# The Sialome of the Retina, Alteration in Age-Related Macular Degeneration Pathology, and Potential Impacts on Complement Factor H

Jaclyn Swan,[1] Christopher B. Toomey,[2,3] Max Bergstrand,[1] Hector A. Cuello,[3,4] Jesse Robie,[1] Hai Yu,[5] Yue Yuan,[5] Anoopjit Singh Kooner,[5] Xi Chen,[5] Jutamas Shaughnessy,[6] Sanjay Ram,[6] Ajit Varki,[3,4,7–9] and Pascal Gagneux[1,8,9]

[1]Department of Pathology, University of California – San Diego, La Jolla, California, United States
[2]Shiley Eye Institute, Viterbi Family Department of Ophthalmology University of California – San Diego, La Jolla, California, United States
[3]Glycobiology Research and Training Center, University of California – San Diego, La Jolla, California, United States
[4]Department of Cellular & Molecular Medicine, University of California – San Diego, La Jolla, California, United States
[5]Department of Chemistry, University of California – Davis, Davis, California, United States
[6]Division of Infectious Diseases and Immunology, University of Massachusetts, Chan Medical School, Worcester, Massachusetts, United States
[7]Department of Medicine, University of California – San Diego, La Jolla, California, United States
[8]Department of Anthropology, University of California – San Diego, La Jolla, California, United States
[9]Center for Academic Research and Training in Anthropogeny (CARTA), La Jolla, California, United States

Correspondence: Jaclyn Swan, Department of Pathology, University of California – San Diego, 9500 Gilman Dr, La Jolla, CA 92093, USA; jaswan@ucsd.edu, j.swan@latrobe.edu.au.
Pascal Gagneux, Department of Pathology and Department of Anthropology, University of California – San Diego, 9500 Gilman Dr, La Jolla, CA 92093, USA; pgagneux@ucsd.edu.

**Received:** March 15, 2025
**Accepted:** May 21, 2025
**Published:** June 27, 2025

Citation: Swan J, Toomey CB, Bergstrand M, et al. The sialome of the retina, alteration in age-related macular degeneration pathology, and potential impacts on complement factor H. *Invest Ophthalmol Vis Sci.* 2025;66(6):81. https://doi.org/10.1167/iovs.66.6.81

**Purpose.** Little is known about sialic acids of the human retina, despite their integral role in self /non-self-discrimination by complement factor H (FH), the alternative complement pathway inhibitor.

**Methods.** A custom sialoglycan microarray was used to characterize the sialic acid-binding specificity of native FH or recombinant molecules where IgG Fc was fused to FH domains 16 to 20 (which contains a sialic acid-binding site), domains 6 and 7 (which contains a glycosaminoglycan-binding site), or the FH-related proteins (FHRs) 1 and 3. We analyzed macular and peripheral retinal tissue from postmortem ocular globes for the amount, type, and presentation (glycosidic linkage type) of sialic acid in individuals with age-related macular degeneration (AMD) and age-matched controls using fluorescent lectins and antibodies to detect sialic acid and endogenous FH. Released sialic acids from neural retina, retinal pigmented epithelium (RPE) cells, and the Bruch's membrane (BrM) were labeled with 1,2-diamino-4,5-methylenedioxybenzene-2HCl (DMB), separated and quantified by high-performance liquid chromatography (HPLC).

**Results.** Both native FH and the recombinant FH domains 16 to 20 recognized Neu5Ac and Neu5Gc that is $\alpha$2-3-linked to the underlying galactose. 4-*O*-Acylation of sialic acid and sulfation of GlcNAc did not inhibit binding. Different linkage types of sialic acid were localized at different layers of the retina. The greatest density of $\alpha$2-3-sialic acid, which is the preferred ligand of FH, did not colocalize with endogenous FH. The level of sialic acids at the BrM/choroid interface of the macula and peripheral retina of individuals with AMD were significantly reduced.

**Conclusions.** The sialome of the human retina is altered in AMD. This may affect FH binding and, consequently, alternative complement pathway regulation.

Keywords: sialic acid, glycans, complement factor H (FH)

Limited information exists regarding the complex carbohydrates (glycans) present in the human retina, despite their crucial role in immune system regulation and physiology.[1] Age-related macular degeneration (AMD) is the leading cause of blindness in individuals over the age of 60 years.[2] Dysregulation of the alternative complement system contributes to the pathology of this disease.[3,4] The glycocalyx forms the outer surface of the cells, provides one of the first points of contact for cell-to-cell communication, and defines molecular patterns on each cell. As these glycans are involved in self- and non-self-recognition, alteration of glycosylation has the potential to modulate interactions with immune regulatory proteins such as complement factor H (FH), which is known to interact with negatively charged cell surface glycans, including sialic acids[5] and select glycosaminoglycans,[6] and malondialdehyde epitopes

that result from oxidative stress.[7] Previous studies have explored changes in IgG glycosylation and serum N-glycans as potential biomarkers for AMD[8] but glycosylation changes within the retina have not been investigated. Understanding the normal and abnormal glycosylation patterns of the human retina with a focus on sialic acid will provide much needed insight into this multi-factorial disease.

Glycans are found conjugated to proteins, lipids, and even RNA.[9] Apart from providing a source of energy, this class of biomolecules also provides structural integrity, influences the confirmation and activity of proteins, and contributes to defining the molecular identity of cells and tissues. Glycans are attached to biomolecules through the enzymatically controlled glycosylation processes. In humans and other vertebrates, most glycans carry terminal sialic acid. The molecular pattern formed by sialic acids defines the sialome; a key component of the molecular frontier of each cell. Unlike proteins in which the sequence and structure can be predicted based on the genome, glycosylation is not template driven. Instead, glycosylation is the result of the combined action of several gene products, such as glycosyltransferases and glycosidases, and the availability of their substrates, activated monosaccharides (nucleotide sugars). For this reason, small environmental and biological changes can have dramatic effects on glycosylation. To add further complexity, glycans can be branched and different monosaccharides can be joined by different glycosidic linkage types in different positions, giving rise to immense molecular diversity, much of it with distinct biological characteristics. Glycans play essential roles in the cellular organization and development of multicellular organisms. For example, loss of sialic acid from the glycans of serum glycoproteins and red blood cells function as an indicator of aging and signals for their removals from the circulatory system. Similarly, it is hypothesized that overall tissue sialyation also changes with age.

Despite some glycan analyses of the cornea and tear fluid, less is known about the glycan composition of the retina. The corneal epithelium contains densely O-glycosylated glycoproteins, specifically mucin (MUC16).[10] The O-glycans on this mucin are heavily sialylated and hydrated, providing essential boundary lubrication and protection against potential pathogens. We essentially all see through a complex layer of glycoproteins. Alterations in the O-glycans of keratinized cells have been linked to inflammatory conditions such as superior limbic keratoconjunctivitis. Moreover, increased sulfation on O-glycans has been observed in the tear fluid of patients with ocular rosacea.[11,12] Existing retinal glycan studies have utilized lectin staining methods, using glycan-binding proteins (lectins) to reveal the presence of sialic acids on drusen, indicated by positive staining with the lectin *Limax flavus* agglutinin (LFA).[13] Drusen are the characteristic lesions observed in AMD, located between the Bruch's membrane (BrM) and retinal pigmented epithelium (RPE) cells. Drusen exhibit minimal staining for terminal galactose (revealed by peanut agglutinin [PNA]). Treatment with sialidase to remove sialic acid results in decreased LFA staining and increased staining with PNA, suggesting that drusen are rich in sialic acid, predominantly linked to $\beta$1-3 linked galactose.[13,14] Sialic acid can be linked to underlying galactose or *N*-acetylgalactosamine in $\alpha$2-3 and/or $\alpha$2-6 linkage, or attached to another sialic acid with an $\alpha$2-8 linkage, forming di-, oligo, or polysialic acids. LFA is believed to bind sialic acid regardless of its linkage to the underlying monosaccharide. Sialic acid is a key cell surface molecule in vertebrates where it provides most of the negative charge on cell surfaces. It is very important for biomedicine, as humans have evolved distinct sialic acid biology and sialic acids form one of the primary motifs used by our innate immune system to distinguish self from non-self/altered-self.[15]

Multiple immune-regulating lectins within the human innate immune system discern self from non-self by detecting sialic acid. In some cases, the specific linkage of sialic acid is crucial for this recognition process. For example, FH recognizes $\alpha$2-3 linked sialic acid.[16,17] FH is a large, secreted glycoprotein and a key regulator of the alternative complement cascade. Dysregulation of the complement system is believed to be an underlying mechanism associated with AMD, and the Y402H polymorphism in *CFH* accounts for one of the strongest risk factors demonstrating a potential role in the pathology.[18,19] FH is made up of 20 short census repeat (SCR) domains, each containing approximately 60 amino acids joined by short linkers. FH discriminates self from non-self via two separate glycan-binding domains. SCR6-8 is responsible for glycosaminoglycan (GAG) recognition,[20] whereas the SCR20 recognizes sialic acid with the specific linkage of $\alpha$2-3, as well as GAGs.[5,17] In addition to recognizing "self" glycan motifs, FH simultaneously inhibits the alternative pathway by accelerating the decay of C3 convertase (C3bBb) or acting as a co-factor for factor I cleavage of C3b to its hemolytically inactive form, iC3b. Recently, two inhibitors of the complement cascade have been shown to delay the progression of geographic atrophy in patients with advanced AMD.[21–23] However, the efficacy and side-effect profile limits there use in clinical practice. Alternatively, alteration of the sialome (the complete repertoire of sialic acid types and linkages from a particular cell, tissue, or organism) has the potential to suppress overactive immune responses, including complement activation. Thus, we sought to determine whether there is a change in sialylation of human retinal tissue in individuals with AMD.

Little is known about the retinal glycans in the normal aged eye and even less in degenerative conditions like AMD. Due to the non-regenerative nature of retinal cells and the plasticity of glycosylation, changes in glycan composition are possible correlates, or even causes, for immune dysregulation. Presentation of sialic acid in certain linkages on the termini of glycolipids, N-, and O-glycans contributes to the cells, and, in combination, the tissues' presentation of "self." Changes in presentation of sialic acid (linkage) or/and change in the quantity of sialic acid could both have the potential to contribute to AMD pathology. We aimed to further elucidate FH specificities in recognizing sialic acid using a sialoglycan array. Additionally, postmortem human ocular samples were used to investigate the distribution of sialic acid linkage through the neural retina and sub retina and the amount of sialic acid in the neural retina, RPE, and BrM/choroid interface was quantified.

## Methods

### Sialoglycan Array Printing

The sialoglycan arrays were printed as described previously.[24] Briefly, the source plate was prepared by aliquoting 20 µL of 10 mM chemically synthesized sialoglycans (each containing a terminal amino group on the aglycon) diluted in printing buffer (300 mM phosphate buffer pH 8.4) per well according to the plate template. The sialoglycan arrays were printed using ArrayIt SpotBot extreme instrument at

Table 1. An Overview of the Sialoglycan Array Assays Conducted

| Lectin | Primary Antibody | Secondary Antibody |
|---|---|---|
| Native FH (Quidel A410) (10 µg/mL) | Goat poly clonal anti-human FH (0.5 µg/mL) | Anti-goat Cy5 (5 µg/mL) |
| rFH 16–20/mouseIgG2aFc (0.5 µg/mL) | Goat poly clonal anti-human FH (0.5 µg/mL) | Anti-goat Cy5 (5 µg/mL) |
| rFH 6–10/mouseIgG2aFc (10–0.5 µg/mL) | Anti-mouse IgG Cy3 (5 µg/mL) | N/A |
| rFHR1/humanIgG1Fc (5–0.5 µg/mL) | Anti-human IgG Cy3 (5 µg/mL) | N/A |
| rFHR3/humanIgG1Fc (5–0.5 µg/mL) | Anti-human IgG Cy3 (5 µg/mL) | N/A |

20°C 65% humidity, onto PolyAn 3D-NHS (Automate Scientific, PO-10400401).

Once the slides were dried, they were blocked in pre warmed tris ethanolamine buffer (0.1 M Tris and 0.05 M ethanolamine, pH 9, 50°C) for 60 minutes gently rocking. Subsequently, immerse in pre-warmed Milli-Q water (50°C), followed by gentle rocking for 10 minutes in water at the same temperature. The water rinse and wash were then repeated a second time. The slides were dried by centrifuging in a horizontal slide holder for 10 minutes at 700 revolutions per minute (rpm). The slides were vacuumed sealed in a non-transparent container and stored at 4°C until used.

## Ocular Dissection

Human ocular globes were collected from the San Diego Eye Bank with written consent from the individual or the next of kin. Due to the individuals being unidentifiable the collection of this tissue was under institutional review board (IRB) exception from the UC San Diego Human Research Protection Program. The human tissue experiments complied with the guidelines of the ARVO Best Practices for Using Human Eye Tissue in Research (November 2021). Ocular samples were collected from male and female subjects aged between 55 and 99 years of age (Supplementary Table S1). The anterior segment of the globe was first removed and samples, including 2-mm punches beside the fovea within the macular region and 2-mm punches of the peripheral retina, were taken and snap frozen in optimal cutting temperature compound (OCT; Tissue Tek, Sakura Finetek, Torrance, CA, USA) to be used for cryosectioning. These were used for immunohistochemistry. Encompassing the 2-mm punch in each area an 8-mm punch was taken. From this 8-mm punch, the neural retina was removed and the presence of AMD pathology was recored. The RPE cells were carefully brushed off, collected, washed, and snap frozen. Following this, the BrM and choroid were peeled off from the sclera and as much of the choroid as possible was removed from the BrM.

## Histology

Cryosections from the stored OCT blocks were cut at 8-µm thick using a Leica CM1860 at −18°C and placed on to Superfrost Plus microscope slides (Fisherbrand, 12-550-15). Each slide had six sections, with three sections from the macular and three sections from the peripheral retina. All slides were stored at −80°C. Prior to use the slides were defrosted at room temperature and air dried for a minimum of 1 hour and each frozen tissue section was circled using a PAP pen, to be able to concentrate the reagent correctly and prevent desiccation. Three individuals with AMD and five age matched controls were included in the histology experiments (Table 1). A hematoxylin and eosin (H&E) stain was standard in order to confirm tissue morphology. A summary of each of the assays performed on the slides with the corresponding controls is included in Table 2.

## Immunofluorescence Using SGRP3$^{Hsa}$ for α2-3-Linked Sialic Acid and SNA for α2-6-Linked Sialic Acid

*Arthrobacter ureafaciens* sialidase (AUS; Roche, 10269611001) was diluted to 5 mU/mL in 50 mM sodium acetate buffer pH 5.2. Mock treatment included the sodium acetate buffer alone. The mock and sialidase solution were each overlaid on the necessary slides and incubated in a humid chamber overnight at 37°C. The slides were washed three times in PBS. All slides were fixed in 10% buffered formalin for 10 minutes and then washed. Autofluorescence was quenched using 1× TrueBlack (Biotum, 23007) solution in 70% ethanol (v/v), washed, and avidin biotin blocked using the Vector labs kit (SP-2001). Prior to the addition of the primary lectin, all slides were overlaid with diluting buffer. Sialoglycan Recognizing Probe 3 (SGRP3$^{Hsa}$), which is a sialic acid probe specific for α2-3-linked sialic acid, was originally derived from *Streptococcus gordonii* and SGRP3$^{Hsa}$ non-binding (SGRP3$^{Hsa}$NB; carbohydrate binding mutant)[24] were then added at 20 µg/mL but the directly conjugated SNA-FITC (Vector Labs NC9785207) was added at 5 µg/mL in diluting buffer. The slides were incubated for 1 hour at room temperature, washed, and binding detected using streptavidin-Cy3 (Jackson ImmunoResearch, 109-160-084). All slides were washed and overlaid with nuclear Hoechst solution and then mounted with aqueous mounting media for viewing and photomicrography.

## Immunofluorescence Using Anti-PolySialic Acid

After fixation in 10% buffered formalin, the slides were washed. Relevant slides were treated with enzyme activated recombinant EndoN enzyme 7 µg/mL in 2% BSA (w/v) for 2 hours at 37°C or blocked in 2% BSA (w/v). Endogenous fluorescence was removed and mAb735 was diluted 1:1000 in 2% BSA and incubated for 2 hours at room temperature in a humid chamber. The slides were then washed and overlaid with anti-mouse IgG-Cy3 (Jackson ImmunoResearch, 115-165-071) for 45 minutes, and then washed. All slides were treated with nuclear Hoechst stain and mounted using aqueous mounting media for viewing and digital photomicrography.

## Immunofluorescence Using Anti-FH

For the detection of endogenous FH, the cryosections were initially fixed in ice cold acetone for 20 seconds and then washed in PBS. Autofluorescence was quenched using 1× TrueBlack in 70% ethanol for 1 minutes and then washing the slides in PBS. The slides were overlaid with dilut-

**Table 2.** The Lectins Used to Probe the Histology Slides and the Corresponding Controls and Secondaries Used

| Primary Protein | Source | Enzyme Control | Secondary |
|---|---|---|---|
| SNA-FITC | VectorLabs | Sialidase | N/A |
| SGRP3$^{Hsa}$ | Ref. 24 | Sialidase and SGRP3$^{Hsa}$ NB | Streptavidin Cy3 |
| mAb735 | Ref. 25 | EndoN | Anti-mouse IgG Cy3 |
| Goat polyclonal anti-human FH | Ref. 26 | N/A | Anti-goat IgG Cy3 |

ing buffer (0.5% cold water fish gelatin in PBS) followed by goat polyclonal anti-human FH diluted 1:500 in diluting buffer, incubated for 2 hours at room temperature. After washing, the binding was detected using Anti-goat Cy3 (Cy3 AffiniPure Donkey Anti-Goat IgG (H+L) (min X Ck, GP, Sy Hms, Hrs, Hu, Ms, Rb, and Rat Sr) (Product Code: 705-165-147), and diluted 1:500 in diluting buffer for 30 minutes. The nuclei were counter stained with Hoechst and then the slides were mounted with aqueous mounting media for viewing and digital photomicrography.

### Imaging

All microscopy imaging was captured on a Keyence Biorevo BZ-9000 microscope. Post-processing, such as brightening and merging, was conducted with Fiji 2.14.0/1.54F.

### Sialic Acid Identification and Quantification (DMB-HPLC)

Ocular tissue was suspended in Milli-Q water with the addition of 1× Halt protease inhibitor cocktail (Thermo Scientific, 1861278) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The tissue was homogenized by sonication in 15-second bursts at approximately 15% intensity, followed by a 45-second rest, repeated 3 times or until the tissue was fully homogenized. Protein concentration was determined using a BCA assay (Thermo Scientific, 23225). Tissue lysate was stored at −20°C until use the following day.

### Acid Release of Sialic Acids

The starting material type dictated the quantity of protein from which the sialic acid was released. Approximately, 10 µg of BrM lysate, 20 µg of neural retina lysate, and as much RPE cell lysate as possible was incubated in 2 M acetic acid at 80°C for 3 hours, gently shaking at 300 rpm. The released sialic acid was separated from the lysate by passing through a 10 kDa spin filter. The flow through was frozen at −80°C and lyophilized. The free sialic acid was resuspended in 100 µL of Milli-Q water. An aliquot of this was base treated to remove the O-acetyl groups by adding NaOH to a final concentration of 0.1 M and incubating at 37°C for 1 hour. The pH was subsequently adjusted back to approximately pH 7 by dropwise addition of HCl. The resuspended acid released sialic acid and the base treated sialic acid were fluorescently labeled by the addition of 7 mM 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride, 1.6 M glacial acetic acid, 0.75 M $\beta$-mercaptoethanol, and 18 mM sodium hydrosulphite at a 1:1 ratio. The labeling occurred in the dark at 4°C for 48 hours to prevent loss and migration of *O*-acetyl modifications, as described previously.[27] Standards including 1 nmol of Neu5Ac and free sialic acid from bovine submaxillary mucins (BSM) was also labeled in parallel.

Depending on the concentration of sialic acid in the sample, either the maximum amount of labeled sialic acid or 50% was injected into the LaChrome Elite Hitachi HPLC system with a Phenomenex Gemini C18 column (5 µm, 250 mm × 4.6 mm) at room temperature. A gradient was run over 68 minutes switching from solution A (acetonitrile: methanol: water, 7:7:86 [v/v/v]) to solution B (acetonitrile: methanol: water, 11:7:82 [v/v/v]) with an additional 7 minutes of 100% solution B with a consistent flow rate of 0.9 mL/minute.[27] The DMB-labeled sialic acid species were detected a fluorescence detector at an excitation of 373 nm and emission of 448 nm.

Using the Neu5Ac standard to generate a standard curve, the amount of Neu5Ac in each sample was determined, and the concentrations of Neu5Ac were normalized to the protein concentration of the original sample. The types of sialic acid present were determined by comparison to the BSM standard. In the Prism graph pad, multiple unpaired *t*-test with Welch correction and no assumption of variance of each group were performed. We also conducted Pearson correlations between amount of Neu5Ac and age or time between death and dissection.

## Results

### Sialic Acid-Binding Specificity of Complement Factor H

It was evident that rFH16-20 is very specific for sialic acid in an $\alpha$2-3-linkage to galactose (Fig. 1A). Similar to previous results, it binds both Neu5Ac and Neu5Gc[17,28] but did not recognize other sialic acid species such as 2-keto-3-deoxy-D-glycero-D-galactonononic acid (Kdn) or legionaminic acid (Leg). However, there was variability in binding depending on the linkage and monosaccharide attached to the reducing terminal of Sia$\alpha$3Gal and included N-/O-glycan sialoglycan motifs and ganglioside type motifs. For examples, Neu5Ac/Neu5Gc$\alpha$3Gal$\beta$4GlcNAc and their various modifications (9OAc, 4OAc, 4NAc, and 6S) are commonly identified on N- and O-glycans. Recombinant FH was also found to strongly interact with Neu5Gc$\alpha$3Gal$\beta$3GalNAc, which is representative of core 1 O-glycans, and its counterparts containing a 9OAc or 4OAc-modified Neu5Gc. FH also recognized GM3 ganglioside-type glycans, Neu5Ac/Neu5Gc$\alpha$3Gal$\beta$4Glc, and modifications with 4OAc and 4NAc at the sialic acid. However, recognition was lost if the sialic acid was capped with an $\alpha$2-8-linked sialic acid (GD3). The addition of $\beta$1-4 GalNAc to create GM2 also prevented the recognition of FH. Similarly, the presence of fucose in Lewis X structures (Neu5Ac$\alpha$3Gal$\beta$4(Fuc$\alpha$3)GlcNAc) prevented the recognition of FH. Interestingly, FH did not recognize any 7,9-diOAc sialic acid species. In all cases, just the sialylgalactose disaccharide alone was enough to initiate FH binding. This suggests that FH does not specifically recognize one class of glycans.

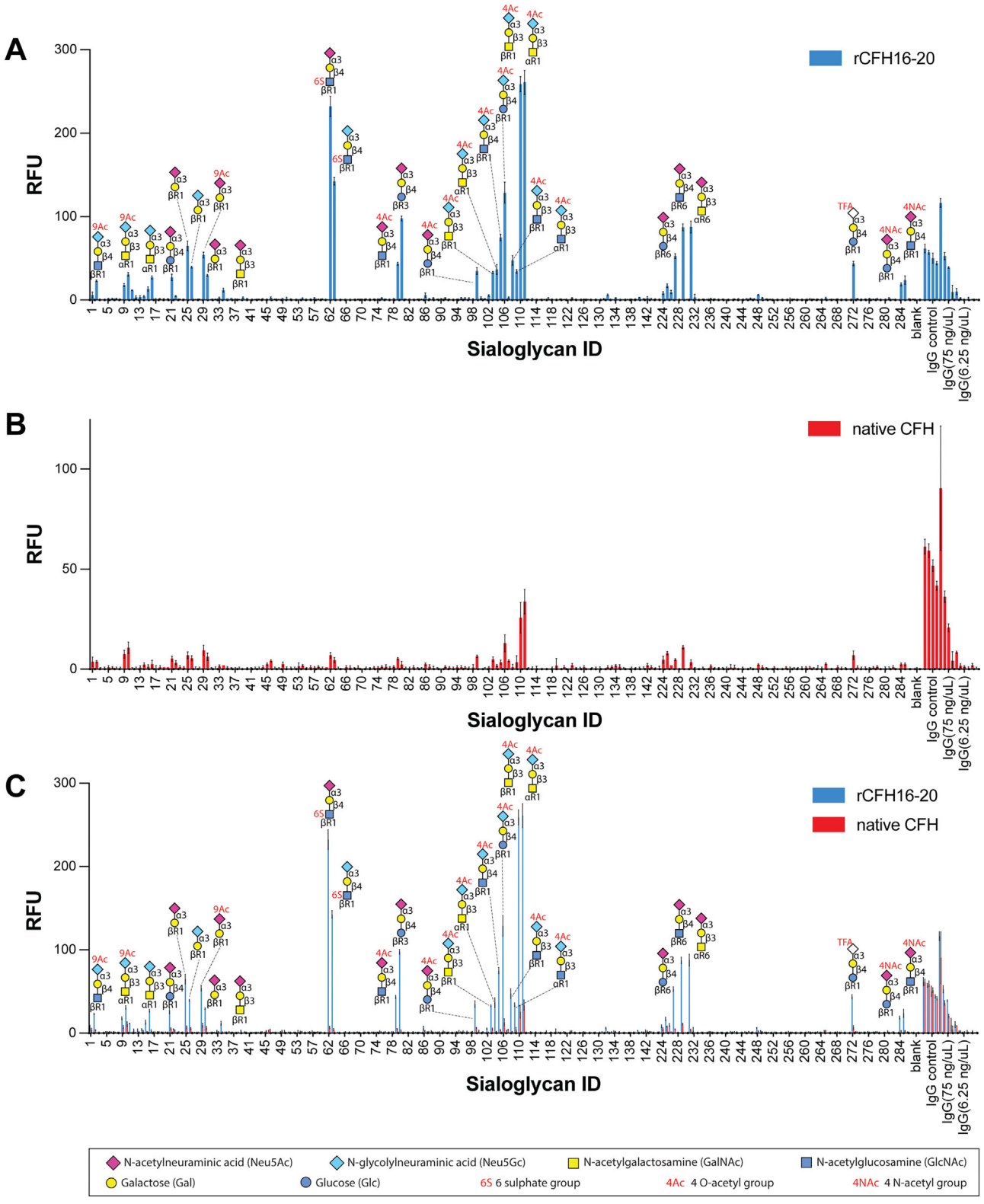


**Figure 1.** Human complement factor H (CFH) recognition of sialic acid. (**A**) Recombinant CFH domains 16 to 20 fused to Fc (rCFH16-20; 0.5 µg/mL) the composition of the sialogycans recognized are depicted. (**B**) Native CFH purified from human serum (10 µg/mL). (**C**) An overlay to compare the recognition of rCFH 16 to 20 and native CFH. Relative fluorescent units (RFUs). Error bars represent standard deviation of four replicate spots. For a list of all glycan compositions included on the array, see Supplementary Table S2.

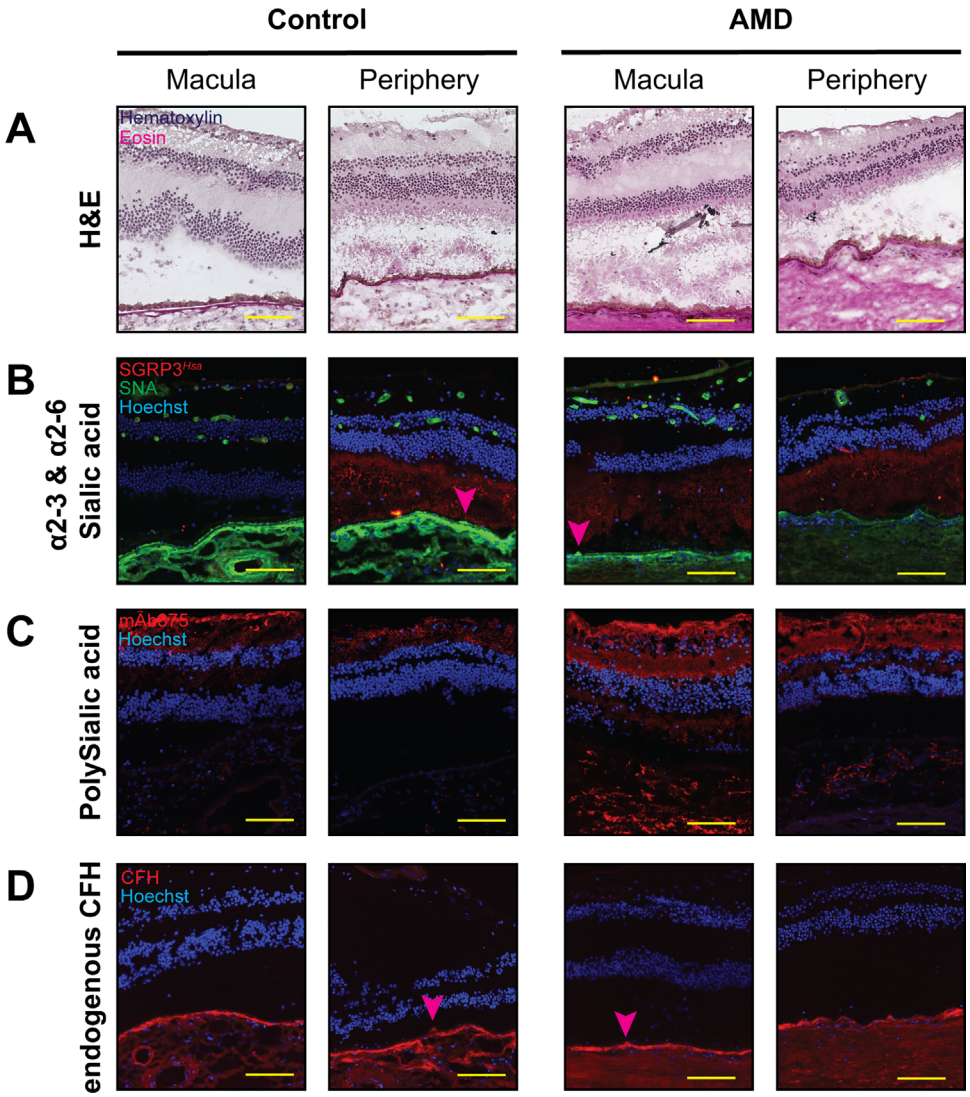


**Figure 2.** The distribution of different sialic acid linkages throughout the retina of individuals with and without age-related macular degeneration (AMD). (**A**) Hematoxylin and eosin stain (H&E). (**B**) SGRP3$^{Hsa}$ recognition of $\alpha$2-3-linked sialic acid and SNA $\alpha$2-6-linked sialic acid. (**C**) Monoclonal antibody 735 (mAb735) binds $\alpha$2-8 polysialic acid. (**D**) Endogenous FH detected with goat polyclonal anti-human FH detecting endogenous FH. The yellow scale bar is 100 µm. The *pink arrowhead* indicated drusen. Comparison of individual variation can be observed in Supplementary Figure S1.

A sulfated GlcNAc (sialoglycan #62) showed a greater amount of rFH16-20 binding than its non-sulphated counterparts. Furthermore, rFH16-20 showed a preference for 4-*O*-acetylated Neu5Gc, although binding to 4-*O*-acetylated Neu5Ac was also observed.

The recombinant FH16-20 and native full-length FH, purified from human serum, showed a very similar binding profile to the sialoglycan array (Fig. 1B), although the dominant interaction for native FH was with Neu4,5Ac$_2$3Gal$\beta$3GalNAc (sialoglycans #110 and #111). The relative fluorescent units reflecting binding of the full-length protein appeared to be much lower than that of the truncated recombinant protein on the glycan array (Fig. 1C). The recombinant FH16-20 was used at 0.5 µg/mL and the native FH as used at 10 µg/mL. The difference in the number of sialic acid binding sites does not account for the difference in the amount of binding. The 2 glycan binding sites of the rFH16-20 molecule, on account of it being expressed as an Fc fusion protein, may have contributed to its greater avidity compared to the monomeric full-length FH.

Recombinant GAG binding domain of FH, rFH6-10, was passed over the sialoglycan micro array at several concentrations, however, no binding was observed. Similarly, recombinant FHR1 or FHR3 fused to Fc did not bind to any of the sialoglycans on the microarray (data not shown).

## Histology

**Hematoxylin and Eosin.** Due to the nature of the collection and the fragility of the retina tissue, mechanical damage artifacts and retinal detachment were commonly observed during collection and cryosectioning. In some cases, the outer and inner photoreceptor segments were missing and, in other cases, the RPE was dislocated as was evident in the H&E staining (Fig. 2A).

**Detection of α2-6 and α2-3 Sialic Acid in Human Retinas.** SNA is a lectin produced in the bark of *Sambucus nigra* (European elder berry tree), it recognizes a variety of sialic acid species but strictly linked to the underlying glycan in an α2-6-sialyl linkage. Commonly, SNA is used to stain the epithelium of blood vessels throughout the body, the use of SNA in this study clearly illustrated the vasculature in the inner layers of the retina, the choriocapillaris, and choroidal vasculature (Fig. 2B). Moderate detection of α2-6-linked sialic acid was observed in the BrM and choroid. Interestingly, individual number 3 showed strong detection of α2-6-linked sialic acid on the inner as well as outer side of the BrM (Supplementary Fig. S1). Strong staining of the drusen in two individuals was observed (see Fig. 2B, pink arrowhead). Very little detection of α2-6-linked sialic acid was observed on the RPE cells of all individuals. No difference was observed in detection of α2-6-linked sialic acid between macula and peripheral regions of AMD individuals or controls. Sialidase treatment appropriately abolished binding of SNA, however, in the mock-treated samples, a comparable amount of binding was observed (Supplementary Fig. S2), demonstrating that none of the SNA binding was due to non-specific protein-protein interactions. The sialic acid probe specific for α2-3-linked sialic acid (SGRP3$^{Hsa}$) was originally derived from *Streptococcus gordonii*. The SGRP3$^{Hsa}$ bound to the photoreceptors very specifically (see Fig 2B). The amount of binding to the photoreceptors in the macula and peripheral region was variable for each individual, however, there was no specific trend. Unfortunately, after overnight sialidase and mock treatment, the majority of the SGRP3$^{Hsa}$ binding was lost but the SNA binding remained unchanged (see Supplementary Fig. S2). Potentially the long exposure to the low pH and temperature could have hydrolyzed some of the sialic acid. However, no recognition was observed for the non-binding SGRP3$^{Hsa}$ which has a mutated sialic acid binding domain. TrueBlack successfully quenched the autofluorescence observed due to the accumulation of lipofuscins in the RPE cells as no signal was detected in any of the negative controls.

**Detection of Polysialic Acid in Human Retinas.** Polysialic acid was identified and mapped in the human retina. This was achieved using a monoclonal mouse antibody (mAB735) developed against colominic acid (α2-8 polysialic acid) produced by a strain of pathogenic *Escherichia coli* (*E. coli*) K1 bacterium. These results were corroborated by staining with SGRP8 which recognizes α2-8-linked di-sialic acid (SGRP8 data not shown). As a negative control, the tissue section was treated with an active recombinant EndoN enzyme, which specifically cleaves α2-8-linked sialic acid, to demonstrate the mAb was specific for polysialic acid identified in the human retina (Supplementary Fig. S3). The distribution of α2-8-linked sialic acid appears to be very specific, for the innermost layers of the retina (ganglion cell layers and the inner plexiform layer; Fig. 2C). However, in 2 of the 3 AMD individuals evaluated, a strong detection of α2-8-linked sialic acid was observed in the localized area of the choroid (see Fig. 2C).

**Detection of Endogenous FH in Human Retinas.** The presence of endogenous FH was observed throughout the choroid and sclera for all individuals in tissue collected from both the macula and peripheral regions (Fig. 2D). Very strong detection of endogenous FH was seen in the choriocapillaris of most individuals. Although detection in the choroidal vasculature was most prominent in individual number 1 (see Supplementary Fig. S1). Consistent with previous work,[29] endogenous FH was detected on drusen. Considerable interindividual variation was observed and there was no difference between patients with AMD and healthy controls, and no obvious trend when comparing the peripheral tissue to the macula. No endogenous FH was detected in the neural retina of any of the individuals regardless of AMD status or the region the tissue was taken from, suggesting systemic FH cannot cross the blood retina barrier, and if RPE or neural retina cells produce FH, it is below the level of detection.

### Comparison of Sialic Acid Content in the Sialome of Patients With AMD (DMB-HPLC)

The amount of sialic acid in the neural retina, RPE cells, and the BrM/choroid interface was measured by releasing sialic acid with mild acid, labeling with DMB followed by HPLC separation. Each sample was analyzed in comparison to bovine BSM standard (dark blue chromatogram; Fig. 3A) and base treated (red chromatogram; see Fig. 3A) to determine if any *O*-acetylated sialic acid species were present. A very small amount of 7-*O*-acetylated and 9-*O*-acetylated Neu5Ac was detected in some of the samples. However, this proportion made up less than 1% of the total sialic acid. As expected, no Neu5Gc was detected (humans have lost the capacity to produce this sialic acid derived from Neu5Ac due to a loss-of function mutation of the gene encoding the enzyme CMAH, which is now fixed in the human population).

For each batch, a standard curve was generated by running known concentration of Neu5Ac standards, enabling the quantification of Neu5Ac in each sample. Subsequently, the Neu5Ac levels were normalized to the initial protein concentration. No significant correlation was found between the amount of Neu5Ac detected and either the age of the subject or the time elapsed between death and dissection. There was no difference between the amount of Neu5Ac in the macula compared to the peripheral retina for any of the different retinal tissues collected. However, a lower amount of sialic acid was detected in the neural retina and the RPE cells compared to the BrM/choroid interface (Fig. 3B). Although there was individual variation, there is no significant difference between the amount of sialic acid between individuals with and without AMD in the neural retina and RPE cells. However, individuals with AMD had significantly less sialic acid in the BrM/choroid interface compared with aged-matched individuals in both the macula and peripheral retina ($P = 0.02$ and $P = 0.004$, respectively; see Fig. 3B).

## Discussion

To our knowledge, this is the first indepth study of the human ocular sialome in combination with the distribution of endogenous FH protein. Utilizing a sialoglycan array has illustrated the intricacies of the siaclic acid-binding domain of FH recognition for different presentations of sialic acid, with various modifications. Eyes from healthy subjects and patients with AMD revealed variation in sialic acid linkage across the different layers of the retina. Last, the quantity of sialic acid in BrM/choroid interface is significantly reduced in individuals who had AMD, in both the macula and peripheral region of the retina. This suggests that sialic acid may

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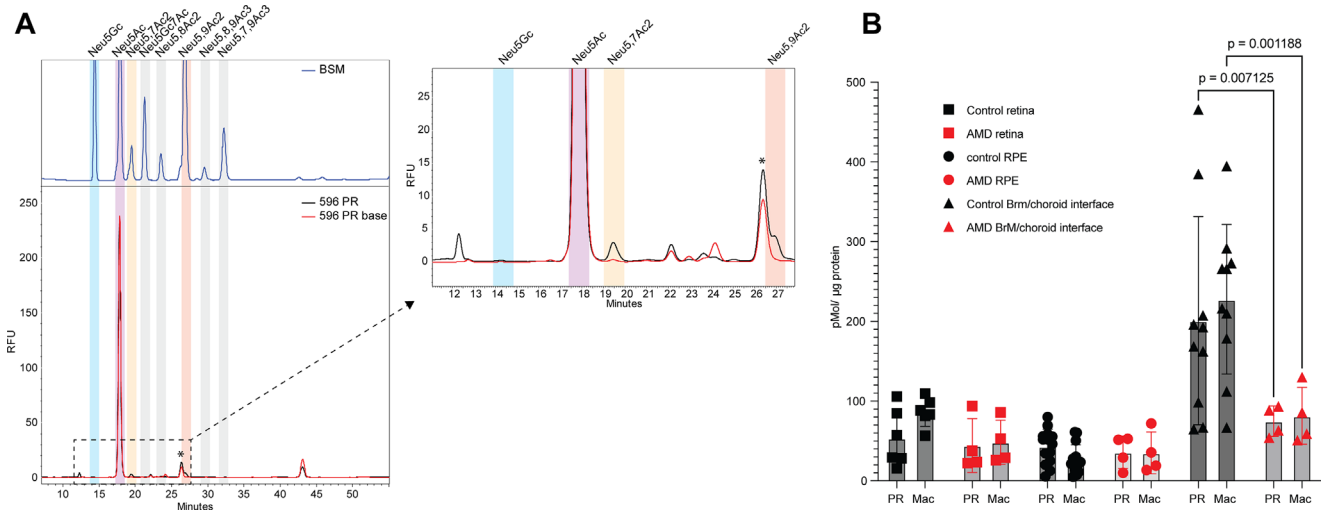


**Figure 3.** Identification and quantification of sialic acid in the human retina with and without age-related macular degeneration (AMD). (**A**) Representative DMB-HPLC profile. Retention time (RT) of peaks detected in the retinal samples (*lower panel, black*) are compared to RT of well- characterized distinct types of sialic acids present in bovine submaxillary mucins (BSM; *upper panel, blue*). Additionally, the released sialic acids are base treated (*lower panel, red*) to remove any potential *O*-acetyl groups, confining the modification of the sialic acid. (**B**) The quantity of Neu5Ac in each sample from both the peripheral retina (PR) and macula (Mac) was determined by comparisons to standards of known concentration. An unpaired *t*-test with Welch correction and no assumption of variance of each group, error bars represent the standard error of the mean.

play a role in the organization of the retina and in AMD pathology.

Through the use of a diverse sialoglycan array it was confirmed that both native FH and rFH16-20 specifically bind $\alpha$2-3-linked sialic acids, which is consistent with previous literature.[16,17] However, a preference for 4-*O*-acetylated sialic acid was observed, which has not been demonstrated before. Each of the sialoglycans that had previously shown weak but positive interaction with full-length FH (in the micromolar and millimolar ranges) through the saturation transfer difference (STD) nuclear magnetic resonance (NMR) studies was identified on the sialoglycan array.[16,17] Unexpectedly, interactions with 9-*O*-acetylated Neu5Ac and 9-*O*-acetylated Neu5Gc were observed, contrary to previous findings which demonstrated increased FH binding to murine erythroleukemia cells following treatment with a 9-*O*-acetylesterase[30] and markedly decreased FH binding to lacto-*N*-neotetraose (LNnT) in gonococcal lipooligosaccharide (LOS) capped with Neu5,9Ac$_2$ compared to LOS bearing Neu5Ac on LNnT.[28] However, this difference could be due to the way the modified sialic acid is presented on biological surfaces, where it may interact with other surface molecules, versus in purified form on microarrays. Additionally, the presence of additional FH ligand, such as C3b/C3d[31] or PorB.1A[32] (both non-glycan mediated), have previously been demonstrated to enhance avidity, this level of complexity is lacking from this assay. As demonstrated in the past, it does not appear that human FH has any particular preference over Neu5Ac or Neu5Gc.[17] Similarly, the sialic acid binding domain of many Siglecs has rapidly evolved allowing Siglecs to preference both Neu5Ac and Neu5Gc, instead of only recognizing Neu5Gc, which is the case for non-human primate Siglecs.[33,34] Humans are unable to synthesize Neu5Gc from its precursor Neu5Ac and the source of Neu5Gc on any human tissue is due to dietary uptake and metabolic incorporation.[35] Therefore, the FH recognition of Neu5Gc could reflect an ancestral condition in human evolution, from when our ancestors still possessed a functional CMP-Neu5Ac hydroxylase gene, required for the synthesis of Neu5Gc.[36,37]

The glycan array also demonstrated that sulfation of the GlcNAc in the LacNAc motif underlying the terminal sialic acid increased recognition of FH. Similar fine-tuning of sialic acid binding has also been observed for Siglec 3 (CD33, sialic acid-binding Ig superfamily lectin 3) another immune modulating sialic acid receptor.[38] This is the first time that FH has been shown to recognize 4-*O*-acetylated sialic acid. Previously, Neu4,5Ac$_2$ has been found in horses, guinea pigs, monotremes, and several fish species but its presence in humans has not been definitively established to date.[39–42] Although, Neu4Ac5Gc has been detected in human patients with colon cancer.[43] Therefore, it is interesting that FH, a self-recognition complement regulator, is recognizing this modified version of sialic acid. The distribution of this sialic acid modification is likely to be under-represented in the literature due to the difficulty of its detection by HPLC and lack of commercially available probes or antibodies.[44] It will be interesting to speculate what role this interaction could possibly be having considering its distributions in humans is predicted to be minimal, if not absent.

It is important to consider that FH does not recognize sialic acid in isolation, the glycocalyx, and extra cellular matrix, further away from the cell surface, consists of many different types of glycans and glycoconjugates. Their density and specific combinations could each play a role in FH recognition (even other glycans which do not directly interact[45]). Additionally, FH can recognize both sialic acid and GAGs, the relationship between sialic acid and GAG recognition of FH has not been investigated. Technically FH could recognize both sialic acid and GAGs at the same time through simultaneous interactions with both SCR 7 and SCR 18 to 20, and the effect this dual interaction may have on protein function or surface avidity is unknown. It is conceivable that the sialome could change but then be compensated by the GAGome and therefore have little effect on FH recognition.

The retina is a highly organized tissue, consisting of distinct layers, each defined by different cell types. We believe the different cell types in each layer is what is contributing to the very specific localization of each of the different sialic acid linkage types throughout the tissue. The only region where there appears to be considerable overlap is in the BrM and choriocapillaris that appear to be highly sialylated with both $\alpha$2-3 and $\alpha$2-6-linkages, but no polysialic acid. Although not apparent in the histology, the amount of sialic acid present in the neural retina and RPE cells is significantly less than what was detected in the BrM/choroid interface, demonstrating the advantage of combining histology with biochemical analysis of the analytes. Interestingly none of the sialic acid linkage-specific lectins recognized Muller cells, which span the whole neural retina. The photoreceptors appear to have a very distinct sialome consisting of only $\alpha$2-3-linked sialic acid. Previous lectin studies have also demonstrated that the photoreceptors and more specifically the cone photoreceptors stain very strongly with PNA suggesting an abundance of uncapped O-glycans.[46]

FH has been shown to bind to oxidized lipids and bisretinoids on drusen.[47] Although intense staining of SNA and, to some extent, SGRP3$^{Hsa}$ was observed on the drusen, along with previous LFA staining,[13] the interaction between FH and drusen is not necessarily a glycan-protein interaction. Oxidative stress leads to the formation of malondialdehyde, increasing the interaction of FH, and preventing pro-inflammatory events.[7,47,48] This interaction occurs at the polyanion binding site of FH within SCR 19 and 20, which could compete with negatively charged glycans for binding.[48] We found that the localization of endogenous FH does not align with the areas showing the highest staining for $\alpha$2-3-linked sialic acid in the photoreceptors. However, our results also illustrate that systemic FH does not appear to be crossing the blood retina barrier, although accumulation on and in drusen was observed. The amount of FHFH reported to be produced by the RPE cells must be below the level of detection by our specific antibodies or in line with current presumptions FHFH produced by RPE is secreted basally. Despite the change in sialic acid observed in the BrM, a change in the amount of endogenous FH was not observed. However, the goat polyclonal antibody is not capable of differentiating between full length FH and the truncated splice variant FH like (FHL) which consists only of SCR 1 to 7. It also cross reacts with FHR1 and FHR3 (FHRs 2, 4, and 5 have not been tested). FHL and the FHRs can compete with FH for binding to surfaces. Therefore, variable expression of each of these FH family members could affect complement regulation as only the full-length FH and FHL are capable of inhibiting the alternative complement system.[49]

Although the amount and type of sialic acid in the neural retina and RPE cells does not change between individuals with AMD and those without, there is a significant difference in quantity of sialic acid in the BrM/choroid interface. Lack of terminal sialic acid is thought to be a marker for complement activation and apoptosis.[50] Interestingly, in individuals without AMD, the quantity of sialic acid in the BrM/choroid interface is much greater than other regions of the retina. In individuals with AMD, this reverts to a level comparable with the neural retina and RPE cells. Whether there is increased sialidase activity in this region or downregulation of sialyltransferases is yet to be determined. In a previous model of uveitis, another inflammatory eye disease, an upregulation of NEU1 in Muller cells has been reported.[51] The factors contributing to the reduced quantity of Neu5Ac in the BrM is currently unknown. Possible explanations include a reduction in sialylated N-glycans, O-glycans, or gangliosides. Many proteins which make up the BrM are potentially glycosylated including collagen, laminin, and integrins.[52–54] Unfortunately, this reduction in sialic acid quantity in the BrM/choroid interface was not observable in the histology. How this change in the sialome could affect AMD pathology remains speculative. To date, the role of sialic acid in the blood-retina/brain-barrier remains unclear. It would be interesting to know if sialome alteration could induce leakage of this crucial selectively permeable layer. However, cell bound, rapidly evolving sialic acid-binding lectins, the Siglecs, which are expressed in different combinations on most immune cells, are likely to be involved in differential responses to altered sialomes.[55] We hypothesize that a reduced amount of sialic acid would result in less inhibition of microglia (Siglec 1, 3, 11, and 16) and macrophages (Siglec 3, 7, and 9), however, immune regulation was beyond the scope of this study.[55,56] Recently, Gne± knockout mice, with reduced amounts of the key enzyme in sialic acid synthesis, showed reduced protein-bound polysialic acid in the neural retina.[57] This led to increased lysophagosomal activity in retinal microglia, upregulation of pro-inflammatory IL-1$\beta$, and loss of rod bipolar cells. Knocking out complement factor C3 partially reversed these effects, underscoring the connection between sialic acid and the alternative complement system.[57] Nanoparticles decorated in polysialic acid are currently in human trials.[58] It is believed to inhibit microglia/macrophage by interacting with Siglecs.[59]

The GAG-binding domain of FH did not interact with any sialoglycans on the array even those with an $\alpha$2-8-linkage. A recent study showed a weak interaction between polysialic nanoparticles and FH and FH domains 6 and 7 fused to Fc.[60] The polysialic acid nanoparticles could enhance FH binding to C3b in surface plasmon resonance assays and modestly reduced hemolytic activity of the alternative and classical pathways of complement.[60] This contrasts with our data and prior studies.[60] Although this study and prior studies have not shown a direct interaction between FH and $\alpha$2-8 polysialic acid or the ability of colominic acid to enhance the interaction between FH and C3b,[6,61] differences in experimental outcomes may have resulted from differences in the formulations of colominic acid (i.e. nanoparticles versus soluble colominic acid), the concentration of ligands used (experiments with polysialic acid nanoparticles used ligand concentrations 2 to 3 orders of magnitude higher than prior studies) and/or the type of assay used. Nevertheless, the reduction in the quantity of sialic acid in the BrM of individuals with AMD seen in this study warrants further study of sialic acid-based treatment options that compensate for the reduction of sialic acid in disease eyes.

This study provides novel insight into the spatial distribution of sialylated glycans in healthy and AMD-affected eyes. Using multiple lectins and controlling endogenous auto fluorescence has illustrated clear localization of each of the different sialic acid linkages. A decrease of sialic acid in the BrM/choroid interface of individuals with AMD suggested a change in sialome could be contributing to the progressive disease. Although only a small number of individuals were included in this study due to the limitation of deriving postmortem human ocular tissue.

### *Acknowledgments*

The authors thank Nissi Varki for all her advice regarding the histology component of this project and many helpful discussions. Thank you also to Professor Travis Beddoe for the discussion regarding protein interactions.

Supported by the BrightFocus Postdoctoral Fellowship: M2023003F (to J.S.), NIH grant number: R01AI130684 (to X.C. and A.V.), The G. Harold and Leila Y. Mathers Foundation (to P.G.), and Avecida Therapeutics SRA 306071-00001(to P.G. and J.S.)

Disclosure: **J. Swan**, None; **C.B. Toomey**, None; **M. Bergstrand**, None; **H.A. Cuello**, None; **J. Robie**, None; **H. Yu**, None; **Y. Yuan**, None; **A.S. Kooner**, None; **X. Chen**, None; **J. Shaughnessy**, None; **S. Ram**, None; **A. Varki**, None; **P. Gagneux**, None

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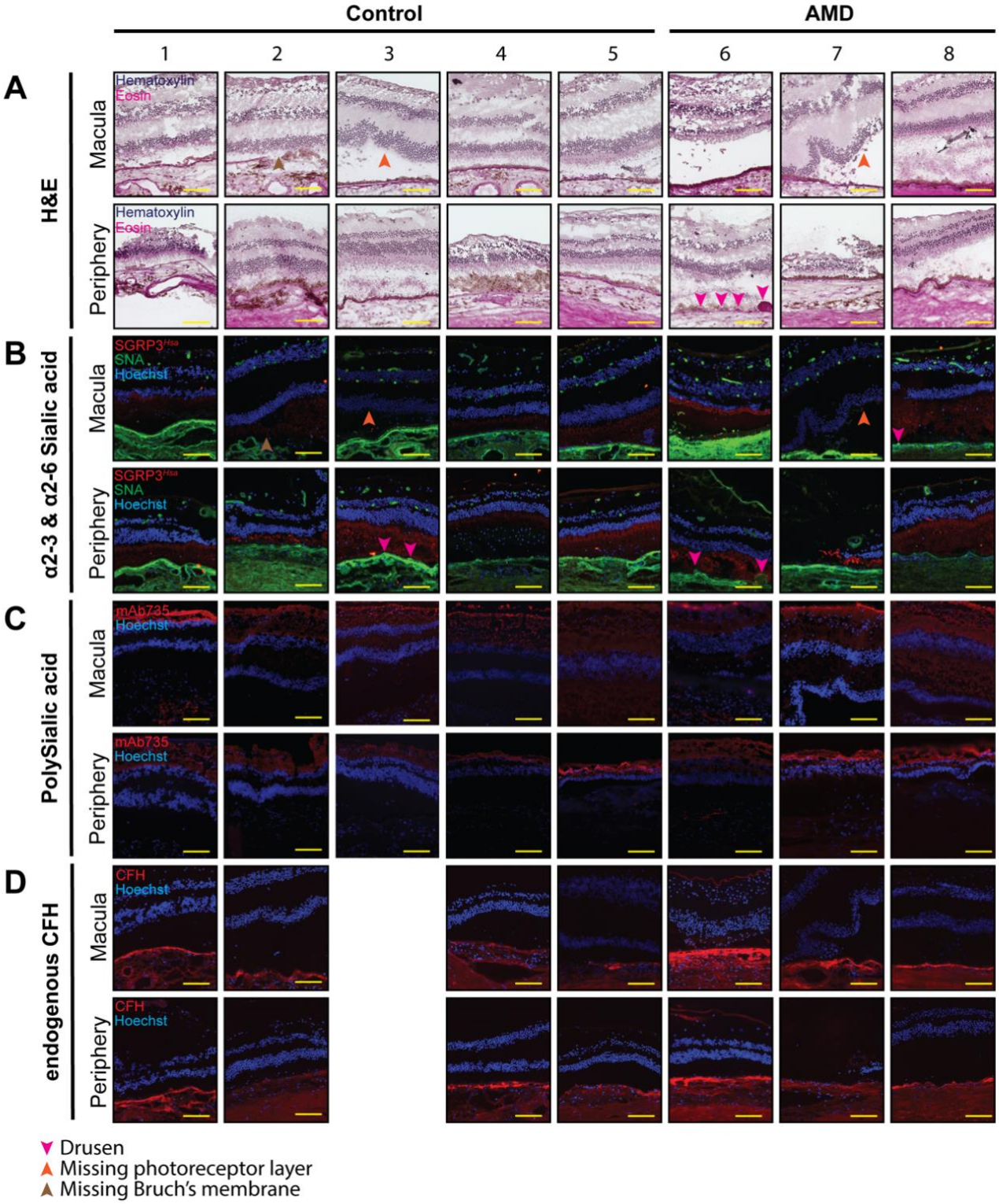
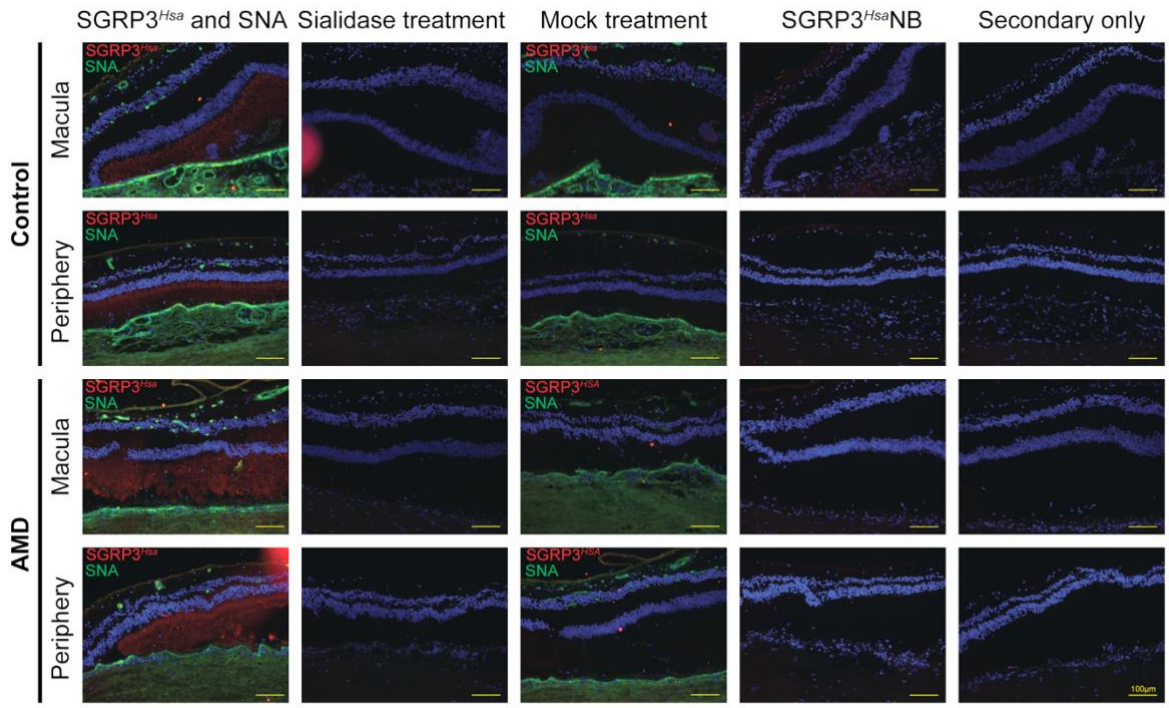
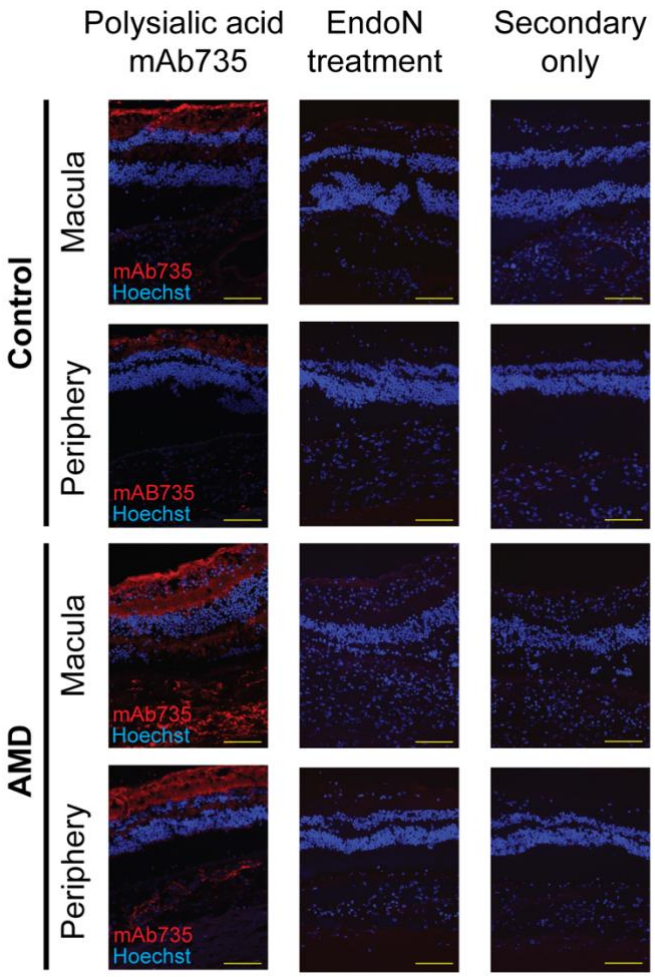



**Supplementary Figure S1.** The distribution of different sialic acid linkages throughout the retina of individuals with (n=3) and without age related macular degeneration (AMD) (n=5). (A) Hematoxylin and eosin stain (H&E). (B) SGRP3$^{Hsa}$ recognizes α2-3-linked sialic acid and SNA α2-6-linked sialic acid. (C) Monoclonal antibody 735 (mAb735) binds α2-8 polysialic acid. (D) Endogenous CFH detected with goat polyclonal anti-human CFH detecting endogenous CFH. The yellow scale bar is 100μm. Pink arrowhead indicated drusen; orange arrowhead shows tissue with missing photoreceptors and the brown arrow heads indicates areas where the Bruch's membrane is missing.




**Supplementary Figure S2.** Multi-lectin staining and negative controls.




**Supplementary Figure S3.** Polysialic acid staining using mAB735 and negative controls which include EndoN treatment to cleave polysialic acid and secondary only control. Yellow scale bar = 100 μm.

**Supplementary Table S1.** Details of the ocular donors and which experiments they were included in.

| ID | sex | age | AMD status | Death to refrigeration (hrs:mins) | Death to enucleation (hrs:mins) | Death to dissection (hrs:mins) | DMB-HPLC | | | Histology | | | | | Cause of death |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Retina | RPE | BrM/ choroid | H&E | SNA | SGRP3$^{Hsa}$ | mAb375 | ahFH | |
| 1 | M | 79 | no | 4:23 | 18:45 | 23:03 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | Myocardial Infraction |
| 2 | M | 69 | no | 7:00 | 26:57 | 32:20 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | Hypertensive Cardiovascular Disease |
| 3 | F | 93 | no | 2:45 | 5:45 | 8:57 | | ■ | ■ | ■ | ■ | ■ | ■ | | Dementia |
| 4 | F | 74 | no | 2:31 | 21:36 | 23:19 | | ■ | ■ | ■ | ■ | ■ | ■ | ■ | Intraparenchymal hemorrhage |
| 5 | M | 80 | no | 6:45 | 17:23 | 19:15 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | Myocardial Infraction |
| 6 | F | 69 | AMD | 10:22 | 11:58 | 15:22 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | N/A |
| 7 | M | 79 | AMD | 1:53 | 23:23 | 28:22 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | Pneumonia |
| 8 | M | 82 | AMD | 2:50 | 20:57 | 27:10 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | Blunt Force Injury d/t Trauma -Peds vs Auto |
| 9 | F | 72 | no | 4:00 | 26:21 | 52:15 | | ■ | | | | | | | Cerebral Vascular Accident |
| 10 | M | 73 | no | 4:00 | 23:08 | 47:25 | | ■ | | | | | | | Cancer- Pancreas |
| 11 | f | 80 | no | n/a | 23:38 | 31:25 | | ■ | | | | | | | Congestive Heart Failure |
| 12 | M | 81 | no | 2:12 | 24:54 | 29:07 | | ■ | | | | | | | Myocardial Infraction |
| 13 | F | 71 | no | 3:55 | 8:40 | 11:27 | | ■ | ■ | | | | | | Jaw Cancer |
| 14 | M | 73 | no | 3:59 | 8:29 | 11:52 | ■ | ■ | ■ | | | | | | Sepsis |
| 15 | F | 69 | no | 8:29 | 13:56 | 16:20 | ■ | ■ | ■ | | | | | | Cervical Cancer |
| 16 | M | 65 | no | n/a | 5:54 | 25:15 | ■ | ■ | ■ | | | | | | Septic Shock |
| 17 | F | 99 | AMD | 2:39 | 13:23 | 16:16 | ■ | ■ | ■ | | | | | | Sepsis |

**Supplementary Table S2.** Inventrory of the sialoglycans and controls used to print the siaolglycan arrays.

| Glycan ID | Compound | Sialic acid Type | Linkage | Acetylation / Sulfation | Linear / Branched | Glycan Type | MW |
|---|---|---|---|---|---|---|---|
| 1 | Neu5,9$Ac_2$α3Galβ4GlcNAcβR1 | Ac | 2-3 | 9OAc | L | LacNAcβ/Type 2 | 795.72 |
| 2 | Neu5Gc9Acα3Galβ4GlcNAcβR1 | Gc | 2-3 | 9OAc | L | LacNAcβ/Type 2 | 811.72 |
| 3 | Neu5,9$Ac_2$α6Galβ4GlcNAcβR1 | Ac | 2-6 | 9OAc | L | LacNAcβ/Type 2 | 795.72 |
| 4 | Neu5Gc9Acα6Galβ4GlcNAcβR1 | Gc | 2-6 | 9OAc | L | LacNAcβ/Type 2 | 811.72 |
| 5 | Neu5Acα6GalNAcαR1 | Ac | 2-6 | 0 | L | STn | 633.58 |
| 6 | Neu5Gcα6GalNAcαR1 | Gc | 2-6 | 0 | L | (Neu5Gc)-STn | 649.57 |
| 7 | Neu5,9$Ac_2$α3Galβ3GlcNAcβR1 | Ac | 2-3 | 9OAc | L | Type1 | 795.72 |
| 8 | Neu5Gc9Acα3Galβ3GlcNAcβR1 | Gc | 2-3 | 9OAc | L | Type1 | 811.72 |
| 9 | Neu5,9$Ac_2$α3Galβ3GalNAcαR1 | Ac | 2-3 | 9OAc | L | Core1/Type 3 | 795.72 |
| 10 | Neu5Gc9Acα3Galβ3GalNAcαR1 | Gc | 2-3 | 9OAc | L | Core1/Type 3 | 811.72 |
| 11 | Neu5Acα3Galβ4GlcNAcβR1 | Ac | 2-3 | 0 | L | LacNAcβ/Type 2 | 753.68 |
| 12 | Neu5Gcα3Galβ4GlcNAcβR1 | Gc | 2-3 | 0 | L | LacNAcβ/Type 2 | 769.68 |
| 13 | Neu5Acα3Galβ3GlcNAcβR1 | Ac | 2-3 | 0 | L | Type1 | 753.68 |
| 14 | Neu5Gcα3Galβ3GlcNAcβR1 | Gc | 2-3 | 0 | L | Type1 | 769.68 |
| 15 | Neu5Acα3Galβ3GalNAcαR1 | Ac | 2-3 | 0 | L | Core1/Type 3 | 753.68 |
| 16 | Neu5Gcα3Galβ3GalNAcαR1 | Gc | 2-3 | 0 | L | Core1/Type 3 | 769.68 |
| 17 | Neu5Acα6Galβ4GlcNAcβR1 | Ac | 2-6 | 0 | L | LacNAcβ/Type 2 | 753.68 |
| 18 | Neu5Gcα6Galβ4GlcNAcβR1 | Gc | 2-6 | 0 | L | LacNAcβ/Type 2 | 769.68 |
| 19 | Neu5Acα6Galβ4GlcβR1 | Ac | 2-6 | 0 | L | Lactoseβ | 712.63 |
| 20 | Neu5Gcα6Galβ4GlcβR1 | Gc | 2-6 | 0 | L | Lactoseβ | 728.63 |
| 21 | Neu5Acα3Galβ4GlcβR1 | Ac | 2-3 | 0 | L | Lactoseβ | 712.63 |
| 22 | Neu5Gcα3Galβ4GlcβR1 | Gc | 2-3 | 0 | L | Lactoseβ | 728.63 |
| 23 | Neu5,9$Ac_2$α6GalNAcαR1 | Ac | 2-6 | 9OAc | L | GalNAcα | 633.58 |
| 24 | Neu9Ac5Gcα6GalNAcαR1 | Gc | 2-6 | 9OAc | L | GalNAcα | 649.57 |
| 25 | Neu5Acα3GalβR1 | Ac | 2-3 | 0 | L | Galactoseβ | 550.49 |
| 26 | Neu5Gcα3GalβR1 | Gc | 2-3 | 0 | L | Galactoseβ | 566.49 |
| 27 | Neu5Acα6GalβR1 | Ac | 2-6 | 0 | L | Galactoseβ | 550.49 |
| 28 | Neu5Gcα6GalβR1 | Gc | 2-6 | 0 | L | Galactoseβ | 566.49 |

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|---|---|---|---|---|---|---|---|
| 29 | Neu5,9$Ac_2$α3GalβR1 | Ac | 2-3 | 9OAc | L | Galactoseβ | 592.52 |
| 30 | Neu9Ac5Gcα3GalβR1 | Gc | 2-3 | 9OAc | L | Galactoseβ | 608.52 |
| 31 | Neu5,9$Ac_2$α6GalβR1 | Ac | 2-6 | 9OAc | L | Galactoseβ | 592.52 |
| 32 | Neu9Ac5Gcα6GalβR1 | Gc | 2-6 | 9OAc | L | Galactoseβ | 608.52 |
| 33 | Neu5Acα3Galβ3GalNAcβR1 | Ac | 2-3 | 0 | L | Type 4 | 753.68 |
| 34 | Neu5Gcα3Galβ3GalNAcβR1 | Gc | 2-3 | 0 | L | Type 4 | 769.68 |
| 35 | Neu5,9$Ac_2$α3Galβ3GalNAcβR1 | Ac | 2-3 | 9OAc | L | Type 4 | 795.72 |
| 36 | Neu9Ac5Gcα3Galβ3GalNAcβR1 | Gc | 2-3 | 9OAc | L | Type 4 | 811.72 |
| 37 | Neu5,9$Ac_2$α6Galβ4GlcβR1 | Ac | 2-6 | 9OAc | L | | 754.66 |
| 38 | Neu9Ac5Gcα6Galβ4GlcβR1 | Gc | 2-6 | 9OAc | L | | 770.66 |
| 39 | Neu5,9$Ac_2$α3Galβ4GlcβR1 | Ac | 2-3 | 9OAc | L | | 754.66 |
| 40 | Neu9Ac5Gcα3Galβ4GlcβR1 | Gc | 2-3 | 9OAc | L | | 770.66 |
| 41 | Neu5Acα8Neu5Acα3Galβ4GlcβR1 | Ac-Ac | 2-8/2-3 | 0 | L | GD3-short linker | 1025.86 |
| 42 | Neu5Acα8Neu5Acα8Neu5Acα3Galβ4GlcβR1 | Ac-Ac-Ac | 2-8/2-8/2-3 | 0 | L | GT3- short linker | 1339.1 |
| 43 | Galβ4GlcβR1 | Sc | 0 | 0 | L | Lactose (Lac)- short linker | 399.39 |
| 44 | Galβ4Glcβ-$NH_2$ | Sc | 0 | 0 | L | Lac- amine | 341.31 |
| 45 | Galβ4GlcNAcβR1 | Sc | 0 | 0 | L | N-acetyl Lactosamine (LacNAc) | 440.44 |
| 46 | Galβ-$NH_2$ | Sc | 0 | 0 | L | | 179.17 |
| 47 | GalNAcαR1 | Sc | 0 | 0 | L | | 278.3 |
| 48 | GalβR2 | Sc | 0 | 0 | L | | 443.49 |
| 49 | GalβR1 | Sc | 0 | 0 | L | | 237.25 |
| 50 | Galβ4GlcβR2 | Sc | 0 | 0 | L | Lac- long linker | 605.63 |
| 51 | Galβ3GalNAcβR1 | Sc | 0 | 0 | L | T-antigen Core 1 (β) | 440.44 |
| 52 | Galβ3GalNAcαR1 | Sc | 0 | 0 | L | T-antigen Core 1 | 440.44 |
| 53 | Galβ3GlcNAcβR1 | Sc | 0 | 0 | L | | 440.44 |
| 54 | Galβ4GlcNAc6SβR1 | Sc | 0 | SO3 | L | 6-*O*-sulfo LacNAc | 542.49 |
| 55 | Neu5Acα3Galβ4(Fucα3)GlcNAcβR1 | Ac | 2-3 | 0 | B | (Neu5Ac)-Lewis$^X$ | 899.82 |
| 56 | Neu5Gcα3Galβ4(Fucα3)GlcNAcβR1 | Gc | 2-3 | 0 | B | (Neu5Gc)-Lewis$^X$ | 915.82 |
| 57 | Neu5Acα3Galβ4(Fucα3)GlcNAc6SβR1 | Ac | 2-3 | SO3 | B | (Neu5Ac)-6-*O*-sulfo Lewis$^X$ | 1001.86 |
| 58 | Neu5Gcα3Galβ4(Fucα3)GlcNAc6SβR1 | Gc | 2-3 | SO3 | B | (Neu5Gc)-6-*O*-sulfo Lewis$^X$ | 1017.86 |
| 60 | Neu5Acα3Galβ3GlcNAcβ3Galβ4GlcβR1 | Ac | 2-3 | 0 | L | (Neu5Ac)-LNT | 1077.96 |
| 61 | Neu5Gcα3Galβ3GlcNAcβ3Galβ4GlcβR1 | Gc | 2-3 | 0 | L | (Neu5Gc)-LNT | 1093.96 |

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|---|---|---|---|---|---|---|---|
| 62 | Neu5Acα3Galβ4GlcNAc6SβR1 | Ac | 2-3 | SO3 | L | (Neu5Ac)-6-O-sulfo LacNAc | 855.72 |
| 63 | Neu5Gcα3Galβ4GlcNAc6SβR1 | Gc | 2-3 | SO3 | L | (Neu5Gc)-6-O-sulfo LacNAc | 871.72 |
| 64 | Neu5Acα8Neu5Acα3Galβ4GlcβR4 | Ac-Ac | 2-8/2-3 | 0 | L | GD3- long linker | 1347.23 |
| 65 | Neu5Acα8Neu5Acα8Neu5Acα3Galβ4GlcβR4 | Ac-Ac-Ac | 2-8/2-8/2-3 | 0 | L | GT3-long linker | 1660.47 |
| 66 | Neu5Acα3(Neu5Acα6)Galβ4GlcβR1 | Ac(Ac) | 2-3(2-6) | 0 | B | | 1025.86 |
| 67 | Neu5Gcα3(Neu5Acα6)Galβ4GlcβR1 | Gc(Ac) | 2-3(2-6) | 0 | B | | 1041.86 |
| 68 | Kdnα3(Neu5Acα6)Galβ4GlcβR1 | Kdn(Ac) | 2-3(2-6) | 0 | B | | 984.81 |
| 69 | Neu5Gcα8Neu5Acα3Galβ4GlcβR1 | Gc-Ac | 2-8/2-3 | 0 | L | | 1041.86 |
| 70 | Kdnα8Neu5Acα3Galβ4GlcβR1 | Kdn-Ac | 2-8/2-3 | 0 | L | | 984.81 |
| 71 | Neu5Acα8Kdnα6Galβ4GlcβR1 | Ac-Kdn | 2-8/2-6 | 0 | L | | 984.81 |
| 72 | Neu5Acα8Neu5Gcα3Galβ4GlcβR1 | Ac-Gc | 2-8/2-3 | 0 | L | | 1041.86 |
| 73 | Neu5Acα8Neu5Gcα6Galβ4GlcβR1 | Ac-Gc | 2-8/2-6 | 0 | L | | 1041.86 |
| 74 | Kdnα8Neu5Gcα3Galβ4GlcβR1 | Kdn-Gc | 2-8/2-3 | 0 | L | | 1000.81 |
| 75 | Neu5Gcα8Neu5Gcα3Galβ4GlcβR1 | Gc-Gc | 2-8/2-3 | 0 | L | | 1057.86 |
| 77 | Neu5Gc$^{Me}$α8Neu5Acα3Galβ4GlcβR1 | Gc$^{Me}$-Ac | 2-8/2-3 | Me | L | | 1055.89 |
| 78 | Galα3Galβ4GlcNAcβR1 | Sc | 0 | 0 | L | | 602.58 |
| 79 | Neu4,5Ac$_2$α3Galβ4GlcNAcβR1 | Ac | | 4OAc | L | | |
| 80 | Neu5Acα3Galβ4GlcβR3 | Ac | | | L | | |
| 81 | Legα3Galβ4GlcβR3 | Leg | | | L | | |
| 82 | Neu5Ac9NAcα3Galβ4GlcβR1 | Ac | | 9NAc | L | | |
| 83 | Gal6Sβ4(Fucα3)GlcNAcβR1 | | | SO3 | B | 6'-*O*-Sulfo Lewis$^X$ | 688.63 |
| 84 | Neu5Acα3Gal6Sβ4(Fucα3)GlcNAcβR1 | Ac | | SO3 | B | 6'-O-Sulfo Neu5Ac-Lewis$^X$ | 1001.86 |
| 85 | Neu5Gcα3Gal6Sβ4(Fucα3)GlcNAcβR1 | Gc | | SO3 | B | 6'-O-Sulfo Neu5Gc-Lewis$^X$ | 1017.86 |
| 86 | Gal6Sβ4(Fucα3)GlcNAc6SβR1 | | | SO3 | B | 6,6'-di-O-Sulfo Lewis$^X$ | 790.67 |
| 87 | Neu5Acα3Gal6Sβ4(Fucα3)GlcNAc6SβR1 | Ac | | SO3 | B | 6,6'-di-O-Sulfo Neu5Ac Lewis$^X$ | 1103.9 |
| 88 | Neu5Gcα3Gal6Sβ4(Fucα3)GlcNAc6SβR1 | Gc | | SO3 | B | 6,6'-di-O-Sulfo Neu5Ac Lewis$^X$ | 1119.9 |
| 89 | Galβ4GlcNAcβ3Galβ4GlcβR1 | | | | L | | 764.73 |
| 90 | Neu5Acα3Galβ4GlcNAcβ3Galβ4GlcβR1 | Ac | | | L | | 1077.97 |
| 91 | Neu5Gcα3Galβ4GlcNAcβ3Galβ4GlcβR1 | Gc | | | L | | 1093.96 |
| 92 | Neu5,9Ac$_2$α3Galβ4GlcNAcβ3Galβ4GlcβR1 | Ac | | 9OAc | L | | 1120 |
| 93 | Neu5Gc9Acα3Galβ4GlcNAcβ3Galβ4GlcβR1 | Gc | | 9OAc | L | | 1136 |
| 94 | Kdnα3Galβ4GlcβR1 | Kdn | | | L | | |

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|---|---|---|---|---|---|---|---|
| 95 | Kdnα6Galβ4GlcβR1 | Kdn | | | L | | 754.67 |
| 96 | Neu5Acα3(GalNAcβ4)Galβ4GlcβR1 | Ac | | | B | GM2 | 1120 |
| 97 | Neu5Gcα3(GalNAcβ4)Galβ4GlcβR1 | Gc | | | B | GM2 | 795.72 |
| 98 | Neu5Acα8Neu5Acα3(GalNAcβ4)Galβ4GlcβR1 | Ac-Ac | | | B | GD2 | 795.72 |
| 99 | Neu4,5$Ac_2$α3Galβ4GlcβR1 | Ac | 2-3 | 4OAc | L | | 795.72 |
| 100 | Neu4,5$Ac_2$α3Galβ4GlcNAcβ3Galβ4GlcβR1 | Ac | 2-3 | 4OAc | L | | 795.72 |
| 101 | Neu4,5$Ac_2$α3Galβ3GlcNAcβR1 | Ac | 2-3 | 4OAc | L | | 811.72 |
| 102 | Neu4,5$Ac_2$α3Galβ3GlcNAcαR1 | Ac | 2-3 | 4OAc | L | | 770.67 |
| 103 | Neu4,5$Ac_2$α3Galβ3GalNAcβR1 | Ac | 2-3 | 4OAc | L | | 1136 |
| 104 | Neu4,5$Ac_2$α3Galβ3GalNAcαR1 | Ac | 2-3 | 4OAc | L | | 811.72 |
| 105 | Neu4Ac5Gcα3Galβ4GlcNAcβR1 | Gc | 2-3 | 4OAc | L | | 811.72 |
| 106 | Neu4Ac5Gcα3Galβ4GlcβR1 | Gc | 2-3 | 4OAc | L | | 811.72 |
| 107 | Neu4Ac5Gcα3Galβ4GlcNAcβ3Galβ4GlcβR1 | Gc | 2-3 | 4OAc | L | | 811.72 |
| 108 | Neu4Ac5Gcα3Galβ3GlcNAcβR1 | Gc | 2-3 | 4OAc | L | | 753.68 |
| 109 | Neu4Ac5Gcα3Galβ3GlcNAcαR1 | Gc | 2-3 | 4OAc | L | | 591.54 |
| 110 | Neu4Ac5Gcα3Galβ3GalNAcβR1 | Gc | 2-3 | 4OAc | L | | 591.54 |
| 111 | Neu4Ac5Gcα3Galβ3GalNAcαR1 | Gc | 2-3 | 4OAc | L | | 794.74 |
| 112 | Neu5Ac9NAcα6Galβ4GlcβR1 | Ac | 2-6 | 9NAc | L | | 794.74 |
| 113 | Neu5Ac9NAcα3GalβR1 | Ac | 2-3 | 9NAc | L | | 794.74 |
| 114 | Neu5Ac9NAcα6GalβR1 | Ac | 2-6 | 9NAc | L | | 794.74 |
| 115 | Neu5Ac9NAcα3Galβ4GlcNAcβR1 | Ac | 2-3 | 9NAc | L | | 794.74 |
| 116 | Neu5Ac9NAcα6Galβ4GlcNAcβR1 | Ac | 2-6 | 9NAc | L | | 794.74 |
| 117 | Neu5Ac9NAcα3Galβ3GlcNAcβR1 | Ac | 2-3 | 9NAc | L | | 794.74 |
| 118 | Neu5Ac9NAcα6Galβ3GlcNAcβR1 | Ac | 2-6 | 9NAc | L | | 632.6 |
| 119 | Neu5Ac9NAcα3Galβ3GalNAcαR1 | Ac | 2-3 | 9NAc | L | | |
| 120 | Neu5Ac9NAcα6Galβ3GalNAcαR1 | Ac | 2-6 | 9NAc | L | | |
| 121 | Neu5Ac9NAcα3Galβ3GlcNAcαR1 | Ac | 2-3 | 9NAc | L | | |
| 122 | Neu5Ac9NAcα6GalNAcαR1 | Ac | 2-6 | 9NAc | L | | |
| 123 | Neu5Acα3Galβ4GlcNAcβR5 | Ac | 2-3 | | L | | 988.98 |
| 124 | Neu5Acα6Galβ4GlcNAcβR5 | Ac | 2-6 | | L | | 988.98 |
| 125 | Neu5Acα6Galβ4GlcβR5 | Ac | 2-6 | | L | | 947.93 |
| 126 | Neu5Acα3Galβ4GlcβR5 | Ac | | | L | | 947.93 |

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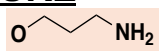
|---|---|---|---|---|---|---|---|
| 127 | Neu5Ac9NAcα3Galβ3GalNAcβR1 | Ac | | 9NAc | L | | 794.74 |
| 128 | Neu5Ac9NAcα6Galβ3GalNAcβR1 | Ac | | 9NAc | L | | 794.74 |
| 129 | Neu5Ac9NAcα6Galβ3GlcNAcαR1 | Ac | | 9NAc | L | | 794.74 |
| 130 | Neu5Ac9NAcα8Neu5Acα3Galβ4GlcβR1 | Ac-Ac | | 9NAc | L | GD3 | 1066.92 |
| 131 | Neu5Ac7NAcα3Galβ4GlcβR1 | Ac | | 7NAc | L | | 753.68 |
| 132 | Kdnα3GalβR1 | Kdn | | | L | | 509.44 |
| 133 | Kdnα6GalβR1 | Kdn | | | L | | 509.44 |
| 134 | Kdnα3Galβ4GlcNAcβR1 | Kdn | | | L | | 712.63 |
| 135 | Kdnα6Galβ4GlcNAcβR1 | Kdn | | | L | | 712.63 |
| 136 | Kdnα3Galβ3GlcNAcβR1 | Kdn | | | L | | 712.63 |
| 137 | Kdnα6Galβ3GlcNAcβR1 | Kdn | | | L | | 712.63 |
| 138 | Kdnα3Galβ3GlcNAcαR1 | Kdn | | | L | | 712.63 |
| 139 | Kdnα6Galβ3GlcNAcαR1 | Kdn | | | L | | 712.63 |
| 140 | Kdnα3Galβ3GalNAcαR1 | Kdn | | | L | | 712.63 |
| 141 | Kdnα6Galβ3GalNAcαR1 | Kdn | | | L | | 712.63 |
| 142 | Kdnα6GalNAcαR1 | Kdn | | | L | | 550.49 |
| 143 | Kdnα3Galβ4GlcNAcβ3Galβ4GlcβR1 | Kdn | | | L | | 1036.91 |
| 144 | Kdnα3Galβ4(Fucα3)GlcNAcβR1 | Kdn | | | B | $Lewis^X$ | 858.77 |
| 145 | Kdnα3Galβ3(Fucα4)GlcNAcβR1 | Kdn | | | B | $Lewis^A$ | 858.77 |
| 224 | Galβ4GlcβR6 | | | | L | | 1100.16 |
| 225 | Galβ4GlcNAcβR6 | | | | L | | 1141.21 |
| 226 | Galβ3GalNAcαR6 | | | | L | | 1141.21 |
| 227 | Neu5Acα3Galβ4GlcβR6 | Ac | 2-3 | | L | | 1391.41 |
| 228 | Neu5Acα6Galβ4GlcβR6 | Ac | 2-6 | | L | | 1391.41 |
| 229 | Neu5Acα3Galβ4GlcNAcβR6 | Ac | 2-3 | | L | | 1432.47 |
| 230 | Neu5Acα6Galβ4GlcNAcβR6 | Ac | 2-6 | | L | | 1432.47 |
| 231 | Neu5Acα3Galβ3GalNAcαR6 | Ac | 2-3 | | L | | 1432.47 |
| 232 | Legα3Galβ4GlcβR6 | Leg | 2-3 | | L | | 1416.47 |
| 233 | Legα3Galβ4GlcNAcβR6 | Leg | 2-3 | | L | | 1457.52 |
| 234 | Legα6Galβ4GlcNAcβR6 | Leg | 2-6 | | L | | 1457.52 |
| 235 | Legα3Galβ3GalNAcαR6 | Leg | 2-3 | | L | | 1457.52 |
| 236 | Galβ3(Fucα4)GlcNAcβR1 | | | | B | $Lewis^A$ | 586.59 |

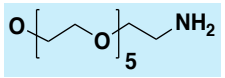
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 237 | Fucα2Galβ3(Fucα4)GlcNAcβR1 | | | | B | $Lewis^B$ | 732.73 |
| 238 | Neu5Acα3Galβ3(Fucα4)GlcNAcβR1 | Ac | 2-3 | | B | $Lewis^A$ | 899.83 |
| 239 | Neu5Gcα3Galβ3(Fucα4)GlcNAcβR1 | Gc | 2-3 | | B | $Lewis^A$ | 915.82 |
| 240 | Neu5,9$Ac_2$α3Galβ3(Fucα4)GlcNAcβR1 | Ac | 2-3 | 9OAc | B | $Lewis^A$ | 941.86 |
| 241 | Neu5Ac9NAcα3Galβ3(Fucα4)GlcNAcβR1 | Ac | 2-3 | 9NAc | B | $Lewis^A$ | 940.87 |
| 242 | GlcNAcβ4Galβ4GlcβR1 | | | | L | GA2 | 602.58 |
| 243 | Neu5,9$Ac_2$α8Neu5Acα3Galβ4GlcβR1 | Ac-Ac | 2-8 | 9OAc | L | GD3 | 1044.92 |
| 244 | Neu5Acα8Neu5Acα3(Galβ3GalNAcβ4)Galβ4GlcβR1 | Ac-Ac | 2-8 | | B | GD1b | 1368.21 |
| 245 | Neu5Acα3(Galβ3GalNAcβ4)Galβ4GlcβR1 | Ac | 2-3 | | B | GM1 | 1077.97 |
| 246 | Neu5Gcα3(Galβ3GalNAcβ4)Galβ4GlcβR1 | Gc | 2-3 | | B | GM1 | 1093.96 |
| 247 | Neu5Acα3(Fucα2Galβ3GalNAcβ4)Galβ4GlcβR1 | Ac | 2-3 | | B | Fuc-GM1 | 1224.11 |
| 248 | Neu5Gcα3(Fucα2Galβ3GalNAcβ4)Galβ4GlcβR1 | Gc | 2-3 | | B | Fuc-GM1 | 1240.11 |
| 249 | Neu5Ac7NAcα3Galβ4GlcNAcβR1 | Ac | 2-3 | 7NAc | L | LacNAcβ/Type2 | 772.76 |
| 250 | Neu5Acα3Galβ3GlcNAcαR1 | | | | L | | 753.68 |
| 251 | Neu5Acα6Galβ3GlcNAcβR1 | | | | L | | 753.68 |
| 252 | Neu5Gcα6Galβ3GlcNAcβR1 | | | | L | | 769.68 |
| 253 | Neu5Acα6Galβ3GalNAcβR1 | | | | L | | 753.68 |
| 254 | Neu5Gcα6Galβ3GalNAcβR1 | | | | L | | 769.68 |
| 255 | Neu5Ac8Meα3Galβ4GlcβR1 | | | | L | | 726.66 |
| 256 | Neu5Ac8Meα6Galβ4GlcβR1 | | | | L | | 726.66 |
| 257 | $Neu5Gc^{Me}$α3Galβ4GlcβR1 | | | | L | | 742.66 |
| 258 | $Neu5Gc^{Me}$α6Galβ4GlcβR1 | | | | L | | 742.66 |
| 259 | Kdn5Meα3Galβ4GlcβR1 | | | | L | | 685.6 |
| 260 | Kdn5Meα6Galβ4GlcβR1 | | | | L | | 685.6 |
| 261 | Kdn5Acα6Galβ4GlcβR1 | | | | L | | 713.61 |
| 262 | $Neu5Gc^{Ac}$α6Galβ4GlcβR1 | | | | L | | 770.67 |
| 263 | Kdn9Acα6Galβ4GlcβR1 | | | | L | | 713.61 |
| 264 | Neu5Ac9Ltα6Galβ4GlcβR1 | | | | L | | 784.69 |
| 265 | Kdn9Meα3Galβ4GlcβR1 | | | | L | | 685.6 |
| 266 | Kdn9Meα6Galβ4GlcβR1 | | | | L | | 685.6 |
| 267 | $Kdn^{8\text{-deoxy}}$α3Galβ4GlcβR1 | | | | L | | 655.58 |
| 268 | Kdn9Fα3Galβ4GlcβR1 | | | | L | | 673.57 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 269 | Kdn9Fα6Galβ4GlcβR1 | | | | L | | 673.57 |
| 270 | Kdn5Fα3Galβ4GlcβR1 | | | | L | | 673.57 |
| 271 | Kdn5Fα6Galβ4GlcβR1 | | | | L | | 673.57 |
| 272 | Neu5TFAα3Galβ4GlcβR1 | | | | L | | 766.6 |
| 273 | 4,6-*bis*-epi-Kdoα6Galβ4GlcβR1 | | | | L | | 641.55 |
| 274 | $Kdn^{8\text{-deoxy}}$α6Galβ4GlcβR1 | | | | L | | 655.58 |
| 275 | Neu5,7,9triNAca2-3LacbPro$NH_2$ | Ac | | | L | | 794.74 |
| 276 | Neu5,7,9triNAca2-6LacbPro$NH_2$ | Ac | | | L | | 794.74 |
| 277 | Neu5,7diNAca2-6LacbPro$NH_2$ | Ac | | | L | | 753.68 |
| 278 | Neu5,7diNAca2-3LacbPro$NH_2$ | Ac | | | L | | 753.68 |
| 279 | Neu5,7diNAca2-6GalNAcaPro$NH_2$ | Ac | | | L | | 632.6 |
| 280 | Neu5,7,9triNAca2-6GalNAcaPro$NH_2$ | Ac | | | L | | 673.65 |
| 281 | Leg5,7diNAca2-6LacbPro$NH_2$ | Leg | | | L | | 737.68 |
| 282 | Leg5,7diNAca2-3LacNAcbPro$NH_2$ | Leg | | | L | | 778.74 |
| 283 | Leg5,7diNAca2-3Galb1-3GalNAcbPro$NH_2$ | Leg | | | L | | 778.74 |
| 284 | Neu4,5diNAca2-3LacbPro$NH_2$ | Ac | | | L | | 753.68 |
| 285 | Neu4,5diNAca2-3LacNAcbPro$NH_2$ | Ac | | | L | | 794.74 |

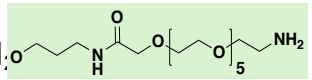
blank
IgG control
IgG(100 ng/uL)
IgG(12.5 ng/uL)
IgG(150 ng/uL)
IgG(25 ng/uL)
IgG(3.12 ng/uL)
IgG(50 ng/uL)
IgG(6.25 ng/uL)
IgG(75 ng/uL)
water

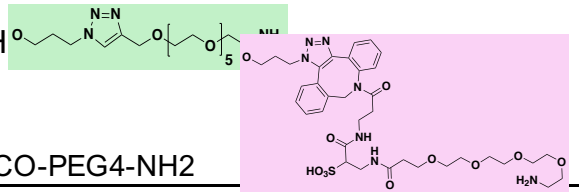
## LINKER STRUCTURE

R1 = Pro-$NH_2$ 

R2 = HEG-$NH_2$ 

R3 = Aryl amine

R4 = Pro-HEG-$NH_2$ 

R5 = Pro-Htrz-TEG-NH 

R6 = Pro-Htrz-Sulfo-DBCO-PEG4-NH2