Bioconjugate Chemistry

Article

Cite This: *Bioconjugate Chem.* 2019, 30, 161−168

pubs.acs.org/bc

Presentation Mode of Glycans Affect Recognition of Human Serum anti-Neu5Gc IgG Antibodies

Salam Bashir,[†] Shani Leviatan Ben Arye,[†] Eliran Moshe Reuven,[†] Hai Yu,[‡] Cristina Costa,[§] Manuel Galiñanes,[∥] Tomaso Bottio,[#] Xi Chen,[‡] and Vered Padler-Karavani*[,†]

[†]The George S. Wise Faculty of Life Sciences, Department of Cell Research and Immunology, Tel Aviv University, Tel Aviv, 69978, Israel

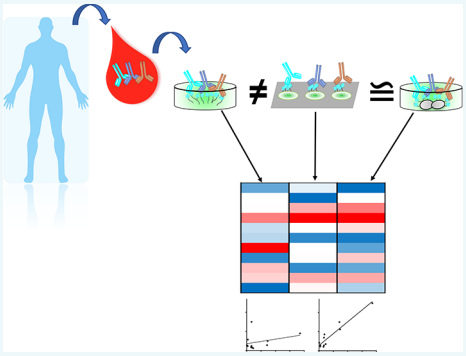
[‡]Department of Chemistry, University of California-Davis, Davis, California 95616, United States

[§]Infectious Pathology and Transplantation Division, Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), Hospitalet de Llobregat 08908, Spain

[∥]Reparative Therapy of the Heart, Vall d'Hebron Research Institute (VHIR) and Department of Cardiac Surgery, University Hospital Vall d'Hebron, Autonomous University of Barcelona (AUB), Barcelona, 08035, Spain

[#]Department of Thoracic and Cardiovascular Sciences and Public Health, University of Padova, Padova, 35122, Italy

ABSTRACT: Recognition of carbohydrates by antibodies can be affected by antigen composition and density. This had been investigated in a variety of controllable multivalent systems using synthetic carbohydrate antigens, yet such effects on anticarbohydrate antibodies in circulating human serum have not been fully addressed thus far. All humans develop a polyclonal and diverse response against carbohydrates containing a nonhuman sialic acid form, *N*-glycolylneuraminic acid (Neu5Gc). This red meat-derived monosaccharide is incorporated into a diverse collection of human glycans resulting in circulating anti-Neu5Gc antibodies in human sera. Such antibodies can cause exacerbation of diseases mediated by chronic inflammation such as cancer and atherosclerosis. We aimed to evaluate how different presentation modes of Neu5Gc-glycans can affect the detection of anti-Neu5Gc IgGs in human serum. Here, we compare serum IgG recognition of Neu5Gc-containing glycoproteins, glycopeptides, and synthetic glycans. First, Neu5Gc-positive or Neu5Gc-deficient mouse strains were used to generate glycopeptides from serum glycoproteins. Then we developed a reproducible ELISA to screen human sera against Neu5Gc-positive glycopeptides for detection of human serum anti-Neu5Gc IgGs. Finally, we evaluated ELISA screens against glycopeptides in comparison with glycoproteins, as well as against elaborated arrays displaying synthetic Neu5Gc-glycans. Our results demonstrate that the presentation mode and diversity of Neu5Gc-glycans are critical for detection of the full collection of human serum anti-Neu5Gc IgGs.



INTRODUCTION

Carbohydrates are major biomolecules that play key structural and functional roles, such as affecting protein folding and trafficking, involved in cell recognition, development, and signaling.[1,2] Carbohydrate chains (glycans) either free or conjugated to proteins and lipids decorating the surfaces of all living cells are potential candidates or targets for diagnostics and therapeutics.[3−5] However, antibodies targeting glycan determinants commonly have low affinity and some have mixed specificities in comparison to antibodies targeting proteins.[4,6,7] Glycan density, valency, and flexibility could affect antiglycan antibodies binding characteristics.[8−10] In fact, binding of two or more carbohydrate determinants could result in antibodies of higher avidity and specificity,[10] and such interactions rely on antigen density for optimal spacing and orientation. Several studies investigated the importance of glycan density for avidity and selectivity of antibodies and lectins in a variety of controllable multivalent systems.[11−16] For example, modulation of glycan densities on a carrier protein (e.g., bovine serum albumin or BSA) revealed different binding patterns of serum antibodies,[9] and similarly affected binding preferences of antibodies and lectins against dendrimers[17] and synthetic polymer[13] with different glycan densities.

There are diverse anticarbohydrate antibodies in human serum, some involved in human health (e.g., blood group antibodies, anti-Gal).[18−20] Likewise, incorporation of the dietary nonhuman sialic acid, *N*-glycolylneuraminic acid (Neu5Gc), into human glycans results in a polyclonal serum anti-Neu5Gc response that targets diverse Neu5Gc-glycoconjugates.[21] Circulating serum antibodies against Neu5Gc-containing structures have been suggested to contribute to chronic inflammation-related diseases in humans,[3,22−25] some of which were observed in a dose-dependent manner.[26−28]

Received: November 14, 2018
Published: November 30, 2018

DOI: 10.1021/acs.bioconjchem.8b00817
Bioconjugate Chem. 2019, 30, 161−168

Moreover, the levels of circulating human anti-Neu5Gc antibodies could potentially be used to assess patients clinical condition.[23,29,30] However, detection and quantification of human serum anti-Neu5Gc IgGs are challenging due to the high diversity of Neu5Gc-containing epitopes and resulting humoral responses.

We aimed to evaluate how different presentation modes of Neu5Gc-glycans can affect the detection of anti-Neu5Gc IgGs in human serum. Current methods include enzyme-linked immunosorbent assay (ELISA) and sialoglycan microarray displaying selected synthetic or natural targets.[21,29,31,32] Here, we compare serum IgG recognition of Neu5Gc-containing glycoproteins, glycopeptides, and synthetic glycans. Our results demonstrate that the presentation mode and diversity of Neu5Gc-glycans are critical for detection of human serum anti-Neu5Gc IgGs.

RESULTS AND DISCUSSION

Preparation and Characterization of Sialo-Glycopeptides. To generate sialo-glycopeptides (GPs), serum from wild-type (WT; Neu5Gc-positive) or *Cmah*-knockout (*Cmah*-KO; Neu5Gc-negative) mice were collected and digested with Pronase protease, a mixture of endopeptidases and exopeptidases, to yield GPs and free amino acids (Figure 1).[33]

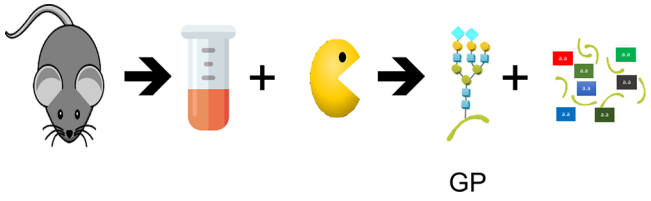


Figure 1. Generating glycopeptides (GP) from mouse serum. Schematic diagram of method. Mouse serum was collected, then digested with Pronase and GPs purified.

Digestion was allowed to proceed for 5 days with daily addition of fresh Pronase to ensure complete breakdown of serum sialo-glycoproteins. Progression of digestion was monitored by silver staining and showed that after 3 days most proteins had been digested (Figure 2A). Yet, according to 1,2-diamino-4,5-methylenedioxybenzene-high performance liquid chromatography (DMB-HPLC) analysis, the Sia content had not been altered throughout the 5 days of digestion (Figure 2B). Free amino acids were separated from the digested GPs by 3 kDa centrifugal filters, with minimal loss of sialylated GPs in the run-through (Figure 3A). To further characterize Sia type and linkage, the resulting GPs were coated onto ELISA plates and examined by chicken anti-Neu5Gc IgY, and the Sia-specific lectins SNA (binds α2-6-

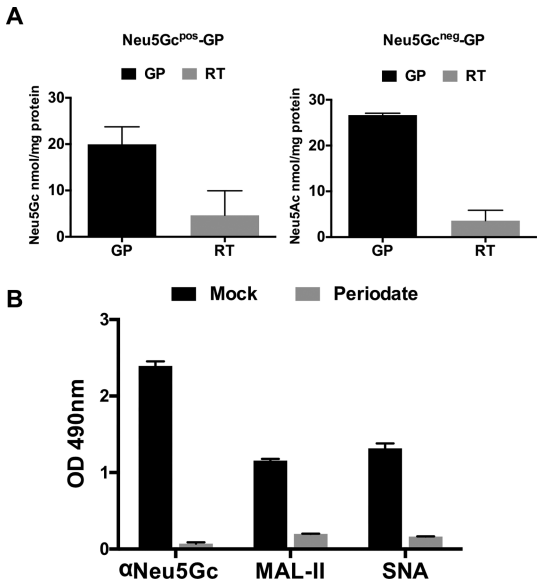


Figure 3. Characterization of Sia in glycopeptides. (A) Pronase digest was separated by size though Amicon 3 kDa filters, and Sia content quantified by DMB-HPLC (top fraction contains most GPs; bottom fraction runthrough, RT, shows minimal Sia loss). (B) Qualitative characterization of Sia in GPs from WT mouse sera by ELISA (coated at 1 μg/well protein) confirmed presence of Neu5Gc (by anti-Neu5Gc-IgY), and demonstrated Siaα2-6 (SNA) and Siaα2-3 (MAL-II) GP-glycoconjugates. Antibody/lectins signal was dramatically reduced after mild periodate treatment (that cleaves C8−C9 in side chain of Sia), demonstrating their Sia specific-recognition (representative of two independent experiments; mean ± SD of triplicates).

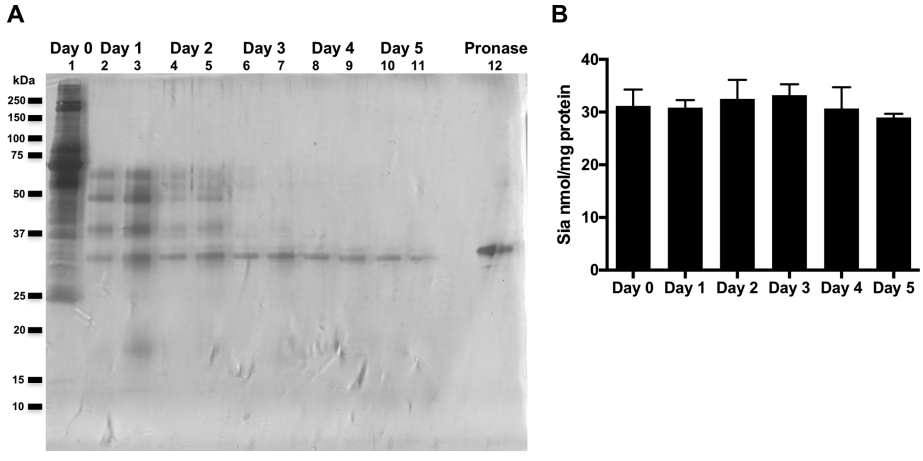


Figure 2. Characterization of Pronase digest. Mouse serum with total 80 mg protein was used per digestion. (A) 12.5% gel was loaded with serum digest at 2.5 μg/lane (lanes 1,2,4,6,8,10), 5 μg/lane (lanes 3,5,7,9,11), or 6 μg of Pronase (lane 12), then daily protein digestion was evaluated by silver staining (protein quantified by BCA assay). (B) Total sialic acid content in Pronase digest (15 μL) was analyzed daily by DMB-HPLC, showing no significant loss during digestion (representative of at least two independent experiments; mean ± SEM).

DOI: 10.1021/acs.bioconjchem.8b00817
Bioconjugate Chem. 2019, 30, 161−168

linked Sia) and MAL-2 (binds α2-3-linked Sia). GPs prepared from WT mice sera contained Neu5Gc (Neu5Gc^{pos}-GP) and both Siaα2-3/6-linked glycoconjugates (Figure 3B), consistent with data obtained for undigested WT mouse sera glycoproteins.[29] GPs obtained from *Cmah*-KO mice sera lacked Neu5Gc expression (Neu5Gc^{neg}-GP), but showed similar detection of Siaα2-3/6-linked glycoconjugates that contained only Neu5Ac (data not shown). Importantly, mild periodate treatment of the coated GPs abolished antibody/lectins binding (Figure 3B), demonstrating their Sia-specific recognition (mild periodate treatment would truncate the Sia side chain at carbons C8 and C9, leaving a terminal hydroxyl at C7 while maintaining the carboxylate on C1).[34]

Optimizing Reproducible Detection of Serum anti-Neu5Gc IgGs Using Sialo-Glycopeptides. Establishing minimal batch-to-batch variability is critical for further use of digested GPs, hence separate batches were examined as ELISA targets (Figure 4). We evaluated reproducibility of human

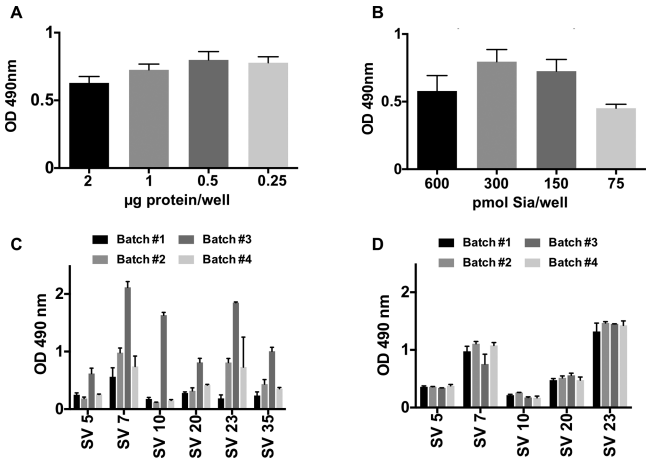


Figure 4. Evaluating reproducibility of human serum IgG reactivity against Neu5Gc^{pos}-GPs by ELISA. (A,B) 96-well Costar plates were coated with Neu5Gc^{pos}-GPs in several concentrations based on protein (A; BCA) or Sia (B; DMB-HPLC) content, then binding of human serum IgG (SV7) was evaluated by ELISA. (C,D) 96-well Costar plates were coated with optimal Neu5Gc^{pos}-GPs 0.25 μg protein/well (C) or 150 pmol Sia/well (D) from four different GPs batches, then reproducibility of recognition by human serum IgG was evaluated by ELISA with several samples (serum diluted 1/100). Batch-to-batch variability was minimal when plates were coated according to Sia content (representative of two independent experiments; mean ± SD of triplicates).

serum IgG binding to Neu5Gc^{pos}-GPs coated onto 96-well plates either according to their protein content (determined by BCA assay) or according to their Sia content (determined by DMB-HPLC). Initially, to determine optimal coating concentration for maximal detection of serum binding, GPs were coated at serial dilutions (Figure 4). There was similar serum binding against GPs coated at 0.25−2 μg protein/well (Figure 4A); however, when coated at 75−600 pmol Sia/well, maximal binding occurred at 150 or 300 pmol Sia/well (Figure 4B). Subsequently, we examined serum binding to four different batches of GPs preparations coated with the selected optimal concentrations to yield maximal signal at the lowest possible concentration (either 0.25 μg protein/well or 150 pmol Sia/well). Analysis of five different sera samples showed that when GPs were coated according to protein content there was variable serum IgG binding to different GPs batches (Figure 4C). However, when GPs were coated according to Sia content there was reproducible serum binding, across all five human sera samples against different batches of GPs preparations (Figure 4D). Thus, running ELISA assay with sialylated GPs-target coating according to Sia concentration resulted in a reproducible human serum IgG binding.

Subsequently, four human serum samples with expected high, medium, or low anti-Neu5Gc IgG reactivity were examined by ELISA against Neu5Gc^{pos}-GPs coated targets, with or without preabsorption with serially diluted Neu5Gc^{pos}-GPs (0.25−0.03 mM Sia). All human serum samples showed maximal inhibition already at the lowest concentration of 0.03 mM GPs (Figure 5A). Of note, maximal inhibition readout did not reach absolute zero despite the fact that the coated target and inhibitor were identical, suggesting that antibody recognition of GPs against surface-coated targets is slightly higher than free GPs. For specific detection of serum anti-Neu5Gc IgGs using GPs, 28 human serum samples were examined against Neu5Gc^{pos}-GPs coated plates with or without preabsorption with *Cmah*-KO sialo-glycoproteins (of mouse serum selected to lack mouse-anti-human IgG reactivity[29]), or with 0.03/0.06 mM Neu5Gc^{pos}-GPs (Figure 5B). Preabsorption with *Cmah*-KO sialo-glycoproteins aimed to remove any human-anti-mouse reactivity; however, it did not inhibit human serum IgG binding (Figure 5B), suggesting that the digestion process was efficient at removing such potential background reactivity. Furthermore, in all examined human sera, free Neu5Gc^{pos}-GPs (either 0.03 or 0.06 mM) significantly inhibited reactivity, but did not abolish it completely (Figure 5A,B). To estimate specific human anti-Neu5Gc IgGs reactivity, the binding signal of human sera IgGs against Neu5Gc^{pos}-GP (GP assay) was used as the maximal signal, from which the minimal signal after inhibition with 0.03 mM Neu5Gc^{pos}-GPs was deducted (GP ELISA Inhibition Assay; GP-EIA; Figure 5C). While the absolute anti-Neu5Gc IgG value was slightly lower in the GP-EIA, there was a very strong correlation between GP versus GP-EIA signals (R^2 = 0.977; Figure 5D). Hence, using Neu5Gc^{pos}-GPs as targets in ELISA (GP assay) seemed to provide a good estimate for anti-Neu5Gc IgGs reactivity in human serum, and it was similar to the specific reactivity calculated after deducting the inhibition with free GPs (GP-EIA).

Comparing Different Methods for Detection of Human Serum anti-Neu5Gc IgGs. Mouse sera sialo-glycoproteins or their resulting digested sialo-glycopeptides are expected to contain identical glycans. Nevertheless, there was a clear difference between the recognition of serum IgG against whole (WT) versus digested glycoproteins (Neu5Gc^{pos}-GPs) in 39 human sera samples (Figure 6A,B). This could result from background human anti-mouse reactivity against mouse glycoproteins in the examined human sera samples. However, comparison of the more specific assays for detection of human anti-Neu5Gc IgGs revealed a similar lack of correlation between either EIA (plates coated with WT glycoproteins and preabsorption of human serum against *Cmah*-KO[29]) versus GP-EIA (plates coated with Neu5Gc^{pos}-GPs with preabsorption of human serum against Neu5Gc^{pos}-GPs) (Figure 6C,D). Thus, despite the fact that identical glycan structures are presented in both assays, it seems that different populations of anti-Neu5Gc IgGs in human sera are detected by the different assays. The major difference between the coating Neu5Gc-glycoproteins versus the digested Neu5Gc^{pos}-GPs is in their mode of presentation, where the

DOI: 10.1021/acs.bioconjchem.8b00817
Bioconjugate Chem. 2019, 30, 161−168

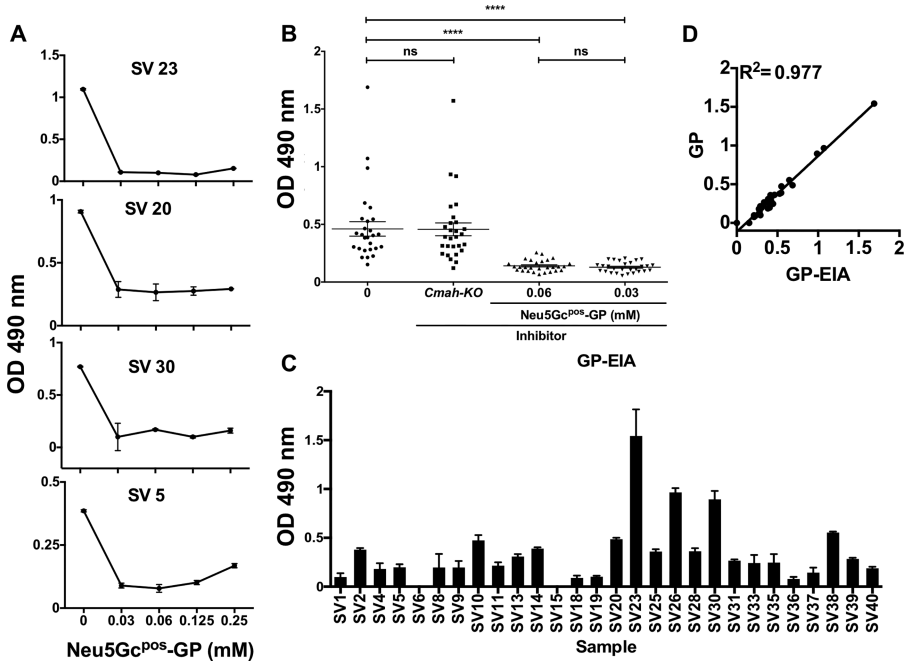


Figure 5. Evaluating specific detection of human serum anti-Neu5Gc IgGs by ELISA against Neu5Gc^{pos}-GPs. (A) 96-well Costar plates were coated with Neu5Gc^{pos}-GPs, then binding of human serum IgG (diluted 1/100) was evaluated by ELISA, either as is (0), or after preincubation with competing Neu5Gc^{pos}-GPs (0.03−0.25 mM Sia). This demonstrated specific anti-Neu5Gc IgG reactivity in all samples, and signal was maximally inhibited already at 0.03 mM Sia. (B) 28 human serum samples were examined (at 1/100 dilution) by ELISA against coated Neu5Gc^{pos}-GPs, either as is (0) or after preincubation with *Cmah*-KO mouse sera (1/4000) or Neu5Gc^{pos}-GPs (0.03 mM and 0.06 mM). The *Cmah*-KO sera did not absorb any human serum reactivity, while Neu5Gc^{pos}-GPs at both concentrations inhibited most reactivity (One-Way ANOVA). (C) Anti-Neu5Gc IgGs specific reactivity was calculated by deducting the signal obtained after preincubation with 0.03 mM Neu5Gc^{pos}-GPs from the signal with no inhibition (glycopeptides ELISA inhibition assay; GP-EIA). (D) Comparing the human serum reactivity against Neu5Gc^{pos}-GPs as is (GP) versus the GP-EIA signal showed strong correlation (Pearson r = 0.977). (Representative of at least two independent experiments for A-D; mean ± SD of triplicates.)

glycans are found much closer and densely packed in the digested glycopeptides rather than on the native glycoproteins.

Another method for detection of anti-Neu5Gc IgGs in human sera is by a sialoglycan microarray. In this method, a collection of individual Neu5Gc-glycans with a terminal primary amine are each covalently conjugated onto epoxide-coated slides allowing a more detailed analysis of the different pools of anti-Neu5Gc IgGs in human serum.[32,34] Eleven human serum samples were examined on a sialoglycan microarray revealing the diversity in anti-Neu5Gc IgGs in different donors ranging from high-medium-low reactivity, against different Neu5Gc-glycans (Figure 7A). While there was a lack of correlation between EIA and GP-EIA (Figure 7B, Figure 6D), there was a strong correlation between the average anti-Neu5Gc IgGs response on the array in each human serum sample to the response revealed by the EIA (Figure 7B,D), and lack of correlation between the array and GP-EIA (Figure 7C). In this array format, di-, tri-, or tetra-saccharides are presented in each spot in a highly dense manner (100 μM each glycan per ∼70 nm spot),[32] yet each spot is dedicated to a specific glycan. This is in contrast to the densely packed Neu5Gc^{pos}-GPs that contain a heterogeneous mixture of glycans. Hence the GP-EIA assay is likely detecting only a fraction of the serum anti-Neu5Gc IgGs. Other studies also suggest that the three-dimensional organization of glycans and their spatial organization could affect antibody recognition.[34−36] Antibody binding could be enhanced or rather reduced by different presentation modes of glycan epitopes,[7,34,37,38] and "clustered saccharide patches" of multiple glycans in close proximity have important biological roles.[35,36]

CONCLUSIONS

Our findings highlight the importance of glycans' density and composition (homogeneous/heterogeneous) in defining anti-carbohydrate antibody recognition. Here we used the diverse and polyclonal anti-Neu5Gc IgGs response in human sera to demonstrate how the presentation mode of glycans can dramatically affect anti-carbohydrate antibody recognition. In this case, the GP-EIA assay could only detect a fraction of the circulating anti-Neu5Gc IgGs. In contrast, the array provided a more holistic view of the response and, when averaged, seemed comparable with the simpler EIA assay that involves a mixture of Neu5Gc glycoproteins. Such insights could be important for understanding the biology and implications of this response in humans, given the importance of anti-Neu5Gc antibodies in cancer, xenotransplantation, autoimmunity and biotherapeutics.[3,22−25,39,40]

MATERIALS AND METHODS

Human Sera Samples. Human sera were obtained from the Israeli Blood Bank and used in accordance with the Helsinki Declaration and Tel Aviv University Institutional Review Board.

Mice. Wild-type (WT) and *Cmah*$^{-/-}$ (*Cmah*-KO) mice[41] were bred in a congenic C57BL/6 background and maintained according to the Tel Aviv University Institutional Animal Care and Use Committee guidelines for laboratory animals.

DOI: 10.1021/acs.bioconjchem.8b00817
Bioconjugate Chem. 2019, 30, 161−168

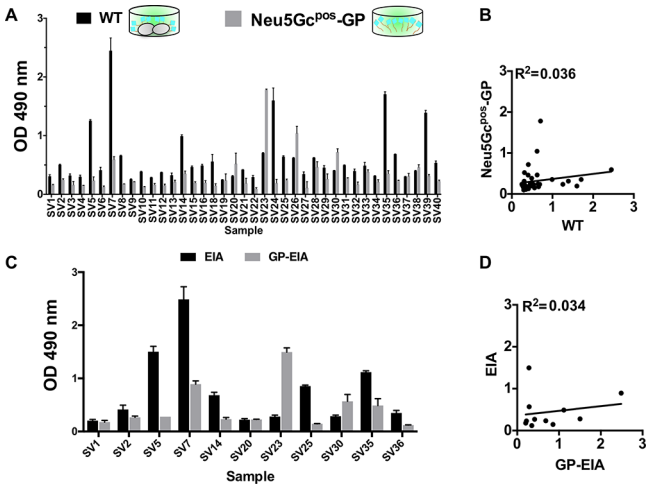


Figure 6. Human sera anti-Neu5Gc IgGs detection against mouse sera sialo-glycoproteins versus sialo-glycopeptides. (A) 96-well Costar plates were coated either with Neu5Gc^{pos}-GPs (150 pmol/well) or undigested WT mouse serum (1 μg/well; selected to be negative for mouse-anti-human IgG reactivity), then binding of 39 human sera samples was evaluated by ELISA, detected by HRP-anti-human IgG (Representative of two independent experiments; mean ± SD of duplicates). (B) Comparing human sera IgG reactivity against Neu5Gc^{pos}-GP or undigested WT mouse serum shows no correlation (Pearson r = 0.036). (C) Anti-Neu5Gc IgG reactivity in 11 human sera samples (expected to have high/medium/low reactivity) were evaluated either by EIA or GP-EIA. In EIA assay,[29] undigested WT mouse serum (1 μg/well; selected to be negative for mouse-anti-human IgG reactivity) was coated to 96-well plates, then examined with human serum samples (diluted 1/100) that were preincubated with *Cmah*-KO mouse sera (1/4000; to absorb human anti-mouse reactivity). In GP-EIA assay, the plates were coated with Neu5Gc^{pos}-GP, and human sera preincubated with Neu5Gc^{pos}-GP (0.03 mM). Representative of at least two independent experiments; mean ± SD of duplicates). (D) Comparing specific anti-Neu5Gc IgG signal obtained with EIA versus GP-EIA showed lack of correlation (Pearson r = 0.034).

Antibodies and Lectins. Affinity-purified polyclonal chicken anti-Neu5Gc IgY (Biolegend), horseradish peroxidase (HRP)−streptavidin, HRP−goat-anti-human IgG, HRP−AffiniPure donkey-anti-chicken IgY (IgG) (H+L), HRP-Streptavidin, and Cy3−goat-anti-human IgG (H+L) (Jackson ImmunoResearch), HRP−goat-anti-human IgG (Bio-Rad), biotinylated SNA (*Sambucus nigra*), and biotinylated MAL-II (*Maackia amurensis* lectin II) (Vector Laboratories).

Generating Glycopeptides by Digestion of Mouse Sera. WT or *Cmah*-KO mouse serum (80 mg) was diluted in 8 mL of 0.1 M Tris-HCl pH 8.0 with 10 mM $CaCl_2$, and then filter sterilized. Pronase solution (Calbiochem; 10 mg/mL in distilled water) was incubated at 60 °C for 30 min to inactivate any contaminating sialidase activity, then filter sterilized. To initiate serum digestion, 500 μL of sterile Pronase solution was added to 9 mL sterile serum solution and the mixture was incubated at 37 °C for 5 days, with daily additions of 250 μL sterile Pronase. To examine progress of digestion, 40 μL aliquots were collected prior to addition of Pronase (Day 0) and every 24 h. After 5 days, Pronase digest was separated on 3 kDa Amicon filters, at 4700 rpm for 1 h at 4 °C. The glycopeptides containing upper solution (GP) was stored at 4 °C until used.

Silver Staining. Samples of mouse serum digest (25 μL/day) were dissolved in 20% sample buffer (0.35 M Tris pH 6.8, 10.4% SDS, 30% glycerol, 0.6 M DTT, 0.0012% bromophenol blue), then boiled at 100 °C for 5 min. Samples (15 μL/lane) were separated on 12.5% SDS-PAGE, then gel transferred to freshly prepared fixation buffer (10% acetic acid, 40% ethanol, 0.0185% formaldehyde, and 50% water) and incubated for 20 min. The gel was washed with 50% ethanol 3 times for 10 min each, then incubated in 0.02% $Na_2S_2O_3$ in water for 1 min. The gel was washed 3 times with water for 20 s each, then incubated in silver solution (9.4 mM $AgNO_3$, 0.02% Formaldehyde in water) for 12 min. The gel was washed 3 times with water 20 s each, then incubated in fixation buffer 2 (0.0005% $Na_2S_2O_3$, 0.015% Formaldehyde, 0.47 M Na_2CO_3 in water). When staining had developed, gel was transferred to a new container with stopping solution (10% acetic acid, 40% ethanol and 50% water).

DMB-HPLC Sia Analysis. To quantitate Sia, these were released from glycoconjugates by acid hydrolysis using 0.1 N NH_2SO_4 for 1.5 h at 80 °C, then neutralized with 0.1 N of NaOH. Released Sias were then derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB; Sigma) for 2.5 h at 50 °C, separated by Microcon-10 centrifugal filters and analyzed by fluorescence detection by reverse-phase high-pressure liquid chromatography (DMB-HPLC) (Hitachi HPLC, Chromaster), on C18 column (Phenomenex C18 Gemini 250 × 4.6 mm) at 24 °C in running buffer (84.5% ddH_2O, 8.5% acetonitrile, 7% methanol (Merck) for 60 min (min) at a flow rate of 0.9 mL/min. Quantification of Sia was carried out by comparison with known quantities of DMB-derivatized Neu5Ac.[42]

Enzyme-Linked Immunosorbent Assay (ELISA). We tested binding of human serum and lectins to mouse serum glycoconjugates and GPs by ELISA. Costar 96-well were coated overnight at 4 °C with 1 μg/well of WT pooled mouse sera (lacking mouse-anti-human IgG) or 150 pmol/well GP in coating buffer (50 mM sodium carbonate-bicarbonate buffer, pH 9.5). Wells were blocked for 2 h at RT with blocking buffer (PBS pH 7.3, 1% chicken ovalbumin (Sigma); PBS/OVA). After removal of buffer, human sera (diluted 1:100 in PBS/OVA) or lectins (0.1 mg/mL Bio-SNA, 4 mg/mL Bio-MAL-1, 4 mg/mL Bio-MAL-2) were added to triplicate wells at 100 μL/well then incubated at RT for 2 h. Wells were washed three times with PBST (PBS pH 7.3, 0.1% Tween-20), detection antibody was then added (100 μL/well, 1:7000 HRP-goat-anti-human IgG or 1:10 000 HRP-streptavidin (0.1 mg/mL) diluted in PBS) and incubated for 1 h at RT. After washing three times with PBST, wells were developed with 0.5 mg/mL *O*-phenylenediamine in citrate-PO_4 buffer, pH 5.5, reaction was stopped with H_2SO_4 and absorbance was measured at a 490 nm wavelength on a SpectraMax M3 (Molecular Devices).

Mild Periodate Treatment to Determine Sia Specificity. Binding of human sera, lectins, or antibodies to tissue homogenates was tested by ELISA as described.[21] Costar 96-well plates were coated overnight at 4 °C with mouse sera at saturating concentration (1 μg/well) of WT/*Cmah*-KO pooled mouse sera or (150 pmol/well) GP. Next, wells were decanted and freshly prepared periodate solution (cold 2 mM sodium metaperiodate in PBS pH 6.5, 200 μL/well) was added followed by gentle shaking for 20 min at 4 °C in the dark. The reaction was stopped by addition of 50 μL/well of freshly prepared 100 mM sodium borohydride in PBS pH 6.5 (final concentration of 20 mM) followed by 10 min incubation at RT with gentle shaking (the borohydride inactivates the periodate). Concurrently, as a mock control, periodate and

DOI: 10.1021/acs.bioconjchem.8b00817
Bioconjugate Chem. 2019, 30, 161−168

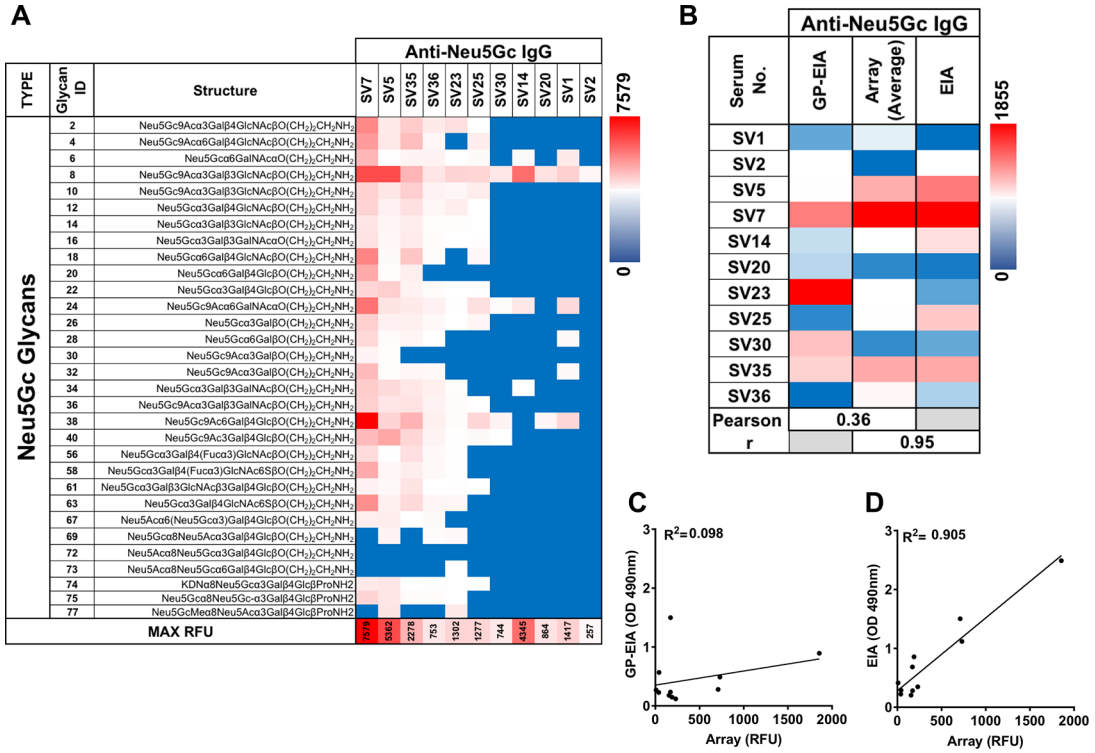


Figure 7. Comparison of human serum anti-Neu5Gc IgG reactivity analyzed by sialoglycan microarray versus EIA and GP-EIA. (A) Sialoglycan microarray analysis of sera from healthy donors (diluted 1/100). Relative fluorescent units (RFU) are presented as a heatmap. (B–D) Correlation analysis of anti-Neu5Gc IgG reactivity obtained by GP-EIA and EIA versus sialoglycan microarray (average of all Neu5Gc-glycans per serum sample). (C) No correlation between anti-Neu5Gc IgG antibody reactivity between GP-EIA and sialoglycan microarray (Pearson r = 0.098). (D) Strong correlation observed between anti-Neu5Gc IgG antibody levels between EIA and sialoglycan microarray (Pearson r = 0.905).

borohydride solutions were premixed at the same ratio (4:1) and wells incubated (250 μL/well) side-by-side with the periodate treated wells. To remove resulting borates, wells were washed three times (10 min each wash) with cold wash buffer (50 mM sodium acetate pH 5.5, 100 mM NaCl), then three times with PBS pH 7.3. Subsequently, wells were further processed as described (ELISA assay).

ELISA Inhibition Assay (EIA). Anti-Neu5Gc IgGs were evaluated by an ELISA using mouse serum glycoproteins as coating targets as described.[29] Briefly, Costar 96-well were coated overnight at 4 °C with 1 μg/well WT pooled mouse sera (lacking mouse-anti-human IgG) in coating buffer (50 mM sodium carbonate-bicarbonate buffer, pH 9.5). Wells were blocked for 2 h at RT with PBS/OVA (PBS pH 7.3, 1% chicken ovalbumin). During the blocking, human serum was diluted 1:100 in EIA buffer (PBS pH 7.3, 1% chicken ovalbumin and *Cmah*-KO pooled sera that lacks mouse-anti-human reactivity, diluted at 1:4000, thus containing 2.3 μg protein/100 μL and 57 pmol Sia/100 μL) and incubated on ice for 2 h. Next, PBS/OVA (PBS pH 7.3, 1% chicken ovalbumin). During the blocking, human serum was diluted 1:100 in EIA buffer (PBS pH 7.3, 1% chicken ovalbumin and *Cmah*-KO pooled sera that lacks mouse-anti-human reactivity, diluted at 1:4000, thus containing 2.3 μg protein/100 μL and 57 pmol Sia/100 μL) and incubated on ice for 2 h. Next, PBS/OVA was removed from wells and preincubated human serum was added to triplicate wells at 100 μL/well, then incubated at RT for 2 h. Wells were washed three times with PBST (PBS pH 7.3, 0.1% Tween-20), detection antibody was then added (100 μL/well, 1:7000 HRP-goat-anti-human IgG diluted in PBS) and incubated for 1 h at RT. After washing three times with PBST, wells were developed with 0.5 mg/mL *O*-phenylenediamine in citrate-PO_4 buffer, pH 5.5, reaction was stopped with H_2SO_4, and absorbance was measured at a 490 nm wavelength on a SpectraMax M3 (Molecular Devices).

Glycopeptides ELISA Inhibition Assay (GP-EIA). We tested human serum binding specifically to $Neu5Gc^{pos}$-GPs by GP-EIA. Costar 96-well were coated overnight at 4 °C with saturating concentration (150 pmol Sia/well). $Neu5Gc^{pos}$-GPs in coating buffer (50 mM sodium carbonate-bicarbonate buffer, pH 9.5). Wells were blocked for 2 h at RT with PBS/OVA (PBS pH 7.3, 1% chicken ovalbumin). During the blocking, human serum was diluted 1:100 in GP-EIA buffer (PBS pH 7.3, 1% chicken ovalbumin and serial dilution of $Neu5Gc^{pos}$-GPs) and incubated on ice for 2 h. Next, PBS/OVA was removed from wells and pretreated human serum was added to triplicate wells at 100 μL/well then incubated at RT for 2 h. Subsequently, wells were further processed as described (ELISA/EIA assay).

Sialoglycan Microarray Fabrication. Arrays were fabricated with NanoPrint LM-60 Microarray Printer (Arrayit) on epoxide-derivatized slides (Corning) with 16 subarray blocks on each slide. Glycoconjugates were distributed into 384-well source plates using 4 replicate wells per sample and 8 μL per well (Version 2). Each glycoconjugate was prepared at 50 μM in an optimized print buffer (300 mM phosphate buffer pH 8.4, supplemented with 0.005% Tween-20). To monitor printing quality AlexaFluor-555-Hydraside (Invitrogen, at 2 ng/μL in 178 mM phosphate buffer, pH 5.5) was used for each printing run. The arrays were printed with four SMP3 pins (5 μm tip, 0.25 μL sample channel, ~100 μm spot diameter; Arrayit), with spot to spot spacing of 275 μm. The humidity

DOI: 10.1021/acs.bioconjchem.8b00817
Bioconjugate Chem. 2019, 30, 161–168

level in the arraying chamber was maintained at about 70% during printing. Printed slides were left on arrayer deck overnight, allowing humidity to drop to ambient levels (40–45%). Next, slides were packed, vacuum-sealed, and stored at room temperature (RT) until used.

Sialoglycan Microarray Binding Assay. Slides were developed and analyzed as previously described,[32] with some modifications. Slides were rehydrated with ddH_2O and incubated for 30 min in a staining dish with 50 °C prewarmed ethanolamine (0.05 M) in Tris-HCl (0.1 M, pH 9.0) to block the remaining reactive epoxy groups on the slide surface, then washed with 50 °C prewarmed ddH_2O. Slides were centrifuged at 200 × g for three min then fitted with ProPlate Multi-Array 16-well slide module (Invitrogen) to divide into the subarrays (blocks). Slides were washed with PBST (0.1% Tween 20), aspirated, and blocked with 200 μL/subarray of blocking buffer (PBS/OVA, 1% w/v ovalbumin, in PBS, pH 7.3) for 1 h at RT with gentle shaking. Next, the blocking solution was aspirated and 100 μL/block of 1:100 diluted sera diluted in PBS/OVA were incubated with gentle shaking for 2 h at RT. Slides were washed three times with PBST, then with PBS for 2 min. Bound antibodies were detected by incubating with secondary detection diluted in PBS, 200 μL/block at RT for 1 h, Cy3-anti-human IgG 1.2 μg/mL (Jackson Immunoresearch). Slides were washed three times with PBST, then with PBS for 10 min, followed by removal from ProPlate Multi-Array slide module and immediately dipping in a staining dish with ddH_2O for 10 min with shaking, then centrifuged at 200 × g for 3 min, and scanned immediately.

Array Slide Processing. Processed slides were scanned and analyzed as described at 10 μm resolution with a Genepix 4000B microarray scanner (Molecular Devices) at 350 gain. Image analysis was carried out with Genepix Pro 6.0 analysis software (Molecular Devices). Spots were defined as circular features with a variable radius using with local background subtraction.

Statistical Analysis. Statistical analysis conducted with Prism 6 with the specific methods as indicated in the figure legends.

AUTHOR INFORMATION

Corresponding Author

*E-mail: vkaravani@tauex.tau.ac.il. Tel: +972-3-640-6737. Fax: +972-3-642-2046.

ORCID

Hai Yu: 0000-0002-4378-0532

Xi Chen: 0000-0002-3160-614X

Vered Padler-Karavani: 0000-0002-4761-3571

Author Contributions

V.P.-K. designed the experiments, S.B. conducted the research with assistance of S.L.B.-A. and E.M.R. H.Y. and X.C. provided critical reagents. V.P.-K. and S.B. wrote the manuscript, and all authors read and approved the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the European Union Seventh Framework Program (FP7/2007/2013) under the Grant agreement 603049 for Translink consortium (http://www.translinkproject.com/) (to V.P.-K, X.C., C.C., M.G., T.B.). This work was also partially supported by the European Union H2020 Program grants (ERC-2016-STG-716220) (to V.P.-K.).

REFERENCES

(1) Varki, A. (2017) Biological roles of glycans. *Glycobiology 27*, 3–49.

(2) Varki, A., and Gagneux, P. (2015) in *Essentials of Glycobiology* (Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G., Kinoshita, T., Packer, N. H., and Prestegard, J. H., Eds.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

(3) Padler-Karavani, V. (2014) Aiming at the sweet side of cancer: Aberrant glycosylation as possible target for personalized-medicine. *Cancer Lett. 352*, 102–112.

(4) Sterner, E., Flanagan, N., and Gildersleeve, J. C. (2016) Perspectives on Anti-Glycan Antibodies Gleaned from Development of a Community Resource Database. *ACS Chem. Biol. 11*, 1773–1783.

(5) Fuster, M. M., and Esko, J. D. (2005) The sweet and sour of cancer: glycans as novel therapeutic targets. *Nat. Rev. Cancer 5*, 526–542.

(6) Haji-Ghassemi, O., Blackler, R. J., Martin Young, N., and Evans, S. V. (2015) Antibody recognition of carbohydrate epitopes†. *Glycobiology 25*, 920–952.

(7) Manimala, J. C., Roach, T. A., Li, Z., and Gildersleeve, J. C. (2007) High-throughput carbohydrate microarray profiling of 27 antibodies demonstrates widespread specificity problems. *Glycobiology 17*, 17C–23C.

(8) Kiessling, L. L., and Grim, J. C. (2013) Glycopolymer probes of signal transduction. *Chem. Soc. Rev. 42*, 4476–4491.

(9) Oyelaran, O., Li, Q., Farnsworth, D., and Gildersleeve, J. C. (2009) Microarrays with varying carbohydrate density reveal distinct subpopulations of serum antibodies. *J. Proteome Res. 8*, 3529–3538.

(10) Lundquist, J. J., and Toone, E. J. (2002) The cluster glycoside effect. *Chem. Rev. 102*, 555–578.

(11) Wang, L., Cummings, R. D., Smith, D. F., Huflejt, M., Campbell, C. T., Gildersleeve, J. C., Gerlach, J. Q., Kilcoyne, M., Joshi, L., Serna, S., et al. (2014) Cross-platform comparison of glycan microarray formats. *Glycobiology 24*, 507–517.

(12) Rho, J. H., Mead, J. R., Wright, W. S., Brenner, D. E., Stave, J. W., Gildersleeve, J. C., and Lampe, P. D. (2014) Discovery of sialyl Lewis A and Lewis X modified protein cancer biomarkers using high density antibody arrays. *J. Proteomics 96*, 291–299.

(13) Godula, K., and Bertozzi, C. R. (2012) Density variant glycan microarray for evaluating cross-linking of mucin-like glycoconjugates by lectins. *J. Am. Chem. Soc. 134*, 15732–15742.

(14) Artigas, G., Hinou, H., Garcia-Martin, F., Gabius, H. J., and Nishimura, S. I. (2017) Synthetic Mucin-Like Glycopeptides as Versatile Tools to Measure Effects of Glycan Structure/Density/Position on the Interaction with Adhesion/Growth-Regulatory Galectins in Arrays. *Chem. - Asian J. 12*, 159–167.

(15) Mammen, M., Choi, S. K., and Whitesides, G. M. (1998) Polyvalent Interactions in Biological Systems: Implications for Design and Use of Multivalent Ligands and Inhibitors. *Angew. Chem., Int. Ed. 37*, 2754–2794.

(16) Gildersleeve, J. C., and Wright, W. S. (2016) Diverse molecular recognition properties of blood group A binding monoclonal antibodies. *Glycobiology 26*, 443–448.

(17) Branderhorst, H. M., Ruijtenbeek, R., Liskamp, R. M., and Pieters, R. J. (2008) Multivalent carbohydrate recognition on a glycodendrimer-functionalized flow-through chip. *ChemBioChem 9*, 1836–1844.

(18) Muthana, S. M., and Gildersleeve, J. C. (2016) Factors Affecting Anti-Glycan IgG and IgM Repertoires in Human Serum. *Sci. Rep. 6*, 19509.

(19) Schneider, C., Smith, D. F., Cummings, R. D., Boligan, K. F., Hamilton, R. G., Bochner, B. S., Miescher, S., Simon, H. U., Pashov, A., Vassilev, T., et al. (2015) The human IgG anti-carbohydrate repertoire exhibits a universal architecture and contains specificity for microbial attachment sites. *Sci. Transl. Med. 7*, 269ra1.

DOI: 10.1021/acs.bioconjchem.8b00817
Bioconjugate Chem. 2019, 30, 161–168

(20) Galili, U. (2013) Discovery of the natural anti-Gal antibody and its past and future relevance to medicine. *Xenotransplantation*, n/a.
(21) Padler-Karavani, V., Yu, H., Cao, H., Chokhawala, H., Karp, F., Varki, N., Chen, X., and Varki, A. (2008) Diversity in specificity, abundance, and composition of anti-Neu5Gc antibodies in normal humans: potential implications for disease. *Glycobiology 18*, 818−830.
(22) Okerblom, J., and Varki, A. (2017) Biochemical, Cellular, Physiological, and Pathological Consequences of Human Loss of N-Glycolylneuraminic Acid. *ChemBioChem 18*, 1155−1171.
(23) Alisson-Silva, F., Kawanishi, K., and Varki, A. (2016) Human risk of diseases associated with red meat intake: Analysis of current theories and proposed role for metabolic incorporation of a non-human sialic acid. *Mol. Aspects Med. 51*, 16−30.
(24) Amon, R., Reuven, E. M., Leviatan Ben-Arye, S., and Padler-Karavani, V. (2014) Glycans in immune recognition and response. *Carbohydr. Res. 389*, 115−122.
(25) Salama, A., Evanno, G., Harb, J., and Soulillou, J. P. (2015) Potential deleterious role of anti-Neu5Gc antibodies in xenotransplantation. *Xenotransplantation 22*, 85−94.
(26) Pearce, O. M., Laubli, H., Verhagen, A., Secrest, P., Zhang, J., Varki, N. M., Crocker, P. R., Bui, J. D., and Varki, A. (2014) Inverse hormesis of cancer growth mediated by narrow ranges of tumor-directed antibodies. *Proc. Natl. Acad. Sci. U. S. A. 111*, 5998−6003.
(27) Padler-Karavani, V., Hurtado-Ziola, N., Pu, M., Yu, H., Huang, S., Muthana, S., Chokhawala, H. A., Cao, H., Secrest, P., Friedmann-Morvinski, D., et al. (2011) Human xeno-autoantibodies against a non-human sialic acid serve as novel serum biomarkers and immunotherapeutics in cancer. *Cancer Res. 71*, 3352−3363.
(28) Samraj, A. N., Pearce, O. M., Läubli, H., Crittenden, A. N., Bergfeld, A. K., Banda, K., Gregg, C. J., Bingman, A. E., Secrest, P., Diaz, S. L., et al. (2015) A red meat-derived glycan promotes inflammation and cancer progression. *Proc. Natl. Acad. Sci. U. S. A. 112*, 542−547.
(29) Padler-Karavani, V., Tremoulet, A. H., Yu, H., Chen, X., Burns, J. C., and Varki, A. (2013) A simple method for assessment of human anti-Neu5Gc antibodies applied to Kawasaki disease. *PLoS One 8*, e58443.
(30) Samraj, A. N., Bertrand, K. A., Luben, R., Khedri, Z., Yu, H., Nguyen, D., Gregg, C. J., Diaz, S. L., Sawyer, S., Chen, X., et al. (2018) Polyclonal human antibodies against glycans bearing red meat-derived non-human sialic acid N-glycolylneuraminic acid are stable, reproducible, complex and vary between individuals: Total antibody levels are associated with colorectal cancer risk. *PLoS One 13*, e0197464.
(31) Nguyen, D. H., Tangvoranuntakul, P., and Varki, A. (2005) Effects of natural human antibodies against a nonhuman sialic acid that metabolically incorporates into activated and malignant immune cells. *J. Immunol. 175*, 228−236.
(32) Leviatan Ben-Arye, S., Yu, H., Chen, X., and Padler-Karavani, V. (2017) Profiling Anti-Neu5Gc IgG in Human Sera with a Sialoglycan Microarray Assay. *J. Visualized Exp. 125*, 1 DOI: 10.3791/56094.
(33) Solís, D., and Díaz-Mauriño, T. (1992) Does fibrinogen contain populations with different degree of sialylation. *Thromb. Res. 67*, 631−641.
(34) Padler-Karavani, V., Song, X., Yu, H., Hurtado-Ziola, N., Huang, S., Muthana, S., Chokhawala, H. A., Cheng, J., Verhagen, A., Langereis, M. A., et al. (2012) Cross-comparison of protein recognition of sialic acid diversity on two novel sialoglycan microarrays. *J. Biol. Chem. 287*, 22593−22608.
(35) Cohen, M., and Varki, A. (2014) Modulation of glycan recognition by clustered saccharide patches. *Int. Rev. Cell Mol. Biol. 308*, 75−125.
(36) Cohen, M., and Varki, A. (2010) The sialome-far more than the sum of its parts. *OMICS 14*, 455−464.
(37) Grant, O. C., Smith, H. M., Firsova, D., Fadda, E., and Woods, R. J. (2014) Presentation, Presentation, Presentation! Molecular Level Insight into Linker Effects on Glycan Array Screening Data. *Glycobiology 24*, 17−25.
(38) Liang, C. H., Wang, S. K., Lin, C. W., Wang, C. C., Wong, C. H., and Wu, C. Y. (2011) Effects of neighboring glycans on antibody-carbohydrate interaction. *Angew. Chem., Int. Ed. 50*, 1608−1612.
(39) Reuven, E. M., Leviatan Ben-Arye, S., Marshanski, T., Breimer, M. E., Yu, H., Fellah-Hebia, I., Roussel, J. C., Costa, C., Galiñanes, M., Mañez, R., et al. (2016) Characterization of immunogenic Neu5Gc in bioprosthetic heart valves. *Xenotransplantation 23*, 381−392.
(40) Ekser, B., Li, P., and Cooper, D. K. C. (2017) Xenotransplantation: past, present, and future. *Curr. Opin. Organ Transplant. 22*, 513−521.
(41) Hedlund, M., Tangvoranuntakul, P., Takematsu, H., Long, J. M., Housley, G. D., Kozutsumi, Y., Suzuki, A., Wynshaw-Boris, A., Ryan, A. F., Gallo, R. L., et al. (2007) N-glycolylneuraminic acid deficiency in mice: implications for human biology and evolution. *Mol. Cell. Biol. 27*, 4340−4346.
(42) Hara, S., Yamaguchi, M., Takemori, Y., Furuhata, K., Ogura, H., and Nakamura, M. (1989) Determination of mono-O-acetylated N-acetylneuraminic acids in human and rat sera by fluorometric high-performance liquid chromatography. *Anal. Biochem. 179*, 162−166.

DOI: 10.1021/acs.bioconjchem.8b00817
Bioconjugate Chem. 2019, 30, 161−168