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Characterization and Quantitation of Human Milk Oligosaccharides
using LC-MS based methods- Impacts on Fetal Development and Infant
Health

By

ANITA VINJAMURI
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

_____
Carlito B. Lebrilla, Chair

_____
Gang-Yu Liu

_____
Carolyn Slupsky

Committee in Charge

2022

i

DEDICATION

To my mom, my husband, my many pets, and my family and friends

for their love and support

ACKNOWLEDGEMENTS

I'd like to first thank my mom who raised me to never give up on my dreams and always keep going. Without your sacrifice of moving to the United States I would not be where I am today. I know that my life changed completely in just a couple of years, but I will always be your little girl. Thank you for teaching me to take life a little less seriously and reminding me that there is so much more to look forward to. Your enthusiasm, passion and lighthearted energy has helped me overcome a lot of obstacles. Because of you: the way you raised me, supported me, and nurtured me; I have accomplished what I have to this day.

Marques, thank you for being the best husband, best friend, and teammate a women could ask for. I know that this journey has not been easy, but you have always believed in me even when I didn't and that means so much to me. I know my experiments often made little sense to you but thank you for caring, for listening, and for being my number one cheerleader. Thank you for calming me down when things didn't go my way in the lab and for always making me see things in a different perspective. I value your opinion so much and you always find the right words to put me at ease. You make light of every situation and can make me laugh through the toughest times. Thank you for staying by my side through the tears, late nights in lab, weekends away, and exhaustion. While this accomplishment was not easy, it would have been impossible without you.

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all my ideas (even if they weren't the best). If it wasn't for you, I would not have found my passion in science, and I most definitely would not have gotten my PhD.

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Characterization and Quantitation of Human Milk Oligosaccharides using LC-MS based methods- impacts on fetal development and infant health

Abstract

Human Milk Oligosaccharides (HMOs) are the third most abundant solid component in human breast milk, consisting of hundreds of unique structures. HMOs are indigestible by the infant but have shown to be very beneficial to the infant's development, making them an intriguing constituent. However, due to the lack of standards, reliable methods for quantitation, and complexity of performing large scale analysis, there are limits to our general knowledge of their abundances and functions. This dissertation focuses on the development and implication of mass spectrometry-based methods to characterize and quantitate HMOs to address unanswered questions in the field.

Chapter 1 provides an overview of HMO structures and the functions they collectively play in infant development. Chapter 2 details the development of a comprehensive library and a high-throughput method that allowed for accurate quantitation of HMOs using high resolution mass spectrometry. Optimized methods were applied to breast milk samples collected from over 2000 mothers from 19 geographically diverse sites to investigate how HMO profiles vary across the globe. The results revealed significant phenotypic variations in the mother's milk and secretor status globally. Chapter 3 reports discovery of several oligosaccharides, inclusive of 8 HMOs, found in amniotic fluid. This chapter detailed the methodologies from sample preparation to data analysis and was applied to a cohort of over 500 mothers, making it the most comprehensive

study of amniotic fluid to date. Analysis revealed compositional changes in HMO profiles across

gestation. Chapter 4 studies demonstrated that select HMOs, including linkage-specific sialylated

structures, can act as decoys to prevent SARS-Cov2 infection. This study also investigated the

mechanism of binding between the spike protein and the ACE2 receptor on the cell surface. The

results detailed here exemplify the important role that cell surface glycosylation plays in host-

pathogen interactions.

Glossary of Abbreviations

ACN – acetonitrile

CID – collision induced dissocation

FA – Formic Acid

Fuc – Fucose

EIC – extracted ion chromatogram

FUT2 – α(1-2)-fucosyltransferase

Gal – Galactose

Glc – Glucose

GlcNAc – N-acetylglucosamine

Hex – Hexose

HexNAc – N-acetylhexosamine

HMO – Human Milk Oligosaccharide

LC – liquid chromatography

MS – mass spectrometry

Neu5Ac – N-acetylneuraminic acid

PGC – Porous Graphitized Carbon

Ppm – Parts per million

Q-TOF – quadrupole orthogonal time-of-flight

RT – Retention time

SD – Standard deviation

SPE – Solid phase extraction

TOF – time-of-flight

TFA – Trifluoracetic acid

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CHAPTER ONE

INTRODUCTION

Human breast milk is recognized by the World Health Organization as the ideal source of nutrition for infants in at least the first six months of life.[1] Mother's milk contains all the components essential for the healthy development of a growing infant. Among food and diet, human milk is unique in that it is tailored to the nutritional needs of the baby, constantly adapting in compositions to the infant's developmental requirements.

The composition of human milk is complicated but well-regulated and consists of macronutrients from three major groups: fats, proteins, and carbohydrates. The most abundant component are the carbohydrates, which are predominantly composed of lactose corresponding to approximately 70 g/L of the constituents in milk.[2] A unique subset of the carbohydrates is comprised of longer oligomers based on a lactose core known collectively as human milk oligosaccharides (HMOs). While HMOs are significantly lower in abundance than lactose, they still comprise a large fraction of the dry mass of mother's milk and can even be more abundant than proteins particularly during early lactation. Although lactose is composed of a galactose bound to a reducing glucose through an β(1,4) linkage, human milk oligosaccharides (HMOs) are built by adding further to the lactose core with the addition of galactoses and glucose as well as terminal decorations of fucose and sialic acids. Endogenous human enzymes in the gut readily break down lactose to provide fuel and mass for the growth of the infant. However, there are no human enzymes in the gut in appreciable amounts that break down HMOs. Thus, the mystery since

the characterization of the first HMO structure in 1930[3] was – why do mothers produce these compounds in large amounts when they provide no direct nutritional value to the infant?

The answer to this question has been debated since the discovery of HMOs. However, new analytical and rapid genome sequencing tools have provided definitive answers that are even more remarkable, namely that mothers use HMOs to recruit bacteria by feeding them so that they can provide benefits to the infant. While the advancements in genome sequencing are discussed in detail elsewhere, less is known regarding the major advances in analytical techniques for macromolecular analysis, in particular liquid chromatography – mass spectrometry (LC-MS). LC-MS based methods have revealed many unknown HMO structures, and additionally provided rapid profiling with quantitation. These methods have provided HMO abundances in human milk as well as other bodily fluids and tissues including blood[4] and urine.[5] This contribution attempts to summarize a general view of HMOs through their structural analyses, compositions, and functions.

This work has been published in the Encyclopedia of Cellular Biology and is included with permission in this thesis.

Structures of HMO

HMOs are composed of five monosaccharides: L-fucose, D-glucose, D-galactose, N-acetylglucosamine and N-acetylneuraminic (sialic) acid (**Figure 1.1**). The foundation of every HMO consists of lactose core (a disaccharide comprised of glucose and galactose) at the reducing end, which is then further elongated with additional monosaccharides to provide linear and branched structures with the branching occurring at the galactose of the lactose core. Despite the decades of studies on HMOs, their biosynthesis is still not fully resolved. As glycans, HMOs are most similar in structure to O-glycans on proteins and some glycolipids (some having a lactose

core) than to N-glycans, therefore it is commonly acknowledged that the syntheses of HMOs is likely to proceed through similar pathways. Genomic studies have also been performed to examine the variations in genes that code the same or similar transferases.[6] Further studies have attempted to correlate the gene and corresponding glycosyl transferase to HMO structures, identifying perhaps the sets of glycosyl transferases responsible for the structural diversity of these compounds.[7]

D-Glucose	D-Galactose	L-Fucose	N-Acetyl neuraminic acid	N-Acetyl glucosamine (GlcNAc)
Empirical Formula: $C_6H_{12}O_6$	Empirical Formula: $C_6H_{12}O_6$	Empirical Formula: $C_6H_{12}O_5$	Empirical Formula: $C_{11}H_{19}NO_9$	Empirical Formula: $C_8H_{15}NO_6$
Monoisotopic Mass: 180.0634	Monoisotopic Mass: 180.0634	Monoisotopic Mass: 164.0685	Monoisotopic Mass: 309.1060	Monoisotopic Mass: 221.0899
Symbol:	Symbol:	Symbol:	Symbol:	Symbol:

Figure 1.1 Five building blocks of human milk oligosaccharides.

The addition of even a single monosaccharide residue readily differentiates the trisaccharide structure from the lactose core in several important ways. For example, the addition of a fucose to lactose produces fucosyllactose, while the addition of a sialic acid to lactose yields sialyllactose. Although lactose is readily digestible by human enzymes, adding a monosaccharide with no corresponding enzymes makes it indigestible to the infant. Most glycosyl hydrolases are exoglycosidase, which means they start at the nonreducing end or away from the lactose core. The

addition of either a sialic acid or fucose significantly alters the chemical characteristics and the biological functions of resulting compounds from each other and from lactose. Sialylated species contain a carboxylic acid group, making sialylated oligosaccharides acidic or anionic. Fucosylated and undecorated oligosaccharides are considered electronically neutral. The addition of even one monosaccharide can add significant variability in the resulting structures particularly with how it connects to the lactose core. The addition of fucose can occur on the 2, 3, 4, and 6 positions of the galactose. Additionally, the orientation of the linkage (α or β) at these positions results in even greater variability. However, the large number of possible structures are limited by the small number of glycosyl transferases. Thus, for example, although the addition of fucose may occur in 14 possible combinations, in reality only two of those are found in HMOs. The addition of fucose to lactose can occur either to the 2-position of the galactose to produce 2'-fucosyllactose (2'FL) or on the 3-position of the glucose to yield 3-fucosyllactose (3-FL).

HMOs may contain from 3 to more than 20 monosaccharides, however the most abundant structures have degree of polymerization between three and seven.[8] The potential structural diversity represented by all these variables were previously extrapolated to predict a large number of potential structures, with some estimates suggesting that as many as 10^9 structures were possible. However, LC-MS eventually resolved this issue by showing that in a single mother, there are only about 100 structures over the five orders of magnitude in dynamic range of the analytical method. When the milk from five mothers were combined, less than 300 structures were obtained. [9] The glycans that make up the ABO blood type are also shown in **Figure 1.2** for comparison illustrating the shared similarities between HMOs and glycans on glycolipids that define the blood type. With the analogy to the blood type, HMOs stratify individuals so that we should also develop a method for typing milk.
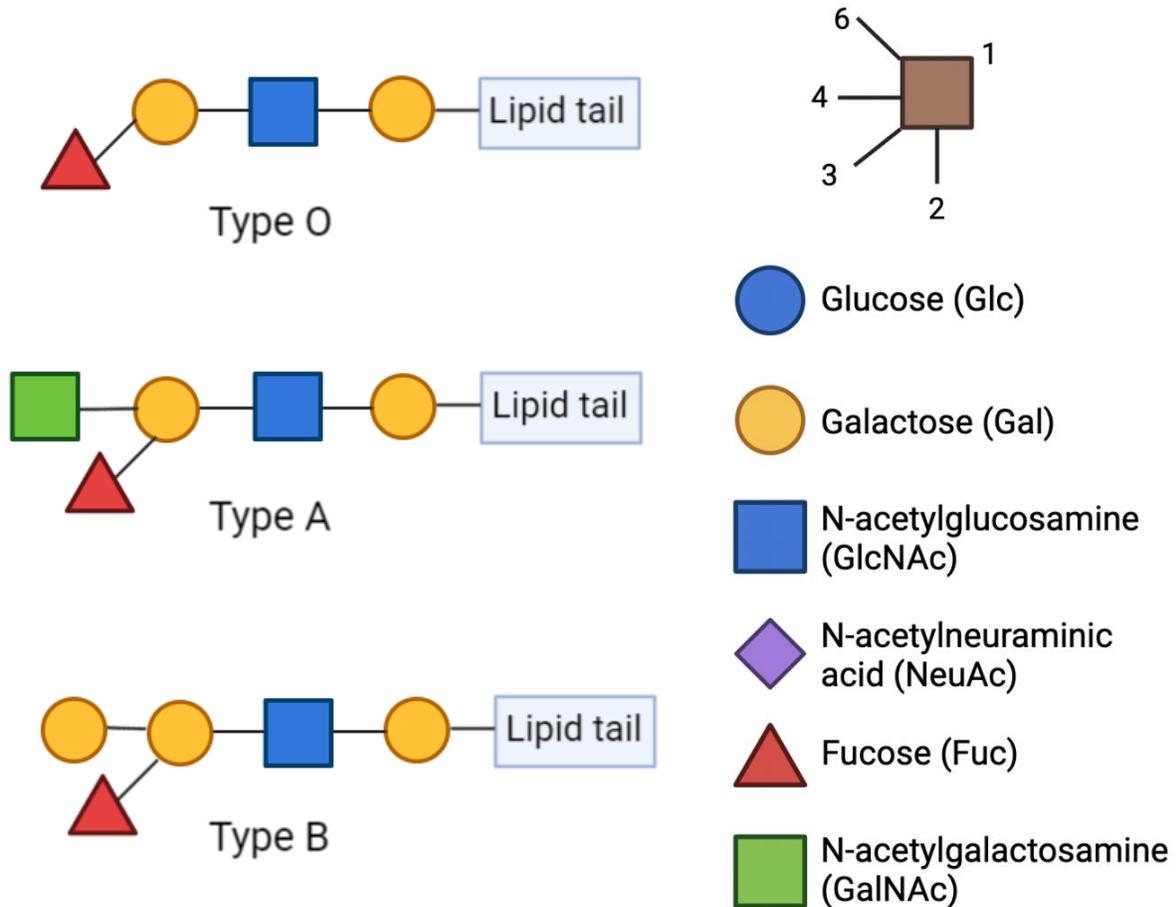
Figure 1.2 Glycan composition on red blood cell distinguishes A, B, AB, and O blood groups.

HMOs can be classified into four structural subgroups based on their compositions: fucosylated, sialylated, sialofucosylated, and undecorated. Oligosaccharides with at least one fucose are fucosylated. Likewise, oligosaccharides with at least one sialic acid (N-acetylneuraminic acid) unit are sialylated, and structures with both are sialofucosylated. Undecorated structures contain neither a fucose nor a sialic acid and are only composed of glucose, galactose, and GlcNac (N-acetylglucosamine). In general, mothers will have 35-60% fucosylated HMOs, 12-14% sialylated HMOs, 40-50% undecorated HMOs and 0-15% sialofucosylated.[10] As

discussed below, various factors such as geographical origin, genetic, and infant's age contribute to the variations in absolute and relative abundances of HMOs. However, the fraction of these distinct group of components varies greatly among mammalian species.

Structural Analysis of HMOs yields large numbers of structures

Structural analysis of individual structures and quantitative profiling methods were key to elucidating the functions of HMOs. While there are general functional activities associated with the entire collections of structures, individual structures or group of structures are found to have unique and specific biological function. Thus, despite the complexity and heterogeneity of the structures, systematic structural analysis was important in elucidating the roles of individual HMOs. There have been individual efforts in determining specific structures, however there was no consistent earlier effort to analyze oligosaccharide structures comprehensively.[11, 12] Even now there remains no universal method for HMO analysis, however there are currently more systematic methods that provide good representation of the collection.

Isolation of HMOs from milk was complicated by the large abundances of lactose impeding the analysis of HMOs. However, solid phase extraction methods have made this more routine. A method for extensive profiling of HMOs structures now involves separation with liquid chromatography using a mass spectrometry detector. Preparation of HMOs using this method require four major steps: defatting, protein precipitation, reduction of HMOs, and solid phase extraction. The result is the production of chemically reduced structures or alditols that removes the anomeric reducing end to produce a single peak in the chromatogram.

A method developed for the comprehensive structural elucidation of many structures includes the use of liquid chromatography – mass spectrometry (LC-MS), tandem MS (MS/MS), and enzymatic digestion. In this approach, LC is used to isolate the individual compounds and MS to determine mass. LC-MS/MS in combination with exoglycosidases were used to determine the monosaccharide components and their linkages. Exoglycosidases are glycan cleaving enzymes that selectively cleave terminal residues and are highly specific to linkage, stereochemistry, and configuration of the anomeric carbon at the linkage. By systematically probing each HMO, the monosaccharides that make up the oligosaccharide and the specific linkages between each saccharide were deduced.[13] This LC-MS method provided a more systematic analysis by allowing the monitoring of hundreds of structures simultaneously with quantitative information making large clinical trials feasible with comprehensive HMO analyses (**Figure 1.3**).[14]
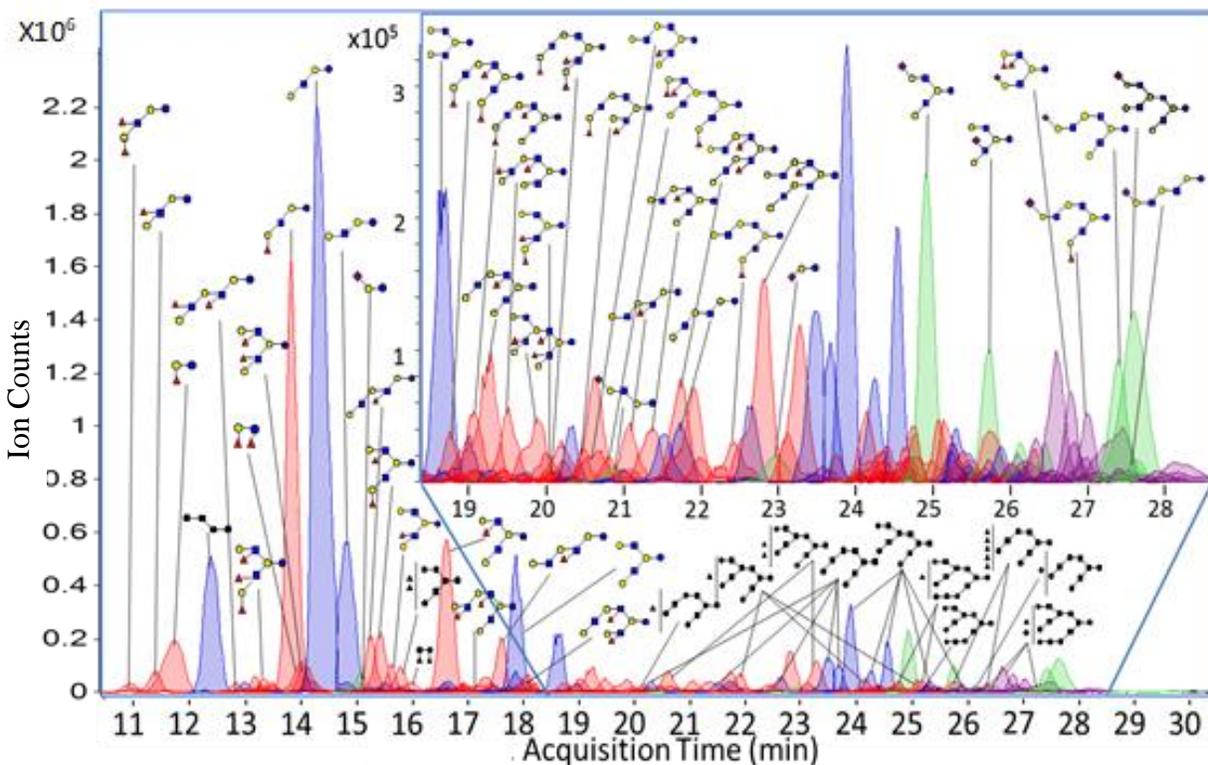
Figure 1.3 LC-MS chromatogram of human milk oligosaccharides.

While semi-quantification (relative abundances) of HMOs more readily achieved, absolute quantitation of each structure remains problematic. There are very few HMO standards of sufficient quantity and purity for producing standard solutions. While LC-MS profiling provides relative quantitation, there are differences in ionization and detection efficiencies with LC-MS. Methods that employ spectrometric detection such as fluorescence and ultraviolet-visible are used for HMO analysis and are attractive because they are generally cheaper and provide similar detection efficiencies for each structure. These methods are however structurally non-specific, thus a shift in LC retention time or coeluting structures can complicate the identification of the compounds. Additionally, they require the addition of a label. While the detection efficiency is similar for the different labeled compounds facilitating quantitation, the reactivity of the HMOs toward the label can vary slightly. The labeling method also must be highly efficient. Even with 99% efficiency, this would still render the much less abundant species unidentifiable and more difficult to quantitate further decreasing the dynamic range to two as opposed to four or five for LC-MS. HMO methods for analysis are still constantly being refined. New methods that require no extensive enrichment, derivatization, with high quantitation have become available.[8] LC-MS, using a triple quadrupole detector, was employed with multiple reaction monitoring (MRM) to identify specific structures with quantitation. This method is fast and provides absolute quantitation and will likely play a greater role in the future HMO analysis.

A systematic analysis of the structures and their abundances showed that a small number of structures dominate the abundances.[15] **Table 1.1** provides a schematic representation of the 30 most common HMO structures representing 80% of the abundances in mother's milk. Each mother has approximately 100 structures, with about 110 representing 100% of the HMO abundances.[15] About 50 structures represent 90% of the abundances, while 18 structures represent 70% of the

abundances.   The accumulation of milk from five mothers may produce more than 300 unique structures. The smaller structures contain mono-fucosylation or sialylation with unique antigens such as Lewis b and Lewis x, while the larger ones can contain multi-fucosylation and sialylation with multiple antigens present in the same structure.

Table 1.1 The 30 most abundant oligosaccharides found in human breast milk. Monosaccharide composition of structures are given as Hex_HexNAc_Fuc_Neu5Ac and represented as glucose (●), galactose (●), N-acetylglucosamine (■), and fucose(▲).

HMO	Structure	Mass	Composition			
			Hex	HexNAc	Fuc	NeuAc
6'SL		635.2272	2	0	0	1
3'SL		635.2272	2	0	0	1
3'FL		490.190	2	0	1	0
2'FL		490.190	2	0	1	0
LDFT		363.248	2	0	2	0
LNT		709.264	3	1	0	0
LNnT		709.264	3	1	0	0
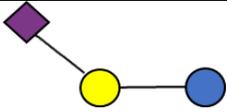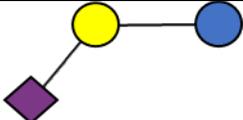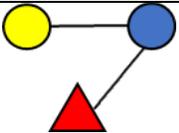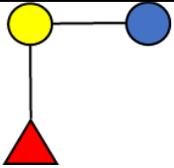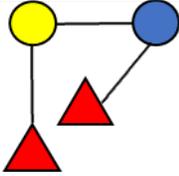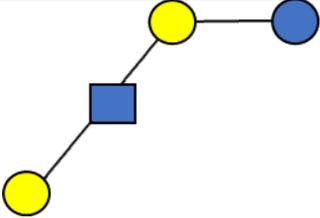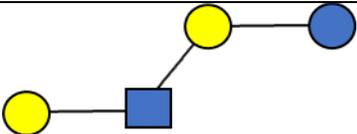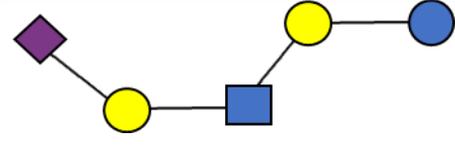
LST c		1000.359	3	0	1	1
LST b		1000.359	3	0	1	1
LST a		1000.359	3	0	1	1
LNFP II		855.322	3	1	1	0
LNFP III		855.322	3	1	1	0
LNFP I		855.322	3	1	1	0
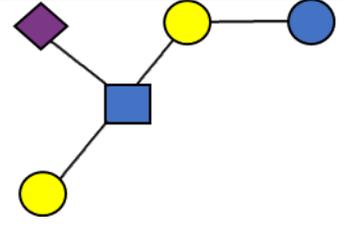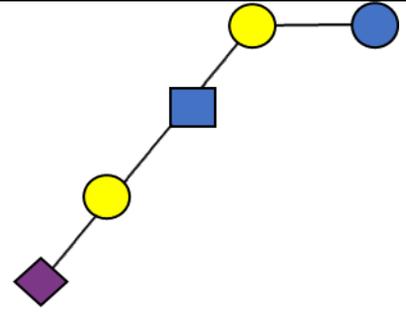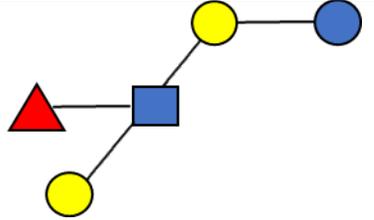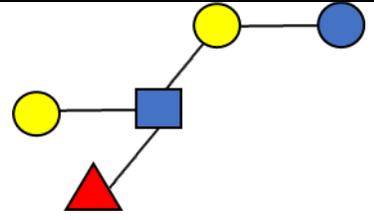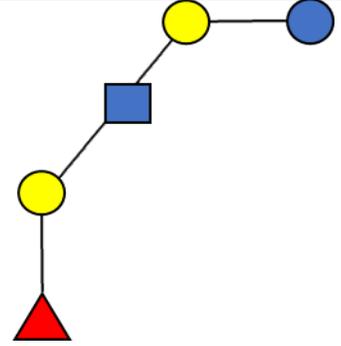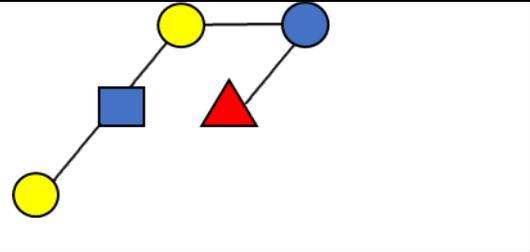
LNFP V		855.322	3	1	1	0
LNDFH I		1001.380	3	1	2	0
LNDFH II		1001.380	3	1	2	0
LNH		1074.396	4	2	0	0
LNnH		1074.396	4	2	0	0
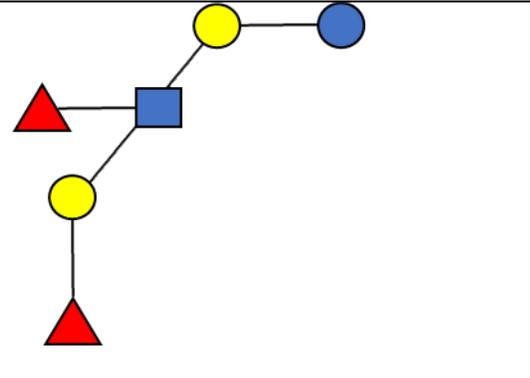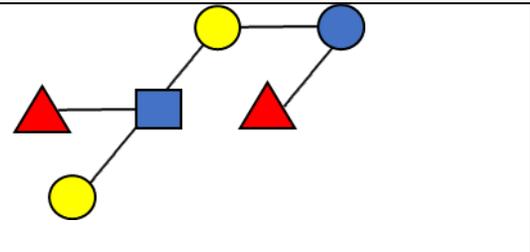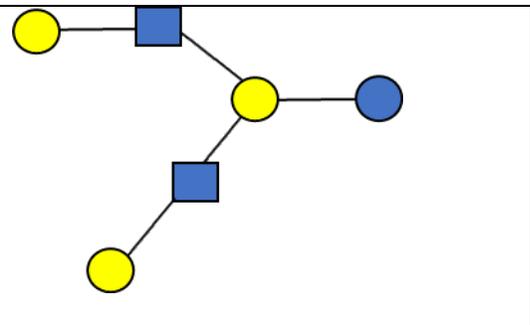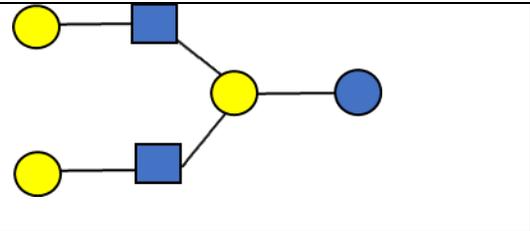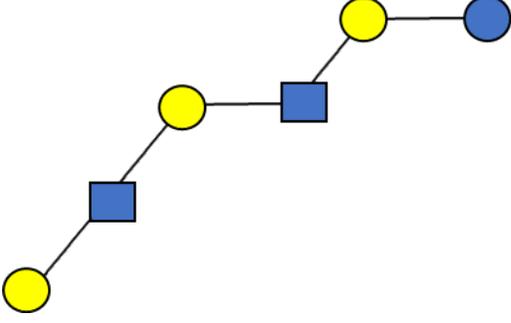
P-LNH		1074.396	4	2	0	0
MFpLNH IV		1220.454	4	2	1	0
MFLNH I		1220.454	4	2	1	0
MFLNH III		1220.454	4	2	1	0
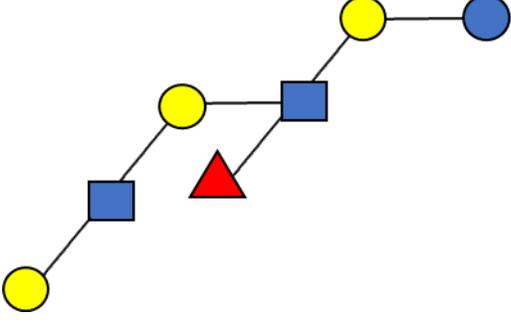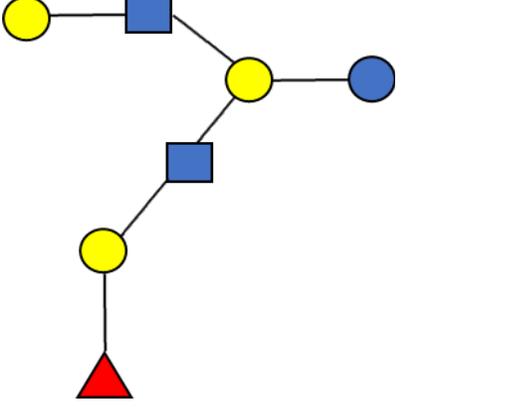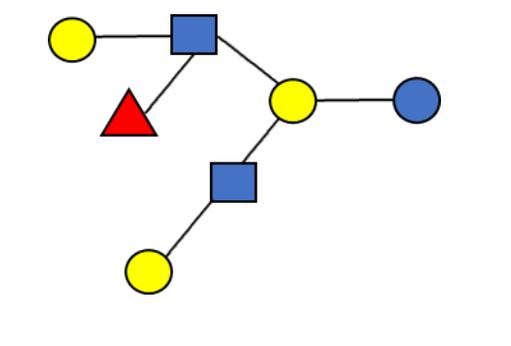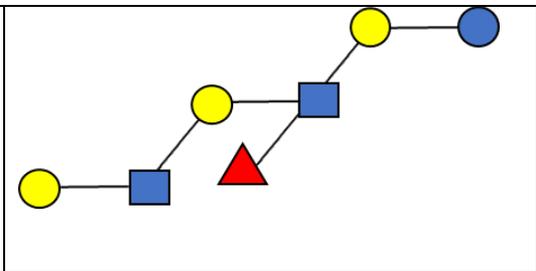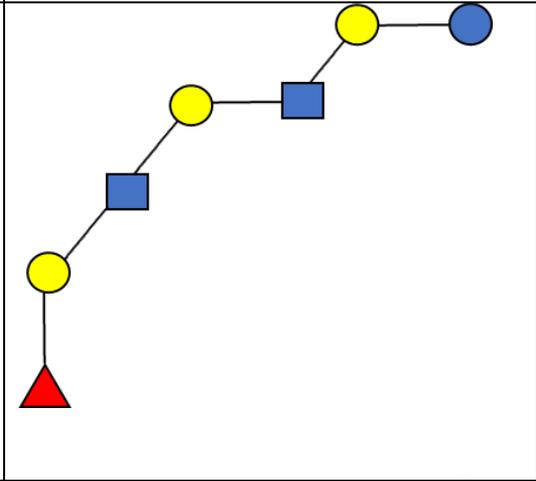
IFLNH III		1220.454	4	2	1	0
IFLNH I		1220.454	4	2	1	0
DFpLNH II		1366.512	4	2	2	0
DFLNHb		1366.512	4	2	2	0

14
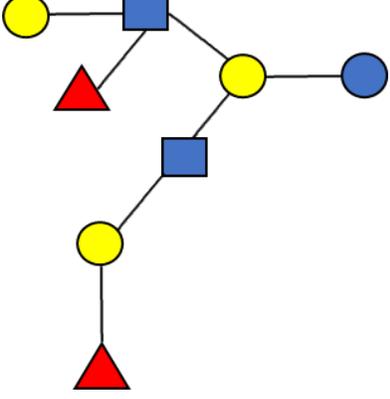
DFLNHa		1366.512	4	2	2	0
TFLNH		1512.570	4	2	3	0
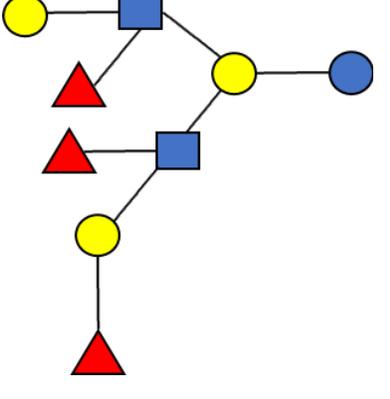
Compositions of HMOs and their abundances in mothers' milk

The abundances of the components of milk are dynamic during lactation with several factors that directly affect the total and relative abundances of the structures. The first few days after birth the mother's milk is the richest, containing the highest amounts of proteins, fats, and HMOs. This nutrient rich milk is known as colostrum. The colostrum contains on average 22g/L protein, 29 g/L fat and 20-23 g/L HMOs.[16] The most significant factor in the total abundances of HMOs is the month post-partum. The concentration of HMOs decreases monthly and reaches a stable concentration at 6 months postpartum corresponding to 7-12 g/L.[17] The standard recommendation of exclusive breast feeding for at least the first 6 months matches with the

moment where HMOs reach a stable concentration. Beyond month six, the abundances of HMO remain stable even for children who are nursed for up to two years. It is debatable whether the infant gets more HMO earlier and less later. In the later period as the infant grows bigger, the mother is also producing more milk while the infant is also consuming more milk.

Although total abundances of HMOs in the mother's milk decrease in the first six months of lactation, the abundances of individual components vary little relative to each other throughout lactation. There is an increase in the relative abundances of fucosylated structures during the first six months but this trend is observed for all fucosylated structures and is not readily observed with individual structures.[8] The direct effect of this increased fucosylation on the infant health is unknown, however it has been observed that high concentrations of fucose containing structures in the mother's milk correlates to higher levels of *Bifidobacterium* and *Bacteroides*.[18] Sialylated structures appear to be constant and generally remain low during the first six months.

Exceptions to the above general behavior are milk for infants born preterm. Preterm or "premature" infants are born before the 37th week of pregnancy. The prevalence of preterm births is relatively high in the United States with nearly 1 in every 10 infants being born prematurely. The HMOs from the milk of mothers with preterm infant may not fit the common profile and may themselves be immature.[19] While some of the milks have normal HMO profiles, those that are born weeks early have milk that are lower in some structures such as *lacto N tetraose* with total fucosylation considerably reduced by as much as 75%. In general, preterm milk does not follow general trends of term births regarding total concentrations. Interestingly, even when the putative birth date is reached, the HMO concentrations remain in the same deficient state. Most premature infants receive processed breast milk, which contain HMO, and are further supplemented by

formula milk, which currently contain little or no HMO. However, this situation is being remedied as manufacturers are rapidly adding one or two structures in limited amounts.

Variations in HMO structures between individuals are due primarily to the specific genotype of the mother. Variations in HMO abundances from different mothers depends highly on the Secretor (Se) and Lewis blood group (Le) loci. The relationships between the genes and the structures they produce are shown in **Table 1.2**.

Table 1.2 Secretor (Se) and Lewis blood group (Le) loci affect HMO abundances and structures in mothers milk.

Secretor Genotype (FUT2)	Lewis Genotype (FUT3)	Phenotype	Glycan Epitope, Fucose Linkage
se/se non-sec	le/le	Le (a-b-), Se-	Type 1 chain
se/se non-sec	Le/Le or Le/le	Le (a+b-), Se-	Le^a Fuc α1-4
Se/Se secretor	Le/Le or Le/le	Le (a-b+), Se+	Le^b Fuc α1-4 α1-2
Se/Se secretor	le/le	Le (a-b-), Se+	H(O) Fuc α1-2
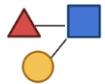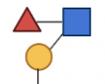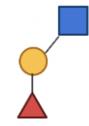
There are several genotypes that affect the phenotypic HMO abundances. Between these various possibilities, the secretor genotype provides the greatest differentiation among HMO structures. A functional FUT2 allele produces an α(1,2)-fucosyltransferase enzyme that adds a fucose in the α(1,2) position of the nascent oligosaccharide. When this gene is active (secretor mother), it produces HMOs containing α(1,2)-fucose in large abundances. Non- Secretors have an inactive FUT2 gene and subsequently produce little to no α(1,2)-fucosylated structures. Among the fucosylated structures, α(1,2)-fucosyllactose or 2'fucosyllatose (2'FL) is structurally the simplest and one of the more abundant oligosaccharides found in the milk of secretor mothers. LC-MS chromatograms highlight the differences in breast milk composition between secretor and non-secretor mothers for a few selected structures (**Figure 1.4**).
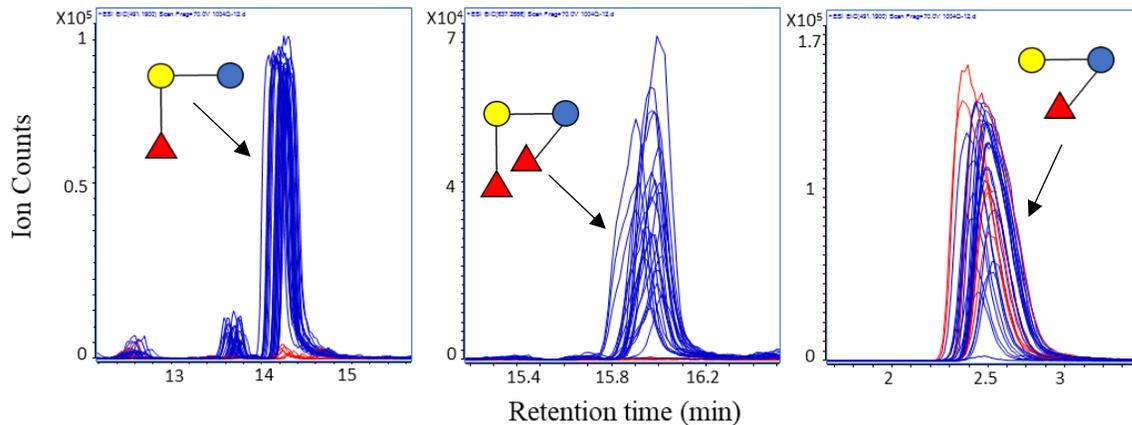
Figure 1.4. Extracted ion chromatograms (EICs) displaying differences in abundances of HMO markers (a) 2'FL (b) LDFT (c) 3'FL between mothers whom are secretors (–) and Non-secretors (–) from a select group of mothers. Monosaccharide composition of structures are given as Hex_HexNAc_Fuc_Neu5Ac and represented as glucose (●), galactose (●), and fucose (▲).

Milk from secretor mothers contain significantly more 2'FL, LNFP I, LDFT and other α(1,2)-fucosylated structures in their milk compared to non-secretors. Nonsecretor mothers produce nearly no α(1,2)-fucosylated structures, although in some cases there are small amounts of 2'FL

produced. In general, the LC-MS chromatograms yield very different profiles between secretors and nonsecretors. If measured precisely and reproducibly, HMOs can be used to phenotype the milk and determine the mother's phenotypic secretor status.[20] It is possible to phenotype the mother based on the abundance of α(1,2)-fucosylated species with nearly 100% accuracy.

Non-secretor mothers have an inactivated FUT2 gene, with the nature of the inactivation differing between different populations.[21] Interestingly, the inactivation of a major fucosyl transferase gene should decrease the amount of fucosylation in nonsecretor mothers; however, fucose is such an important monosaccharide that other fucosyl transferases make up for the absence of the specific enzyme by producing other fucose linked structures such as α(1,3), α(1,4) (FUT3 gene) and α(1,6), which indeed are in greater abundance in nonsecretor mothers. The representation of the general abundances and structures that distinguish between secretors and nonsecretors are provided in **Figure 1.5** Although fucosylated structures do correlate with secretors and nonsecretor mothers, there are non-fucosylated structures that also differ consistently in abundances between the phenotypes.
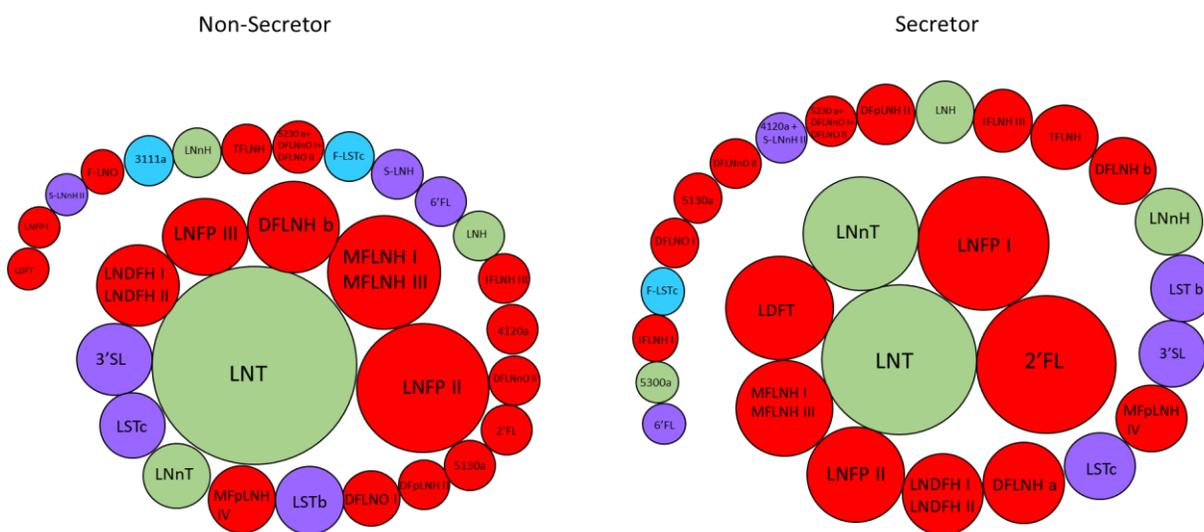
Figure 1.5 Differences in oligosaccharide abundances in human milk between secretor (right) and non-secretors (non-secretors). Size of bubble represents the relative abundance of the oligosaccharide in the mother's milk.

The total fraction of nonsecretor mothers is smaller compared to secretor mothers. A value of 20% nonsecretors is often cited in literature, however this value varies with different populations and is not broadly maintained. In Europe and much of North America with a large population of European descendants, the fraction of non-secretors generally remains within the 20% range. HMO analysis of milk from mothers in West Africa show a considerably greater fraction of nonsecretors corresponding to nearly 40% nonsecretors, perhaps representing the upper limit for the fraction of non-secretors. Broader genomic studies, not related to human milk, have examined genotypic variations throughout various populations and found that the fraction of nonsecretors can reach near zero particularly among indigenous peoples.[22]

There is currently little evidence that indicates that one phenotype is generally more advantageous than the other. However, from the evolutionary standpoint there must have been local pressures that selected one phenotype over the other. While there have been several studies that compare the microbiota of an infant fed with secretor vs non-secretor milk, there is still a lack of understanding how these differences directly correlate to the health of the infant in the developmental stages and later in life. However, there has been some research that suggests that there are advantages and disadvantages that associate with each when comparing rates and severity of some diseases. For example, secretors are more likely to get stomach ulcers but are less likely to get yeast infections. Non secretors on the other hand have been shown to have a greater chance of developing type I diabetes and Ulcerative colitis but are less likely to get Crohn's disease.

There are other factors that affect HMO abundances but much less strongly than the mother's secretor status or period of lactation. Studies on the mode of delivery, the diet of the mother, the sex of the infant, the age of the mothers show they are all important considerations and have been found to affect the total concentrations of HMOs.[23] Diet is the natural potential source

for HMO variations. Indeed, mothers who go through seasonal changes in weight due to periods of under-nutrition produce lower abundances of HMOs with under-nutrition.[24] The variations have been further correlated to their impact on the gut microbiome of the infants. However, many of these studies are on limited cohorts with overlapping confounding factors that make it difficult to parse out small changes in abundances. Larger cohorts and more systematic studies are needed but are difficult to perform due to the sensitive nature of the subjects.

HMOs provide health benefits in its interactions with the gut microbiome

The unique structures of HMOs and their abundances in human milk have provided early indications as to their roles in the infant. The structures resemble other glycan structures such as those on the cell membrane or the glycocalyx, which are known to interact with viruses and bacteria. For this reason, studies of HMO-microbe interactions have focused on the potential role primarily as decoys, binding to bacteria and virus to prevent pathogens from colonizing the gut. However, both commensal and pathogens bind HMOs so selectivity may be difficult to achieve. Furthermore, free oligosaccharides do not bind as strongly as conjugated ones. Thus, glycoproteins such as IgA and lactoferrin, which are highly glycosylated with similar Lewis epitopes as those found in HMOs and with similarly large abundances, are better suited for this role as they can coordinate more extensively to the surface of pathogens.[25] Additionally, many of the correlative studies regarding protection through HMO behavior as decoys can be better attributed to the enhancement of specific bacteria that are favored by their consumption of HMOs allowing them to better compete.

HMOs transit through the upper gastrointestinal tract (GIT) intact to reach the lower intestine where they interact with millions of bacteria. The distal gut is where HMOs provide the greatest benefit to the infant. A recent study reported high levels of HMOs, and in particular those

that are sialylated, promoted growth in mice suffering from under-nutrition.[26] There is a large number of different bacteria that colonize the infant's gut, but few have the enzymes that bind, transport and simultaneously catabolize HMOs. *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) are gram-positive prokaryotes that are found in the distal gut of healthy infants and contain the gene *cassette* that have evolved to interact specifically with HMOs. These bacteria have the complement of solute binding proteins that bring in HMOs intact to be digested by glycosyl hydrolases in the bacteria with specificities that correspond to the linkages and monosaccharide compositions of HMOs. Indeed, monitoring the feces of infants in early lactation shows that much of the HMOs provided in milk are lost in feces.[27] However, when the bifidobacterial population increases in the gut and becomes established, much of the HMOs in feces are no longer there suggesting consumption of HMOs by the bacterial population. Other *Bifidobacterium* species consume HMOs but not to the extent of *B. infantis*. With the aid of HMOs, *B. infantis* can become the dominant bacterium and become the master colonizer of the gut.[28] The mother's secretor status also plays a role in the establishment of the gut microbiota. Secretor mothers producing the corresponding milk tend to establish bifidobacteria earlier than nonsecretor mothers.[29]

The broad benefits of *B. infantis* is still an evolving subject, but the bacteria's contribution to the infant's health already appears to be significant. *B. infantis* can lower pathogen concentrations in the gut by decreasing pH through the production of short chain fatty acids (SFCAs). *B. infantis* secretes acidic metabolites such as acetate, butyrate, and lactate that lower the pH of the gut making it inhospitable to various bacterial pathogens.[30] The presence of *B. infantis* in the gut has been shown to significantly decrease the amount of antimicrobial resistant genes belonging primarily to *Escherichia*, *Clostridium*, and *Staphylococcus* in the infant gut.[31]

Additionally, SCFA further aid the development of the gut by increasing intestinal barrier function thereby promoting immune maturation.

Aside from its interaction with the gut microbiome, there are other roles attributed to HMOs. The brain is highly sialylated, and thus sialic acid in HMOs have been proposed to play a role in the development of the infant brain. Sialic acids are a major component of brain tissues and are integral in influencing the brain development in the early stages of life, affecting neuronal transmissions, synaptogenesis, and memory formation.[32] In the first 6 months of life, the infant undergoes a critical period of growth where the brain has great plasticity to account for rapid physical and cognitive brain development. In the first 90 days alone, the infant brain grows approximately 1% per day, reaching 64% of an adult's brain volume by the end of 3 months.[33] This rapid expansion requires a continues supply of nutrients. As several of the structures are sialylated, HMOs are one of the richest sources of sialic acid containing molecules.[34] The most abundant sialic acid containing structures are 6'-siallactose and 3'-siallactose; however larger structures with multiple sialic acids are also present. While HMOs themselves have not been reported intact in the brain, studies have shown that there is an increase in sialic acid containing molecules such as gangliosides in breastfed infants compared to formula fed further indicating the link between breastfeeding and neurological development. Compared to other mammals, the human brain has more sialic acid-containing molecules. In fact, humans have roughly 2-4 times the amount of sialylated glycoconjugates compared to other mammals such as our closest relatives, the chimpanzees.[35] Furthermore, the concentration of sialylated molecules increase with age confirming the association that these structures have with maturation and evolutionary development.[35]

Immune development occurs in the first few weeks of life when immune cells peak in circulation. HMOs have been proposed to play a key role in developing and modulating the immune system.[36] Selected HMO structures have the ability to promote epithelial barrier maturation and the production of mucins. The benefits that HMOs impart to the infant may also be found on the mother who produces them. Indeed, several in vitro studies do point to additional benefits of HMO alone. For example, the addition of 6'SL to intestinal cells increased secretions of cytokines such as Il-9 and CCL20.[36] Other observations included increased production of TNFα with cells subjected to the same compounds. Similarly, villi formation in intestinal cells were increased with the supplementation of 2'FL.

There are other attributes often associated with HMO supplementation, but many are difficult to separate from the symbiotic relationship of HMO and *B. infantis*. Clinical studies on the effects of HMO supplementation are observed with similar supplementation of *B. infantis*. For example, the supplementation of HMO has been suggested to decrease necrotizing enterocolitis; however, similar observations were observed with supplementation with *B. infantis* alone. Because of the key role that HMOs play in the colonization of the infant gut, the effects of HMOs alone are often clinically difficult to separate from the effects of *B. infantis* or other microbes.

Milk Oligosaccharides from other mammals

A comparison of HMOs with other mammalian milk oligosaccharides provides further insight into the unique structures found in human milk and the potential evolutionary path of HMOs. While this topic can be significantly larger, the discussion here is brief highlighting the broad differences between HMO and other mammalian MOs. Many of the structures in other mammals are also found in humans. The structures found in most mammalian milks are typically

less abundant and smaller in numbers than human milk, with the exception of some primates. For example, bovine milk has only 1 g/L of oligosaccharides in colostrum, and this drops to 100 mg/L in mature milk.

A major structural difference between mammals is in the lactose core itself. In bovine milk (BMO), a fraction of the lactose core is modified with glucose replaced by an N-acetylglucosamine.[37] The milk oligosaccharides can also be elongated from the modified core. A broader difference between mammals is in the distribution of fucosylated, sialylated, and undecorated structures. For example, bovine milk has the highest level of sialylation and very low fucosylation in their milk oligosaccharides, in contrast to human milk which has very high levels of fucosylation and low levels of sialylation **(Figure 1.6)**.[38] The most abundant BMOs are the isomers of sialyl lactose, namely 6'-siallactose and 3'-siallactose with the ratio of the two inverting between breeds.[39] An additional oligosaccharide structure of sialic acid found in bovine milk is not found in human milk, namely N-glycolylneuraminic acid (NeuGc).[40] Although this structure is found in bovine milk, its abundance is considerably lower than in bovine tissue. In general, fucosylated structures are in very low abundance in bovine milk and may not be detectable by modern analytical tools unless sufficiently enriched. In general, BMO abundances are significantly lower than other mammals, perhaps due to selective breeding.[41] The value of bovine milk is generally based on the protein content and not BMO. Selection of high protein yields may have decreased the abundances of BMO. Oligosaccharides in porcine milk have a somewhat greater amount of fucosylation, making it more similar to human milk.[42] Other mammalian milks, such as murine milk, have similar structures to bovine milk with little to no fucosylated structures.

The milk oligosaccharides of selected primates have been systematically studied and were found to have a greater overall similarity with the structures found in humans.[15] The numbers vary between species, with humans and chimpanzees having similar numbers (approximately 100) and gorilla having the least (approximately 50). Stratification of the oligosaccharides did not necessarily follow the phylogenetic tree. Rhesus and humans had much greater similarities compared to humans and gorillas. It was concluded that the milk oligosaccharides separated according to social structures, with those that had the more complicated social structures sharing more similar compounds, while more solitary animals, such as the gorilla, having different milk oligosaccharides.
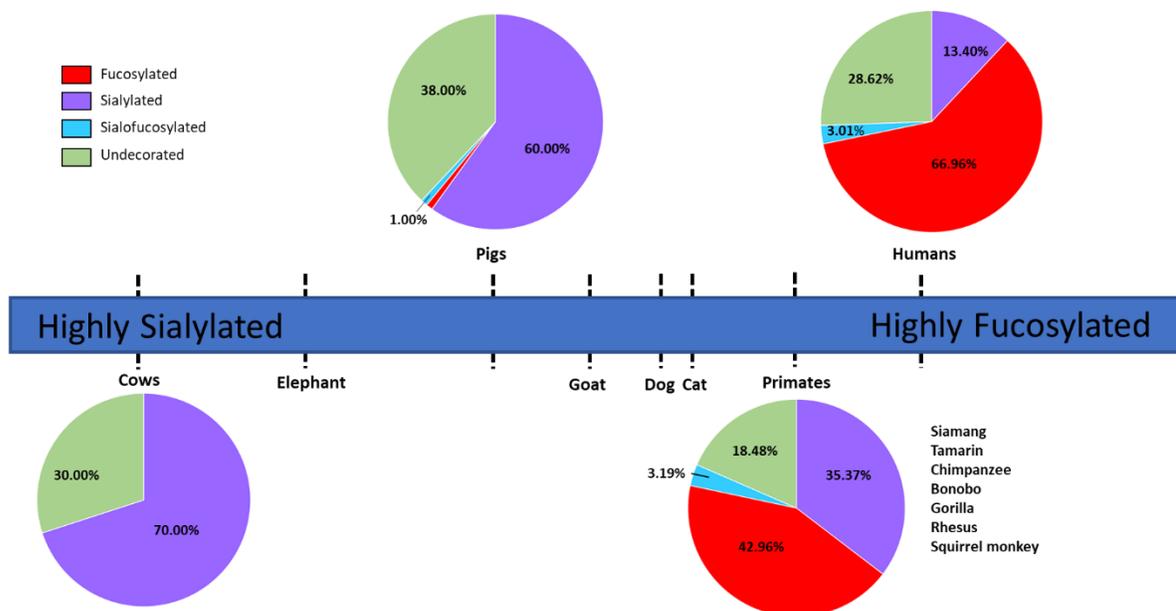
Figure 1.6 Comparison of breast milk composition of different mammalian species from highly sialylated (left) to highly fucosylated (right).

The milk oligosaccharides of dogs and cats have levels of fucosylation and sialylation that are intriguingly closer to primates.[43] Indeed, canines and felines share many structures found in humans consistent with shared microbiomes between the species. Canines have milk oligosaccharides that are dominated with 3'SL, 6'SL and 2'FL representing over 90% of the

abundances. Felines have several more structures dominated by DFLNHb and 3'SL and others with very little 2'FL in the milk. Interestingly, dogs and cats appear to be similar to different secretor phenotypes. Dogs appear more like secretors, while cats seem to be more similar to nonsecretors.

HMO in Infant formula

Infant formula has traditionally been produced from cow's milk, with the abundances of milk oligosaccharide extremely low as those observed in BMOs. Trace amount of sialyllactose and other trisaccharides are typically found in commercial products. However, with the significant interest in HMO, infant formula has now been enriched with HMOs, although generally not in abundances and complexity that would replicate breast milk. Supplementation has focused on simple structures that are easier to synthesize, specifically 2'FL and 6'SL. Mother's milk contains relatively large concentrations of lacto-*N*-tetraose (LNT), Lacto-N-fucopentaose (LNFP) and 6-Siallactose (6'SL), however these oligosaccharides are not yet commonly supplemented in infant formula because they have yet to be adapted for large scale production [44], although this situation is rapidly changing.

CONCLUSION

Human breast milk contains a unique blend of milk oligosaccharides. These oligosaccharides assist in the growth of a healthy infant throughout the most important developmental stages of life. Their main function in the infant is to establish the gut microbiome by feeding and enriching the proper gut bacteria. HMOs play critical roles not just in establishing a healthy gut microbiome, but also in protection by inhibiting pathogenic infections and in aiding

the development of the brain. These actions, while important to the infant, also have direct consequences on the child's well-being later in later life by decreasing the chances of childhood obesity, diabetes, and gastrointestinal diseases. Human milk oligosaccharides are therefore an important class of compounds that play critical roles in the development of the infant and their health trajectory throughout life.

The studies of HMOs further reveal the role of food. Human milk is the perfect food for a human infant, refined by millions of years of evolution. Human milk also serves as a model of the ideal food for adults. The ideal food therefore contains more than just fuel and material for building body mass. It contains nondigestible fiber that works with the gut microbiome to provide important compounds not produced by the person or the food alone. A more thorough understanding of HMOs have been enabled by new sequencing and analytical tools. These tools will similarly reveal the role of fiber in later life.

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CHAPTER TWO

Human Milk Oligosaccharide Compositions Illustrate Global Variations in Early Nutrition

ABSTRACT

Human milk oligosaccharides (HMOs) are an abundant class of compounds found in human milk and have been linked to the development of the infant and specifically the brain, immune system, and gut microbiome. Liquid chromatography mass spectrometry-based analytical methods that profile these structures with broad structural coverage and quantitative information reveal their structural heterogeneity and potentially their biological roles. These methods were used to obtain relative quantitation of specific structures in over 2000 samples from over 1000 mothers in urban, semi-rural and rural sites across geographically diverse countries. A common behavior found among all sites was a decrease in HMO abundances during lactation until approximately month six postnatal, where they remained relatively constant. The greatest variations in structural abundances were associated with the presence of $\alpha(1,2)$-fucosylated species. Because genomic analyses of the mothers were not performed, milk was phenotyped according to the abundances of $\alpha(1,2)$-fucosylated structures. Those with high abundances were termed S+ milk, while those with low abundances were S- milk. However, previous studies have shown that the $\alpha(1,2)$-fucosylated structures correlated with the genetic secretor status. The fraction of mothers S- milk differed among various sites. Geographic variations in the representation of $\alpha(1,2)$-fucosylated as well as other fucosylated and sialylated carbohydrate HMO structures within and between sites and as a function of time of lactation were similarly observed. This study represents the largest structural HMO study to date and reveals the general behavior of HMOs during lactation among different populations.

INTRODUCTION

Human milk for infants is a manifestation of a highly adapted, dynamic and personalized process of human postnatal development. Human milk is 'dynamic' in the sense that time-dependent changes occur in the presence and concentration of milk bioactives within and across mothers and 'personalized' in the sense that maternal genotype, health status, and environmental exposures, including diet, can impact milk compositions.[1-5]

Human milk oligosaccharides (HMOs) are among the most abundant and diverse components of breast milk, with hundreds of unique structures identified to date.[6-9] HMOs serve as nutrients for highly-adapted early bacterial colonizers of the infant gut, including specific strains of bifidobacteria endowed with suites of gene encoded proteins dedicated to the import and utilization of HMOs.[10-14] While the prebiotic effect is believed to be a major function, a small fraction of HMOs are absorbed in the small intestine and detectable in plasma [15] and urine[16] suggesting the potential for direct effects on host physiology including immunomodulation[17-19] and brain development.[20]

HMOs are assembled by glycosyltransferases to form either branched or linear structures. HMOs generally consist of a lactose [glucose (Glc) and galactose (Gal)] core, with variable combinations of N-acetylglucosamine (GlcNAc) and can be further bound to monosaccharides including sialic acids (N-acetylneuraminic acid or Neu5Ac) and fucose (Fuc).[21] The process yields an extensive number of oligosaccharides that in many cases are unique to human milk.[22]

Variations in HMOs are greatest among secretor genotypes. Secretors are individuals with a functioning FUT2 gene encoding α(1,2)-fucosyltransferase that attaches fucose via an α(1,2)-linkage to terminal Gal residues thereby producing blood antigens into secreted fluids (e.g., sweat, tears, semen, and milk).[21, 23-25] Non-secretors have diminished ability to produce

ABH or Lewis b antigens (Le[b]; Fucα1,2Galβ1,3[Fucα1,4]GlcNAcβ) due to mutations in the

FUT2 gene.[21, 23-25] Genetic studies have documented variations in the prevalence of the wild-

type and mutant *FUT2* alleles around the world.[26-28] The prevailing existence of different

genotypes in populations show that there are unique advantages to individuals. This notion is

consistent with evidence suggesting a protective effect against, for example, otitis media[29] and

autoimmune diseases[30, 31] for secretors and against viral diarrhea for non-secretors.[32] While

different alterations in the FUT2 gene among various populations determine the secretor status of

the mother, the milk and its function is guided by the abundances of HMO structures. Thus,

classifying the milk phenotype, for example the amount of sialylation and fucosylation, is a more

direct approach in surveying the differences between mothers' milks and correlating infant health

outcomes. Advanced analytical methods now make it possible to accurately determine the

phenotypic secretor status by directly quantitating the abundances of α(1,2)-fucosylated

structures present in the breast milk.[23] For the purpose of this study, we refer to milk that

corresponds to high amounts of α(1,2)-fucosylated structures as S+ milk, and milk corresponding

to low abundances as S- milk. HMOs can therefore be used to type the milk according to the

presence or absence of α(1,2)-fucosylated structures regardless of the genotype.[8, 23]

Mass spectrometry-based analytical methods have enabled more rapid and precise

characterization of human milk, including the capability to simultaneously determine the

abundances of hundreds of distinct carbohydrate structures. However, determination of HMOs

are largely limited to studies of cohorts living in a small number of sites or geographic locales.[33-35] In this report, we describe the results of a cross-sectional analysis that quantified the

abundances of HMOs to determine the natural variations during lactation among various sites

involving mothers from different ethnic groups. Milk samples were obtained and analyzed from

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over 1000 mothers living in 15 countries encompassing six continents and representing a diverse set of ecological and cultural backgrounds.

This work has been published in the Journal of Nutrition and is included with permission in this thesis.

MATERIALS AND METHODS

Sources of breast milk samples

Breast milk samples (N=2234) were collected from mothers (N=1090) in 16 global sites, including urban, rural and semi-rural communities in 15 sites spanning Africa, Eurasia, the Americas, and Australia. Samples and resulting data were collected from different studies as detailed in the **Table 2.1**. However, the quality controls and analytical methods for each sample remained the same. A detailed summary of sample information including country, population, collection procedures, sample size, and infant age is provided in **Table 2.1** and **Table 2.2**.

Table 2.1 Summary of successful milk collections as a function of location and lactation month**.**

Location																										

Location	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	21	24	26	Total
Argentina			1	1		2	1	3	2		3	3	2	2											20
Bangladesh		15	15	14	12	12	12	12																		92
Bolivia		1	1	3	7	4	1	6	4	2	5	3	2	1	7	2	3	2	3	1	2			1	61
Boston				2	1	4	2	2	2	1	1		4	1											20
Brazil			22	18	14	20	18	17																	109
Davis	8	59	40	31	37	38		30					1	11											255
Gambia			33			33	33																		99
India			42	42	42	42	40	39																	247
Malawi			73		84			652																	809
Namibia			3	2		1			1	2					1	1				1					12
Nepal			1	3		3	2	4	4	2	3	2			1							1	2		28
Perth		28	29																						57
Peru		35	36	33	34	32	31	32																	233
Philippines		5	1	1	2	2	2	1	2	2	1	1	2	1											18
Poland			1	2	1	4	2	2	4	1		3		2	1										23
South Africa			26	26	27	26	25	26																	151
Total	8	143	324	178	261	223	169	816	19	10	13	12	11	18	10	3	3	2	3	2	2	1	2	1	2234

Lactation Month

Table 2.2 Summary of all study sites and variations in milk types.

Country	Site Location	Setting	No. of mothers	No. of samples	S+ milk producers	S- milk producers	% S+ milk producers
Argentina	Namqom	Rural	20		17	3	85
Bolivia	Amazonian Lowlands	Rural	52		52	0	100
Brazil	Fortaleza, Ceará	Urban	23	109	21	2	91.3
Peru	Loreto	Rural	37	236	36	1	97.3
Australia	Perth		26		20	6	76.9
Bangladesh	Mirpur, Dhaka	Urban	15	92	12	3	80
India	Vellore, Tamil Nadu	Urban	43	247	29	14	67.4
Nepal	Nubri Valley	Rural	28		20	8	71.4
Philippines	Cebu	Rural & Urban	18		13	5	72.2
Gambia		Rural	33	99	21	12	63.6
Malawi	Mangochi district	Rural	658	809	491	165	74.9
Namibia	Omuhonga Basin	Rural	12		10	2	83.3
South Africa	Thohoyandou, Limpopo Province	Rural	27	151	17	10	63
Poland	Beskid Wyspowy Mtns	Rural	23		18	5	78.3
United States	Boston, Massachusetts	Urban	20		15	5	75
United States	Davis, California	Urban	55	252	40	14	74.1

[1]Values presented correspond to the number of samples collected

All infants were delivered full term. Written informed consent was obtained from all parents/guardians prior to study enrollment. Samples were collected using standardized protocols for all populations. The samples from the Gambia were analyzed from a sub-study embedded within a randomized trial to investigate the effects of pre-natal and infancy nutritional supplementation on infant immune development, The Early Nutrition and Immune Development (ENID) Trial, ISRCTN49285450, http://www.isrctn.com/ISRCTN49285450)64, registered on December 11, 2009. Ethical approval for the ENID Trial and the 'ENID-Bioactives' sub-study was obtained from the joint Gambian Government/MRC Unit. The Gambia Ethics Committee and the George Washington University Institutional Research Board (#13-0441). Full informed consent was obtained from each participant, prior to inclusion in the study.

There were 20 mothers from Argentina (approval provided by the University of Pennsylvania Institutional Review Board #811200), 52 from Bolivia (approval provided by the University of California, Santa Barbara, two study periods: 2009: IRB ANTH-GU-MI-010-3U #09-312 and ANTH-GU-MI-010-10R #10-290; 2012-2013: ANTH-GU-MI-010 #19-13-0206 and #3-15-067), 20 from Boston, USA (approval provided by the Harvard Committee on the Use of Human Subjects, File Number 23868), 12 from Namibia (approval provided by the Harvard Committee on the Use of Human Subjects, IRB13-0900 and the University of California, Los Angeles Institutional Review Board, IRB#13-000881), 28 from Nepal (approval provided by the Washington University in St. Louis, IRB 201302059 and the Nepal Health Research Council, IRB 1329), 18 from the Philippines (approval provided by Northwestern University and the University of San Carlos, Philippines, IRB STU00001299), and 23 from Poland (approval provided by the Harvard Committee on the Use of Human Subjects IRB21979) who provided breast milk samples (N=182). Informed consent was provided by all subjects. Samples were collected using a

standardized protocol for all populations. Premature births, multiple births, and those infants being exclusively bottle fed were excluded from the sample set. Participants were asked to nurse from the sample breast approximately two hours prior to sampling (except for the 2009 sampling period in Bolivia, in which case mothers were asked to not feed from either breast for one hour prior to sampling) and refrain from nursing from the sampled breast again until collection. At the time of sample collection, participants nursed their infants for approximately two to two and a half minutes before hand-expressing a small, mid-feed sample. Those from Bolivia evacuated the whole breast with a manual breast pump instead of hand expression from the 2009 study. Consent for data collection was secured at multiple levels. The Tsimane Health and Life History Project maintains formal agreements with the local municipal government of San Borja and the Tsimane governing body to conduct research in Tsimane territories. Researchers held community meetings to explain study aims and obtain community-wide consent to stay in and work with members of participating villages. Individual informed consent was obtained verbally from participants at recruitment and again before providing milk samples. Meetings, participant interviews, and explanation of study aims and procedures were conducted in the language preferred by the participant. An informational sheet with the main elements of the informed consent process was left with the prospective participants and sample collection was conducted by a team that included a local field assistant who could translate any questions and answers.

The Malnutrition and Enteric Disease (MAL-ED) Study (ClinicalTrials.gov Identifier: NCT02441426) was an observational birth cohort study conducted at multiple sites from November 2009 to February 2014, to investigate the linkages between malnutrition and intestinal infections and their effects on children in the developing world. Each of the sites, which included Bangladesh (Dhaka), India (Vellore), Brazil (Fortaleza), Peru (Loreto) and South Africa (Venda),

obtained ethical approval from their respective institutions and written informed consent from participants. iLiNS-DYAD-M (ClinicalTrials.gov Identifier: NCT01239693) was a randomized, controlled, single-blind, parallel group clinical trial of micronutrient fortified lipid-based nutrient supplements conducted in southern Malawi. The study was performed between February 2011 and April 2015 and approved by institutional review boards of the University of Malawi and Pirkanmaa Hospital (Finland); informed consent was provided by all participants. Breast milk samples from these studies that were included in the analysis presented here were collected under Bill & Melinda Gates Foundation Grant OPP1033518 with approval from the institutional review board at Washington University School of Medicine in St. Louis.

HMO extraction and mass spectrometric analysis

HMOs were extracted from breast milk samples using previously reported methods.[7, 8, 36, 37] Briefly, whole milk samples were aliquoted into 96-well plates, diluted, and then defatted via centrifugation. Proteins were precipitated with ethanol, and the resulting glycans were reduced with sodium borohydride (Sigma-Aldrich, St. Louis, MO). Solid phase extraction was performed on graphitized carbon cartridges (Glygen, Columbia, MD) to remove lactose and salts. After the solvent was evaporated, purified HMOs were reconstituted and diluted prior to analysis. Standard solutions were made of HMO in water, with concentrations ranging from 0.05 mg/mL-0.2 mg/mL.

Extracted HMOs were analyzed on a nano-HPOC-TOF-MS The HPLC unit (Model series 1200, Agilent Technologies, Santa Clara, CA) that utilizes a capillary pump for sample loading (4 µL/min) and a nano pump for analyte separation (0.3 µL/min). Loading and separation were performed on a microfluidic chip packed with porous graphitized carbon via enrichment and analytical columns, respectively, using a binary gradient of solvent A [3% acetonitrile (ACN) in 0.1% formic acid] and solvent B (90% ACN in 0.1% formic acid). This system was coupled to an

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Agilent 6220 series TOF mass spectrometer. Detection was performed in the positive mode, and calibration was achieved with a dual nebulizer electrospray source with calibrant ions ranging from mass-to-charge (*m/z*) 118.086 to 2721.895.

Structural annotation of HMOs

Data were collected using Agilent MassHunter Workstation Data Acquisition software (B.02.01) and analyzed using Agilent MassHunter Qualitative Analysis software B.03.01 and B.06.00. The 'Find Compound by Molecular Feature' function was used to extract ion abundances to within 20 ppm of theoretical HMO masses. Individual HMOs were identified by accurate mass, retention time, and elution order defined previously developed HMO libraries.[7, 8] An in-house software program was used to align peaks due to minor retention time shift.[37] HMOs were grouped into classes as follows: fucosylated HMOs (any structure with Fuc), sialylated HMOs (any structure with Neu5Ac), undecorated HMOs (neither Fuc nor Neu5Ac present), and fucosylated plus sialylated HMOs (both Fuc and Neu5Ac present). Relative abundances (%) were calculated by normalizing class and individual compound abundances to total HMO abundance in each breast milk sample. Compounds that were not identified in individual samples but were present in at least 50% of all samples were given a LOD/2 abundance.

Classification of milk secretor phenotype as S+ and S- based on HMO abundances

Phenotypic secretor status was determined following our previously published method.[23] Structures with known α(1,2)-Fuc linkages were identified by matching exact masses and retention times to previously developed annotated HMO libraries.[7, 8] The abundances of the most abundant α(1,2)-fucosylated structures, namely 2'-fucosyllactose(2'FL), lactodifucotetraose(LDFT), difucosyllacto-N-hexaose a(DFLNHa) and trifucosyllacto-N-hexaose (TFLNH) were summed and normalized to the total HMO abundances in a given sample, so that a relative α(1,2)-fucosylation

value could be determined. Secretor status was assigned based on a previously established and validated threshold of 6%.[23] If this value exceeded the threshold. the mother was deemed a secretor (S+), conversely if the value fell below this threshold the mother was deemed a non-secretor (S-). The number of mothers producing S+ and S- milk in each location was determined and the proportion of S- mothers was calculated for each location. If a mother provided multiple samples from different postpartum time points, her secretor status was determined based on the secretor status determination in the majority of her samples. If there was no 'majority milk type', she was excluded from the statistical analysis (one mother from Davis, USA, two mothers from Malawi, and three mothers from Perth, Australia).

Statistical analyses

Mann-Whitney tests were used to determine the differences between absolute and relative abundances of the HMO classes. Furthermore, the data was grouped to show how secretor status, geographical location, age, sex, and lactation month affected the HMO profiles. An alpha correction of $\alpha=0.05$ was used for the statistical analysis. Differences were determined when all samples from all time points were combined (S+ $N=1709$, S- $N=524$), and when samples were split by location including all time points (Argentina: S+ $N=17$, S- $N=3$; Boliva: S+ $N=52$, S- $N=0$; Bangladesh: S+ $N=72$, S- $N=20$; USA[Boston]: S+ $N=15$, S- $N=5$; Brazil: S+ $N=97$, S- $N=12$; USA[Davis]: S+ $N=194$, S- $N=58$; Gambia: S+ $N=63$, S- $N=36$; India: S+ $N=166$, S- $N=81$; Malawi: S+ $N=601$, S- $N=208$; Namibia: S+ $N=10$, S- $N=2$; Nepal: S+ $N=20$, S- $N=8$; Australia: S+ $N=42$, S- $N=15$; Peru: S+ $N=227$, S- $N=8$; Philippines: S+ $N=13$, S- $N=5$; Poland: S+ $N=18$, S- $N=5$; South Africa: S+ $N=93$, S- $N=58$). Due to the vast changes in milk composition throughout lactation, samples were binned based on lactation month, therefore milk collected from a single mother at different time points were treated as independent samples.

Data Availability

Data that support the findings of this study are available upon request from the corresponding author (C.B.L.).

RESULTS

HMO-based classification of milk into S+ and S- phenotypes

Over 2000 breast milk samples were collected from 1,090 mothers in 15 geographical sites. The samples obtained from six continents were analyzed under one protocol allowing direct comparison of abundances by classes and individual structures (**Table 2.2**). Using nano-HPLC-qTOF-MS, we identified 60 structures that were common to most samples. However, the total number of unique structures varied for each mother, with the average count of nearly 100 structures in a single mother. The abundances for these structures varied widely, spanning four orders of magnitude.

Table 2.2 The 60 most common HMO structures in S+ and S- milks across all study sites.

Neutral Mass (Da)	Composition (Hex_HexNAc_Fuc_Neu5Ac)	HMO	S- type milk mean	S+ type milk mean	*P*-value
490.19	2010	2'FL	0.002 ±0.005	0.07 ±0.04	<0.0001
636.24	2020	LDFT	0.001 ± 0.003	0.04 ± 0.03	<0.0001
709.26	3100	LNT + LNnT	0.2 ± 0.09	0.1 ± 0.06	<0.0001
855.32	3110	LNFP II	0.04 ± 0.03	0.02 ± 0.02	<0.0001
855.32	3110	LNFP I + LNFP III	0.02 ± 0.02	0.06 ± 0.04	<0.0001
1074.39	4200	LNH	0.009 ± 0.01	0.009 ± 0.007	0.06
1074.39	4200	LNnH	0.009 ± 0.01	0.01 ± 0.01	<0.0001
1074.39	4200	p-LNH	0.004 ± 0.007	0.004 ± 0.005	<0.0001
1220.45	4210	MFpLNH IV	0.02 ± 0.01	0.02 ± 0.009	0.1
1220.45	4210	412Oa	0.007 ± 0.01	0.003 ± 0.006	<0.0001
1220.45	4210	MFLNH III + MFLNH I	0.03 ± 0.02	0.02 ± 0.01	0.02
1220.45	4210	IFLNH III	0.008 ± 0.006	0.01 ± 0.006	<0.0001
1220.45	4210	IFLNH I	0.001 ± 0.003	0.004 ± 0.004	<0.0001
1366.51	4220	DFpLNH II	0.01 ± 0.007	0.01 ± 0.006	<0.0001
1366.51	4220	DFLNH b	0.02 ± 0.01	0.01 ± 0.007	<0.0001
1366.51	4220	DFLNHa	0.001 ± 0.002	0.01 ± 0.01	<0.0001
1512.57	4230	TFLNH	0.004 ± 0.004	0.006 ± 0.005	<0.0001
1585.58	5310	5130a	0.006 ± 0.006	0.004 ± 0.003	<0.0001
1585.58	5310	F-LNO	0.004 ± 0.003	0.003 ± 0.002	0.002
1731.64	5320	DFLNO I	0.007 ± 0.005	0.003 ± 0.003	<0.0001
1731.64	5320	DFLNnO II	0.004 ± 0.005	0.003 ± 0.003	0.9
1731.64	5320	5230a + DFLNnO I/DFLNO II	0.004 ± 0.003	0.006 ± 0.004	<0.0001
635.22	2001	6'SL	0.003 ± 0.003	0.002 ± 0.003	<0.0001
635.22	2001	3'SL	0.01 ± 0.008	0.01 ± 0.007	0.4
1000.36	3101	LSTc + LSTb	0.03 ± 0.02	0.03 ± 0.01	<0.0001
1000.36	3101	LSTa	0.003 ± 0.002	0.002 ± 0.002	<0.0001
1365.49	4201	S-LNH	0.003 ± 0.004	0.002 ± 0.002	<0.0001
1365.49	4201	4021a + S-LNnH II	0.004 ± 0.005	0.006 ± 0.005	<0.0001
490.19	2010	%2'FL	0.4 ± 0.9	11.1 ± 4.6	<0.0001
636.24	2020	%LDFT	0.2 ± 0.5	5.8 ± 5.1	<0.0001
709.26	3100	%LNT + LNnT	30.3 ± 10	21.7 ± 5.9	<0.0001
855.32	3110	%LNFP II	6.1 ± 5	2.9 ± 2.6	<0.0001
855.32	3110	%LNFP I + LNFP III	3.8 ± 2.5	8.2 ± 5	<0.0001
1074.39	4200	%LNH	1.5 ± 1.6	1.2 ± 0.9	0.5
1074.39	4200	%LNnH	1.4 ± 1.8	1.7 ± 1.4	<0.0001
1074.39	4200	%p-LNH	0.7 ± 1.2	0.6 ± 0.7	<0.0001
1220.45	4210	%MFpLNH IV	2.8 ± 1.5	2.3 ± 1.2	<0.0001
1220.45	4210	%412Oa	1.1 ± 1.7	0.6 ± 1	<0.0001

1220.45	4210	%MFLNH III + MFLNH I	4.3 ± 2.6	3.3 ± 1.8	<0.0001
1220.45	4210	%IFLNH III	1.3 ± 0.9	1.4 ± 0.9	0.007
1220.45	4210	%IFLNH I	0.2 ± 0.5	0.5 ± 0.6	<0.0001
1366.51	4220	%DFpLNH II	2.2 ± 1.3	1.5 ± 0.8	<0.0001
1366.51	4220	%DFLNH b	3.3 ± 2.6	1.6 ± 1.2	<0.0001
1366.51	4220	%DFLNHa	0.2 ± 0.3	2 ± 1.8	<0.0001
1512.57	4230	%TFLNH	0.7 ± 0.6	0.9 ± 0.7	<0.0001
1585.58	5310	%5130a	0.9 ± 0.8	0.5 ± 0.4	<0.0001
1585.58	5310	%F-LNO	0.6 ± 0.4	0.4 ± 0.3	<0.0001
1731.64	5320	%DFLNO I	1.1 ± 0.7	0.5 ± 0.4	<0.0001
1731.64	5320	%DFLNnO II	0.7 ± 0.7	0.5 ± 0.4	0.0007
1731.64	5320	%5230a + DFLNnO I/DFLNO II	0.7 ± 0.5	0.8 ± 0.4	<0.0001
635.22	2001	%6'SL	0.5 ± 0.5	0.3 ± 0.4	<0.0001
635.22	2001	%3'SL	2.6 ± 1.7	2.2 ± 1.4	<0.0001
1000.36	3101	%LSTc + LSTb	5.2 ± 2	3.8 ± 1.6	<0.0001
1000.36	3101	%LSTa	0.5 ± 0.3	0.3 ± 0.3	<0.0001
1365.49	4201	%S-LNH	0.5 ± 0.5	0.3 ± 0.3	<0.0001
1365.49	4201	%4021a + S-LNnH II	0.6 ± 0.7	0.8 ± 0.6	<0.0001

[1]Values are presented as Mean Abundance ± SD. All data collected was used for this analysis including data from the same mother at different time points of lactation. Monosaccharide composition represented by four-digit code (Hex_HexNAc_Fuc_Neu5Ac). Common HMO abbreviations were used to name oligosaccharides. Oligosaccharides with two compound names are isomers that were difficult to resolve chromatographically, the data presented are the sum of their combined abundances. Oligosaccharide names proceeding a percentage indicates that the values presented are mean relative abundances. P values were obtaining using Mann-Whitney tests.

HMOs containing Lewis b structures (α(1,2)-fucose), a feature of the secretor genotype (homozygous or heterozygous for the functional *FUT2* allele)[23,38,25], were most variable between mothers. We defined milk rich in Lewis b structures [2'-fucosyllactose(2'FL), lactodifucotetraose(LDFT), difucosyllacto-*N*-hexaose a(DFLNHa) and trifucosyllacto-*N*-hexaose (TFLNH)] that were consistently represented in samples collected within and across the different geographic sites S+ milk, belonging to a secretor mother. Milk containing a total relative abundance of <6% of these four Lewis b structures was defined as S+ milk, thus belonging to a non-secretor mother. This criterion was developed previously and has been validated with genomic data.[23] Phenotyping the milk addresses what the infant receives, while genotyping the mother does not necessarily translate to HMO abundances.

Figure 2.1 shows extracted ion chromatograms (EICs) of the most abundant α(1,2)-fucosylated compounds in human milk, namely 2'-fucosyllactose (2'FL) and lactodifucotetraose (LDFT) (**Figure 2.1a, b**). These compounds were consistently higher in S+ milk (blue) compared to S- (red, near baseline) across all sites (P<0.05). Five other α(1,2)-Fuc-containing structures, namely lacto-*N*-fucopentaose I (LNFP I), lacto-*N*-difucohexaose I (LNDFH I), monofucosyllacto-*N*-hexaose I (MFLNH I), isomer I fucosyl-paralacto-*N*-hexaose (IFLNH I), and difucosyllacto-*N*-hexaose c (DFLNH c), were much less ubiquitous, and when detected, were present at much lower abundances than the four structures used for determination of secretor status (**Table 2.2**). Conversely, there were structures, particularly α(1,3)- and α(1,4)-fucosylated HMOs, that were significantly higher in S- milk (**Figure 2.1c-f, Table 2.2**). Three isomers with composition 3Hex:1HexNAc:1Fuc and having α(1,3/4)-Fuc linkages were produced in higher abundances in mothers with S- milk (**Figure 2.1e**).
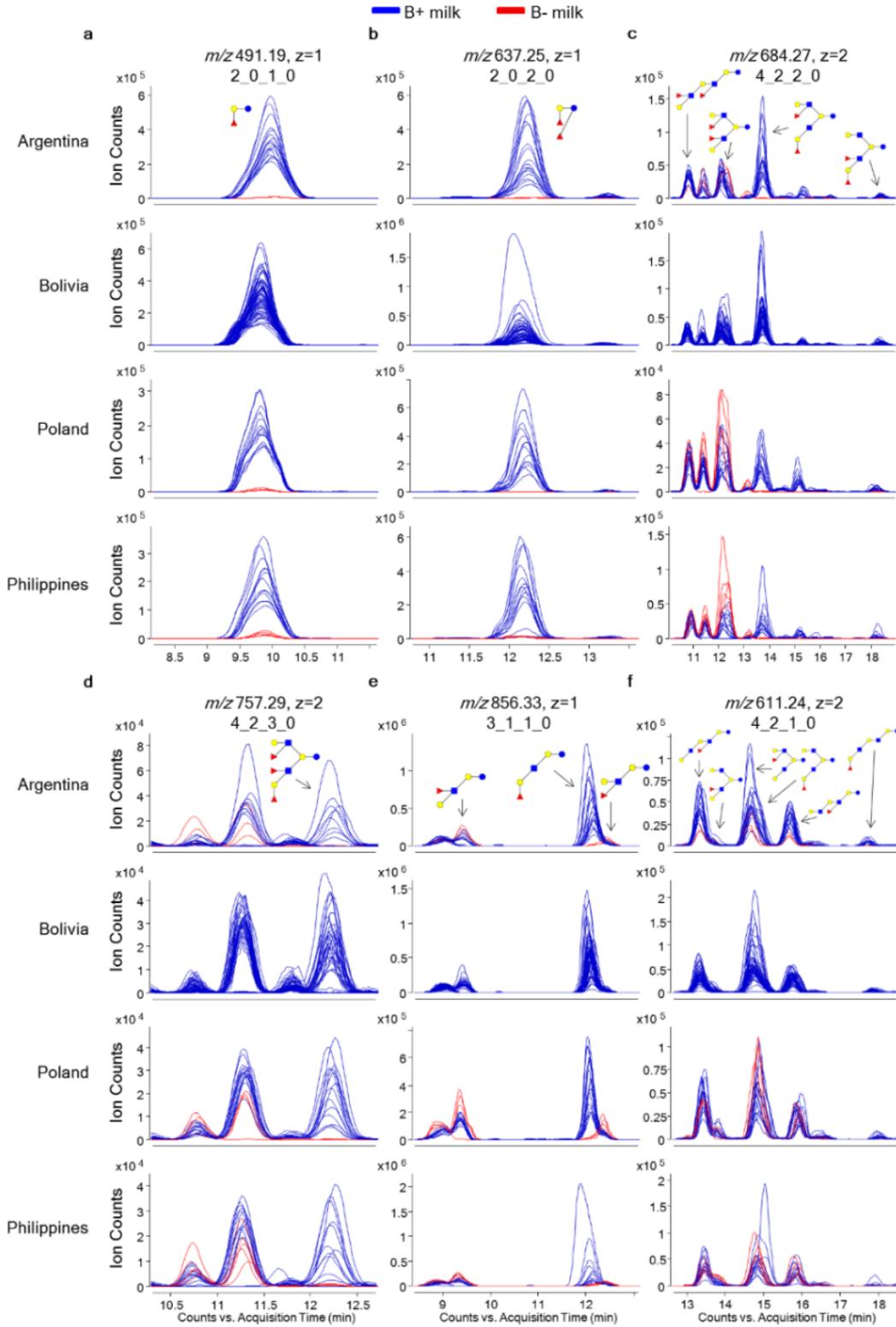
Figure 2.1 Extracted ion chromatograms (EICs) displaying differences in abundances of HMO markers with α(1-2)-linked Fuc between mothers with S+ (—) and S- (—) milk from different locations around the world. Locations were chosen to represent different areas with both S+ and

S- milk producers. Bolivia was also chosen to display EICs of 100% S+ milk producers. Monosaccharide composition of structures are given as Hex_HexNAc_Fuc_Neu5Ac and represented as glucose (●), galactose (●), N-acetylglucosamine (■), and fucose (▲). (**a**) EIC of 2'-fucosyllactose (2'FL) with *m/z* 491.19. (**b**) EIC of lactodifucotetraose (LDFT) with *m/z* 637.25. (**c**) EIC of isomers difucosyl-*parap-lacto-N*-hexaose (DFpLNH II), difucosyllacto-*N*-hexaose (b) (DFLNH b), difucosyllacto-*N*-hexaose (a) (DFLNHa), and difucosyllacto-*N*-hexaose (c) (DFLNHc) with *m/z* 684.27. (**d**) EIC of trifucosyllacto-*N*-hexaose (TFLNH) with *m/z* 757.29. (**e**) EIC of isomers lacto-*N*-fucopentaose II (LNFP II), lacto-*N*-fucopentaose I (LNFP I), and lacto-*N*-fucopentaose III (LNFP III) with *m/z* 856.33. (**f**) EIC of isomers fucosyl-*para*-lacto-*N*-hexaose (MFpLNH IV), 4120a, monofucosyllacto-*N*-hexaose III (MFLNH III), monofucosyllacto-*N*-hexaose I (MFLNH I), isomer III fucosyl-*para*-lacto-*N*-hexaose (IFLNH III), and isomer I fucosyl-*para*-lacto-*N*-hexaose (IFLNH I) with *m/z* 611.24.

The relative abundances of the 60 most common structures were shown for S+ milk and S- in **Figure 2.2** (see **Table 2.2**).

The abundance of each HMO was normalized to the total abundances of the selected group, which made up approximately 97% of all abundances. For mothers that produce S+ milk, the most abundant HMOs were Lacto-N-Tetraose/Lacto-N-Neotetraose (LNT/LNnT). These two compounds are isomers and were difficult to resolve chromatographically, and the data presented are the sum of their combined abundances. At all sites, mothers with S- milk had higher abundances of LNFP II, suggesting this HMO is a potential marker of S- milk (P<0.0001; Mann-Whitney). Other HMOs, including MFLNH I, MFLNH III and DFpLNH II were also higher in S- milk achieving statistical significance (P< 0.05; Mann-Whitney) at all sites except Brazil (P<0.38; Mann-Whitney).
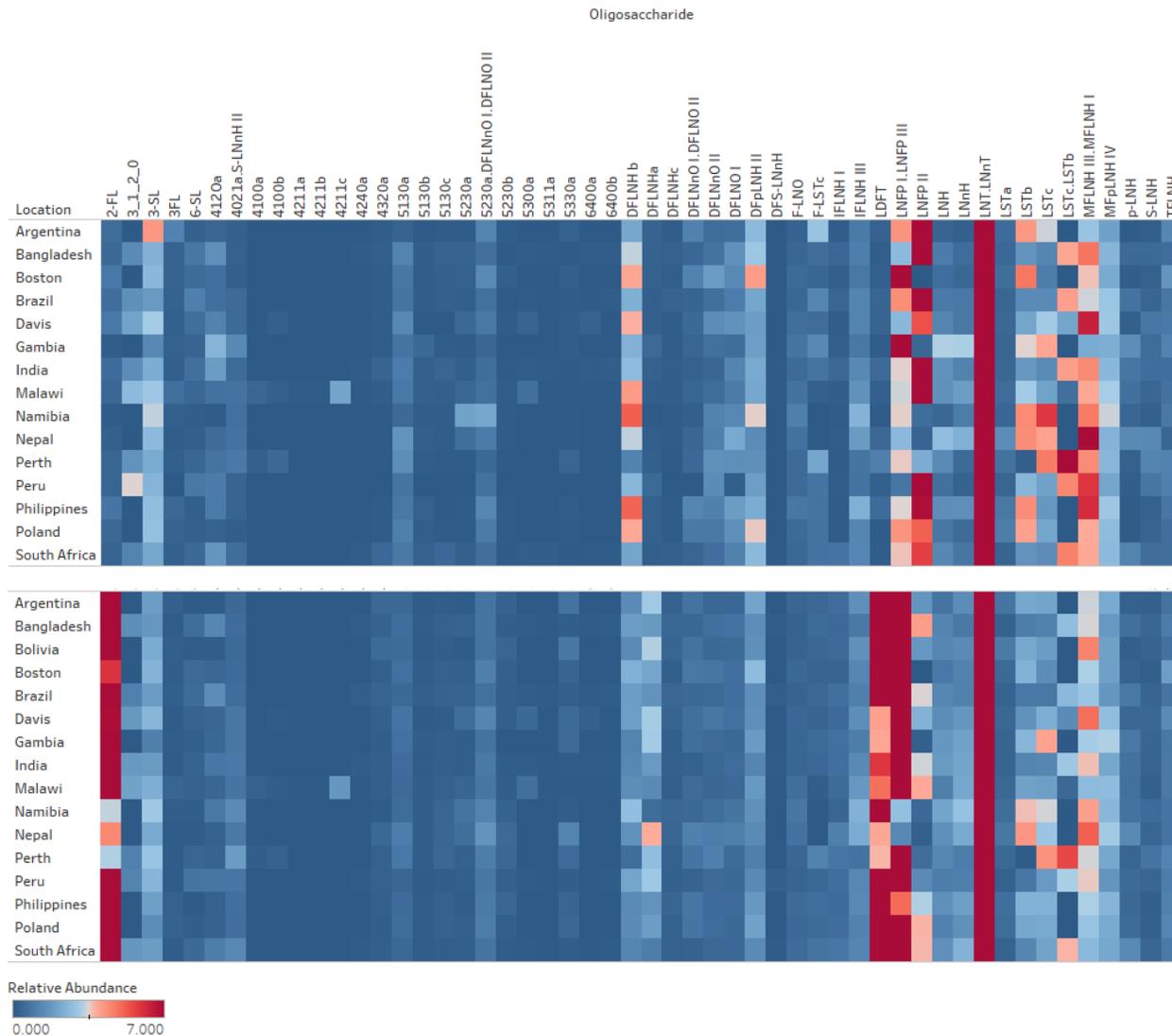
Figure 2.2 Heatmap of relative abundances of the most common (60) HMOs across 15 geographically diverse sites. Comparison of abundances from mothers who are (top) S-producers and (bottom) S+ producers. HMO abundance values correspond to HPLC-qTOF MS spectral abundance normalized to the mean of the total abundance of counts from each sample. HMOs that were not baseline separated (resolution >1.5) were grouped together and labeled accordingly.

Total HMO abundances between sites and during lactation

The total abundances of HMOs between mothers from various sites were compared as a function of months postpartum. **Figure 2.3** compared total HMOs from mothers living in Brazil, Bangladesh, Davis-USA, Peru, India and South Africa sampled between postpartum months 1-6.

These sites were selected because they provided the most extensive longitudinal sampling. The highest abundances were observed at month 1 and decreased uniformly thereafter. Each site showed similar behavior and similar decreases in total abundances with some variations in months 3 and 4. For example, in both the Peru and India sites total abundances decreased at month 3 and remained nearly constant thereafter, while in the other sites HMO abundances dropped uniformly between month 1 and 6. When comparing total absolute abundance in secretors between month 1 and 6 statistical significance was achieved across all selected sites, were higher significance was achieved in sites with more sampling **(Figure 2.3b).** Similarly, statistical significance was similarly achieved when comparing abundances in non-secretors between month 1 and 6 where there were sufficient number of samples. HMO abundances were also comparing between S+ and S- milk across these sites by averaging the means of all time points within one site. In all counties except Brazil, S+ milk possessed higher abundances of HMOs (P<0.0001, Mann-Whitney).
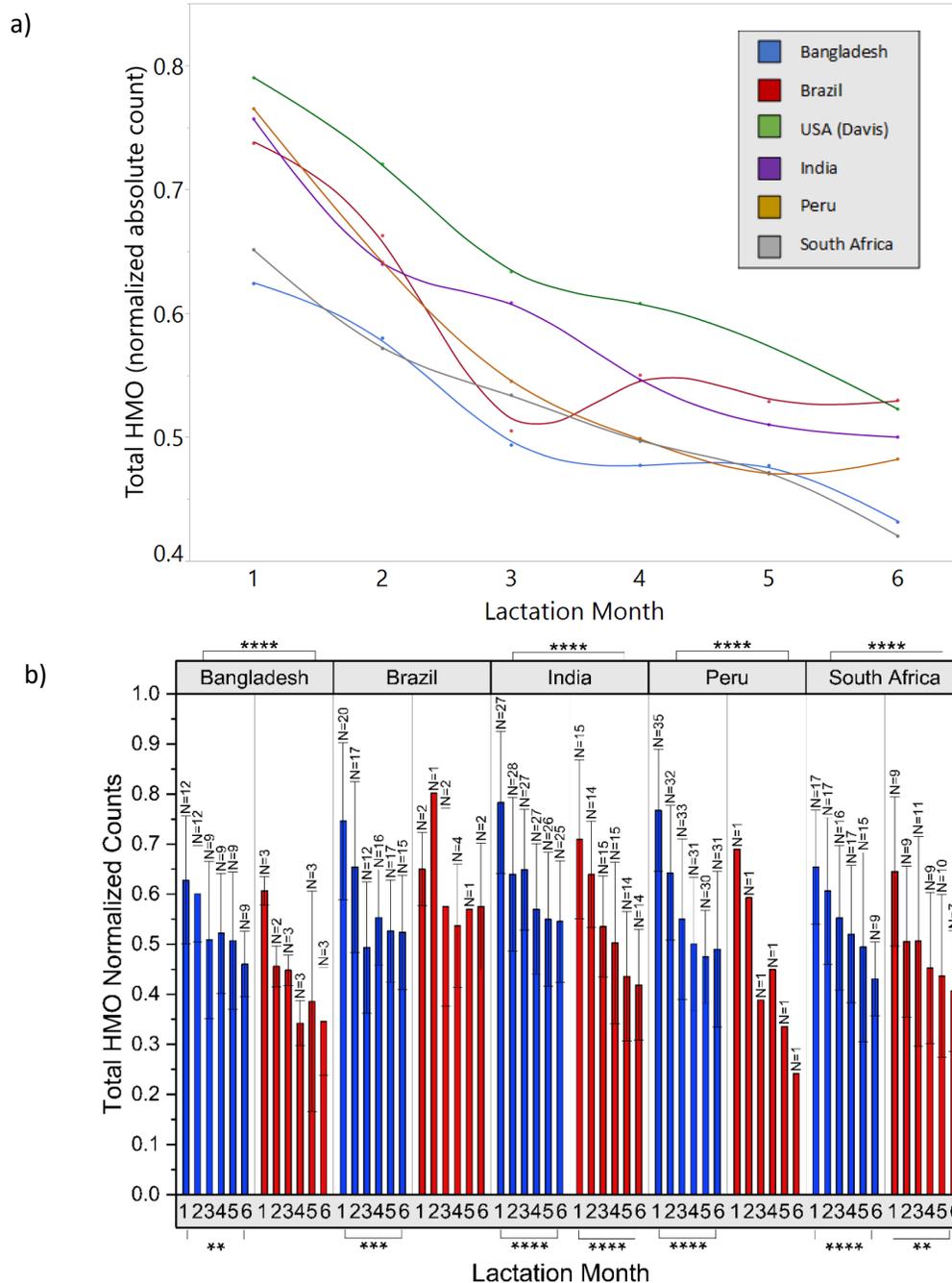
Figure 2.3 (A) Mean changes in HMO concentrations in breastmilk samples collected monthly during the first 6 months postpartum at sites with extensive longitudinal sampling. (B) Total HMO abundances as a function of location, lactation month, and secretor status. HMO abundance values correspond to HPLC-qTOF MS spectral abundance normalized to the mean of the total abundance of ion counts from each sample. N values correspond to the number of samples. Error bars represent standard deviation. P values were obtaining using Mann-Whitney tests with an α correction of α=0.05.

Total HMO abundances and maternal age

Variations in HMOs by maternal age were examined at sites with large numbers of samples [Malawi, Davis-USA, India, Peru]. The milk samples were binned into age groups <20, 21-30, 31- 40 and 41-50 years. Total absolute abundances of HMOs were not different between the age groups **(Figure 2.4)** or between S+ and S- milk (Data not shown). There were also no maternal age-related differences in total fucosylation, total sialylation or levels of 2'FL (the latter among S+ mothers).
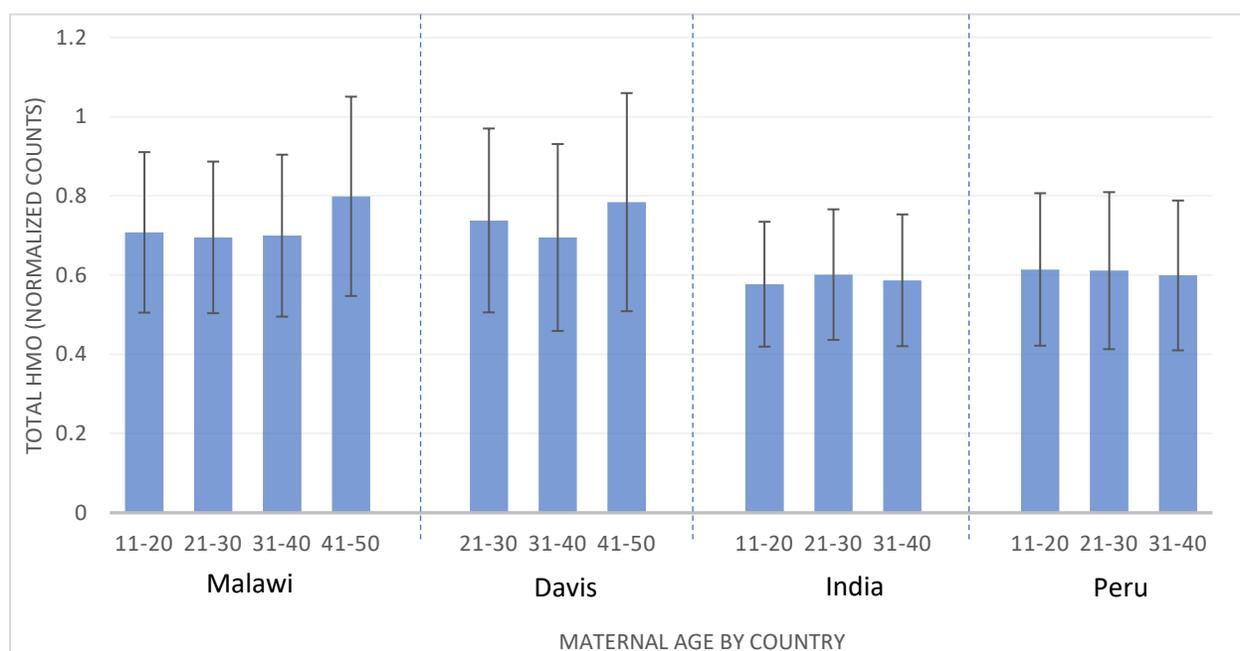
Figure 2.4 Total abundance of HMOs categorized by maternal age groups and countries. HMO abundance values correspond to HPLC-qTOF MS spectral abundance normalized to the mean of the total abundance of ion counts from each sample. Error bars represent standard error.

Total HMO abundances and infant sex

HMOs were compared to determine whether the sex of the infant affected total HMO abundances. Comparisons were made within sites (**Figure 2.5**). We found no statistically significant differences in absolute HMO abundances in milk from mothers of male compared to mothers of female infants. Similarly, total fucosylation and total sialylation yielded no differences based on sex of the offspring (not shown).
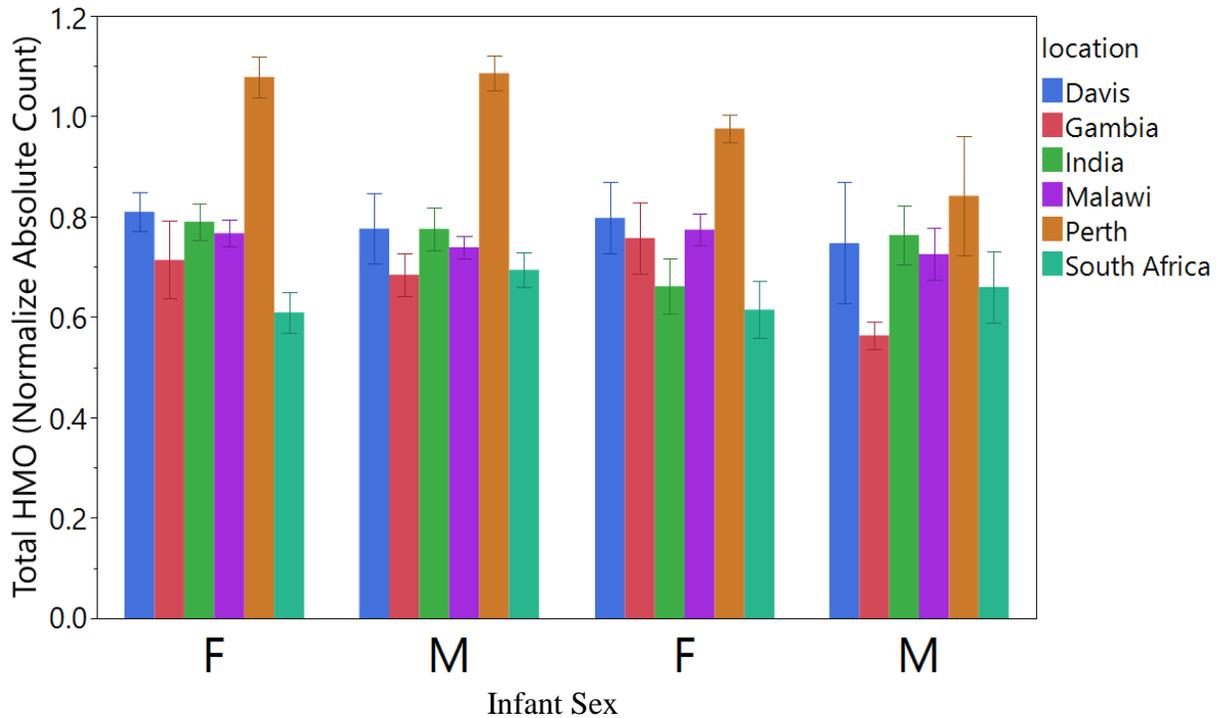
Figure 2.5 Total Abundances of HMOs based on infant gender from regions with the most complete longitudinal samples, where F corresponds to Female infants and M corresponds to male infants. Error bars represent standard error.

Variations in HMO subtypes across study sites and secretor status

Fucosylated HMOs. Fucosylated oligosaccharides comprised the largest and most abundant groups of HMO structures. We first compared the total abundances of α(1-2)-fucosylated HMOs in milk collected monthly during the first 6 months from mothers at study sites with longitudinal sampling, namely Bangladesh, Bolivia, India, Peru, and South Africa (**Figure 2.6a and Table 2.3)**.

Table 2.3 Mean levels of total HMO, α(1,2)-fucosylated HMOs, total fucosylation, total sialylation and total undecorated HMOs by study site and secretor status.

Country	Site Location	GDP per capita	Total HMO (Normalized Counts)		Total Fucosylation (%)		Total Sialylation (%)		Total Undecorated (%)		α(1,2)-Fucosylated (%)	
			B+	B-	B+	B-	B+	B-	B+	B-	B+	B-
Argentina	Namqom	14,508	0.71 ± 0.14	0.37 ± 0.07	69.04 ± 4.69	64.66 ± 5.62	10.40 ± 2.62	24.16 ± 2.86	23.47 ± 5.03	22.44 ± 5.07	24.48 ± 4.94	2.22 ± 0.53
Bolivia	Amazonian Lowlands	3,351	0.61 ± 0.13	0 ± 0	68.90 ± 6.39	0 ± 0	11.14 ± 2.69	0 ± 0	23.20 ± 6.39	0 ± 0	24.12 ± 8.87	0 ± 0
Brazil	Fortaleza, Ceará	9,881	0.59 ± 0.16	0.59 ± 0.12	65.18 ± 6.19	54.80 ± 10.01	11.74 ± 3.64	14.76 ± 5.66	27.68 ± 6.64	36.94 ± 11.55	20.51 ± 7.32	1.72 ± 1.01
Peru	Loreto	6,723	0.61 ± 0.19	0.48 ± 0.17	71.26 ± 4.79	64.15 ± 5.57	13.63 ± 3.22	15.99 ± 3.46	20.35 ± 4.92	26.74 ± 6.41	24.64 ± 7.65	1.98 ± 0.68
Australia	Perth	53,831	0.98 ± 0.24	0.94 ± 0.16	63.24 ± 4.58	58.02 ± 10.77	29.43 ± 6.73	34.17 ± 6.98	21.42 ± 6.08	26.32 ± 10.71	10.84 ± 5.06	2.61 ± 0.82
Bangladesh	Mirpur, Dhaka	1,564	0.58 ± 0.15	0.47 ± 0.15	65.30 ± 5.42	56.16 ± 12.40	11.44 ± 2.95	15.17 ± 3.19	27.76 ± 5.79	34.31 ± 12.66	18.45 ± 7.10	1.28 ± 0.90
India	Vellore, Tamil Nadu	1,980	0.62 ± 0.16	0.54 ± 0.16	62.94 ± 7.43	54.15 ± 9.78	10.78 ± 2.87	15.13 ± 4.15	29.92 ± 7.18	36.37 ± 9.16	19.32 ± 6.49	1.17 ± 2.07
Nepal	Nubri Valley	900	0.64 ± 0.13	0.63 ± 0.24	62.23 ± 6.64	49.66 ± 19.50	14.31 ± 2.88	19.19 ± 4.04	28.42 ± 6.12	38.71 ± 19.37	12.82 ± 3.68	0.99 ± 0.37
Philippines	Cebu	2,982	0.62 ± 0.09	0.51 ± 0.11	64.11 ± 9.35	60.90 ± 5.89	11.54 ± 2.34	16.11 ± 4.35	27.65 ± 9.94	28.74 ± 6.34	20.09 ± 5.37	2.24 ± 0.36
Gambia		673	0.58 ± 0.18	0.55 ± 0.17	53.63 ± 8.49	42.60 ± 16.81	13.85 ± 5.07	16.34 ± 5.76	36.62 ± 7.68	45.96 ± 16.20	18.49 ± 65.85	1.69 ± 1.47
Malawi	Mangochi district	357	0.72 ± 0.20	0.66 ± 0.20	62.01 ± 9.91	54.22 ± 16.59	12.21 ± 3.37	14.31 ± 5.61	29.92 ± 9.32	36.37 ± 15.11	20.54 ± 7.20	1.32 ± 0.74
Namibia	Omuhonga Basin	5,516	0.52 ± 0.25	0.47 ± 0.02	63.63 ± 10.08	57.52 ± 1.67	17.92 ± 4.0	25.43 ± 1.54	26.07 ± 8.41	26.93 ± 0.67	15.10 ± 10.04	1.11 ± 0.03
South Africa	Thohoyandou, Limpopo Province	6,120	0.56 ± 0.15	0.51 ± 0.18	60.15 ± 7.71	50.95 ± 10.45	13.16 ± 3.26	15.20 ± 3.08	31.32 ± 7.69	39.58 ± 9.85	17.88 ± 5.84	1.03 ± 0.74
Poland	Beskid Wyspowy Mtns	13,871	0.70 ± 0.22	0.54 ± 009	63.58 ± 6.60	51.46 ± 6.98	10.69 ± 3.66	17.35 ± 6.01	29.13 ± 7.25	36.68 ± 9.3	18.86 ± 5.06	1.20 ± 0.29
United States	Boston, Massachusetts	59,939	0.76 ± 0.25	0.58 ± 0.27	62.44 ± 3.88	58.95 ± 4.89	12.73 ± 3.47	16.45 ± 2.88	29.40 ± 5.35	30.87 ± 5.70	18.41 ± 6.77	2.87 ± 0.97
United States Davis	Davis, California	59,939	0.72 ± 0.24	0.65 ± 0.24	64.00 ± 5.86	59.92 ± 9.23	13.57 ± 4.40	19.41 ± 6.36	27.50 ± 5.98	29.10 ± 9.19	15.44 ± 7.19	2.11 ± 1.52

[1]Gross Domestic Product (GDP) values for each country are representative of the year of collection. Values are presented as mean ± SD. All data collected was used for this analysis including data from the same mother at different time points of lactation. P values were obtaining using Mann-Whitney tests.
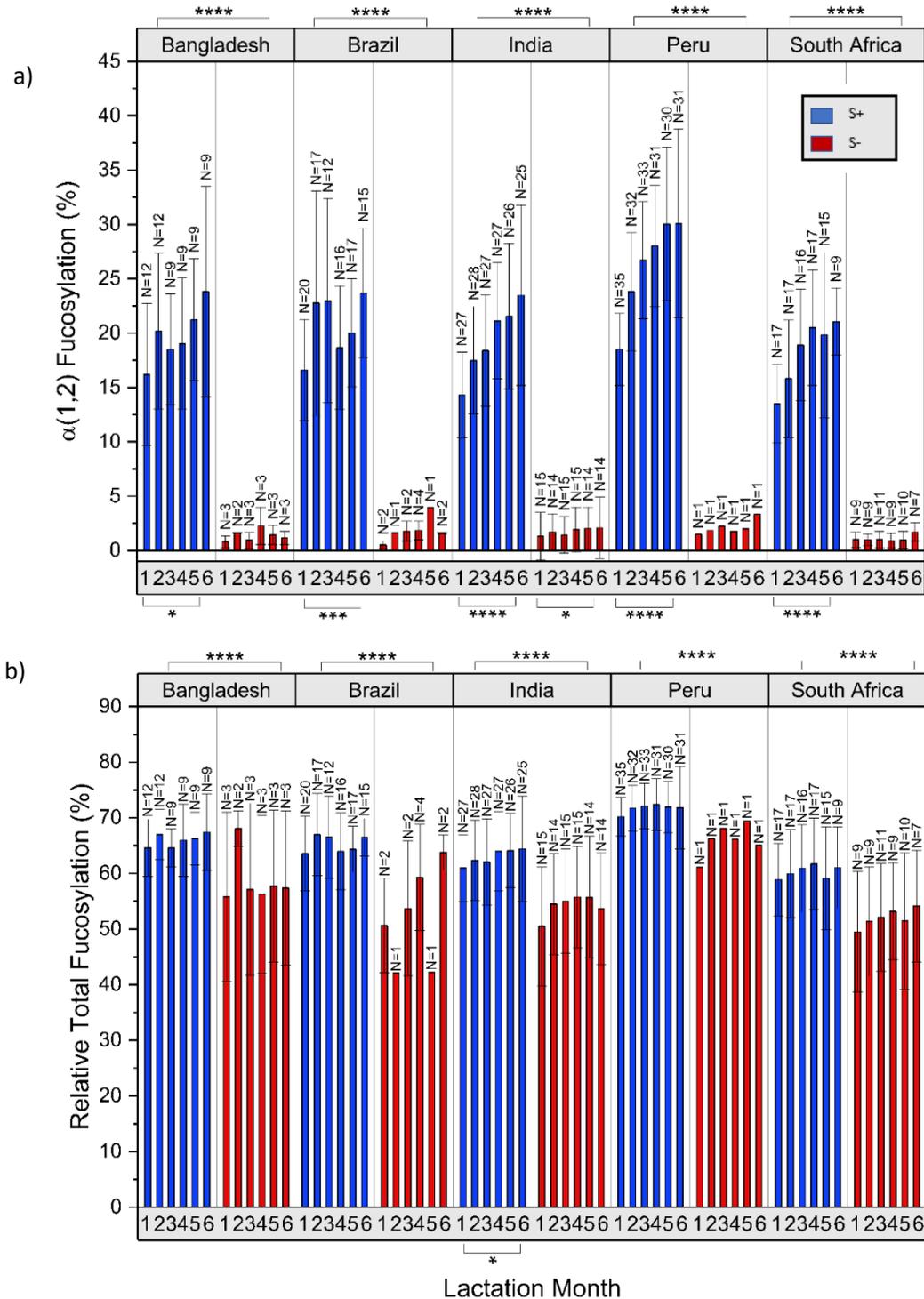
Figure 2.6 Variations in Fucosylated HMOs during Lactation Between Geographical Sites. (A) Mean relative abundance of total α(1,2)-fucose-containing HMOs in breastmilk samples as a function of lactation month (child's postnatal age) at sites with extensive longitudinal sampling. (B) Summed mean relative abundance of all fucose containing HMOs in the same samples (%

Total Fucosylation). S- type producers (■) and S+ type producers (■). HMO abundance values correspond to HPLC-qTOF MS spectral abundance normalized to the mean of the total abundance of counts from each sample. Samples from multiple timepoints provided by a single mother as well as samples which only one time point was provided were included in this analysis. N values correspond to the number of samples. Error bars represent standard deviation. P values were obtaining using Mann-Whitney tests with an α correction of α=0.05.

The proportion of α(1,2)-fucosylated structures in secretors increased significantly between postnatal months 1-6, with statistical significance across all selected sites (P<0.05-P<0.0001, Mann-Whitney). However, for S- milk, only the site with large sample size (India) achieved statistical significance. S+ milk had consistently higher abundances of α(1,2)-fucosylated HMOs (ranging from 15-20% of total HMOs) compared to S- milk (<5% of total HMOs; P<0.0001, Mann-Whitney)). Interestingly S- milk, while containing very low abundances of α(1,2)-fucosylated HMOs, at times had nonzero abundances slightly above the baseline (P<0.5; Mann-Whitney). As 2-fucosyllactose is the most abundant of these structures, it increased over time as expected (**Figure 2.7**). Total fucosylation (oligosaccharides that contain fucose regardless of the linkage) increased between months 1 and 6 in all sites, however significance was only achieved in the India site and only for S+ milk (P<0.05, Mann-Whitney) (**Figure 2.6b**). Total fucosylation differed slightly between S+ and S- milk (**Figure 2.6b**). In general, S- milk had low abundances of fucosylated structures compared to S+ milk, reflecting the deficit in α(1,2)-fucosylated HMOs, but partially compensated by increases in (1,3/4)-fucose linkages.
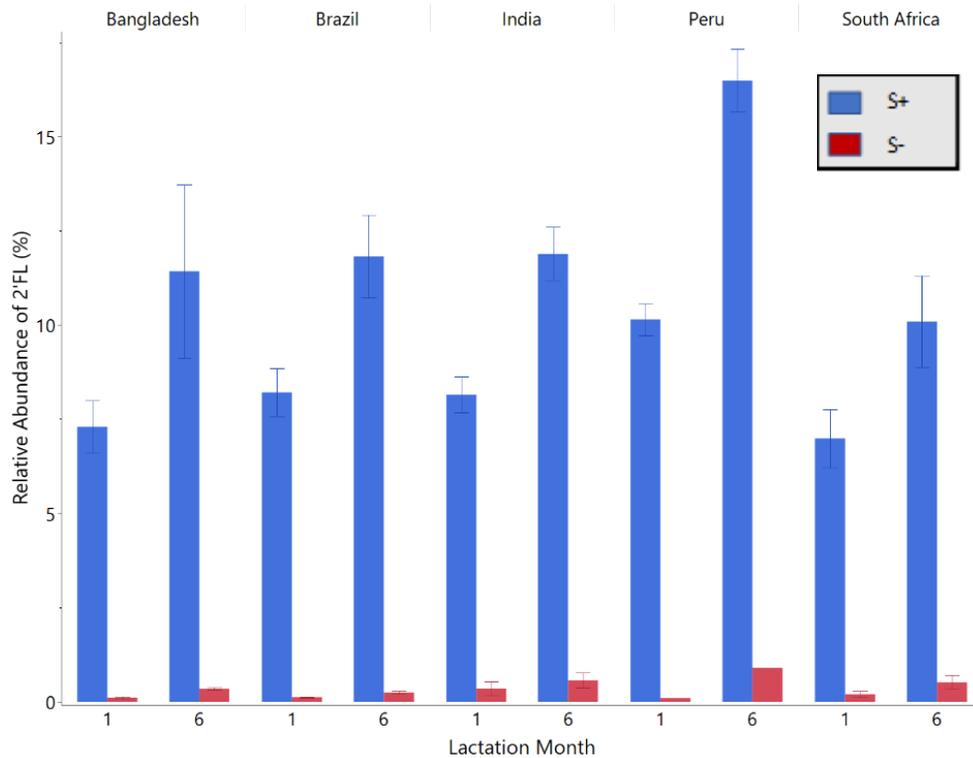
Figure 2.7 Relative abundance of 2'FL at 1 and 6 month postpartum across 5 geographically diverse sites, where (■) corresponds to S+ producers and (■) corresponds to S- producers.

Variations in fucosylated structures were observed between milk from different countries within each secretor phenotype (**Figure 2.2**). Mothers with S+ milk from Nepal had significantly higher levels of DFLNHa (P<0.0002, Mann-Whitney) and lower levels of 2'FL (P<0.0001) (both S+, secretor markers) compared to the other sites. Human milk obtained from mothers residing in the Boston-USA and Gambia sites had high relative abundances of LNFP I and LNFP III compared to all other sites, and low abundances of LNFP II. Milk from mothers residing in Perth-Australia had relatively high abundances of LSTc and LSTb compared to other sites. Interestingly, 2-fucosyllactose was not generally the most abundant in S+ milk among all countries. Australia, Boston-USA, and Namibia all had S+ milk with the most abundant α(1-2)-fucosylated compound being LDFT (P = 0.005, Mann-Whitney).

60

Sialylated HMOs. Sialylated oligosaccharides generally had much lower abundances in human breast milk than fucosylated species. The mean abundances of sialylated HMOs in samples collected over the first 6 months postnatal from different study sites are shown in **Figure 2.8** and **Table 2.3.** Total abundances of sialylated HMOs were relatively constant throughout lactation for both S+ and S- phenotypes. Likewise, the relative abundances of sialylated HMOs were comparable across study sites with extensive sampling (Bangladesh, Bolivia, India, Peru, and South Africa) averaging 12.3% ± 3.4 of the total HMOs in S- milk and 15.2% ± 3.8 in S+ milk (P<0.05-P<0.0001, Mann-Whitney). The most abundant sialylated HMOs when considering all samples were the combined group of LSTc+LSTb (in both S+ and S- samples) (**Figure 2.2**). Note however that the values from Peru for S- milk were obtained from samples provided by a single mother due to the very low prevalence of S- milk in this population. In populations with greater frequencies of S- milk, there was a greater relative abundance of sialylated structures in S- milk compared to S+ milk. In samples from India, Bangladesh and South Africa, the absolute abundances of sialylated HMOs in month 1 was nearly 15% greater in S- compared to S+ milk.

It is noteworthy that the sialylated HMO DSLNT, which has been shown to be protective against necrotizing enterocolitis in an animal model[39] and in a human preterm infant cohort [40] was often found in low abundance and was not one of the 60 HMOs found in all sites.
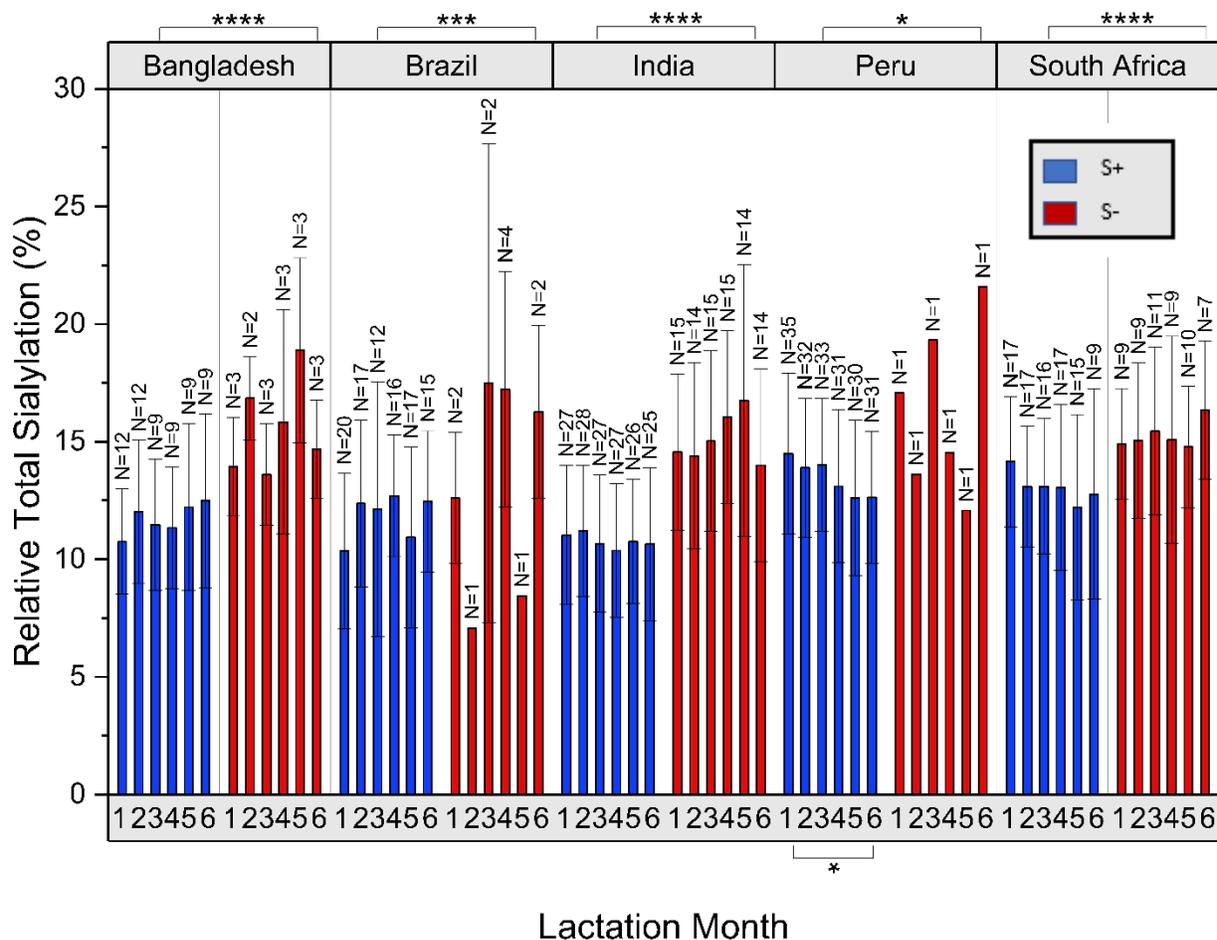
Figure 2.8 Mean relative abundances of total sialic acid (Neu5Ac)-containing HMOs (% Total Sialylation) in breastmilk samples as a function of lactation month (child's postnatal age) at sites with extensive longitudinal sampling. Samples from multiple timepoints provided by a single mother as well as samples from multiple mothers with one time point were all included in this analysis. HMO abundances corresponded to HPLC-qTOF MS abundances normalized to the mean of the total ion counts from each sample. Error bars represented standard deviation. P values were obtaining using Mann-Whitney tests with an α correction of α=0.05.

Undecorated (nonfucosylated and nonsialylated) HMOs. The total abundances of

undecorated HMOs (lacking both fucose and sialic acid) in milk from mothers at the various

study sites were compared in **Figure 2.9** and **Table 2.3.** Undecorated HMOs were lower in S+

milk compared to S- milk (P=0.002, Mann-Whitney).[41] Between sites, overall relative

abundances of undecorated HMOs were comparable (31.6±8.9%) with the exception of Peru,

which exhibited significantly lower levels (20.5± 5.2%) (P=<0.0001) due to the higher amounts

of fucosylation and diminished numbers of S- milk. Across all samples the most abundant

undecorated HMO was the LNT/LNnT group (**Figure 2.2**).
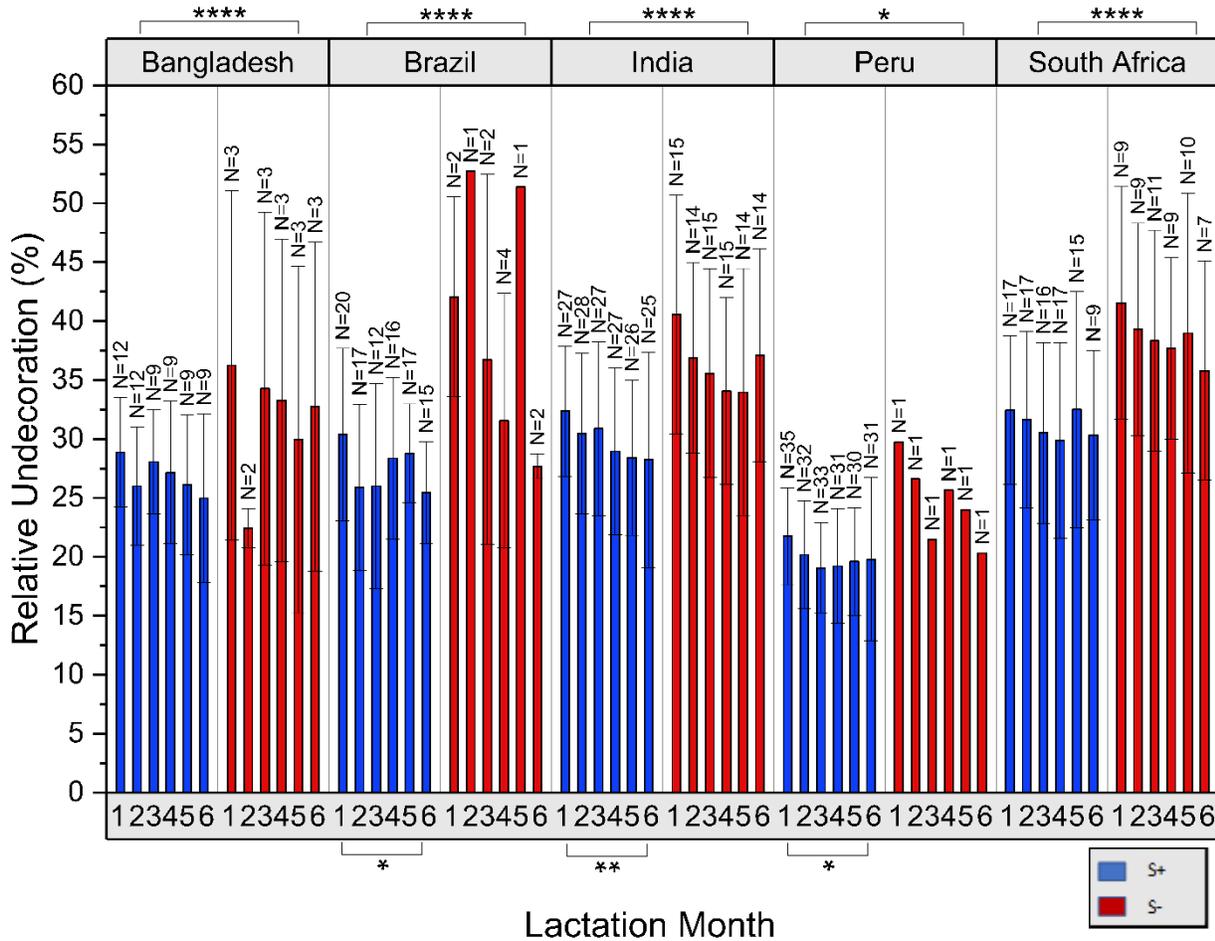
Figure 2.9 Relative abundance of undecorated HMOs from regions with the most complete
longitudinal samples, where (■) corresponds to S+ producers and (■) corresponds to S-
producers. Samples from multiple timepoints provided by a single mother as well as samples
which only one time point was provided were included in this analysis. N values correspond to
the number of samples. Error bars represent standard deviation. P values were obtaining using
Mann-Whitney tests with an α correction of α=0.05.

Global Variations of S+ and S- milk

With the samples from different locations and the ability to distinguish S+ and S- milk, we determined the distribution of the two phenotypes globally. The sites are located on the map along with the fraction of S+ (blue) and S- (red) milk (**Figure 2.10**). The fraction of the mothers that produced S- milk was lower than those that produced S+ at every geographical site. High proportions of S- milk was found in Africa (37% in South Africa and 36% in The Gambia). These values contrast with other sites in Africa including Namibia (17%) and Malawi (25%) that had lower proportions of S- milk. Other sites with high levels of S- milk included those in India 35% and Nepal 29%. Bangladesh was lower with only 20% S- mothers. The USA sites (Davis, CA (22%) and Boston MA, 25%) were similar to the Poland (22%) site and the Australian site (19%). Low values were found in South America sites with Bolivia (0%), Peru (3%), Brazil (10%). The samples from these sites were obtained from indigenous populations. The other South American site, Argentina (15%) included nonindigenous populations.
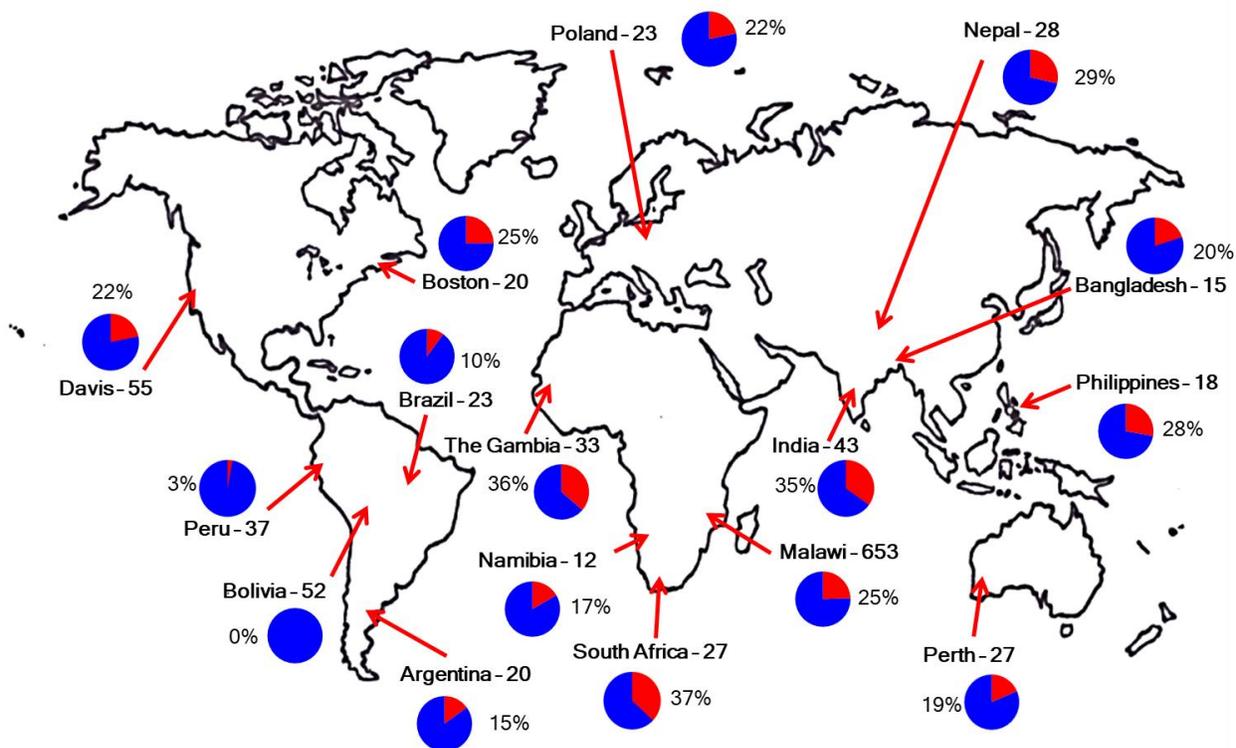
Figure 2.10 Proportion of samples tested from each study location that were S+ type (i.e. secretor mothers, ■) and S- type (non-secretor mothers, ■). Labels presented as Country-Number of Mothers. If samples from multiple timepoints were provided by the same mother, the secretor status determination was concluded based on the majority of her samples. If there was no 'majority milk type', she was excluded from the statistical analysis (one mother from Davis, USA, two mothers from Malawi, and three mothers from Perth, Australia).

DISCUSSION

The analyses of the HMO compositions of human milk samples collected from 16 sites around the world using a sensitive HPLC-qTOF-MS-based approach provided the most extensive dataset reported to date. While the samples were collected from different studies, the analytical method for each sample was the same. Unfortunately, absolute quantitation of total HMO and specific structures was not possible due to the low availability of standards in the earliest analysis.

This study significantly expands on our previous work at a single site in The Gambia.[23] The large number of samples from various geographical locations allowed us to further explore factors that may affect HMO production during lactation. Factors such as the age of the mother and the sex of the infant do not affect HMO abundances. The sex of the infant had been previously reported to affect milk compositions, suggesting that some components of human milk may be tailored to sex-differentiated developmental priorities.[42] However, examination of milk among all populations and within each site yielded no significant variations in total HMO abundances based on sex of a mother's child. Because neither dietary data nor maternal nutritional status were available for this study, we were not able to determine the extent to which these factors might influence abundances of individual structures nor levels of fucosylation and sialylation. However, the comparison of per capita gross domestic product (GDP) was obtained by comparing total abundances with published GDP. Interestingly, we observed some correlation between per capita GDP and total levels of HMOs from mothers living at the different sites. (**Figure 2.11**). As shown, countries with high GDP per capita tended to have milk with the most abundant levels of HMOs. Likewise, mothers from sites with the low GDP per capita tended to have lower levels of HMOs. Among countries with low GDPs, there was a common minimum level of HMOs, while the trend towards higher abundances of HMOs did not appear to manifest until significantly higher GDPs were obtained. It would be difficult to make conclusions regarding GDP and HMO production as the amounts of HMOs fed to the infant can vary depending on feeding local feeding practices. Furthermore, the sample size, though large in totality, is still small at the local level and cannot fully represent the respective nation. However, we encourage further studies on societal effects on milk production.
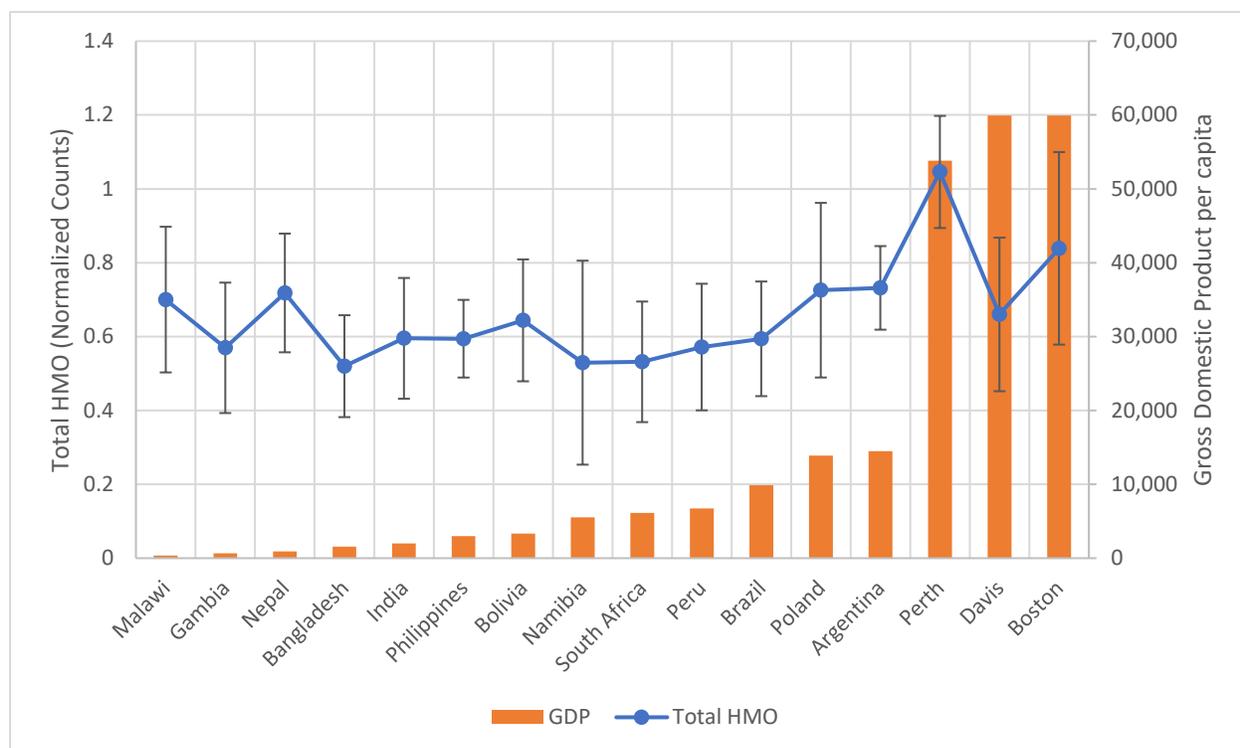
Figure 2.11 (■) Gross domestic product per capita across all study sites (■) average abundance of HMOs found in mothers across all study sites. Samples from multiple timepoints provided by a single mother as well as samples which only one time point was provided were included in this analysis. Error bars represent standard deviation.

Genomic analysis has been previously used to obtain global distributions of secretors and nonsecretors.[43] While this approach uses obtains the genotypic status, the phenotype, i.e., the actual abundances of different structures and structural types in the milk, are those that affect infant health and developmental outcomes.[44] Hence, the concentrations of HMOs are crucial to understanding the health outcomes of infants providing information of distinct nutritional components that align with secretor status. Additionally, among secretors there are large variations in the abundances of fucosylated and sialylated species that are important contributors to infant development. The concentrations can then be used to phenotype milk based on the abundances of specific structures, namely α(1,2)-fucosylated species, without genomic data. For

the purposes of this study, we therefore refer to milk that corresponds to high amounts of α(1,2)-fucosylated structures as S+ milk, and milk corresponding to low abundances as S- milk.

The fraction of mothers who produce S- milk was nearly 40% in West and South Africa and South Asia, while nearly zero in parts of Latin America. The fraction of nonsecretor mothers (and hence S- milk) is often cited to be ~20%. However, this number is based primarily on studies of European and Euro-American mothers, which is consistent with our own results for sites in Europe and the USA. The USA sites (Davis CA, Boston MA), and Poland (the sole European site) had S- milk in proportions similar to those previously reported.[34] The rarity of S- milk among indigenous populations in South America suggests either founder effects during human migration into Beringia or selection from pathogen pressure. Many infectious diseases including cholera, whose severity is associated with blood type, can have devastating effects on populations.[17] As previously discussed, blood typing is similar to human milk in that the presence or absence of α(1,2)-fucosylated structures is the key determinant. A study of the cholera outbreak in Peru in 1991 found that those with blood type O, which occurs at very high frequency among South American indigenous populations, had more severe symptoms and were eight times more likely to be hospitalized, emphasizing the relevance of glycosylation on infectious diseases.[45]

A distinguishing feature of humans and primates compared to other mammals is the high level of fucosylated structures with humans having the highest abundances.[46] Fucosylated structures, or the presence of at least one fucose, increased in relative abundances throughout lactation. Previous studies similarly noted that fucosylation increased throughout lactation for the first six months regardless of secretor status.[41] Fucosylation was generally higher in S+ milk. As a consequence of higher fucosylation in S+ milk, the amount of undecorated HMOs was lower

compared to S- negative milk, consistent with earlier findings.[41] In contrast, sialylation is the lowest among humans but is significantly greater in bovine and porcine milk .[47] In this study, though sialylated oligosaccharides in the human milk samples were still low, they were relatively higher in S- compared to S+ milk. Previous studies have also reported the higher abundances of sialylated HMOs in nonsecretor (S-) milk.[41]

The structural variety of HMOs and their different relative abundances have the potential to endow human milk from different mothers with distinct functional properties, including modulating its effect on the developing microbiota and its effects on infection/colonization with enteropathogens.[48] Members of the infant gut microbiota have specific glycosyl hydrolases and glycan-binding proteins that either promote the fitness of specific community members or block the host from infection. For example, HMOs promote the growth of *Bifidobacterium longum* subsp. *infantis*, a gut symbiont that is richly endowed with a suite of genes specifically adapted to import and utilize HMOs. The HMO composition of breastmilk thus has the potential to influence the fitness of strain-level variants of this and other related bifidobacterial species and to shape a program of normal postnatal community development (succession) that has been identified in healthy individuals and that is impaired in those with undernutrition [13, 49] Preclinical studies in gnotobiotic animals and clinical studies of the effects of repairing microbial community immaturity in children with acute malnutrition support the notion that healthy development of the microbiota is causally linked to healthy growth.[13, 49] Similarly, HMOs with α(1-2) linked Fuc (S+ milk) are associated with decreased incidence and severity of diarrhea caused by *Campylobacter jejuni* and enteropathogenic *E. coli* - enteropathogens[50, 51] that are ubiquitous in many low income countries where childhood undernutrition is prevalent.[52] S- milk is enriched in HMOs that bind to *Helicobacter pylori*, and enteropathogenic *E. coli* preventing their attachment to gut epithelial

cells.[53] Individuals homozygous for *FUT2* mutations (non-secretors) also show resistance to

norovirus infection [54, 55] or considering this relationship from the viewpoint of the pathogen, both

rotavirus and norovirus (two of the most common causes of viral diarrhea in infants) appear to

prefer the secretor host.[32] These mothers could in turn provide protection to their infant by

delivering S- milk. Determining infant infectious disease risk should ideally include consideration

of the secretor status of both the mother and the infant to address such key question as whether S+

milk is particularly beneficial for the non-secretor infant and vice versa.

The global results suggest that secretor status and the complement of HMO produced by

a mother during lactation are influenced in part by adaptations shaped by ancestral nutritional

and disease ecologies experienced by diverse human populations. Indeed, immunofactors in

breastmilk are associated with subsistence practices that affect nutritional intake and pathogen

exposure of diverse traditional societies and demonstrate the importance of considering

populations within their contemporary, historical, and prehistorical contexts.[45] The "first-step"

findings described here highlight the importance of multi-population studies to better

characterize the relationships among maternal characteristics, HMO composition, early gut

community development, the products of microbial metabolism of these HMOs, and measures of

infant health status.[46] Delineation of these relationships, along with those mediated by other key

constituents of breastmilk, e.g., secreted immunoglobulins and antibacterial proteins, will help

guide the design of future prebiotic approaches based on purified milk components (or

synthetically-produced mimetics) and/or synbiotics (prebiotics combined with a probiotic micro-

organisms) that promote healthy gut community development healthy growth of infants , and

even healthy immune and inflammatory responses over a lifetime.

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J.C.C.D. performed statistical analyses on HMO data and infant disease metadata. C.B.L. designed the study and oversaw the analyses. A.V. and C.B.L. interpreted results and wrote the manuscript together with J.C.C.D., M.B. and J.I.G

Data and materials availability: Materials and correspondence should be directed to Carlito B. Lebrilla, cblebrilla@ucdavis.edu.

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CHAPTER THREE

Fetus Receives Breast Milk Carbohydrates in Utero: An LC-MS based method for HMO analysis in Amniotic Fluid

ABSTRACT

Human Milk Oligosaccharides (HMOs) are an abundant component of breast milk and have shown to be beneficial to the infant's development. Previous studies have found that HMOs are not only present in maternal milk but also present in the mother's serum and urine. Herein, we report the first comprehensive study of oligosaccharides present in amniotic fluid demonstrating direct interaction of HMOs with the developing fetus. An extensive library and analytical methods were developed that allow for accurate quantitation of oligosaccharides using high resolution mass spectrometry. This method was applied to over 500 mothers from different gestational periods, ethnicity/race, ages, and birth outcomes. We observed over 30 oligosaccharide structures present in the amniotic fluid, eight of which are exclusive to maternal milk. Structure specific changes in concentration of amniotic fluid throughout gestation were revealed. HMOs 2'FL and LDFT increased with increasing gestational age (p=9.9x10^{-8} and 0.002, respectively) while other HMOs such as 3'SL decreased in concentration throughout gestation. These results provide insight into the potential roles of HMOs and provides evidence that the developing fetus is receiving breast milk components in utero.

INTRODUCTION

Human breast milk consists of a diverse group of bioactive molecules that aid in the development of the infant. Nutrients can be categorized primarily into three major components: fats, proteins, and carbohydrates. Carbohydrates are the most abundant nutrient accounting for roughly 40% of the total calories provided by breast milk.[1] A unique subset of carbohydrates found in human breast milk are called human milk oligosaccharides (HMOs). HMOs are unique among the macronutrients because unlike lactose, proteins, or fats, they are not digestible nor absorbed, yet they remain as one of the most abundant components.[2] The base structure of every HMO consists of lactose (a disaccharide comprised of glucose and galactose) at the reducing end and elongated with further additions of L-fucose (Fuc), D-galactose (Gal), N-acetylglucosamine (GlcNAc) and N-acetylneuraminic acid (Sia). These additions very in sequence and orientation, which accounts for the structural diversity of hundreds of unique oligosaccharides that have been identified in human milk to date.[3, 4]

The presence and considerable abundance of oligosaccharides in maternal milk strongly suggests an important functional role for these sugars in infant development. The structure-function relationships between individual HMO structures and infant health have not been completely explored, however scientists have shown that HMOs collectively provide many benefits to the developing infant including establishing gastrointestinal microbiome, [5-11] acting as a decoy for pathogenic infection,[12, 13] and aiding in neurological and cognitive development.[14] More recent investigations have suggested that HMOs may benefit even the developing neonate as HMOs have been found in material blood,[15] urine,[16] and more recently amniotic fluid[17] of pregnant mothers. The presence of HMOs in amniotic fluid is particularly important because

amniotic fluid not only surrounds the fetus during pregnancy but is also involved in various intermembranous pathways that transfer fluid and solutes from maternal plasma to the fetus.[18-20]

Amniotic fluid initially was thought to provide primarily physical support and other benefits such as allowing room for fetal growth and development, acting as a mechanical cushion for protection, and temperature regulation, but more recently researchers have observed potential nutritive functions of amniotic fluid providing vital nutrients such as proteins, lipids, and carbohydrates.[21, 22] Unfortunately, due to the complexity of the matrix, low concentrations of the analytes and the difficulty in obtaining samples, there are limited studies on the composition of amniotic fluid and even less on the direct effects of the composition on fetal development at different stages of gestation.

Herein, we report the methodologies for extraction, isolation, and characterization of all quantifiable oligosaccharides (including non-HMO structures) in N=516 maternal amniotic fluid samples utilizing LC-MS, making this the most comprehensive study of amniotic fluid oligosaccharides to date. The methods and results detailed here aim to provide the first steps to understanding the biological role of oligosaccharides in the uterus and provide the analytical tools for further investigations to address the many unanswered questions regarding the role and composition of amniotic fluid.

METHODS

Amniotic Fluid Collection

Under IRB approval from the University of Texas (HSC-MS-07-0109), this study recruited gravid participants who were undergoing clinically indicated and/or maternal requested

genetic amniocentesis for prenatal diagnosis. Consistent with genetic diagnostic amniocentesis being performed in the mid-trimester, participants were recruited and sampled at 15-29 weeks gestation (mean gestational age 17.5 +\- 1.9) and underwent full and informed consent to allow an additional 3-5 ml of amniotic fluid to be sterilely collected as part of their planned clinical procedure using aseptic technique, transferred under sterile conditions to 1.5 ml screw top cryopreservation vials, and stored at -80 °C until future use in the current research studies. Briefly, after sterile preparation and draping of the lower gravid abdomen, a 20 to 22 G spinal needle was advanced perpendicular to the skin with the transducer beam at a 15- to 20-degree angle. In a single passage under continuous ultrasound visualization and guidance using aseptic techniques and with sterile handling maintained throughout the entirety of the procedure, a total of 30 to 45 mL of sterile amniotic fluid was collected in three steps. As a first step, an initial sterile 3 or 5 cc syringe was used to aspirate a small amount (1 to 3 mL) of amniotic fluid through the sheath to discard any potential maternal or skin contaminants. Caution was taken when entering the amniotic cavity to ensure no direct contact with the fetus. Second, without repositioning the sheath, sequential aspirations with 10 or 15 cc sterile syringes collected the 25 to 35 cc of amniotic fluid to be sent for clinical diagnostic purposes. Third, and again without repositioning the sheath, a final aliquot of 3-5 mL of amniotic fluid was aspirated with a 5 cc sterile syringe, using identical aseptic sterile technique under continuous ultrasound visualization. This final 3-5 mL of amniotic fluid was immediately transferred and aliquoted to sterile cryotubes for preservation at -80 °C. The prospective cohort was followed for later outcomes during the pregnancy, which were abstracted into an anonymized (coded, without identifying data) repository that accompanied the biobank. Data recorded included the week of amniocentesis, self-reported race and ethnicity of the mother, parity, indication for

amniocentesis, history of preterm birth, and delivery outcome inclusive of gestational age at delivery, spontaneous or indicated preterm birth and including preterm premature rupture of membranes. The outcome of the fetal karyotype was also included.

Oligosaccharide Extraction from Amniotic Fluid

A total of 514 amniotic fluid (AF) samples were prepared for oligosaccharide profiling. Surrogate internal standard mix (xylo-oligosaccharides) was prepared in a large batch and added to individual samples prior to preparation. 100 µL of AF was purified using a 10K allowance ultracentrifuge filter. The flow through was then cleaned up with solid phase extraction packed with C18 and sequentially processed through PGC. Eluant was dried and concentrated with water prior to analyses on a nano-HPLC-qTOF-MS (nano-HPLC: Model series 1200, QTOF: Model series 6220, Agilent Technologies). Separation was performed on a microfluidic chip packed with PGC via enrichment and analytical columns, respectively, using a binary gradient of solvent A [3% acetonitrile (ACN) in 0.1% formic acid] and solvent B (90% ACN in 0.1% formic acid). Optimized gradient composition as follows: 0.00–2.50 min, 2–7% B; 2.50–10 min, 7–10% B; 10.00–25.00 min, 10–16% B; 25.0– 40.00 min, 16– 35% B, 40.00– 45.00 min, 35-100% B. Mass spec was set to operate in positive mode with parameters optimized in previous publication.[23, 24] Standard solutions of the most abundant HMOs where ran prior to each batch with concentrations ranging from (0.03 fmol/mL to 15.75 nmol/mL). Blanks and quality controls were run every 6 samples.

Statistical Analysis and Data Processing

Data was collected using Agilent MassHunter Workstation Data Acquisition and then analyzed with Agilent MassHunter Qualitative Analysis software. Peak areas were assigned

using Profinder B.08.00. Calibration curves were constructed using Microsoft Excel 2016.

Matrix correction factors were calculated by dividing the intensity of pure internal standard by

the mean internal standard signal in the samples. Cohort normalization was performed between

all batches to reduce batch-to-batch variability by normalizing each batch to the cohort mean.

Limits of Quantitation (LOQ) and Limits of Detection (LOD) were defined as 6X and 3X the

signal-to-noise ratio of the blanks respectively.

RESULTS

Analysis of HMOs in amniotic fluid poses various challenges due to the diversity of

structures and complexity of the fluid matrix. Additionally, due to their importance in fetal

development and proximity to the fetus, samples even in minimal amounts, are difficult to

obtain.
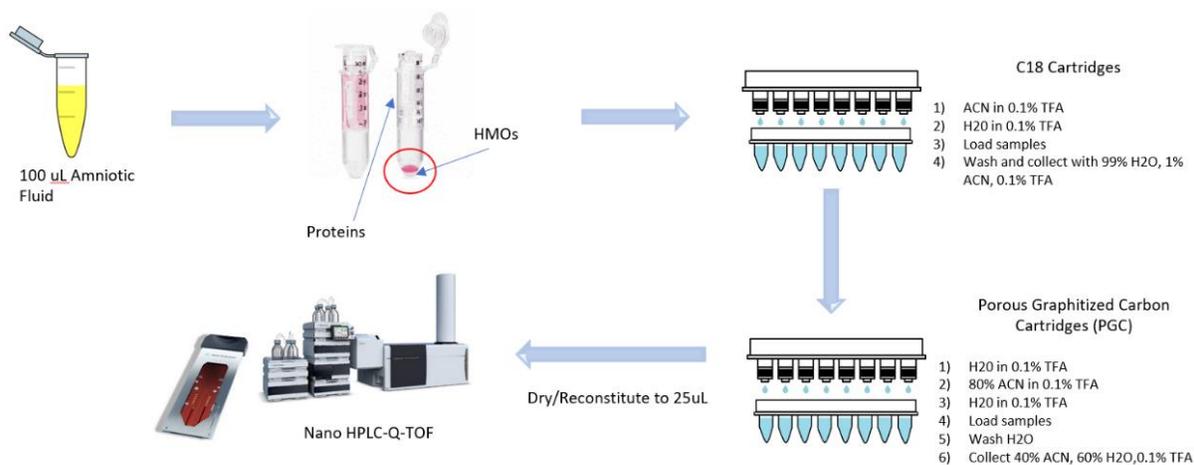
Figure 3.1 Workflow for extraction, isolation, and profiling of oligosaccharides in amniotic
fluid.

A method for isolation and extraction of oligosaccharides was developed specifically for amniotic fluid. A schematic of the extraction procedure is presented in **Figure 3.1** The extraction procedure is unique from those published analyzing oligosaccharides in breast milk[3, 5, 23-27], serum[15, 28], plasma[29], or urine[16, 30, 31] in that it required several isolation steps to separate the oligosaccharides from the other biological components. A 100 µL aliquot of the fluid was first purified through a 10K allowance filter allowing the smaller molecules (<10 kDa), such as the oligosaccharides, to pass through. Larger molecules, such as proteins, were retained and separated from the oligosaccharides. The flow through (containing the HMOs) then went through two solid phase extractions, the first extraction with C18 and then sequentially with PGC. The C18 cartridge removed proteins, peptides or lipids potentially remaining in the sample after the filtration. PGC is commonly used for glycan retention through dispersive interactions and polar retention, allowing most nonpolar species and salts to be removed. Isolation of oligosaccharides from other biological fluids such as breast milk or serum often required a single SPE step; however, because of the complexity of the matrix, we found that performing two clean up steps provided the best glycan coverages **(Figure 3.2)**. As shown, we observed up to a seven-fold increase in peak intensity when performing both C18 and PGC cleanups sequentially compared to using just one extraction technique. The enriched HMOs were dried down and concentrated to 4X the original concentration prior to analyses on a nano-HPLC-qTOF-MS.
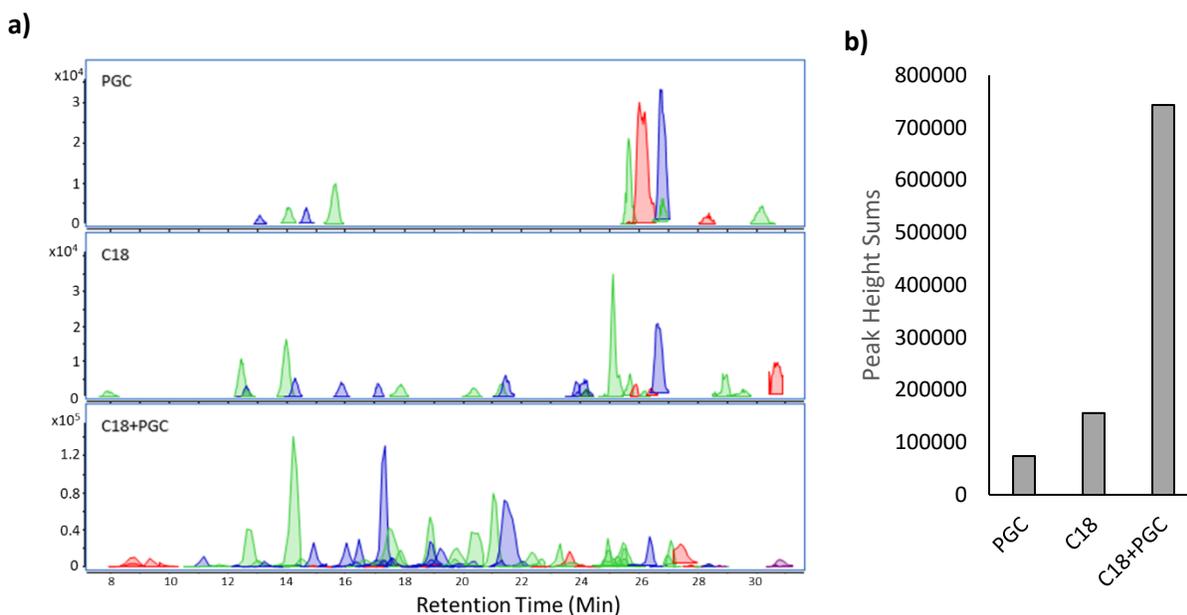
Figure 3.2 (a) Extracted Ion Chromatograms of amniotic fluid samples extracted with PGC (top), C18 (middle), and C18 + PGC (bottom). Red indicates fucosylated oligosaccharides, green indicates sialylated oligosaccharides, blue indicates undecorated oligosaccharides. **(b)** Total peak height of oligosaccharides detected from the corresponding chromatograms.

Amniotic fluid from N=4 mothers were pooled and analyzed on a nano-HPLC-Q-TOF-MS. Oligosaccharide peaks were filtered by matching the output data obtained on the LC-MS with an in-house library of exact masses and chemical compositions. Analysis on nano-HPLC-Q-TOF-MS revealed around 64 oligosaccharides present in amniotic fluid. **Figure 3.3** shows a representative chromatogram of the oligosaccharides detected from the pooled amniotic fluid sample.
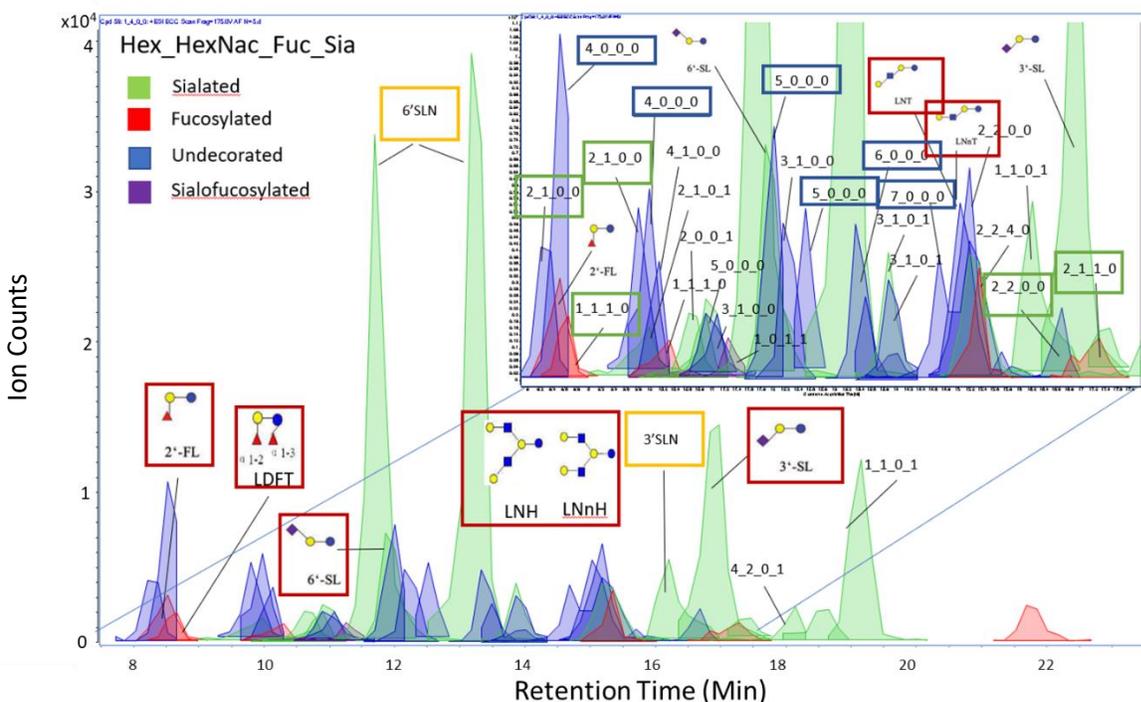
Figure 3.3 Extracted ion chromatograms of oligosaccharides in amniotic fluid. Monosaccharide composition of structures are given as Hex _HexNAc_Fuc_Neu5Ac and represented as glucose (●), galactose (●), N-acetylglucosamine (■), and fucose (▲). Color of chromatographic peak is characterized by decoration, green-sialylated, red-fucosylated, blue- undecorated, purple-sialofucosylated.

Of the total identified oligosaccharides, eight were common to human breast milk

(represented by the red boxed structures in the chromatogram) including 2'-Fucosyllactose (2'-

FL), Lactodifucotetraose (LDFT), Lacto-N-tetraose (LNT), Lacto-N-neotetraose (LNnT), 3'-

Sialyllactose (3'-SL), 6'-Sialyllactose (6'-SL), Lacto-N-neohexaose (LNnH), Lacto-N-hexaose

(LNH). Interestingly, we observed several non-HMO structures that had structures similar to

HMOs as they contained a lactose core and were similarly elongated with sialic acid and fucose.

The most abundant of these structures were 2_1_0_0 and 2_2_0_0. Surprisingly, 3'-Sialyl-N-

acetyllactosamine (3'-SLN) and 6'-Sialyl-N-acetyllactosamine (6'-SLN) (represented by the

yellow boxed structures in the chromatogram) were found to be one of the most abundant

oligosaccharides in the amniotic fluid samples and have also been previously reported in infant urine and serum but are generally not found in maternal milk.[15, 28] Hexose containing structures, represented by the blue boxed structures in the chromatogram were also found in large abundance in a majority of the samples analyzed. We also observed the presence of oligosaccharides that appeared as fragments of larger HMO species (green boxed structures). They did not correspond to HMO structures but were similar in composition while missing one or two terminal sugars. Several more oligosaccharides were identified; however, many were either too low in abundance to elucidate the structure or were only found in a small subset of samples (not shown).
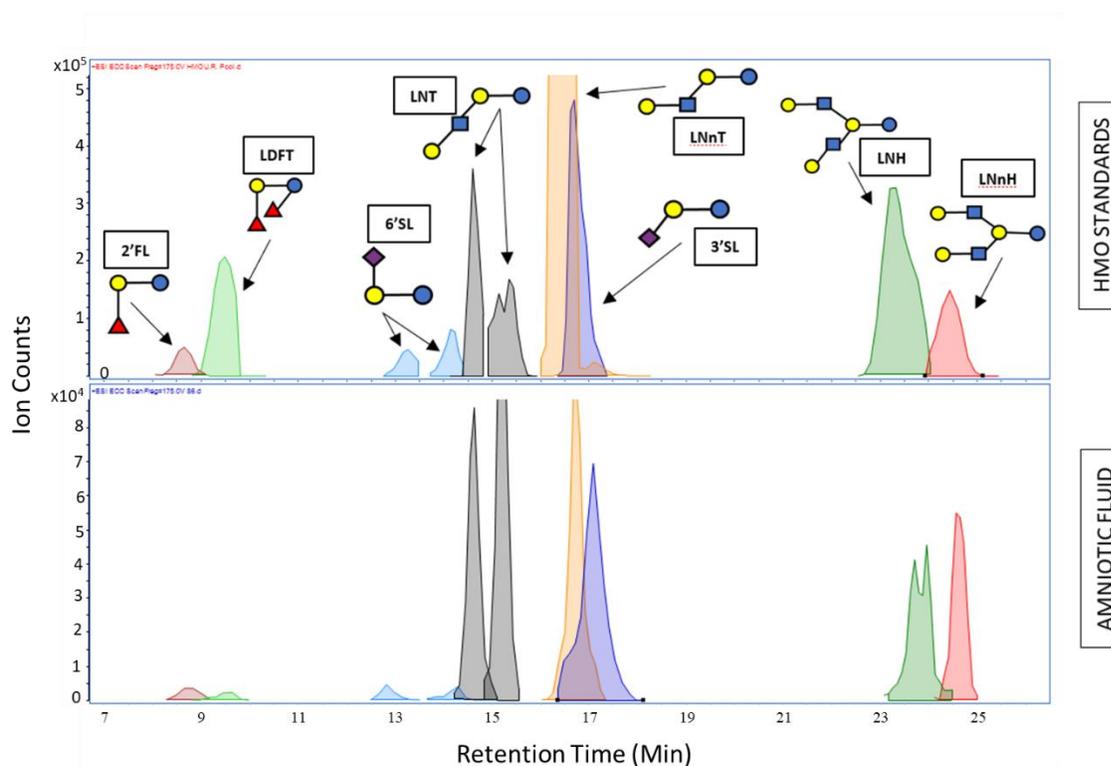
Figure 3.4 nano-HPLC-Q-TOF-MS extracted ion chromatograms of (top) human milk oligosaccharide standard pool (bottom) human milk oligosaccharides in a representative amniotic fluid sample.

Identified human milk oligosaccharides were validated and measured against purified standards by comparing retention times (**Figure 3.4)** and fragmentation spectra (**Figure 3.5**). Retention time matching was conducted by comparing a pooled sample of purchased human milk oligosaccharides with the oligosaccharides found in amniotic fluid. Standards and samples were not reduced during sample preparation therefore some structures were observed to have two characteristic peaks representing both the α and β configuration of the reducing end. Due to the nature of low flow systems, any small variations in temperature, solvent composition, or column compactness can cause significant retention time shifts. Thus, an HMO reference pool was run between every 6 samples to monitor and account for any retention time shifts.
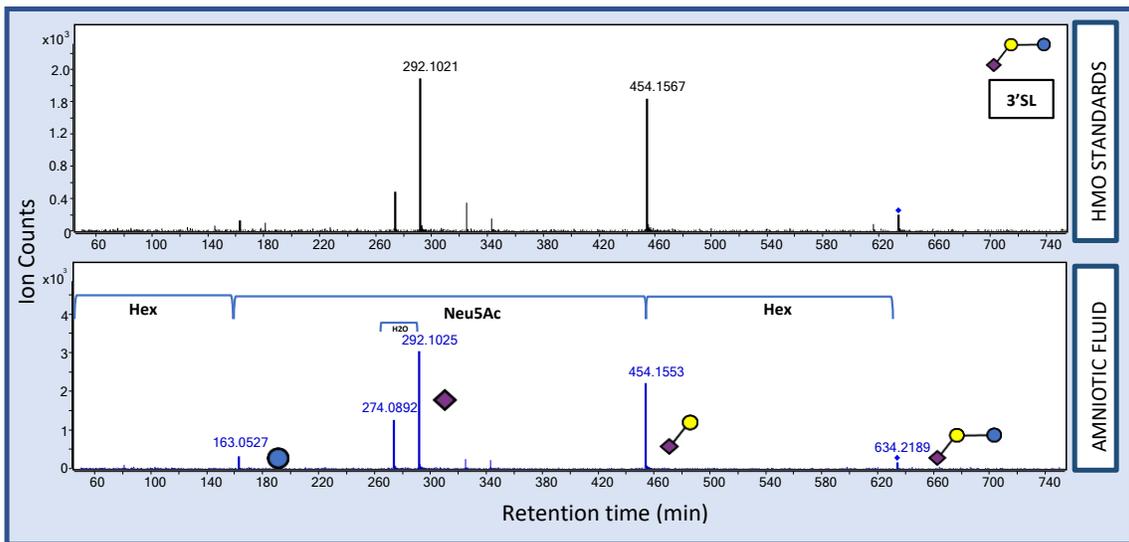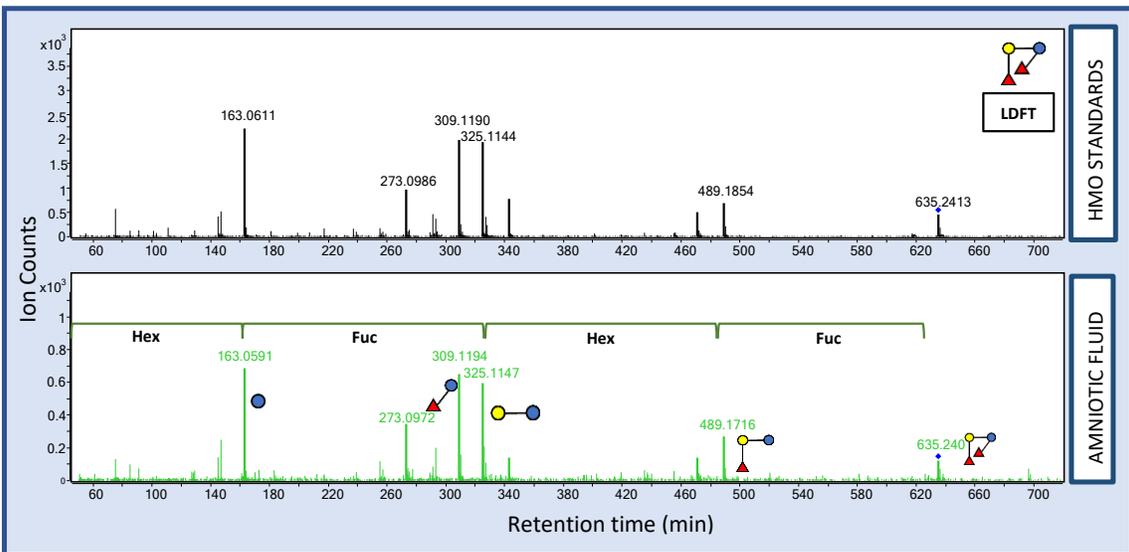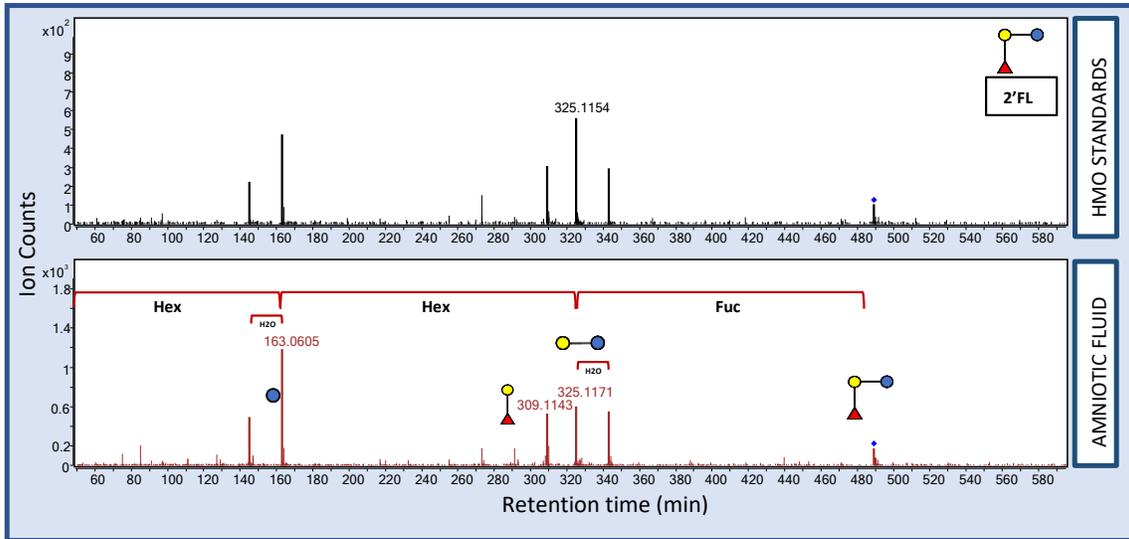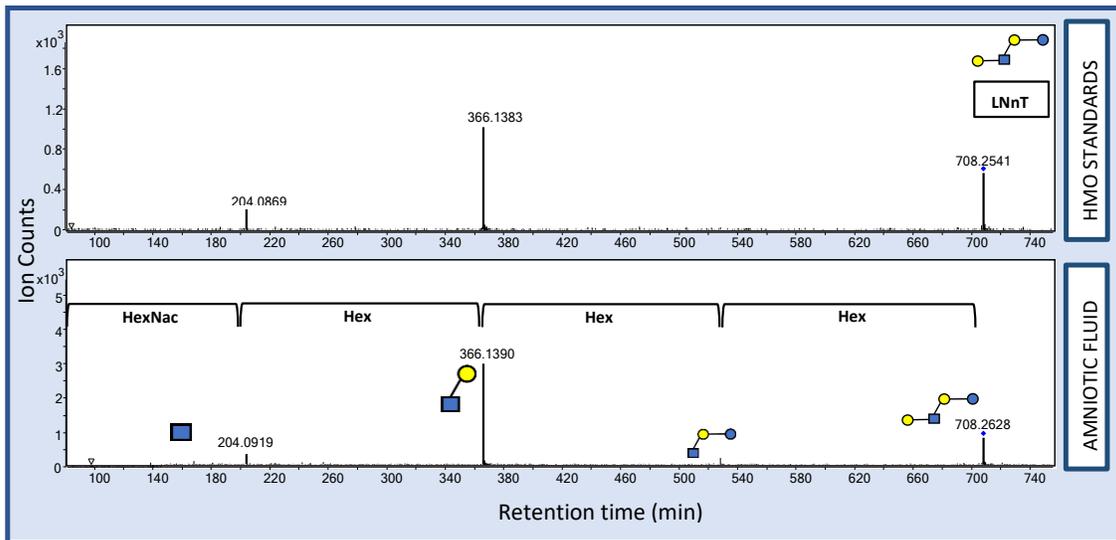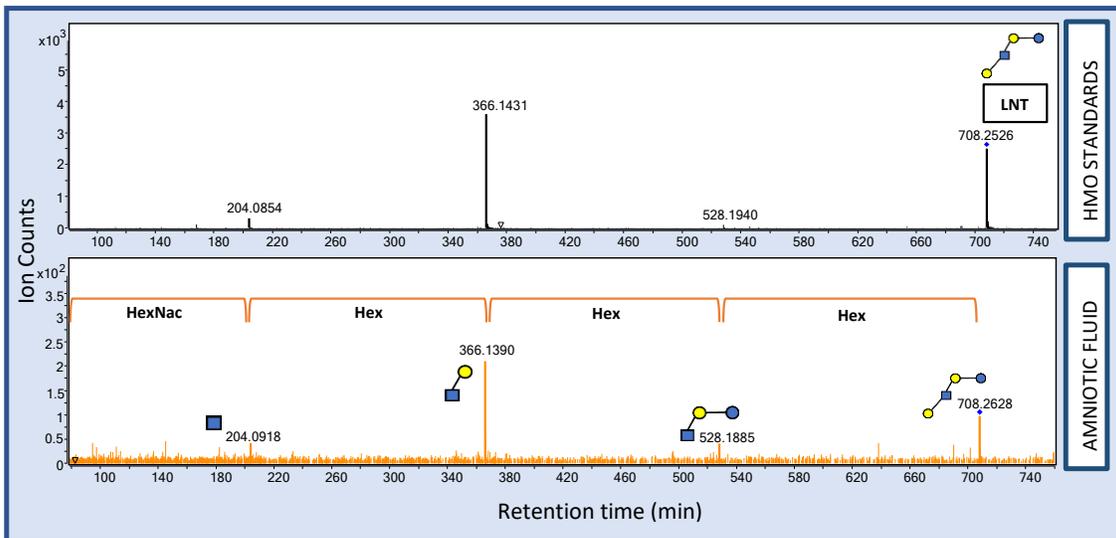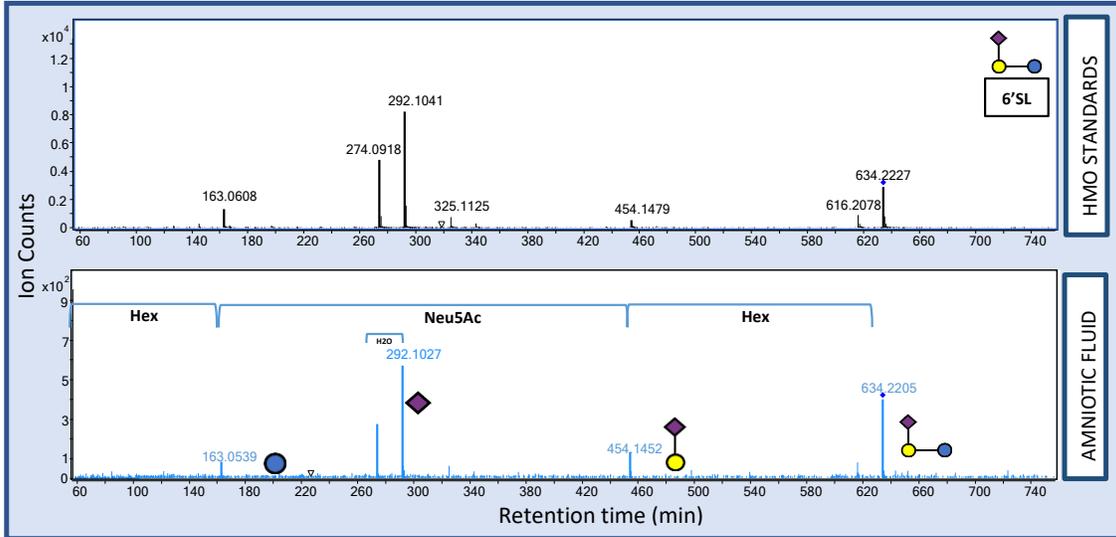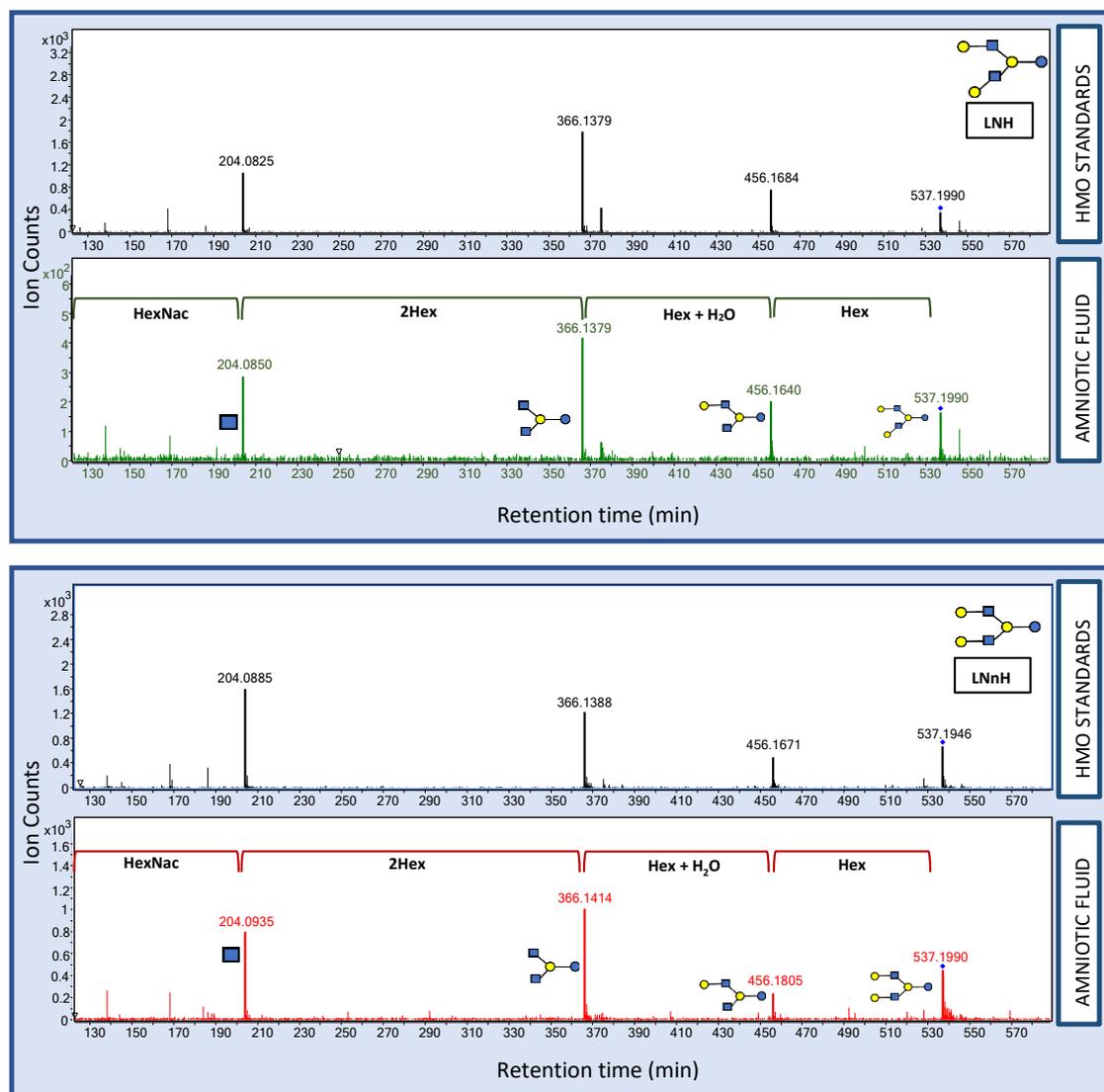
Figure 3.5 Tandem MS/MS spectra comparing mass spectra of (top) purified HMO standard to (bottom) HMOs in amniotic fluid.

Tandem MS data of the standards validated the structures and compositions of the respective HMOs present in amniotic fluid samples. Due to the limitation of working with low abundance analytes, MS/MS data were not obtainable to validate peaks in every sample. In these instances, validation relied solely on exact mass, isotopic distributions, and retention time. **Table**

3.1 presents a comprehensive list of observed oligosaccharides. A mass filter of 20 ppm was applied to the library scan. Retention times were averaged across samples but can shift up to 6 minutes between batches. Structures where standards were not available were confirmed by MS/MS fragmentation and labeled as follows: Hex_HexNAc_Fuc_Neu5Ac.

Table 3.1 comprehensive list of oligosaccharides found in amniotic fluid.

hex	hexnac	fuc	sia	Neutral Mass	(M+H)	(M+2H)/2	Compound	Formula	MS/MS	RT	Structure
1	1	0	1	674.2381774	675.24599	338.126895	1_1_0_1	C25H42O19N2	yes	13	6'SLN
1	1	0	1	674.2381774	675.24599	338.126895	1_1_0_1	C25H42O19N2	yes	16	
1	1	0	1	674.2381774	675.24599	338.126895	1_1_0_1	C25H42O19N2	yes	14	
1	1	0	1	674.2381774	675.24599	338.126895	1_1_0_1	C25H42O19N2	yes	20	3'SLN
2	0	0	1	634.2320294	635.23984	318.12382	2_0_2_0	C24H42O19N0	yes	10	LDFT
2	0	0	1	634.2320294	635.23984	318.12382	2_0_2_0	C24H42O19N0	yes	11	
2	0	1	0	634.2320294	635.23984	318.12382	2_0_2_0	C24H42O19N0	no	28	
2	0	1	0	488.1741206	489.18193	245.094865	2_0_1_0	C18H32O15N0	yes	1.2	3'FL
2	0	1	0	488.1741206	489.18193	245.094865	2_0_1_0	C18H32O15N0	yes	8	
2	0	2	0	488.1741206	489.18193	245.094865	2_0_1_0	C18H32O15N0	yes	9	2'FL
2	0	2	0	545.1955843	546.2034	273.6056	2_1_0_0	C20H35O16N1	yes	9	
2	0	2	0	545.1955843	546.2034	273.6056	2_1_0_0	C20H35O16N1	yes	12	
2	0	2	2	837.311402	838.31922	419.66351	2_1_2_0	C32H55O24N1	yes	4	
2	0	2	2	633.2116283	634.21944	317.61362	2_0_0_1	C23H39O19N1	yes	13	6'SL
2	1	0	0	633.2116283	634.21944	317.61362	2_0_0_1	C23H39O19N1	yes	17	3'SL
2	1	0	0	1216.422863	1217.43068	609.21924	2_0_2_2	C46H76O35N2	no	18.5	
2	1	2	0	1216.422863	1217.43068	609.21924	2_0_2_2	C46H76O35N2	yes	18	
2	2	0	1	1039.370374	1040.3782	520.693	2_2_0_1	C39H65O29N3	yes	26	
2	4	0	0	1154.433702	1155.44154	578.22467	2_4_0_0	C44H74O31N4	yes	25.5	
3	0	0	0	504.1690352	505.17685	253.092325	3_0_0_0	C18H32O16N0	yes	1.5	
3	0	0	0	504.1690352	505.17685	253.092325	3_0_0_0	C18H32O16N0	yes	5	
3	0	0	0	504.1690352	505.17685	253.092325	3_0_0_0	C18H32O16N0	yes	17	
3	0	0	0	504.1690352	505.17685	253.092325	3_0_0_0	C18H32O16N0	yes	10	
3	0	1	0	650.2269441	651.23476	326.12128	3_0_1_0	C24H42O20N0	yes	3	
3	1	0	0	707.2484078	708.25623	354.632015	3_1_0_0	C26H45O21N1	yes	17	LNT & LNnT
3	1	0	1	853.3063167	854.31414	427.66097	3_1_1_0	C32H55O25N1	yes	11	LNFP II

											LNFP III + I
3	1	1	0	853.3063167	854.31414	427.66097	3_1_1_0	C32H55O25N1	yes	15	I
3	1	1	0	910.3277804	911.33561	456.171705	3_2_0_0	C34H58O26N2	yes	11	
3	2	0	0	910.3277804	911.33561	456.171705	3_2_0_0	C34H58O26N2	yes	17	
3	2	0	0	1056.385689	1057.39352	529.20066	3_2_1_0	C40H68O30N2	yes	17	
3	2	1	0	998.3438244	999.35165	500.179725	3_1_0_1	C37H62O29N2	yes	26	
3	2	2	1	1493.539015	1494.54685	747.777325	3_2_2_1	C57H95O42N3	yes	24	
4	0	0	0	1161.417049	1162.42488	581.71634	4_1_2_0	C44H75O34N1	yes	26	
4	0	0	0	1363.476021	1364.48386	682.74583	4_2_0_1	C51H85O39N3	yes	25	
4	1	0	0	869.3012313	870.30906	435.65843	4_1_0_0	C32H55O26N1	yes	23	LNH
4	1	0	0	869.3012313	870.30906	435.65843	4_1_0_0	C32H55O26N1	yes	24	LNnH
4	1	0	0	869.3012313	870.30906	435.65843	4_1_0_0	C32H55O26N1	yes	18	
4	1	2	0	1363.476021	1364.48386	682.74583	4_2_0_1	C51H85O39N3	yes	18	
4	2	0	0	1363.476021	1364.48386	682.74583	4_2_0_1	C51H85O39N3	yes	25	
4	2	0	0	1072.380604	1073.38844	537.19812	4_2_0_0	C40H68O31N2	yes	14	
4	2	0	0	1072.380604	1073.38844	537.19812	4_2_0_0	C40H68O31N2	yes	17	
4	2	0	1	1072.380604	1073.38844	537.19812	4_2_0_0	C40H68O31N2	yes	22	
4	2	0	1	1363.476021	1364.48386	682.74583	4_2_0_1	C51H85O39N3	yes	18	
4	2	0	1	1363.476021	1364.48386	682.74583	4_2_0_1	C51H85O39N3	yes	25	
4	2	0	1	1510.554331	1511.56217	756.284985	4_2_3_0	C58H98O43N2	yes	18	
4	2	0	1	702.24296	667.22968	334.11874	4_0_0_0	C24H42O21N0	yes	10	
4	2	3	0	702.24296	667.22968	334.11874	4_0_0_0	C24H42O21N0	yes	25	
5	0	0	0	1437.5128	1438.52065	719.764225	5_3_0_0	C54H91O41N3	yes	22	
5	2	0	2	1437.5128	1438.52065	719.764225	5_3_0_0	C54H91O41N3	yes	18	
5	2	0	2	1437.5128	1438.52065	719.764225	5_3_0_0	C54H91O41N3	yes	27	
5	2	2	0	1437.5128	1438.52065	719.764225	5_3_0_0	C54H91O41N3	yes	11	
5	3	0	0	1437.5128	1438.52065	719.764225	5_3_0_0	C54H91O41N3	yes	17	
5	3	0	0	828.2746822	829.28251	415.145155	5_0_0_0	C30H52O26N0	yes	28	
5	3	0	0	1526.549245	1527.55709	764.282445	5_2_2_0	C58H98O44N2	yes	21	
5	3	0	0	1728.608217	1729.61607	865.311935	5_3_0_1	C65H108O49N4	yes	22	
5	3	0	0	1816.624261	1817.63211	909.319955	5_2_0_2	C68H112O52N4	yes	25	

5	3	0	1	1816.624261	1817.63211	909.319955	5_2_0_2	C68H112O52N4	yes	29
5	4	0	1	1931.687589	1932.69545	966.851625	5_4_0_1	C73H121O54N5	yes	23
6	0	0	0	1599.565624	1600.57348	800.79064	6_3_0_0	C60H101O46N3	no	18
6	1	2	0	990.3275057	991.33534	496.17157	6_0_0_0	C36H62O31N0	yes	14
6	3	0	0	1485.522696	1486.53054	743.76917	6_1_2_0	C56H95O44N1	yes	21
7	0	0	0	1152.380329	1153.38817	577.197985	7_0_0_0	C42H72O36N0	yes	6
7	0	0	0	1152.380329	1153.38817	577.197985	7_0_0_0	C42H72O36N0	yes	20
7	2	1	2	2286.787817	2287.79568	1144.40174	7_2_1_2	C86H142O66N4	yes	23
8	2	1	2	2448.84064	2449.84851	1225.428155	8_2_1_2	C92H152O71N4	yes	24

Analysis of all 514 samples remained the goal of this project, however a small set was performed in a pilot study to validate the method and evaluate feasibility of performing a larger scale analysis. A small set of N=51 (out of a total of 514) were analyzed for this validation study. Mothers selected for the pilot study varied in maternal characteristics (ethnicity/race, age), sampling characteristic (gestational age), and birth outcomes (spontaneous preterm birth (SPTB), indicated preterm birth (IPTB) and normal birth).

In order to evaluate the contribution of performing a larger scale analysis, we assessed whether any associations could be made with selected cohort characteristics and oligosaccharide profiles of the amniotic fluid samples that would warrant further investigation. Oligosaccharides were rapidly identified by aligning retention times, isotopic distributions, and exact masses with previously validated structures from **Table 3.1**. As a first assessment we compared the concentration of HMOs present in the amniotic fluid as a function of gestational age (**Figure 3.6**).
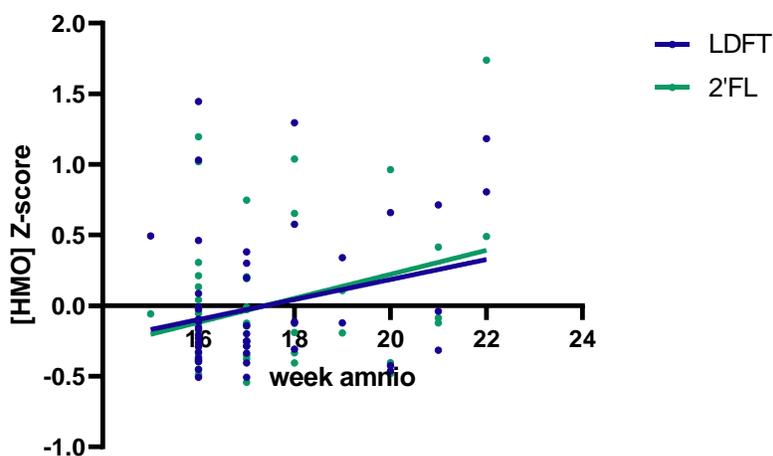
Figure 3.6. LDFT and 2'FL trends throughout gestation. HMOs were normalized via Z-scores such that the mean of HMO concentrations is zero. For LDFT, R = 0.26, p = 0.06. For 2'FL, R=0.32, p=0.02.

Preliminary findings showed that from the eight quantifiable HMOs, the most abundant, 2'FL and LDFT, significantly correlated with increasing gestational age (r= 0.32 and 0.26, p=0.02 and 0.06, respectively, inclusive of linear analysis [2'FL, p=0.04]) (**Figure 3.6**). Statistical significance was not achieved for the remaining HMOs (LNH, LNT, LNnT, 6'SL, 3'SL); however, these structures were present in much lower abundance making the search for systematic variations more difficult. Similarly, comparing other cohort characteristics such as race, maternal age, reason for 2nd trimester amniocentesis, or subsequent occurrence of PTB showed no associations with HMO abundance (not shown).

Inspired by the preliminary data showing compositional changes in oligosaccharides across gestation, we applied our methods to the larger cohort of N=516 in efforts to further assess and identify potential trends and correlations that could have been lost due to the small sample size. **Table 3.2** summarizes important characteristics of the cohort. The average maternal age was 34.3 which is significantly higher than the nation average of 25.6. Maternal background, such as previous pregnancy (nulliparous) and history of premature deliveries, was also included. Amniocentesis was performed in the second trimester from 14 to 29 weeks and averaging 17.5 weeks. Amniocentesis can cause complications in the pregnancy and is only performed if certain indications are observed. These indications are detailed in **Table 3.2**.

Table 3.2 Summary of demographics, gestational age and observed indications for study cohort (N=516).

	Cohort	U.S. National Averages
Maternal Age (years)	34.3 +\- 5.4 (17 to 44)	25.6
Ethnicity		
Asian	227 (31.1%)	
Hispanic	139 (19.1%)	
African American	144 (19.8%)	
White	219 (30.0%)	
Nulliparous	200 (27.4%)	~40%
History of preterm birth	78 (10.7%)	
Preterm Delivery	92 (12.6%)	9.6%
Gestational Age at Sampling (weeks)	17.5 +\- 1.9 (14 to 29)	
Amniocentesis Indication		
Advanced Maternal Age (AMA)	389	
+Mat. Serum Screen (+MSS)	218	
Abnormal Ultrasound	40	
Multiples	82	

Absolute concentrations of HMOs in human milk have been measured previously, however these structures have not yet been quantified in amniotic fluid using LC-MS. The concentration of oligosaccharides in amniotic fluid can provide insight into the origin of these structures in the uterus. Thus, absolute quantitation was performed using external calibrations to quantify the most abundant HMOs observed. Calibration curves were run alongside every sample set. Because of the large matrix effects and retention time shifts observed in the pilot study, a surrogate plant-based internal standard mix, consisting of xylo-oligosaccharides (DP 4-7) was used to monitor retention times and normalize peak areas. **Figure 3.7** plots the instrument

responses versus concentration of six standards used to determine the concentrations present in amniotic fluid. LNT and LNnT were not able to be chromatographically separated in all samples, thus LNnT was used to approximate the concentrations of LNT and LNnT.
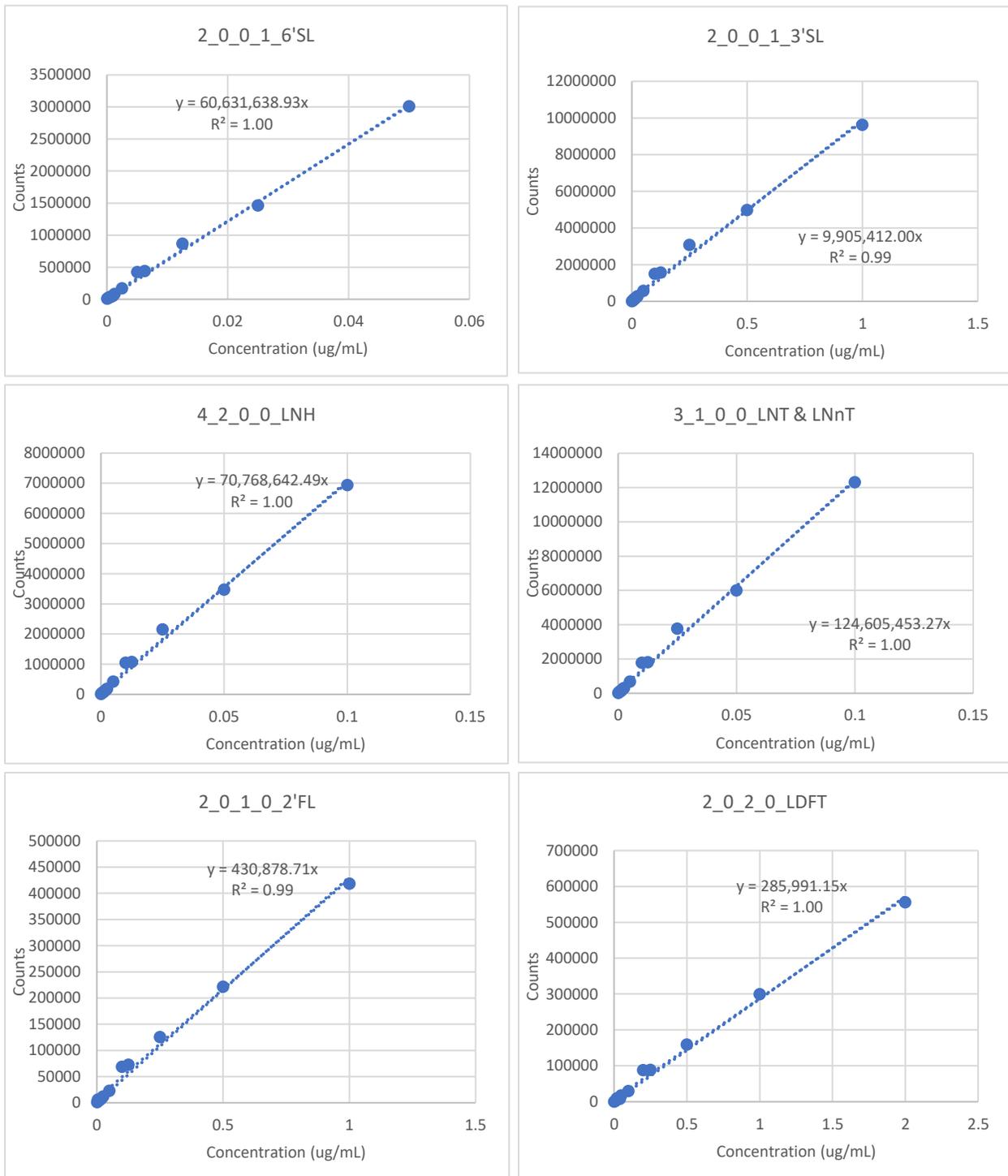
Figure 3.7 Example Calibration curves for the six external standards used to quantify HMOs in amniotic fluid.

The linear range of the oligosaccharide standards spanned 3-4 orders of magnitude (approx. $2X10^2$-$3X10^6$ units). The LOD and LOQs were determined to be in the pico-femto molar ranges allowing for quantitation of low abundant compounds. Regression coefficients were excellent for all compounds ($R^2> 0.95$). **Table 3.3** presents the average concentration of each quantitated HMO observed in amniotic fluid. Total HMO concentrations observed ranged from 0.014-2.87 nmol/mL with an average concentration of 0.33 nmol/mL

Table 3.3 Concentration of HMOs in Amniotic fluid. Limits of Quantitation (LOQ) and Limits of Detection (LOD) were defined as 6X and 3X the signal-to-noise ratio of the blanks respectively. Concentration given as ng/mL ± SD.

HMO	Concentration (ng/mL)	LOD (counts)	LOQ (pg/mL)
2'FL	88.52±93.79	2472	11.474
LDFT	88.95±88.69	2934	20.518
6'SL	0.39±.389	9630	0.3176
3'SL	5.49±6.03	7791	1.57
LNH	0.65±0.77	28248	0.15
LNT/LNnT	0.68±0.81	9405	0.80

Correlations observed in the pilot study were also observed in the larger cohort. Namely, the most abundant HMOs (2'FL and LDFT) increased with increasing gestational age (r= 0.23 and 0.14), $p=9.9x10^{-8}$ and 0.002, respectively **(Figure 3.8)** Interestingly, analysis of the larger cohort revealed that 3'SL decreased in concentration throughout gestation (r=-0.14, p=0.002),

which was not observed in the pilot study. No significant correlation was found with LNH or LNT/LnNT.
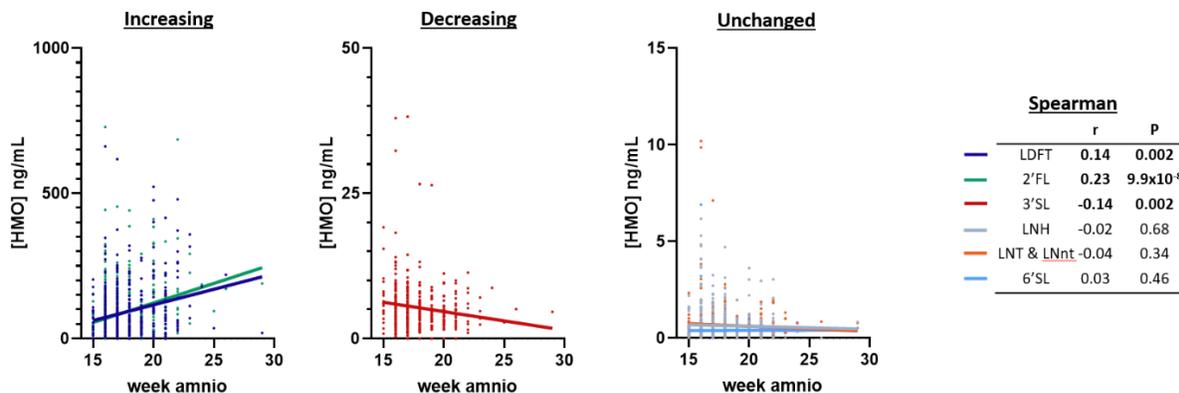
Figure 3.8 Structure specific HMO correlations to gestational age. For LDFT, R = 0.14, p = 0.002. For 2'FL, R=0.23, p=9.9x10^{-8}. For 3'Sl, R=-0.014, p=0.002. For LNH R=-0.02, p=0.68. For LNT & LNnT, R=-0.04, p=0.34. For 6'SL, R=0.03, p=0.46.

Additionally, we investigated how maternal characteristics affected the oligosaccharide abundances and profile (**Figure 3.9**). Analysis of HMOs with maternal age, parity, race, history of premature delivery (Hx PTD), and reason for performing the amniocentesis revealed little to no correlation with HMO abundance. However, correlational analysis revealed HMO levels to be intercorrelated within subjects based on their glycan type. For example, samples that tended to have higher levels of 6'SL also tended to have higher levels of 3'SL (both sialylated species) (r=0.68). Similar trends are observed with fucosylated species such as 2'FL and LDFT (r= 0.74) and undecorated structures LNH and LNT (r=0.48).
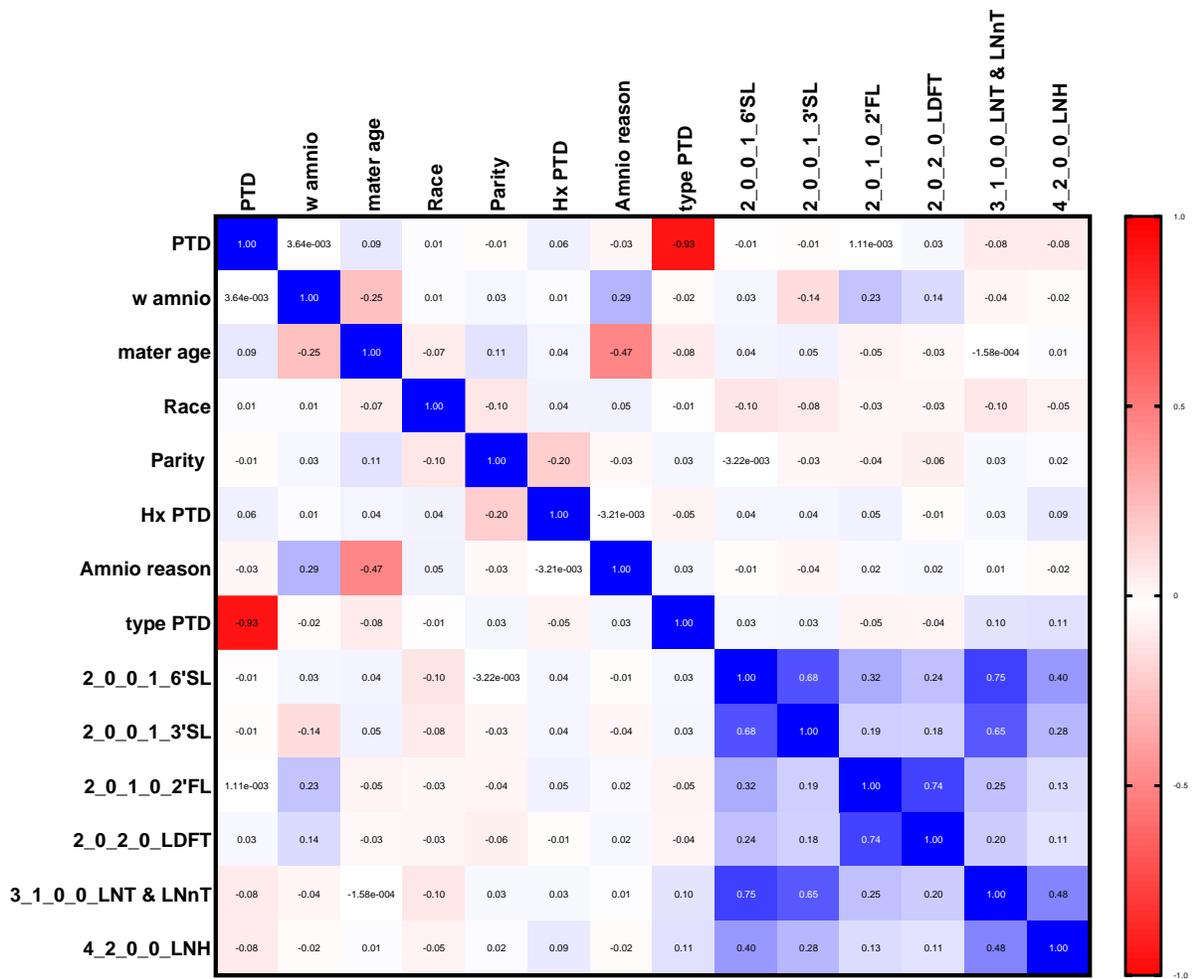
Figure 3.9 Correlation analysis between subjects and study characteristics.

DISCUSSION

Oligosaccharides in mammalian milk are a highly evolutionarily preserved nutrient but are not digestible by the offspring that consume them. Rather, they are known to provide a growth advantage to beneficial microbes, act as "decoys" for pathogenic microbes, and promote a healthy gut epithelial barrier. HMOs have been presumed to be found and function solely in the postnatal period of infant development. [5-14, 32, 33] We demonstrate here that oligosaccharides, and HMOs in particular, are present in human amniotic fluid as early as the 2nd trimester.

The source or mechanism of transportation of these HMOs in the amniotic fluid has yet to be elucidated. The HMOs observed in amniotic fluid, although alike in structure, differ greatly in abundance compared to those found in maternal milk. Concentrations of HMOs in amniotic fluid were found to range from 0.014-2.87 nmol/mL, which is several orders of magnitude less than those found in maternal milk (approx. 20 μmol/mL).[34] More so, only a small subset of the oligosaccharides commonly observed in milk was identified in the amniotic fluid, suggesting a distinct and important functional role of these select structures. Previous studies conducted in our lab have shown evidence of HMOs in maternal circulation during pregnancy.[28] Both the structures and concentrations of these HMOs in the blood resemble those found in amniotic fluid. This points towards the hypothesis that HMOs could be transported through permeability of the epithelial membrane.

The role that these HMOs play in fetal development is not yet clear. In this study, we report the first observation and characterization of other non-HMO oligosaccharides found in amniotic fluid, which could potentially provide further insight into the biological role of these structures in the uterus. We observed approximately 20 oligosaccharides found across most samples (>80%) that have structures similar to those found in maternal milk, in that they contain

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a lactose core with terminations of fucose and sialic acid but differ by monosaccharide composition and/or linkage. Of these structures, a handful were found to resemble partial HMOs in that they contain the same monosaccharide composition as those found in maternal milk but lack one or two terminal sugars. Glycosylation involves several stepwise processes leading to groups of structures such as HMOs. The absence or down regulation of any transferase associated with the synthesis of any structure could affect the final product. The presence of partial structures could be due to an incomplete process of the formation of these oligosaccharides. For example, two "incomplete" structures were found in relatively large abundance in the amniotic fluid: 2hex_2hexNac_0Fuc_0Sia (2_2_0_0) and 2hex_1hexNac_0Fuc_0Sia (2_1_0_0). The absence or down regulation of a galactotransferase could potentially hinder the addition of terminal galactoses and therefore partially inhibit the formation of LNH (4_2_0_0) and LNT (3_1_0_0). Alternatively, if in fact amniotic fluid is not sterile[35], these structures could be digestion products that are formed by an exogalactosidase which cleaved both terminal galactoses of LNH (4_2_0_0) to form 2_2_0_0 and the single terminal galactose in LNT (3_1_0_0) to form (2_1_0_0). Previously, our lab has analyzed fecal samples from exclusively breastfed infants and found that digested oligosaccharides in feces correspond to the action of glycosidases on HMOs.[36] Among other products, 2_2_0_0 and 2_1_0_0 was found in relatively large abundances in most fecal samples.

Additionally, 3'SLN and 6'SLN were the most abundant sialylated species found in amniotic fluid. Towards the end of the first trimester of gestation, as the infant begins to develop kidneys, amniotic fluid composition starts to resemble that of infant urine.[20] Our lab as well as others, have studied urine composition of exclusively breastfed infants.[30] In these studies, several sialylated HMOs including 3'SLN and 6'SLN were found in significant abundance in infant

urine that are not typically found in maternal milk suggesting synthesis in the proximal intestine.[30]

CONCLUSION

We demonstrate for the first time a robust comprehensive method for quantitation of oligosaccharides in amniotic fluid using nano-LC chip-TOF MS. This study covers methodologies from sample preparation to data analysis. Analysis of 516 amniotic fluid samples revealed trends throughout gestation and opened the door to the possible roles that HMOs play in developmental nutrition; however, many unknowns remain. Further studies that elucidate the function of these HMOs in fetal development and explore the mechanism behind the formation of these structures are required.

data and metadata. C.B.L. and K.M.A. designed the study and oversaw the analyses. A.V. and

C.B.L. interpreted results and wrote the manuscript.

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CHAPTER FOUR

Host Cell Glycocalyx Remodeling Reveals SARS-Cov-2 Spike Protein Glycomic Binding Sites

ABSTRACT

Glycans on the host cell membrane and viral proteins play critical roles in pathogenesis. Highly glycosylated epithelial cells represent the primary boundary separating embedded host tissues from pathogens within the respiratory and intestinal tracts. SARS-CoV-2, the causative agent for the COVID-19 pandemic, reaches into the respiratory tract. We found purified human milk oligosaccharides (HMOs) inhibited the viral binding on cells. Spike (S) protein receptor binding domain (RBD) binding to host cells were partly blocked by co-incubation with exogenous HMOs, most by 2-6-sialyl-lactose (6'SL), supporting the notion that HMOs can function as decoys in defense against SARS-Cov2. To investigate the effect of host cell glycocalyx on viral adherence, we metabolically modified and confirmed with glycomic methods the cell surface glycome to enrich specific N-glycan types including those containing sialic acids, fucose, mannose, and terminal galactose. Additionally, Immunofluorescence studies demonstrated that the S protein preferentially binds to terminal sialic acids with α-(2,6)-linkages. Furthermore, site-specific glycosylation of S protein RBD and its human receptor ACE2 were characterized using LC-MS/MS. We then performed molecular dynamics calculations on the interaction complex to further explore the interactive complex between ACE2 and the S protein. The results showed that hydrogen bonds mediated the interactions between ACE2 glycans and S protein with desialylated glycans forming significantly fewer hydrogen bonds. These results supported a mechanism where the virus binds initially to glycans on host cells preferring α-(2,6)-sialic acids and finds ACE2 and with the proper orientation infects the cell.

INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of COVID-19[1], encodes an extensively glycosylated spike (S) protein that protrudes from the viral surface and binds angiotensin-converting enzyme 2 (ACE2) on host cells.[2-6] This novel SARS-CoV-2 was found to share similarities with the SARS-CoV, which was responsible for the SARS pandemic that occurred in 2002.[7, 8] ACE2 serves as the entry point for several coronaviruses into cells, including SARS-CoV and SARS-CoV-2.[9, 10] The receptor binding domain (RBD) of SARS-CoV-2 S protein has been limited to amino acid residues Arg319 to Phe541.[11-13] *In vitro* binding measurements also showed that the SARS-CoV-2 RBD binds to ACE2 with an affinity in the low nanomolar range, indicating that the RBD is a key functional component within the S1 subunit responsible for the binding of SARS-CoV-2 to ACE2.[2, 13] The plasma membrane protein ACE2 is abundantly expressed in humans tissues, including respiratory and intestinal epithelia, liver arteries, heart and kidney.[14]

Mammalian epithelial cells are highly glycosylated[15, 16] due to glycoproteins and glycolipids found on the cell membrane. Both the ACE2 receptor and the S protein are similarly extensively glycosylated. Several glycosylation sites are found near the binding interface.[12, 17-19] The role of glycosylation in the interaction between human ACE2 and SARS-CoV-2 S protein has been extensively studied, primarily using molecular dynamics (MD) simulations.[12, 20, 21] Human ACE2 variants have also been modeled, characterized, and examined for susceptibility to coronavirus interactions.[22, 23] Among ACE2 glycosylation sites, one of the most characterized positions for its role in S protein binding and viral infectivity is the asparagine on position 90 (N90). Recent genetic and biochemical studies showed that mutations that removed glycosylation on N90 site directly increased the susceptibility to SARS-CoV-2 infection.[21, 23] In contrast, glycans

present on N322 and N90 have the opposite effects on S protein binding. The N322 glycan interacts tightly with the RBD of the ACE2-bound S protein and strengthens the complex.[20] The S protein also contains glycosaminoglycan (GAG) binding motifs so that host surface GAGs contribute to cell entry by SARS-CoV-2.[24] Additionally, heparan sulfate has also been shown to promote Spike-ACE2 interaction.[25]

Pathogen adhesion is often mediated by highly specific lectin-glycan interactions. For example, *Escherichia coli* with type 1 fimbriae binds to cell surfaces exhibiting preference for high mannose glycans, while *Escherichia coli* with type S fimbriae has binding specificity for α-(2,3)-linked sialic acids. Cell surface glycans have also been shown to act as a shield to mask its identity as a viable host to the pathogen. It was recently proposed that HMOs can prevent viral adhesion to intestinal epithelial cells via binding to the epithelial surface, causing structural changes in the receptor thereby impeding the virus from hijacking the host cell.[26] Breast-fed infants have significant amounts of HMOs lining the mucosal surface of their gastrointestinal tract. While the viral binding to glycans and HMO in particular have been studied, the direct interaction between the virus and host glycans remain relatively unexplored.

In this study, the role of host glycosylation and its effect on S protein binding was examined by identifying the host glycans that are involved in the binding. The study began with HMOs in a rapid assay to determine the broad details of the oligosaccharide that bind the virus. We then examined the impact of host cell glycosylation on S protein binding, by modifying the host glycosylation while leaving protein expression unchanged using transferase inhibitors. Using newly developed glycomic tools, we found that specific glycans on the host cell facilitate S protein binding and that binding depends more on the nature of glycans than it does on the membrane proteins.

This work has been published in the Frontiers in Molecular Biology and is included with permission in this thesis.

METHODS AND MATERIALS

HMO Purification

HMOs were obtained from breast milk samples using previously reported methods.[27, 28] Briefly, breast milk samples from 7 mothers were pooled. The pooled sample was defatted through centrifugation, proteins were precipitated with ethanol, and the resulting glycans were reduced with sodium borohydride (Sigma-Aldrich, St. Louis, MO). Solid phase extraction was performed on 25 mg graphitized carbon cartridges (ThermoFisher). Solvents were dried in vacuo using miVac (SP Scientific, PA) and purified HMOs were reconstituted and diluted prior to analysis.

Inhibition of HMO against SARS-CoV-2

All HMO screens were performed with Vero E6 cells. Cells were plated in 96 well plates at 5e3 cells/well one day prior to infection. HMOs were diluted from stock to 50 μM and an 8-point 1:2 dilution series was prepared in duplicate in Vero Media. Every compound dilution and control was normalized to contain the same concentration of drug vehicle (e.g., DMSO). Cell plates were pre-treated with drug for 2 h at 37 °C (5% CO_2) prior to infection with diluted SARS-CoV-2 GFP for a final MOI of 0.1. In addition to plates that were infected, parallel plates were left uninfected to monitor cytotoxicity of drug alone, measured by CellTiter-Glo (CTG) assays as per the manufacturer's instructions (Promega, Madison, WI). Plates were then incubated at 37 °C (5% CO_2) for 48 hours, followed by fixation with 4.0% paraformaldehyde, nuclear staining with Hoechst (Invitrogen, Carlsbad, CA), and data acquisition on a Celigo 5-channel Imaging

Cytometer (Nexcelom Bioscience, Lawrence, MA). The percent of infected cells was determined for each well based on GFP expression by manual gating using the Celigo software. For the CTG assays, luminescence was read on a BioTek Synergy HTX plate reader (BioTek Instruments Inc., Winooski, VT) using the Gen5 software (v7.07, Biotek Instruments Inc., Winooski, VT).

Cell culture and glycocalyx remodeling treatments

Human liver hepatocellular carcinoma HepG2, lung carcinoma epithelial Calu-3, urinary bladder epithelial RT4 cells were obtained from American Type Culture Collection (ATCC, VA).

HepG2 and Calu-3 cells were grown in Eagle's Minimum Essential Medium (EMEM). RT4 cells were cultured in McCoy's 5a Medium. All media were supplemented with 10% (v/v) fetal bovine serum and 100 U mL^{-1} penicillin and streptomycin. Cells were sub-cultured at 90% confluency and maintained at 37 °C in a humidified incubator with 5% CO_2. At 50% cell confluency, the cells were either treated with 150 μM kifunensine, 2-fluoro-L-fucose, or 3-fluorinated sialic acid for 48 hours.

Sample Information

Recombinant human angiotensin-converting enzyme 2(ACE2), SARS-CoV-2 Spike protein S1 Subunit RBD (Arg319-Phe541) and Spike protein S1 subunit (Val16-Arg685) derived from transfected human HEK293 cells were obtained from RayBiotech (Georgia, Product Number 230-30165, 230-30162) and Sino Biological (China, Product Number 40591-V08H), respectively.

Immunofluorescence

The cells were seeded into FluoroDish™ cell culture dishes (WPI, FL) coated with poly-d-lysine with appropriate density using EMEM cell culture media. At 40% confluency, cells were treated with media either supplemented with 150 μM kifunensine, 2-fluoro-L-fucose, or 3-

fluorinated sialic acid for 48 hours. Control cell culture without treatment and treated cells were rinsed with phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde (Affymetrix, OH). Recombinant SARS-CoV-2 spike protein RBD and S1 subunits were conjugated to a fluorescent label with Alexa Fluor™ 555 according to manufacturing instructions (Microscale Protein Labeling Kit, Invitrogen, MA). Fixed control and glyco-modified cells were then incubated with fluorescent labelled S proteins or Anti-ACE2 antibody (Santa Cruz Biotechnology, TX) in PBS at 4 °C for 18 hours. Cells were stained for the nucleus with 1.6 μM Hoechst 33342 (Thermo Fisher Scientific, MA) followed by the staining for the plasma membrane with 1000-fold diluted CellMask™ Deep Red Plasma Membrane Stain (Thermo Fisher Scientific, MA), respectively at 37 °C for 10 min. Fluorescence images were captured using a Leica TCS SP8 STED 3X Super-Resolution Confocal Microscope (Wetzlar, Germany). Fluorescence intensity was quantified for selected cell area. Quantification was performed with software ImageJ.

Cell membrane extraction

Cell membrane fractions were prepared as previously described.[16, 29, 30] Briefly, control and glycoengineered cells were collected and resuspended in homogenization buffer containing 0.25 M sucrose, 20 mM HEPES-KOH (pH 7.4), and protease inhibitor mixture (1:100; Calbiochem/EMD Chemicals). Cells were lysed on ice with five alternating on and off pulses in 5 and 10 second intervals using a probe sonicator (Qsonica, CT). Nuclear and mitochondrial fractions and cellular debris were pelleted and isolated by centrifugation at $2000 \times g$ for 10 min. The supernatants were then ultra-centrifuged at $200\,000 \times g$ for 45 min at 4 °C to extract the plasma membrane. The pellets of the cell membrane were resuspended in 500 μL of 0.2 M Na_2CO_3 solution and 500 μL of water followed by two more ultracentrifugation treatments at $200\,000 \times g$ for 45 min to wash off the endoplasmic reticulum (ER) and cytoplasmic fraction.

Enzymatic N-glycan release and purification of N-glycans

Extracted cell membrane fractions or RNase B were suspended with 100 μL of 100 mM NH$_4$HCO$_3$ in 5 mM dithiothreitol and heated in boiling water for 2 minutes to denature the proteins. Solutions of with 2 μL of peptide N-glycosidase F (New England Biolabs, MA) were added to the samples to release the N-glycans from proteins, and the resulting solutions were then incubated in a microwave reactor (CEM Corporation, NC) at 20 watts, 37 °C for 10 min. The samples were further placed in a 37 °C water bath for 18 hours. Ultracentrifugation at 200 000 x g for 30 min was performed to precipitate membrane fractions, and the supernatant containing N-glycans was collected and purified using porous graphitic carbon (PGC) on a 96-well SPE plate (Grace, IL). The plate was equilibrated with 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The samples were loaded onto the plate and washed with nanopure water. N-Glycans were eluted with a solution of 40% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid, and the samples were dried in vacuo using miVac (SP Scientific, PA) prior to mass spectrometric analysis.

Glycoprotein digestion and enrichment

Details of the protein digestion have been described previously.[30] Extracted cell membrane proteins were reconstituted in 60 μL of 8 M urea at room temperature. Recombinant proteins and dissolved cell membrane proteins were reduced with 2 μL of 550 mM dithiothreitol, alkylated with 4 μL of 450 mM iodoacetamide. A 420 μL of 50 mM ammonium bicarbonate solution was added to dilute the urea concentration to 1 M and to adjust the pH value. The samples were incubated with 2 μg trypsin at 37 °C for 18 hours. The resulting peptides were concentrated *in vacuo* using miVac (SP Scientific, PA). Glycopeptides were enriched by solid-phase extraction using iSPE®-HILIC cartridges (HILICON, Sweden). The cartridges were conditioned with 0.1% (v/v)

trifluoroacetic acid in acetonitrile, followed by 1% (v/v) trifluoroacetic acid and 80% (v/v) acetonitrile in water. The samples were loaded and washed with 1% (v/v) trifluoroacetic acid and 80% (v/v) acetonitrile in water. The enriched glycopeptides were eluted with water containing 0.1% (v/v) trifluoroacetic acid and dried prior to mass spectrometric analysis.

Glycomic analysis with LC-MS/MS

Glycan samples were reconstituted with 30 μL nanopure water and analyzed using an Agilent 6520 Accurate Mass Q-TOF LC/MS equipped with a PGC nano-chip (Agilent Technologies, CA). The glycan separation was performed at a constant flow rate of 300 nL min −1 , and a binary gradient was applied using (A) 0.1% (v/v) formic acid in 3% acetonitrile and (B) 1% (v/v) formic acid in 90% acetonitrile: 0–2 min, 0–0% (B); 2–20 min, 0–16% (B); 20–40 min, 16%-72% (B); 40–42 min, 72–100% (B); 42–52 min, 100–100% (B); 52–54 min, 100–0% (B); 54–65 min, 0–0% (B). MS spectra were collected with a mass range of m/z 600–2000 at a rate of 1.5 s per spectrum in positive ionization mode. The most abundant precursor ions in each MS1 spectrum were subjected to fragmentation through collision-induced dissociation (CID) based on the equation V collision =1.8 x (m/z) /100 V - 2.4 V.

Glycomic data analysis

Extraction of the compound chromatographs of glycans from cells was obtained via the MassHunter Qualitative Analysis B08 software (Agilent, CA). N-Glycan compositions were identified according to accurate masses using an in-house library constructed based on the knowledge of N-glycan biosynthetic pathways and previously obtained in-house structures of N-glycans. Relative abundances were determined by integrating peak areas for observed glycan masses and normalizing to the summed peak areas of all glycans detected.

Glycoproteomic analysis with LC-MS/MS

The enriched glycopeptide samples were reconstituted with nanopure water and directly characterized using UltiMate™ WPS-3000RS nanoLC 980 system coupled to the Nanospray Flex ion source of an Orbitrap Fusion Lumos Tribrid Mass Spectrometer system (Thermo Fisher Scientific, MA). The analytes were separated on an Acclaim™ PepMap™ 100 C18 LC Column (3 µm, 0.075 mm x 150 mm, ThermoFisher Scientific). A binary gradient was applied using 0.1% (v/v) formic acid in (A) water and (B) 80% acetonitrile: 0–5 min, 4–4% (B); 5–133 min, 4–32% (B); 133–152 min, 32%-48% (B); 152–155 min, 48–100% (B); 155–170 min, 100–100% (B); 170–171 min, 100–4% (B); 171–180 min, 4–4% (B). The instrument was run in data-dependent mode with 1.8kV spray voltage, 275 °C ion transfer capillary temperature, and the acquisition was performed with the full MS scanned from 700 to 2000 in positive ionization mode. Stepped higher-energy C-trap dissociation (HCD) at 30±10% was applied to obtain tandem MS/MS spectra with m/z values starting from 120.

Glycoproteomic data analysis

Glycopeptide fragmentation spectra were annotated using Byonic software (Protein Metrics, CA) against the reviewed UniProt Severe acute respiratory syndrome coronavirus 2 spike protein database. Carbamidomethyl modification at cysteine residues and oxidation at methionine were assigned as the modification.

Molecular dynamic simulation of S protein on ACE2

The 3D structure of S protein and ACE2 complex was obtained from PDB (PDB code 7DF4).[31] The most abundant glycans for each ACE2 glycosite were modeled and attached to the protein using CHARMM-GUI.[32] Additionally, the fully-desialylated glycans were modeled and

attached to generate a fully-desialylated homolog of the ACE2 glycoprotein. The models were solvated using the TIP3P water model, and counterions were added to neutralize the system. The CHARMM carbohydrate force field[33] and CHARMM36m force field[34] were used for the carbohydrate and protein structures. Equilibration was performed at 303.15 K over 10 ps. Molecular dynamics simulation was performed using NAMD software package version 2.13[35] at 303.15 K under NPT conditions over 5 ns with an output every 10 ps. Long-range electrostatics were evaluated using the particle-mesh Ewald (PME) method.[36] Covalent bonds involving hydrogen were constrained with the SHAKE algorithm.[37] After dynamics simulations, trajectories were loaded onto VMD for visualization and analysis.[38] Specifically, the intermolecular hydrogen-bonding interactions (donor-acceptor distance 3.0 Å, angle cutoff 20°) of each glycan in the fully-sialylated and desialylated forms were compared over the simulation period.

RESULTS

Inhibition of virus binding by human milk oligosaccharides

Human milk oligosaccharides (HMOs) contain a number of unique structures that can be used to rapidly screen the glycan specificity of the virus. We tested whether SARS-CoV-2 virus could be inhibited by HMOs. We first examined whether pooled samples of purified HMOs from seven different mothers could affect the binding of SARS-CoV-2 virus on Vero E6 cells. **Figure 4.1a** showed that the binding capability was affected by the HMO mixture to about 25%. HMOs contain compounds with terminal fucose, sialic acid and galactose. To identify the functional components that could specifically affect binding, we further tested individual compounds that contained these terminal saccharides. The HMOs 2′-fucosyllactose (2′-FL), 6′- sialyllactose (6′-SL), and lacto-N-neotetraose (LNnT) were selected for this study because they represent many of

the structures and are abundant in mothers' milk. 2′-FL and 6′-SL were produced by adding fucose or a N-acetylneuraminic acid (Neu5Ac)[39] to the lactose core, respectively. Lacto-N-neotetraose (LNnT) is a neutral HMO with a galactose terminus and contained neither fucose nor sialic acid. The infection studies showed that 2'-FL did not diminish infection, however both 6'-SL and LNnT showed some diminished infection to a similar extent as the pooled sample (**Figure 4.1**).
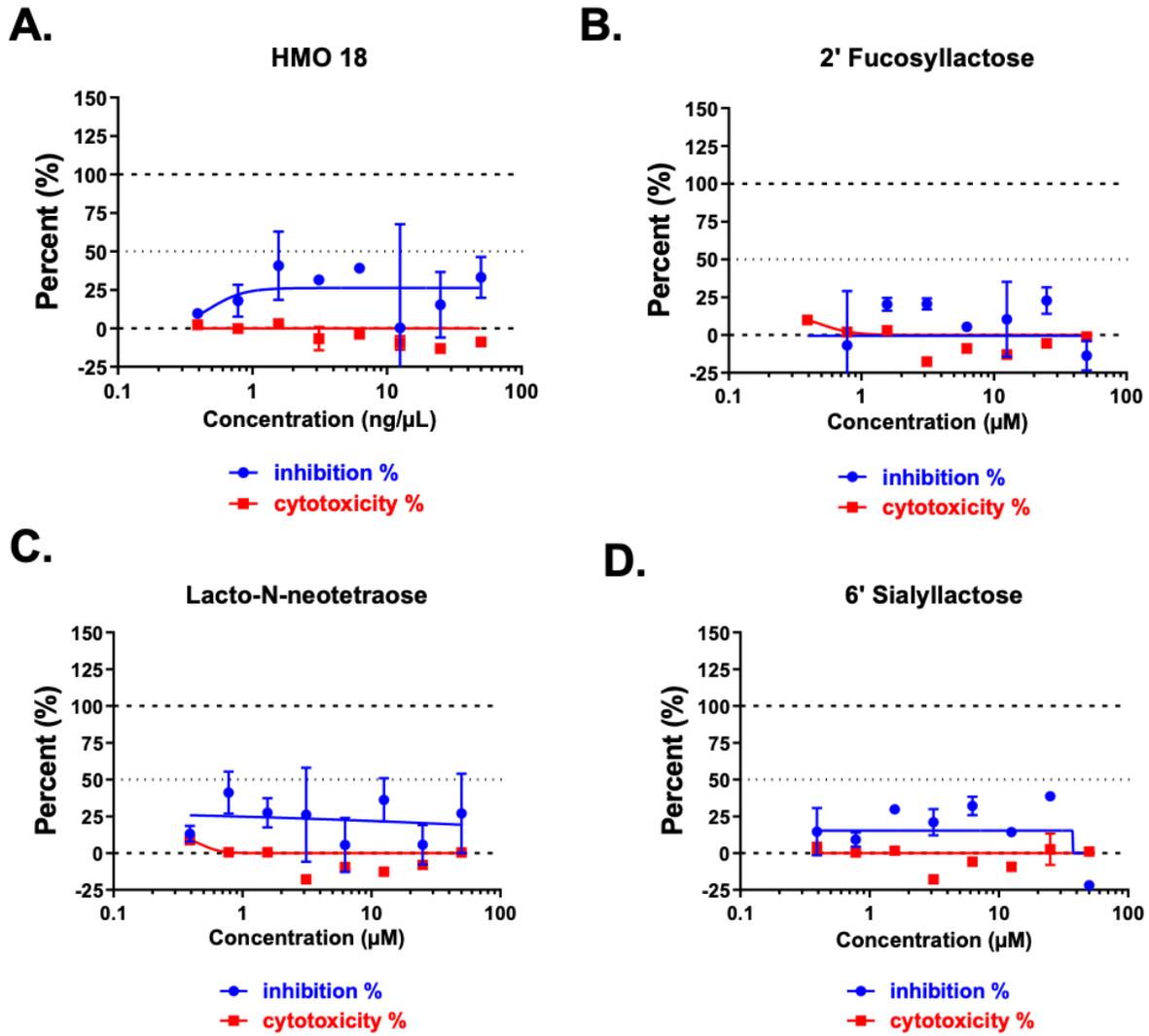
Figure 4.1 Viral infection on cells and cytotoxicity assays. Cell plates were pre-treated with pooled HMOs(A), 2′-FL(B), LNnT(C), and 6′-SL(C), respectively. The treatment was performed for 2 h at 37°C (5% CO2) prior to infection. The percent of infected cells was determined for each well based on GFP expression. All samples were run in triplicate on both an assay plate and a toxicity plate.

Due to limitations with working on the whole intact virus, we used the S protein as a surrogate for the virus. To validate this model, we performed the experiments on the S protein using the fluorescent labeling and immunofluorescence imaging. SARS-CoV-2 enters host cells via the angiotensin-converting enzyme 2 (ACE2) receptor, which binds the receptor binding domain (RBD) of the S protein.[12] *The Human Protein Atlas* (HPA), a website resource for protein expression profiles in cells, tissues and organs (https://www.proteinatlas.org/)[40, 41] was used to select the host cell with ACE2 expression. HepG2 was selected after confirming ACE2 expression with labeled antibody and immunofluorescence on the cell membrane (**Figure 4.2**).
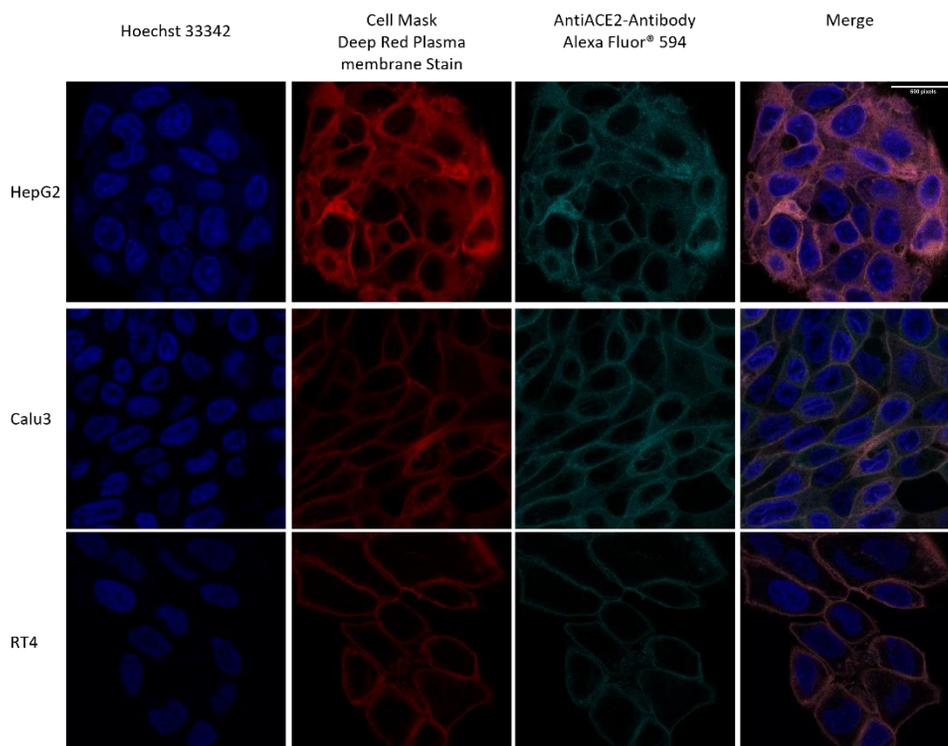
Figure 4.2 Validating the expression of ACE2 in HepG2 cells using immunofluorescence. Cells were incubated with mouse monoclonal antibody. The imagines show HepG2, Calu3 and RT4 cells expressing huACE2. The columns (from left to right) show staining of nuclear acid (Hoechst 33342), plasma membrane (CellMask™ Deep Red), anti-ACE2 antibody, and merged image. Scale bar, 600 pixels.

To verify further whether HMOs block viral adhesion, we tested the ability of the selected HMO compounds to inhibit RBD binding to HepG2 cells with immunofluorescence. Preincubating HepG2 cells with HMOs did not decrease the binding between the RBD and the cells suggesting that the HMOs did not block binding sites on the host cell surface (**Figure 4.S2**). We then tested whether the HMOs could block or alter the RBD of the virus by preincubating the RBD and the HMOs before introduction to HepG2 cells. Fluorescently labelled RBD was preincubated with 2′-FL, LNnT and 6′-SL separately then allowed to interact with host cells (**Figure 4.3A**). Quantitation of fluorescent signal intensity showed that HMOs blocked binding of RBD to cells presumably reflecting the behavior of the intact virus. The RBD was blocked only slightly by 2'FL (not statistically significant), more by LNnT (significant), and the most by 6'SL (**Figure 4.3B**). The data further showed that HMOs can potentially function as decoys to affect SARS-CoV-2 adherence.
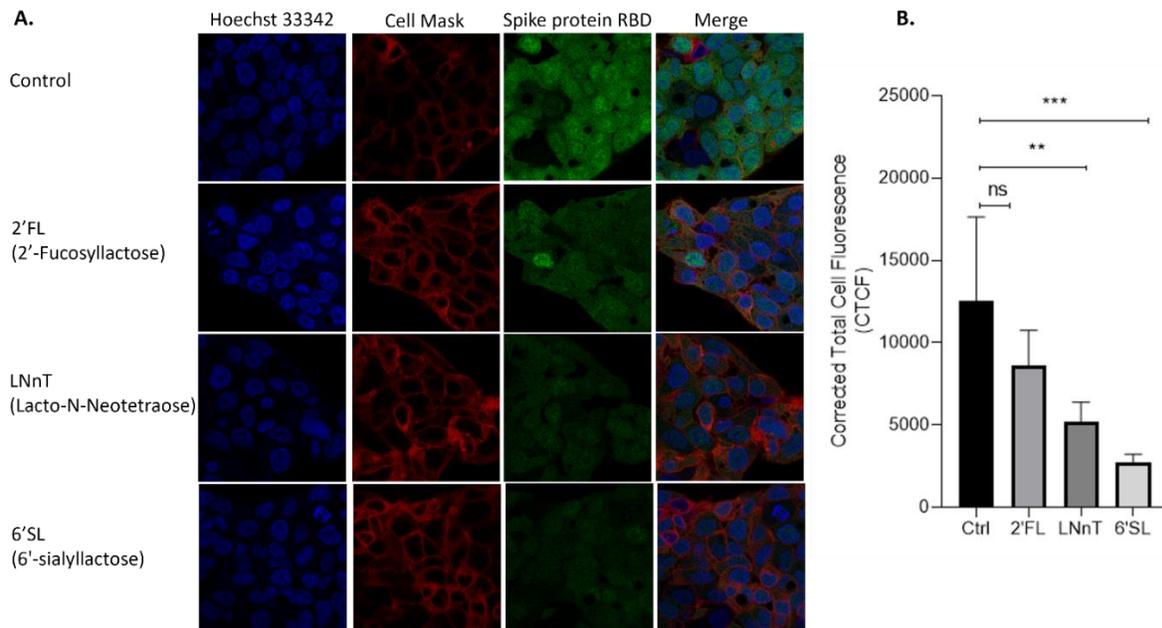
Figure 4.3 Inhibition of HMOs on the binding between HepG2 cells and Spike protein RBD. Fluorescent labelled proteins were preincubated with 1 mg per mL 2′-FL, 6′-SL, and LNnT, respectively. The preincubation was performed at room temperature for 30 min. (A) Immunofluorescence for S protein RBD binding. The columns (from left to right) show staining of nuclear acid (Hoechst 33342), plasma membrane (CellMask™ Deep Red), S protein RBD, and merged image. Scale bar, 496 pixels. (B) Quantification of fluorescent intensity of Spike protein RBD binding. Fluorescence intensity was quantified for selected cell area. Quantification was performed with software ImageJ. Asterisks indicate the statistical significance between groups compared (**p< 0.01%; ***p< 0.001%; ns p<0.05).

Determining SARS-CoV-2 binding through variable glycocalyx expression

The notable decrease in binding caused by 6′-SL drew our attention to sialic acids as potential receptors on the cell surface. To further investigate the effects of cell surface glycans on RBD binding, we altered the cell membrane glycans through transferase inhibitors. We first characterized the glycan of the cell membrane and ACE2 on the native cell line. For this analysis, complex and hybrid type glycans were combined to distinguish them from oligomannose type. The N-glycan profile showed a notable abundance of sialylated and sialyfucosylated structures (**Figure 4.4A**). The most abundant N-glycan compositions had multiple fucose and sialic acid (N-acetylneuraminic acid or Neu5Ac) residues such as $Hex_6HexNAc_5Fuc_2NeuAc_3$, $Hex_6HexNAc_5Fuc_1NeuAc_3$ and $Hex_5HexNAc_4Fuc_1NeuAc_2$. Glycoproteomic analysis of the cell membrane revealed seven glycosites on the ACE2 protein of HepG2 cells. The *N*-glycoforms of the ACE2 protein extracted from HepG2 cells were diverse and the most common structures were both fucosylated and sialylated (**Figure 4.4B**, **Table S4.1**). For comparison, we analyzed the glycosylation of commercial recombinant ACE2 protein expressed from HEK293 (**Figure 4.5**, **Table S4.2**) and found them to be similar to those expressed by HepG2 (**Table 4.1**). Both proteins were highly sialylated and fucosylated with limited amounts of high-mannose glycans.

Table 4.1 Summary of Glycoproteomic Profiles of ACE2 proteins. The number of glycoforms is shown in Table 1.

Subtype of N-Glycans detected on glycosites	Fucosylated	Sialyfucosylated	Undecorated	Sialylated	HM
Recombinant Human ACE2	147	175	68	119	16
ACE2 from HepG2 cells	100	120	38	65	5

A. Chromatogram of N-Glycome of HepG2 cells

B. N-Glycoforms from ACE2 Protien

...FNHEAEDLFYQSSLASWNYNTN_{53}ITEENVQNMNNAGDK...EQSTLAQMYPLQEIQN_{90}L

TVKLQLQALQQN_{103}GSSVLSEDK..........FFVSVGLPN_{322}MTQGFWENSMLTDPGNVQK

....SIGLLSPDFQEDN_{432}ETEINFLLK.................

...CDISN_{546}STEAGQK...N_{690}VSDIIPR......

- Sialylated
- Sialyfucosylated
- Undecorated
- Fucosylated
- HighMannose
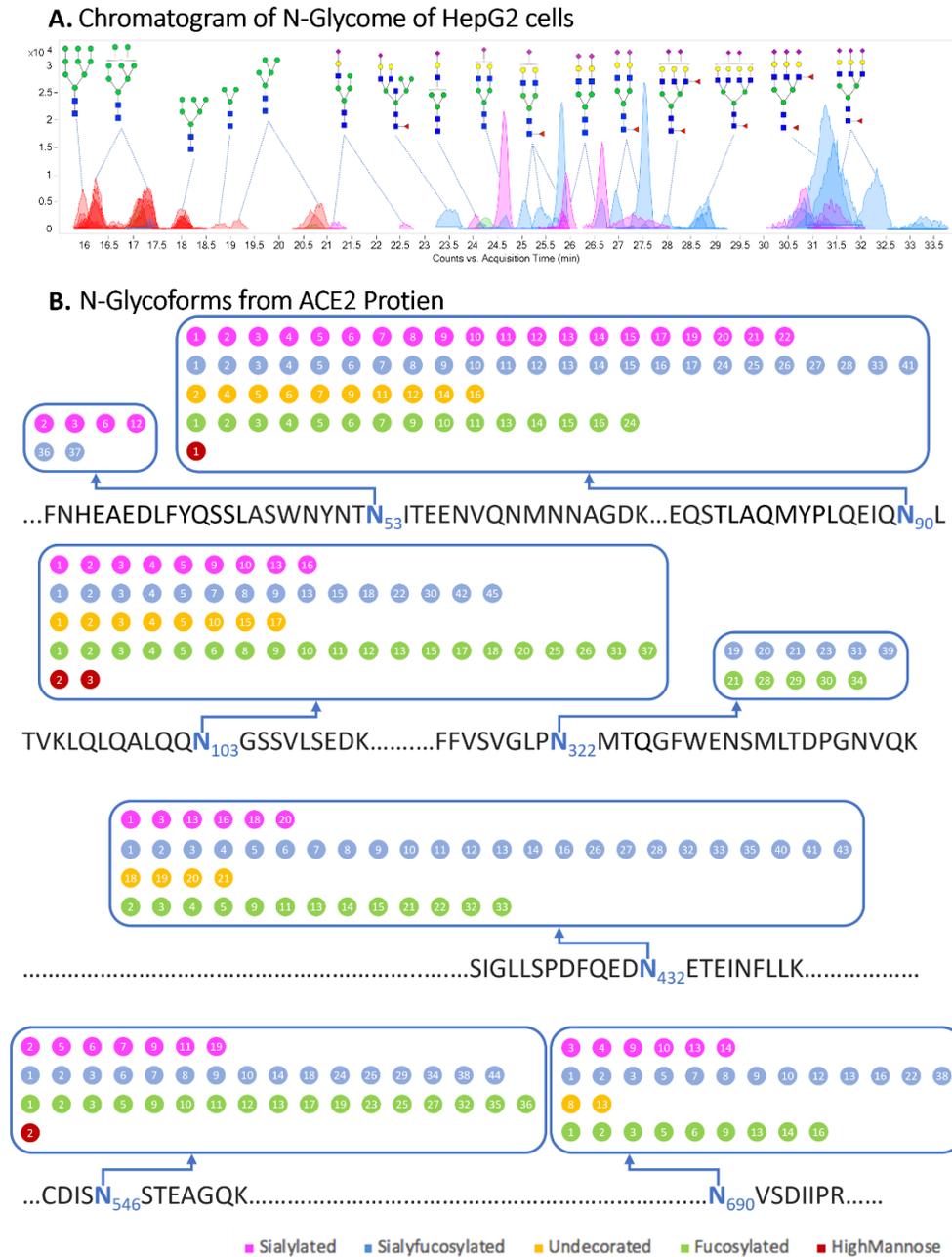
Figure 4.4 Cell membrane N-glycome and site-specific occupancy of ACE2 receptor in HepG2 cells. (A) Individual N-glycan species of HepG2 host cells. LC-MS peaks were color coded to assign glycan subtype. Abundant peaks are annotated with putative structures. Symbol nomenclature is used for representing glycan structures (https://www.ncbi.nlm.nih.gov/glycans/snfg.html). (B) Site-specific occupancy of ACE2 receptor in HepG2 cells. The N-glycoforms from ACE2 protien extracted from HepG2 cells are distributed on 7 glycosites. The labeled numbers inside dots denote identified individual glycan and the details were shown in Table S2.
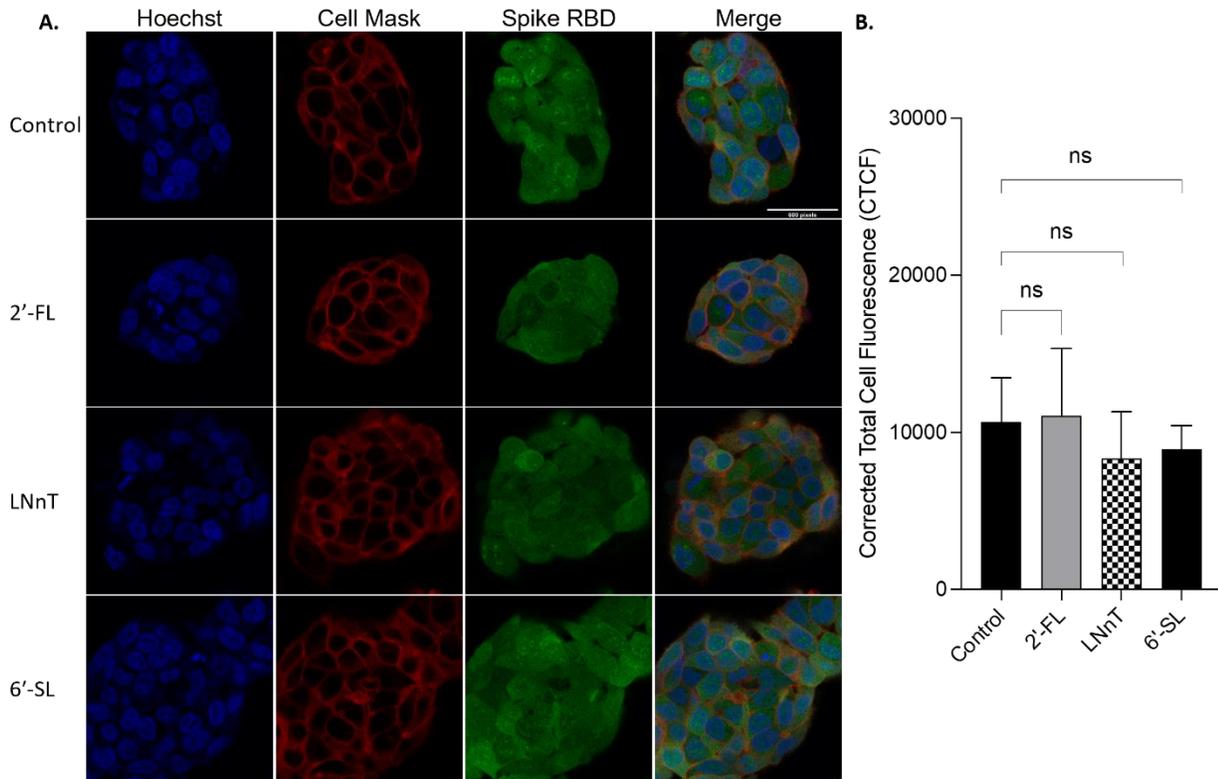
Figure 4.5 Effect of HMOs on the binding between HepG2 cells and Spike protein RBD. HepG2 cells were preincubated with 1 mg per mL 2′-FL, 6′-SL, and LNnT, respectively. After 30 min-preincubation at room temperature, cells were washed with PBS to remove exogenous decoys before fixation. (A) Immunofluorescence for S protein RBD binding with HepG2 cells. The columns (from left to right) show staining of nuclear acid (Hoechst 33342), plasma membrane (CellMask™ Deep Red), S protein RBD, and merged image. Scale bar, 600 pixels. (B) Quantification of fluorescent intensity of Spike protein RBD binding. Fluorescence intensity was quantified for selected cell area. Quantification was performed with software ImageJ. Asterisks indicate the statistical significance between groups compared (ns $p < 0.05$).

We metabolically altered the cell surface glycome to enrich for sialic acids, fucoses and mannoses, respectively. To determine whether these changes in glycosylation affected ACE2 expression on the cell membrane, we probed the cells with fluorescently labeled antibodies (**Figure 4.6**) These experiments showed no significant changes in protein expression for ACE2 in any of the glycan modification procedures. To diminish fucosylation on the HepG2 cell surface, we employed a fucosyltransferase inhibitor, 2-fluoro-L-fucose (2F-Fucose). To inhibit sialylation, a sialyltransferase inhibitor 3-fluorinated sialic acid(3-F-Sia) was used.

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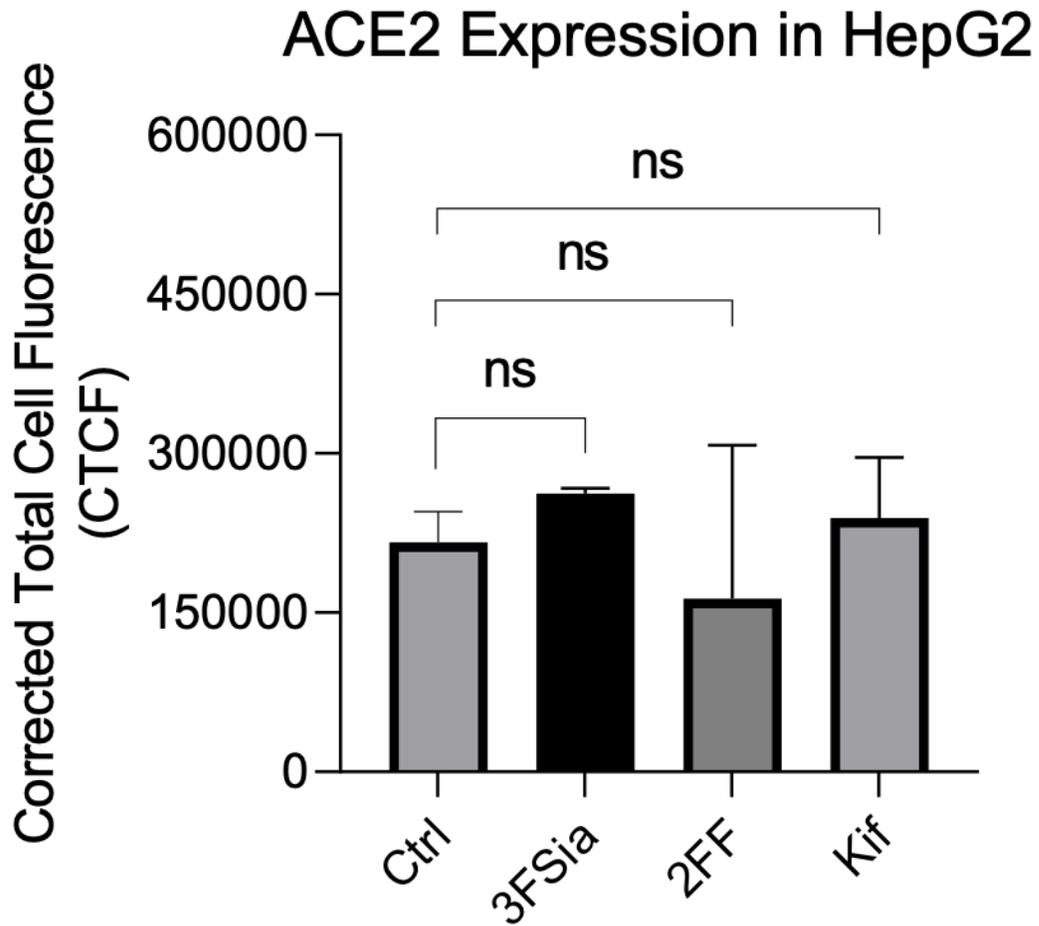
Figure 4.6 Host cell glycocalyx remodeling not altering ACE2 expression on HepG2 cells. Quantification of fluorescent intensity of anti-ACE2 antibodies on HepG2 cells. Fluorescence intensity was quantified for selected cell area. Quantification was performed with software ImageJ. Asterisks indicate the statistical significance between groups compared (ns $p<0.05$).

The predicted behavior of each substrate is shown in **Figure 4.7**. Compositional profiles were generated for the modified cells, using the sum of the intensities for similar glycan types from the LC-MS analysis. These inhibitors have recently been applied for altering cell surface glycosylation to yield similar results (**Figure 4.7B**).[42] 2F-Fucose inhibits fucosylation by being converted to the sugar nucleotide GDP-2F-Fuc.[43] It then accumulates in the cell and binds to the transferase and prohibits the enzyme from adding fucose to the nascent chain, thereby decreasing fucose expression on the cell surface.[44] The sialyfucosylated N-glycans decreased from 75% to 10% after inhibition with 2F-Fucose treatment. The sialyfucosylated N-glycans were converted to sialylated (only) ones. For example, the abundant sialyfucosylated compound $Hex_5HexNAc_4Fuc_1NeuAc$ decreased (9.6% to 1.9%, relative abundance) relative to the unfucosylated species $Hex_5HexNAc_4Fuc_0NeuAc_2$ which increased in abundance from 3.7% to 19% (**Table 4.S3**). The sialylation pathway was inhibited using 3-F-Sia, a fluorinated sialic acid substrate [cytidine monophosphate (CMP)–SiaFAc][45], which binds more strongly to the enzyme thereby prohibiting the transfer of sialic acids. Treatment with 3-F-Sia decreased the relative abundance of all sialyfucosylated N-glycans from 75% to 34%. Simultaneously, the relative abundance of fucosylated (only) species increased from 1 % to 27 %. Thus, it appears that the inhibitors are highly effective diminishing fucosylated and sialylated structures, respectively.

A. Schematic Representation of Modifying Host Cell Surface Glycosylation
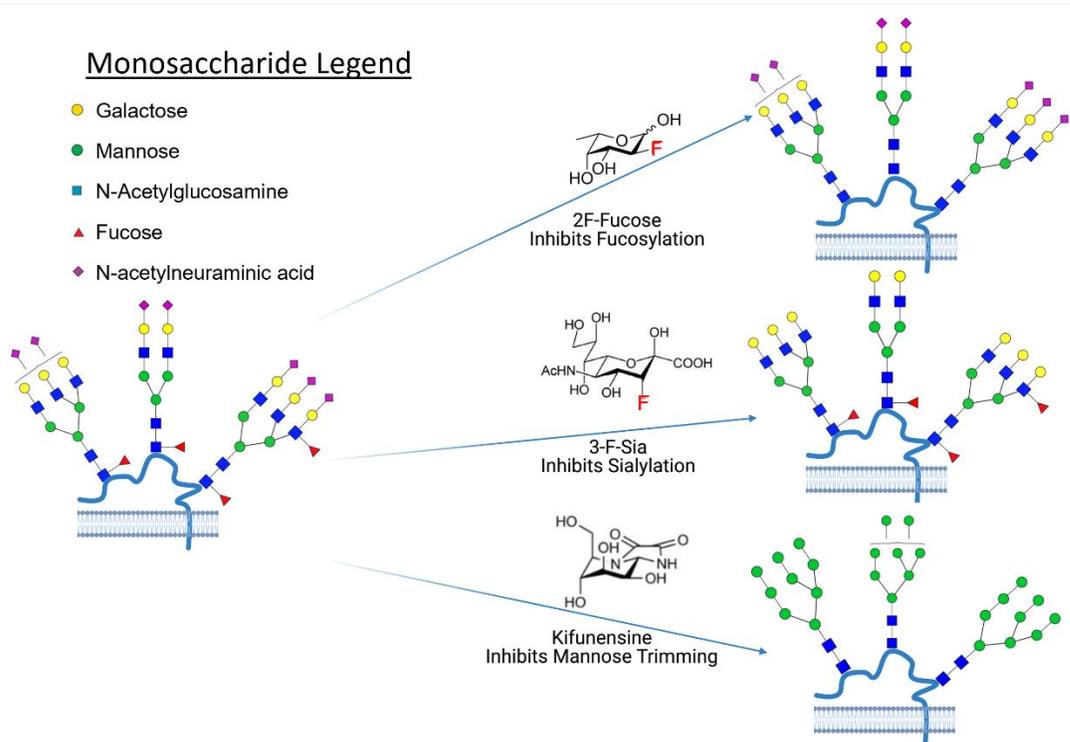
B. Relative Abundance of N-Glycan Subtypes from Modified HepG2 Cells
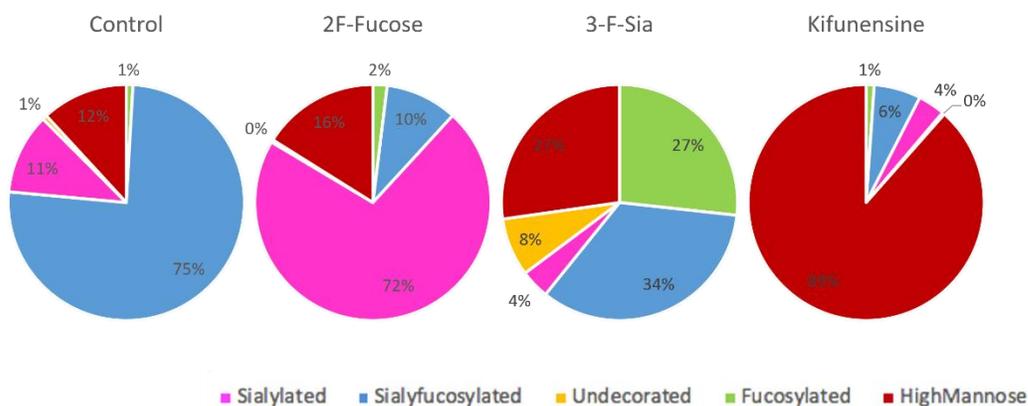
Figure 4.7 Host Cell Surface Glycome Modification. 2F-Fucose (Fucosyltransferase Inhibitor); 3-F-Sia (Sialyltransferase Inhibitor). (A) Metabolic engineering stargey for altering host cell glycosulation. Symbol nomenclature is used for representing glycan structures (https://www.ncbi.nlm.nih.gov/glycans/snfg.html). (B) N-Glycome Profiles of unmodified and modified HepG2 cells from LC-MS analysis. Compound list and details are shown in Table S3. Pie charts were color coded to assign glycan subtype. Numbers inside pie charts denote the relative abundance of each identified glycan subtype.

After confirming that glycan alteration had taken place in host cell, immunofluorescence analysis was used to observe the effect of the host glycome on viral binding. Treatment of 2F-Fucose did not affect RBD binding to the cell significantly as observed by immunofluorescence imaging (**Figure 4.8A**). However, inhibition of sialylation by 3-F-Sia decreased the S protein RBD binding with HepG2 cells by 64% (**Figure 4.8B**), indicating that the binding was likely mediated by sialic acid residues on the host cell surface. Similar trends were observed in other cell lines with ACE2 expression, namely Calu3 and RT4 (**Figure 4.8B**). Desialylation inhibited the binding from S protein RBD significantly, and decreased fucosylation did not change the extent of the binding.
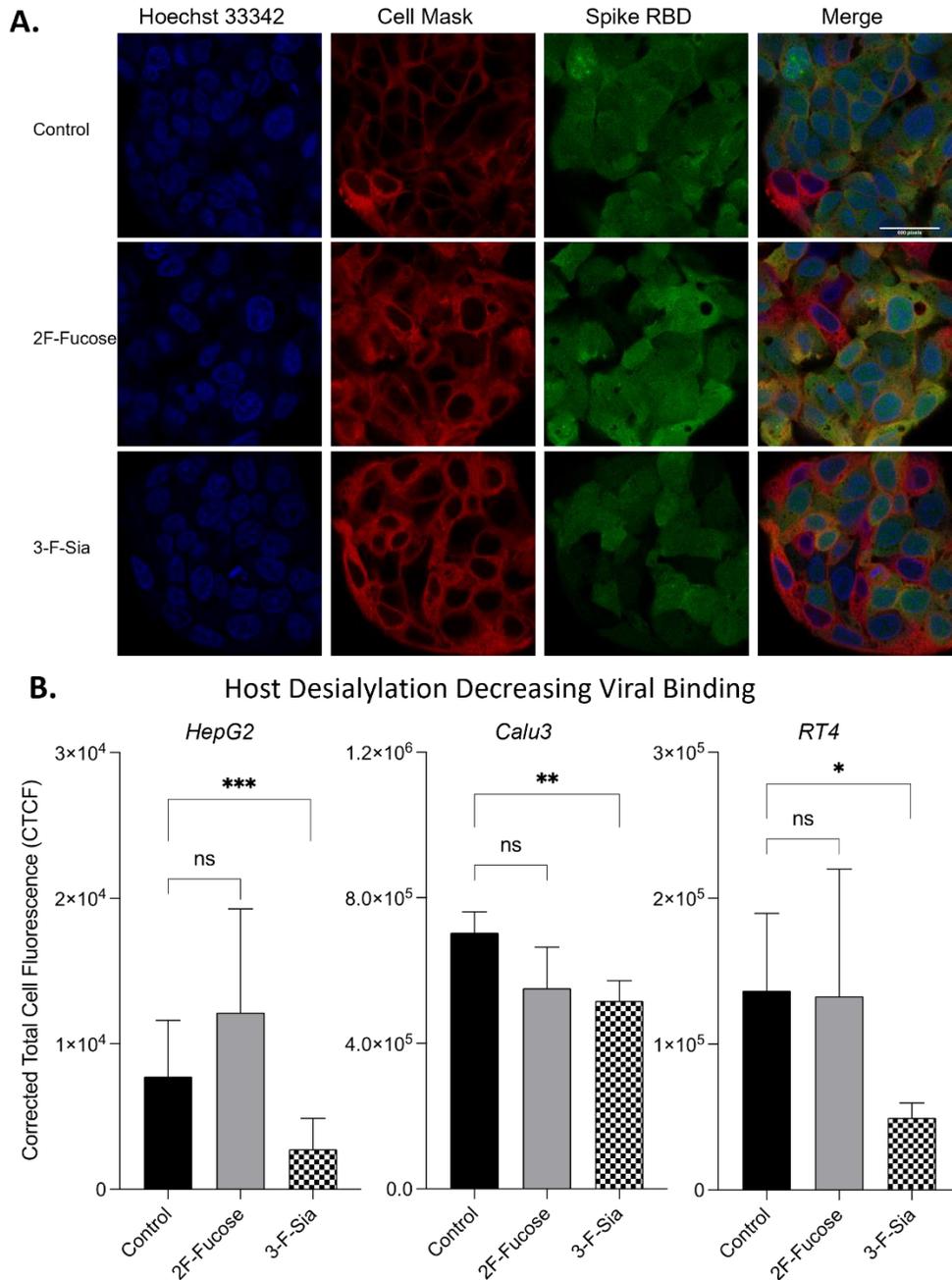
Figure 4.8 Remodeling host glycome alters binding between host cells and Spike protein RBD. 2F-Fucose (Fucosyltransferase Inhibitor); 3-F-Sia (Sialyltransferase Inhibitor). (A) Immunofluorescence for S protein RBD binding with modified cells. The columns (from left to right) show staining of nuclear acid (Hoechst 33342), plasma membrane (CellMask™ Deep Red), S protein RBD, and merged image. Scale bar, 600 pixels. (B) Quantification of fluorescent intensity of Spike protein RBD binding. Fluorescence intensity was quantified for selected cell area. Quantification was performed with software ImageJ. Asterisks indicate the statistical significance between groups compared (*p< 0.05%; **p< 0.01%; ***p< 0.001%; ns p<0.05).

In mammalian cells, terminal sialic acids are commonly found in α-glycosidic linkages to the C-3 or C-6 hydroxyl of galactose via α-(2,3)- or α-(2,6)-linkage for N-glycans.[46] In nasal mucosa, α-(2-6)-sialic acids are dominant[47] with significantly less detected in the lung.[48] We further investigated linkage specificities for RBD binding by preincubating the RBD with sialylated HMOs. Preincubation with 3′- sialyllactose (3′-SL) did not decrease the binding, while a significant decrease was observed after preincubation with 6′-SL, confirming S protein RBD prefers binding with α-(2-6) sialic acids (**Figure 4.9**).
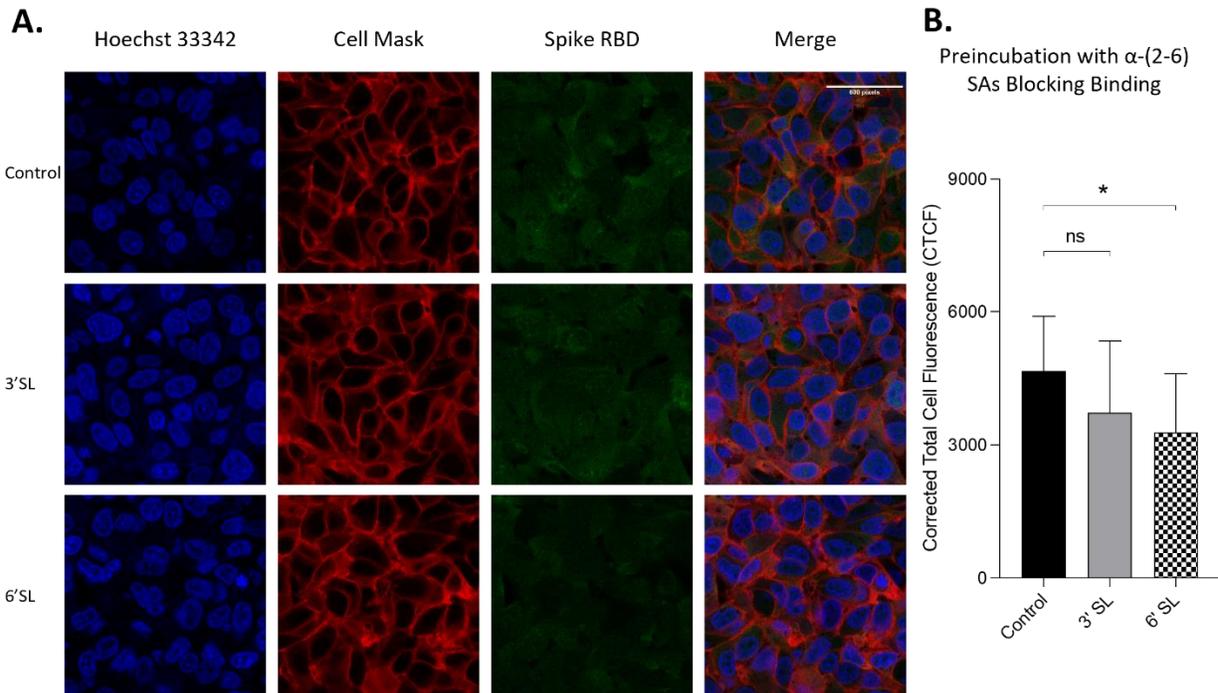
Figure 4.9 Sialylated 6′-SL HMOs inhibited binding of Spike protein RBD to HepG2 Cells. Fluorescent labelled proteins were preincubated with 1 mg per mL 3′-FL(3'-sialyllactose) and 6′-SL(6'-sialyllactose) respectively at room temperature. (A) Immunofluorescence for S protein RBD binding with cells. The columns (from left to right) show staining of nuclear acid (Hoechst 33342), plasma membrane (CellMask™ Deep Red), S protein RBD, and merged image. Scale bar, 600 pixels. (B) Quantification of fluorescent intensity of Spike protein RBD binding. Fluorescence intensity was quantified for selected cell area. Quantification was performed with software ImageJ. Asterisks indicate the statistical significance between groups compared (*p< 0.05%; ns p<0.05).

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Fucosylated glycans were also observed on ACE2 proteins in HepG2 cells (**Table S1**). Terminal α-(1,2) and α-(1,3)-fucose residues are commonly found in mammalian cells.[49, 50] To confirm that fucosylation is less important, 2′-FL and 3′-fucosyllactose (3′-FL) , components of HMOs, were used.[39] Preincubating the S protein RBD with 2′-FL or 3′-FL did not significantly alter binding (**Figure 4.10**). The S protein RBD again showed little affinity to terminal fucose residues on host cells.
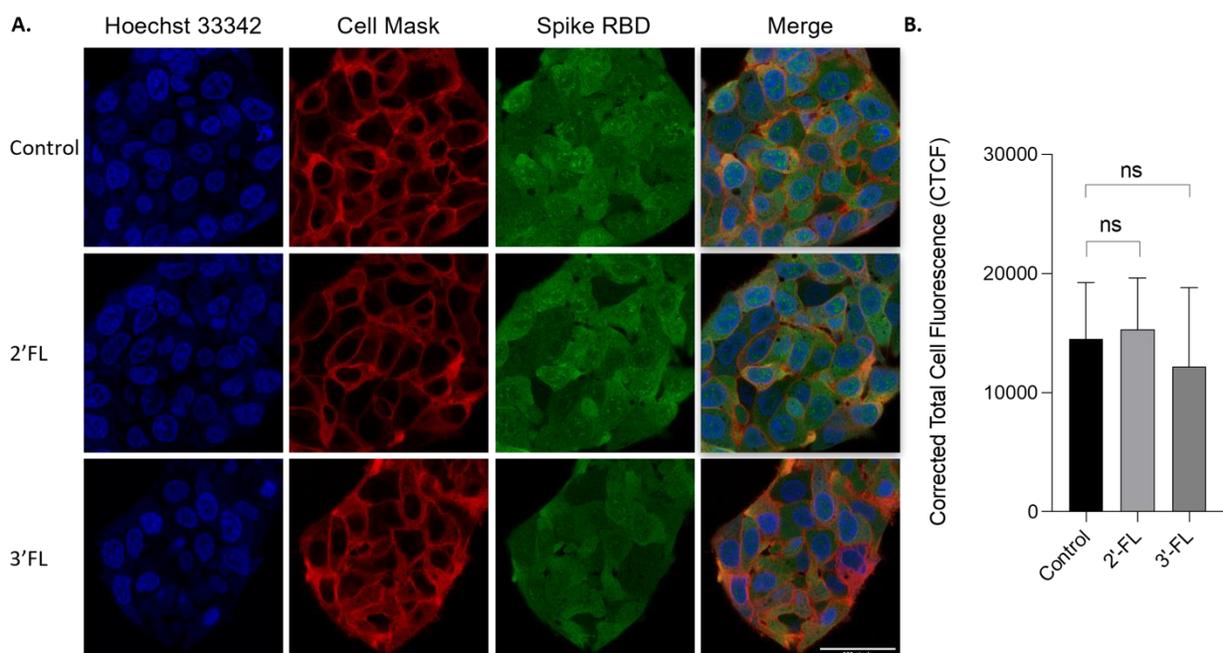
Figure 4.10 Effect of fucosylated HMOs on the binding between HepG2 cells and Spike protein RBD. S protein RBD with 2′-FL or 3′-FL for 30 min at room temperature. (**A**) Immunofluorescence for S protein RBD binding with HepG2 cells. The columns (from left to right) show staining of nuclear acid (Hoechst 33342), plasma membrane (CellMask™ Deep Red), S protein RBD, and merged image. Scale bar, 600 pixels. (**B**) Quantification of fluorescent intensity of Spike protein RBD binding. Fluorescence intensity was quantified for selected cell area. Quantification was performed with software ImageJ. Asterisks indicate the statistical significance between groups compared (ns $p < 0.05$).

Glycosylation of HepG2 included primarily complex and hybrid type structures with fewer high mannose structures. The latter have been reported as important mediators in host-virus binding for human coronaviruses HKU1[51] and severe acute respiratory syndrome (SARS).[52] We remodeled the cell surface to produce primarily oligomannose and determined its effects on SARS-CoV-2 binding. Kifunensine (Kif) is commonly used to inhibit the α-mannosidase-I[53], thereby preventing mannose trimming to increase oligomannose-type glycans.[52, 54] Our LC/MS data also proved its increasing the relative abundance of oligomannose to 89% in whole cell N-glycome as shown in **Figure 4.4**. Introduction of Kif to the cell resulted in a fourfold increase in the binding as measured by immunofluorescence imaging (**Figure 4.11A, 4.11B**). N-Glycans, released from RNase B, were also employed to examine high mannose type binding. Preincubation with the oligomannose decreased the binding of S protein RBD with host cells (**Figure 4.11C**). This effect was dose dependent with higher concentrations preventing binding more strongly. High mannose glycans on the host cell surface can therefore increase the adherence of S protein RBD.
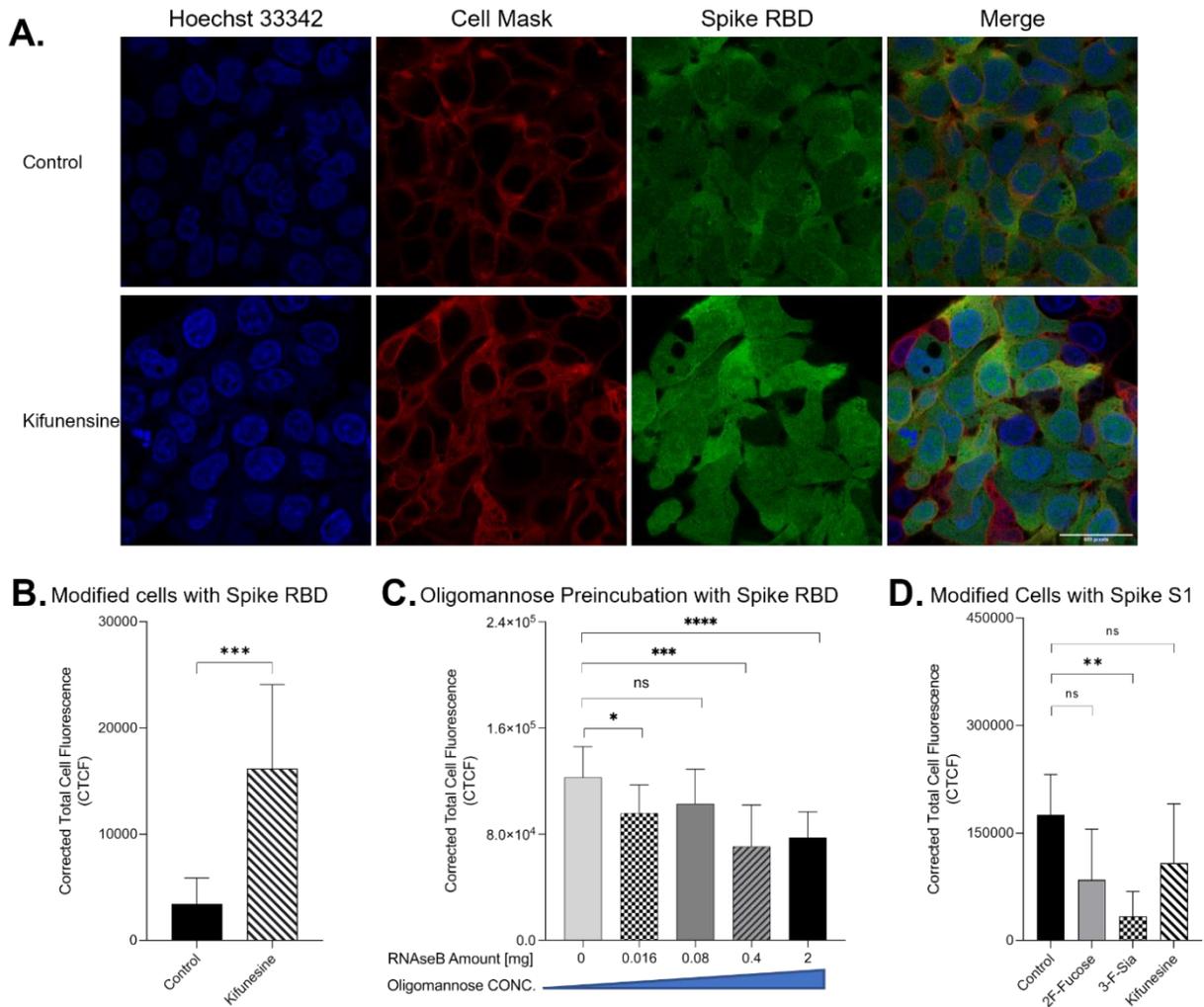
Figure 4.11 Introducing high mannose glycans into viral binding. (A) Immunofluorescence for S protein RBD binding with modified cells. The columns (from left to right) show staining of nuclear acid (Hoechst 33342), plasma membrane (CellMask™ Deep Red), S protein RBD, and merged image. Scale bar, 600 pixels. Quantification of fluorescent intensity of Spike protein RBD (B) (C) or S1 subunit (D) binding. (C) Fluorescent labelled proteins were preincubated with purified high mannose at room temperature for 30 min before binding. Fluorescence intensity was quantified for selected cell area. Quantification was performed with software ImageJ. Asterisks indicate the statistical significance between groups compared ($*p < 0.05\%$; $**p < 0.01\%$; $***p < 0.001\%$; $****p < 0.0001\%$; ns $p < 0.05$).

To further validate the binding of the spike protein with the host glycocalyx, we used spike protein S1 subunit, a longer polypeptide segment of the S protein and includes the sequence of RBD. Treatment of the cell line with 2F-Fucose did not change the binding between S1 subunit and host cells (**Figure 4.11D**). Similarly, the use of 3-F-Sia significant decreased the fluorescent intensity of the assay demonstrating again that the spike protein binds to sialic acids. Surprisingly, the use of Kif on the S1 subunit no longer increased binding. The binding studies showed that there was no significant change in binding relative to the control.

Molecular Dynamics Calculations of ACE2 and S Protein Interactions

To gain further insight into the interactions between the primary receptor ACE2 [12, 55, 56] and the SARS-CoV-2 S protein, we performed molecular dynamics calculations on the interacting complex. Based on the glycoproteomic results for ACE2 from the HepG2 cell line (**Table S4.1**), we constructed a model with selected glycoforms on ACE2. ACE2 contained seven occupied N-glycan sites corresponding to Asn 53, 90, 103, 322, 432, 546, and 690 (**Figure 4.12A**).
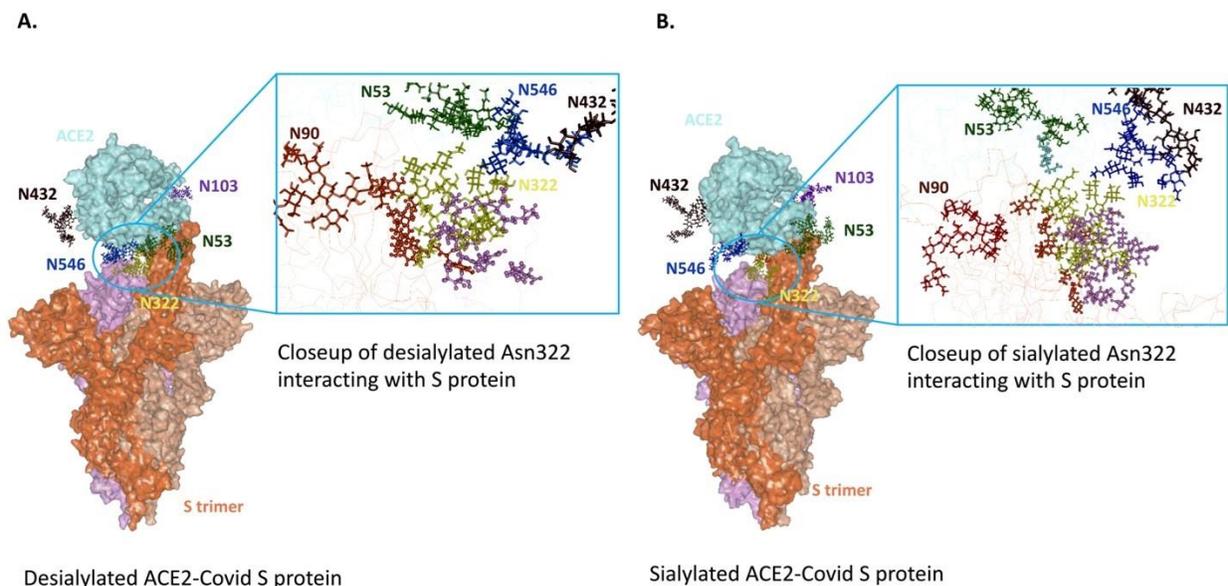
Figure 4.12 Modelled sialylated and desialylated ACE2-Covid S protein complexes. 3D structural modeling of glycosylated ACE2 interacting with S-protein. Results from glycomics and glycoproteomics of HEPG2 cell lines were used to generate (A) fully-desialylated and (B) fully-sialylated homologs of ACE2, interacting with S-protein.

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From the quantitative glycoproteomic results and the Protein Data Bank-derived complex (PDB ID: 7DF4)[31], the most abundant glycan at each site were modelled with CHARMM-GUI.[32] The resulting structure, shown in the "up" conformation, was selected because it represented the activated complex prior to invasion. Molecular dynamics simulations were performed on the complex with solvent and associated ions for 5 ns (See Methods Section). After the simulations, the number of hydrogen bonds formed between the ACE2 glycans and S protein determined. For comparison, the same calculations were performed on the fully desialylated ACE2 homologs (**Figure 4.12B**). The results showed that many of the glycans on ACE2 interacted with the S protein through hydrogen bonding interactions. Comparison of the fully sialylated and desialylated glycans showed significantly lower number of hydrogen bonds (based on 3 Å, donor-acceptor

distances) particularly on Asn 90 (22 hydrogen bonds by glycan) and Asn 322 (51 hydrogen bonds by glycan) of the desialylated homolog (**Figure 4.13A**). These results are consistent with earlier simulations performed by Zhao et al on ACE2 - S who noted that both glycan sites were also the most interactive in the complex.[21] Furthermore, when the sialic acids were considered relative to other monosaccharide residues (3 by sialic acid at Asn 90, 15 by sialic acid at Asn 322), their contributions to the overall interactions were proportionally larger (**Figure 4.9B**).
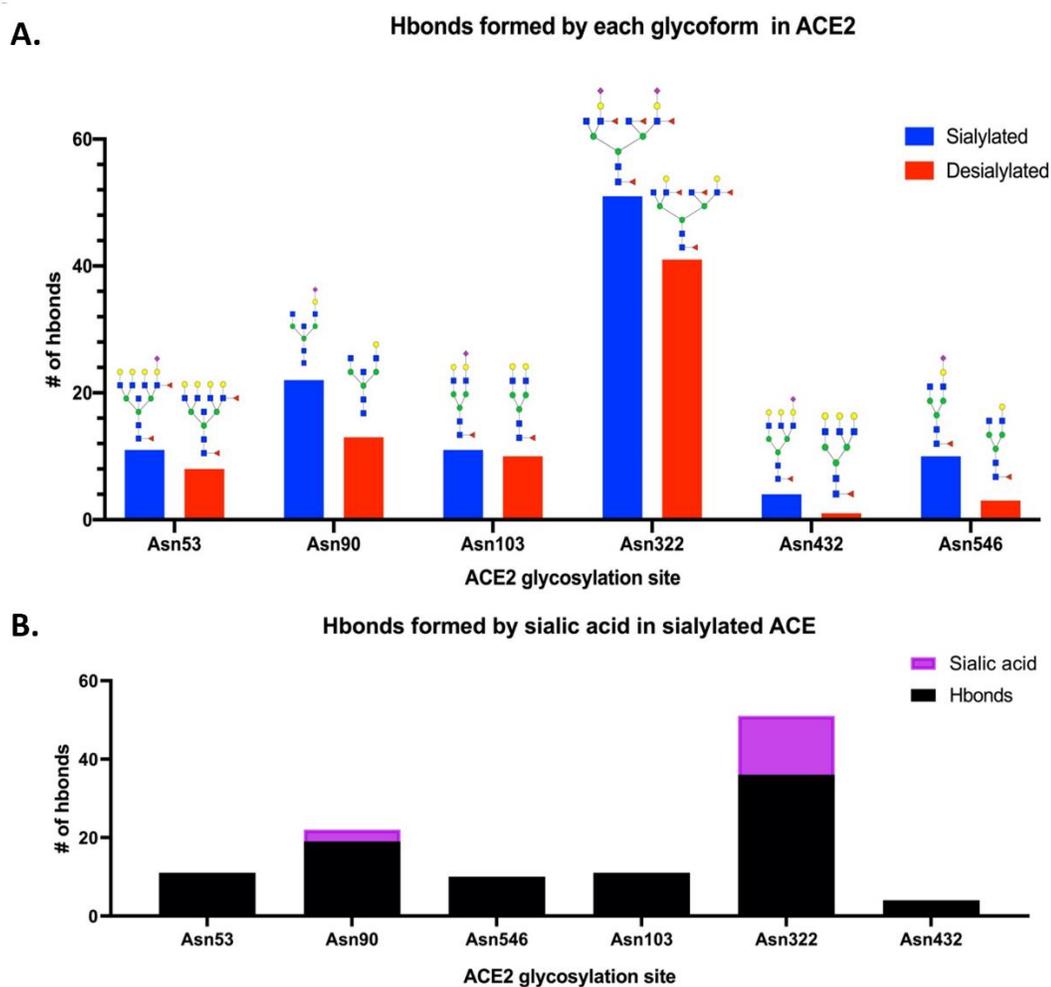
Figure 4.13 Interactions of glycosylated ACE2 and S-protein were revealed using molecular dynamics simulations. (A) The number of intramolecular hydrogen bonds was drastically higher for each fully-sialylated N-glycan compared to the desialylated glycoform. (B) For Asn 90 and Asn 322, the sialic acid residue in the glycoform accounted for ~10% of hydrogen bonds.

DISCUSSION

Glycans on the host cell membrane and on viral proteins play key roles in the infection of SARS-CoV-2. Viral glycosylation has been the primary focus of glycomic studies related to the virus. Indeed, the virus is highly glycosylated with at least 17 N-glycosylation and 2 O-glycosylation sites identified.[19] We found two occupied N-glycosites on Spike RBD (**Table S4.4**) consistent with earlier findings. However, the host cells were also highly glycosylated. The LC-MS glycomic profile of HepG2 shows cell membrane with an abundance of high mannose-type glycans as well as complex-type structures with a high degree of sialylation. These structures are also branched with a combination of bi, tri, and higher antennary structures. The HepG2 cell lines was selected for its expression of ACE2, and these highly sialylated branched structures were similarly present in the protein further alluding to the importance of sialylation in at least the host-virus adhesion process.

The results showed that sialic acid in human milk oligosaccharides (HMOs) can block the binding of virus on the cell membrane. These results are further supported by recent findings that show similar deflecting properties of sialylated HMOs toward the S protein of SARS-CoV-2[26] and illustrating further the protective nature of human milk against these pathogens. HMOs are similar to O-glycans in structure; however, N-glycans on membrane proteins similarly provide sialic acid on their termini. Altering the glycans on the cell membrane, while maintaining the expression levels of proteins such as ACE2, shows that sialic acid on the cell surface induces stronger binding to the virus. ACE2 is itself highly sialylated, in the cell line used in this study and from commercial sources (mainly from HEK293). ACE2 expressed recombinantly in other cell lines have similar glycosylation profiles that are similarly rich in sialylation. Deeper structural analysis showed that the binding prefers a specific linkage, namely α-(2,6)-sialic acids. Interestingly, the human

influenza virus has a similar preference for binding.[57-59] Perhaps not coincidently, the human epithelium is rich in α-(2,6)-sialic acid, which is also more abundant than the isomer α-(2,3)-sialic acid, the binding site of avian bird flu.

The binding of sialic acid point to specific protective measures by the host. In breastfed infants, HMOs provide some protection. Human milk is also full of proteins that are highly sialylated, such as the immunoglobulins and lactoferrins.[60-63] In adults, pathogen deflection is performed by the mucus layer in the lungs and gastrointestinal tract. SARS-CoV-2 is a respiratory disease reaching deep into the respiratory tract and the lungs. It also infects the intestine [64], with both types of tissues protected by a mucus layer constructed around high molecular weight glycoproteins called mucins.[65] Mucin are expressed in epithelial surfaces of gastrointestinal, genitourinary, and respiratory tracts, where they also shield the surface against chemical and physical damages[46]. While mucins are covered primarily by O-glycans that are similar to human milk oligosaccharides, they contain the same sialic acid termini as N-glycans. The mucus layer therefore presents a myriad of potential binding sites for commensal and pathogenic microbes[66, 67], and shedding mucins is a defense strategy against pathogen infection.

High mannose glycans were also investigated as potential inhibitors of viral infections. The high mannose glycans were also strongly bound in the shorter version (RBD) of the S protein. However, in the longer homolog (S1 subunit) this binding was diminished. These results suggest that that there is a high mannose binding site on the S protein that is potentially shielded in the longer homolog. On the other hand, high mannose glycans are typically not found on epithelial cells[16] and are not abundant in the blood. However, they are much more abundant in the tissue samples compared to serum. These glycans are found in cancer cells [68, 69] and stem cells.[70] The levels of several oligomannose type glycans are upregulated in tumor tissue.[71, 72] The role of

mannose residue as a host receptor has been studied and proved in the microbe-host interactions, such as *Salmonella enterica* subsp. *enterica* serovar typhimurium (*S. typhimurium*)[73], influenza virus[74, 75], dengue virus[76] and human immunodeficiency virus (HIV).[77] Mannan is usually employed for studying mannose binding with virus.[51, 52, 75, 78] The mannans are highly heterogeneous in length and branching. The repeating α-(1,6)-linked mannose backbone is usually branched by short chains of α-(1-2) and α-(1-3)-linked mannose structures.[79] In this study, we used oligomannose released form RNase B[80] instead of mannan. The released high mannose glycans were determined with mass spectrometry **(Figure 4.14),** and all those structures have been found in human cell glycomes.
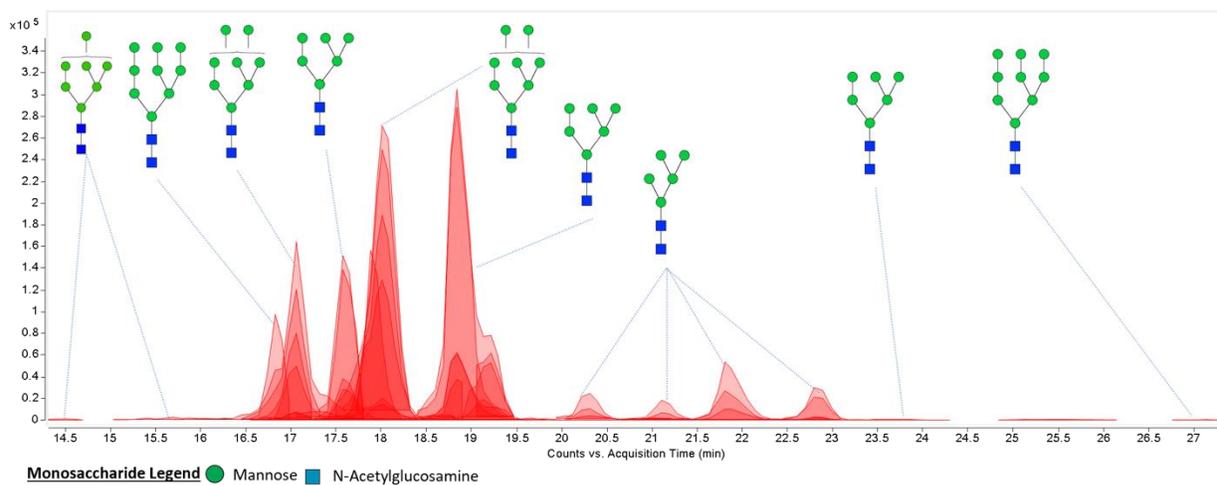
Figure 4.14 Chromatogram of high mannose N-glycans released form RNase B. Abundant peaks are annotated with putative structures. Symbol nomenclature is used for representing glycan structures (https://www.ncbi.nlm.nih.gov/glycans/snfg.html).

The integrated method developed here, which includes alteration of cell surface glycan products through specific inhibitors, coupled with the enrichment of the membrane proteins and extensive glycomic and glycoproteomic analysis provides a new platform for obtaining structural specificity in host-microbe interactions. Glycans are common targets for many commensals and pathogens. This method will have great utility in identifying glycan targets of individual microbes

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and even toxins that bind glycans. The method is made possible by recent advancements in novel glycosyl transferase inhibitors that produce specifically glycosylated membrane proteins. I should be noted that the conversion to a glycan type is never fully complete. There are residual endogenous glycans due to the differences in turnover of different glycoproteoform.[81] However, the ability to perform glycomic profiling with LC-MS provides a guiding assay to examine the extent of the glycomic transformation.

CONCLUSIONS

The study supports a mechanism for binding of SARS-CoV-2 to the cell membrane that is primarily mediated by glycans. The preferred target of the S protein is sialylated glycans with α-(2,6)-sialic acids on the termini positions. The virus likely binds to cells and tissues rich in sialylated glycans, whether N-, O-, and potentially even glycolipids that are found in the surface of the epithelial surface. The airway epithelium is rich in sialic acids and in particular, α-(2,6)-sialic acids. In this regard, the human influenza virus and SARS-CoV-2 have the same binding preference on host membranes. Invasion of SARS-CoV-2 likely occurs when the virus fortuitously binds to the ACE2 protein, which itself is highly sialylated. The alignment between the S and the ACE2 protein is further facilitated by hydrogen binding interactions between the sialylated glycans of the host cell and the polypeptide of the S protein.

Table S4.1 Glycoproteomic information of ACE2 derived from HepG2 cells

Peptide Sequence	Modification	Modified Amino Acid	Modification Position	Glycans	Label in Figure 3	Relative Abundance	Glycan Subtype
FNHEAEDL FYQSSLAS WNYNTNIT EENVQNM NNAGDK	NGlycan/31 39.1366	N	53	HexNAc(7)Hex(7)Fuc(2)NeuAc(1)	36	0.0326	Sialyfuc osylated
FNHEAEDL FYQSSLAS WNYNTNIT EENVQNM NNAGDK	NGlycan/29 92.0834	N	53	HexNAc(5)Hex(5)Fuc(4)NeuAc(2)	37	0.0298	Sialyfuc osylated
FNHEAEDL FYQSSLAS WNYNTNIT EENVQNM NNAGDK	NGlycan/19 54.7036	N	53	HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	2	0.0235	Sialylate d
FNHEAEDL FYQSSLAS WNYNTNIT EENVQNM NNAGDK	NGlycan/21 57.7829	N	53	HexNAc(6)Hex(4)Fuc(0)NeuAc(1)	12	0.0227	Sialylate d
FNHEAEDL FYQSSLAS WNYNTNIT EENVQNM NNAGDK	NGlycan/23 19.8358; Deamidated /0.9840	N,N	53,58	HexNAc(6)Hex(5)Fuc(0)NeuAc(1)	6	0.0053 9	Sialylate d
FNHEAEDL FYQSSLAS WNYNTNIT EENVQNM NNAGDK	NGlycan/21 16.7564	N	53	HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	3	0.0253	Sialylate d

EQSTLAQM YPLQEIQN LTVK	NGlycan/20 59.7349	N	90	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	1	0.592	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/20 59.7349	E,N	75,90	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	1	0.0441	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/23 50.8304	N	90	HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	2	0.275	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/23 50.8304	E,N	75,90	HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	2	0.0259	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/18 97.6821	N	90	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	3	0.7	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/18 97.6821	E,N	75,90	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	3	0.0349	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Deamidated /0.9840; NGlycan/18 97.6821	Q,N	76,90	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	3	0.0126	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/23 67.8457	N	90	HexNAc(4)Hex(6)Fuc(2)NeuAc(1)	4	0.0617	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/23 67.8457	E,N	75,90	HexNAc(4)Hex(6)Fuc(2)NeuAc(1)	4	0.0223	Sialyfuc osylated

EQSTLAQM YPLQEIQN LTVK	NGlycan/22 05.7928	N	90	HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	5	0.0237	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/27 89.9993	N	90	HexNAc(6)Hex(7)Fuc(1)NeuAc(1)	6	0.328	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/24 24.8671	N	90	HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	7	0.218	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro-Glu/- 18.0106; NGlycan/24 24.8671	E,N	75,90	HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	7	0.0155	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/27 15.9625	N	90	HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	8	0.221	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro-Glu/- 18.0106; NGlycan/27 15.9625	E,N	75,90	HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	8	0.0392	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/22 62.8143	N	90	HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	9	0.212	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/21 00.7615	N	90	HexNAc(5)Hex(4)Fuc(1)NeuAc(1)	10	0.149	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/30 81.0947	N	90	HexNAc(6)Hex(7)Fuc(1)NeuAc(2)	11	0.0402	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro-Glu/- 18.0106; NGlycan/27 32.9779	E,N	75,90	HexNAc(5)Hex(7)Fuc(2)NeuAc(1)	12	0.177	Sialyfuc osylated

EQSTLAQM YPLQEIQN LTVK	NGlycan/27 32.9779	N	90	HexNAc(5)Hex(7)Fuc(2)NeuAc(1)	12	0.127	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/30 07.0580	N	90	HexNAc(5)Hex(6)Fuc(1)NeuAc(3)	13	0.0612	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/25 53.9097	N	90	HexNAc(5)Hex(5)Fuc(1)NeuAc(2)	14	0.153	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/25 53.9097	E,N	75,90	HexNAc(5)Hex(5)Fuc(1)NeuAc(2)	14	0.0247	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/22 21.7878	N	90	HexNAc(4)Hex(6)Fuc(1)NeuAc(1)	15	0.0205	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/30 24.0733	N	90	HexNAc(5)Hex(7)Fuc(2)NeuAc(2)	16	0.034	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/30 82.1151	E,N	75,90	HexNAc(6)Hex(7)Fuc(3)NeuAc(1)	17	0.124	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/24 65.8937	N	90	HexNAc(6)Hex(5)Fuc(1)NeuAc(1)	24	0.0757	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/27 74.0044	E,N	75,90	HexNAc(6)Hex(6)Fuc(2)NeuAc(1)	25	0.075	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/26 27.9465	N	90	HexNAc(6)Hex(6)Fuc(1)NeuAc(1)	26	0.0389	Sialyfuc osylated

EQSTLAQM YPLQEIQN LTVK	NGlycan/33 72.1902	N	90	HexNAc(6)Hex(7)Fuc(1)NeuAc(3)	27	0.0246	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/29 19.0419	N	90	HexNAc(6)Hex(6)Fuc(1)NeuAc(2)	28	0.0411	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/25 70.9250	N	90	HexNAc(5)Hex(6)Fuc(2)NeuAc(1)	33	0.0272	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro-Glu/-18.0106; NGlycan/25 70.9250	E,N	75,90	HexNAc(5)Hex(6)Fuc(2)NeuAc(1)	33	0.0225	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/32 10.1373	N	90	HexNAc(6)Hex(6)Fuc(1)NeuAc(3)	41	0.0106	Sialyfuc osylated
EQSTLAQM YPLQEIQN LTVK	NGlycan/20 75.7298	N	90	HexNAc(4)Hex(6)Fuc(0)NeuAc(1)	1	0.0551	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/19 54.7036	N	90	HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	2	2.02	Sialylate d
EQSTLAQM YPLQEIQN LTVK	Glu->pyro-Glu/-18.0106; NGlycan/19 54.7036	E,N	75,90	HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	2	0.123	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/21 16.7564	N	90	HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	3	2	Sialylate d
EQSTLAQM YPLQEIQN LTVK	Glu->pyro-Glu/-18.0106; NGlycan/21 16.7564	E,N	75,90	HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	3	0.11	Sialylate d

EQSTLAQM YPLQEIQN LTVK	NGlycan/19 13.6770	N	90	HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	4	0.51	Sialylate d
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/19 13.6770	E,N	75,90	HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	4	0.0556	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/24 07.8518	N	90	HexNAc(5)Hex(5)Fuc(0)NeuAc(2)	5	0.748	Sialylate d
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/24 07.8518	E,N	75,90	HexNAc(5)Hex(5)Fuc(0)NeuAc(2)	5	0.134	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/23 19.8358	N	90	HexNAc(6)Hex(5)Fuc(0)NeuAc(1)	6	0.587	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/27 72.9840	N	90	HexNAc(6)Hex(6)Fuc(0)NeuAc(2)	7	0.552	Sialylate d
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/27 72.9840	E,N	75,90	HexNAc(6)Hex(6)Fuc(0)NeuAc(2)	7	0.0723	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/24 81.8886	N	90	HexNAc(6)Hex(6)Fuc(0)NeuAc(1)	8	0.49	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/17 51.6242	N	90	HexNAc(4)Hex(4)Fuc(0)NeuAc(1)	9	0.47	Sialylate d
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106;	E,N	75,90	HexNAc(4)Hex(4)Fuc(0)NeuAc(1)	9	0.0279	Sialylate d

	NGlycan/17 51.6242						
EQSTLAQM YPLQEIQN LTVK	NGlycan/22 04.7724	N	90	HexNAc(4)Hex(5)Fuc(0)NeuAc(2)	10	0.125	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/26 10.9312	N	90	HexNAc(6)Hex(5)Fuc(0)NeuAc(2)	11	0.437	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/21 57.7829	N	90	HexNAc(6)Hex(4)Fuc(0)NeuAc(1)	12	0.418	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/22 78.8092	N	90	HexNAc(5)Hex(6)Fuc(0)NeuAc(1)	13	0.136	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/25 69.9046	N	90	HexNAc(5)Hex(6)Fuc(0)NeuAc(2)	14	0.122	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/29 76.0634	N	90	HexNAc(7)Hex(6)Fuc(0)NeuAc(2)	15	0.0988	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/27 31.9575	N	90	HexNAc(5)Hex(7)Fuc(0)NeuAc(2)	17	0.0226	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/15 48.5448	N	90	HexNAc(3)Hex(4)Fuc(0)NeuAc(1)	19	0.0133	Sialylate d
EQSTLAQM YPLQEIQN LTVK	Deamidated /0.9840; NGlycan/24 40.8620	Q,N	89,90	HexNAc(5)Hex(7)Fuc(0)NeuAc(1)	20	0.0193	Sialylate d
EQSTLAQM YPLQEIQN LTVK	Deamidated /0.9840; NGlycan/24 40.8620	Q,N	81,90	HexNAc(5)Hex(7)Fuc(0)NeuAc(1)	20	0.0181	Sialylate d

EQSTLAQM YPLQEIQN LTVK	NGlycan/29 35.0368	N	90	HexNAc(6)Hex(7)Fuc(0)NeuAc(2)	21	0.0184	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/28 61.0001	N	90	HexNAc(5)Hex(6)Fuc(0)NeuAc(3)	22	0.0142	Sialylate d
EQSTLAQM YPLQEIQN LTVK	NGlycan/14 44.5339	N	90	HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	1	0.112	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/16 06.5867	N	90	HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	2	0.428	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/17 68.6395	N	90	HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	3	0.251	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/20 76.7502	N	90	HexNAc(4)Hex(6)Fuc(2)NeuAc(0)	4	0.297	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro-Glu/-18.0106; NGlycan/21 33.7717	E,N	75,90	HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	5	1.13	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/21 33.7717	N	90	HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	5	0.0772	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/19 14.6974	N	90	HexNAc(4)Hex(5)Fuc(2)NeuAc(0)	6	0.315	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro-Glu/-18.0106; NGlycan/26 44.9618	E,N	75,90	HexNAc(6)Hex(7)Fuc(2)NeuAc(0)	7	0.365	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/18 09.6661	N	90	HexNAc(5)Hex(4)Fuc(1)NeuAc(0)	9	0.224	Fucosyl ated

EQSTLAQM YPLQEIQN LTVK	NGlycan/17 52.6446	N	90	HexNAc(4)Hex(4)Fuc(2)NeuAc(0)	10	0.0827	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/22 79.8296	E,N	75,90	HexNAc(5)Hex(6)Fuc(2)NeuAc(0)	11	0.334	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/22 79.8296	N	90	HexNAc(5)Hex(6)Fuc(2)NeuAc(0)	11	0.101	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/19 71.7189	N	90	HexNAc(5)Hex(5)Fuc(1)NeuAc(0)	13	0.136	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/19 71.7189	E,N	75,90	HexNAc(5)Hex(5)Fuc(1)NeuAc(0)	13	0.0362	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/24 41.8824	N	90	HexNAc(5)Hex(7)Fuc(2)NeuAc(0)	14	0.0567	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/21 17.7768	E,N	75,90	HexNAc(5)Hex(5)Fuc(2)NeuAc(0)	15	0.138	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/21 17.7768	N	90	HexNAc(5)Hex(5)Fuc(2)NeuAc(0)	15	0.0945	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	Deamidated /0.9840; NGlycan/21 17.7768	Q,N	86,90	HexNAc(5)Hex(5)Fuc(2)NeuAc(0)	15	0.0191	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/19 30.6923	N	90	HexNAc(4)Hex(6)Fuc(1)NeuAc(0)	16	0.135	Fucosyl ated

EQSTLAQM YPLQEIQN LTVK	Glu->pyro-Glu/-18.0106; NGlycan/1930.6923	E,N	75,90	HexNAc(4)Hex(6)Fuc(1)NeuAc(0)	16	0.0975	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/2092.7452	N	90	HexNAc(4)Hex(7)Fuc(1)NeuAc(0)	24	0.0493	Fucosyl ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/1622.5816	N	90	HexNAc(4)Hex(5)Fuc(0)NeuAc(0)	2	0.455	Undecor ated
EQSTLAQM YPLQEIQN LTVK	Deamidated /0.9840; NGlycan/1622.5816	Q,N	86,90	HexNAc(4)Hex(5)Fuc(0)NeuAc(0)	2	0.181	Undecor ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/1663.6082	N	90	HexNAc(5)Hex(4)Fuc(0)NeuAc(0)	4	1.22	Undecor ated
EQSTLAQM YPLQEIQN LTVK	Deamidated /0.9840; NGlycan/1663.6082	Q,N	89,90	HexNAc(5)Hex(4)Fuc(0)NeuAc(0)	4	0.015	Undecor ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/1825.6610	N	90	HexNAc(5)Hex(5)Fuc(0)NeuAc(0)	5	0.659	Undecor ated
EQSTLAQM YPLQEIQN LTVK	Deamidated /0.9840; NGlycan/1825.6610	Q,N	86,90	HexNAc(5)Hex(5)Fuc(0)NeuAc(0)	5	0.0298	Undecor ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/1501.5553	N	90	HexNAc(5)Hex(3)Fuc(0)NeuAc(0)	6	0.433	Undecor ated
EQSTLAQM YPLQEIQN LTVK	Deamidated /0.9840; NGlycan/1501.5553	Q,N	89,90	HexNAc(5)Hex(3)Fuc(0)NeuAc(0)	6	0.00379	Undecor ated

EQSTLAQM YPLQEIQN LTVK	NGlycan/14 60.5288	N	90	HexNAc(4)Hex(4)Fuc(0)NeuAc(0)	7	0.351	Undecor ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/18 66.6875	N	90	HexNAc(6)Hex(4)Fuc(0)NeuAc(0)	9	0.195	Undecor ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/20 28.7404	N	90	HexNAc(6)Hex(5)Fuc(0)NeuAc(0)	11	0.133	Undecor ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/21 90.7932	N	90	HexNAc(6)Hex(6)Fuc(0)NeuAc(0)	12	0.0716	Undecor ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/19 87.7138	N	90	HexNAc(5)Hex(6)Fuc(0)NeuAc(0)	14	0.0451	Undecor ated
EQSTLAQM YPLQEIQN LTVK	NGlycan/12 98.4760	N	90	HexNAc(4)Hex(3)Fuc(0)NeuAc(0)	16	0.0407	Undecor ated
EQSTLAQM YPLQEIQN LTVK	Glu->pyro- Glu/- 18.0106; NGlycan/17 02.5813	E,N	75,90	HexNAc(2)Hex(8)Fuc(0)NeuAc(0)	1	0.114	High Mannos e
EQSTLAQM YPLQEIQN LTVKLQLQ ALQQNGSS VLSEDK	Glu->pyro- Glu/- 18.0106; NGlycan/25 69.9046	E,N	75,90	HexNAc(5)Hex(6)Fuc(0)NeuAc(2)	14	0.0386	Sialylate d
LQLQALQQ NGSSVLSE DK	NGlycan/20 59.7349	N	103	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	1	5.19	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/20 59.7349	Q,N	101,10 3	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	1	0.0322	Sialyfuc osylated

LQLQALQQ NGSSVLSE DK	NGlycan/23 50.8304	N	103	HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	2	3.91	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	NGlycan/18 97.6821	N	103	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	3	1.79	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/18 97.6821	Q,N	98,103	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	3	0.0119	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/18 97.6821	Q,N	101,10 3	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	3	0.0118	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/18 97.6821	Q,N	102,10 3	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	3	0.0075 8	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	NGlycan/23 67.8457	N	103	HexNAc(4)Hex(6)Fuc(2)NeuAc(1)	4	1.47	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/23 67.8457	Q,N	101,10 3	HexNAc(4)Hex(6)Fuc(2)NeuAc(1)	4	0.0161	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	NGlycan/22 05.7928	N	103	HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	5	0.595	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	NGlycan/24 24.8671	N	103	HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	7	0.0542	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	NGlycan/27 15.9625	N	103	HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	8	0.0695	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	NGlycan/22 62.8143	N	103	HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	9	0.0782	Sialyfuc osylated

LQLQALQQ NGSSVLSE DK	NGlycan/30 07.0580	N	103	HexNAc(5)Hex(6)Fuc(1)NeuAc(3)	13	0.0252	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/22 21.7878	Q,N	101,10 3	HexNAc(4)Hex(6)Fuc(1)NeuAc(1)	15	0.153	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	NGlycan/16 94.6027	N	103	HexNAc(3)Hex(4)Fuc(1)NeuAc(1)	18	0.0109	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	NGlycan/27 16.9829	N	103	HexNAc(5)Hex(6)Fuc(3)NeuAc(1)	22	0.0145	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	NGlycan/23 91.8569	N	103	HexNAc(5)Hex(4)Fuc(1)NeuAc(2)	30	0.0604	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	NGlycan/24 08.8722	N	103	HexNAc(5)Hex(5)Fuc(2)NeuAc(1)	42	0.0177	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/24 08.8722	Q,N	101,10 3	HexNAc(5)Hex(5)Fuc(2)NeuAc(1)	42	0.0098 4	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	NGlycan/25 86.9200	N	103	HexNAc(5)Hex(7)Fuc(1)NeuAc(1)	45	0.0061 9	Sialyfuc osylated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/20 75.7298	Q,N	98,103	HexNAc(4)Hex(6)Fuc(0)NeuAc(1)	1	2.24	Sialylate d
LQLQALQQ NGSSVLSE DK	NGlycan/19 54.7036	N	103	HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	2	0.0519	Sialylate d
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/21 16.7564	Q,N	101,10 3	HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	3	0.0274	Sialylate d

LQLQALQQ NGSSVLSE DK	NGlycan/21 16.7564	N	103	HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	3	0.174	Sialylate d
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/19 13.6770	Q,N	98,103	HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	4	0.44	Sialylate d
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/19 13.6770	Q,N	102,10 3	HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	4	0.429	Sialylate d
LQLQALQQ NGSSVLSE DK	NGlycan/19 13.6770	N	103	HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	4	0.0206	Sialylate d
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/19 13.6770	Q,N	101,10 3	HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	4	0.0057	Sialylate d
LQLQALQQ NGSSVLSE DK	NGlycan/24 07.8518	N	103	HexNAc(5)Hex(5)Fuc(0)NeuAc(2)	5	0.0204	Sialylate d
LQLQALQQ NGSSVLSE DK	NGlycan/17 51.6242	N	103	HexNAc(4)Hex(4)Fuc(0)NeuAc(1)	9	0.317	Sialylate d
LQLQALQQ NGSSVLSE DK	NGlycan/22 04.7724	N	103	HexNAc(4)Hex(5)Fuc(0)NeuAc(2)	10	0.469	Sialylate d
LQLQALQQ NGSSVLSE DK	NGlycan/22 78.8092	N	103	HexNAc(5)Hex(6)Fuc(0)NeuAc(1)	13	0.0777	Sialylate d
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/22 78.8092	Q,N	98,103	HexNAc(5)Hex(6)Fuc(0)NeuAc(1)	13	0.0382	Sialylate d
LQLQALQQ NGSSVLSE DK	NGlycan/23 66.8253	N	103	HexNAc(4)Hex(6)Fuc(0)NeuAc(2)	16	0.012	Sialylate d

LQLQALQQ NGSSVLSE DK	NGlycan/14 44.5339	N	103	HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	1	5.02	Fucosyl ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/14 44.5339	Q,N	101,10 3	HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	1	0.0661	Fucosyl ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/14 44.5339	Q,N	98,103	HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	1	0.0119	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/16 06.5867	N	103	HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	2	3.26	Fucosyl ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/16 06.5867	Q,N	101,10 3	HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	2	0.0406	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/17 68.6395	N	103	HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	3	2.65	Fucosyl ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/17 68.6395	Q,N	101,10 3	HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	3	1.14	Fucosyl ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/17 68.6395	Q,N	96,103	HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	3	0.0296	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/20 76.7502	N	103	HexNAc(4)Hex(6)Fuc(2)NeuAc(0)	4	2.04	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/21 33.7717	N	103	HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	5	0.0536	Fucosyl ated

LQLQALQQ NGSSVLSE DK	NGlycan/19 14.6974	N	103	HexNAc(4)Hex(5)Fuc(2)NeuAc(0)	6	0.497	Fucosyl ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/19 14.6974	Q,N	101,10 3	HexNAc(4)Hex(5)Fuc(2)NeuAc(0)	6	0.0298	Fucosyl ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/19 14.6974	Q,N	98,103	HexNAc(4)Hex(5)Fuc(2)NeuAc(0)	6	0.0209	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/22 22.8082	N	103	HexNAc(4)Hex(6)Fuc(3)NeuAc(0)	8	0.348	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/18 09.6661	N	103	HexNAc(5)Hex(4)Fuc(1)NeuAc(0)	9	0.187	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/17 52.6446	N	103	HexNAc(4)Hex(4)Fuc(2)NeuAc(0)	10	0.334	Fucosyl ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/17 52.6446	Q,N	102,10 3	HexNAc(4)Hex(4)Fuc(2)NeuAc(0)	10	0.141	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/22 79.8296	N	103	HexNAc(5)Hex(6)Fuc(2)NeuAc(0)	11	0.0819	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/16 47.6132	N	103	HexNAc(5)Hex(3)Fuc(1)NeuAc(0)	12	0.212	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/19 71.7189	N	103	HexNAc(5)Hex(5)Fuc(1)NeuAc(0)	13	0.121	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/21 17.7768	N	103	HexNAc(5)Hex(5)Fuc(2)NeuAc(0)	15	0.0524	Fucosyl ated

LQLQALQQ NGSSVLSE DK	NGlycan/12 41.4545	N	103	HexNAc(3)Hex(3)Fuc(1)NeuAc(0)	17	0.0676	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/20 60.7553	N	103	HexNAc(4)Hex(5)Fuc(3)NeuAc(0)	18	0.114	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/22 63.8347	N	103	HexNAc(5)Hex(5)Fuc(3)NeuAc(0)	20	0.0615	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/14 03.5073	N	103	HexNAc(3)Hex(4)Fuc(1)NeuAc(0)	25	0.0489	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/24 25.8875	N	103	HexNAc(5)Hex(6)Fuc(3)NeuAc(0)	26	0.0459	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/19 55.7240	N	103	HexNAc(5)Hex(4)Fuc(2)NeuAc(0)	31	0.0246	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/23 45.8878	N	103	HexNAc(7)Hex(3)Fuc(3)NeuAc(0)	37	0.0007 33	Fucosyl ated
LQLQALQQ NGSSVLSE DK	NGlycan/14 60.5288	N	103	HexNAc(4)Hex(4)Fuc(0)NeuAc(0)	1	4.24	Undecor ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/14 60.5288	Q,N	101,10 3	HexNAc(4)Hex(4)Fuc(0)NeuAc(0)	1	2.61	Undecor ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/14 60.5288	Q,N	98,103	HexNAc(4)Hex(4)Fuc(0)NeuAc(0)	1	2.61	Undecor ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/14 60.5288	Q,N	102,10 3	HexNAc(4)Hex(4)Fuc(0)NeuAc(0)	1	2.55	Undecor ated

LQLQALQQ NGSSVLSE DK	NGlycan/16 22.5816	N	103	HexNAc(4)Hex(5)Fuc(0)NeuAc(0)	2	3.29	Undecor ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/16 22.5816	Q,N	98,103	HexNAc(4)Hex(5)Fuc(0)NeuAc(0)	2	1.93	Undecor ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/16 22.5816	Q,N	101,10 3	HexNAc(4)Hex(5)Fuc(0)NeuAc(0)	2	1.88	Undecor ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/17 84.6344	Q,N	101,10 3	HexNAc(4)Hex(6)Fuc(0)NeuAc(0)	3	1.75	Undecor ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/16 63.6082	Q,N	102,10 3	HexNAc(5)Hex(4)Fuc(0)NeuAc(0)	4	0.142	Undecor ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/16 63.6082	Q,N	98,103	HexNAc(5)Hex(4)Fuc(0)NeuAc(0)	4	0.142	Undecor ated
LQLQALQQ NGSSVLSE DK	NGlycan/16 63.6082	N	103	HexNAc(5)Hex(4)Fuc(0)NeuAc(0)	4	0.094	Undecor ated
LQLQALQQ NGSSVLSE DK	NGlycan/18 25.6610	N	103	HexNAc(5)Hex(5)Fuc(0)NeuAc(0)	5	0.319	Undecor ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/18 25.6610	Q,N	101,10 3	HexNAc(5)Hex(5)Fuc(0)NeuAc(0)	5	0.113	Undecor ated
LQLQALQQ NGSSVLSE DK	Deamidated /0.9840; NGlycan/18 25.6610	Q,N	102,10 3	HexNAc(5)Hex(5)Fuc(0)NeuAc(0)	5	0.113	Undecor ated

LQLQALQQNGSSVLSEDK	NGlycan/1501.5553	N	103	HexNAc(5)Hex(3)Fuc(0)NeuAc(0)	10	0.135	Undecorated
LQLQALQQNGSSVLSEDK	Deamidated/0.9840; NGlycan/1257.4494	Q,N	98,103	HexNAc(3)Hex(4)Fuc(0)NeuAc(0)	15	0.0438	Undecorated
LQLQALQQNGSSVLSEDK	NGlycan/1298.4760	N	103	HexNAc(4)Hex(3)Fuc(0)NeuAc(0)	17	0.036	Undecorated
LQLQALQQNGSSVLSEDK	Deamidated/0.9840; NGlycan/1298.4760	Q,N	101,103	HexNAc(4)Hex(3)Fuc(0)NeuAc(0)	17	0.0216	Undecorated
LQLQALQQNGSSVLSEDK	NGlycan/2188.7398	N	103	HexNAc(2)Hex(11)Fuc(0)NeuAc(0)	3	0.00855	High Mannose
LQLQALQQNGSSVLSEDK	Deamidated/0.9840; NGlycan/1216.4229	Q,N	101,103	HexNAc(2)Hex(5)Fuc(0)NeuAc(0)	2	0.00151	High Mannose
LQLQALQQNGSSVLSEDK	Deamidated/0.9840; NGlycan/1216.4229	Q,N	102,103	HexNAc(2)Hex(5)Fuc(0)NeuAc(0)	2	0.00151	High Mannose
LQLQALQQNGSSVLSEDKSK	NGlycan/2059.7349	N	103	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	1	0.175	Sialyfucosylated
LQLQALQQNGSSVLSEDKSK	NGlycan/2350.8304	N	103	HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	2	0.143	Sialyfucosylated
LQLQALQQNGSSVLSEDKSK	NGlycan/1897.6821	N	103	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	3	0.0589	Sialyfucosylated
LQLQALQQNGSSVLSEDKSK	NGlycan/2205.7928	N	103	HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	5	0.0213	Sialyfucosylated

LQLQALQQ NGSSVLSE DKSK	NGlycan/14 44.5339	N	103	HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	1	0.21	Fucosyl ated
LQLQALQQ NGSSVLSE DKSK	Deamidated /0.9840; NGlycan/14 44.5339	Q,N	102,10 3	HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	1	0.0048 9	Fucosyl ated
LQLQALQQ NGSSVLSE DKSK	NGlycan/16 06.5867	N	103	HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	2	0.153	Fucosyl ated
LQLQALQQ NGSSVLSE DKSK	NGlycan/17 68.6395	N	103	HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	3	0.113	Fucosyl ated
LQLQALQQ NGSSVLSE DKSK	NGlycan/19 14.6974	N	103	HexNAc(4)Hex(5)Fuc(2)NeuAc(0)	6	0.0273	Fucosyl ated
LQLQALQQ NGSSVLSE DKSK	NGlycan/17 52.6446	N	103	HexNAc(4)Hex(4)Fuc(2)NeuAc(0)	10	0.0213	Fucosyl ated
FFVSVGLP NMTQGFW ENSMLTDP GNVQK	NGlycan/31 95.1628	N	322	HexNAc(6)Hex(5)Fuc(4)NeuAc(2)	19	0.0995	Sialyfuc osylated
FFVSVGLP NMTQGFW ENSMLTDP GNVQK	NGlycan/25 38.9352	N	322	HexNAc(5)Hex(4)Fuc(4)NeuAc(1)	20	0.0957	Sialyfuc osylated
FFVSVGLP NMTQGFW ENSMLTDP GNVQK	NGlycan/28 31.0259	N	322	HexNAc(7)Hex(6)Fuc(1)NeuAc(1)	21	0.0878	Sialyfuc osylated
FFVSVGLP NMTQGFW ENSMLTDP GNVQK	NGlycan/24 98.9039; Deamidated /0.9840	N,Q	322,32 5	HexNAc(6)Hex(7)Fuc(1)NeuAc(0)	21	0.0039 7	Fucosyl ated

FFVSVGLP NMTQGFW ENSMLTDP GNVQK	NGlycan/27 42.0146	N	322	HexNAc(6)Hex(4)Fuc(4)NeuAc(1)	23	0.0757	Sialyfuc osylated
FFVSVGLP NMTQGFW ENSMLTDP GNVQK	NGlycan/20 85.7870	N	322	HexNAc(5)Hex(3)Fuc(4)NeuAc(0)	28	0.0423	Fucosyl ated
FFVSVGLP NMTQGFW ENSMLTDP GNVQK	NGlycan/22 88.8663	N	322	HexNAc(6)Hex(3)Fuc(4)NeuAc(0)	29	0.0334	Fucosyl ated
FFVSVGLP NMTQGFW ENSMLTDP GNVQK	NGlycan/24 50.9192	N	322	HexNAc(6)Hex(4)Fuc(4)NeuAc(0)	30	0.0261	Fucosyl ated
FFVSVGLP NMTQGFW ENSMLTDP GNVQK	NGlycan/29 04.0674	N	322	HexNAc(6)Hex(5)Fuc(4)NeuAc(1)	31	0.0576	Sialyfuc osylated
FFVSVGLP NMTQGFW ENSMLTDP GNVQK	NGlycan/26 12.9720	N	322	HexNAc(6)Hex(5)Fuc(4)NeuAc(0)	34	0.0181	Fucosyl ated
FFVSVGLP NMTQGFW ENSMLTDP GNVQK	NGlycan/22 46.8194	N	322	HexNAc(5)Hex(4)Fuc(2)NeuAc(1)	39	0.0279	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/20 59.7349	N	432	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	1	0.253	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/23 50.8304	N	432	HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	2	0.223	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/18 97.6821	N	432	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	3	0.0157	Sialyfuc osylated

SIGLLSPDF QEDNETEI NFLLK	NGlycan/23 67.8457	N	432	HexNAc(4)Hex(6)Fuc(2)NeuAc(1)	4	0.0248	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/22 05.7928	N	432	HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	5	0.0243	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/27 89.9993	N	432	HexNAc(6)Hex(7)Fuc(1)NeuAc(1)	6	0.141	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/24 24.8671	N	432	HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	7	0.287	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/27 15.9625	N	432	HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	8	0.282	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/22 62.8143	N	432	HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	9	0.193	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/21 00.7615	N	432	HexNAc(5)Hex(4)Fuc(1)NeuAc(1)	10	0.0708	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/30 81.0947	N	432	HexNAc(6)Hex(7)Fuc(1)NeuAc(2)	11	0.206	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/27 32.9779	N	432	HexNAc(5)Hex(7)Fuc(2)NeuAc(1)	12	0.189	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/30 07.0580	N	432	HexNAc(5)Hex(6)Fuc(1)NeuAc(3)	13	0.181	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/25 53.9097	N	432	HexNAc(5)Hex(5)Fuc(1)NeuAc(2)	14	0.062	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/30 24.0733	N	432	HexNAc(5)Hex(7)Fuc(2)NeuAc(2)	16	0.139	Sialyfuc osylated

SIGLLSPDF QEDNETEI NFLLK	NGlycan/26 27.9465	N	432	HexNAc(6)Hex(6)Fuc(1)NeuAc(1)	26	0.0683	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/33 72.1902	N	432	HexNAc(6)Hex(7)Fuc(1)NeuAc(3)	27	0.0659	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/29 19.0419	N	432	HexNAc(6)Hex(6)Fuc(1)NeuAc(2)	28	0.0651	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/29 36.0572	N	432	HexNAc(6)Hex(7)Fuc(2)NeuAc(1)	32	0.049	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/25 70.9250	N	432	HexNAc(5)Hex(6)Fuc(2)NeuAc(1)	33	0.0436	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/32 27.1527	N	432	HexNAc(6)Hex(7)Fuc(2)NeuAc(2)	35	0.0339	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/28 62.0205	N	432	HexNAc(5)Hex(6)Fuc(2)NeuAc(2)	40	0.0234	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/32 10.1373	N	432	HexNAc(6)Hex(6)Fuc(1)NeuAc(3)	41	0.021	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/28 79.0358	N	432	HexNAc(5)Hex(7)Fuc(3)NeuAc(1)	43	0.0164	Sialyfuc osylated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/20 75.7298; Deamidated /0.9840	N,N	432,43 7	HexNAc(4)Hex(6)Fuc(0)NeuAc(1)	1	0.0065 7	Sialylate d
SIGLLSPDF QEDNETEI NFLLK	Deamidated /0.9840; NGlycan/21 16.7564	Q,N	429,43 2	HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	3	0.0045 8	Sialylate d

SIGLLSPDF QEDNETEI NFLLK	NGlycan/22 78.8092; Deamidated /0.9840	N,N	432,43 7	HexNAc(5)Hex(6)Fuc(0)NeuAc(1)	13	0.0152	Sialylate d
SIGLLSPDF QEDNETEI NFLLK	NGlycan/23 66.8253; Deamidated /0.9840	N,N	432,43 7	HexNAc(4)Hex(6)Fuc(0)NeuAc(2)	16	0.0239	Sialylate d
SIGLLSPDF QEDNETEI NFLLK	NGlycan/26 43.9414	N	432	HexNAc(6)Hex(7)Fuc(0)NeuAc(1)	18	0.0218	Sialylate d
SIGLLSPDF QEDNETEI NFLLK	Deamidated /0.9840; NGlycan/24 40.8620	Q,N	429,43 2	HexNAc(5)Hex(7)Fuc(0)NeuAc(1)	20	0.0046 2	Sialylate d
SIGLLSPDF QEDNETEI NFLLK	NGlycan/16 06.5867	N	432	HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	2	0.0226	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/17 68.6395	N	432	HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	3	0.0729	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/20 76.7502	N	432	HexNAc(4)Hex(6)Fuc(2)NeuAc(0)	4	0.113	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/21 33.7717	N	432	HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	5	0.164	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	Deamidated /0.9840; NGlycan/21 33.7717	Q,N	429,43 2	HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	5	0.0263	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/18 09.6661	N	432	HexNAc(5)Hex(4)Fuc(1)NeuAc(0)	9	0.0374	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/22 79.8296	N	432	HexNAc(5)Hex(6)Fuc(2)NeuAc(0)	11	0.0759	Fucosyl ated

SIGLLSPDF QEDNETEI NFLLK	NGlycan/19 71.7189	N	432	HexNAc(5)Hex(5)Fuc(1)NeuAc(0)	13	0.0896	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/24 41.8824	N	432	HexNAc(5)Hex(7)Fuc(2)NeuAc(0)	14	0.173	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/21 17.7768	N	432	HexNAc(5)Hex(5)Fuc(2)NeuAc(0)	15	0.0209	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/24 98.9039	N	432	HexNAc(6)Hex(7)Fuc(1)NeuAc(0)	21	0.0564	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/23 36.8511	N	432	HexNAc(6)Hex(6)Fuc(1)NeuAc(0)	22	0.0541	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/21 74.7983	N	432	HexNAc(6)Hex(5)Fuc(1)NeuAc(0)	32	0.0231	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/22 95.8245	N	432	HexNAc(5)Hex(7)Fuc(1)NeuAc(0)	33	0.0235	Fucosyl ated
SIGLLSPDF QEDNETEI NFLLK	Deamidated /0.9840; NGlycan/21 49.7666	Q,N	429,43 2	HexNAc(5)Hex(7)Fuc(0)NeuAc(0)	18	0.0203	Undecor ated
SIGLLSPDF QEDNETEI NFLLK	NGlycan/17 84.6344; Deamidated /0.9840	N,N	432,43 7	HexNAc(4)Hex(6)Fuc(0)NeuAc(0)	19	0.0164	Undecor ated
SIGLLSPDF QEDNETEI NFLLK	Deamidated /0.9840; NGlycan/18 25.6610	Q,N	429,43 2	HexNAc(5)Hex(5)Fuc(0)NeuAc(0)	20	0.0116	Undecor ated
SIGLLSPDF QEDNETEI NFLLK	Deamidated /0.9840; NGlycan/23 52.8460	Q,N	429,43 2	HexNAc(6)Hex(7)Fuc(0)NeuAc(0)	21	0.0039 6	Undecor ated

CDISNSTE AGQK	NGlycan/20 59.7349	N	546	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	1	0.463	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/23 50.8304	N	546	HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	2	0.126	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/18 97.6821	N	546	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	3	0.691	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/27 89.9993	N	546	HexNAc(6)Hex(7)Fuc(1)NeuAc(1)	6	0.0137	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/24 24.8671	N	546	HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	7	0.0957	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/27 15.9625	N	546	HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	8	0.0422	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/22 62.8143	N	546	HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	9	0.17	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/21 00.7615	N	546	HexNAc(5)Hex(4)Fuc(1)NeuAc(1)	10	0.208	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/25 53.9097	N	546	HexNAc(5)Hex(5)Fuc(1)NeuAc(2)	14	0.0278	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/16 94.6027	N	546	HexNAc(3)Hex(4)Fuc(1)NeuAc(1)	18	0.112	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/24 65.8937	N	546	HexNAc(6)Hex(5)Fuc(1)NeuAc(1)	24	0.0382	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/26 27.9465	N	546	HexNAc(6)Hex(6)Fuc(1)NeuAc(1)	26	0.0266	Sialyfuc osylated

CDISNSTE AGQK	NGlycan/18 56.6556	N	546	HexNAc(3)Hex(5)Fuc(1)NeuAc(1)	29	0.0629	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/23 03.8409	N	546	HexNAc(6)Hex(4)Fuc(1)NeuAc(1)	34	0.0426	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/23 51.8508	N	546	HexNAc(4)Hex(5)Fuc(3)NeuAc(1)	38	0.0296	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/27 56.9891	N	546	HexNAc(6)Hex(5)Fuc(1)NeuAc(2)	44	0.0135	Sialyfuc osylated
CDISNSTE AGQK	NGlycan/19 54.7036	N	546	HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	2	0.289	Sialylate d
CDISNSTE AGQK	NGlycan/24 07.8518	N	546	HexNAc(5)Hex(5)Fuc(0)NeuAc(2)	5	0.144	Sialylate d
CDISNSTE AGQK	NGlycan/23 19.8358	N	546	HexNAc(6)Hex(5)Fuc(0)NeuAc(1)	6	0.0090 9	Sialylate d
CDISNSTE AGQK	NGlycan/27 72.9840	N	546	HexNAc(6)Hex(6)Fuc(0)NeuAc(2)	7	0.0157	Sialylate d
CDISNSTE AGQK	NGlycan/17 51.6242	N	546	HexNAc(4)Hex(4)Fuc(0)NeuAc(1)	9	0.0345	Sialylate d
CDISNSTE AGQK	NGlycan/26 10.9312	N	546	HexNAc(6)Hex(5)Fuc(0)NeuAc(2)	11	0.0285	Sialylate d
CDISNSTE AGQK	NGlycan/15 48.5448	N	546	HexNAc(3)Hex(4)Fuc(0)NeuAc(1)	19	0.0206	Sialylate d
CDISNSTE AGQK	NGlycan/14 44.5339	N	546	HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	1	0.418	Fucosyl ated

CDISNSTE AGQK	NGlycan/16 06.5867	N	546	HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	2	1.06	Fucosyl ated
CDISNSTE AGQK	NGlycan/17 68.6395	N	546	HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	3	0.251	Fucosyl ated
CDISNSTE AGQK	NGlycan/21 33.7717	N	546	HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	5	0.0436	Fucosyl ated
CDISNSTE AGQK	NGlycan/18 09.6661	N	546	HexNAc(5)Hex(4)Fuc(1)NeuAc(0)	9	0.343	Fucosyl ated
CDISNSTE AGQK	NGlycan/17 52.6446	N	546	HexNAc(4)Hex(4)Fuc(2)NeuAc(0)	10	0.0208	Fucosyl ated
CDISNSTE AGQK	NGlycan/22 79.8296	N	546	HexNAc(5)Hex(6)Fuc(2)NeuAc(0)	11	0.0738	Fucosyl ated
CDISNSTE AGQK	NGlycan/16 47.6132	N	546	HexNAc(5)Hex(3)Fuc(1)NeuAc(0)	12	0.141	Fucosyl ated
CDISNSTE AGQK	NGlycan/19 71.7189	N	546	HexNAc(5)Hex(5)Fuc(1)NeuAc(0)	13	0.182	Fucosyl ated
CDISNSTE AGQK	NGlycan/12 41.4545	N	546	HexNAc(3)Hex(3)Fuc(1)NeuAc(0)	17	0.129	Fucosyl ated
CDISNSTE AGQK	NGlycan/15 65.5601	N	546	HexNAc(3)Hex(5)Fuc(1)NeuAc(0)	19	0.0644	Fucosyl ated
CDISNSTE AGQK	NGlycan/18 50.6926	N	546	HexNAc(6)Hex(3)Fuc(1)NeuAc(0)	23	0.0496	Fucosyl ated
CDISNSTE AGQK	NGlycan/14 03.5073	N	546	HexNAc(3)Hex(4)Fuc(1)NeuAc(0)	25	0.0427	Fucosyl ated

CDISNSTE AGQK	NGlycan/20 12.7454	N	546	HexNAc(6)Hex(4)Fuc(1)NeuAc(0)	27	0.0454	Fucosyl ated
CDISNSTE AGQK	NGlycan/21 74.7983	N	546	HexNAc(6)Hex(5)Fuc(1)NeuAc(0)	32	0.024	Fucosyl ated
CDISNSTE AGQK	NGlycan/18 98.7025	N	546	HexNAc(4)Hex(4)Fuc(3)NeuAc(0)	35	0.0086 6	Fucosyl ated
CDISNSTE AGQK	NGlycan/17 27.6130	N	546	HexNAc(3)Hex(6)Fuc(1)NeuAc(0)	36	0.0072 1	Fucosyl ated
CDISNSTE AGQK	NGlycan/12 16.4229	N	546	HexNAc(2)Hex(5)Fuc(0)NeuAc(0)	2	0.0209	High Mannos e
NVSDIIPR	NGlycan/20 59.7349	N	690	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	1	1.75	Sialyfuc osylated
NVSDIIPR	NGlycan/23 50.8304	N	690	HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	2	0.52	Sialyfuc osylated
NVSDIIPR	NGlycan/18 97.6821	N	690	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	3	0.61	Sialyfuc osylated
NVSDIIPR	NGlycan/22 05.7928	N	690	HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	5	0.0253	Sialyfuc osylated
NVSDIIPR	NGlycan/24 24.8671	N	690	HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	7	0.202	Sialyfuc osylated
NVSDIIPR	NGlycan/27 15.9625	N	690	HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	8	0.179	Sialyfuc osylated
NVSDIIPR	NGlycan/22 62.8143	N	690	HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	9	0.0468	Sialyfuc osylated

NVSDIIPR	NGlycan/2100.7615	N	690	HexNAc(5)Hex(4)Fuc(1)NeuAc(1)	10	0.0335	Sialyfucosylated
NVSDIIPR	NGlycan/2732.9779	N	690	HexNAc(5)Hex(7)Fuc(2)NeuAc(1)	12	0.134	Sialyfucosylated
NVSDIIPR	NGlycan/3007.0580	N	690	HexNAc(5)Hex(6)Fuc(1)NeuAc(3)	13	0.0296	Sialyfucosylated
NVSDIIPR	NGlycan/3024.0733	N	690	HexNAc(5)Hex(7)Fuc(2)NeuAc(2)	16	0.0197	Sialyfucosylated
NVSDIIPR	NGlycan/2716.9829	N	690	HexNAc(5)Hex(6)Fuc(3)NeuAc(1)	22	0.0858	Sialyfucosylated
NVSDIIPR	NGlycan/2351.8508	N	690	HexNAc(4)Hex(5)Fuc(3)NeuAc(1)	38	0.00339	Sialyfucosylated
NVSDIIPR	NGlycan/2116.7564	N	690	HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	3	0.0204	Sialylated
NVSDIIPR	NGlycan/1913.6770	N	690	HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	4	0.756	Sialylated
NVSDIIPR	NGlycan/1751.6242	N	690	HexNAc(4)Hex(4)Fuc(0)NeuAc(1)	9	0.193	Sialylated
NVSDIIPR	NGlycan/2204.7724	N	690	HexNAc(4)Hex(5)Fuc(0)NeuAc(2)	10	0.101	Sialylated
NVSDIIPR	NGlycan/2278.8092	N	690	HexNAc(5)Hex(6)Fuc(0)NeuAc(1)	13	0.0671	Sialylated
NVSDIIPR	NGlycan/2569.9046	N	690	HexNAc(5)Hex(6)Fuc(0)NeuAc(2)	14	0.0422	Sialylated

NVSDIIPR	NGlycan/1444.5339	N	690	HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	1	0.0189	Fucosylated
NVSDIIPR	NGlycan/1606.5867	N	690	HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	2	0.322	Fucosylated
NVSDIIPR	NGlycan/1768.6395	N	690	HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	3	0.817	Fucosylated
NVSDIIPR	NGlycan/2133.7717	N	690	HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	5	0.113	Fucosylated
NVSDIIPR	NGlycan/1914.6974	N	690	HexNAc(4)Hex(5)Fuc(2)NeuAc(0)	6	0.0243	Fucosylated
NVSDIIPR	NGlycan/1809.6661	N	690	HexNAc(5)Hex(4)Fuc(1)NeuAc(0)	9	0.0703	Fucosylated
NVSDIIPR	NGlycan/1971.7189	N	690	HexNAc(5)Hex(5)Fuc(1)NeuAc(0)	13	0.0882	Fucosylated
NVSDIIPR	NGlycan/2441.8824	N	690	HexNAc(5)Hex(7)Fuc(2)NeuAc(0)	14	0.0876	Fucosylated
NVSDIIPR	NGlycan/1930.6923	N	690	HexNAc(4)Hex(6)Fuc(1)NeuAc(0)	16	0.0822	Fucosylated
NVSDIIPR	NGlycan/1622.5816	N	690	HexNAc(4)Hex(5)Fuc(0)NeuAc(0)	8	0.2	Undecorated
NVSDIIPR	NGlycan/1460.5288	N	690	HexNAc(4)Hex(4)Fuc(0)NeuAc(0)	13	0.0604	Undecorated

Table S4.2 Site-specific occupancy of recombinant ACE2 derived from HEK293 cells

Glycans	Peptide Sequence	Glyco site	Glycan Subtype
HexNAc(5)Hex(5)Fuc(3)NeuAc(0)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Fucosylated
HexNAc(5)Hex(4)Fuc(1)NeuAc(0)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Fucosylated
HexNAc(9)Hex(9)Fuc(1)NeuAc(0)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Fucosylated
HexNAc(6)Hex(5)Fuc(4)NeuAc(2)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(4)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(2)NeuAc(2)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(4)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Sialyfucosylated
HexNAc(7)Hex(7)Fuc(1)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(3)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Sialyfucosylated
HexNAc(7)Hex(7)Fuc(2)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Sialyfucosylated
HexNAc(7)Hex(8)Fuc(1)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Sialyfucosylated
HexNAc(5)Hex(7)Fuc(3)NeuAc(2)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Sialyfucosylated
HexNAc(7)Hex(8)Fuc(0)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Sialylated
HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEE NVQNMNNAGDK.W	53	Sialylated

HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(6)Hex(4)Fuc(0)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(4)Hex(7)Fuc(0)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(6)Hex(5)Fuc(0)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(7)Hex(4)Fuc(0)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(2)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(7)Hex(5)Fuc(0)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(6)Hex(5)Fuc(0)NeuAc(2)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(2)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(7)Hex(5)Fuc(0)NeuAc(2)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(7)Hex(7)Fuc(0)NeuAc(1)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(7)Hex(6)Fuc(0)NeuAc(2)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(3)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(7)Hex(7)Fuc(0)NeuAc(2)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated
HexNAc(7)Hex(7)Fuc(0)NeuAc(3)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Sialylated

HexNAc(5)Hex(3)Fuc(0)NeuAc(0)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Undecorated
HexNAc(5)Hex(4)Fuc(0)NeuAc(0)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Undecorated
HexNAc(6)Hex(3)Fuc(0)NeuAc(0)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Undecorated
HexNAc(6)Hex(4)Fuc(0)NeuAc(0)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Undecorated
HexNAc(7)Hex(3)Fuc(0)NeuAc(0)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Undecorated
HexNAc(4)Hex(7)Fuc(0)NeuAc(0)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Undecorated
HexNAc(7)Hex(4)Fuc(0)NeuAc(0)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	53	Undecorated
HexNAc(5)Hex(4)Fuc(3)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(5)Hex(3)Fuc(3)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(6)Hex(3)Fuc(3)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(6)Hex(4)Fuc(3)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(6)Hex(6)Fuc(3)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(4)Hex(5)Fuc(3)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(6)Hex(5)Fuc(3)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(5)Hex(5)Fuc(3)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(4)Hex(4)Fuc(2)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated

HexNAc(5)Hex(3)Fuc(2)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(5)Hex(4)Fuc(2)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(4)Hex(6)Fuc(2)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(7)Hex(7)Fuc(2)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(5)Hex(6)Fuc(2)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(6)Hex(6)Fuc(2)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(6)Hex(7)Fuc(2)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(6)Hex(3)Fuc(2)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(4)Hex(3)Fuc(2)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(6)Hex(4)Fuc(2)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(5)Hex(4)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(6)Hex(6)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(5)Hex(3)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated

HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(4)Hex(6)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(4)Hex(7)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(7)Hex(6)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(6)Hex(5)Fuc(1)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Fucosylated
HexNAc(2)Hex(8)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	High Mannose
HexNAc(2)Hex(9)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	High Mannose
HexNAc(6)Hex(4)Fuc(4)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(3)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosylated
HexNAc(6)Hex(5)Fuc(3)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosylated
HexNAc(6)Hex(4)Fuc(3)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosylated
HexNAc(7)Hex(6)Fuc(3)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(3)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(2)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosylated

HexNAc(4)Hex(6)Fuc(2)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(6)Hex(5)Fuc(2)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(6)Hex(4)Fuc(2)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(5)Hex(5)Fuc(2)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(5)Hex(7)Fuc(2)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(5)Hex(4)Fuc(1)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(5)Hex(6)Fuc(1)NeuAc(3)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(5)Hex(5)Fuc(1)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(5)Hex(4)Fuc(1)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated
HexNAc(6)Hex(5)Fuc(1)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosy lated

HexNAc(3)Hex(4)Fuc(1)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(0)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVKLQLQALQQNGSSVLSEDK.S	90	Sialylated
HexNAc(4)Hex(4)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(4)Hex(6)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(4)Hex(5)Fuc(0)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(5)Hex(7)Fuc(0)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(6)Hex(5)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(7)Hex(5)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(6)Hex(4)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(6)Hex(5)Fuc(0)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated

HexNAc(5)Hex(6)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(3)Hex(4)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(4)Hex(6)Fuc(0)NeuAc(2)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(4)Hex(7)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(7)Hex(4)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(6)Hex(7)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(3)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(6)Hex(3)Fuc(0)NeuAc(1)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Sialylated
HexNAc(4)Hex(4)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(4)Hex(5)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(4)Hex(3)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(5)Hex(3)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(4)Hex(6)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(5)Hex(4)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(5)Hex(5)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(6)Hex(3)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated

HexNAc(6)Hex(4)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(6)Hex(5)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(6)Hex(6)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(3)Hex(3)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(7)Hex(6)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(7)Hex(3)Fuc(0)NeuAc(0)	K.EQSTLAQMYPLQEIQNLTVK.L	90	Undecorated
HexNAc(7)Hex(4)Fuc(4)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(5)Hex(5)Fuc(4)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(5)Hex(3)Fuc(4)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(7)Hex(3)Fuc(4)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(4)Hex(4)Fuc(3)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(4)Hex(5)Fuc(3)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(4)Hex(3)Fuc(3)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(4)Hex(6)Fuc(3)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(6)Hex(5)Fuc(3)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(7)Hex(4)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated

HexNAc(4)Hex(4)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(4)Hex(5)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(5)Hex(4)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(3)Hex(4)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(5)Hex(5)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(4)Hex(6)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(5)Hex(6)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(6)Hex(6)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(5)Hex(7)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(3)Hex(5)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(6)Hex(7)Fuc(2)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(3)Hex(4)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(3)Hex(3)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(5)Hex(4)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated

HexNAc(5)Hex(3)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(6)Hex(5)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(3)Hex(6)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(6)Hex(4)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(4)Hex(6)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(7)Hex(4)Fuc(1)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Fucosylated
HexNAc(5)Hex(4)Fuc(3)NeuAc(0)	K.LQLQALQQNGSSVLSEDKSK.R	103	Fucosylated
HexNAc(2)Hex(11)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	High Mannose
HexNAc(2)Hex(5)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	High Mannose
HexNAc(2)Hex(7)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	High Mannose
HexNAc(2)Hex(10)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	High Mannose
HexNAc(2)Hex(9)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	High Mannose

HexNAc(2)Hex(8)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	High Mannose
HexNAc(4)Hex(4)Fuc(3)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(3)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(3)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(6)Fuc(3)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(3)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(7)Fuc(3)NeuAc(2)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(9)Hex(9)Fuc(2)NeuAc(4)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(6)Fuc(2)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(2)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(4)Fuc(2)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(2)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(2)NeuAc(2)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(2)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(6)Fuc(2)NeuAc(2)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated

HexNAc(6)Hex(4)Fuc(2)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(3)Hex(4)Fuc(1)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(1)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(6)Fuc(1)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(6)Fuc(1)NeuAc(2)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(3)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(2)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(7)Hex(4)Fuc(1)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(1)NeuAc(2)	K.LQLQALQQNGSSVLSEDK.S	103	Sialyfucosylated
HexNAc(4)Hex(4)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated

HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(7)Hex(4)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(4)Hex(5)Fuc(0)NeuAc(2)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(2)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(2)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(3)Hex(4)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(4)Hex(5)Fuc(0)NeuAc(3)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(5)Hex(6)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(6)Hex(4)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(6)Hex(5)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(4)Hex(6)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(6)Hex(3)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated
HexNAc(5)Hex(7)Fuc(0)NeuAc(1)	K.LQLQALQQNGSSVLSEDK.S	103	Sialylated

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HexNAc(5)Hex(3)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(4)Hex(3)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(4)Hex(4)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(4)Hex(5)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(5)Hex(4)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(6)Hex(7)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(5)Hex(5)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(7)Hex(8)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(6)Hex(3)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(6)Hex(4)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(6)Hex(5)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(4)Hex(6)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(3)Hex(4)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(3)Hex(5)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(6)Hex(6)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated
HexNAc(5)Hex(6)Fuc(0)NeuAc(0)	K.LQLQALQQNGSSVLSEDK.S	103	Undecorated

HexNAc(7)Hex(6)Fuc(5)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(5)Hex(3)Fuc(4)NeuAc(0)	K.EAEKFFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(5)Hex(4)Fuc(4)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(6)Hex(4)Fuc(4)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(6)Hex(6)Fuc(3)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(6)Hex(3)Fuc(3)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(7)Hex(3)Fuc(2)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(3)Hex(4)Fuc(2)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(5)Hex(3)Fuc(2)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(7)Hex(4)Fuc(2)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(4)Hex(4)Fuc(2)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(4)Hex(7)Fuc(1)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(4)Hex(6)Fuc(1)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(3)Hex(5)Fuc(1)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Fucosylated
HexNAc(2)Hex(6)Fuc(0)NeuAc(0)	K.EAEKFFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	High Mannose

HexNAc(2)Hex(11)Fuc(0)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	High Mannose
HexNAc(2)Hex(4)Fuc(0)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	High Mannose
HexNAc(7)Hex(5)Fuc(5)NeuAc(2)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(6)Hex(4)Fuc(5)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(6)Hex(4)Fuc(4)NeuAc(1)	K.EAEKFFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(4)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(6)Hex(5)Fuc(4)NeuAc(2)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(6)Hex(5)Fuc(4)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(7)Hex(5)Fuc(4)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(3)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(6)Hex(4)Fuc(3)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(9)Hex(9)Fuc(3)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(6)Hex(6)Fuc(3)NeuAc(2)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(7)Hex(5)Fuc(3)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(4)Hex(7)Fuc(3)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(6)Hex(4)Fuc(2)NeuAc(1)	K.EAEKFFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated

HexNAc(6)Hex(3)Fuc(2)NeuAc(1)	K.EAEKFFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(4)Hex(4)Fuc(2)NeuAc(1)	K.EAEKFFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(6)Hex(5)Fuc(2)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(2)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(8)Hex(8)Fuc(2)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(5)Hex(7)Fuc(2)NeuAc(2)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(7)Hex(5)Fuc(2)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(4)Hex(7)Fuc(2)NeuAc(2)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(3)Hex(4)Fuc(2)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(7)Hex(4)Fuc(2)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(3)Hex(5)Fuc(1)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(6)Hex(3)Fuc(1)NeuAc(2)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(7)Hex(5)Fuc(1)NeuAc(2)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(7)Hex(4)Fuc(1)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated
HexNAc(3)Hex(6)Fuc(1)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialyfucosylated

HexNAc(4)Hex(6)Fuc(0)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialylated
HexNAc(3)Hex(4)Fuc(0)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialylated
HexNAc(4)Hex(4)Fuc(0)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialylated
HexNAc(4)Hex(6)Fuc(0)NeuAc(2)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialylated
HexNAc(4)Hex(7)Fuc(0)NeuAc(2)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialylated
HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialylated
HexNAc(7)Hex(5)Fuc(0)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialylated
HexNAc(6)Hex(3)Fuc(0)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialylated
HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(2)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialylated
HexNAc(3)Hex(5)Fuc(0)NeuAc(1)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Sialylated
HexNAc(6)Hex(3)Fuc(0)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Undecorated
HexNAc(7)Hex(6)Fuc(0)NeuAc(0)	K.FFVSVGLPNMTQGFWENSMLTDPGNVQK.A	322	Undecorated
HexNAc(4)Hex(5)Fuc(3)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(5)Hex(4)Fuc(3)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(5)Hex(6)Fuc(3)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated

HexNAc(5)Hex(5)Fuc(3)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(5)Hex(6)Fuc(2)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(4)Hex(6)Fuc(2)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(5)Hex(5)Fuc(2)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(5)Hex(7)Fuc(2)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(6)Hex(5)Fuc(1)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(5)Hex(4)Fuc(1)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(6)Hex(6)Fuc(1)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(5)Hex(3)Fuc(1)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(6)Hex(4)Fuc(1)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated
HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Fucosylated

HexNAc(6)Hex(7)Fuc(4)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(3)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(4)Hex(4)Fuc(3)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(2)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(2)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(4)Hex(6)Fuc(2)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(2)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(6)Fuc(2)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(2)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(2)NeuAc(3)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(5)Hex(7)Fuc(2)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(5)Hex(7)Fuc(2)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(2)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated

HexNAc(6)Hex(6)Fuc(1)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(3)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(6)Fuc(1)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(3)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(4)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(1)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(1)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(5)Fuc(1)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(6)Fuc(1)NeuAc(3)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated

HexNAc(6)Hex(5)Fuc(1)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialyfucosylated
HexNAc(6)Hex(5)Fuc(0)NeuAc(2)	K.HLKSIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(7)Hex(6)Fuc(0)NeuAc(2)	K.HLKSIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(5)Hex(6)Fuc(0)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(4)Hex(4)Fuc(0)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(6)Hex(7)Fuc(0)NeuAc(3)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(7)Hex(7)Fuc(0)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(3)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(7)Hex(7)Fuc(0)NeuAc(3)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(5)Hex(6)Fuc(0)NeuAc(2)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated

HexNAc(6)Hex(7)Fuc(0)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(6)Hex(5)Fuc(0)NeuAc(1)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Sialylated
HexNAc(5)Hex(4)Fuc(0)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Undecorated
HexNAc(6)Hex(4)Fuc(0)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Undecorated
HexNAc(5)Hex(5)Fuc(0)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Undecorated
HexNAc(4)Hex(6)Fuc(0)NeuAc(0)	K.SIGLLSPDFQEDNETEINFLLK.Q	432	Undecorated
HexNAc(4)Hex(4)Fuc(3)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(5)Hex(5)Fuc(3)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(4)Hex(5)Fuc(3)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(5)Hex(6)Fuc(3)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(5)Hex(4)Fuc(3)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(4)Hex(4)Fuc(2)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(5)Hex(6)Fuc(2)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(5)Hex(4)Fuc(2)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(4)Hex(5)Fuc(2)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(3)Hex(4)Fuc(2)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated

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HexNAc(4)Hex(6)Fuc(2)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(5)Hex(3)Fuc(2)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(3)Hex(4)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(3)Hex(5)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(3)Hex(3)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(5)Hex(4)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(6)Hex(4)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(6)Hex(6)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(5)Hex(3)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(6)Hex(3)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated

HexNAc(6)Hex(5)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(3)Hex(6)Fuc(1)NeuAc(0)	K.CDISNSTEAGQK.L	546	Fucosylated
HexNAc(2)Hex(8)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	High Mannose
HexNAc(2)Hex(5)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	High Mannose
HexNAc(2)Hex(7)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	High Mannose
HexNAc(2)Hex(6)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	High Mannose
HexNAc(2)Hex(11)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	High Mannose
HexNAc(5)Hex(4)Fuc(4)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(6)Hex(4)Fuc(4)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(3)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(3)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(3)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(3)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(4)Hex(6)Fuc(2)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	K.CDISNSTEAGQK.L	546	Sialyfucosylated

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HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(3)Hex(4)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(6)Hex(5)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(6)Hex(4)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(3)Hex(5)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(6)Hex(6)Fuc(1)NeuAc(2)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(2)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(6)Hex(6)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(2)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(3)	K.CDISNSTEAGQK.L	546	Sialyfucosylated

HexNAc(5)Hex(6)Fuc(1)NeuAc(3)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(3)Hex(6)Fuc(1)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(6)Hex(6)Fuc(1)NeuAc(3)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(6)Hex(5)Fuc(1)NeuAc(2)	K.CDISNSTEAGQK.L	546	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(4)Hex(4)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(2)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(6)Hex(5)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(3)Hex(4)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(7)Hex(5)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(6)Hex(3)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(6)Hex(5)Fuc(0)NeuAc(2)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(6)Hex(4)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(2)	K.CDISNSTEAGQK.L	546	Sialylated

HexNAc(7)Hex(4)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(3)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(3)Hex(5)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(7)Hex(6)Fuc(0)NeuAc(1)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(7)Hex(7)Fuc(0)NeuAc(3)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(7)Hex(6)Fuc(0)NeuAc(2)	K.CDISNSTEAGQK.L	546	Sialylated
HexNAc(5)Hex(3)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(6)Hex(3)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(4)Hex(4)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(5)Hex(5)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(5)Hex(4)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(4)Hex(3)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(3)Hex(5)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(6)Hex(5)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(6)Hex(4)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated

HexNAc(3)Hex(4)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(3)Hex(6)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(4)Hex(5)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(6)Hex(6)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(7)Hex(5)Fuc(0)NeuAc(0)	K.CDISNSTEAGQK.L	546	Undecorated
HexNAc(7)Hex(5)Fuc(4)NeuAc(0)	K.FNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDK.W	690	Fucosylated
HexNAc(5)Hex(5)Fuc(4)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(4)Hex(5)Fuc(2)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(5)Hex(7)Fuc(2)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(3)Hex(4)Fuc(1)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(4)Hex(3)Fuc(1)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(4)Hex(4)Fuc(1)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(3)Hex(3)Fuc(1)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated

HexNAc(5)Hex(4)Fuc(1)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(5)Hex(7)Fuc(1)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(0)	K.NVSDIIPR.T	690	Fucosylated
HexNAc(5)Hex(6)Fuc(3)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(2)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(2)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(5)Hex(7)Fuc(2)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(2)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(4)Hex(4)Fuc(1)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(5)Hex(4)Fuc(1)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(5)Hex(5)Fuc(1)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(5)Hex(6)Fuc(1)NeuAc(3)	K.NVSDIIPR.T	690	Sialyfucosylated

HexNAc(6)Hex(7)Fuc(1)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(2)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(6)Hex(6)Fuc(1)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(6)Hex(6)Fuc(1)NeuAc(2)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(6)Hex(5)Fuc(1)NeuAc(1)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(6)Hex(7)Fuc(1)NeuAc(3)	K.NVSDIIPR.T	690	Sialyfucosylated
HexNAc(4)Hex(5)Fuc(0)NeuAc(1)	K.NVSDIIPR.T	690	Sialylated
HexNAc(4)Hex(4)Fuc(0)NeuAc(1)	K.NVSDIIPR.T	690	Sialylated
HexNAc(4)Hex(5)Fuc(0)NeuAc(2)	K.NVSDIIPR.T	690	Sialylated
HexNAc(5)Hex(6)Fuc(0)NeuAc(1)	K.NVSDIIPR.T	690	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(1)	K.NVSDIIPR.T	690	Sialylated
HexNAc(5)Hex(4)Fuc(0)NeuAc(1)	K.NVSDIIPR.T	690	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(2)	K.NVSDIIPR.T	690	Sialylated
HexNAc(5)Hex(5)Fuc(0)NeuAc(2)	K.NVSDIIPR.T	690	Sialylated
HexNAc(6)Hex(6)Fuc(0)NeuAc(1)	K.NVSDIIPR.T	690	Sialylated
HexNAc(5)Hex(6)Fuc(0)NeuAc(2)	K.NVSDIIPR.T	690	Sialylated

HexNAc(6)Hex(7)Fuc(0)NeuAc(1)	K.NVSDIIPR.T	690	Sialylated
HexNAc(6)Hex(5)Fuc(0)NeuAc(1)	K.NVSDIIPR.T	690	Sialylated
HexNAc(6)Hex(4)Fuc(0)NeuAc(1)	K.NVSDIIPR.T	690	Sialylated
HexNAc(6)Hex(5)Fuc(0)NeuAc(2)	K.NVSDIIPR.T	690	Sialylated
HexNAc(3)Hex(4)Fuc(0)NeuAc(0)	K.NVSDIIPR.T	690	Undecorated
HexNAc(4)Hex(5)Fuc(0)NeuAc(0)	K.NVSDIIPR.T	690	Undecorated
HexNAc(4)Hex(4)Fuc(0)NeuAc(0)	K.NVSDIIPR.T	690	Undecorated
HexNAc(4)Hex(3)Fuc(0)NeuAc(0)	K.NVSDIIPR.T	690	Undecorated
HexNAc(5)Hex(5)Fuc(0)NeuAc(0)	K.NVSDIIPR.T	690	Undecorated
HexNAc(6)Hex(7)Fuc(0)NeuAc(0)	K.NVSDIIPR.T	690	Undecorated
HexNAc(3)Hex(3)Fuc(0)NeuAc(0)	K.NVSDIIPR.T	690	Undecorated
HexNAc(5)Hex(6)Fuc(0)NeuAc(0)	K.NVSDIIPR.T	690	Undecorated
HexNAc(5)Hex(4)Fuc(0)NeuAc(0)	K.NVSDIIPR.T	690	Undecorated
HexNAc(5)Hex(3)Fuc(0)NeuAc(0)	K.NVSDIIPR.T	690	Undecorated
HexNAc(6)Hex(6)Fuc(0)NeuAc(0)	K.NVSDIIPR.T	690	Undecorated

Table S4.3 HepG2 N-Glycome Profiles

Treatment	Mass (exp.)	Glycan Subtype	Hex	HexNAc	Fuc	NeuAc	RT	Relative Abundance
Control	3171.1077	Sialyfucosylated	6	5	2	3	33.79	17.09
Control	3025.05215	Sialyfucosylated	6	5	1	3	33.435	11.45
Control	2368.82772	Sialyfucosylated	5	4	1	2	27.272	9.59
Control	2880.01648	Sialyfucosylated	6	5	2	2	28.099	6.53
Control	3536.23075	Sialyfucosylated	7	6	2	3	32.88	4.60
Control	2733.95668	Sialyfucosylated	6	5	1	2	26.792	4.43
Control	2222.77629	Sialylated	5	4	0	2	26.406	3.73
Control	1720.58797	High Mannose	8	2	0	0	16.527	2.81
Control	1558.53507	High Mannose	7	2	0	0	16.535	2.30
Control	2878.99649	Sialylated	6	5	0	3	30.249	2.25
Control	3099.08627	Sialyfucosylated	7	6	1	2	28.928	2.25
Control	1882.63764	High Mannose	9	2	0	0	14.876	2.06
Control	1396.48489	High Mannose	6	2	0	0	16.534	1.90
Control	1234.43361	High Mannose	5	2	0	0	15.055	1.73
Control	3245.14147	Sialyfucosylated	7	6	2	2	28.276	1.68

Control	2077.74411	Sialyfucosylated	5	4	1	1	25.034	1.53
Control	3901.36548	Sialyfucosylated	8	7	2	3	32.562	1.52
Control	4047.42108	Sialyfucosylated	8	7	3	3	32.674	1.26
Control	2807.99108	Sialyfucosylated	7	6	1	1	25.401	1.20
Control	4412.54442	Sialyfucosylated	9	8	3	3	31.832	1.11
Control	2587.9302	Sialylated	6	5	0	2	26.246	1.11
Control	4558.60993	Sialyfucosylated	9	8	4	3	32.39	1.10
Control	4265.47873	Sialylated	9	8	0	4	33.248	0.78
Control	2514.90407	Sialyfucosylated	5	4	2	2	28.523	0.75
Control	3026.06317	Sialyfucosylated	6	5	3	2	28.563	0.73
Control	3682.2928	Sialyfucosylated	7	6	3	3	33.002	0.73
Control	3683.31196	Sialylated	9	8	0	2	33.296	0.64
Control	4048.42004	Sialyfucosylated	8	7	5	2	32.699	0.59
Control	3756.32838	Sialyfucosylated	8	7	3	2	28.07	0.57
Control	3391.20449	Sialyfucosylated	7	6	3	2	28.027	0.53
Control	3101.09265	Undecorated	9	8	0	0	28.415	0.49

Control	2881.00488	Sialyfucosylated	6	5	4	1	27.692	0.42
Control	2571.90946	Sialyfucosylated	5	5	1	2	26.295	0.42
Control	2734.96851	Sialyfucosylated	6	5	3	1	33.651	0.39
Control	1931.68258	Sialylated	5	4	0	1	23.753	0.39
Control	2954.05527	Sialyfucosylated	7	6	2	1	26.097	0.38
Control	2044.68308	High Mannose	10	2	0	0	17.203	0.38
Control	1728.60378	Sialylated	5	3	0	1	21.681	0.38
Control	3431.13406	Sialyfucosylated	6	7	1	3	31.982	0.36
Control	2223.80247	Sialyfucosylated	5	4	2	1	25.502	0.35
Control	1072.37862	High Mannose	4	2	0	0	16.521	0.31
Control	1890.65345	Sialylated	6	3	0	1	22.723	0.30
Control	2953.05105	Sialylated	7	6	0	2	26.139	0.29
Control	3757.30547	Sialyfucosylated	8	7	5	1	32.822	0.27
Control	2589.91795	Fucosylated	6	5	4	0	28.085	0.23
Control	2409.86881	Sialyfucosylated	4	5	1	2	27.005	0.23
Control	2661.94723	Sialylated	7	6	0	1	25.696	0.23

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Control	4339.52848	Sialyfucosylated	8	7	5	3	33.275	0.20
Control	2296.80193	Sialylated	6	5	0	1	25.705	0.20
Control	3319.13534	Sialyfucosylated	8	7	2	1	28.781	0.19
Control	4119.41958	Sialyfucosylated	7	6	4	4	35.184	0.19
Control	4485.67268	Sialyfucosylated	8	7	6	3	38.828	0.19
Control	3828.35852	Sialyfucosylated	7	6	4	3	34.004	0.18
Control	1462.5422	Fucosylated	3	4	1	0	20.95	0.18
Control	3100.10068	Sialyfucosylated	7	6	3	1	25.852	0.17
Control	4015.44804	Sialyfucosylated	6	7	5	3	31.983	0.17
Control	2206.75588	High Mannose	11	2	0	0	18.813	0.16
Control	2692.9092	Sialyfucosylated	7	4	1	2	38.675	0.15
Control	2572.96644	Sialyfucosylated	5	5	3	1	29.548	0.15
Control	910.32508	High Mannose	3	2	0	0	17.598	0.15
Control	3390.16773	Sialyfucosylated	7	6	1	3	33.259	0.14
Control	3448.21784	Sialyfucosylated	7	7	2	2	33.233	0.14
Control	3244.1124	Sialylated	7	6	0	3	26.78	0.14
Control	2442.8558	Sialyfucosylated	6	5	1	1	25.67	0.13
Control	5067.7955	Sialyfucosylated	8	7	6	5	36.386	0.13

Control	3830.32217	Sialyfucosylated	9	8	3	1	30.32	0.12
Control	2458.8865	Sialylated	7	5	0	1	37.857	0.12
Control	3522.23455	Sialyfucosylated	8	8	2	1	32.887	0.12
Control	2524.90341	Sialyfucosylated	4	7	1	1	32.428	0.12
Control	1712.61544	Sialyfucosylated	4	3	1	1	25.716	0.12
Control	2614.92315	Fucosylated	4	6	5	0	33.298	0.11
Control	3611.26524	Sialyfucosylated	8	7	4	1	38.783	0.11
Control	2790.97739	Sialylated	6	6	0	2	32.444	0.11
Control	2425.82883	Sialylated	5	5	0	2	35.078	0.10
Control	2921.07879	Sialyfucosylated	5	6	2	2	35.268	0.10
Control	4046.42392	Sialyfucosylated	8	7	1	4	35.085	0.10
Control	3975.39019	Sialyfucosylated	9	8	2	2	31.281	0.10
Control	3318.16418	Sialylated	8	7	0	2	30.164	0.09
Control	2882.10681	Fucosylated	8	7	1	0	30.816	0.09
Control	3027.05103	Sialylated	8	7	0	1	28.578	0.09
Control	2588.89877	Sialyfucosylated	6	5	2	1	36.527	0.09

Control	2384.83165	Sialylated	6	4	0	2	27.744	0.08
Control	2906.09018	Sialyfucosylated	4	6	5	1	38.744	0.08
Control	1275.44533	Undecorated	4	3	0	0	35.01	0.08
Control	3083.10218	Sialyfucosylated	6	6	2	2	26.23	0.07
Control	3593.25052	Sialyfucosylated	7	7	1	3	31.955	0.07
Control	3539.23755	Fucosylated	9	8	3	0	27.747	0.06
Control	2939.01167	Undecorated	8	8	0	0	36.393	0.06
Control	2645.92309	Sialyfucosylated	6	6	1	1	31.998	0.06
Control	2240.85387	Fucosylated	6	4	3	0	37.912	0.06
Control	2305.78103	Sialyfucosylated	3	6	2	1	37.219	0.06
Control	2834.03916	Fucosylated	5	7	4	0	38.018	0.06
Control	2838.97667	Sialyfucosylated	7	4	2	2	37.941	0.05
Control	2513.90677	Sialylated	5	4	0	3	35.88	0.04
Control	2955.06296	Fucosylated	7	6	4	0	28.231	0.04
Control	2368.82969	High Mannose	12	2	0	0	29.213	0.04
Control	2809.03324	Fucosylated	7	6	3	0	37.715	0.04

Control	2297.81088	Fucosylated	6	5	2	0	29.713	0.03
2F-Fucose	2222.77505	Sialylated	5	4	0	2	25.676	19.62
2F-Fucose	2878.99658	Sialylated	6	5	0	3	26.037	16.78
2F-Fucose	2953.03182	Sialylated	7	6	0	2	27.705	6.79
2F-Fucose	2587.90476	Sialylated	6	5	0	2	26.533	6.78
2F-Fucose	1720.58802	High Mannose	8	2	0	0	16.566	3.77
2F-Fucose	2661.94302	Sialylated	7	6	0	1	25.267	3.75
2F-Fucose	1931.67633	Sialylated	5	4	0	1	23.079	3.42
2F-Fucose	3244.11253	Sialylated	7	6	0	3	28.846	2.92
2F-Fucose	1558.53822	High Mannose	7	2	0	0	15.041	2.90
2F-Fucose	2296.80792	Sialylated	6	5	0	1	24.025	2.77
2F-Fucose	1882.647	High Mannose	9	2	0	0	14.123	2.75
2F-Fucose	1234.43339	High Mannose	5	2	0	0	15.19	2.45
2F-Fucose	1396.48898	High Mannose	6	2	0	0	16.568	2.42
2F-Fucose	2368.82765	Sialyfucosylated	5	4	1	2	27.779	1.86
2F-Fucose	3318.16515	Sialylated	8	7	0	2	29.098	1.74

2F-Fucose	3609.25396	Sialylated	8	7	0	3	33.503	1.32
2F-Fucose	3683.29518	Sialylated	9	8	0	2	29.583	1.25
2F-Fucose	3974.38315	Sialylated	9	8	0	3	33.786	1.13
2F-Fucose	2879.99453	Sialyfucosylated	6	5	2	2	30.617	0.89
2F-Fucose	1728.60884	Sialylated	5	3	0	1	21.694	0.88
2F-Fucose	1890.65652	Sialylated	6	3	0	1	22.729	0.78
2F-Fucose	2588.91014	Sialyfucosylated	6	5	2	1	24.98	0.72
2F-Fucose	3025.04442	Sialyfucosylated	6	5	1	3	36.657	0.71
2F-Fucose	2733.93209	Sialyfucosylated	6	5	1	2	28.397	0.62
2F-Fucose	2954.02543	Sialyfucosylated	7	6	2	1	28.189	0.60
2F-Fucose	3099.09833	Sialyfucosylated	7	6	1	2	28.855	0.58
2F-Fucose	2044.68014	High Mannose	10	2	0	0	17.216	0.53
2F-Fucose	3026.06469	Sialyfucosylated	6	5	3	2	26.219	0.52
2F-Fucose	1072.38139	High Mannose	4	2	0	0	16.577	0.50
2F-Fucose	2589.95618	Fucosylated	6	5	4	0	26.636	0.50
2F-Fucose	2881.99063	Fucosylated	8	7	1	0	25.486	0.46

2F-Fucose	3685.28046	Fucosylated	9	8	4	0	29.58	0.46
2F-Fucose	3027.06498	Sialylated	8	7	0	1	26.755	0.44
2F-Fucose	2425.85821	Sialylated	5	5	0	2	25.171	0.43
2F-Fucose	3245.11443	Sialyfucosylated	7	6	2	2	33.647	0.36
2F-Fucose	3976.36614	Sialyfucosylated	9	8	4	1	34.047	0.32
2F-Fucose	910.3236	High Mannose	3	2	0	0	17.626	0.29
2F-Fucose	2790.9762	Sialylated	6	6	0	2	25.61	0.28
2F-Fucose	1566.56591	Sialylated	4	3	0	1	23.026	0.27
2F-Fucose	2206.74945	High Mannose	11	2	0	0	18.823	0.26
2F-Fucose	2660.97388	Sialyfucosylated	5	4	3	2	21.898	0.24
2F-Fucose	3682.2912	Sialyfucosylated	7	6	3	3	29.574	0.24
2F-Fucose	2077.73078	Sialyfucosylated	5	4	1	1	25.728	0.24
2F-Fucose	2368.80395	High Mannose	12	2	0	0	27.192	0.21
2F-Fucose	4048.40764	Sialyfucosylated	8	7	5	2	29.825	0.21
2F-Fucose	2735.92624	Undecorated	8	7	0	0	28.945	0.18
2F-Fucose	3611.25006	Sialyfucosylated	8	7	4	1	35.844	0.18

2F-Fucose	4413.4887	Sialyfucosylated	9	8	5	2	31.14	0.18
2F-Fucose	2264.80701	Sialyfucosylated	4	5	2	1	25.913	0.17
2F-Fucose	2881.00685	Sialyfucosylated	6	5	4	1	33.124	0.17
2F-Fucose	2734.96526	Sialyfucosylated	6	5	3	1	27.874	0.14
2F-Fucose	2061.72161	Sialyfucosylated	4	4	2	1	37.573	0.14
2F-Fucose	3684.29203	Sialyfucosylated	9	8	2	1	31.638	0.14
2F-Fucose	3320.11472	Fucosylated	8	7	4	0	35.419	0.12
2F-Fucose	3101.14769	Fucosylated	7	6	5	0	39.219	0.12
2F-Fucose	4339.50078	Sialyfucosylated	8	7	5	3	33.904	0.10
2F-Fucose	3246.11681	Sialyfucosylated	7	6	4	1	35.726	0.10
2F-Fucose	2426.89513	Sialyfucosylated	5	5	2	1	35.333	0.10
2F-Fucose	1802.6326	Undecorated	6	4	0	0	33.999	0.10
2F-Fucose	2808.03381	Sialyfucosylated	7	6	1	1	26.387	0.10
2F-Fucose	2499.9437	Sialylated	6	6	0	1	38.209	0.10
2F-Fucose	3392.17516	Sialylated	9	8	0	1	33.655	0.09
2F-Fucose	2297.83217	Fucosylated	6	5	2	0	35.682	0.08

2F-Fucose	1316.48659	Undecorated	3	4	0	0	18.27	0.07
2F-Fucose	2719.93553	Fucosylated	7	7	1	0	37.565	0.07
2F-Fucose	2662.94794	Fucosylated	7	6	2	0	26.188	0.07
2F-Fucose	2304.85609	Sialylated	3	6	0	2	26.475	0.07
2F-Fucose	2994.00598	Sialylated	6	7	0	2	30.169	0.06
2F-Fucose	4705.61901	Sialyfucosylated	9	8	7	2	35.79	0.06
2F-Fucose	2078.76857	Fucosylated	5	4	3	0	25.122	0.06
2F-Fucose	3319.13729	Sialyfucosylated	8	7	2	1	30.648	0.05
2F-Fucose	2546.8532	Sialylated	7	4	0	2	26.248	0.05
2F-Fucose	3903.47421	Sialyfucosylated	8	7	6	1	37.479	0.05
2F-Fucose	2458.91249	Sialylated	7	5	0	1	26.117	0.04
2F-Fucose	2385.89524	Sialyfucosylated	6	4	2	1	35.948	0.04
2F-Fucose	2571.87902	Sialyfucosylated	5	5	1	2	26.287	0.04
Kifunensine	1882.63654	High Mannose	9	2	0	0	18.42	30.04
Kifunensine	1720.58961	High Mannose	8	2	0	0	18.552	23.46
Kifunensine	1558.53554	High Mannose	7	2	0	0	18.571	15.68

Kifunensine	1396.48489	High Mannose	6	2	0	0	18.571	8.29
Kifunensine	1234.43341	High Mannose	5	2	0	0	21.976	5.19
Kifunensine	2044.69275	High Mannose	10	2	0	0	18.422	2.46
Kifunensine	2368.82775	Sialyfucosylated	5	4	1	2	28.266	1.89
Kifunensine	2036.71566	Sialyfucosylated	6	3	1	1	25.084	1.64
Kifunensine	1072.37197	High Mannose	4	2	0	0	18.571	1.52
Kifunensine	1890.65922	Sialylated	6	3	0	1	25.073	0.98
Kifunensine	3318.19664	Sialylated	8	7	0	2	31.922	0.88
Kifunensine	910.32426	High Mannose	3	2	0	0	18.574	0.79
Kifunensine	2206.73848	High Mannose	11	2	0	0	20.126	0.70
Kifunensine	1874.66193	Sialyfucosylated	5	3	1	1	24.953	0.57
Kifunensine	3521.25843	Sialylated	8	8	0	2	37.869	0.48
Kifunensine	2401.83446	Sialyfucosylated	7	4	1	1	35.177	0.41
Kifunensine	3084.13601	Sialyfucosylated	6	6	4	1	35.582	0.40
Kifunensine	1728.60681	Sialylated	5	3	0	1	25.007	0.38
Kifunensine	2368.77118	High Mannose	12	2	0	0	33.846	0.36

Kifunensine	2662.96639	Fucosylated	7	6	2	0	35.27	0.34
Kifunensine	2906.16005	Sialyfucosylated	4	6	5	1	28.748	0.31
Kifunensine	2540.93262	Sialylated	5	7	0	1	39.016	0.30
Kifunensine	2255.75782	Sialylated	7	4	0	1	31.505	0.29
Kifunensine	2880.00539	Sialyfucosylated	6	5	2	2	28.562	0.28
Kifunensine	2222.77518	Sialylated	5	4	0	2	27.397	0.27
Kifunensine	2735.99792	Undecorated	8	7	0	0	38.275	0.26
Kifunensine	2217.87719	Fucosylated	3	7	2	0	37.524	0.20
Kifunensine	1915.70408	Sialyfucosylated	4	4	1	1	36.118	0.18
Kifunensine	2459.89419	Fucosylated	7	5	2	0	36.872	0.17
Kifunensine	2160.76488	Fucosylated	3	6	3	0	34.838	0.16
Kifunensine	2524.98709	Sialyfucosylated	4	7	1	1	38.967	0.15
Kifunensine	2450.86613	Sialyfucosylated	3	6	1	2	25.093	0.13
Kifunensine	4179.44551	Sialyfucosylated	9	9	4	1	35.099	0.12
Kifunensine	2134.72875	Sialylated	5	5	0	1	33.378	0.11
Kifunensine	2369.84004	Sialyfucosylated	5	4	3	1	32.443	0.11

Kifunensi ne	2379.8438 6	Fucosylated	4	7	2	0	32.92 3	0.11
Kifunensi ne	1891.6451 6	Fucosylated	6	3	2	0	21.91 8	0.11
Kifunensi ne	2734.0219 7	Sialyfucosylated	6	5	1	2	30.82 2	0.09
Kifunensi ne	2547.8558	Sialyfucosylated	7	4	2	1	35.57 6	0.09
Kifunensi ne	2530.8255 2	Sialyfucosylated	6	4	1	2	32.54 4	0.07
Kifunensi ne	2573.9273 6	Undecorated	7	7	0	0	30.66 6	0.05
3-F-Sia	1720.586	High Mannose	8	2	0	0	17.35 3	6.88
3-F-Sia	2516.9077	Fucosylated	7	6	1	0	25.94 1	5.50
3-F-Sia	1558.5353 8	High Mannose	7	2	0	0	17.35 2	5.39
3-F-Sia	2077.7428 6	Sialyfucosylated	5	4	1	1	28.38 9	5.18
3-F-Sia	2368.8336 4	Sialyfucosylated	5	4	1	2	30.59 3	4.85
3-F-Sia	1882.6382 9	High Mannose	9	2	0	0	17.21 9	4.26
3-F-Sia	1396.4847 6	High Mannose	6	2	0	0	17.44 3	4.25
3-F-Sia	1234.4309	High Mannose	5	2	0	0	21.10 6	3.94
3-F-Sia	1786.6409 7	Fucosylated	5	4	1	0	25.25 8	3.48
3-F-Sia	2370.8360 5	Undecorated	7	6	0	0	25.01 9	3.23

3-F-Sia	2808.00462	Sialyfucosylated	7	6	1	1	27.074	2.99
3-F-Sia	2954.04937	Sialyfucosylated	7	6	2	1	26.213	2.36
3-F-Sia	2297.81326	Fucosylated	6	5	2	0	25.431	2.09
3-F-Sia	2882.02771	Fucosylated	8	7	1	0	26.098	2.05
3-F-Sia	2588.92525	Sialyfucosylated	6	5	2	1	26.396	1.94
3-F-Sia	2223.79455	Sialyfucosylated	5	4	2	1	25.675	1.90
3-F-Sia	1932.67907	Fucosylated	5	4	2	0	24.976	1.87
3-F-Sia	2442.86866	Sialyfucosylated	6	5	1	1	26.62	1.74
3-F-Sia	3028.09659	Fucosylated	8	7	2	0	28.437	1.71
3-F-Sia	1640.58591	Undecorated	5	4	0	0	35.013	1.71
3-F-Sia	2880.04654	Sialyfucosylated	6	5	2	2	38.959	1.70
3-F-Sia	2809.00778	Fucosylated	7	6	3	0	23.875	1.63
3-F-Sia	3099.08639	Sialyfucosylated	7	6	1	2	29.617	1.59
3-F-Sia	2005.71767	Undecorated	6	5	0	0	23.093	1.45
3-F-Sia	3100.09993	Sialyfucosylated	7	6	3	1	29.537	1.43
3-F-Sia	3245.14437	Sialyfucosylated	7	6	2	2	30.516	1.40

3-F-Sia	2662.94933	Fucosylated	7	6	2	0	25.466	1.38
3-F-Sia	3247.15	Fucosylated	9	8	1	0	26.713	1.32
3-F-Sia	2222.7747	Sialylated	5	4	0	2	29.578	1.08
3-F-Sia	2044.69093	High Mannose	10	2	0	0	20.016	0.92
3-F-Sia	3319.16483	Sialyfucosylated	8	7	2	1	26.542	0.87
3-F-Sia	1072.37752	High Mannose	4	2	0	0	17.437	0.79
3-F-Sia	1599.55907	Undecorated	6	3	0	0	19.552	0.70
3-F-Sia	3539.26307	Fucosylated	9	8	3	0	25.305	0.65
3-F-Sia	3465.23302	Sialyfucosylated	8	7	3	1	26.339	0.57
3-F-Sia	1712.62035	Sialyfucosylated	4	3	1	1	28.383	0.51
3-F-Sia	3537.25022	Sialyfucosylated	7	6	4	2	28.274	0.49
3-F-Sia	2661.94328	Sialylated	7	6	0	1	25.8	0.49
3-F-Sia	3246.15536	Sialyfucosylated	7	6	4	1	25.689	0.49
3-F-Sia	3976.40533	Sialyfucosylated	9	8	4	1	26.483	0.48
3-F-Sia	2078.75633	Fucosylated	5	4	3	0	21.163	0.48
3-F-Sia	2206.73801	High Mannose	11	2	0	0	20.112	0.48

3-F-Sia	3391.19318	Sialyfucosylated	7	6	3	2	28.495	0.47
3-F-Sia	3464.2053	Sialyfucosylated	8	7	1	2	30.585	0.47
3-F-Sia	2587.90396	Sialylated	6	5	0	2	29.646	0.45
3-F-Sia	1275.4572	Undecorated	4	3	0	0	22.809	0.45
3-F-Sia	1421.51798	Fucosylated	4	3	1	0	21.978	0.41
3-F-Sia	2734.97407	Sialyfucosylated	6	5	3	1	26.182	0.41
3-F-Sia	1462.54135	Fucosylated	3	4	1	0	20.631	0.40
3-F-Sia	1728.60241	Sialylated	5	3	0	1	23.495	0.39
3-F-Sia	1437.5089	Undecorated	5	3	0	0	19.535	0.37
3-F-Sia	2719.98218	Fucosylated	7	7	1	0	20.622	0.35
3-F-Sia	2384.85617	Sialylated	6	4	0	2	28.267	0.35
3-F-Sia	1890.65742	Sialylated	6	3	0	1	24.28	0.33
3-F-Sia	3393.20671	Fucosylated	9	8	2	0	26.06	0.31
3-F-Sia	2093.73431	Sialylated	6	4	0	1	25.876	0.29
3-F-Sia	3903.37858	Sialyfucosylated	8	7	6	1	28.421	0.29
3-F-Sia	4123.46536	Fucosylated	9	8	7	0	26.959	0.28

3-F-Sia	2645.93503	Sialyfucosylated	6	6	1	1	32.336	0.27
3-F-Sia	2953.02357	Sialylated	7	6	0	2	28.453	0.25
3-F-Sia	1259.46769	Fucosylated	3	3	1	0	24.147	0.25
3-F-Sia	3685.28777	Fucosylated	9	8	4	0	37.541	0.24
3-F-Sia	2589.9414	Fucosylated	6	5	4	0	22.834	0.20
3-F-Sia	910.32577	High Mannose	3	2	0	0	21.013	0.20
3-F-Sia	3174.14215	Fucosylated	8	7	3	0	25.208	0.20
3-F-Sia	3831.35379	Fucosylated	9	8	5	0	26.683	0.19
3-F-Sia	1915.67879	Sialyfucosylated	4	4	1	1	26.613	0.19
3-F-Sia	1665.6196	Fucosylated	3	5	1	0	21.993	0.19
3-F-Sia	3026.06328	Sialyfucosylated	6	5	3	2	29.012	0.19
3-F-Sia	1868.70072	Fucosylated	3	6	1	0	21.446	0.18
3-F-Sia	1583.57208	Fucosylated	5	3	1	0	20.471	0.18
3-F-Sia	3466.24153	Fucosylated	8	7	5	0	26.414	0.18
3-F-Sia	3536.22715	Sialyfucosylated	7	6	2	3	31.09	0.17
3-F-Sia	2354.8569	Fucosylated	6	6	1	0	19.958	0.17

3-F-Sia	2630.99329	Fucosylated	5	6	4	0	36.596	0.15
3-F-Sia	2955.02886	Fucosylated	7	6	4	0	28.724	0.15
3-F-Sia	1989.71387	Fucosylated	5	5	1	0	23.973	0.14
3-F-Sia	3757.31879	Sialyfucosylated	8	7	5	1	25.677	0.14
3-F-Sia	2368.80585	High Mannose	12	2	0	0	21.669	0.14
3-F-Sia	2134.71958	Sialylated	5	5	0	1	37.628	0.13
3-F-Sia	1931.69133	Sialylated	5	4	0	1	29.624	0.13
3-F-Sia	2264.81909	Sialyfucosylated	4	5	2	1	25.283	0.12
3-F-Sia	3684.2584	Sialyfucosylated	9	8	2	1	33.301	0.10
3-F-Sia	1745.61187	Fucosylated	6	3	1	0	18.619	0.10
3-F-Sia	3612.25987	Fucosylated	8	7	6	0	28.504	0.09
3-F-Sia	2897.03326	Sialyfucosylated	7	5	3	1	36.039	0.09
3-F-Sia	2151.77901	Fucosylated	6	5	1	0	26.534	0.09
3-F-Sia	2094.74813	Fucosylated	6	4	2	0	36.069	0.08
3-F-Sia	2547.90255	Sialyfucosylated	7	4	2	1	35.668	0.08
3-F-Sia	4414.56797	Sialyfucosylated	9	8	7	1	28.255	0.07

3-F-Sia	2822.93785	Sialyfucosylated	6	4	3	2	37.122	0.07
3-F-Sia	2087.80714	Undecorated	4	7	0	0	32.639	0.06
3-F-Sia	4996.74472	Sialyfucosylated	9	8	7	3	36.324	0.06
3-F-Sia	2572.88395	Sialyfucosylated	5	5	3	1	26.79	0.06
3-F-Sia	2280.8109	Sialyfucosylated	5	5	1	1	23.018	0.06
3-F-Sia	2426.86719	Sialyfucosylated	5	5	2	1	26.83	0.06
3-F-Sia	2978.06525	Sialyfucosylated	5	7	1	2	34.581	0.05
3-F-Sia	2239.79153	Sialyfucosylated	6	4	1	1	25.629	0.05
3-F-Sia	2646.93468	Fucosylated	6	6	3	0	34.239	0.05
3-F-Sia	2790.96657	Sialylated	6	6	0	2	33.314	0.04
3-F-Sia	3010.05383	Sialyfucosylated	5	5	4	2	37.026	0.04
3-F-Sia	2369.84416	Sialyfucosylated	5	4	3	1	18.8	0.03
3-F-Sia	2864.97364	Sialylated	7	7	0	1	36.659	0.03
3-F-Sia	2530.87403	Sialyfucosylated	6	4	1	2	37.909	0.02
3-F-Sia	2499.88748	Sialylated	6	6	0	1	28.377	0.02

Table S4.4 Site-specific occupancy of recombinant Spike protein RBD derived from HEK293 cells

Glycans	Peptide Sequence	Glycosite	Glycan Subtype
HexNAc(8)Hex(9)Fuc(3)NeuAc(0)	R.FPNITNLCPFGEVFNATR.F	331	Fucosylated
HexNAc(9)Hex(9)Fuc(3)NeuAc(0)	R.FPNITNLCPFGEVFNATR.F	331	Fucosylated
HexNAc(9)Hex(9)Fuc(6)NeuAc(0)	R.FPNITNLCPFGEVFNATR.F	331	Fucosylated
HexNAc(9)Hex(9)Fuc(2)NeuAc(1)	R.FPNITNLCPFGEVFNATR.F	331	Sialyfucosylated
HexNAc(7)Hex(6)Fuc(5)NeuAc(1)	R.FPNITNLCPFGEVFNATR.F	331	Sialyfucosylated
HexNAc(9)Hex(9)Fuc(5)NeuAc(1)	R.FPNITNLCPFGEVFNATR.F	331	Sialyfucosylated
HexNAc(9)Hex(9)Fuc(1)NeuAc(1)	R.FPNITNLCPFGEVFNATR.F	331	Sialyfucosylated
HexNAc(9)Hex(9)Fuc(4)NeuAc(2)	R.FPNITNLCPFGEVFNATR.F	331	Sialyfucosylated
HexNAc(7)Hex(7)Fuc(4)NeuAc(2)	R.FPNITNLCPFGEVFNATR.F	331	Sialyfucosylated
HexNAc(7)Hex(6)Fuc(5)NeuAc(2)	R.FPNITNLCPFGEVFNATR.F	331	Sialyfucosylated
HexNAc(8)Hex(8)Fuc(0)NeuAc(2)	R.FPNITNLCPFGEVFNATR.F	331	Sialylated
HexNAc(8)Hex(9)Fuc(7)NeuAc(2)	R.FPNITNLCPFGEVFNATR.F	331	Sialyfucosylated
HexNAc(7)Hex(6)Fuc(4)NeuAc(2)	R.FPNITNLCPFGEVFNATR.F	331	Sialyfucosylated
HexNAc(7)Hex(8)Fuc(6)NeuAc(3)	R.FPNITNLCPFGEVFNATR.F	331	Sialyfucosylated

HexNAc(7)Hex(7)Fuc(2)Neu Ac(3)	R.FPNITNLCPFGEVFNA TR.F	331	Sialyfucosylate d
HexNAc(8)Hex(9)Fuc(2)Neu Ac(1)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(9)Hex(9)Fuc(3)Neu Ac(1)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(6)Hex(7)Fuc(4)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(9)Hex(9)Fuc(2)Neu Ac(1)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(9)Hex(9)Fuc(6)Neu Ac(0)	R.FPNITNLCPFGEVFNA TR.F	343	Fucosylated
HexNAc(8)Hex(9)Fuc(3)Neu Ac(0)	R.FPNITNLCPFGEVFNA TR.F	343	Fucosylated
HexNAc(9)Hex(9)Fuc(4)Neu Ac(1)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(9)Hex(9)Fuc(3)Neu Ac(0)	R.FPNITNLCPFGEVFNA TR.F	343	Fucosylated
HexNAc(7)Hex(7)Fuc(2)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(8)Hex(8)Fuc(6)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(9)Hex(9)Fuc(2)Neu Ac(0)	R.FPNITNLCPFGEVFNA TR.F	343	Fucosylated
HexNAc(8)Hex(8)Fuc(6)Neu Ac(3)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(7)Hex(6)Fuc(2)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(7)Hex(8)Fuc(6)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(9)Hex(9)Fuc(4)Neu Ac(0)	R.FPNITNLCPFGEVFNA TR.F	343	Fucosylated

HexNAc(9)Hex(9)Fuc(1)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(8)Hex(9)Fuc(5)Neu Ac(1)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(8)Hex(8)Fuc(3)Neu Ac(0)	R.FPNITNLCPFGEVFNA TR.F	343	Fucosylated
HexNAc(9)Hex(9)Fuc(2)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(8)Hex(9)Fuc(2)Neu Ac(0)	R.FPNITNLCPFGEVFNA TR.F	343	Fucosylated
HexNAc(8)Hex(8)Fuc(5)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(7)Hex(8)Fuc(5)Neu Ac(3)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(8)Hex(9)Fuc(6)Neu Ac(0)	R.FPNITNLCPFGEVFNA TR.F	343	Fucosylated
HexNAc(7)Hex(6)Fuc(4)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(8)Hex(8)Fuc(2)Neu Ac(0)	R.FPNITNLCPFGEVFNA TR.F	343	Fucosylated
HexNAc(9)Hex(9)Fuc(5)Neu Ac(0)	R.FPNITNLCPFGEVFNA TR.F	343	Fucosylated
HexNAc(8)Hex(9)Fuc(3)Neu Ac(1)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(9)Hex(9)Fuc(2)Neu Ac(3)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(8)Hex(8)Fuc(5)Neu Ac(3)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(9)Hex(9)Fuc(6)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(7)Hex(6)Fuc(3)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d

HexNAc(8)Hex(9)Fuc(3)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(7)Hex(8)Fuc(6)Neu Ac(3)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(8)Hex(8)Fuc(2)Neu Ac(1)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(8)Hex(9)Fuc(7)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(9)Hex(9)Fuc(3)Neu Ac(2)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d
HexNAc(8)Hex(8)Fuc(6)Neu Ac(1)	R.FPNITNLCPFGEVFNA TR.F	343	Sialyfucosylate d

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