Anti-CD20 monoclonal antibodies are generated in the Golgi of animal cell lines. As a result of biotherapeutic manufacturing, anti-CD20 monoclonal antibodies may produce the sialic acid, N-glycolylneuraminic acid (Neu5Gc). Cells produce this monosaccharide when the enzyme cytidine monophosphate N-acetylmuraminic acid hydroxylase (CMAH) adds an oxygen atom to N-acetylmuraminic acid (Neu5Ac). Neu5Gc must be activated via linkage to cytidine monophosphate for sialyl transferases to add them to glycan termini.1

The levels of Neu5Gc incorporated into monoclonal antibody–based drugs significantly depend on the host cell line, the expression levels of CMAH and cytidine monophosphate, and the cell culture process used. The chimeric monoclonal antibody anti-CD20 rituximab and the fully human anti-CD20 monoclonal antibody ofatumumab are produced in CHO and NSO cells, respectively. CHO express lower CMAH than NSO. Therefore, the proportion of N-linked glycans capped with Neu5Gc is higher in ofatumumab than in rituximab, with a consequent significantly high immunogenic profile.2

Humans do not have activated Neu5Gc due to a deletion of a critical exon in the CMAH gene.3 However, despite humans’ inability to synthesize it, Neu5Gc may become a so-called xeno-autoantigen, because of its presence on human glycoproteins and glycolipids through metabolic incorporation after dietary consumption of mammalian-derived products, such as dairy and meat.4 Therefore, in humans, anti-Neu5Gc antibodies (anti-Neu5Gc Abs) are usually generated during early infancy, minimally persist during adulthood, and could be of concern when administering a therapeutic containing Neu5Gc.1

In an extensive randomized clinical trial, we compared the efficacy of a single infusion of ofatumumab versus rituximab in 140 children and young adults (aged 2–24 years) with steroid- and calcineurin inhibitor–dependent nephrotic syndrome (NCT02394119).5 The main result we reported was that ofatumumab was not superior to rituximab in maintaining remission in such subjects. However, rituximab was more efficient in subjects <9 years old.5

Dr. Lemaire, in a comment about the clinical trial, suggested that the serum titer of anti-Neu5Gc Abs may have affected the clinical outcome of subjects.6 To test this hypothesis, we developed a homemade ELISA test for the serum detection of circulating anti-Neu5Gc Abs. We tested sera at enrollment (before infusions) and at 6–9 months of follow-up in 39 and 62 subjects receiving ofatumumab and rituximab, respectively (no significant differences of baseline characteristics were reported).

Briefly, Nunc-MaxiSorp plates (Thermo Fisher Scientific) were coated with bovine sialomucin (Sigma-Aldrich). Then, plates were washed and blocked with PBS with 1% wt/vol chicken ovalbumin (chick protein lacks Neu5Gc). After further washes, serum samples (1:100 in PBS-T and 1% wt/vol chicken ovalbumin) were added. Plates were washed and we added rabbit anti-Human IgG conjugated to horseradish peroxidase (Invitrogen, 1:5000 in PBS-T) and 1% wt/vol chicken ovalbumin. After adequate washes, the peroxidase substrate (TMB, Bio-Rad) was added, and the reaction was stopped with an H2SO4 solution. An iMark microplate reader (Bio-Rad) read the absorbance at 450 nm. To standardize the response of the antibodies, we used a pool of strongly positive controls.

As Figure 1A shows, serum levels of anti-Neu5Gc Abs significantly decreased after infusion of both rituximab and
ofatumumab (P<0.0001), with no differences between the two arms (0.62 RU/ml [SD 0.49–0.94] in ofatumumab versus 0.62 RU/ml [SD 0.49–0.94] in rituximab at 6–9 months after infusion). Previous infusions of anti-CD20 antibodies did not correlate with anti-Neu5Gc Ab levels. Moreover, through the Youden’s index, we identified a cutoff value to define high and low levels of anti-Neu5Gc Abs in our population.

Figure 1B shows that serum titers of anti-Neu5Gc Abs at infusion do not affect response to rituximab or ofatumumab in terms of percentage of relapse. Therefore, in our cohort of subjects with steroid-dependent nephrotic syndrome, receiving a single infusion of anti-CD20 monoclonal antibodies, the speculated immunogenic profile of such therapies, in particular of ofatumumab, was not confirmed. We administered low doses of antibodies compared with other therapeutic schemes, and this may, in part, justify the lack of immunogenicity. As a major limitation, not all subjects enrolled in the study were tested for anti-Neu5Gc Abs. However, anti-Neu5Gc Abs are reported to be increased in several autoimmune diseases, such as multiple sclerosis. Therefore, the decrement of anti-Neu5Gc Abs after anti-CD20 monoclonal antibodies infusion may be of relevance and may suggest that we consider administration of therapies targeting B cells also in these diseases.

In conclusion, Neu5Gc recently raised clinical concerns due to its immunogenic potential and pre-existing anti-Neu5Gc Abs in humans. In biotherapeutic manufacturing, anti-CD20 monoclonal antibodies may introduce Neu5Gc. Therefore, pre-existing anti-Neu5Gc Abs in patients may limit the efficacy of such treatments. We report the absence of clinical effect of pre-existing anti-Neu5Gc Abs in pediatric and young adults with steroid-dependent nephrotic syndrome and treated with anti-CD20 monoclonal antibodies.

**DISCLOSURES**

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**AUTHOR CONTRIBUTIONS**

A. Angeletti and G. Ghiggeri conceptualized the study; A. Angeletti, M. Bruschi, X. Kajanal, and G. Ghiggeri were responsible for the data curation; M. Bruschi and X. Kajanal were responsible for the formal analysis; G. Ghiggeri was responsible for the funding acquisition; A. Angeletti was responsible for the validation; A. Angeletti and G. Ghiggeri provided supervision; F. Lugani was responsible for the methodology; G. Ghiggeri was responsible for the resources; A. Angeletti and G. Ghiggeri provided supervision; F. Lugani was responsible for the validation; A. Angeletti wrote the original draft; and A. Angeletti, G. Candiano, G. Ghiggeri, and F. Lugani reviewed and edited the manuscript.

**DATA SHARING STATEMENT**

All data used in this study are available.

**REFERENCES**


