



# Glycosylated Biotherapeutics: Immunological Effects of N-Glycolylneuraminic Acid

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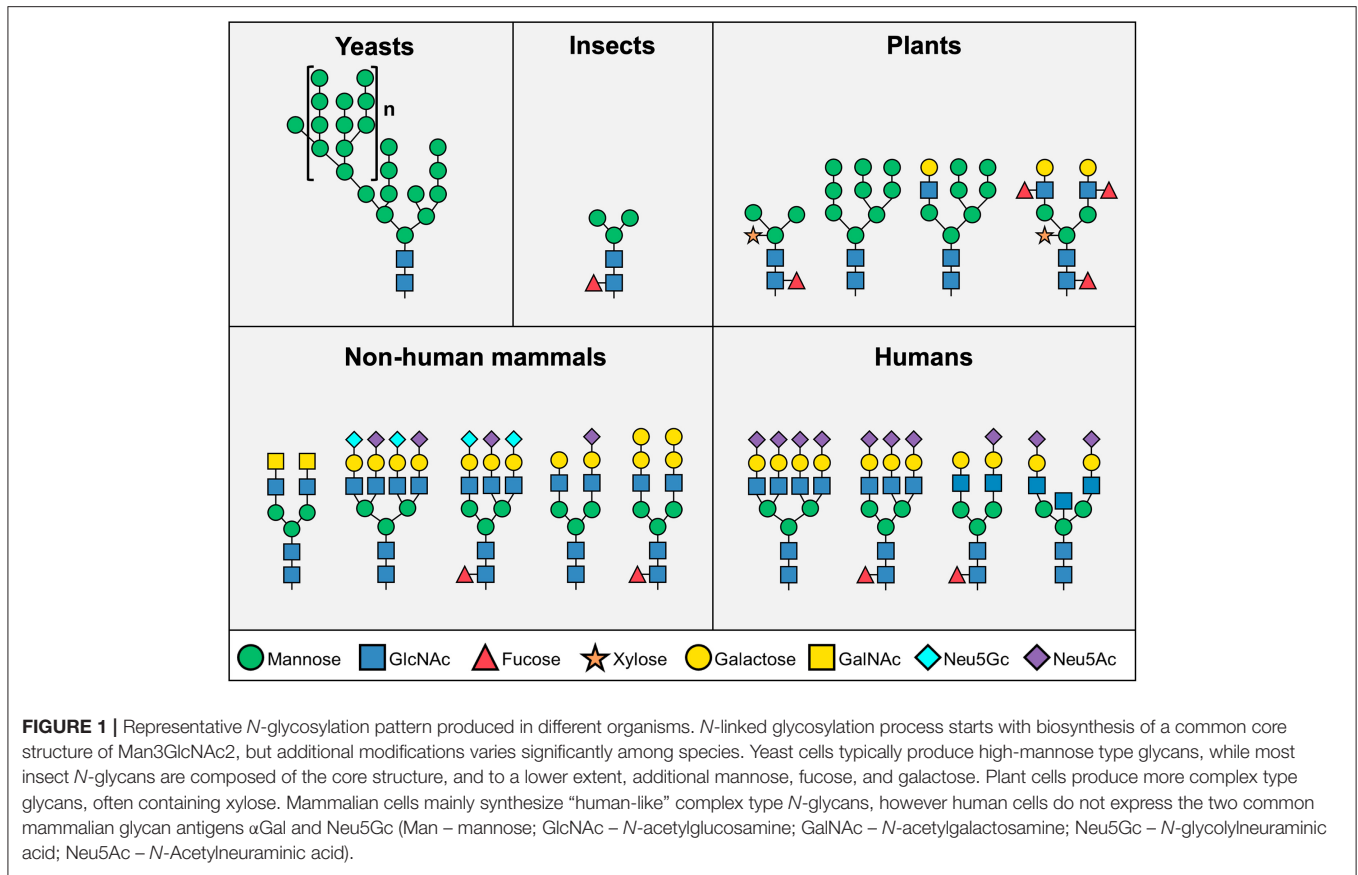
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The emerging field of biotherapeutics provides successful treatments for various diseases, yet immunogenicity and limited efficacy remain major concerns for many products. Glycosylation is a key factor determining the pharmacological properties of biotherapeutics, including their stability, solubility, bioavailability, pharmacokinetics, and immunogenicity. Hence, an increased attention is directed at optimizing the glycosylation properties of biotherapeutics. Currently, most biotherapeutics are produced in non-human mammalian cells in light of their ability to produce human-like glycosylation. However, most mammals produce the sialic acid *N*-glycolylneuraminic acid (Neu5Gc), while humans cannot due to a specific genetic defect. Humans consume Neu5Gc in their diet from mammalian derived foods (red meat and dairy) and produce polyclonal antibodies against diverse Neu5Gc-glycans. Moreover, Neu5Gc can metabolically incorporate into human cells and become presented on surface or secreted glycans, glycoproteins, and glycolipids. Several studies in mice suggested that the combination of Neu5Gc-containing epitopes and anti-Neu5Gc antibodies could contribute to exacerbation of chronic inflammation-mediated diseases (e.g., cancer, cardiovascular diseases, and autoimmunity). This could potentially become complicated with exposure to Neu5Gc-containing biotherapeutics, bio-devices or xenografts. Indeed, Neu5Gc can be found on various approved and marketed biotherapeutics. Here, we provide a perspective review on the possible consequences of Neu5Gc glycosylation of therapeutic protein drugs due to the limited published evidence of Neu5Gc glycosylation on marketed biotherapeutics and studies on their putative effects on immunogenicity, drug efficacy, and safety.

**Keywords:** antibody, biotherapeutics, glycosylation, sialic acid, *N*-glycolylneuraminic acid (Neu5Gc), immunology, anti-carbohydrate antibodies

## INTRODUCTION

Biotherapeutics are a rapidly increasing portion of the pharmaceutical market, with over a 100 new products approved and marketed in the U.S. and the European Union over the past decade (1). Among the commonly used biotherapeutics are antibodies, cytokines, enzymes, and hormones, originally purified from living organisms and characterized by their therapeutic potential, with limited evaluation of their potential immunological effects in recipient patients. Large-scale manufacturing of these therapeutic products involves expression of recombinant DNA in biological



systems such as bacteria, yeast, insect and mammalian cells, as well as transgenic animals (1, 2). Being produced in living systems, therapeutic proteins often undergo post-translational modifications, most notably glycosylation.

Glycosylation is an important and ubiquitous modification, in which sugar chains (glycans) are covalently attached to proteins or lipids. Glycan biosynthesis is a template-independent process, which rely on a complex network of serially operating glycan-modifying enzymes (3, 4). The variety of possible monosaccharide compositions and modifications, linkage configurations and branching points gives rise to a tremendous diversity of glycan structures (Varki et al., 2015). Since this is not a template-driven process, proteins with identical amino acid sequences would typically differ in the degree of occupancy of their glycosylation sites (macro-heterogeneity), and would carry different glycans in a specific glycosylation site (micro-heterogeneity) (5). The glycosylation pattern of a cell changes through development and differentiation, under different environmental conditions, and during pathologies such as inflammation and malignancy, indicating the involvement of glycans in numerous processes in physiology and in disease (6).

Glycosylation of biotherapeutics has a substantial impact on their pharmacological properties and biological activity (7–10). Biotherapeutics glycosylation is largely determined by their production system (**Figure 1**). While non-mammalian cells (i.e.,

yeast, insect, or plant cells) are attractive due to their high yields, production of most biopharmaceutical products have shifted into mammalian expression platforms (i.e., hamster, human, or mouse cells) largely owing to consideration of their different glycosylation patterns (1). While yeast cells contain mostly high-mannose structures, mammalian-derived systems carry more complex glycans that include galactose, fucose and sialic acids (**Figure 1**)—all dramatically affecting the pharmacodynamics and pharmacokinetics of the drugs, most notable in glycosylated-antibodies (11–13). Higher levels of sialic acid at the tips of glycan chains generally improves serum half-life and stability of biotherapeutics (12, 14, 15), partly since in the presence of terminal sialic acid glycosylated-biotherapeutics are not recognized by liver asialoglycoprotein receptors (ASGR1) or mannose receptors (MR; CD206), thereby preventing their rapid removal from circulation (12, 16). In addition, the negative charge of sialic acids positively contribute to their thermal stability and solubility (17, 18). Monoclonal antibodies constitute a major class of biotherapeutics, and in many of these antibodies the functionality is directly regulated by the glycosylation on their Fc domain. All IgG antibodies are glycosylated at a conserved asparagine residue (Asn297) in the Fc region (19), and some are also glycosylated at their Fab region (20–22). The glycan on Asn297 site modulates the shape of the Fc domain in a way that it alters its ability to interact with various Fc receptors (10, 15, 20, 23, 24). Remarkably, IgG

Fc glycosylation is altered in pathological conditions such as autoimmunity (25), infection (10), and cancer (26–28), thereby modulating their effector functions (29). Interestingly, removal of the whole *N*-glycan revokes the ability of the Fc domain to interact with Fc receptors, thus Fc glycosylation is essential for the IgG effector functions (13, 30). The absence of fucose residues enhances antibody-dependent cellular cytotoxicity (31). In addition, higher presence of galactose promotes complement-dependent cytotoxicity, while decreased galactosylation leads to alternative complement cascade activation (32, 33). IgG antibodies with higher amount of terminal  $\alpha$ 2–6-linked sialic acids are recognized by DC-SIGN on dendritic cells, leading to anti-inflammatory activity (34, 35), while on the other hand activation of dendritic cells through antibody aggregates may induce immunogenicity and development of anti-drug antibodies (36). Aiming to optimize glycosylation properties, currently most biotherapeutics are produced in mammalian expression systems, with their ability to produce human-like glycosylation (1, 2, 37). Major efforts had been put into various methods for cell-glycoengineering to control antibody glycosylation (1, 35, 38–40), or to predict the glycosylation based on computational modeling (13, 38, 41–44).

Although humans and most other mammals have relatively similar glycosylation patterns, two major differences have been identified. Unlike most other mammals, humans lack the enzymes to synthesize the Gal $\alpha$ 1–3Gal $\beta$ 1–(3)4GlcNAc ( $\alpha$ Gal) epitope and the common sialic acid *N*-glycolylneuraminic acid (Neu5Gc) (45) (**Figure 1**). In addition to the inability to naturally express these sugar structures, all humans produce circulating antibodies against both antigens (45–49). In contrast to  $\alpha$ Gal, exogenous Neu5Gc can be metabolically incorporated into newly synthesized glycans and become presented on human cells (50, 51). Co-existence of Neu5Gc-containing epitopes and circulating anti-Neu5Gc antibodies have been suggested to exacerbate chronic inflammation-mediated diseases (52–57). This immune-conflict may be further complicated with exposure to Neu5Gc-containing biotherapeutics, bio-devices or xenografts. Indeed, recent studies have suggested that Neu5Gc-glycans have an enormous diversity (58–60), and predicted to be widely found on various approved and marketed biotherapeutics (2, 61), such as Cetuximab (61) and anti-thymocyte globulin (62–65). Although biotherapeutics provide effective treatment for a variety of clinical conditions, suboptimal efficacy and safety are major concerns for many of these products. Herein, we discuss the unique situation of Neu5Gc-containing biotherapeutics in the face of anti-Neu5Gc responses in humans, and the current knowledge on the effects of Neu5Gc on immunogenicity, efficacy, and safety of biotherapeutics.

## Neu5Gc IS IMMUNOGENIC IN HUMANS

Sialic acids are 9-carbon  $\alpha$ -keto acidic sugars usually present at the outermost part of glycans in animals (5, 66). The two most common sialic acids in mammals are *N*-acetylneuraminic acid (Neu5Ac) and its hydroxylated form, Neu5Gc. Conversion of

CMP-Neu5Ac to CMP-Neu5Gc is catalyzed by the enzyme CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) that is inactive in humans (66). In contrast to all other mammals, humans cannot synthesize Neu5Gc due to irreversible mutation in the *CMAH* gene that occurred  $\sim$ 3 million years ago, before the appearance of the genus *Homo* (67–70). Nevertheless, consumption of Neu5Gc-containing mammalian-derived products (e.g., red meat and dairy) results in uptake of Neu5Gc-glycoproteins through micropinocytosis (71–73) and metabolic incorporation of Neu5Gc epitopes into newly synthesized glycans (50, 56, 72–74). Thus, low levels of Neu5Gc are present in human tissues, mostly on endothelium and epithelium, and are known to accumulate in certain pathological conditions, mostly in cancer (52, 56, 71, 75).

This unique phenomenon results in presentation of foreign antigen in the context of self (Neu5Gc is replacing the self Neu5Ac on existing cellular glycans), termed “Xeno-autoantigen” (47, 57). Hence, Neu5Gc is foreign in humans and mediates production of a complex anti-Neu5Gc antibodies response, or “Xeno-autoantibodies” (47, 51, 57, 76). Neu5Gc is a 325 Dalton molecule and cannot by itself fill the paratope of an antibody, yet Neu5Gc-containing glycan-epitopes are highly diverse (58–60) and are recognized by polyclonal anti-Neu5Gc IgM, IgA, and mostly IgG antibodies that make up 0.1–0.2% of total circulating antibodies in humans (47, 49, 77–79). Anti-Neu5Gc antibodies in humans arise already in infants, soon after the introduction of dietary Neu5Gc (e.g., cow milk in baby formula, meat-containing grinded foods), and have been suggested to be induced through uptake of dietary Neu5Gc by non-typeable *Haemophilus influenzae* (NTHi) during infection in infants (80), and through micropinocytosis of Neu5Gc-glycoproteins into human cells followed by recycling into the cells surface glycoproteins and glycolipids (71–74). In fact, all healthy humans examined thus far had anti-Neu5Gc antibodies, although sometimes at low levels and with limited repertoires (47, 49, 78, 81). This antibody response against Neu5Gc can be higher in certain pathologies and may remain high for decades (82–84).

Studies in mice had suggested that the co-existence of Neu5Gc-glycans and serum anti-Neu5Gc antibodies may lead to immune-driven chronic inflammation, termed “xenosialitis,” thereby exacerbating chronic inflammation-related diseases such as cancer, cardiovascular disease and autoimmunity (52, 53, 57, 84–86). For example, high anti-Neu5Gc IgG titers were shown to be associated with increased risk for colorectal cancer (84), which also fits the reported association of red meat consumption and higher carcinoma risk (55, 87–89). Similarly, in a human-like mouse model (*Cmah*-KO) high consumption of Neu5Gc resulted in an inflammatory phenotype, and together with circulating anti-Neu5Gc antibodies (in *Cmah/Ldlr*-DKO mouse model) resulted in increased atherosclerosis (52, 86, 90). These findings in mice fit the reported high risk of cardiovascular disease that is associated with consumption of red meat and processed meat (91, 92), although clear evidence in humans is still controversial, at least through *in vitro* studies on effects of anti-Neu5Gc antibodies on human endothelial cells that express authentic Neu5Gc levels (65). Neu5Gc and anti-Neu5Gc antibodies had also been suggested to participate in autoimmunity (54, 55, 93). Altogether,

this unique human-specific immune conflict could help explain the susceptibility to numerous chronic inflammation-related diseases, which conspicuously occur in humans (94). The consequences of Neu5Gc/anti-Neu5Gc responses in humans could potentially be further exacerbated by exposure to Neu5Gc-containing biotherapeutics, bio-devices, or xenografts.

## Neu5Gc ON MARKETED BIOTHERAPEUTICS ASSOCIATED WITH THEIR PRODUCTION SYSTEM

Production of many biotherapeutics involves non-human mammalian cells, serum or serum-derived substances, thus are likely to contain some levels of Neu5Gc. Generalizations cannot be made since glycosylation properties, including Neu5Gc levels, are influenced by many factors during the production process. Yet, it is reasonable to assume the relative Neu5Gc levels in biotherapeutics according to their production systems (2). The most common platform for biotherapeutics is Chinese hamster ovary (CHO) cells (1, 2, 95). Several studies have suggested the presence of Neu5Gc on biotherapeutics produced in CHO cells, though in relatively low levels (2, 95, 96). Baby hamster kidney cells (BHK-21) are also often used for production of biotherapeutics and are expected to express low levels of Neu5Gc (2). By contrast, murine myeloma cell lines (e.g., NS0 and Sp2/0) are known to produce Neu5Gc at significantly higher levels (2, 97, 98). Drugs produced in animals (non-human mammals that are known to synthesize Neu5Gc intrinsically; e.g., cow, pig, goat, sheep, and rabbit) are also likely to contain Neu5Gc, since they were shown to express high amounts of Neu5Gc (50, 60). For example, antithrombin produced in goat milk and anti-thymocyte globulin derived from rabbit, are known to contain high levels of Neu5Gc (2, 62, 63). Similarly, Neu5Gc is also widely found in xenografts that are used for organ and tissue replacement in humans, as demonstrated with tissues derived from cows and pigs (99–102). These findings also prompted the generation of Neu5Gc-deficient animals as novel platforms (103–107).

Human cell lines represent the ideal production platform in terms of glycosylation properties, but their high risk of viral transmission and low protein yield make them less popular for production of biotherapeutics (37). Nevertheless, several products derived from human cells (HEK293 and HT-1080) have been approved in recent years (1). Utilization of these cells may become more common in the future, yet the presence of Neu5Gc in their products remains a significant concern, as it can also be metabolically incorporated from exogenous sources (i.e., from the growth media). Hence, even human cells can produce Neu5Gc-containing biotherapeutics if Neu5Gc is unintentionally supplemented in their growth media, for example through the addition of animal serum or serum-derived substances (2, 45). Although it was well-known that humans cannot express Neu5Gc, its immunogenic potential was under-rated for years, and accordingly its presence on biotherapeutics was largely disregarded. With the accumulating information about Neu5Gc and anti-Neu5Gc antibodies in humans, the presence of Neu5Gc

on biotherapeutics should be re-evaluated. While the effect of Neu5Gc on biotherapeutics remains poorly characterized, several recent studies addressed possible consequences (61–65, 108–111), as described below.

## EFFECTS ON SERUM ANTI-Neu5Gc IgG RESPONSES IN HUMANS

Treatment of human patients with Neu5Gc-containing biotherapeutics can significantly alter the pre-existing immune response against Neu5Gc, both quantitatively and qualitatively. Yet, some studies failed to show human immune response against Neu5Gc-containing biotherapeutics, as in the case of recombinant erythropoietin that was produced in CHO cells (96, 112). Of note, these conclusions were based on the human response evaluated against Neu5Gc-containing gangliosides. It is possible that with current technologies as glycan microarrays it would be possible to revisit these findings. More recent studies were able to clearly demonstrate immunological effects in humans. Anti-thymocyte globulin (ATG) is an immunosuppressive biotherapeutic commonly used in transplantation and autoimmune diseases (113). ATG is a polyclonal IgG produced in rabbits and was shown to contain Neu5Gc (62, 63). One of the side effects during treatment with this drug is the development of an immune reaction against the non-human animal-derived immunoglobulins. This is characterized by immune complex formation that can develop into a serum sickness disease (62, 114). In fact, without strong immunosuppression most patients will develop serum sickness (114). Furthermore, it was shown that ~10% of first-kidney graft recipients treated with the immunosuppressive drug ATG developed serum sickness disease, and in addition had increased serum anti-Neu5Gc IgG responses (62). The serum sickness was associated with late graft loss, and these patients exhibited further elevated titers of anti-drug and anti-Neu5Gc IgG in blood samples >4 years post-transplantation compared to patients without serum sickness (62). In another study, ATG treatment was found to be associated with a shift in anti-Neu5Gc IgG repertoire in transplantation patients over time (64). Similarly, analyzing early events in another prospective study of kidney-graft recipients within their first year showed that patients with ATG induction treatment had a highly significant increase in anti-Neu5Gc IgG levels compared to patients not treated with ATG. In addition, these antibodies shifted their response repertoire over time to recognize different Neu5Gc-glycans, even in the face of a strong immunosuppression in those patients, but no effect on the graft function was observed within the limit of this study (110).

While mostly used in transplantation, ATG therapy was also explored as a therapeutic drug in young adults within the Study of Thymoglobulin to arrest Type 1 Diabetes (START clinical trial) (114). In these diabetic patients, ATG treatment also resulted in a highly significant increase in levels of serum anti-Neu5Gc IgM and IgG that peaked after 1 month and remained detectable even 1 year after treatment (108). Further characterization of the top responders by elaborated glycan

microarrays demonstrated the rapid increase in responses against multiple Neu5Gc-glycans after 1 month, persistence over 2 years, and further demonstrated altered repertoires of serum anti-Neu5Gc IgG (63). In fact, ATG treatment changed the pre-existing response to induce anti-Neu5Gc IgG of higher affinity with extended diversity. Interestingly, in some patients there was *de-novo* recognition of various Neu5Gc-containing glycan epitopes, including of Neu5Gc-glycans normally expressed on glycolipids that were not present on the ATG drug (63). Overall, these findings suggested that Neu5Gc-containing biotherapeutics are immunogenic reagents, and once injected into humans that already express circulating anti-Neu5Gc antibodies, act as triggers of extended immune responses. In fact, current data support their role as inducers of secondary anti-Neu5Gc immune responses. In some individuals possibly also triggering a recall of memory responses inducing antibody recognition of Neu5Gc-glycans that had not been presented on the drug.

## ANTI-Neu5Gc ANTIBODIES IN DISEASE

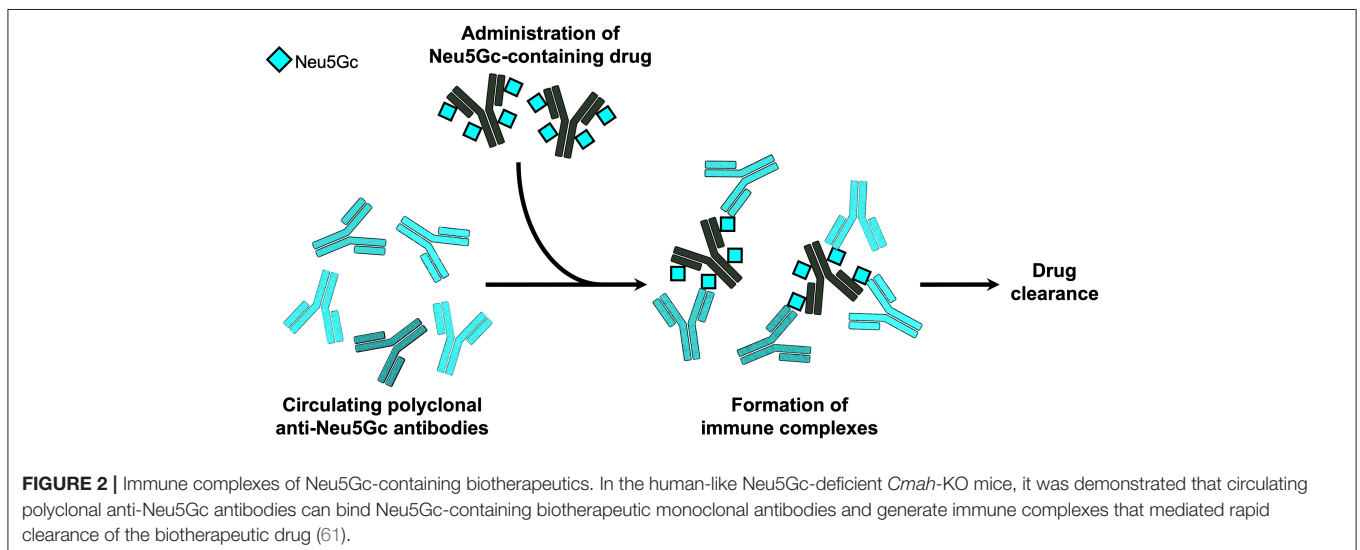
It was postulated that such elevated anti-Neu5Gc responses could potentially increase Neu5Gc-mediated xenosialitis and chronic inflammation-mediated diseases, as cancer and atherosclerosis (53). High pre-existing total anti-Neu5Gc IgG levels measured by glycan microarrays were associated with increased risk of colorectal cancer (in a cohort of 71 colorectal cancer cases and matched controls of the EPIC-Norfolk cohort plasma samples) (84). However, based on a large cohort of ~200,000 kidney allograft recipients, average anti-Neu5Gc IgG responses measured by EIA method did not show increased colon cancer risk in the ~18% ATG-treated patients compared to those not treated with ATG (111). Of note, these studies evaluated different pools of blood anti-Neu5Gc IgG antibodies and measured by different methods: pre-existing antibodies by arrays (84) vs. drug-induced antibodies by EIA (111). Currently, different methods are available to measure anti-Neu5Gc antibody

responses (49, 115), and there are clear differences between pre-existing vs. ATG-induced anti-Neu5Gc IgG (63, 65), that together could perhaps explain the different analysis outcome regarding cancer risk.

Likewise, contradicting reports exist regarding anti-Neu5Gc antibodies in the context of cardiovascular disease risks. Aiming to examine gene expression profiles by *in vitro* studies, human endothelial cells that were engineered to express low levels of surface Neu5Gc (mimicking the levels expected to be present from dietary intake in humans) were exposed to different pools and dose of affinity-purified anti-Neu5Gc antibodies. This analysis showed differential gene expression when cells were exposed to ATG-induced compared to pre-existing anti-Neu5Gc antibodies or in the absence of such antibodies. Interestingly, drug-induced anti-Neu5Gc antibodies did not significantly upregulate inflammation-related genes that would be expected in xenosialitis (65). However, other *in vivo* studies in the human-like Neu5Gc-deficient mice also lacking the LDL receptor showed increased atherosclerosis propensity only when both high levels of diet-derived Neu5Gc-antigens and induced anti-Neu5Gc antibodies were present, thus supporting xenosialitis (90). Altogether, these findings suggest that anti-Neu5Gc antibody responses in humans are complex and further studies are needed to better understand their relationship with various diseases in humans.

## RAPID CLEARANCE OF Neu5Gc-CONTAINING BIOTHERAPEUTICS *IN VIVO*—EVIDENCE IN MICE

Besides the immunogenicity of Neu5Gc on biotherapeutics, it was postulated that these Neu5Gc-drugs could potentially be recognized by circulating anti-Neu5Gc antibodies in humans, and by that affect drug levels and/or efficacy in patients. This was directly investigated using the top selling cancer biotherapeutic monoclonal antibodies targeting EGFR (61).



Consistent with their production system, it was shown that Cetuximab produced in murine myeloma cells contains Neu5Gc, while Panitumumab expressed in CHO cells lack Neu5Gc (61). Human serum anti-Neu5Gc antibodies could bind the Neu5Gc-containing Cetuximab and generate immune complexes, but did not bind Panitumumab. Furthermore, consistent with their expected immunogenic properties, injection of these drugs to the human-like Neu5Gc-deficient *Cmah*-KO mice induced serum anti-Neu5Gc antibody only in the Neu5Gc-containing Cetuximab-treated group. In these mice, circulating serum anti-Neu5Gc antibodies resulted in a rapid clearance of the Neu5Gc-containing Cetuximab, but not of Panitumumab (61). Together, these data suggest that Neu5Gc on biotherapeutics could potentially affect drug levels in circulation through immune complex formation (Figure 2), at least in human-like mice. Currently, there is no evidence of drug neutralizing activity of anti-Neu5Gc antibodies. It remains to be investigated whether Neu5Gc/anti-Neu5Gc could affect drug clearance in patients, hence alter drug efficacy and as such play a role in the variability of the clinical responses observed across a population for a given biotherapeutic.

## CONCLUSIONS AND PERSPECTIVE

Biotherapeutics have revolutionized the treatment for numerous clinical conditions, yet immunogenicity and efficacy issues remain to be addressed. Currently, most biotherapeutics are produced in non-human mammalian cells to allow human-like glycosylation, as it was shown to dramatically affect pharmacological properties of these products. Yet, despite the fact that it was recognized that humans cannot produce the non-human carbohydrate Neu5Gc, its immunogenic potential was much ignored, and accordingly its expression on biotherapeutics was largely overlooked. In fact, non-human mammals produce Neu5Gc-glycans, against which all humans have circulating

polyclonal antibodies. Moreover, Neu5Gc can be metabolized by human cells and become presented on cell surface glycans, glycoproteins and glycolipids. In addition, all humans examined thus far had serum anti-Neu5Gc responses at variable levels and repertoires. Neu5Gc on biotherapeutics may induce the pre-existing anti-Neu5Gc responses in humans, and these could potentially contribute to increased xenosialitis and related diseases, yet further evidence is needed to fully understand the developed responses and their effects in humans. Drug-induced or pre-existing anti-Neu5Gc antibody responses could potentially contribute to drug clearance from circulation through immune complex formation, thereby reducing drug efficacy, although clear evidence in humans is yet to be provided. While not discussed in this review, similar effects could be expected by  $\alpha$ Gal glycosylation on biotherapeutics since all humans have circulating anti-Gal antibodies. Thus, much of the mechanistic insights into the outcome of the co-existence of anti-Neu5Gc antibodies and antigenic Neu5Gc-containing biotherapeutics (or anti-Gal antibodies and antigenic  $\alpha$ Gal-containing biotherapeutics) in humans is largely unknown and warrants further investigation.

## AUTHOR CONTRIBUTIONS

SY and VP-K wrote the manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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