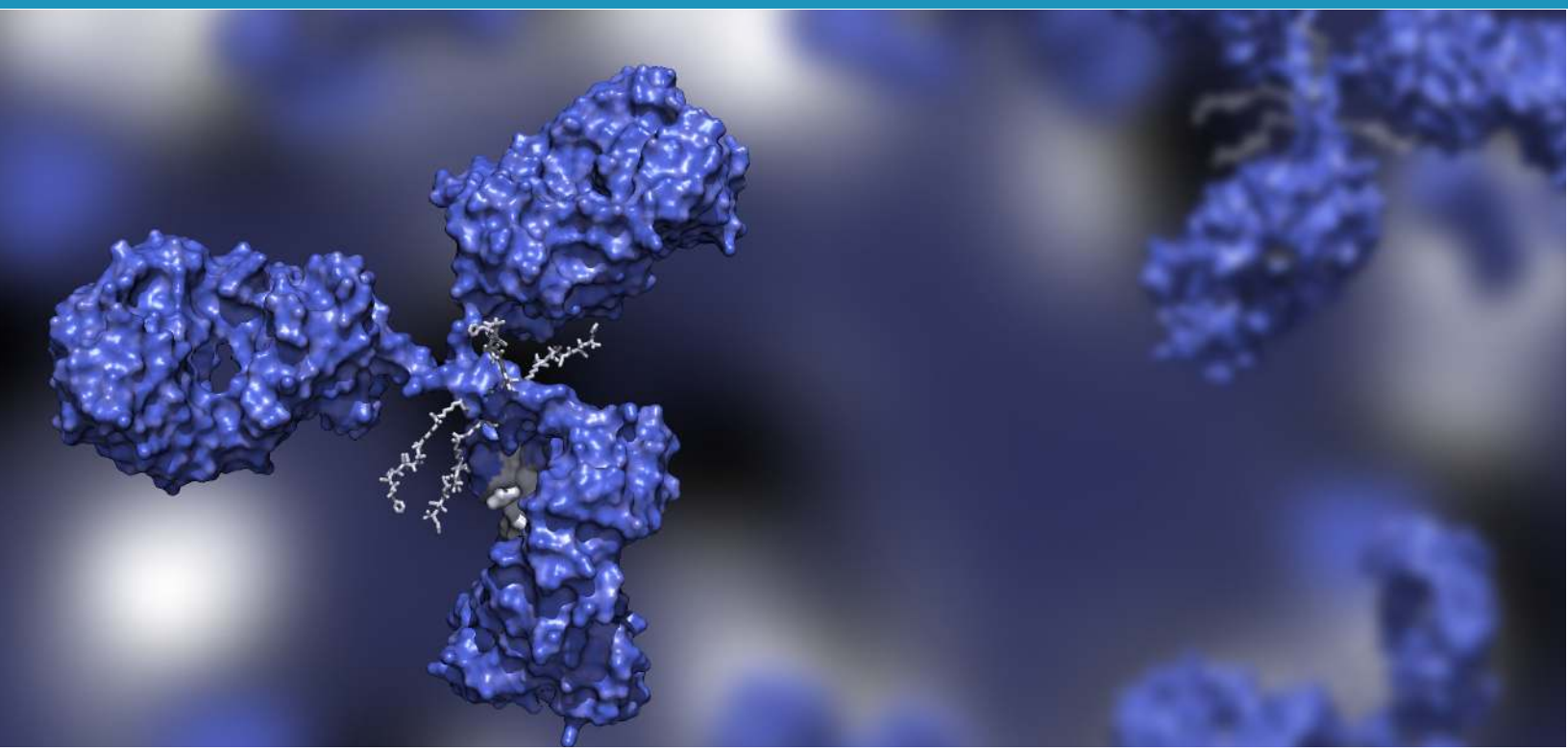

Characterisation of N-linked glycosylation patterns of IgG antibodies in PEAKS GlycanFinder

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Abstract:

Monoclonal antibodies are emerging as excellent therapeutic agents for drug targeting and immunotherapies. Critical steps in their development as a therapeutic involve characterizing binding affinity, stability, and function. These properties are often regulated by site-specific N- or O-linked glycosylation of antibodies, therefore it is essential to define the composition, structure, and glycosylation sites when developing an effective therapeutic. PEAKS GlycanFinder provides a software solution for this step of the development process in accurately and specifically identifying glycans, along with their structures and modification sites across antibody sequences.

Introduction:

Antibodies contain two functional regions: the variable region (Fvab) or antigen binding domain and the constant region (Fc), which regulates the immune response (1). The biological activity of the Fc region can be regulated at the genetic level (isotype and subclass) or at the PTM level via glycosylation. Studies have revealed the importance of antibody glycosylation as a regulator of antibody stability, half life, secretion, immunogenicity, and function, therefore changes in antibody glycosylation patterns may alter intrinsic properties and stability (2-3). The use of recombinant monoclonal antibodies (mAbs) as a therapeutic in the treatment of disease is gaining traction in the pharmaceutical industry do to their increased success rate and efficacy when compared to chemically synthesized drugs. Currently the majority of marketed mAbs belong to the immunoglobin G (IgG) class (4). Within the IgG isotype there are four subclasses: IgG1-IgG4. Most IgG antibodies contain a conserved N-linked glycan in the Fc region. The glycan motifs are highly heterogenous (high mannose, complex or hybrid) and significantly affect the Fc mediated effector functions (5). Therefore, the specific glycan profile of an antibody is unique and plays a critical role in antibody function. Thus, antibody glycan profiling provides essential information for antibody function. PEAKS GlycanFinder is a software solution that offers comprehensive glycan profiling across multiple samples with high sensitivity and accuracy. Furthermore, it can differentiate glycosylation sites of peptides with highly similar amino acid sequences (isomers) and properties as with the IgG subclasses (Fig. 1). Here we report an MS-based glycoproteomic workflow using PEAKS GlycanFinder software to determine human IgG subclass specific glycosylation (Fig. 2).

Methods:

For this experiment, human IgG mixture (≥95%, Sigma) was reduced, alkylated, and digested with Trypsin. The glycopeptides were enriched using a manufactured microcrystalline cellulose SPE cartridge before injection into UHPLC tandem timsTOF pro2 mass spectrometer and Orbitrap Fusion Lumos. In the case of timsTOF, to increase the quality of glycopeptides, 10 PASEF scans were combined to one MS/MS scan and Nanobooster was enabled to improve the ionization efficiency of glycopeptides. The data presented here was generated using the DDA and analyzed with PEAKS GlycanFinder. All parameters used for the glycan analysis are indicated in Tables #1 and Table #2. Data generated from the timsTOF were used to analyze IgG subclass glycan profiles and the data generated from the Orbitrap Fusion Lumos were used for LFQ analysis across multiple IgG subclass replicates.

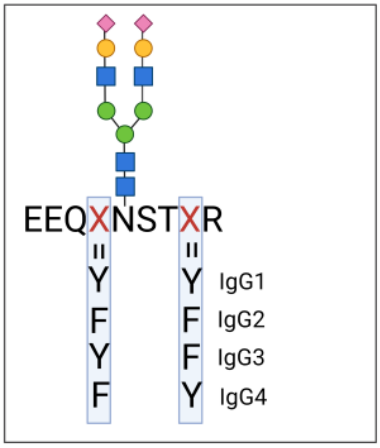


Fig 1. High sequence similarity of human IgG glycopeptides. The sequences of human IgG1-IgG4 surrounding the conserved N-linked glycan site in the Fc region of the heavy chain.

Parameter	Setting
Instrument	timsTOF
Activation Method	CID
Enzyme	Trypsin
Precursor mass tolerance	20 ppm
Fragment mass tolerance	0.05 DA
Glycan Fragment mass tolerance	40 ppm
Ion Mobility tolereance	0.05 1/k0
Digest mode	Semi-specific
Missed cleavage	1
Fixed PTM	Carbamidomethylation
Variable PTM	Deamidation (NQ), Oxidation (M)
Max allowed variable PTM per peptide	2
Glycan database source	Built-in N-linked
Database source	Custom built IgG
Taxa	All species
Searched entries	9
N-linked searched entries	2537
Adduct	NH4
Max adduct per peptide	1
Max fucose count	1
Peptide FDR	1.0%
Protein group FDR	1.0%
Protein unique peptides	≥1
Retention time shift tolerance	2.0 min

Table 1: PEAKS Parameters for timsTOF data

Parameter	Setting
Instrument	Orbi-Orbi
Activation Method	HCD
Enzyme	Trypsin
Precursor mass tolerance	20 ppm
Fragment mass tolerance	0.05 DA
Glycan Fragment mass tolerance	40 ppm
Digest mode	Semi-specific
Missed cleavage	1
Fixed PTM	Carbamidomethylation
Variable PTM	Deamidation (NQ), Oxidation (M)
Max allowed variable PTM per peptide	2
Glycan database source	Built-in N-linked
Database source	Custom built IgG
Taxa	All species
Searched entries	9
N-linked searched entries	2537
Adduct	NH4
Max adduct per peptide	1
Max fucose count	1
Peptide FDR	1.0%
Protein group FDR	1.0%
Protein unique peptides	≥1
Normalization method	Use TIC
Retention time shift tolerance	2.0 min

Table 2: PEAKS Parameters for Orbitrap Fusion Lumos data

Results:

Accurate IgG glycopeptide differentiation

Glycopeptide spectra from purified human IgG1-IgG4 shows GlycanFinder can accurately determine the glycosylation site, the attached glycan moiety and differentiate IgG glycopeptides with high precision. The associated glycan on the glycopeptide is given a S-score (%), which indicates the confidence in the matched glycan structure. For glycan candidates with the same composition, the candidate is sorted by matched glycan Y-ion count. S-Score = (most Y-ion count – 2nd most Y-ion count)/ (most Y-ion count). The higher the score the better. One hundred percent indicates only 1 possible glycan structure, while 0% is given when there are no reporter ions to differentiate between possible glycan structures. (HexNAc)4(Hex)3 and (HexNac)4(Hex)3(Fuc)1 were the glycans detected on IgG1 glycopeptide (Fig. 3a) and IgG2 glycopeptide (Fig. 3b), respectively, and their matched glycan structures with S-Scores can be viewed in the top right corner of the spectrum. The amino acid sequence of the IgG3 (Fig. 3c) glycopeptide (EEQFNSTYR) is an isomer of the IgG4 (Fig. 3d) glycopeptide (EEQYNSTFR). GlycanFinder can differentiate the peptide sequences of these isomers with site-specific glycosylation analysis. The supporting ions for the glycosylation site are shown above the spectra for both IgG3 and IgG4. Both IgG3 and IgG4 glycopeptides were associated with the glycan (HexNAc)4(Hex)5(Fuc)1, however, the matched glycan structure in the IgG4 has a higher S-Score, suggesting that it is the more likely glycan structure.

Glycan profiling of IgG subclasses

In addition to structural information, GlycanFinder provides glycan site profiling. By clicking on the N-linked glycan site you can view the distributions of glycan moieties at that site. Pie charts show the unique glycan profiles of each IgG sample. These results highlight the distinct glycan profiles unique to human IgG subclasses. Fifty-three different glycans moieties were detected on IgG1 (Fig. 4a), 29 different glycan moieties were detected on IgG2 (Fig. 4b), 10 different glycans were detected on IgG3 (Fig. 4c), and 13 different glycans were associated with IgG4 (Fig. 4d). (HexNAc)4(Hex)3(Fuc)1 was the most abundant glycan on IgG1 (23.6%), IgG2 (27.7%) and IgG4 (20.3%). The most abundant glycan detected on IgG3 was (HexNAc)4(Hex)5(Fuc)1 (26.2%).

Label free Quantification of IgG2 glycan moetities

GlycanFinder offers a feature-based approach to LFQ glycan analysis and allows you to quantify glycopeptide abundance and compare across samples. The software displays a comprehensive list and quantitative bar graph showing all detected glycans at the glycosylation site in the Fc of IgG2 across 3 independent LC-MS/MS runs (Fig. 5a). In addition, a pie chart illustrates IgG2 glycan site profiling across across 3 replicates (Fig. 5b). These results show (HexNAc)4(Hex)3(Fuc)1 is the most abundant N-linked glycan in the conserved glycan site of the Fc. By selecting the Glycan Profile tool, relative abundances for each glycopeptide are shown across samples, along with peptide precursor ion profiles (Fig. 5c). Lastly, in the Feature tab, you can compare glycopeptide feature profiles across multiple samples (Fig. 5d).

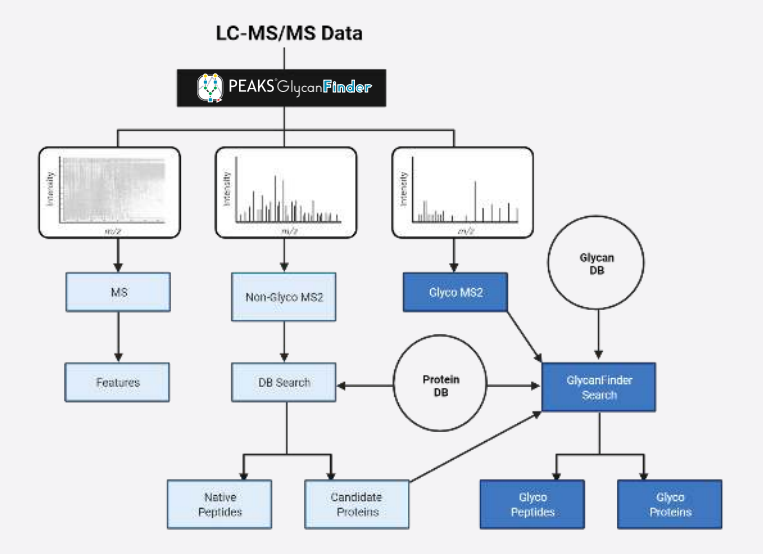


Fig 2. PEAKS GlycanFinder Workflow

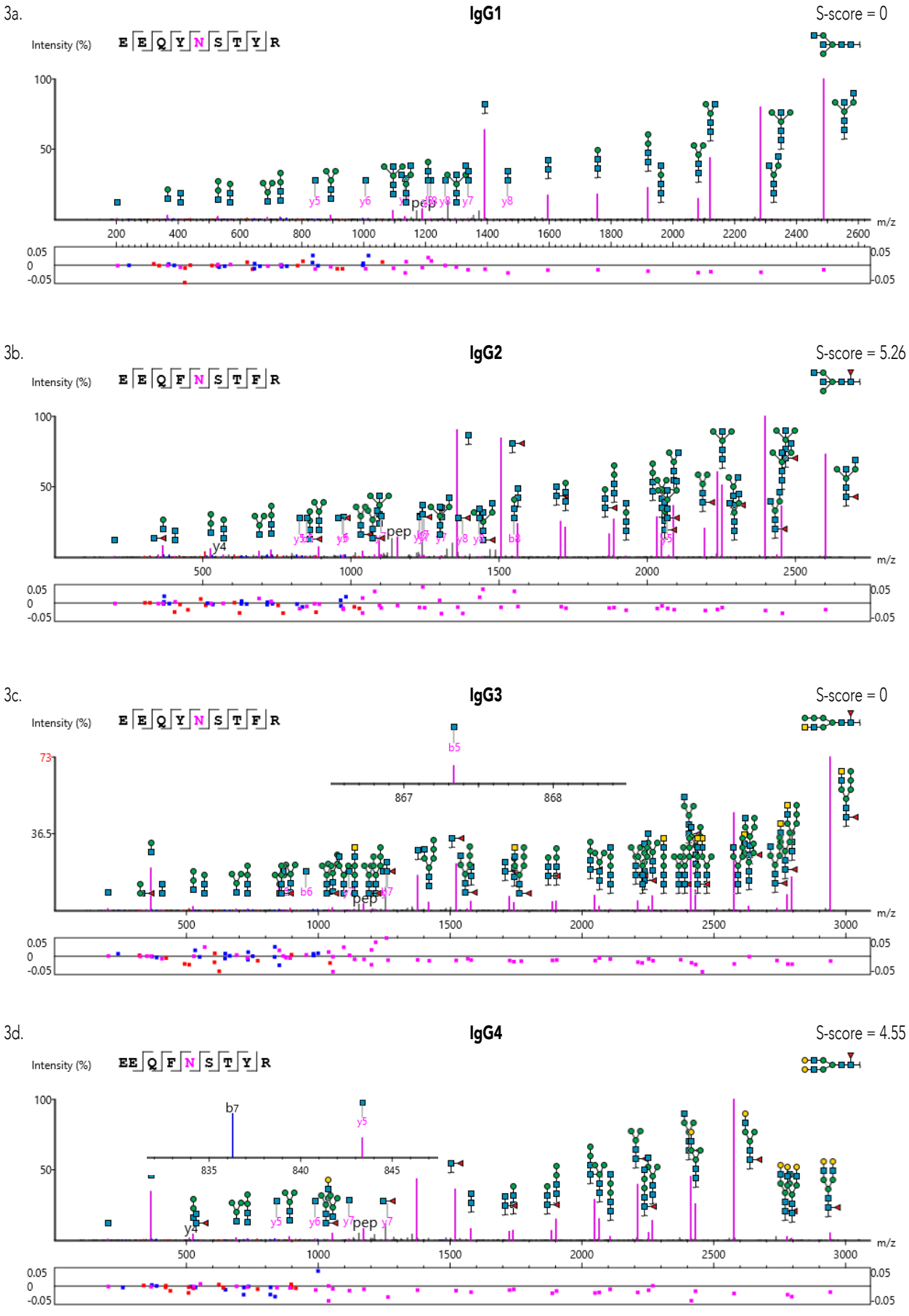
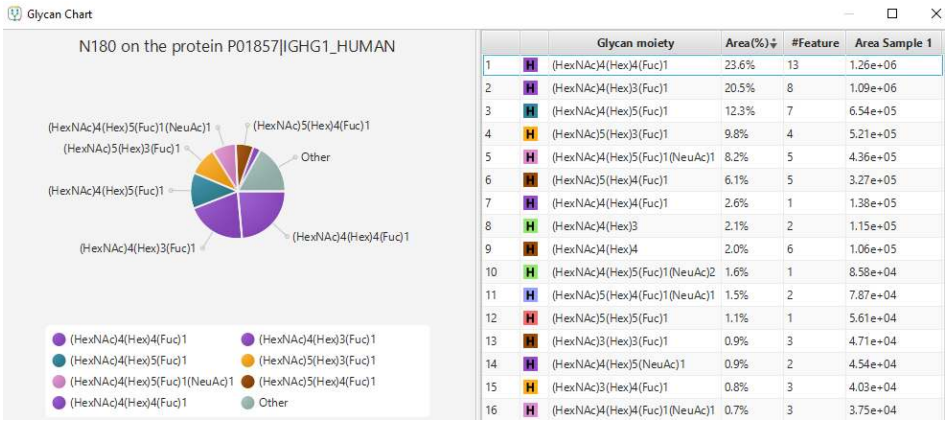


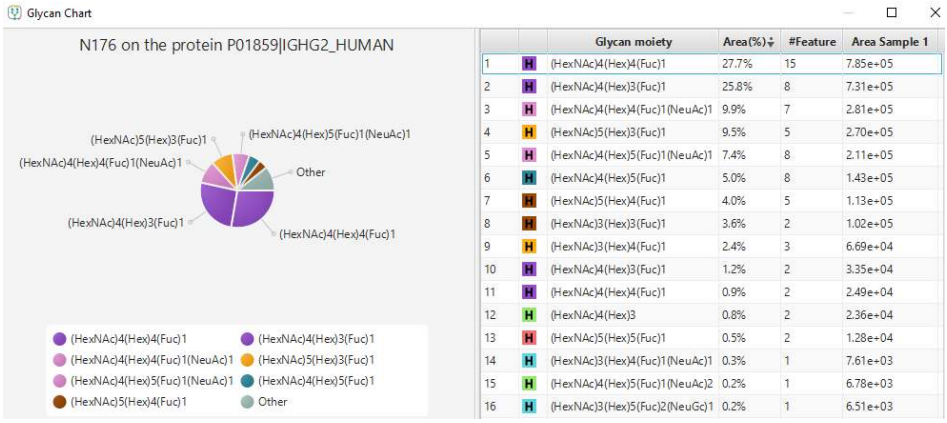
Fig 3. High accuracy in differentiating human IgG glycopeptides

4a. **IgG1**



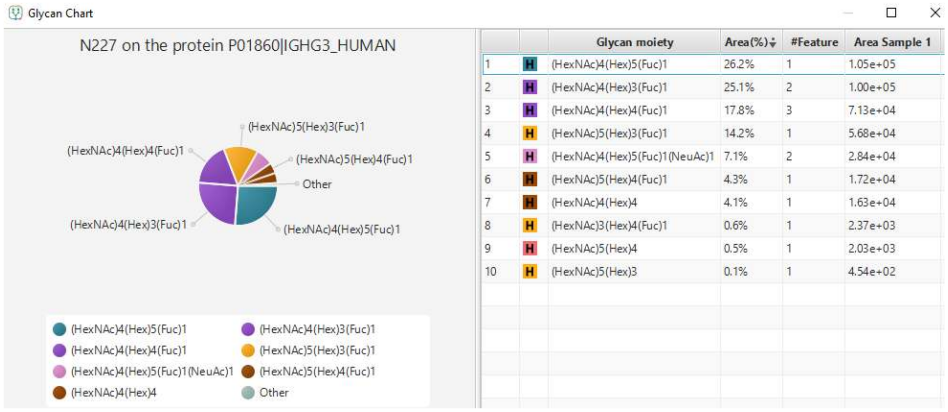
		Glycan moiety	Area(%)	#Feature	Area Sample 1
1	H	(HexNAc)4(Hex)4(Fuc)1	23.6%	13	1.26e+06
2	H	(HexNAc)4(Hex)3(Fuc)1	20.5%	8	1.09e+06
3	H	(HexNAc)4(Hex)5(Fuc)1	12.3%	7	6.54e+05
4	H	(HexNAc)5(Hex)3(Fuc)1	9.8%	4	5.21e+05
5	H	(HexNAc)4(Hex)5(Fuc)1(NeuAc)1	8.2%	5	4.36e+05
6	H	(HexNAc)5(Hex)4(Fuc)1	6.1%	5	3.27e+05
7	H	(HexNAc)4(Hex)4(Fuc)1	2.6%	1	1.38e+05
8	H	(HexNAc)4(Hex)3	2.1%	2	1.15e+05
9	H	(HexNAc)4(Hex)4	2.0%	6	1.06e+05
10	H	(HexNAc)4(Hex)5(Fuc)1(NeuAc)2	1.6%	1	8.58e+04
11	H	(HexNAc)5(Hex)4(Fuc)1(NeuAc)1	1.5%	2	7.87e+04
12	H	(HexNAc)5(Hex)5(Fuc)1	1.1%	1	5.61e+04
13	H	(HexNAc)3(Hex)3(Fuc)1	0.9%	3	4.71e+04
14	H	(HexNAc)4(Hex)5(NeuAc)1	0.9%	2	4.54e+04
15	H	(HexNAc)3(Hex)4(Fuc)1	0.8%	3	4.03e+04
16	H	(HexNAc)4(Hex)4(Fuc)1(NeuAc)1	0.7%	3	3.75e+04

4b. **IgG2**



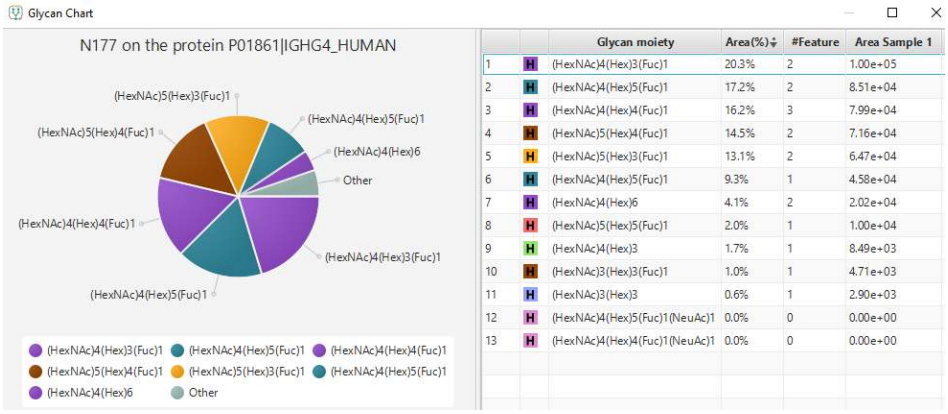
		Glycan moiety	Area(%)	#Feature	Area Sample 1
1	H	(HexNAc)4(Hex)4(Fuc)1	27.7%	15	7.85e+05
2	H	(HexNAc)4(Hex)3(Fuc)1	25.8%	8	7.31e+05
3	H	(HexNAc)4(Hex)4(Fuc)1(NeuAc)1	9.9%	7	2.81e+05
4	H	(HexNAc)5(Hex)3(Fuc)1	9.5%	5	2.70e+05
5	H	(HexNAc)4(Hex)5(Fuc)1(NeuAc)1	7.4%	8	2.11e+05
6	H	(HexNAc)4(Hex)5(Fuc)1	5.0%	8	1.43e+05
7	H	(HexNAc)5(Hex)4(Fuc)1	4.0%	5	1.13e+05
8	H	(HexNAc)3(Hex)3(Fuc)1	3.6%	2	1.02e+05
9	H	(HexNAc)3(Hex)4(Fuc)1	2.4%	3	6.69e+04
10	H	(HexNAc)4(Hex)3(Fuc)1	1.2%	2	3.35e+04
11	H	(HexNAc)4(Hex)4(Fuc)1	0.9%	2	2.49e+04
12	H	(HexNAc)4(Hex)3	0.8%	2	2.36e+04
13	H	(HexNAc)5(Hex)5(Fuc)1	0.5%	2	1.28e+04
14	H	(HexNAc)3(Hex)4(Fuc)1(NeuAc)1	0.3%	1	7.61e+03
15	H	(HexNAc)4(Hex)5(Fuc)1(NeuAc)2	0.2%	1	6.78e+03
16	H	(HexNAc)3(Hex)5(Fuc)2(NeuGc)1	0.2%	1	6.51e+03

4c. **IgG3**



		Glycan moiety	Area(%)	#Feature	Area Sample 1
1	H	(HexNAc)4(Hex)5(Fuc)1	26.2%	1	1.05e+05
2	H	(HexNAc)4(Hex)3(Fuc)1	25.1%	2	1.00e+05
3	H	(HexNAc)4(Hex)4(Fuc)1	17.8%	3	7.13e+04
4	H	(HexNAc)5(Hex)3(Fuc)1	14.2%	1	5.68e+04
5	H	(HexNAc)4(Hex)5(Fuc)1(NeuAc)1	7.1%	2	2.84e+04
6	H	(HexNAc)5(Hex)4(Fuc)1	4.3%	1	1.72e+04
7	H	(HexNAc)4(Hex)4	4.1%	1	1.63e+04
8	H	(HexNAc)3(Hex)4(Fuc)1	0.6%	1	2.37e+03
9	H	(HexNAc)5(Hex)4	0.5%	1	2.03e+03
10	H	(HexNAc)5(Hex)3	0.1%	1	4.54e+02

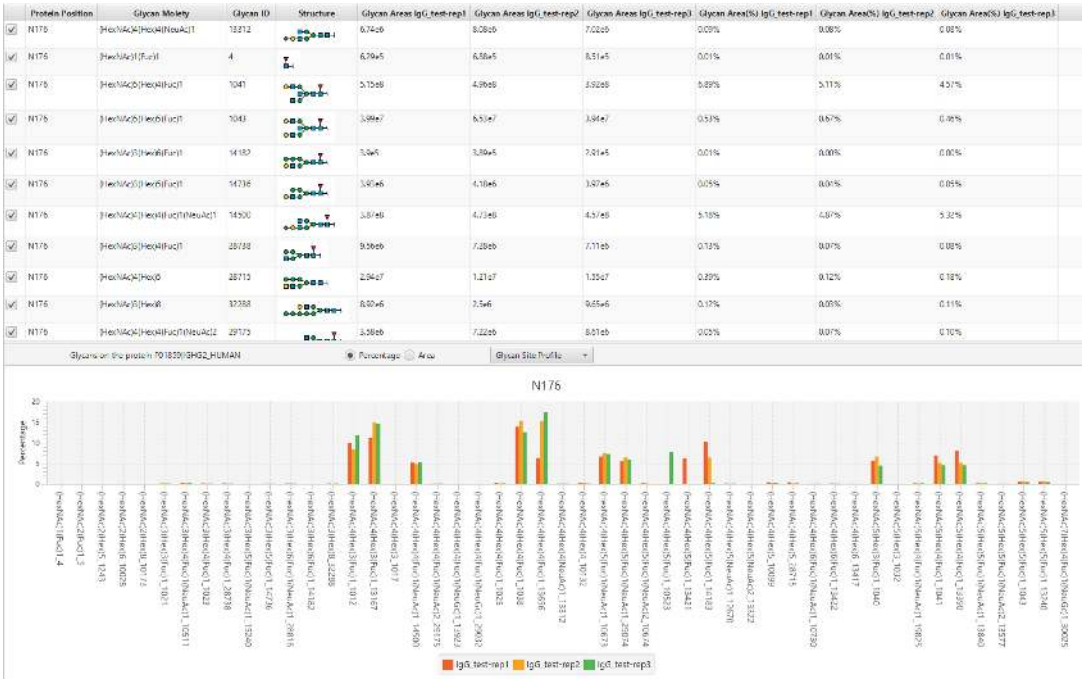
4d. **IgG4**



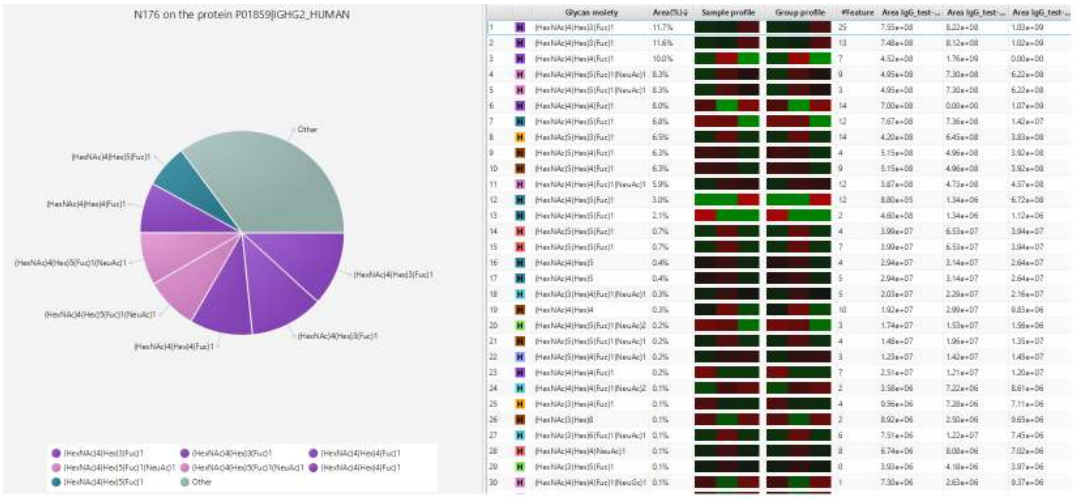
		Glycan moiety	Area(%)	#Feature	Area Sample 1
1	H	(HexNAc)4(Hex)3(Fuc)1	20.3%	2	1.00e+05
2	H	(HexNAc)4(Hex)5(Fuc)1	17.2%	2	8.51e+04
3	H	(HexNAc)4(Hex)4(Fuc)1	16.2%	3	7.99e+04
4	H	(HexNAc)5(Hex)4(Fuc)1	14.5%	2	7.16e+04
5	H	(HexNAc)5(Hex)3(Fuc)1	13.1%	2	6.47e+04
6	H	(HexNAc)4(Hex)5(Fuc)1	9.3%	1	4.58e+04
7	H	(HexNAc)4(Hex)6	4.1%	2	2.02e+04
8	H	(HexNAc)5(Hex)5(Fuc)1	2.0%	1	1.00e+04
9	H	(HexNAc)4(Hex)3	1.7%	1	8.49e+03
10	H	(HexNAc)3(Hex)3(Fuc)1	1.0%	1	4.71e+03
11	H	(HexNAc)3(Hex)3	0.6%	1	2.90e+03
12	H	(HexNAc)4(Hex)5(Fuc)1(NeuAc)1	0.0%	0	0.00e+00
13	H	(HexNAc)4(Hex)4(Fuc)1(NeuAc)1	0.0%	0	0.00e+00

Fig 4. Glycan profiling of IgG subclasses

5a.



5b.



5c.

Fig 5. Label Free Quantification (LFQ) of IgG2 glycan moieties

5d.

	Peptide	Glycan Type	Glycan	Quality	Significance	m/z	z	RT	RT Begin	RT End	Avg. Area	Area IgG_test-rep1	Area IgG_test-rep2	Area IgG_test-rep3	Sample Profile
18	EEQFN(+1403.51)STFR	NLink	(HexNAc)3(Hex)4(Fuc)1	45.89	0.00	1281.0198	2	35.08	34.43	37.13	4.27e+06	5.43e+06	3.31e+06	4.07e+06	
19	EEQFN(+1403.51)STFR	NLink	(HexNAc)3(Hex)4(Fuc)1	79.29	0.00	854.0202	3	27.64	27.12	28.44	3.72e+06	4.13e+06	3.97e+06	3.04e+06	
20	EEQFN(+1444.53)STFR	NLink	(HexNAc)4(Hex)3(Fuc)1	88.17	0.00	1301.5331	2	36.66	35.02	37.77	5.27e+08	4.24e+08	8.02e+08	3.55e+08	
21	EEQFN(+1444.53)STFR	NLink	(HexNAc)4(Hex)3(Fuc)1	84.51	0.00	868.0249	3	36.19	34.92	38.26	3.32e+08	3.24e+08	1.01e+07	6.63e+08	
22	EEQFN(+1444.53)STFR	NLink	(HexNAc)4(Hex)3(Fuc)1	93.52	0.00	1301.5334	2	36.68	35.06	37.77	6.49e+08	5.06e+08	8.55e+08	5.87e+08	
23	EEQFN(+1444.53)STFR	NLink	(HexNAc)4(Hex)3(Fuc)1	92.71	0.00	868.0255	3	36.68	34.48	38.26	5.24e+08	3.24e+08	5.85e+08	6.63e+08	

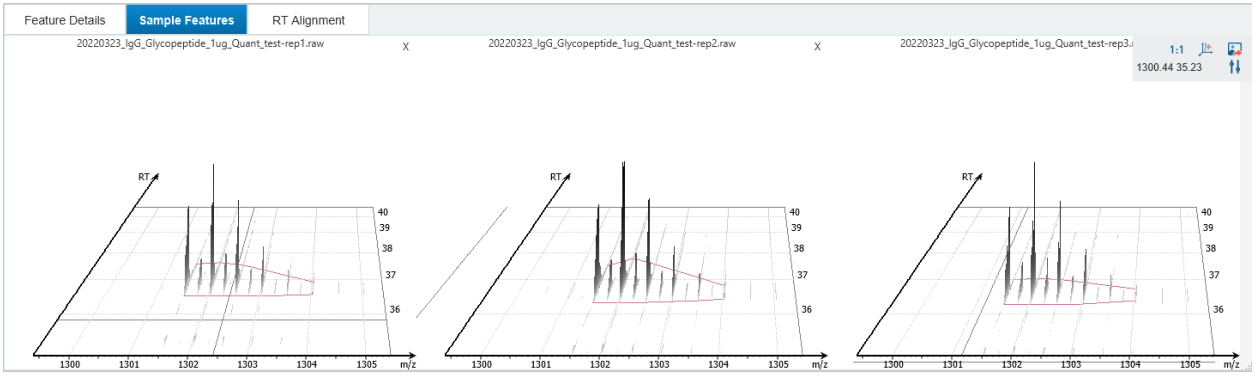


Fig 5. LFQ of IgG2 glycan moieties (cont'd)

Discussion:

Despite the complexity of glycan compositions, structures, and sites of attachment, PEAKS GlycanFinder can differentiate between isomeric glycopeptides, glycosylation sites within the same peptide, and provide structure information from LC-MS/MS data. This has also been aided by the advancement of MS instrument methods, optimizing glycopeptide enrichment and fragmentation, and use of structural glycan databases. Our analysis of IgG glycopeptides demonstrates the accuracy of GlycanFinder in differentiating glycosylation sites among highly similar peptide sequences within the same sample (Fig. 3). We also show that S-Score can provide the user with confidence in determining the glycan structure when there are multiple possibilities of glycans having the same composition. Furthermore, glycan site profiling tools within GlycanFinder display quantitative information on the occupancy of different glycans at a particular site and compares the relative abundances of glycopeptides across samples (Fig. 4). This tool can be used to assess changes in glycan abundances across conditions or recombinant protein expression systems (Fig. 5). Taken together, GlycanFinder is a comprehensive software solution for glycoproteomics and is an excellent tool for characterizing the different glycosylation states of mAbs.

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