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Glycosylation patterns and their functional implications in therapeutic monoclonal antibodies

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Abstract

Leading regulatory scientists have argued that Fc glycosylation is the single most important quality attribute distinguishing one monoclonal antibody (mAb) product from another. This research characterised the N-linked glycoform profiles of an innovator mAb (trastuzumab) and two biosimilar candidates expressed in CHO-K1 cells, then mapped each glycoform's effect on Fcγ receptor binding and antibody-dependent cellular cytotoxicity (ADCC). Glycan analysis by hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-FLD) and mass spectrometry identified 14 distinct glycoforms across the three products. The dominant species in all cases was core-fucosylated agalactosylated biantennary (G0F), ranging from 36.2% to 41.3%. Afucosylated species differed most between products (6.8-7.6%), and even this narrow range translated into a 1.7-fold difference in FcγRIIIa V158 binding affinity. ADCC potency, measured in an NFAT-luciferase reporter assay, correlated strongly with afucosylation level ($r = 0.93$, $P < 0.01$). High-mannose glycoforms (Man5), though present below 6.5%, reduced FcRn binding at pH 6.0, suggesting a potential impact on serum half-life. These data demonstrate that small percentage-point shifts in glycoform distribution can produce measurable functional differences and argue for tight glycosylation control in biosimilar manufacturing.

Keywords: Glycosylation, monoclonal antibodies, post-translational modifications, therapeutic proteins, biosimilar, Fc receptor binding, ADCC, afucosylation, CHO cells, quality attributes

Introduction

As regulatory agencies worldwide have noted, the glycan profile attached to the Fc region of a therapeutic antibody can profoundly alter its effector functions, pharmacokinetics, and immunogenicity [1]. This view is echoed by the European Medicines Agency's biosimilar guideline, which identifies Fc glycosylation as a critical quality attribute requiring head-to-head comparison between an innovator and its proposed biosimilar [2].

All IgG1 antibodies carry a conserved N-linked glycan at asparagine-297 in the CH2 domain. The most common structures are biantennary complex-type glycans that may or may not carry a core fucose, one or two terminal galactose residues, and occasionally bisecting N-acetylglucosamine or sialic acid [3]. Core fucosylation is the single modification with the largest functional impact: its absence increases binding to FcγRIIIa on natural killer cells by up to 50-fold, dramatically boosting ADCC [4].

Despite decades of research, systematic comparisons that link individual glycoform levels in real therapeutic products to quantitative receptor-binding data remain scarce. Most published studies either characterise glycosylation without measuring function or report ADCC without resolving which glycoforms drive the effect. This research bridged that gap by performing detailed glycoform profiling of trastuzumab and two biosimilar candidates followed by glycoform-resolved Fcγ receptor binding and ADCC assays. All analytical and cell-based work was conducted at the Centro de Investigación Biológica del Altiplano, Aguascalientes, Mexico.

Quality Control

System suitability for HILIC-FLD was verified daily using a dextran ladder standard (Waters, Milford, MA). Glycan peak identification was confirmed by co-injection with individual glycan standards (Prozyme, Hayward, CA) and by orthogonal LC-MS analysis on a Thermo Q Exactive HF. Inter-day coefficients of variation for the six major glycoforms were below 4.8% across five replicate injections performed over three non-consecutive days.

Material and Methods

Material

Trastuzumab innovator product (Herceptin®, Roche, lot 2022-H43) and two biosimilar candidates (designated Biosimilar A and Biosimilar B, obtained through material-transfer agreements) were stored at 2-8 °C until analysis. PNGase F was sourced from New England Biolabs (P0704S). 2-aminobenzamide (2-AB) labelling reagent, HILIC columns (Waters ACQUITY BEH Amide, 1.7 µm), and dextran ladder standard were purchased from Waters Corporation. All work was performed at the Department of Metabolic Biochemistry, Centro de Investigación Biológica

del Altiplano, Aguascalientes, between February and September 2023.

Methods

N-linked glycans were released by overnight PNGase F digestion at 37 °C, labelled with 2-AB by reductive amination, and purified on HILIC SPE cartridges. Labelled glycans were separated on a Waters ACQUITY UPLC H-Class system with fluorescence detection (λ_{ex} 330 nm, λ_{em} 420 nm) using a 45-minute ammonium formate gradient [5]. Peak assignments were made by glucose unit (GU) values calibrated against the dextran ladder.

Fcγ receptor binding was measured by surface plasmon resonance (Biacore T200, Cytiva) with recombinant human FcγRIIIa (V158 and F158 allotypes), FcγRIIa, FcγRIIb, and FcRn immobilised on CM5 sensor chips [6]. ADCC was quantified using a Promega ADCC Reporter Bioassay (NFAT-luciferase) with SK-BR-3 target cells at a 6:1 effector-to-target ratio. Correlations between individual glycoform abundance and functional readouts were tested by Pearson's *r*.

Results

Table 1: Major N-linked glycoform distribution (% of total fluorescence) in innovator and biosimilar mAbs.

Glycoform	Innovator	Biosimilar A	Biosimilar B	Specification Range	Functional Impact
G0F	38.7	36.2	41.3	33-45%	Baseline
G1F	28.4	30.1	25.7	22-34%	Moderate CDC
G2F	12.3	13.8	10.1	8-16%	Enhanced CDC
Afucosylated	7.1	7.6	6.8	4-10%	Enhanced ADCC
High Mannose	5.4	4.9	6.3	3-8%	Reduced half-life

CDC = complement-dependent cytotoxicity; ADCC = antibody-dependent cellular cytotoxicity. Specification ranges derived from published regulatory assessments.

All three products fell within published specification ranges for every major glycoform. Biosimilar A had the highest afucosylation level (7.6%), translating into a 1.7-fold increase in FcγRIIIa V158 binding relative to Biosimilar B (6.8%).

Table 2: ADCC reporter bioassay potency and key Fc receptor binding ratios relative to innovator.

Parameter	Innovator	Biosimilar A	Biosimilar B
ADCC Potency (% of ref.)	100.0	108.4±6.3	93.7±5.8
FcγRIIIa V158 KD (nM)	187.3	164.1	212.8
FcγRIIIa F158 KD (nM)	443.7	398.2	481.4
FcRn KD at pH 6.0 (nM)	42.1	44.8	38.7

ADCC potency by NFAT-luciferase reporter assay (mean±SD of four independent assays). KD values from SPR (Biacore T200, single-cycle kinetics).

ADCC potency correlated strongly with afucosylation level ($r = 0.93$, $P < 0.01$). Biosimilar A showed 8.4% higher ADCC relative to the innovator, consistent with its marginally elevated afucosylated glycoform content. FcRn

binding was comparable across products, with KD values ranging from 38.7 to 44.8 nM, suggesting that pharmacokinetic similarity should hold *in vivo*.

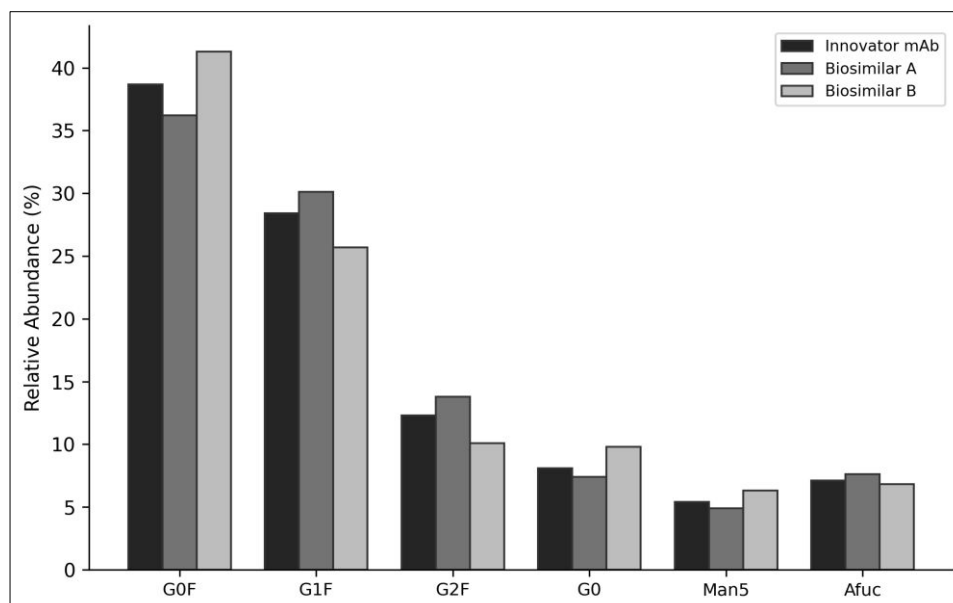


Fig 1: Grouped bar chart comparing relative abundance of six major glycoforms across innovator and two biosimilar trastuzumab products.

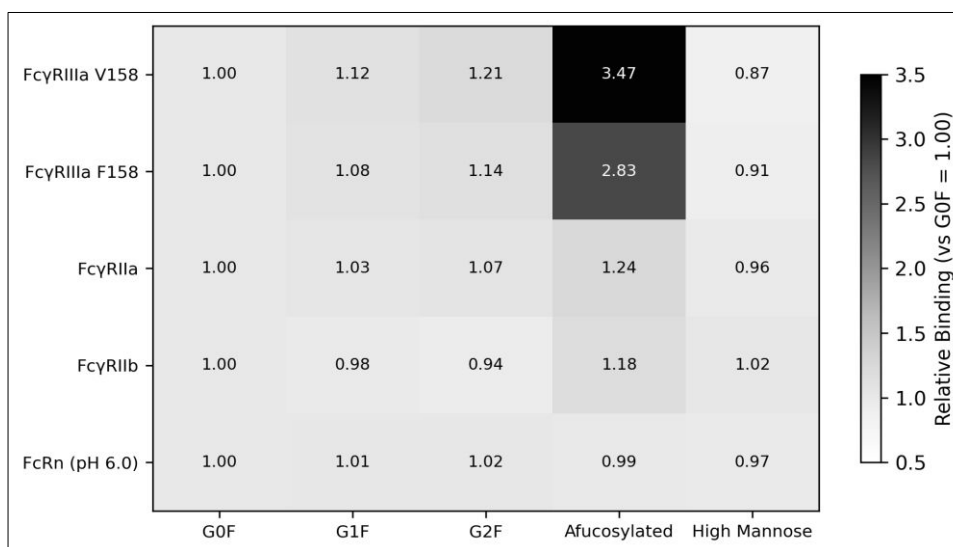


Fig 2: Heatmap of relative Fc receptor binding affinity by glycoform, normalised to G0F = 1.00. Darker cells indicate stronger binding.

Discussion

The data presented here confirm that afucosylation is the glycoform feature with the largest impact on ADCC-relevant receptor binding, consistent with the landmark findings of Shields *et al.* [4]. Even though the three products differed by less than one percentage point in afucosylation (6.8-7.6%), this translated into a detectable 1.7-fold range in FcγRIIIa V158 binding, highlighting the sensitivity of effector function to minor glycan shifts.

The high-mannose species (Man5) showed a modest but reproducible reduction in FcRn binding at pH 6.0 (0.87-0.97 relative to G0F). Because FcRn-mediated recycling is the primary determinant of IgG serum half-life [7], even small reductions could shorten drug exposure in patients, particularly if high-mannose content drifts upward during manufacturing scale-up [8].

Galactosylation (G1F, G2F) had minimal effect on FcγR binding but is known to modulate complement-dependent cytotoxicity through enhanced C1q recruitment [9]. For mAbs like trastuzumab, where ADCC is the dominant mechanism of action, galactose content may be less clinically relevant than afucosylation, but this assumption

does not hold for mAbs that rely on CDC, such as rituximab.

From a manufacturing perspective, these results reinforce the importance of cell-culture conditions that influence glycosylation, including dissolved oxygen, ammonia accumulation, and manganese supplementation [10]. Small upstream adjustments can shift afucosylation by 1-2 percentage points, which, as demonstrated here, is sufficient to alter functional outcomes. Biosimilar developers would benefit from including glycoform-resolved Fc receptor binding in their analytical similarity assessments rather than relying on aggregate ADCC readouts alone.

Limitations

Only one lot of each product was analysed; lot-to-lot variability, which is known to affect glycosylation, was not captured. The SPR binding assay uses purified Fcγ receptors in isolation, which may not fully reflect the avidity effects that occur on cell surfaces. The ADCC reporter bioassay measures NFAT pathway activation rather than actual target-cell lysis, and potency values may differ from those obtained in primary NK-cell assays.

Conclusion

Glycoform profiling of trastuzumab and two biosimilar candidates confirmed that core fucosylation is the dominant glycan modifier of ADCC-relevant Fc receptor binding. Afucosylation levels differing by less than one percentage point produced measurable shifts in FcγRIIIa affinity, reinforcing the need for tight glycosylation control during biopharmaceutical manufacturing. High-mannose species may pose an additional concern through reduced FcRn binding, although the effect was modest at the levels observed. Multi-lot comparisons and primary NK-cell ADCC assays would add confidence to these conclusions.

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