS instrumentation (see the 'Equipment setup') and program its autosampler to inject 5 µl of each sample. Note that due to the void volume of the injection needle, 7.4 µl of sample is used per injection.

    ▲ **CRITICAL STEP** Consecutive injections should contain either comparable amounts of IgG (glycopeptides) or lower concentrations should be injected first.
13. Provide a list with the injection order and identifiable sample names to the LC–MS instrumentation.
14. Perform LC–MS measurements according to the settings described in the 'Experimental setup'. Figure 3 provides an example of the data generated.

    ◆ **TROUBLESHOOTING**

Data processing

● **TIMING file conversion <1 min per sample + preparation of LaCyTools analysis 4 h + LaCyTools analysis ~3 min per sample + data curation and calculation of glycosylation traits 4 h**

▲ **CRITICAL** The data processing is centered around our open-source software package LaCyTools[31], available via GitLab (https://git.lumc.nl/cpm/lacytools) and GitHub (https://github.com/Tarskin/LaCyTools). The GitHub has been maintained since 2016 with regular critical updates and will remain so for the foreseeable future. All additional software performs routine manipulations or visualizations of standardized data formats and should therefore be exchangeable. Figure 4 gives an overview of the data processing workflow.

15. Convert vendor data files from the LC–MS analysis to 32 bit mzXML files using the MSConvert function of ProteoWizard (version 3.0.8708). The 'zeroSamples' filter should be

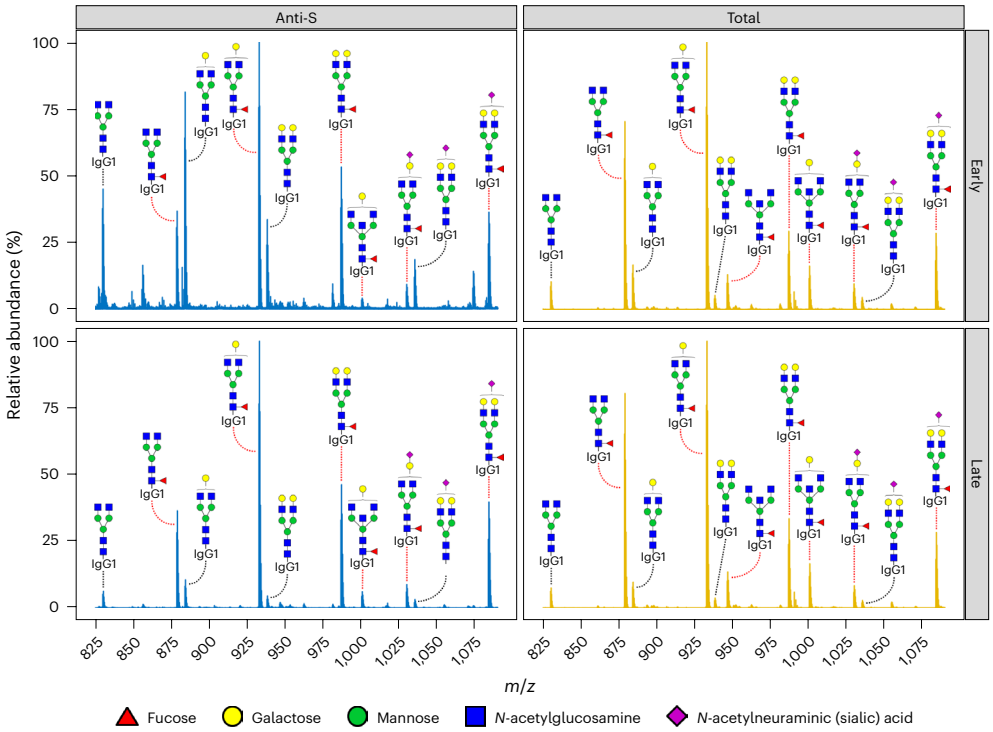

Fig. 3 | Representative mass spectra. Spectra showing a zoom of the *m/z* range containing the $[M+3H]^{3+}$ glycopeptide ions derived from sum spectra of the elution range of either anti-S IgG1 (left) or total IgG1 glycopeptides (right). The comparison exemplifies the lower fucosylation of early anti-S IgG1 responses in SARS-CoV-2 in comparison with the total IgG1 pool and to later stages of the infection. Reprinted from ref. 60, Springer Nature Limited.

used to reduce the file size. If MS/MS data was acquired simultaneously, make sure to select only the MS1 level data using the 'msLevel' filter.

16. Determine the retention time and alignment features by checking a representative set of samples for which IgG subclass glycopeptides are observed with sufficient quality (see further explanation below). There are two alternative sets of alignment features depending on the data:
    - In samples where only one subclass is present (probably IgG1), use the compositions H3N4F1, H4N4F1, H3N5F1, H5N4F1, H4N5F1 and H5N4F1S1 of the relevant subclass as alignment features
    - In samples where two or all subclasses are present, use H3N4F1, H4N4F1 and H5N4F1 of all represented subclasses as alignment features

Furthermore, determine the variation in the retention time of the alignment features, by opening all files in MZmine (version 2.53) and making extracted ion currents of the exact mass ±0.1 Th of all alignment features. If more than two 96-well plates are analyzed, use a dozen files per plate and add all files from the plates that show the highest and lowest retention. Write down the lowest and highest occurring retention time per alignment feature. The middle of each feature is its reference retention time and its distance to the border the ALIGNMENT_TIME_WINDOW used by LaCyTools. Create an alignment .txt file with the reference retention times. Importantly, if the alignment window is ~2 s or below for all features, alignment is not necessary and should be omitted for efficiency reasons.

▲ **CRITICAL STEP** Based on visual inspection of the extracted ion currents, there should be no other features of the same *m/z* with a comparable or higher intensity within the determined alignment region (reference ± window). Alignment on incorrect features might cause analytes to fall outside of the retention time window targeted for extraction.

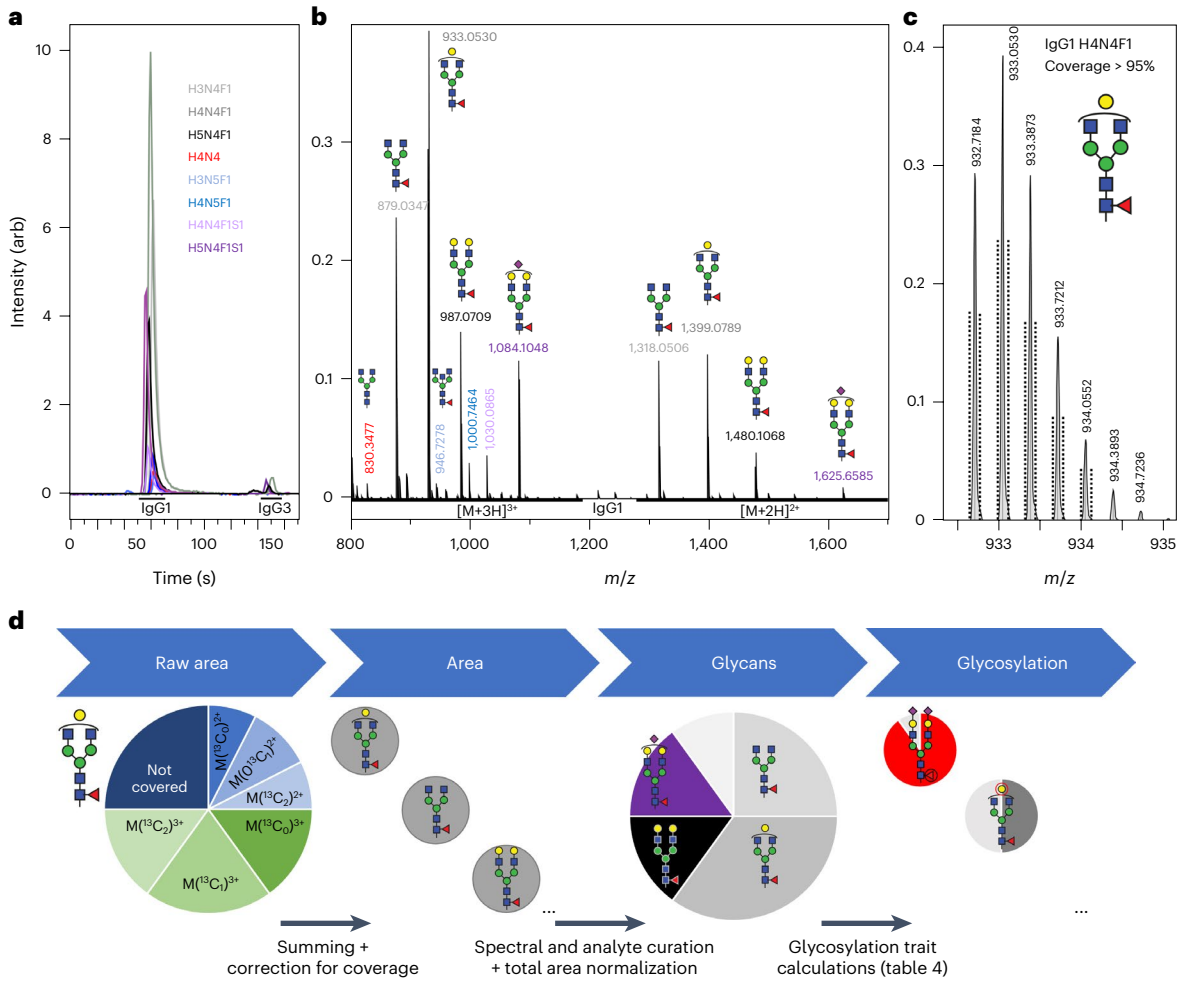

Fig. 4 | Data structure and processing. A figure exemplifying the raw data obtained from the LC–MS measurements and how these are processed into subclass-specific and site-specific glycosylation profiles. **a**, Extracted ion chromatograms of glycopeptides representing major IgG1 and IgG3 glycoforms. Separation of the subclass-specific glycopeptides facilitates differential glycoprofiling by resolving isomeric/isobaric overlaps between subclasses. **b**, A sum mass spectrum is created for the elution range of each subclass. **c**, Signals belonging to abundant isotopologues and charges states are integrated using LaCyTools. The software provides a background subtracted area per target signal, as well as quality parameters that can be used for data curation. **d**, In several additional processing steps, the raw peak areas are converted into relative abundance of glycans and glycosylation traits. For an example of the output data of this workflow, see Fig. 5.

17. Determine integration width (TIME_WINDOW) per cluster by first selecting a high-quality LC–MS analysis where the retention time of the alignment features match the determined reference values (Step 16). Make extracted ion currents of the analyte with the shortest and longest retention time in each cluster using the *m/z* and *m/z* width as in the calibration (using, for example, the vendor software or MZmine). Usually, these would be H5N4F1S2 and H3N4 (H3N4F1 for IgG4) if they are sufficiently abundant. Determine the shortest retention time of the cluster as the left-hand signal at 10% peak height of H5N4F1S2. Determine the longest retention time of the cluster as the right-hand signal at 10% peak height of H3N4. The middle is the retention time of the cluster and half the distance is the TIME_WINDOW.
18. Generate the analyte reference file. We have observed the following glycan compositions on IgG and therefore recommend this list as default for IgG1 and IgG2/3 (for associated glycan structures, see Extended Data Table 1): nonglycosylated, H3N4F1, H4N4F1, H5N4F1,

H4N4F1S1, H5N4F1S1, H5N4F1S2, H3N5F1, H4N5F1, H5N5F1, H4N5F1S1, H5N5F1S1, H3N4, H4N4, H5N4, H4N4S1, H5N4S1, H5N4S2, H3N5, H4N5, H5N5, H4N5S1, H5N5S1, H3N3F1, H4N3F1, H4N3F1S1, H5N3F1, H6N3F1, H6N3F1S1, H6N4F1. Importantly, most IgG4 afucosylated compositions can generally not be determined as the peak asymmetry (peak 'tailing') of IgG1 fucosylated species overwhelms these isomeric signals. In cases where extreme disturbances of glycan synthesis are expected, such as malignancies or congenital orders of glycosylation, a check for additional compositions should be performed. H3N4F1, H4N4F1, H5N4F1, H5N4F1S1 and H5N4F1S2 serve well as calibrants (per cluster).

19. In LaCyTools, load the created files, check the 'Calibration' box and select at minimum the following outputs: 'Analyte Intensity' and 'Analyte QC'. Check the boxes 'Intensities per Charge State' and 'Background subtracted Intensities'. Check that mass accuracy is within 0.2 *m/z* (default setting of 'CALIB_MASS_WINDOW'. Otherwise, adjust 'CALIB_MASS_WINDOW' to meet this condition. Run LaCyTools with the parameters given in Table 3.
▲ **CRITICAL STEP** These parameters are optimized for medium-quality data, which would typically be obtained with nanogram amounts of well-purified antibodies. If the data quality is lower, for example due to less available material or more (non-antibody) contaminants, parameters may need to be adapted. Figure 3 provides a reference for expectable data quality. The main criterium for data quality is how many analytes can be reliably quantified, as judged for example, by the passing of the analyte curation criteria outlined in the next step.
◆ **TROUBLESHOOTING**
20. Perform analyte and spectral curation using the quality criteria provided by LaCyTools (for more information, see the 'data processing' section in the 'Experimental design'). Consider an analyte quantifiable in a given spectrum if it fulfills the following criteria: $-20 < \text{ppm} > 20$, $S/N \geq 9$ and $IPQ \leq 0.2$. Include an analyte if it is quantifiable in at least a certain percentage of spectra (cutoff), typically 80%. Calculate the mean and three times standard deviation of the number of quantifiable analytes and the total analyte intensity in the negative controls. Exclude a given spectrum if either variable falls within this range. Renormalize to obtain the final glycosylation profiles (Fig. 5a).

Table 3 | LaCyTools parameters

Parameter name	Value	Unit	Comments
ALIGNMENT_TIME_WINDOW	*X*	s	Step 16
ALIGNMENT_MASS_WINDOW	0.1	Th	NA
ALIGNMENT_S_N_CUTOFF	27	NA	NA
ALIGNMENT_MIN_PEAK	5	NA	NA
SUM_SPECTRUM_RESOLUTION	100	Th^{-1}	NA
CALIB_MASS_WINDOW	0.2	Da	Step 19
CALIB_S_N_CUTOFF	27	NA	NA
CALIB_MIN_PEAK	4	NA	NA
TIME_WINDOW	*X*	s	Step 17. If needed, use reference file to set per cluster
EXTRACTION_TYPE	2	NA	NA
MASS_WINDOW	0.04	Th	NA
MIN_CHARGE	2	e	Set to 3+ in ref file for analytes above *m/z* 1785
MAX_CHARGE	3	e	Set to 2+ in ref file for analytes below *m/z* 565
MIN_TOTAL	0.8	NA	NA
BACKGROUND_WINDOW	10	Da	NA
EXTRACTION_PADDING	0	NA	NA

Th, Thompson, Da/e; NA, not applicable.

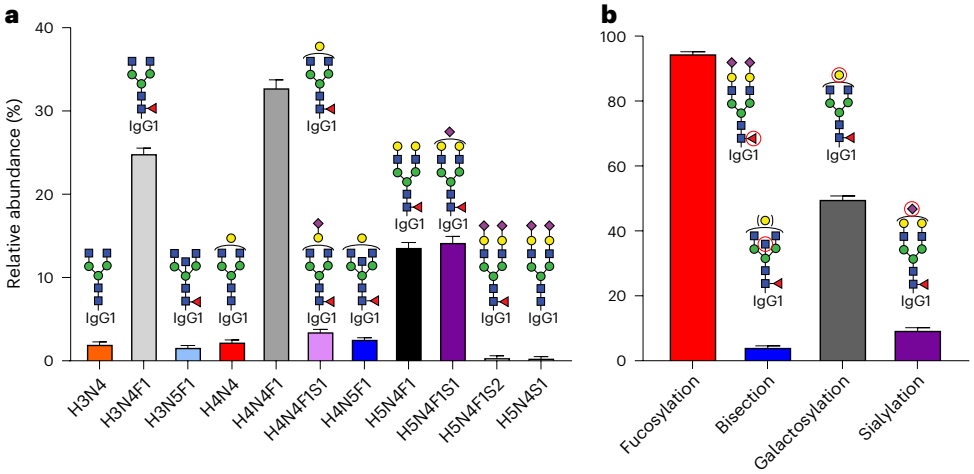

Fig. 5 | Intermediate precision of the method. Glycan abundance (**a**) and glycosylation traits (**b**) are depicted for anti-S IgG from pooled samples of hospitalized patients with COVID-19, used as positive controls in a larger study[20]. The error bars represent the standard deviation of 18 measurements from 6 independent batches prepared and measured over the course of several weeks. The coefficient of variation is generally low for the glycans (3.9% (2.9–5.3%) median (interquartile range)) and even smaller for the glycosylation traits (0.3%, 2.3%, 1.4% and 4.1% from left to right). For the underlying data, refer to Source Data 1.

▲ **CRITICAL STEP** It is important to perform analyte curation per biological/clinical group. The consensus list should then include all analytes passing in any group. This warrants that the most important distinguishing features between groups are included in the analysis. If few analytes pass curation, manually inspect the spectra using the same criteria as provided to LaCyTools for creation of sum spectra to check the quality of excluded analytes.

◆ **TROUBLESHOOTING**

21. Calculate glycosylation traits (Fig. 5b) from the final relative glycan quantities (Fig. 5a) according to the formulas provided in[58] Table 4.

Table 4 | Glycosylation traits

Trait name	Common abbreviation	Formula
Fucosylation[a]	Fuc	(H3N4F1 + H4N4F1 + H5N4F1 + H4N4F1S1 + H5N4F1S1 + H5N4F1S2 + H3N5F1 + H4N5F1 + H5N5F1 + H4N5F1S1 + H5N5F1S1 + H3N3F1 + H4N3F1 + H4N3F1S1)/C
Bisection[a]	Bisec	(H3N5F1 + H4N5F1 + H5N5F1 + H4N5F1S1 + H5N5F1S1 + H3N5 + H4N5 + H5N5 + H4N5S1 + H5N5S1)/C
Galactosylation[a,b]	Gal	(0.5 × (H4N4F1 + H4N4F1S1 + H4N5F1 + H4N5F1S1 + H4N4 + H4N4S1 + H4N5 + H4N5S1 ++ (H5N4F1 + H5N4F1S1 + H5N4F1S2 + H5N5F1 + H5N5F1S1 + H5N4 + H5N4S1 + H5N4S2 + H5N5 + H5N5S1 + H4N3F1 + H4N3F1S1))/C
Sialylation[a,b]	Sia	(0.5 × (H4N4F1S1 + H5N4F1S1 + H4N5F1S1 + H5N5F1S1 + H4N4S1 + H5N4S1 + H4N5S1 + H5N5S1) + (H5N4F1S2 + H5N4S2 + H4N3F1S1))/C
Sialylation per galactose[a]	Sia/Gal	Sia/Gal
Complex glycans[c]	C	H3N4F1 + H4N4F1 + H5N4F1 + H4N4F1S1 + H5N4F1S1 + H5N4F1S2 + H3N5F1 + H4N5F1 + H5N5F1 + H4N5F1S1 + H5N5F1S1 + H3N4 + H4N4 + H5N4 + H4N4S1 + H5N4S1 + H5N4S2 + H3N5 + H4N5 + H5N5 + H4N5S1 + H5N5S1 + H3N3F1 + H4N3F1 + H4N3F1S1
Hybrid type glycans	Hybrid	H5N3F1 + H6N3F1 + H6N3F1S1 + H6N4F1

[a]Traits that are trait *X* of complex type glycans. [b]Gal and Sia are normalized to the available antenna. [c]Used only to aid/clarify calculation as it is redundant with hybrid.

Troubleshooting

Troubleshooting advice can be found in Table 5.

Table 5 | Troubleshooting table

Step	Problem	Possible reason	Solution
10	Dry samples	Inadequate sealing; dried during incubation	Repeat Steps 8–10
		Inadequate sealing; dried during storage:	Repeat Step 8
14	No antigen binding/weak signal	Antigen is degraded or contains incompatible PTMs	Repeat protocol using a new antigen batch or source
	Continuous decrease of signal intensity	Insufficient purification	Repeat protocol and remove sample from plate more thoroughly in Step 5. Tap harder in wash steps
	Weak signals	Sample insufficiently redissolved	Carry out more thorough mixing and add up to 15% ACN in Step 8
	Weak signals; possible clogging of (pre)column	pH outside optimum range for trypsin	Adjust pH to between 7 and 8
		Inactive trypsin	Use new lot of trypsin and prepare fresh
	No or weak signals, also for system suitability standard	No sample injection due to broken needle	Replace needle
		No sample injection due to leaking seal	Replace seals
	No or weak signals	Suboptimal LC–MS system performance	Standard LC–MS troubleshooting; refer to instrument vendor documentation[58]
19	No data, alignment fails	Insufficient quality of alignment features	Consider whether data quality is sufficient for analysis
			Skip alignment using a larger TIME_WINDOW to cover LC peak
			Consider untargeted alignment strategy
	No data, calibration fails	Insufficient quality of calibration features	Integrate without calibration, using a larger MASS_WINDOW to cover MS peak; this decision should be taken per subclass
20	Few analytes passing and excluded analytes have (seemingly) acceptable isotopologue patterns and S/N upon manual inspection of the LC–MS data[a]	High frequency of poor-quality spectra	Lower cutoff, e.g., 50%, for inclusion in consensus list; consider strict spectral curation
		Poor calibration result	Revisit calibrants and settings; if unsuccessful, weaken or remove mass accuracy criterium
		No calibration used	Weaken or remove mass accuracy criterium
		Noisy spectrum/frequent interferences result in poor IPQ	Lower isotopologue coverage (reduce MIN_TOTAL)
	Low ratio of spectra passing	Strong analyte signals in negative control due to improper negative controls	Select a different negative control
		Strong analyte signals in negative control due to nonspecific binding	Optimize capturing to reduce nonspecific binding, for example by washing more stringently or considering alternative supports for the antigen
		Low concentration of ASAs	Repeat protocol using more starting material

[a]Importantly, such inspection should use the same criteria as provided to LaCyTools for creation of sum spectra.

Timing

In all steps, simultaneous handling of two 96-well plates is assumed. Preparation of more or fewer samples will not scale linearly.
Steps 1 and 2, Immobilization of antigen: ~20 min and overnight incubation
Step 3, Washing of immunosorbent plate: ~10 min
Step 4, Capturing of antigen-specific IgG: ~30 min and 1 h incubation
Steps 5 and 6, Washing and elution of antigen-specific IgG: ~45 min
Step 7, Drying: ~2–3 h (hands-off)
Steps 8–10, Proteolytic cleavage: ~30 min and overnight incubation
Steps 11–14, LC–MS measurements: ~45 min preparation and 2 d of measurements (hands-off)
Step 15, File conversion: ~1–2.5 h (hands-off) depending on IT infrastructure
Steps 16–18, Preparation LaCyTools analysis: ~4 h
Step 19, Run LaCyTools: ~10 h (hands-off) depending on IT infrastructure
Steps 20 and 21, Data curation and calculation of glycosylation traits: ~4 h

Anticipated results

Circulatory IgG typically presents a maximum of 26 *N*-glycans on its conserved Fc site (Fig. 1). Depending on specific antibody concentrations, instrument performance and data processing choices (Fig. 4), that is, data quality and quality expectations, ~10–20 glycopeptides are quantified (Figs. 3 and 5a). For a more robust and powerful statistical analysis, these are converted into up to six glycosylation traits (Step 21, Table 4 and Fig. 5b), representing common enzymatic steps in

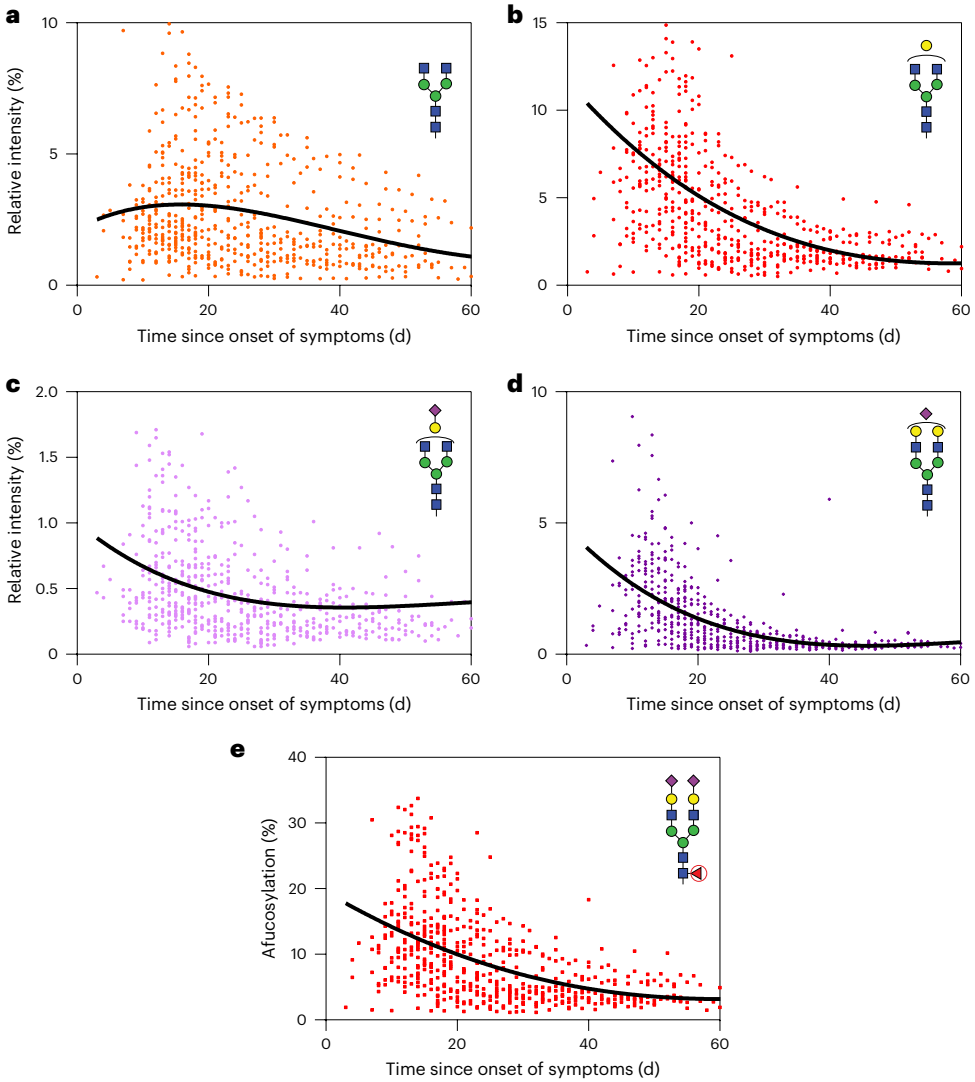

Fig. 6 | Anti-S IgG Fc glycosylation dynamically changes over the course of SARS-CoV-2 infection. Glycan abundance (G0 (**a**), G1 (**b**), G1S (**c**) and G2S (**d**)) and glycosylation traits (afucosylation (**e**)) of specific antibodies, as exemplified in Fig. 5, are obtained through the workflow depicted in Fig. 4 and can be associated with various types of clinical metadata. For example, the integration of GlYcoLISA data with the time passed since the first occurrence of symptoms revealed that early anti-S IgG responses in COVID-19 are characterized by low fucosylation. Initially, anti-S IgG1 fucosylation can be over 10% lower than total IgG1 fucosylation in hospitalized COVID-19 patients[20]. Over time this difference disappears. Depicted is the anti-S IgG glycosylation of 623 individual data points from 156 patients. All individual data points are shown, as well as a fit (solid line; fourth-order polynomial). Trends in individual glycans can be difficult to interpret due to the synergy of multiple biosynthetic pathways and the multifunctional impact. Glycosylation traits, such as fucosylation, help to reduce this complexity to individual biosynthetic steps and clearly defined epitopes with a limited number of biological functions. Nonetheless, as clinical markers individual glycans can be attractive as they may synergistically combine multiple desired associations. Data from ref. 20.

glycan biosynthesis/degradation[59]. Interindividual differences in IgG Fc glycosylation are high. Therefore, it is useful to employ a normalization strategy that corrects for them. In cross-sectional comparisons, the total plasma IgG Fc glycosylation of each individual can serve as a reference. Subtracting the total glycosylation from antigen-specific glycosylation for each trait removes general effects similar for the whole antibody repertoire and yields a Δglycosylation that mainly contains glycosylation changes limited to the ASAs[58]. In longitudinal studies, paired analysis can be used and the various samplings of each patient can serve as each other's reference[20].

IgG Fc glycosylation data provided by GlYcoLISA often show clinical associations and relevance. In the case of alloimmune diseases occurring during pregnancy, including fetal or neonatal alloimmune thrombocytopenia[13] and hemolytic disease of the fetus and newborn[14], low fucosylation of the IgG1 alloantibodies is associated with severe disease outcomes. Antigen-specific IgG1 without core fucose are linked to stronger activation of FcγRIIIa-mediated effector functions, which may make them more pathogenic. It is conceivable that implementation of Fc fucosylation analysis of alloantibodies may be useful for identifying pregnancies with an increased risk of alloimmune complications.

GlYcoLISA results may provide insights into the dynamics of an immune response as reported for SARS-CoV-2 infections. The initial immune response was marked by high galactosylation and sialylation of anti-S IgG1, together with low fucosylation and bisection. These highly skewed glycosylation profiles appeared to be transient, and converted to patterns rather similar to total IgG glycosylation within a few weeks from disease onset[13,20,37–39] (Fig. 6). Furthermore, afucosylation was associated with severe disease and hospitalization[20],

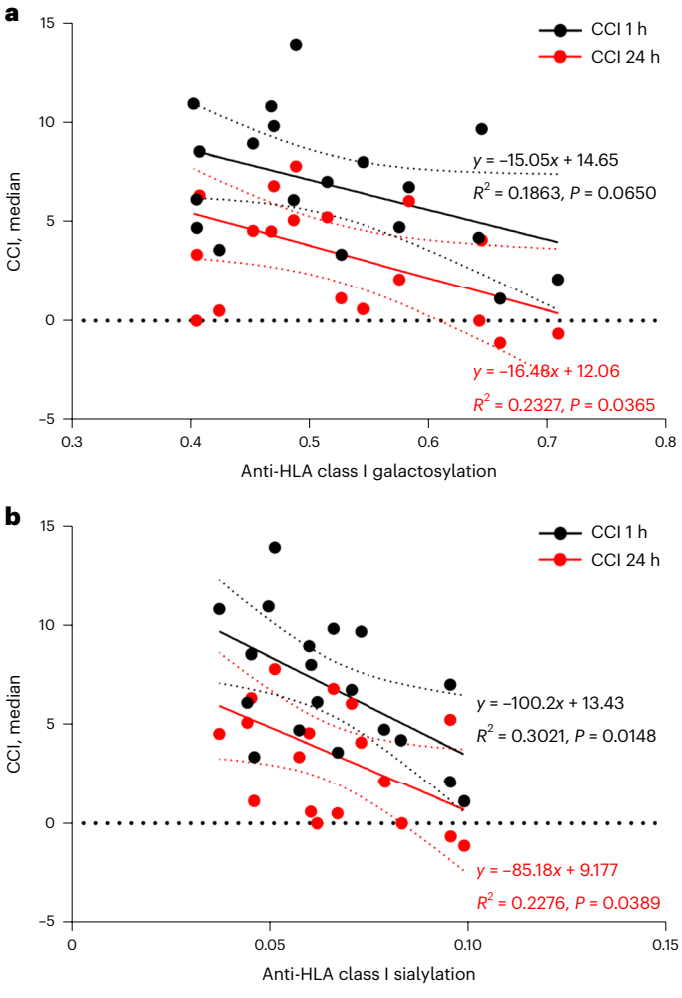

Fig. 7 | High anti-HLA galactosylation and sialylation are associated with the immunological destruction of transfused platelets in patients with chemotherapy-induced thrombocytopenia. The data show a negative correlation between the degree of galactosylation (**a**) and sialylation (**b**) of anti-HLA IgG1 and platelet count after platelet transfusion (presented as corrected count increment (CCI)). The classical complement pathway, which favors galactosylated antibodies (Table 1), was suggested to be the mechanistic link. Solid and dotted lines depict linear regression and 95% confidence interval, respectively. Adapted from ref. 44, Springer Nature Limited.

and low galactosylation and sialylation, and high bisection of anti-S protein IgG1 were associated with disease severity[13].

One powerful approach with the potential to reveal insights into the involvement of antigen-specific glycosylation in disease mechanisms is the correlation of GlYcoLISA results with molecular, cellular or clinical parameters of, for example, inflammation or cell/tissue damage. For example, Fig. 7 depicts the correlation of galactosylation and sialylation of anti- human leukocyte antigen (HLA) IgG1 with the platelet count after platelet transfusion (a measure of damage to transfused platelets)[20]. For SARS-CoV-2 infections a multitude of such correlations has been investigated, for example, with cytokines, chemokines and immune cell markers (clusters of differentiation)[44]. Regarding clinical measures, the DAS28–CRP score for disease severity, used in rheumatoid arthritis, has been correlated inversely with changes in anti-citrullinated protein autoantibody (ACPA)–IgG1 galactosylation during pregnancy[20].

IgG and its glycosylation are key players in adaptive immune responses. However, many questions remain regarding the regulation of IgG glycosylation and its involvement in disease mechanisms. We expect that GlYcoLISA will be a key technology to solve these open questions, especially if combined with tools from immunology and molecular biology. Integration of GlYcoLISA data with (single-cell) B cell transcriptomics or linking of specific signatures to different immune cell subpopulations are exciting examples of the possibilities.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data reported here (Figs. 3 and 5–7) have been previously reported elsewhere or are available in the source data: Fig. 3 and ref. 60 are available upon request from t.pongracz@lumc.nl; Fig. 5, source data; Fig. 6, ref. 20 and Supplementary Table 1; and Fig. 7, ref. 44 and Supplementary Table 1. Source data are provided with this paper.

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Author contributions

D.F. wrote and M.W. corrected the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Related links

Key references in the development of the protocol

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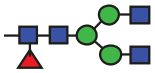
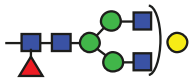
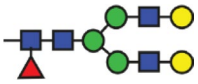
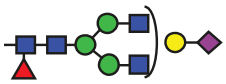
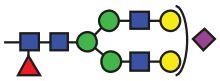
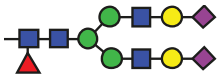
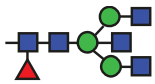
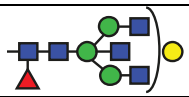
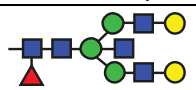
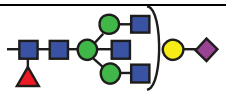
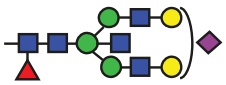
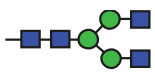
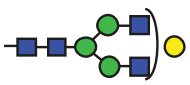
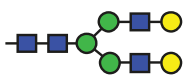
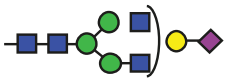
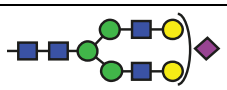
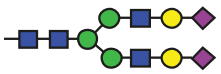
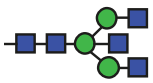
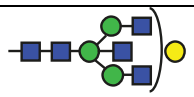
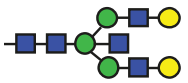
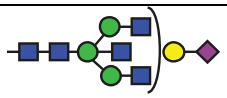
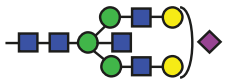
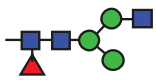
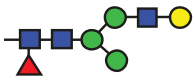
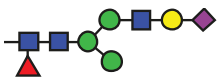
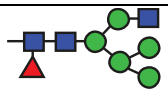
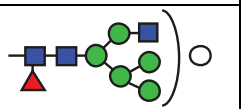
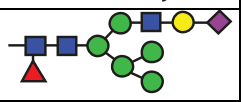
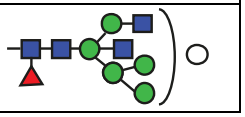
H3N4F1	G0F	
H4N4F1	G1F	
H5N4F1	G2F	
H4N4F1S1	G1S1F	
H5N4F1S1	G2S1F	
H5N4F1S2	G2S2F	
H3N5F1	G0FN	
H4N5F1	G1FN	
H5N5F1	G2FN	
H4N5F1S1	G1S1FN	
H5N5F1S1	G2S1FN	
H3N4	G0	
H4N4	G1	
H5N4	G2	

Composition *	Glycan name	Structure**
H4N4S1	G1S1	
H5N4S1	G2S1	
H5N4S2	G2S2	
H3N5	G0N	
H4N5	G1N	
H5N5	G2N	
H4N5S1	G1S1N	
H5N5S1	G2S1N	
H3N3F1	G0F-N	
H4N3F1	G1F-N	
H4N3F1S1	G1S1F-N	
H5N3F1	Man5G0F hybrid	
H6N3F1	Man5G1F or Man6G0F hybrid	
H6N3F1S1	Man5G1S1F hybrid	
H6N4F1	Man5G1FN/ Man6G0FN hybrid	

* elemental composition deduced from MS measurements expressed as monosaccharide units: H = hexose, N = *N*-acetylhexosamine, F = deoxyhexose (fucose); S = sialic acid (*N*-acetylneuraminic acid)

**CFG notation

Extended Data Table 1 | Compositions, nomenclature and structure

nature portfolio

Corresponding author(s): David Falck and Manfred Wuhrer

Last updated by author(s): 2023-11-28

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All data reported here (Figures 3, 6 and 7) has been previously reported elsewhere or is available as SOURCE DATA:
• Figure 3: https://doi.org/10.1007/s10719-022-10044-0;
available upon request (t.pongracz@lumc.nl)
• Figure 5: SOURCE DATA 1

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Mycoplasma contamination

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○	○	Alter the host range of a pathogen
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☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

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May remain private before publication

Files in database submission

Genome browser session
(e.g. UCSC)

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Replicates

Sequencing depth

Antibodies

Peak calling parameters

Data quality

Software

Flow Cytometry

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Instrument

Software

Cell population abundance

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Magnetic resonance imaging

Experimental design

Design type

Design specifications

Behavioral performance measures

Acquisition

Imaging type(s)

Field strength

Sequence & imaging parameters

Area of acquisition

Diffusion MRI ○ Used ○ Not used

Preprocessing

Preprocessing software

Normalization

Normalization template

Noise and artifact removal

Volume censoring

Statistical modeling & inference

Model type and settings

Effect(s) tested

Specify type of analysis:

Whole brain ROI-based Both

Statistic type for inference (See Eklund et al. 2016)

Correction

Models & analysis

n/a | Involved in the study

- Functional and/or effective connectivity
- Graph analysis
- Multivariate modeling or predictive analysis

Functional and/or effective connectivity

Graph analysis

Multivariate modeling and predictive analysis