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Supplementing Glycosylation A Review of Applying Nucleotide-Sugar Precursors to Growth Medium to Affect Therapeutic Recombinant Protein Glycoform Distributions

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Abstract

Glycosylation is a critical quality attribute (CQA) of many therapeutic proteins, particularly monoclonal antibodies (mAbs), and is a major consideration in the approval of biosimilar biologics due to its effects to therapeutic efficacy. Glycosylation generates a distribution of glycoforms, resulting in glycoproteins with inherent molecule-to-molecule heterogeneity, capable of activating (or failing to activate) different effector functions of the immune system. Glycoforms can be affected by the supplementation of nucleotide-sugar precursors, and related components, to culture growth medium, affecting the metabolism of glycosylation. These supplementations has been demonstrated to increase nucleotide-sugar intracellular pools, and impact glycoform distributions, but with varied results. These variations can be attributed to five key factors: Differences between cell platforms (enzyme/transporter expression levels); differences between recombinant proteins produced (glycan-site accessibility); the fermentation and sampling timeline (glucose availability and exoglycosidase accumulation); glutamine levels (affecting ammonia levels, which impact Golgi pH, as well as UDP-GlcNAc pools); and finally, a lack of standardized metrics for observing shifts in glycoform distributions (glycosylation indices) across different experiments. The purpose of this review is to provide detail and clarity on the state of the art of supplementation strategies for nucleotide-sugar precursors for affecting glycosylation in cell culture processes, and to apply glycosylation indices for standardized comparisons across the field.

Keywords: glycosylation; glycoform; nucleotide sugars; biosimilar; immune effector functions; mAbs; therapeutic protein; glutamine; ammonia; animal cell culture

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1.0 Introduction

Glycosylation is acknowledged to be among the most important critical quality attributes (CQAs) of therapeutic biologicals, particularly for monoclonal antibodies (mAbs), which must be glycosylated for bioactivity (Dordal et al., 1985; Nose and Wigzell, 1983). Glycosylation is performed by a varying succession of enzymes, which regulate the sculpting and pruning of complex oligosaccharides, referred to broadly as 'glycans', onto biomolecules forming glycoconjugates. With respect to glycoprotein therapeutics, glycosylation introduces one of the major forms of molecule to molecule heterogeneity, which is broadly understood to affect a wide range of biotherapeutic efficacy metrics, including function (Dubé et al., 1988), immunogenicity (Bosques et al., 2010; Ghaderi et al., 2010; Padler-Karavani et al., 2008), drug clearance-rate (Morell et al., 1968), protein stability (Mimura et al., 2001; Wyss and Wagner, 1996), solubility (Leavitt et al., 1977), and in the case of mAbs, immune system recognition for effector function (Tao and Morrison, 1989).

Glycan heterogeneity can be influenced broadly by several process conditions including perturbations to temperature, pH and dissolved oxygen; an excellent review of which is presented by Hossler (2012). Other major factors of influence include the up/down regulation of membrane transporters and glycosyltransferases between cell platforms (Chen and Harcum, 2006; McDonald et al., 2016; Wong et al., 2010), and interruptions to the substrate supply chain of nucleotide-sugar metabolism and transport (Liu et al., 2014; McDonald et al., 2016; Nyberg et al., 1999; Pels Rijcken et al., 1995b). Glycan heterogeneity indicates biotherapeutics with molecule-to-molecule quality differences, and in the case of mAbs will activate (or fail to activate) the immune system in different ways. This also creates a challenge for biosimilars to

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match their glycan distributions with that of their reference (innovator) biologics, or at least demonstrate that any differences are not clinically significant (FDA, 2015).

Three major strategies exist to affect the glycosylation of recombinant proteins:

1. glycoengineering of cell platforms generally targeting either the addition or knock-out (including silencing) of glycosyltransferases (Mori et al., 2004; Yamane-Ohnuki et al., 2004), or similarly targeting nucleotide-sugar Golgi transporters (Wright and Morrison, 1998);
2. downstream *in vitro* remodelling of glycans (Hodoniczky et al., 2005);
3. supplementation of nucleotide-sugar precursors and associated components, such as sugars and amine-sugars (Tables 1-3), including nucleosides like uridine and cytidine (Carvalhal et al., 2003; Nyberg et al., 1999), and the metallic ion manganese (Crowell et al., 2007; St Amand et al., 2014; Surve and Gadgil, 2015).

This review will focus on the latter of these methods, providing clarity towards five key factors that have contributed to varied results and interpretations for this promising method of tuning glycosylation in cell culture processes. The first of these factors are the differences in enzyme/transporter expression levels across cell platforms. Secondly, differences between recombinant proteins produced, particularly with respect to the accessibility of their glycan sites by glycosyltransferases. Thirdly, the fermentation and sampling timeline, both with respect to the glucose availability, as well as the accumulation of exoglycosidases at the latter stages of cultures. A fourth factor is the glutamine levels, as increases in ammonia alter the pH of Golgi compartments, and also increase UDP-GlcNAc intracellular pools. Finally, differences in metrics for reporting different glycan attributes, for instance galactosylation, which prevent more standard comparisons of observed shifts in glycoform distributions between reports.

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2.0 Glycosylation

The variability and probabilistic nature of glycosylation (Spahn et al., 2016) lends itself to a security recognition function *in vivo*, mediating countless lectin/ligand binding scenarios for sensing and signaling events, both at the molecular level as well as more broadly for cell signalling. Moremen and colleagues (2012) provide an excellent overview of glycosylation in vertebrates, and the resulting complexity of system-level interactions. From a therapeutics perspective, the significance and potential of glycosylation with respect to current and future biologics continues to grow (Dalziel et al., 2014).

Generally speaking, glycosylation comes in two main forms, N-linked and O-linked, which are designated by the functional group of the amino-acids where the glycans are bound. N-glycans are bound via amide-linkages to asparagine residues, and are typically larger oligosaccharides compared to O-glycans, which are bound to serine or threonine by glycosidic bonds. With respect to therapeutic glycoproteins, N-linked glycosylation receives the most attention in literature, particularly regarding mAbs, as glycosylation of the Fc region is a major factor in immune system effector functions, such as complement activation, and binding to Fcγ receptors of leukocytes towards various immune responses (Tao and Morrison, 1989). O-glycosylation is not yet recognized as notably consequential to effector functions or bioefficacy for many approved glycoprotein therapeutics, but will affect some basic efficacy characteristics like protein stability (Wang et al., 1996).

2.1 Nucleotide-Sugar Metabolism

Substrates of glycosylation generally take the form of monosaccharides paired to particular nucleoside-phosphates (i.e. UDP-GlcNAc, GDP-Fucose, CMP-NeuAc, etc.), and are derived generally from glucose metabolism, as detailed in Figure 1. Nucleotide-sugars are generally

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produced in the cytoplasm, with the exception of cytidine monophosphate N-acetylneuraminic acid (CMP-NeuAc), which is formed in the nucleus (Kean, 1970). Mammalian cells are able to take up a wide diversity of saccharide nutrients through the GLUT and SGLT families of cell membrane transporters. Supplementing nucleotide-sugar precursors circumvents points of regulation and feedback inhibition (Pels Rijcken et al., 1995b), leading to large fold-change increases in intracellular metabolite pools (Tables 1-3). Augustin and Mayoux (2014) present an excellent review of these transporter families, while Freeze and Elbein (2009) provide an exceptional review of the points of regulation controlling this metabolic network. The formation of amine-sugars like glucosamine (GlcN) require an amine donor, such as glutamine, through the action of glutamine-fructose-6-phosphate transaminase (Figure 1, Reaction 3), and these intracellular pools will be starved in glutamine's absence (Nyberg et al., 1999). However, it has also been demonstrated that ammonia can be utilized as a donor towards formation of UDP-GlcNAc and subsequent amine sugar pools (Valley et al., 1999).

With the exception of the endoplasmic reticulum (ER), where dolichol-linked monosaccharide substrates like glucose and mannose are 'flipped' from the cytosol into the organelle lumen (Figure 1, Reaction 24), nucleotide-sugars are typically transported by means of the SLC35 family of transporters, which require the respective nucleotide-monophosphate on the opposing side of the membrane to complete the exchange, as shown in Figure 1. Ishida and Kawakita (2004) have assembled a comprehensive review of the SLC35 family of transporters. Availability of particular nucleoside-monophosphates within the Golgi lumen is dependent on the activity of nucleoside diphosphatase (NDPase) to convert nucleoside-diphosphates to their monophosphate form (Figure 1); which has been identified as a potential bottleneck in glycan metabolism (McDonald et al., 2016).

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2.2 N-Linked Glycosylation

N-linked glycosylation begins in the cytoplasm with the assembly of a seven sugar oligosaccharide onto a dolichol diphosphate anchor (Figure 1, Reaction 23). Once assembled, this oligosaccharide is flipped into the endoplasmic reticulum (ER) where dolichol phosphate linked mannose and glucose species donate the remaining four mannose residues and three glucose residues to complete the fourteen sugar N-glycosylation oligosaccharide precursor. This lipid-linked oligosaccharide is then detached from its dolichol phosphate anchor and bound to an asparagine amino acid of a protein undergoing translation by the oligosaccharyltransferase (OST) enzyme complex (Figure 1, Reaction 26). The resulting precursor to N-linked glycosylation is bound to the protein at a site referred to as a 'sequon'. Once bound to its sequon, in step with protein folding, glycotransferase enzymes of the ER act upon the oligosaccharide precursor glycan, removing the three terminal glucose moieties as well as the centremost terminal mannose (Figure 1, Reaction 27).

2.3 Microheterogeneity

Glycosylation continues into the Golgi, where further glycosyltransferase activity will produce a myriad of glycans that diverge between different cell types. With particular regards to mammalian style complex glycans, forms of microheterogeneity include whether the terminal moieties are mannose (Man) or acetyl-glucosamine (GlcNAc). The degree of GlcNAc branching is referred to as "antennarity", which can be as many as four, plus an extra bisecting GlcNAc on the central mannose. Other forms of microheterogeneity include the binding of core α_{1-6} fucose (Fuc) to the first GlcNAc bound to Asparagine (Asn); as well as the binding of galactose (Gal) to terminal GlcNAc moieties possibly followed by binding of sialic acids (NeuAc/Neu5Gc) to Gal, which are the only glycan moieties to confer a negative charge.

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Differences between glycoforms can be written by abbreviated nomenclatures, such as the Oxford Glycobiology Institute convention, described by Gornik and colleagues (2007), and utilized here. In this convention, the 'F' refers to fucose bound by an $\alpha_{1\text{-}6}$ bond, the 'A' to the degree of antennarity (i.e. GlcNAc branching), 'B' for the presence of a bisecting GlcNAc, 'G' to the degree of galactosylation, and the 'S' for the degree of sialylation.

3.0 Controlling Glycosylation by Supplementation

Typical glycoforms observed for several commercial mAbs are under-galactosylated, hindering their therapeutic potential (Wacker et al., 2011). Research in the field of supplementing nucleotide sugar precursors for affecting glycoforms follows a hypothesis that either substrate-limitation or regulatory feedback bottlenecks prevent more complex glycoforms with terminal galactose and sialic acid moieties, like those observed in endogenous human sera (Flynn et al., 2010). These supplementation strategies, detailed in Tables 1-3, and Figures 3-5, offer a simple means to adjust product glycoforms without having to completely redesign (i.e. glycoengineer) cell platforms.

Nucleotide-sugar precursors and associated components supplemented to growth media have conventionally targeted what were regarded as the most crucial glycan-moieties for mammalian N-glycosylation: N-acetylglucosamine (GlcNAc), galactose (Gal), and the sialic acid N-acetylneuraminic acid (NeuAc). Precursors selected for supplementation favour intermediate species at various points in the intracellular metabolic synthesis of these nucleotide-sugars that would otherwise be synthesized from glucose, as shown in Figure 1.

The three compounds most commonly supplemented in literature include:

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- Glucosamine (GlcN), and more recently N-acetylglucosamine (GlcNAc), often accompanied with uridine (Urd) – as detailed in Table 1 and Figure 3
- Galactose (Gal), often accompanied with uridine (Urd), and occasionally manganese[1] (Mn^{2+}) – as detailed in Table 2 and Figure 4
- N-acetylmannosamine (ManNAc), often accompanied with cytidine (Cyt) – as detailed in Table 3 and Figure 5

These supplements have repeatedly been reported to increase the intracellular pools of nucleotide-sugars, and affect glycoform distributions. Inconsistencies in reported effects can be attributed to differences across five key factors: the host cell platform, the recombinant protein expressed, timeline of fermentation from supplementation to sampling/harvest, the amount of glutamine present in culture media, and a previous lack of standardized metrics for comparison i.e. glycosylation index equations (Figure 2) for observing shifts in glycoform distribution, applied here.

3.1 Key Factors Impacting Supplementation Results

3.1.1 Cell Platform

Differences across cell platforms are expected to produce varying glycan distributions, as it is common even to have clones of the same cell-line that under or over-express an important enzyme or transporter, such as the YB2/0 rat hybridoma cells utilized by Shinkawa and colleagues (2003) that had low levels of fucosyltransferase-8 (FUT8), leading to low-fucose mAbs. Similarly, differences between different mammalian cell lines will commonly produce varying effects under identical nucleotide-sugar precursor supplementation strategies. This can be observed in the work of Baker and colleagues (2001), when the same TIMP-1 protein was

[1] Manganese ions as a supplementation strategy has primarily been utilized in conjunction with uridine and galactose as part of the cocktail recommended by Gramer and colleagues (2011).

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expressed in both GS-CHO and GS-NS0 cells; 10mM glucosamine supplementation resulted in a significant antennarity index increase of 9% in the former ($P < 0.05$), but no change in the latter.

3.1.2 Protein Glycan Site

The type of protein examined is perhaps the greatest factor leading to differences in glycan profiles, as different glycan sequons will have very different solvent accessibility. A literature analysis conducted by Thaysen-Andersen and Packer (2012) determined that core α_{1-6} fucosylation, glycan antennarity, and complex glycoforms vs high mannose, are all notably affected by the solvent-accessibility of glycan sites, and hypothesized the same to be true for terminal galactosylation and sialylation. Therefore, one cannot draw a direct comparison between the glycans derived from EPO and mAbs, without considering that the sequon sites on the two proteins will have very different accessibility to the Golgi infrastructure to modify those glycans. This issue can be observed in Figures 3-5, with respect to the antennarity of different proteins. In Figure 4, the proteins are predominantly IgGs, and glycans are uniformly biantennary or lower, keeping their AI below 40%, while proteins like EPO, TIMP-1, and IL-2 shown in Figures 3&5 can have higher levels of branching due to the accessibility of these sequons to latter N-acetylglucosyltransferases (GnT IV&V) (Thaysen-Andersen and Packer, 2012). Engineering of protein sequences and glycan sites towards affecting therapeutic efficacy further complicates comparisons between systems. Park and colleagues (2016) provide a comprehensive review of methods for modifying Fc sequences to alter glycoforms and generally improve therapeutic potential of biologics.

3.1.3 Fermentation Sampling Timeline

Another very important factor is the fermentation timeline, particularly the time of sampling or harvest of recombinant proteins for glycan analysis. It has been demonstrated by Gramer and

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colleagues (1993, 1995), that CHO cells accumulate sialidase enzymes in their supernatant, and growing cultures past exponential phase until loss of viability can diminish the total sialylation of secreted recombinant proteins. Furthermore, a clear relationship has recently been demonstrated by Liu and colleagues (2014) between availability of glucose and the level of galactosylation and sialylation of mAbs grown in CHO cells. Therefore, in experiments where recombinant proteins are harvested for glycan analysis near the peak of exponential growth, proportions with terminal sialylation will be in greater proportion compared to those harvested when viability is decreasing. This can be observed in Figure 4, comparing the experiments of both Liu and colleagues (2014) and Surve and Gadgil (2015), who sampled their cultures during exponential phase, compared against those of St Amand and colleagues (2014) and Gramer and colleagues (2011), who sampled at the end of the culture. In the case of the former, both controls and final GI levels are generally higher than the latter, despite similar supplementation regimes, cell lines, and IgG proteins. Exceptions can be observed of course in the results of Grainger and James (2013), who sampled at the end of their culture, showing high GI values for both their controls and final galactosylation percentage; however, one can argue these values might have been even higher with earlier sampling.

3.1.4 Glutamine Level

Glutamine is of particular interest due to its relationship with ammonia, which directly impacts glycosylation activities in two ways. First, ammonia is a weak base capable of diffusing across cellular membranes, impacting the intracellular pH of cellular organelles like the Golgi. The cis-, medial-, and trans-Golgi cisternae operate at distinct acidic pH levels, as shown in Figure 1. Glycosyltransferases form reversible homomeric and heteromeric dimers, which migrate as mobile complexes between the Golgi and the ER (Hassinen and Kellokumpu, 2014). Homodimers are favoured closer to the neutral pH of the ER, where these enzymes are

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synthesized, while formation of heteromeric dimers, with enhanced activity, are favoured at the reduced pH points of the respective Golgi cisternae where they operate (Hassinen and Kellokumpu, 2014). This is illustrated in the Golgi glycosyltransferase enzyme pairings in Figure 1. Higher ammonia levels will neutralize the intracellular pH of Golgi cisternae, causing heterodimer pairs to split and reform to their homodimer formats, reducing their functionality (Hassinen and Kellokumpu, 2014). Therefore, elevated supplementation of the labile nutrient glutamine, which increases intracellular ammonia levels, will in turn increase Golgi pH, and perturb the functionality of glycosyltransferases, particularly galactosyltransferase and sialyltransferase, decreasing the GI and SI of glycans. This result was demonstrated directly by Aghamohseni and colleagues (2014), who showed that supplementing increasing concentrations of glutamine to CHO cultures expressing a heavy-chain antibody (HCAb) reduced galactosylation and sialylation of these glycoproteins. This can further be observed in Figure 4, as groups utilizing glutamine synthase (GS) amplified cultures, also tend to have higher GI levels.

The second manner in which glutamine supplementation affects glycoform distributions is by directly increasing UDP-GlcNAc pools producing similar effects and glycoform shifts to those detailed in Table 1. Valley and colleagues (1999) supplementing ammonium ions with ^{15}N tracers to BHK-21 cells produced a significant increase in UDP-GlcNAc intracellular nucleotide sugar pools. Approximately 60% of this pool incorporated the ^{15}N tracers – a proportion approximately equivalent to the increase in the pool itself (Valley et al., 1999). As is demonstrated in Figure 3, elevated glucosamine levels will reduce galactosylation and sialyation of glycoproteins. Therefore, elevated glutamine supplementations to cultures can have a similar

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effect to those reported for either ammonium or glucosamine supplementation (Gawlitzek et al., 1998; Grammatikos et al., 1998; Yang and Butler, 2002).

CHO cells expressing glutamine synthase (GS) require little or no glutamine in their growth medium. St Amand and colleagues (2014), Kildegaard and colleagues (2015), and Surve and Gadgil (2015) all utilized glutamine nutrient in their cultures; 4mM for the former, and 8mM for the latter two respectively (Table 2). Galactose supplementation improves GI for all three of these research groups, but it's typically an increase on the order of only 10%, while researchers supplementing galactose to GS-CHO cultures achieve GI increases for mAbs of 20% or higher (Figure 4). Therefore, addition of glutamine nutrient may limit the potential improvement to GI of galactose supplementation.

3.1.5 Standardized Metrics for Reporting Glycan Shifts

Lastly, given the various experimental differences across reports in literature, it is difficult to make comparisons and draw conclusions regarding the effectiveness of a particular supplementation regime without applying a standard metric and examining glycoform shifts with respect to control cultures. As such, new glycoform distribution index calculations have been created for this review. These calculations are detailed in the following section and have been applied to standardize comparisons between experimental controls and resulting shifts. These comparisons are detailed in Tables 1-3 and Figures 3-5. While glycan index equations have been proposed and utilized by previous researchers, our equations represent the first comprehensive set of equations that can describe N-glycoform characteristics of a range of complexity, including bisecting and multi-antennary glycoforms (Figure 2). A major difference between these equations and those presented by others is that these equations recognize that glycosylation is the result of a sequence of steps that build off one another. In other words, only species that

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have a galactose moiety can be sialylated; therefore the number of sialylated species is compared to the species that have been galactosylated and not all glycoforms. This allows us to interpret the effectiveness of the methodologies to change the glycoform. Consider trying to improve sialylation of glycoforms where all species having galactose moieties are already sialylated. The chance of improving sialylation will be non-existant unless the strategy utilized also improves galactosylation. These equations, however, should not be used to describe the percent glycosylation of an entire population. For example, if the final drug formulation requires a certain percent of all species to contain a sialic acid, then these equations would misrepresent this idea. The equations proposed here have been designed with process improvements in mind.

3.2 Glycoform Distribution Indexes

Our set of indices summarize the degree of glycosylation with respect to glycoform characteristics such as galactosylation (GI), sialylation (SI), core α_{1-6} fucosylation (FI), antennarity (AI), and high-mannose types (MI). These equations can be described generally as a measurement of how much of an available glycan substrate has received a particular glycan moiety as described in the previous section. Figure 2 details each of the five equations, their derivation from a glycan distribution, and provides sample calculations with respect to human serum IgG1 (Flynn et al., 2010) and a recombinant HCAb (Blondeel et al., 2015). Furthermore, Figures 3-5 provide detailed comparisons across the state of the art, regarding both the shifts in the glycosylation of cultures under the reported supplementation regimes, but also comparisons of the starting glycoforms of control cultures. A detailed examination of these is found in the following sections.

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3.3 Supplementation of Nucleotide-Sugar Precursors

3.3.1 Glucosamine (GlcN) & Acetyl-glucosamine (GlcNAc)

Glucosamine (GlcN) supplementation is consistently reported to increase intracellular pools of UDP-HexNAc (i.e. UDP-GlcNAc & UDP-GalNAc), as detailed in Table 1. UDP-GlcNAc/GalNAc intracellular pools form in the cytoplasm and are linked by an epimerase (Figure 1, Reaction 17), which holds them in relatively fixed concentration proportions of approximately 2:1 (Blondeel et al., 2015; Glaser, 1959).

Glucosamine supplementation is commonly reported to reduce the complexity of N-glycan terminal moieties, favouring "G0" glycoforms featuring terminal GlcNAc (i.e with reduced galactosylation and sialylation) (Hills et al., 2001; Pels Rijcken et al., 1995b; Yang and Butler, 2002; Zanghi et al., 1998). GlcN is also reported to increase glycan antennarity (Baker et al., 2001; Gawlitzek et al., 1998; Grammatikos et al., 1998); however, this is only reported for non-IgG proteins with higher-antennarity glycans (Thaysen-Andersen and Packer, 2012), such as TIMP-1 & IL-2 to-date (Table 1, Figure 3). Conversely, Yang and Butler (2002) actually reported the opposite effect, with a reduction in antennarity of EPO from glucosamine feeding. However, Yang and Butler also reported elevated ammonia levels and increased glutamine uptake during GlcN supplementation, while Baker and colleagues (2001) reported minimal increase in ammonia in their GS cells. As such, this effect may depend on how efficiently the cell platform converts ammonia to UDP-GlcNAc (Valley et al., 1999), as opposed to harvesting the glucose from glucosamine via glucosamine-6-phosphate deaminase (Figure 1, Reaction 5). As noted in the previous section, Valley and colleagues (1999) supplementing ^{15}N traced ammonium ions, determined that BHK-21 cells produced a significant increase in UDP-GlcNAc intracellular nucleotide sugar pools. Metabolomic analysis of CHO cultures producing HCAbs supplemented with both GlcN and GlcNAc reported neither elevated ammonia, nor increased

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uptake of glutamine; however, a three-fold increase in acetate was observed after glucose exhaustion, which has been attributed to scavenging of glucose from UDP-GlcNAc pools in stationary phase (Blondeel et al., 2015). Elevated ammonia levels will raise the pH of Golgi compartments, and prevent heteromeric glycosyltransferase dimer complexes, such as GnT-IV/V from functioning properly (Hassinen and Kellokumpu, 2014). Further, increasing glutamine can produce a similar effect as supplementing GlcN and GlcNAc to mammalian cultures (Aghamohseni et al., 2014). Therefore, the effect of increasing antennarity may depend on whether the metabolism favours formation of UDP-GlcNAc pools or scavenging glucose depleting those same pools.

Wong and colleagues (2010) reported the only instance where GlcN supplementation increases the complexity of N-glycans' terminal moieties, with increased sialylation of IFN-γ. It is worth noting that increased sialylation was observed for all of Wong et al.'s experimental conditions including supplementation with galactose and ManNAc, with and without associated nucleosides. Furthermore, the experiment appears to be n=1, with only a single control flask. Therefore, it is possible that the control flask for this experiment is merely an outlier of reduced sialylation. Nyberg and colleagues (1999) similarly cultured CHO cells expressing IFN-γ, and supplemented uridine rather than GlcN, reporting smaller but significant increases to intracellular UDP-GlcNAc pools (Table 1); however, no such increase in sialylation of IFN-γ glycoforms was observed.

While more complex glycans (i.e. those possessing terminal galactose and sialic acid moieties) are therapeutically more desirable, the glycoform distributions of commercial therapeutic mAbs have a greater proportion of terminal GlcNAc "G0" glycoforms (Wacker et al., 2011). As such, reference biologics for the development of biosimilars also favour G0 glycan

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types. Therefore, GlcN/GlcNAc supplementation has been demonstrated as a useful tool for tuning N-glycan distributions towards G0 forms in cultures to achieve a target glycoform distribution (Blondeel et al., 2015). For supplementation strategies, GlcNAc should be considered more favourable compared to GlcN, which has been shown to restrict growth in mammalian cultures producing recombinant proteins (Baker et al., 2001; Blondeel et al., 2015; Grammatikos et al., 1998; Hills et al., 2001; Wong et al., 2010; Yang and Butler, 2002; Zanghi et al., 1998). Glucosamine has further been demonstrated to restrict growth intransformed cancerous cells that are not expressing recombinant proteins (Bekesi and Winzler, 1970; Krug et al., 1984; Oh et al., 2007; Pederson et al., 1992). A similar species to GlcN, galactosamine (GalN) also produces cytotoxic effects when supplemented to cultures (Pels Rijcken et al., 1995a). Restricted growth from supplementation of hexosamines (GlcN/GalN) have been attributed to either competition with glucose transport (Yang and Butler, 2002), or depletion of intracellular ATP and UTP pools (Pels Rijcken et al., 1995a). Pels Rijcken and colleagues (1995a) hypothesized this effect was due to depletion of phosphorylated pyrimidines inhibiting RNA synthesis. However, it has more recently been demonstrated that negative growth from GlcN (and likely GalN) is actually from depleting pools of cytosolic acetyl-CoA, which convert GlcN to GlcNAc (Blondeel et al., 2015), an essential pool for lipid biosynthesis and cell division (Goudar et al., 2010; Quek et al., 2010). Supplementing GlcNAc has been demonstrated to remove this growth inhibition, while still producing an increase in intracellular UDP-GlcNAc pools (Blondeel et al., 2015; Kildegaard et al., 2015). GlcNAc supplementation may still draw phosphorylated pyrimidines away from pools for RNA synthesis leading to more rapid loss of viability in stationary phase; however, the demonstrated improved growth from avoiding acetylation by supplementing GlcNAc makes the activity of glucosamine-phosphate N-

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acetyltransferase the more likely source of growth inhibition for this group of glycosylation supplements.

3.3.2 Galactose

Galactose (Gal) supplementation represents the most successful example of nucleotide-sugar precursor feeding to affect glycoforms to-date. This strategy has been reliably reported across several different research groups, utilizing different cell platforms, expressing different proteins, to increase galactosylation (Figure 4). Even beyond galactosylated glycoforms, supplementation of Gal has also been demonstrated to relieve bottlenecks towards improving the sialylation of Fc-fusion glycoproteins in GS-CHO cultures (Liu et al., 2015). Increased galactosylation is generally accepted to be of therapeutic benefit, with lack of galactosylation associated endogenously with several disease states (discussed in Section 4).

Precursor supplementation strategies often include the complementary nucleoside for a supplemented sugar, such as uridine (Urd) for GlcN and Gal, or cytidine (Cyt) for ManNac. Galactose supplementation strategies are the first to also incorporate manganese ions (Mn^{2+}) to enhance the activity of galactosyltransferase, demonstrated by Gramer and colleagues (2011) in GS-CHO cultures producing IgG. These researchers proposed a synergistic cocktail (in static proportions), featuring uridine, manganese ions, and galactose (UMG), and experimented with increasing concentrations up to 20mM galactose, to achieve more than a 20% increase in galactosylation (Figure 4), with improvements plateauing at 8x UMG (16μM Mn^{2+}, 8mM Urd, and 40mM galactose).

The results of the UMG cocktail were replicated by Grainger and James (2013) utilizing a face-centred designed experiment (i.e. varying the proportions of the UMG supplements), for main effects analysis in two CHO cell lines expressing IgG. These researchers also demonstrated

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more than a 20% increase in IgG galactosylation (Figure 4); however, they determined the best results overall – including growth, mAb titre, mAb galactosylation – from supplementing the low-low-high (LLH) condition (1μM Mn^{2+}, 0.5mM Urd, and 100mM Gal). Variations on the UMG cocktail have continued to be utilized by researchers reporting varying degrees of success for increasing glycoprotein galactosylation (Liu et al., 2014; St Amand et al., 2014; Surve and Gadgil, 2015).

Towards better understanding the main effects of supplementing Gal, Urd, and Mn^{2+}, researchers have pursued various experimental arrangements and concentrations (Table 2, Figure 4). While galactose together with uridine is reported to generate superior results (Grainger and James, 2013; Gramer et al., 2011; Wong et al., 2010), galactose together with manganese alone performs slightly worse (St Amand et al., 2014; Surve and Gadgil, 2015) (Figure 4). Manganese supplementation has been reported to actually reduce fucosylation of IgG in CHO cultures (St Amand et al., 2014; Surve and Gadgil, 2015); however, St Amand and colleagues (2014) report this effect coincides with increased high-mannose type glycans and loss of antennarity (Figure 4).

3.3.3 ManNAc

N-acetylmannosamine (ManNAc), often accompanied by the nucleoside cytidine (Cyt) is supplemented to cultures to increase the intracellular nucleotide-sugar pool of CMP-acetylneuraminic acid (CMP-NeuAc), with the goal of improving the sialylation of glycoproteins (Table 3). Several researchers have reported improvements in the sialylation of glycoproteins in mammalian cell culture following supplementation of ManNAc (Gu and Wang, 1998; Wong et al., 2010; Zanghi et al., 1998). Gu and Wang reported a 15% increase in sialylation, and when supplementing 20mM radiolabelled ManNAc, all of the resulting sialylation included the tracer

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(Gu and Wang, 1998). Interestingly, researchers that do not observe increases in sialylation typically reported a deficit of galactosylation that could be improved with galactose feeding (Hills et al., 2001; Kildegaard et al., 2015; Liu et al., 2015). Liu and colleagues (2015) supplementing galactose improved galactosylation in cultures, which subsequently also lead to increased sialylation by 14%. Synergistic supplementation of galactose and ManNAc is still yet to be reported.

Sialylation of glycoproteins by rodent-derived mammalian cell cultures comes with some risk of the immunogenic glycoform CMP-Neu5Gc, a glycan which does not occur endogenously in humans (Ghaderi et al., 2010; Padler-Karavani et al., 2008). A benefit of ManNAc supplementation reported by Baker and colleagues (2001) is that Neu5Gc moieties were reduced by 22%, making this an intelligent culture addition regardless of whether overall sialylation increases.

3.3.4 Nucleosides

Nucleoside supplementation on its own has been considered a viable strategy for affecting glycoforms, as they have similarly been shown, if more moderately, to increase nucleotide-sugar pools (Tables 1&3). Nyberg and colleagues (1999) reported that supplementing up to 10mM uridine to CHO cultures expressing IFN-γ observed a linear correlation between increased UTP levels and UDP-GlcNAc pools. Pels Rijcken and colleagues (1995b) similarly observed corresponding increases of UTP, UDP-glucose/galactose, and UDP-GlcNAc/GalNAc upon supplementing either 0.5mM uridine or cytidine to primary cultures of rat hepatocytes. Few strategies for supplementation of nucleotides, nucleosides, or nitrogenous bases beyond the pyrimidines exist, as these have been demonstrated to produce negative growth effects (Carvalhal et al., 2003).

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3.3.5 Other Exotic Sugars and Supplements

Beyond the classic nucleotide sugar precursor supplements detailed in Tables 1-3, several additional supplementation strategies have been attempted to exert control over glycan distributions. Inhibitors to particular glycosyltransferases may be utilized to produce hybrid and high-mannose type glycans; however, these present little therapeutic advantage (Section 4).

Several unconventional saccharides have been attempted, including mannose, fucose, and N-acetylneuraminic acid (NeuAc); however, none of these produced any notable shifts to glycoforms (Kildegaard et al., 2015). Hossler and colleagues (2017) have recently reported supplementation of nine unconventional sugars to CHO cultures expressing a mAb glycoprotein. While several of these sugars produced high-mannose and hybrid type glycoforms, melezitose, turanose and to a lesser extent lactose supplemented at 1, 10, 25 and 50mM, created significant increases in galactosylation of the mAb (Hossler et al., 2016). Similarly, these researchers have previously demonstrated that supplementing D-arabinose and L-galactose at 50 mM to CHO cultures expressing a mAb glycoprotein can achieve a complete exchange of fucose moieties for these alternate sugars producing benefits for bioactivity by antibody dependent cell cytotoxicity (ADCC) (Hossler et al., 2017).

4.0 Glycoforms: Bioefficacy and Functionality

Glycoprotein biotherapeutics include mAbs, cytokines, hormones, clotting factors, and growth factors, a thorough listing of these approved by the FDA are available (Ghaderi et al., 2012). Much research has been devoted to understanding how shifts in glycoforms change effector function for mAbs, due the myriad ways IgGs activate the immune system (Tables 4-8). For other glycoproteins, the primary concern is generally terminal sialylation, as this has the greatest impact on extending serum half-life for most glycoprotein therapeutics like tissue

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plasminogen activator (tPA) and erythropoietin (EPO), and reducing how often drugs must be re-administered to patients (Cole et al., 1993; Elliott et al., 2004). Albrecht and colleagues (2014) provide an excellent examination of the impact of glycoform on commercial therapeutics, particularly Darbepoetin-α, a commercial therapeutic recombinant erythropoietin (EPO), which is modified to possess two additional N-glycan sites compared to the usual three in human EPO. Darbepoetin-α carries a much greater proportion of sialic acid moieties compared to the two other commercial forms of EPO, epoetin-α/β (Egrie et al., 2003). These additional sialylated glycan sites effectively double the half-life and bioactivity; however, Darbepoetin's higher aggregate sialylation causes a 5-fold reduced receptor binding, requiring higher dosages (Egrie et al., 2003; Elliott et al., 2004). This result corresponds to reports by Scallon and colleagues (2007), who observed that higher sialylation of mAbs impeded their binding to FcγRIIIa receptors to activate antibody dependent cell cytotoxicity (ADCC), a major bioactivity metric of mAbs (Table 7).

Serum clearance of glycoproteins is often the result of receptors for specific terminal glycans, like mannose binding receptors on liver endothelial cells, or asialoglycoprotein receptors for galactose moieties, also of the liver (Smedsrød and Einarsson, 1990). Conversely to most glycoproteins, it has been reported that sialylation does not extend serum half-life *in vivo* for mAbs (Kaneko et al., 2006; Wright and Morrison, 1998), a result possibly due to the positioning of the glycan site on the Fc region, where IgG glycans are predominantly located.

4.1 Immune Effector Functions for Antibodies

The Fab regions of antibodies allow them to bind to their target antigens with high specificity, but it is their glycosylated Fc regions that interact with and activate immune responses. IgGs possess paired glycosylation sequons at 'asparagine 297' (Asn_{297}) on the heavy

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chain CH2 domain of their Fc region, and produce varying immune response activation capability *in vivo* depending on their glycoform (Tables 4-8). Jefferis (2012) provides an excellent overview of antibody isotypes, their key features, and interactions with the immune system.

The Fc region of antibodies interacts with the immune system by binding to numerous different Fc receptors on the surfaces of immune cells, as well as complexing with C1q, which activates the Complement system cascade. Complement can also be activated by G0 and high-mannose glycans via mannose-binding lectin (MBL) activation; however, such glycoforms also observe rapid clearance from serum (Table 6) (Malhotra et al., 1995; Nimmerjahn et al., 2007). The interaction of immune cell antibody receptors (FcγR) with an antibody-antigen complex mediates a host of immune effector functions, such as antibody-dependent cell phagocytosis (ADCP), cytolysis or cytotoxicity (ADCC), and the release of cytokines and chemokines spurring further cascades of immune responses within the highly regulated immune system. Vidarsson and colleagues (2014) provide a comprehensive review of these effector functions. Therapeutic monoclonal antibody (mAb) glycoforms are often characterized by how well they can recruit immune effector functions, particularly ADCC and Complement dependent cell cytotoxicity (CDC).

Regarding activation of ADCC and CDC endogenously, a typical glycan distribution for human serum IgG1&2 from five healthy volunteers showed a prevalence for five main glycoforms: fucosylated biantennary G0, G1, G2, G2S1, and bisecting G1 (Flynn et al., 2010). While this and similar studies provide a general conception of a "normal" glycan distribution for mAbs, an important characteristic of antibody glycoforms *in vivo*, is their tendency to shift with pathology, as evidenced by Kaneko and colleagues (2006), who reported the downregulation of

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sialylated antibodies *in vivo* during antigenic challenge removing their anti-inflammatory properties (Table 7). Therefore, the optimal distribution of glycans on therapeutic antibodies likely varies from the distribution reported by Flynn and colleagues for treating a particular disorder/pathology. Kapur and colleagues (2014) provide a summary of patterns of IgG-antibody glycosylation with respect to their effects in reported pathologies.

4.2 Glycoforms and Antibody Immune Effector Functions

Recognition and binding of IgGs by C1q and Fc receptors are affected by glycoforms yielding different binding affinities, or steric effects (Ferrara et al., 2006), and therefore significant variation in triggering the immune system. Tables 4-8 demonstrate how changes in glycoforms of antibodies affect their immune effector functions (IEF).

4.2.1 Fucosylation

Core $\alpha_{1\text{-}6}$ fucosylation on the first GlcNAc moiety of the N-glycoform is typical for the majority of human glycans both for antibodies (Flynn et al., 2010) and other secreted glycoproteins (Takeuchi et al., 1988). However, absence of fucosylation has been reported to dramatically increase affinity of IgGs for FcγRIIIa receptors of NK cells, increasing ADCC responses by purified peripheral blood monocytes during *in vitro* assays (Table 4). Steric interaction between the Asn_{297} glycan of the antibody Fc and the Asn_{162} glycan of the FcγRIIIa receptor has been proposed as the mechanism behind this improvement (Ferrara et al., 2006). This also corresponds with research linking individuals with polymorphic FcγRIIIa receptors to improved clinical outcomes when treated with rituximab where core-fucosylation is still present (Cartron et al., 2002). While a clear relationship between fucosylation and ADCC has been reported, several researchers have found no relationship between the core $\alpha_{1\text{-}6}$ fucose moiety and CDC (Chung et al., 2012; Shields et al., 2002; Yamane-Ohnuki et al., 2004).

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4.2.2 Bisecting N-Acetylglucosamine (biGlcNAc)

Core α_{1-6} fucosylation of the N-glycoform is partially inhibited by the GnT-III enzyme, absent in the CHO genome (Xu et al., 2011). GnT-III acts to append a bisecting GlcNAc moiety to the first mannose residue of the glycan (Schachter, 1986). The higher ADCC response from NK cells via FcγRIIIa binding was initially associated with antibodies bearing the bisecting N-GlcNAc (these results are detailed in Table 5); however, it was reported that *in vitro* remodelling of rituximab and trastuzumab glycans to produce antibodies with both core-fucose and bisecting GlcNAc glycoforms demonstrated little improvement (~10%) to ADCC (Hodoniczky et al., 2005). In contrast, Shinkawa and colleagues (2003) column-purified fractions of IgG produced from rat hybridoma YB2/0 cells to obtain fractions high in both bisecting GlcNAc and core α_{1-6} fucosylation, and determined no improvement towards ADCC. Therefore, the benefit of the bisecting glycoform, at least with respect to ADCC mediated by NK cells is not as significant as afucosylation. However, endogenous human IgG1&2 glycoforms *in vivo* possess bisecting GlcNAc with approximately 12% and 9.3% proportions, respectively (Flynn et al., 2010). As such, this glycoform may bear some yet unknown regulatory property for antibodies.

4.2.3 Galactosylation

Terminal galactosylation is not perceived to be an essential factor for stimulating ADCC responses; however, non-galactosylated IgGs have consistently been shown to have weaker C1q binding and complement-dependent cytotoxicity (CDC) responses (Table 6). Reviews of mAb effector function with respect to N-glycoforms have noted that lower galactosylation *in vivo* has been linked to aging, auto-immune disorders and disease states such as rheumatoid arthritis (Harris et al., 2010; Jefferis, 2012; Spearman et al., 2011). Therefore, increased galactosylation is viewed as therapeutically favourable; however, compared to human IgG, commercial monoclonal antibody formulations tend to be under-galactosylated (Wacker et al., 2011).

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4.2.4 Sialylation

Terminal sialylation, unlike other glycoforms, introduces a negative charge to glycans. This glycoform has been reported to reduce binding affinity of mAbs to antigens, FcγR receptors, and reduce ADCC immune responses (Table 7). However, it does possess anti-inflammatory properties, and is credited with the success of intravenous immunoglobulin (IVIG) therapies (Kaneko et al., 2006; Scallon et al., 2007). This seems to mainly be a feature of $\alpha_{2\text{-}6}$ linked sialic glycoforms rather than $\alpha_{2\text{-}3}$ (Anthony et al., 2008). Unlike other therapeutic glycoproteins, mAbs with elevated sialylation have not been reported to receive improvements to serum half-life (Kaneko et al., 2006; Wright and Morrison, 1998). Raju and Lang (2014) provide a thorough examination of the effects of sialyation to a range of commercial glycoproteins.

4.2.5 High Mannose

High mannose glycans typically represent an early departure from glycosylation, either after the endoplasmic reticulum, or the cis-Golgi (Figure 1). This glycoform possesses minimal capability to activate CDC, impaired binding to FcγR receptors, and rapid *in vivo* clearance (Table 8).

4.3 Immunogenic Glycoforms

Several established rodent-derived cell lines such as NS0 and CHO form glycosylation structures immunogenic in humans such as terminal alpha-gal and a sialic acid variant, N-glycolylneuraminic acid (Neu5Gc) (Bosques et al., 2010; Ghaderi et al., 2010; Padler-Karavani et al., 2008). As such, fully human cell lines have grown more popular and attempts have been made with some success to “glycoengineer” human-like glycosylation in alternative production platforms of bacteria, yeast, insect cells, as well as making improvements to mammalian platforms currently in use. Ghaderi and colleagues provide an excellent examination of this subject from the perspective of immunogenic glycoforms (Ghaderi et al., 2012).

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5.0 Conclusions

Glycosylation remains among the most important critical quality attributes (CQAs) of recombinant therapeutic glycoproteins, particularly regarding mAbs, where they directly impact the immune effector function of therapeutics. Galactosylation and afucosylation are the most desirable glycoforms for mAbs towards eliciting immune effector functions, while sialylation is the most desirable for other therapeutic glycoproteins.

Supplementation of nucleotide sugar precursors to tune glycan distributions for more desirable therapeutically active glycoforms continues to demonstrate strong potential as a process improvement strategy. These supplementations, while often effective, are reported with inconsistent results. This is primarily due to five key factors beyond the supplementations themselves. The first is differences across cell platforms, such as the up/down regulation of enzymes and transporters; two different cell platforms expressing the same recombinant protein can produce different effects from the same supplementation regime. Secondly, differences between expressed proteins, and by extension differences in the accessibility of the protein glycan sites, can translate to hard limits on the potential glycan complexity and effectiveness of supplementation; mAbs for instance are naturally limited to only having biantennary glycans compared to other therapeutic glycoproteins. The third factor is the fermentation and sampling timeline, specifically glucose availability, which is inversely correlated to galactosylation and sialylation of glycans. This, together with accumulation of exoglycosidases in cultures, particularly sialidase means that harvesting at the end of exponential phase will result in a different glycan distribution than during stationary phase, following a decrease in cell viability. The fourth factor is the concentration of glutamine supplemented to cultures, as the subsequent increase in ammonia will increase UDP-GlcNAc pools affecting glycoforms, as well as altering

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the pH of Golgi compartments preventing the proper formation of heteromeric dimer glycosyltransferases necessary for complex terminal glycan additions such as galactosylation and sialylation. Finally, a lack of standardized glycosylation index metrics for observing shifts in glycoform distribution, and making suitable comparisons between researchers.

The supplementation of glucosamine (GlcN) and acetylglucosamine (GlcNAc) reduces the terminal complexity of glycans to favour the G0 glycoforms, and can increase antennarity of non-IgG proteins. GlcN causes significant restriction to cell growth, but its acetylated form GlcNAc does not. Galactose supplementation achieves increased galactosylation with the greatest reported consistency, and can be enhanced by the addition of uridine and manganese ions, with best results in a glutamine-synthase cell platform. Acetylmannosamine (ManNAc) supplementation, optionally with cytidine, has been reported to significantly increase sialylation; reports of it not succeeding tend to coincide with cell platforms that already have a deficit in galactosylation. Several other exotic sugars also have the potential to affect factors such as fucosylation in the case of D-arabinose, and galactosylation in the case of melezitose, turanose and to a lesser extent lactose.

While this field continues to present exciting new paths likely to achieve more therapeutic glycoforms, there remains much opportunity for examining the synergistic supplementation of galactose and ManNac to improve sialylation of glycoproteins. Using these supplementation methods to tune glycan distributions towards targeted glycoforms should enter the toolkit of every bioprocess engineer.

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Table 1

Supplement	Cell	Protein	Fermentation Timeline	Gln (mM)	Effect	Researchers
▪ 20mM GlcNac	CHO (DG44)	IgG	▪ Bioreactor, fedbatch ▪ 1L ▪ Addition 48h ▪ Harvest 336h	8	▪ Reduced glycan complexity to favour G0 and FG0 glycoforms ▪ Reduced galactosylation	(Kildegaard et al., 2015)
▪ 7.5mM GlcN ▪ 2.5 - 10mM GlcNAc	CHO (Dukx)	IgG HCAb	▪ Flasks ▪ 125mL ▪ Addition 50h ▪ Harvest 144h-168h	5	▪ Reduced glycan complexity to favour G0 and FG0 glycoforms – both GlcN & GlcNAc supplementation ▪ ~50% drop in growth rate with GlcN supplementation ▪ <5% drop in growth rate with GlcNAc supplementation ▪ proportional increase in UDP-GlcNAc pool up to 2x with GlcNAc supplementation	(Blondeel et al., 2015)
▪ 10mM GlcN	NS0 (GS)	IgG	▪ Flasks ▪ 500mL ▪ Addition 48h ▪ Harvest 96h	0.4 (GS)	▪ 17x UDP HexNAc ▪ -56% Galactosylation ▪ -63% UDP-Hex ▪ Reduced growth	(Hills et al., 2001)
▪ 10mM GlcN	CHO (K1)	EPO	▪ Flasks ▪ 250mL ▪ Addition 0h ▪ Harvest day 4-5	-	▪ 18.5x UDP-GlcNAc ▪ Increased glycan heterogeneity ▪ -22% tetrasialylation	(Yang and Butler, 2002)
▪ 10mM GlcN, glutamine free	BHK	IL-2	▪ Perfusion ▪ 2.5 L ▪ Addition: day 21 ▪ Harvest: days 26-28 and 29-30	0	▪ Greater antennarity, but lower complexity of glycans ▪ 3x UDP-HexNAc ▪ Greater proportion of monosialylation with GlcN ▪ +58% sialylation in std culture without glutamine	(Gawlitzek et al., 1998)
▪ 10mM GlcN + 2mM Urd	CHO (GS) NS0 (GS)	TIMP-1	▪ Flasks ▪ 1000mL ▪ Addition 24h ▪ Harvest 48h	0.4 (GS)	▪ 58x UDP-HexNAc ▪ 8x UDP-Hex ▪ +9% antennarity ▪ -8% sialylation	(Baker et al., 2001)

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▪ 10mM GlcN + 2mM Urd	BHK	IL-2[2]	▪ Perfusion ▪ 2.5 L ▪ Addition 10d ▪ Harvest 14d	0	▪ 12x UDP-GlcNAc ▪ 5x UTP ▪ Rise antennarity	(Grammatikos et al., 1998)
▪ 10mM GlcN ▪ +5mM Urd	CHO (Dukx)	IFN-γ	▪ Flasks (n=1)[3] ▪ 1000mL ▪ Addition 48h ▪ Harvest 96h	4	▪ Reduced growth ▪ +28% sialylation ▪ 6-15x increase UDP-HexNAc	(Wong et al., 2010)
▪ 3.5 & 17.5mM GlcN + 1mM Urd	CHO SCLC	NCAM	▪ 6 & 24-well plates ▪ Addition 12-18h ▪ Harvest 128h	6	▪ 25x UDP GlcNAc ▪ Reduced growth ▪ -90% polysialylation[4]	(Zanghi et al., 1998)
▪ 1-10mM Urd	CHO (Dukx)	IFN-γ	▪ Flasks ▪ 100mL	3	▪ Increased UTP correlated to increased UDP-HexNAc except in absence of glutamine	(Nyberg et al., 1999)
▪ 0.5mM Urd	RH	N/A	▪ Culture dish ▪ 3 mL ▪ Addition 16h ▪ Harvest 24h	2.4[5]	▪ Reduced sialylation ▪ 6.7x UTP ▪ 3.8x UDP-hexose ▪ 4.6x UDP-HexNAc	(Pels Rijcken et al., 1995b)

[2] Modified Il-2 variant featuring an artificial N-glycan site
[3] Only a single flask for each experiment, and only one control flask appears to have been used
[4] NCAM protein glycan results reported only relative to controls
[5] Waymouth culture medium (Morton, 1970)

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Table 2

Supplement	Cell	Protein	Fermentation Timeline	Gln (mM)	Effect	Researchers
▪ 20mM Gal	CHO (DG44)	IgG	▪ Fed-batch bioreactor ▪ 1L ▪ Addition 48h ▪ Harvest 336h	8	▪ +12% galactosylation ▪ No reduction to cell growth or productivity	(Kildegaard et al., 2015)
▪ 10mM galactose	NS0 (GS)	IgG	▪ Flasks ▪ 500mL ▪ Addition 48h ▪ Harvest 96h	0.4 (GS)	▪ 5x UDP-Gal	(Hills et al., 2001)
▪ 10mM Gal ▪ 20mM Gal ▪ 40mM Gal	CHO (GS)	Fc-TNF fusion	▪ Fed-batch bioreactor ▪ 2L ▪ Addition 120h ▪ Harvest 288h	GS	▪ +22% galactosylation ▪ +14% sialylation ▪ No negative growth effects ▪ Glycan distribution scaled well to 200L bioreactor	(Liu et al., 2015)
▪ 20mM galactose	CHO	IL-4/13	▪ Batch bioreactor ▪ 1L ▪ Addition 0h ▪ Harvest 112h	2	▪ No notable effects to sialylation or gene expression	(Clark et al., 2005)
▪ 10mM Gal ▪ +5mM Urd	CHO (Dukx)	IFN-γ	▪ Flasks (n=1)[6] ▪ 1000mL ▪ Addition 48h ▪ Harvest 96h	4	▪ 20x UDP-Gal ▪ +12% sialylation	(Wong et al., 2010)
▪ 0.4, 4, 40μM Mn^{2+}	CHO (dhfr-)	EPO	▪ Roller bottles ▪ $850cm^2$ ▪ Media replaced every 7 days ▪ Addition ▪ Harvest 19d	15	▪ Reduced titres at 40μM Mn^{2+} feed ▪ UDP-Gal pools unchanged ▪ Improved galactosylation and sialylation	(Crowell et al., 2007)
▪ 10, 20, 40mM Gal ▪ 17-68mg/L Mn^{2+}	CHO S	FII	▪ Flasks ▪ Addition 48h ▪ Harvest 144h	-	▪ +26% sialylation from galactose addition ▪ +30% sialylation from addition of $MnSO_4$ ▪ Temperature-shift resulted in similar sialyation improvement for final process	(Lee et al., 2017)
▪ 100mM Gal ▪ 40μM Mn^{2+} ▪ 100mM Gal + 40μM Mn^{2+}	CHO (K1)	IgG	▪ Flasks ▪ 250mL ▪ Addition 0h ▪ Harvest 168h	4	▪ +9% galactosylation ▪ -31% fucosylation and +21% high-mannose glycans for Mn+Gal ▪ -30% fucosylation and +14% high-mannose glycans for Mn alone ▪ 9x UDP-Gal pool ▪ Increased expression of β-Gal II, III, IV and UDP-GalT transcripts	(St Amand et al., 2014)

[6] Only a single flask for each experiment, and only one control flask appears to have been used

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▪ 4μM Mn^{2+} ▪ 16μM Mn^{2+} ▪ 30mM Gal[7] ▪ +4μM Mn^{2+} ▪ +16μM Mn^{2+}	CHO (DG44)	IgG	▪ Flasks ▪ 100mL ▪ Addition 0h ▪ Harvest 72h	8	▪ -30-50% IgG when Replacing /lowering Glc or matching Gal ▪ +11% galactosylation ▪ -5% fucosylation with 16μM Mn^{2+}	(Surve and Gadgil, 2015)
▪ 5-100mM Gal +1-20mM Urd +2-40μM Mn^{2+} ▪ Factors:[8] 1,2,3,4,8,12,16,20 ▪ Factors: 8,12	(2) CHO (GS)	IgG	▪ Fed-batch bioreactor ▪ 2L ▪ Addition 0h ▪ Harvest 360h	GS	▪ +20% galactosylation for 1st cell line at 100mM Gal ▪ +24% galactosylation for 2nd cell line at 40mM Gal ▪ Glycan distribution scaled well to 100L and 1000L bioreactors	(Gramer et al., 2011)
▪ 2.5-100mM Gal[9] +0.5-20mM Urd +1-40μM Mn^{2+}	CHO (GS)	IgG	▪ Flasks ▪ 125mL ▪ Addition 72h ▪ Harvest 192h	GS	▪ +21-24% galactosylatio ▪ +1.5-5% sialylation ▪ Best overall IVCC, MAb titre and galactosylation results with low Mn^{2+}/Urd and high Gal condition ▪ Mab galactosylation correlated well with cell-surface Gal	(Grainger and James, 2013)

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[7] Additional experiments changed the main carbon source to fructose, glucose-free (only gal), and low-glucose delivered from a hydrogel for slow release.

[8] Galactose, urdine and manganese (Mn^{2+}) fed in ratios of 5:1:0.002 mM (UMG cocktail) for two CHO-K1SV cell lines expressing two separate IgGs under GS expression system

[9] Face-centred DOE design for main-effects modeling of UMG cocktail developed by Gramer et al. cocktail, tested on two CHO-K1SV cell lines stably expressing IgG_4 under GS expression system (Gramer et al., 2011).

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Table 3

Feed	Cell	Protein	Fermentation Timeline	Gln (mM)	Effect	Researchers
▪ 20mM ManNac [10]	CHO (DG44)	IgG	▪ 1L - Bioreactor, fedbatch ▪ Addition 48h ▪ Harvest 336h	8	▪ Hypothesis that increases in CMP-NeuAc lead to feedback inhibition, UDP-GlcNAc accumulation, and decrease in galactosylation.	(Kildegaard et al., 2015)
▪ 20mM ManNAc	NS0 (GS)	IgG	▪ 500mL Flasks ▪ Addition 48h ▪ Harvest 96h	0.4 (GS)	▪ 44x CMP-NeuAc ▪ No improvement to sialylation	(Hills et al., 2001)
▪ 20mM ManNAc	CHO (GS) NS0 (GS)	TIMP-1	▪ 1L - Flasks ▪ Addition 24h ▪ Harvest 48h	0.4 (GS)	▪ -22% Neu5Gc content in NS0 cultures ▪ +1% shifts in %-sialylation ▪ 12x increase in CMP-SA (CHO) ▪ 30x increase in CMP-SA (NS0)	(Baker et al., 2001)
▪ 10, 20, 40mM ManNAc	CHO S	FII	▪ Flasks ▪ Addition 48h ▪ Harvest 144h	-	▪ +20% sialylation from ManNAc addition ▪ Temperature-shift resulted in similar sialyation improvement for final process	(Lee et al., 2017)
▪ 0.2, 2, 20, 40mM ManNAc	CHO (Dukx)	IFN-γ	▪ 100mL Flasks ▪ Addition 0h ▪ Harvest 96h	-	▪ 27x increase CMP-SA ▪ +15% complete sialylation ▪ 60% & 100% of sialylation from radiolabelled supplemented ManNAc fed at 2mM & 20mM respectively ▪ No effect to cell growth or productivity	(Gu and Wang, 1998)
▪ 0.5mM Cyt	RH	N/A	▪ 3mL Culture dish ▪ Addition 16h ▪ Harvest 24h	2.4 [11]	▪ 3.0x CTP ▪ 3.2x UDP-HexNAc ▪ 2.6x UDP-hexose	(Pels Rijcken et al., 1995b)
▪ 20mM ManNAc + 10mM Cyt	CHO (GS)	Fc-TNF fusion	▪ 2L Fed-batch bioreactor ▪ Addition 120h ▪ Harvest	GS	▪ Statistically insignificant sialic-acid content increase (terminal galactose determined to be limiting)	(Liu et al., 2015)

[10] Supplementations of mannose, NeuAc, and cytidine were also attempted, but did not vary significantly from control cultures

[11] Waymouth culture medium (Morton, 1970)

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			288h			
▪ 20mM ManNac ▪ + 0.5mM Cyt	CHO SCLC	NCAM	▪ 6 & 24-well plates ▪ Addition 12-18h ▪ Harvest 128h	6	▪ +10-20% polysialylation[12] in CHO ▪ -20-30% NCAM production	(Zanghi et al., 1998)
▪ 20mM ManNAc ▪ 10mM cytidine	CHO (Dukx)	IFN-γ	▪ 1L - Flasks (n=1) ▪ Addition 48h ▪ Harvest 96h	4	▪ 30-120x increase in CMP-SA ▪ +32-36% sialylation -/+ Cyt ▪ +26-52% higher specific productivity	(Wong et al., 2010)

[12] NCAM protein glycan results reported only relative to controls

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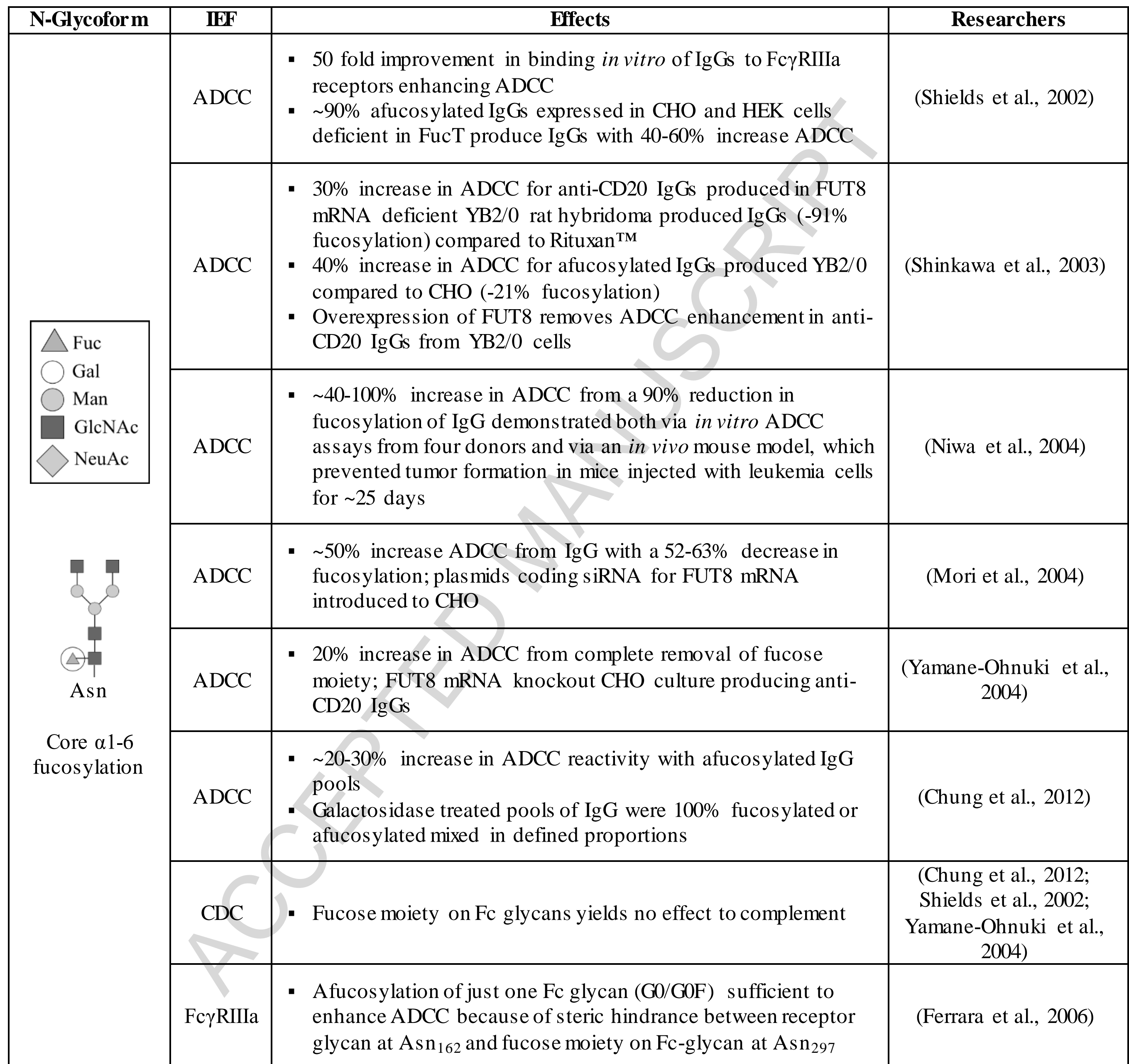
Table 4



N-Glycoform	IEF	Effects	Researchers
Fuc Gal Man GlcNAc NeuAc Asn Core α1-6 fucosylation	ADCC	▪ 50 fold improvement in binding *in vitro* of IgGs to FcγRIIIa receptors enhancing ADCC ▪ ~90% afucosylated IgGs expressed in CHO and HEK cells deficient in FucT produce IgGs with 40-60% increase ADCC	(Shields et al., 2002)
	ADCC	▪ 30% increase in ADCC for anti-CD20 IgGs produced in FUT8 mRNA deficient YB2/0 rat hybridoma produced IgGs (-91% fucosylation) compared to Rituxan™ ▪ 40% increase in ADCC for afucosylated IgGs produced YB2/0 compared to CHO (-21% fucosylation) ▪ Overexpression of FUT8 removes ADCC enhancement in anti-CD20 IgGs from YB2/0 cells	(Shinkawa et al., 2003)
	ADCC	▪ ~40-100% increase in ADCC from a 90% reduction in fucosylation of IgG demonstrated both via *in vitro* ADCC assays from four donors and via an *in vivo* mouse model, which prevented tumor formation in mice injected with leukemia cells for ~25 days	(Niwa et al., 2004)
	ADCC	▪ ~50% increase ADCC from IgG with a 52-63% decrease in fucosylation; plasmids coding siRNA for FUT8 mRNA introduced to CHO	(Mori et al., 2004)
	ADCC	▪ 20% increase in ADCC from complete removal of fucose moiety; FUT8 mRNA knockout CHO culture producing anti-CD20 IgGs	(Yamane-Ohnuki et al., 2004)
	ADCC	▪ ~20-30% increase in ADCC reactivity with afucosylated IgG pools ▪ Galactosidase treated pools of IgG were 100% fucosylated or afucosylated mixed in defined proportions	(Chung et al., 2012)
	CDC	▪ Fucose moiety on Fc glycans yields no effect to complement	(Chung et al., 2012; Shields et al., 2002; Yamane-Ohnuki et al., 2004)
	FcγRIIIa	▪ Afucosylation of just one Fc glycan (G0/G0F) sufficient to enhance ADCC because of steric hindrance between receptor glycan at Asn_{162} and fucose moiety on Fc-glycan at Asn_{297}	(Ferrara et al., 2006)

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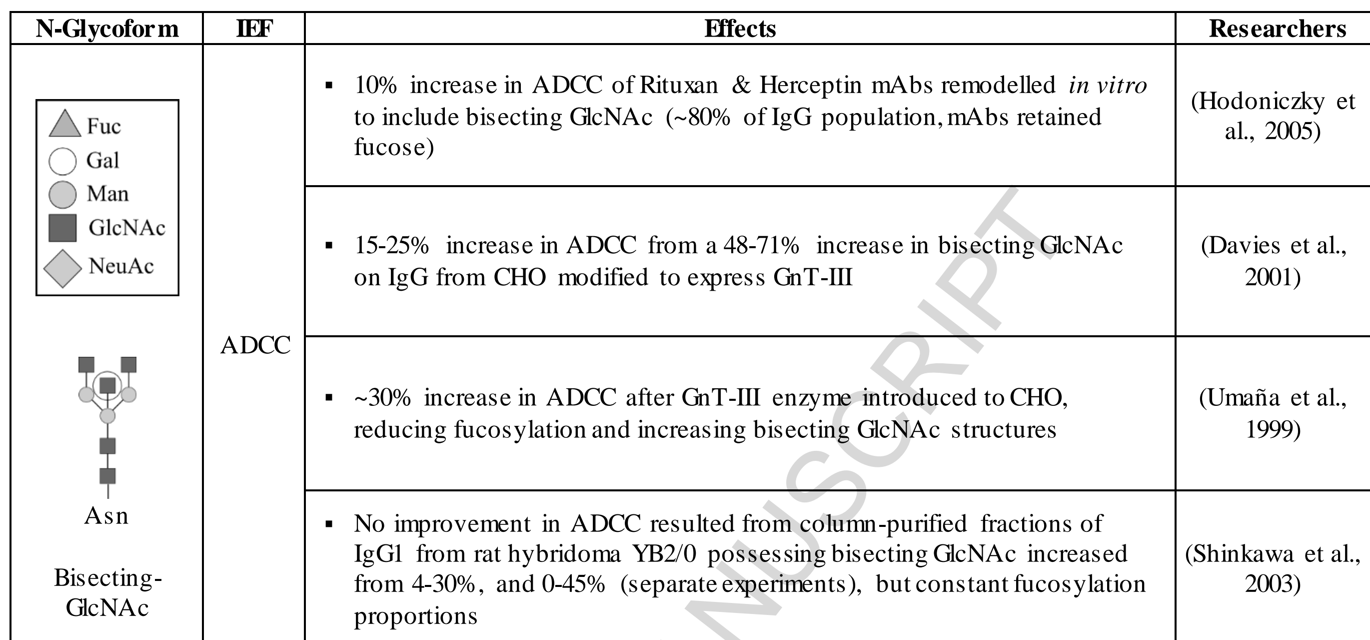
Table 5



N-Glycoform	IEF	Effects	Researchers
Fuc, Gal, Man, GlcNAc, NeuAc; Asn; Bisecting-GlcNAc	ADCC	▪ 10% increase in ADCC of Rituxan & Herceptin mAbs remodelled *in vitro* to include bisecting GlcNAc (~80% of IgG population, mAbs retained fucose)	(Hodoniczky et al., 2005)
		▪ 15-25% increase in ADCC from a 48-71% increase in bisecting GlcNAc on IgG from CHO modified to express GnT-III	(Davies et al., 2001)
		▪ ~30% increase in ADCC after GnT-III enzyme introduced to CHO, reducing fucosylation and increasing bisecting GlcNAc structures	(Umaña et al., 1999)
		▪ No improvement in ADCC resulted from column-purified fractions of IgG1 from rat hybridoma YB2/0 possessing bisecting GlcNAc increased from 4-30%, and 0-45% (separate experiments), but constant fucosylation proportions	(Shinkawa et al., 2003)

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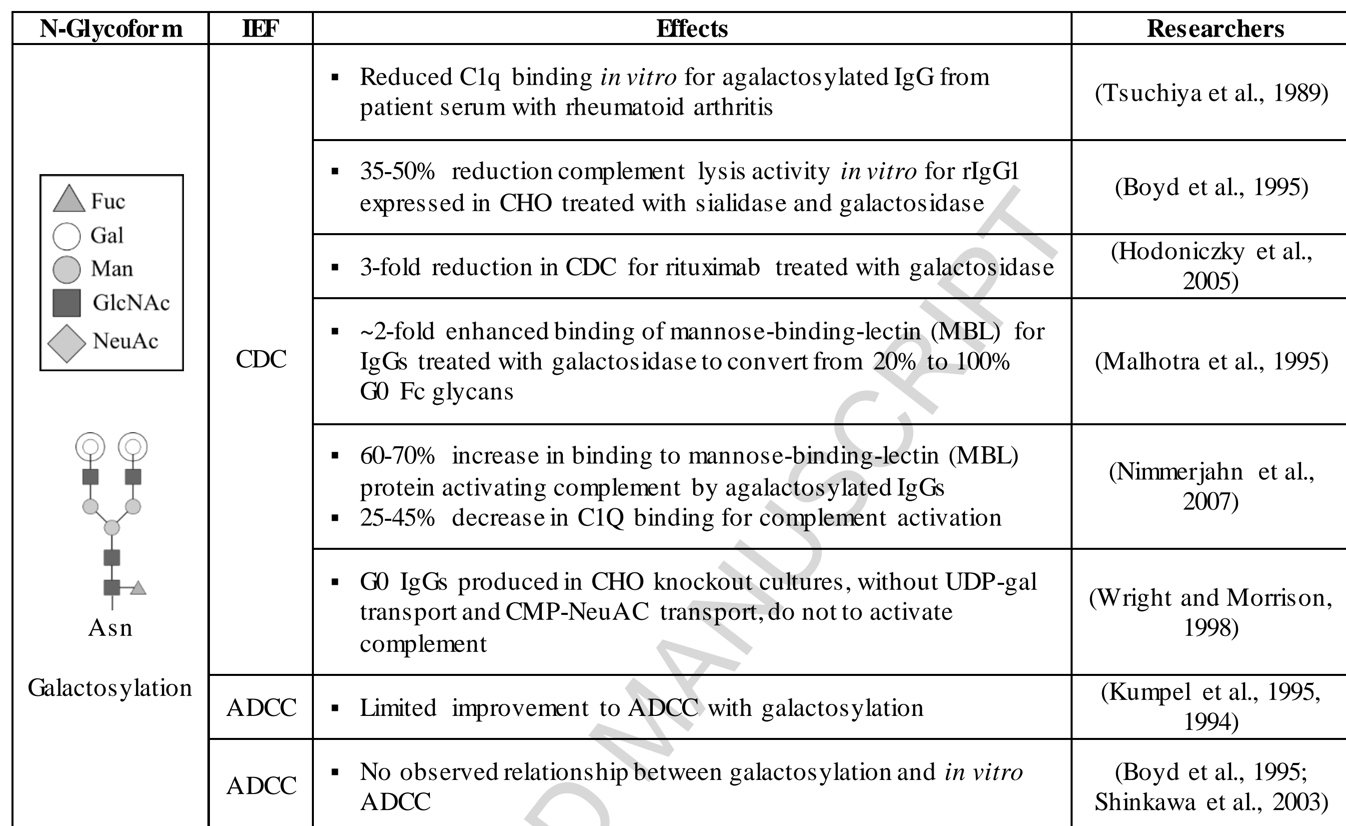
Table 6



N-Glycoform	IEF	Effects	Researchers
Fuc, Gal, Man, GlcNAc, NeuAc Asn Galactosylation	CDC	▪ Reduced C1q binding *in vitro* for agalactosylated IgG from patient serum with rheumatoid arthritis	(Tsuchiya et al., 1989)
		▪ 35-50% reduction complement lysis activity *in vitro* for rIgG1 expressed in CHO treated with sialidase and galactosidase	(Boyd et al., 1995)
		▪ 3-fold reduction in CDC for rituximab treated with galactosidase	(Hodoniczky et al., 2005)
		▪ ~2-fold enhanced binding of mannose-binding-lectin (MBL) for IgGs treated with galactosidase to convert from 20% to 100% G0 Fc glycans	(Malhotra et al., 1995)
		▪ 60-70% increase in binding to mannose-binding-lectin (MBL) protein activating complement by agalactosylated IgGs ▪ 25-45% decrease in C1Q binding for complement activation	(Nimmerjahn et al., 2007)
		▪ G0 IgGs produced in CHO knockout cultures, without UDP-gal transport and CMP-NeuAC transport, do not to activate complement	(Wright and Morrison, 1998)
	ADCC	▪ Limited improvement to ADCC with galactosylation	(Kumpel et al., 1995, 1994)
	ADCC	▪ No observed relationship between galactosylation and *in vitro* ADCC	(Boyd et al., 1995; Shinkawa et al., 2003)

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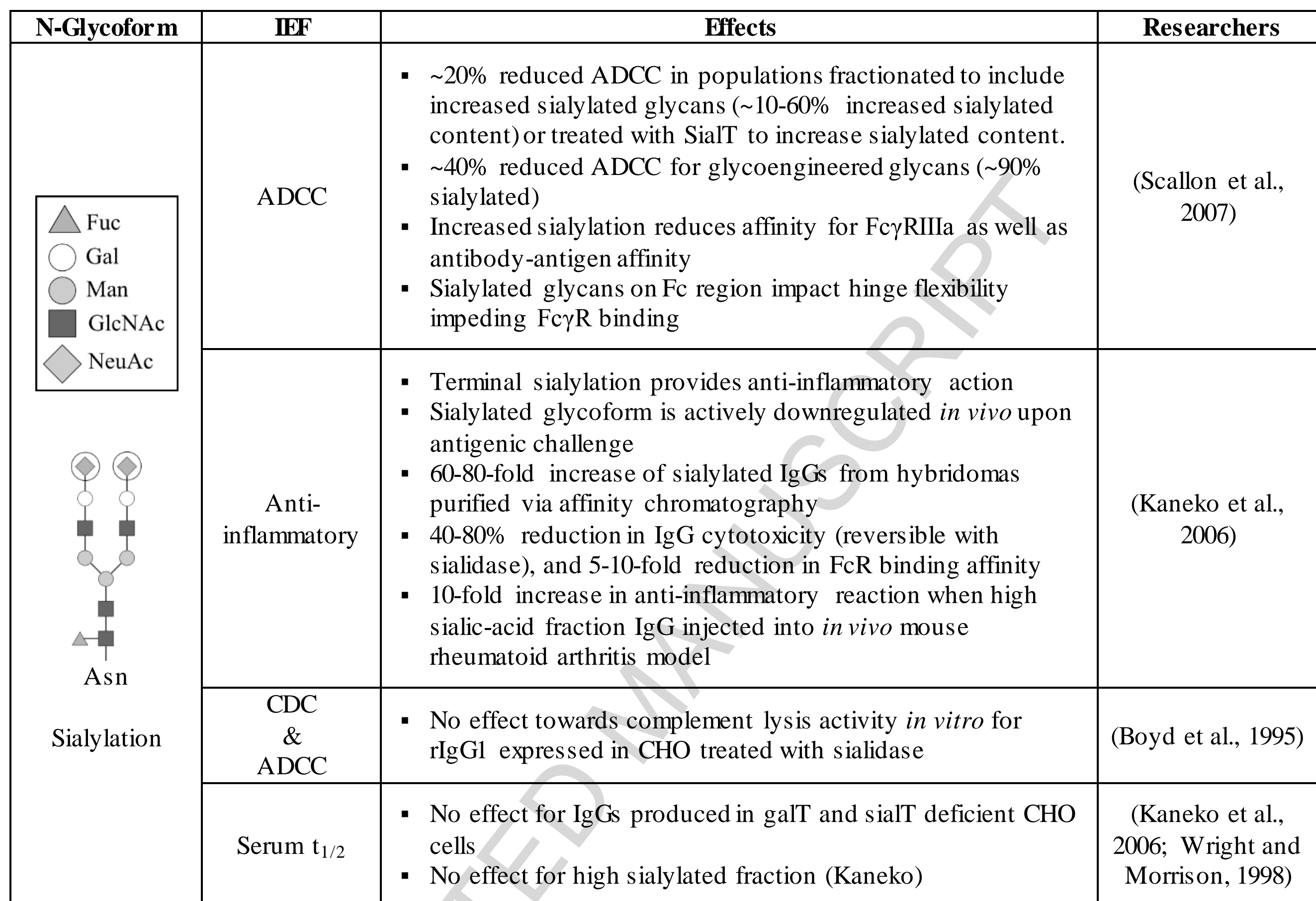
Table 7



N-Glycoform	IEF	Effects	Researchers
Fuc Gal Man GlcNAc NeuAc Asn Sialylation	ADCC	▪ ~20% reduced ADCC in populations fractionated to include increased sialylated glycans (~10-60% increased sialylated content) or treated with SialT to increase sialylated content. ▪ ~40% reduced ADCC for glycoengineered glycans (~90% sialylated) ▪ Increased sialylation reduces affinity for FcγRIIIa as well as antibody-antigen affinity ▪ Sialylated glycans on Fc region impact hinge flexibility impeding FcγR binding	(Scallon et al., 2007)
	Anti-inflammatory	▪ Terminal sialylation provides anti-inflammatory action ▪ Sialylated glycoform is actively downregulated *in vivo* upon antigenic challenge ▪ 60-80-fold increase of sialylated IgGs from hybridomas purified via affinity chromatography ▪ 40-80% reduction in IgG cytotoxicity (reversible with sialidase), and 5-10-fold reduction in FcR binding affinity ▪ 10-fold increase in anti-inflammatory reaction when high sialic-acid fraction IgG injected into *in vivo* mouse rheumatoid arthritis model	(Kaneko et al., 2006)
	CDC & ADCC	▪ No effect towards complement lysis activity *in vitro* for rIgG1 expressed in CHO treated with sialidase	(Boyd et al., 1995)
	Serum $t_{1/2}$	▪ No effect for IgGs produced in galT and sialT deficient CHO cells ▪ No effect for high sialylated fraction (Kaneko)	(Kaneko et al., 2006; Wright and Morrison, 1998)

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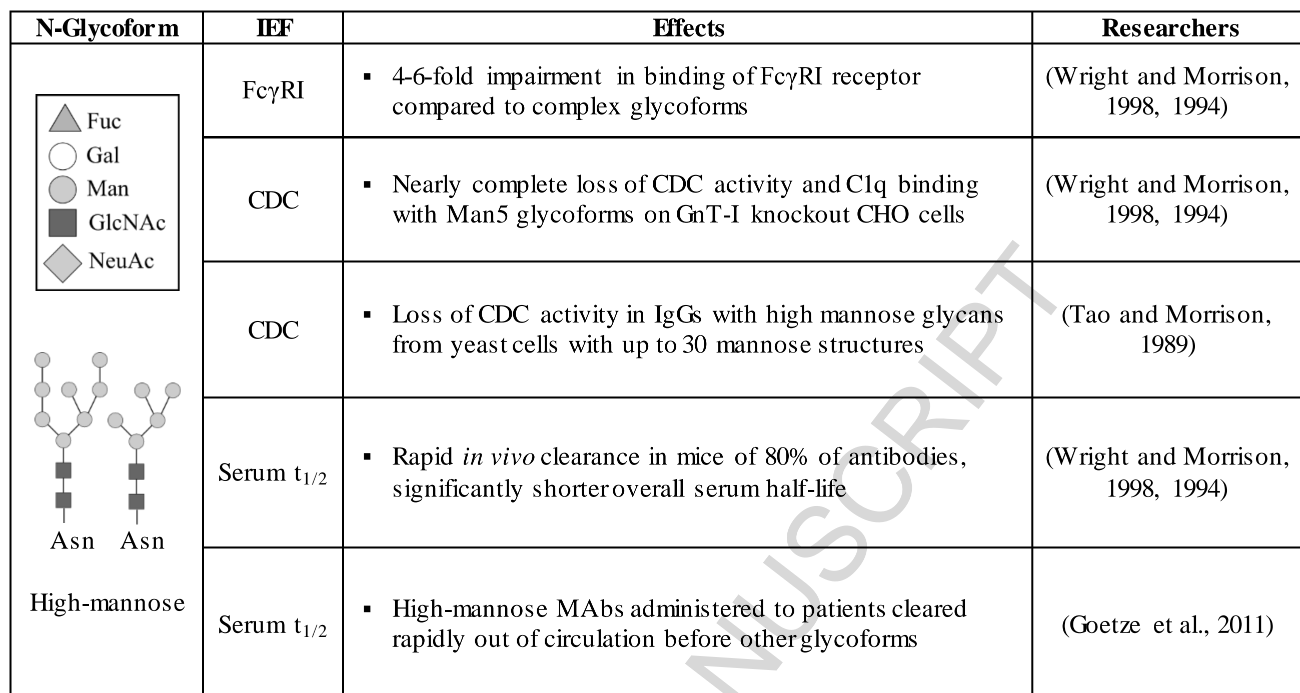
Table 8



N-Glycoform	IEF	Effects	Researchers
High-mannose	FcγRI	▪ 4-6-fold impairment in binding of FcγRI receptor compared to complex glycoforms	(Wright and Morrison, 1998, 1994)
	CDC	▪ Nearly complete loss of CDC activity and C1q binding with Man5 glycoforms on GnT-I knockout CHO cells	(Wright and Morrison, 1998, 1994)
	CDC	▪ Loss of CDC activity in IgGs with high mannose glycans from yeast cells with up to 30 mannose structures	(Tao and Morrison, 1989)
	Serum $t_{1/2}$	▪ Rapid *in vivo* clearance in mice of 80% of antibodies, significantly shorter overall serum half-life	(Wright and Morrison, 1998, 1994)
	Serum $t_{1/2}$	▪ High-mannose MAbs administered to patients cleared rapidly out of circulation before other glycoforms	(Goetze et al., 2011)

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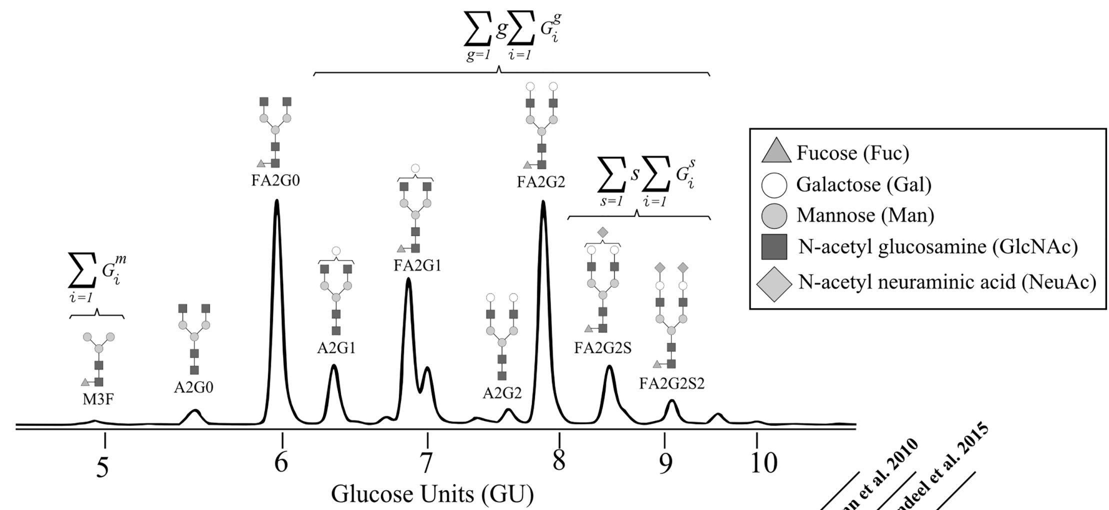
Fucose (Fuc)
Galactose (Gal)
Glucose (Glc)
Glucosamine (GlcN)
Mannose (Man)
N-acetyl glucosamine (GlcNAc)
N-acetyl galactosamine (GalNAc)
N-acetyl mannosamine (ManNAc)
N-acetyl neuraminic acid (NeuAc)
GLUT or SGLT family tranporter
Flippase ER Flippase transporter
SLC35 GDP-Fuc transporter
SLC35 UDP-Gal transporter
SLC35 UDP-GlcNac transporter
SLC35 CMP-NeuAc transporter
Nucleoside diphosphatase (NDP)
GlcNAc-transferases (GlcNAcT)
Fucosyltransferases (FucT)
Galactosyltransferases (GalT)
Sialyltransferases (SiaT)

Plasma Membrane
ATP, ADP, 1a, 1b, 1c, 1d, 1e
-6-P, 2, Fructose-6-P, Glutamine, 3, Glutamate, NH_3, 5, Acetyl-CoA, 4, CoA, 6, Acetate, -6-P, -1-P
Phospho-enolpyruvate (PEP)
7, 8, 9, 10, 11, 12*
-1-P, -6-P, -1-P, 13, Acetyl-CoA, CoA, -1-P, UTP, PP_i, UDP-
14, UTP, PP_i, 15, 16, UTP, PP_i
UDP-, -1-P, UDP-, 17, UDP-
18, 19, GTP, PP_i, 20, UDP, 12*, PEP, 21, CTP, PP_i, CMP-
UDP-, GDP-, Cytosol, 1e, ATP, -6-P, CMP, CMP-, CMP
Nucleus
22*
UDP-, GDP-, UDP-, GDP-, UDP-, CMP-

Golgi
UMP, UDP-, UMP, UDP-, GMP, -GDP, GMP, GDP-, UMP, -UDP, UMP, UDP-
GDP, GMP, 30*, 29*, 28*, CMP, CMP, CMP-, CMP-
I, II
Trans, Medial, Cis
CMP-, UDP-, UDP-, GDP-, UDP, UMP, UDP-
pH 6.3, pH 6.5, pH 6.7

P - P - Dol, 23*, Flippase, Dol-P-P, P - P - Dol, 24*, 25*, - P - Dol, Flippase, Dol-P-, - P - Dol, Flippase, Dol-P-
27*, 26*
ER
pH 7.2

Figure 1



$$GI = \frac{\sum_{g=1} g \sum_{i=1} G_i^g}{\sum_{g=0} \sum_{a=1} a \sum_{j=1} G_j^g} \times 100 \qquad (1)$$

$$SI = \frac{\sum_{s=1} s \sum_{i=1} G_i^s}{\sum_{s=0} \sum_{g=1} g \sum_{j=1} G_j^s} \times 100 \qquad (2)$$

$$FI = \frac{\sum_{f=1} f \sum_{i=1} G_i^f}{\sum_{f=0} \sum_{j=1} G_j^f} \times 100 \qquad (3)$$

$$AI = \frac{\sum_{a=1} a \sum_{i=1} G_i^a}{5 \sum_{a=0} \sum_{j=1} G_j^a} \times 100 \qquad (4)$$

$$MI = \frac{\sum_{m=1} \frac{m}{2} \sum_{i=1} G_i^m}{\sum_{m=0} \sum_{j=1} G_j^m} \times 100 \qquad (5)$$

							Flynn et al. 2010	Blondeel et al. 2015
	Glycoform	g	s	f	a	m	**hIgG1**	**HCAb**
Complex Neutral	FA2G0	0	0	1	2	0	12.5	12.24
	FA2G1	1	0	1	2	0	42.3	25.32
	FA2G2	2	0	1	2	0	21.7	29.46
	A2G0	0	0	0	2	0	0.57	0.97
	A2G1	1	0	0	2	0	1.309	4.12
	A2G2	2	0	0	2	0	1.44	1.83
	A4G1	1	0	0	4	0	-	1.60
	A4G3	3	0	0	4	0	-	1.69
	A4G4	4	0	0	4	0	-	0.78
	FA2BG0	0	0	1	3	0	2.70	-
	FA2BG1	1	0	1	3	0	7.2	-
	FA2BG2	2	0	1	3	0	1.49	-
	A2BG1	1	0	0	3	0	0.341	-
Complex Charged	FA2G1S	1	1	1	2	0	1.21	-
	FA2G2S	2	1	1	2	0	8.6	11.05
	FA2G2S2	2	2	1	2	0	-	5.80
	A2G2S	2	1	0	2	0	0.40	-
	FA2BG1S	1	1	1	3	0	0.174	-
	FA2BG2S	2	1	1	3	0	0.149	-
	A4G3Sn	3	1*	0	4	0	-	1.95
	A4G4Sn	4	1*	0	4	0	-	0.90
Hybrid & Mannose	A1G0	0	0	0	1	1	0.064	0.12
	FA1G1	1	0	1	1	1	-	0.43
	M4FA2G0	0	0	1	2	1	0.138	-
	M4A2G0	0	0	0	2	1	0.053	-
	M4A2G1	1	0	0	2	1	0.248	-
	M4A2G2	2	0	0	2	1	0.150	-
	FA1G1S	1	1	1	1	1	-	-
	M4A2G2S	2	2	0	2	1	0.028	
	M3F	0	0	1	0	2	-	0.09
						GI	58.7%	69.3%
						SI	8.8%	17.5%
						FI	95.4%	84.4%
						AI	39.9%	42.6%
						MI	0.3%	0.9%

$*I =$ glycoform index (%)

$g =$ number of terminal galactose moieties per glycan

$s =$ number of terminal sialic acid moieties per glycan

$f =$ binary number of core $\alpha_{1\text{-}6}$ fucosylation per glycan

$a =$ antennarity/branching; the number of terminal GlcNAc moieties per glycan

$m =$ number per glycan indicating hybrid (1) or high-mannose type (2)

$G_i^* =$ proportion of glycoforms with a particular moiety (g, s, a, f, m)

$G_j^* =$ proportion of glycoforms able to receive a particular moiety (g, s, a, f, m)

Figure 2

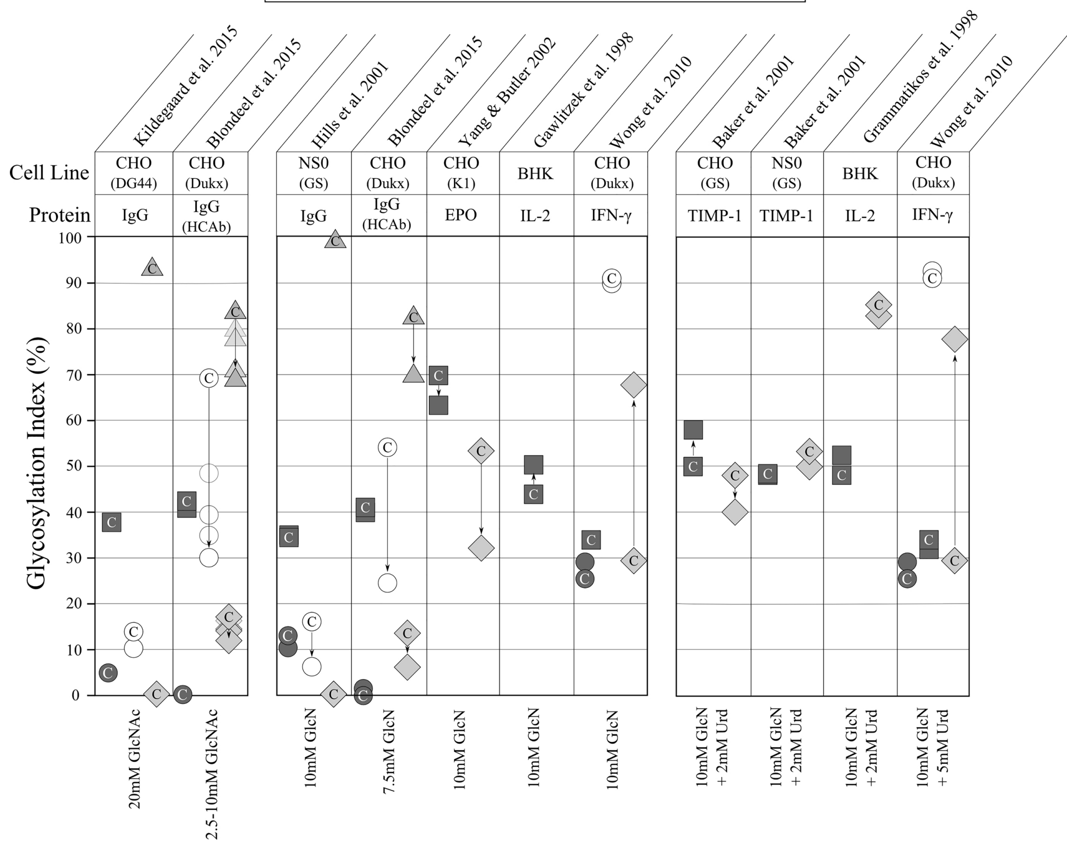


Figure 3

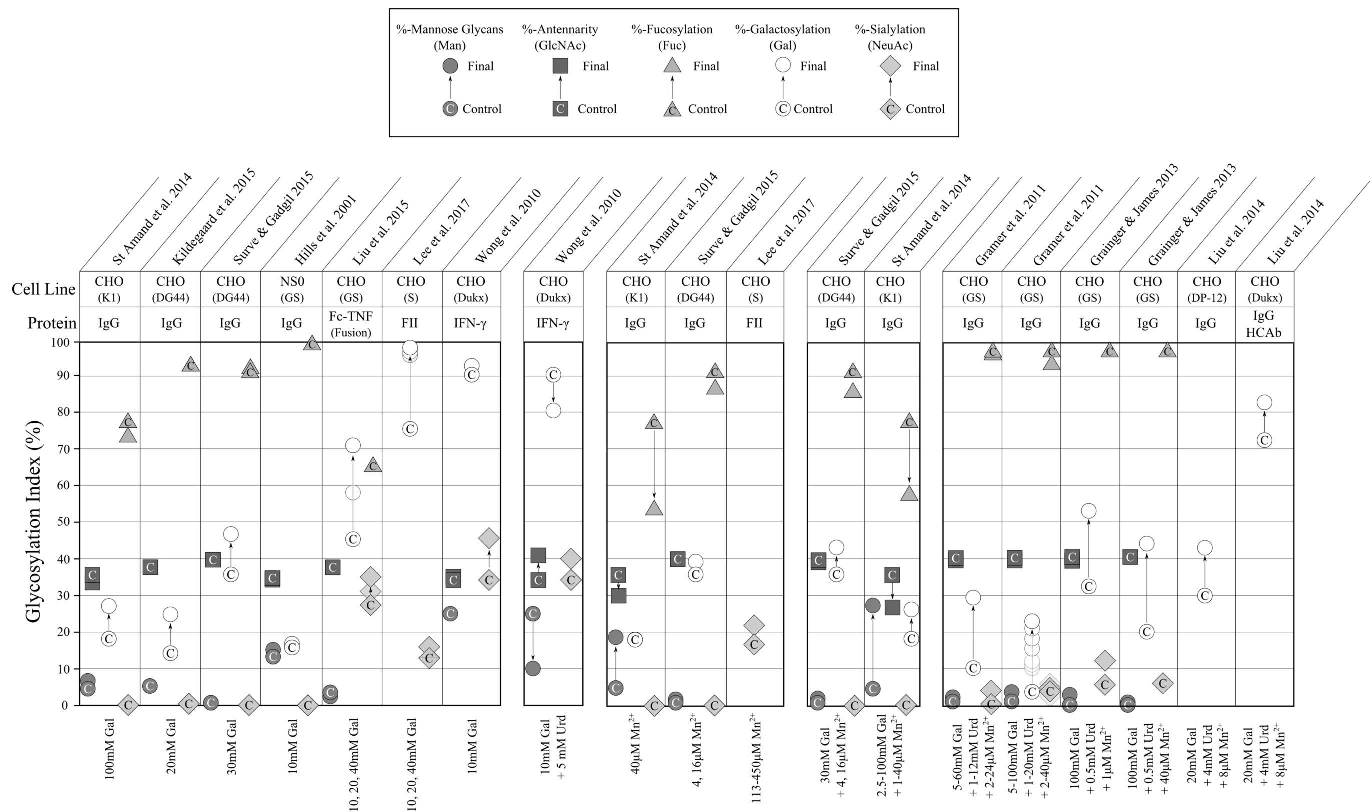
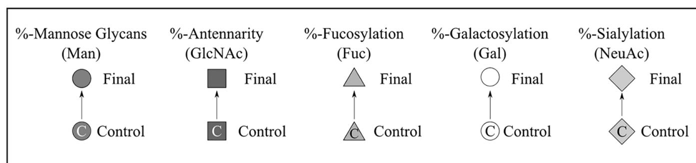


Figure 4



Kildegaard et al. 2015
Hills et al. 2001
Baker et al. 2001
Baker et al. 2001
Lee et al. 2017
Wong et al. 2010
Wong et al. 2010
Liu et al. 2015

Cell Line	CHO (DG44)	NS0 (GS)	CHO (GS)	NS0 (GS)	CHO (S)	CHO (Dukx)	CHO (Dukx)	CHO (GS)
Protein	IgG	IgG	TIMP-1	TIMP-1	FII	IFN-γ	IFN-γ	Fc-TNF (Fusion)
	20mM ManNAc	20mM ManNAc	20mM ManNAc	20mM ManNAc	20-80mM ManNAc	20mM ManNAc	20mM ManNAc + 10mM Cyt	20mM ManNAc + 10mM Cyt

Glycosylation Index (%)
0
10
20
30
40
50
60
70
80
90
100

Figure 5