Diplomarbeit

The Influence of Human Milk Oligosaccharides on the Risk for Preterm Labor and Preterm Birth

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Graz, am 12.02.2020 *Elisabeth Giselbrecht eh.*

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Zusammenfassung

Hintergrund

Humane Milch Oligosaccharide (HMOs) sind bioaktive Glykane, die in der Muttermilch in hoher Konzentration vorkommen. Frühere Studien haben gezeigt, dass HMOs schon in frühen Stadien der Schwangerschaft nachgewiesen werden können und eine Rolle bei der Erhaltung der Gesundheit während der Schwangerschaft für Mutter und Fötus spielen können. Die Frühgeburt und die daraus resultierenden Komplikationen gehören nach wie vor zu den häufigsten Todesursachen bei Neugeborenen weltweit. Die Zahl der Frühgeburten steigt in fast allen Ländern an, die Gründe für den Anstieg sind vielfältig und in vielen Fällen unbekannt.

Hypothese

Zahlreiche Effekte von HMOs auf die Gesundheit von Mutter und Neugeborenen sind bekannt. Wir stellen die Hypothese auf, dass HMOs, - präbiotische, antiinflammatorisch wirkende und das Immunsystem beeinflussende Glykane - die bei der werdenden Mutter in Blut und Urin nachweisbar sind, das Risiko für Frühgeburtlichkeit beeinflussen können. Um diese Hypothese zu überprüfen, untersuchten wir Fälle, die nicht durch Infektionen oder Vorerkrankungen der Mutter, welche mit vorzeitigen Wehen assoziiert sind, erklärt werden können.

Material und Methoden

Wir rekrutierten 60 schwangere Frauen, die im Zeitraum der 23. bis 34. Schwangerschaftswoche aufgrund vermeintlicher vorzeitiger Wehentätigkeit untersucht wurden. Die Patientinnen wurden nach der Untersuchung mittels Kardiotokogramm (CTG), transvaginaler Sonografie sowie abgenommener Blut- und Harnproben in Fälle (n=33) mit nachgewiesener Wehentätigkeit und Kontrollen (n=27) ohne Wehentätigkeit eingeteilt. Aus den abgenommenen Blut- und Harnproben wurden die HMOs zuerst isoliert und danach mittels High Performance Liquid Chromatography (HPLC) quantifiziert.

Ergebnisse

Wir konnten Konzentrationsunterschiede bei den HMOs im Serum der analysierten Gruppen (verkürzte Zervix vs. normale Zervixlänge, Tokolyse vs. keine Tokolyse, Frühgeburt vs. reif geboren) feststellen. Zusätzlich fanden wir einen Zusammenhang zwischen sialylierten HMOs, insbesondere 3’-Sialyllactose (3’SL) und 6’-Sialyllactosamin (6’SLN), mit Frühgeburtlichkeit, verkürztem Gebärmutterhals und erhöhten Entzündungswerten. Der Zusammenhang mit Frühgeburtlichkeit war innerhalb der Fälle sogar noch verstärkt.

Diskussion

Der hier gefundene Zusammenhang der Serumkonzentration von sialylierten HMOs mit erhöhten Entzündungswerten, verkürztem Gebärmutterhals und Frühgeburtlichkeit weist auf ein möglicherweise erhöhtes Risiko für entzündungs-vermittelte Frühgeburten hin. Ob sich spezifische HMOs im mütterlichen Blut tatsächlich als Biomarker eigenen, muss in entsprechenden geplanten prospektiven Studien überprüft werden.

Abstract

Background

Human Milk Oligosaccharides (HMOs) are bioactive glycans that are highly abundant in breast milk. Previous studies have shown that HMOs can be detected in the early stages of pregnancy and may play a role in the mother's and fetus' health during pregnancy.

Preterm birth (PTB) and the resulting complications are still among the most common causes of mortality in newborns worldwide. The number of PTB is rising in nearly every country, but reasons for the increase are varied and, in many cases, unknown.

Hypothesis and Aims

Numerous effects of HMOs on maternal and the newborn's health are known. We hypothesize that HMOs - prebiotic, anti-inflammatory and immune system influencing glycans - which are detectable in the pregnant mother's blood and urine, can influence the risk of preterm birth. We investigated cases that cannot be explained by UTIs, medical conditions or other factors associated with preterm labor.

Material and Methods

We recruited 60 pregnant women who were examined during the 23rd to 34th week of pregnancy for suspected preterm labor. Patients were grouped after Cardiotocography (CTG) examination, transvaginal ultrasound and drawn blood and urine samples in cases (n=33) with confirmed labor and controls (n=27) without labor. From the collected blood samples, HMOs were first isolated and then quantified by means of High Performance Liquid Chromatography (HPLC).

Results

We identified differences in the concentration of HMOs in the serum of the analyzed groups (short cervix vs. normal cervix length, tocolysis vs. no tocolysis treatment, PTB vs. term birth). In addition, we found associations between sialylated HMOs, in particular 3'-Sialyllactose and 6'-Sialyllactosamine, with PTB, shortened cervix, and increased inflammation. This correlation was strengthened in a defined high-risk group and a connection between 3'SL and 6'SLN with inflammation markers was found as well.

Discussion

Our finding that sialylated HMOs were positively associated with higher concentration of inflammation markers, short cervix and PTB indicate that these HMOs might predict an increased risk for inflammation associated PTB. Whether specific HMOs indeed have a potential as biomarkers for PTB remains to be investigated in adequately designed prospective studies.

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List of Abbreviations

2’-FL	2’-Fucosyllactose
2AB	2-Aminobenzamide
3-FL	3-Fucosyllactose
3’-SLN	3’-Sialyllactosamine
3’-SL	3’-Sialyllactose
6’-SLN	6’-Sialyllactosamine
6’-SL	6’-Sialyllactose
AUC	area under the curve
BMI	Body Mass Index
CRP	C-reactive Protein
CST	Community State Type
CTG	Cardiotocography
DNA	Desoxyribonucleic Acid
DSLNT	Disialyllacto-N-tetraose
EQUC	Enhanced Quantitative Urine Culture
FL	Fucosyllactose
Fuc	Fucose
FUT	Fucosyltransferase
Gal	Galactose
GDM	Gestational Diabetes
Glc	Glucose
GlcNAc	N-Acetylglucosamine
HMO	Human Milk Oligosaccharide
HMP	Human Genome Project
HPLC	High Performance Liquid Chromatography
IEC	intestinal epithelial cells
IFN	Interferon
IL	Interleukin
IM	intramuscular
IUGR	Intrauterine Growth Restriction
LDFT	Lactodifucotetraose

Le	Lewis Blood group antigen
LNDFH	Lacto-N-difucohexaose
LNFP	Lacto-N-Fucopentaose
LPS	Lipopolysaccharides
MAPK	mitogen-activated protein kinase
MLN	mesenteric lymph node
mRNA	messenger RNA
mv	missing values
NEC	Necrotizing Enterocolitis
Neu5Ac	N-acetyl neuraminic acid
NF-κB	nuclear factor kappa B
NK	natural killer (cells)
NS	non secretor
OTU	Operational Taxonomic Units
PNC	platelet-neutrophil complex
PPROM	preterm premature rupture of the membranes
PTB	Preterm Birth
rRNA	Ribosomal RNA
SD	Standard Deviation
Se	Secretor
Sia	Sialic acid
SPA	Suprapubic aspiration
SPE	solid phase extraction
Tregs	regulatory T cells
TUC	Transurethral Catheter
UDP-	Uridine diphosphate-
UPEC	Uropathogenic Escherichia coli
UTI	Urinary Tract Infection
WES	Whole Exome Sequencing

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1 Background

1.1 HMOs

Human Milk Oligosaccharides (HMOs) are highly abundant glycans, uniquely found in human breast milk. The first findings on the health benefits of HMOs date back to the early 1900's, when mortality ranged between 20% to 30% in the first year of a newborn's life. At this early stage, pediatrists found a higher resistance against several gut infections including infectious diarrhea in breast fed babies, compared to those fed with bovine milk. The reason for these observations was a difference in the intestinal microbiota of these infants. In 1926, H. Schönfeld detected the "bifidus factor" as a microbial growth factor in human milk, but at the time classified it as a vitamin. In the 1950s, György and Kuhn proved that this growth factor known as the "bifidus factor" for Lactobacillus bifidus in maternal milk consisted of oligosaccharides containing N-acetylglucosamine and polysaccharides. It was also Kuhn who later identified lacto-N-tetraose as a first key component of HMOs (1). Since then further components have been found and several HMOs have consequently been classified.

1.1.1 Structure

More than 150 oligosaccharide structures have been found in human milk so far, and all of them follow a basic scheme in their structure. All HMOs are formed by 5 monosaccharides as basic building blocks (Fig.1). These 5 monosaccharides are: Glucose (Glc), Galactose (Gal), Fucose (Fuc), N-Acetylglucosamine (GlcNAc), Sialic acid (Sia) / N-Acetyl-neuraminic acid (Neu5Ac).

In all HMOs, Lactose (Galactose β-1,4 linked to Glucose) is present on their reducing end. Lactose can also be extended to more complex structures by adding disaccharide repeats of Gal and GlcNAc (Lacto-N-biose or N-Acetyllactosamine) to the backbone (2).

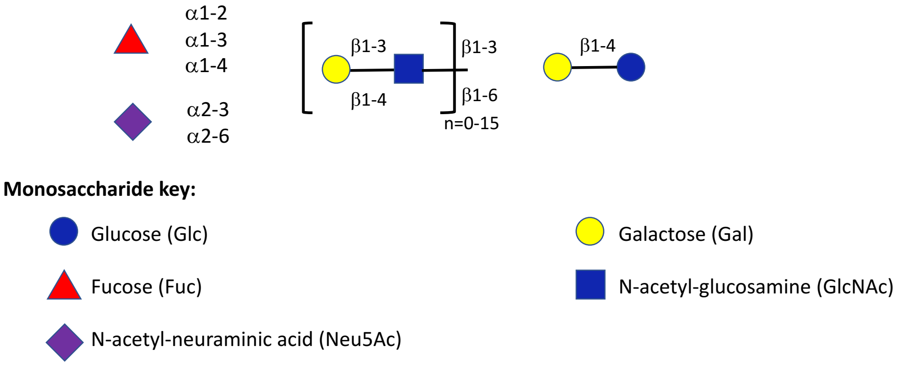


Figure 1. Basic structure of HMOs. The five monosaccharides shown in the key build up the different structures of HMOs. (A) Lactose (Galactose β-1,4 Glucose) is present in all HMOs at their reducing end. For more complex structures, disaccharide repeats (Gal - GlcNAc) can be added to the Lactose backbone. Fucose and N-acetyl-neuraminic acid can be added to further modify HMOs with different resulting functions.

The simplest form of an HMO is a trisaccharide, consisting of Lactose which is sialylated or fucosylated by linking Sialic acid or Fucose (2,3), forming the trisaccharides 2'- and 3-Fucosyllactose (2'-FL and 3FL) or 3'- and 6'-Sialyllactose (3'-SL and 6'-SL) (Fig.2).

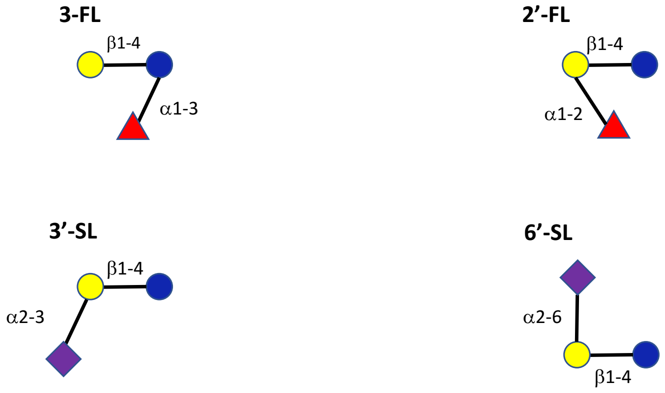


Figure 2. Trisaccharides. 2'-FL = 2'-Fucosyllactose; 3-FL = 3-Fucosyllactose; 3'-SL = 3'-Sialyllactose; 6'-SL = 6'-Sialyllactose. These trisaccharides are formed by linking Fucose or Sialic acid to Lactose.

More complex structures of HMOs are formed by adding the disaccharides N-acetyllactosamine or lacto-N-biose consisting of Gal and GlcNAc to lactose (Fig.1). HMOs with more than a dozen disaccharide units have been described (3).

Sialylated HMOs are formed when sialic acid in form of 5-neuraminic acid is linked in α2-3 or α2-6 linkage to the oligosaccharide backbone or to lactose. Sialic acid containing a carboxylic group adds a negative charge to the resulting HMO (2,3).

As mentioned before, HMOs can also be modified by adding Fucose in a α1-2, α1-3 or α1-4 linkage (see Fig. 3). To add Fucose, specific enzymes, fucosyltransferase-2 or -3 (FUT2 or FUT3) are needed to catalyze the reaction. Fucosylation is genetically determined and follows an all-or-none-law, which means for example that if FUT2 is not expressed by a mother, specific α1-2 fucosylation cannot take place, resulting in large differences in the HMO composition (3–5).

1.1.2 Concentration of HMOs

There is a substantial variation in HMO concentration between individual women, as well as over the course of lactation within one woman. The highest concentrations of HMOs have been found in colostrum, the first form of milk produced by the mammary gland of mammals right after delivery of the newborn. Up to 20-25g/L HMOs are detectable in human colostrum, whilst the concentration declines to 5-20g/L in the following weeks (5,6). Nevertheless, the HMO concentrations in mature human milk still exceed the concentration of proteins. The concentration of oligosaccharides in bovine milk is by 100-1000 fold lower than oligosaccharide concentration in human milk (7). In human milk, the concentration of sialylated HMOs is highest in the first few weeks postpartum and decreases over the lactation period (3). While there is some controversy in the literature, some studies have shown that HMO concentration in the milk after a premature birth is significantly higher than after term birth (3,8).

Research has shown differences in health outcomes in breast fed vs. formula fed infants. This has led to the approach of adding oligosaccharides to infant formula since cow’s milk is the most common base used for infants formula (9).

1.1.3 Secretor Status and Lewis Blood Groups

The exact HMO composition is highly variable in individual women, however, a rough categorization into four separate “milk groups” can be done. The different HMO profiles are also determined by genetics. While one gene locus, the *Secretor Gene* is coding for the α1-2 fucosyltransferase (FUT2), another one, the *Lewis Gene,* encodes for α1-3/4 fucosyltransferase (FUT3). The respective active gene leads to the expression of one of the fucosyltransferases, which are independent from one another and this results in the mother producing a specific set of HMOs. The differences here occur in the fucosylation of HMOs, which differ between secretor and non-secretor on the one hand, and in Lewis positive and Lewis negative on the other (Tab.1) (3). In the European and American population, about 80% of women are secretors, they express FUT2, which links fucose in α1,2 to the backbone (Fig.3) (4,9). In secretor-positive women, 2’-FL and LNFP-I usually show the highest concentrations in milk, but they are virtually absent in secretor-negative women.

The *Lewis blood group Gene* codes for an α1-3/4-fucosyltransferase, FUT3, which is completely independent of FUT2. The production of α1-4-fucosylated HMOs therefore depend on the mother’s Lewis blood group. FUT3 adds fucose via α1-4-binding to the oligosaccharide, leading to Lewis a antigen in the milk of non-secretors, or Lewis b antigen in secretor positive mothers. Lewis negative women do not express FUT3, which means that no α1-3/4-fucosylated HMOs can be synthesized (Lewis a-b-) (e.g. no LNFP-II) (3,5,10). Thus, based on the HMO profile, one can also determine the secretor status and Lewis status (Fig.3). There are 4 different groups, with Group 1 having the most complex HMO composition and Group 4 having the least complex composition. However, the total concentration in Se-Le- breast milk (Group 4) is still several g/L and thus higher than in, for example, cow’s milk (3–5,9).

Table 1. Secretor status and Lewis blood groups. Diversity of human milk in dependence of the mother's genetic background. FL = Fucosyllactose, LNFP = Lacto-N-Fucopentaose, FUT = Fucosyltransferase.

Gene	***Le⁺ (FUT3)***	***Le⁻ (no FUT3)***
Se⁺ (FUT2)	**Lewis positive secretors** Secrete all HMOs	**Lewis negative secretors** Secrete 2'-FL, 3-FL, LNFP-I, LNFP-III
Se⁻ (no FUT2)	**Lewis positive non secretors** Secrete 3-FL, LNFP-II, LNFP-III	**Lewis negative non secretors** Secrete 3-FL, LNFP-III, LNFP-V

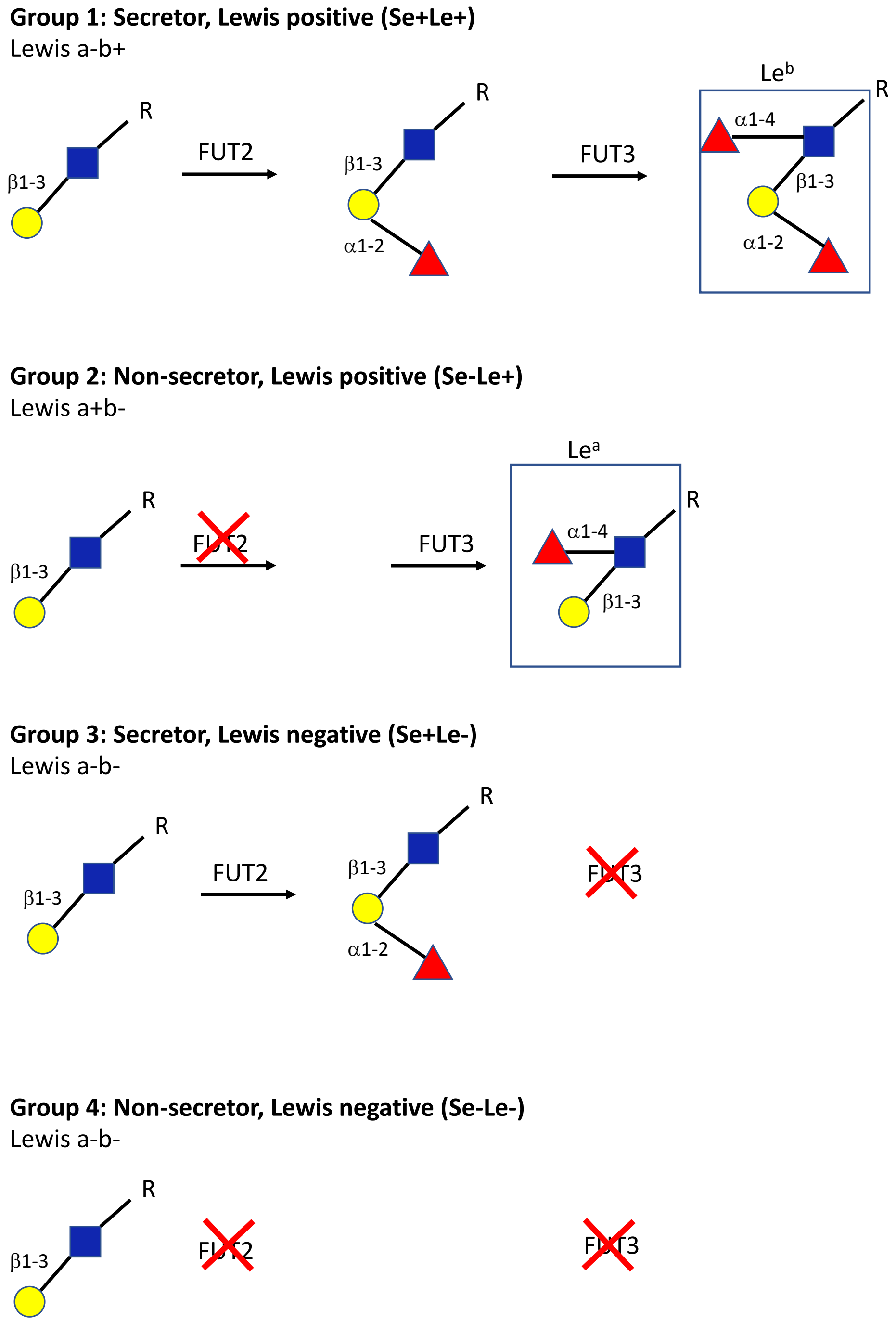


Figure 3. Secretor status and Lewis blood groups. Linkage of Fucose to the HMO-backbone is mainly determined by the mothers Lewis blood group and secretor status; depending on enzyme activity of FUT2 and FUT3, 4 groups can be distinguished; FUT = Fucosyltransferase; Le^b = Lewis b Blood group antigen; Le^a = Lewis a Blood group antigen.

1.1.4 Biosynthesis of HMOs

Up until now, the exact nature of the biosynthesis of HMOs has not been fully understood. It can be assumed that HMOs are an extension of the lactose biosynthesis, which starts in the Golgi apparatus of the lactocytes. Glc becomes activated to UDP-Glc (Uridine diphosphate-Glucose) and further transformed to UDP-Gal (5). Rudloff et al. have shown that exogenous Gal is directly incorporated in HMOs, without firstly being metabolized in the liver, after ^{13}C-labelled galactose was orally applied to lactating mothers (11).

UDP-Gal is then attached to Glc in the Golgi apparatus by a lactose synthase complex. The lactose synthase complex consists of 2 components: The A-protein and the B-protein. The A-protein is an α1-4-galactosyltransferase that transfers UDP-Gal to the terminal GlcNAc during glycoconjugate biosynthesis. The B-protein, so called alpha-lactalbumin, is dependent on lactation hormones and together with the A-protein they shift the acceptor specificity from GlcNAc to Glc to eventually form lactose (5). The further biosynthesis leading from lactose to HMOs is not fully understood yet, as there are great differences in structural composition of milk oligosaccharides compared to other mammalian species, making research in animal models difficult (5).

1.2 Effects of HMOs

More than 70 years of research has shown several health benefits of HMOs in particular for the breastfed neonate. However, the finding that HMOs can be detected in blood and in the urine early during pregnancy indicates further potential benefits for the pregnancy, maternal and fetal health (12,13).

1.2.1 Effects on the Neonate

1.2.1.1 HMOs as Prebiotics

A healthy intestinal microbiome plays a major role in human health. Therefore, efforts are made to manipulate the microbial composition with prebiotics to achieve health benefits. Prebiotics were defined as “a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” (14).

Around 1900, Moro in Heidelberg found a rapid predominance of bifidobacteria in the feces of breastfed infants (1). Later, in the 1950s, HMOs were found to be the bifidogenic factor stimulating the growth of bifidobacteria. The vast majority of HMOs ingested with human milk reaches the colon intact and in high concentrations, therefore they fulfill criteria of being prebiotics (5). In vitro, the oligosaccharide composition of the spent media showed that certain bifidobacteria species can metabolize HMOs. Studies found that *Bifidobacterium longum* subsp. *infantis* ATCC15697 shows very good growth, whereas *Bifidobacterium bifidum* ATCC29521, *Bifidobacterium breve* ATCC15700 and *B. longum* subsp. *longum* ATCC15707 does not grow well or not at all (15).

In co-culture experiments with *B. infantis*, *E. coli* and *Clostridium perfringens, B. infantis* was found to have inhibitory effects on the pathogens. Thus, not only inhibition by the competition for food supply but also so-called "postbiotics" (short-chain fatty acids and other metabolites) can create an environment in which health-promoting bacteria are keeping pathogens in check (5,16). HMOs start shaping the microbiome of a newborn immediately after birth, therefore, they can be called the original prebiotics.

1.2.1.2 Antiadhesive Function

Many body cells carry glycans on their surface, which are used by microorganisms as receptors for recognition and cell attachment. On the other hand, many viruses, bacteria and protozoa can mimic human glycans to bind to lectin receptors on the host's cell surfaces (17). HMOs have similar structures to these glycans on the cell surface, and thus, act as decoy receptors, resulting in an antiadhesive effect against some pathogens (17).

HMOs imitate the receptors for specific pathogens, and thus, their binding sites become occupied, hindering the attachment of the pathogen to the host cell. In various studies, a protective effect against *Streptococcus pneumonia, Listeria monocytogenes, Vibrio cholera, Salmonella fyris*, HIV, *enteropathogenic Escherichia coli*, and *Campylobacter jejuni* was detected. *Escherichia coli* and *Campylobacter jejuni* are responsible for a variety of diarrheal diseases and related to neonatal morbidity and mortality (2,18,19).

Here, especially α1–2-fucosylated HMOs (Fig.4) stand out: *C. jejuni* binds through type 2 H-antigens (2'-fucosylated glycans) to intestinal mucosa cells. In the presence of pooled HMOs or 2'-Fucosyllactose (2'FL), the colonialization of fresh human intestinal mucosa by *C. jejuni* was significantly lower than without HMOs. Studies have shown that *C. jejuni* shows much faster clearance in mice previously fed with pooled HMOs. Similar results were seen in mice from transgenic dams producing 2'-FL, which is not normally present in mouse milk (3). These experimental results were also consistent with observations in humans that showed a negative correlation between high concentrations of 2'-FL in breast milk and the prevalence of diarrhea in the newborn (2,3,5). Not only *C. jejuni* associated diarrhea but also Calicivirus diarrhea is found less often in nursing babies of mothers with a high level of Lacto-N-difucohexaose, which is also an α1–2-fucosylated HMO (Fig.4) (3).

Studies on bladder infections caused by UPEC (uropathogenic *Escherichia coli*) reported controversial results. One study claimed that HMOs can hinder UPEC from adhering to host cells (20), while the other one showed that adhesion of UPEC could not be prevented by HMOs (21). However, a reduced invasion of the bladder epithelium by the pathogens was found. Some HMOs, especially sialylated oligosaccharides, were shown to reduce UPEC mediated MAPK (mitogen-activated protein kinase) and NF-κB-activation (nuclear factor kappa B). The MAPK family can cause apoptosis in bladder and renal epithelial cells and NF-κB-activation, stimulated by *Clostridium difficile*, triggers apoptosis, junction degradation, and cell detachment within the intestinal epithelial monolayer (21).

Chichlowski et al. investigated the interaction between HMO-grown bifidobacteria and intestinal epithelial cells (IEC). In the experiment, the adhesion of *B. bifidum* and *B. infantis* (both HMO-grown) to Caco-2-cells (human colon adenocarcinoma) and HT-29-cells (human colorectal Ca with constant proliferation rate), was examined. Similar to previous reports, the authors showed that cultivation of the bacteria on HMOs had a significant influence on their ability to adhere to intestinal cells. After growing on HMOs, *B. infantis* showed an increased adhesion to HT-29 cells compared to bacteria grown on lactose (22). Other studies also demonstrated a modulated inflammatory response of the epithelial cells through reduced expression of several inflammation-related genes by HMO-grown Bifidus bacteria (23).

In conclusion, HMOs can play an important role in preventing attachment to and invasion of the epithelial lining by pathogens, and by reducing cell cytotoxicity. Previous results indicated that HMOs prevent several microbial infections and support a healthy urinary and gastrointestinal tract in breast fed infants.

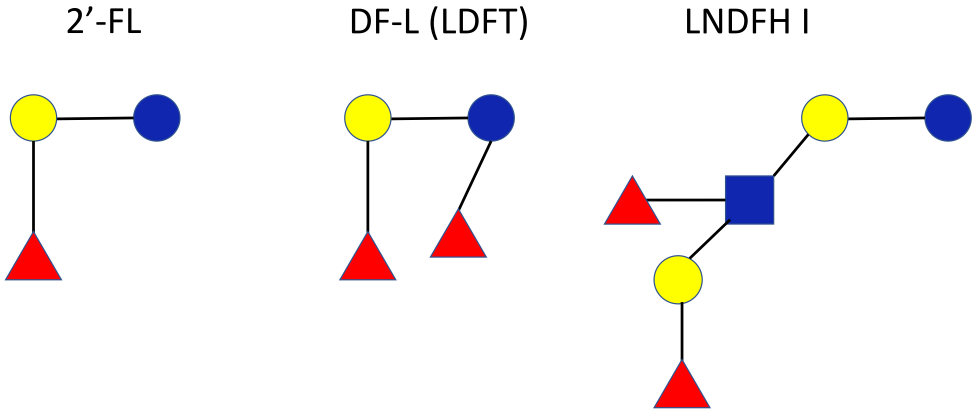


Figure 4. α1-2-fucosylated HMOs. Examples for α1-2-fucosylated HMOs that are known for antiadhesive effects on pathogens binding to epithelial cells. 2'-FL = 2'-Fucosyllactose; LDFT = Lactodifucotetraose; LNDFH = Lacto-N-difucohexaose.

1.2.1.3 Effects on Immune Responses

Newborns do not have a mature immune system. From birth onwards, the innate immune system has to take on the first defense since the adaptive immune system is not yet trained to recognize pathogens due to immature effector B- and T-cells (23,24).

The innate immune system consists of physical barriers and the cellular and humoral immunity. It provides the first line of protection against pathogens but does not generate an immune memory. Again, it is the gastrointestinal tract that plays a major role also in neonatal immunity. The intestinal epithelium functions as a barrier preventing invasion of the host by intestinal microbes (24).

Of all the effects of HMOs mentioned, the modulation of the immune system is perhaps the most remarkable one. As mentioned before, through modulating the gut microbiota, HMOs have an indirect influence on the immune system, but there is also a direct effect. After being absorbed in the intestinal tract, HMOs enter the bloodstream unmodified and operate on a systemic level via the modulation of immune cells (24,25).

Fucose-containing HMOs regulate the immune response by influencing different cell populations. These are primarily Th2-promoting (humoral immunity) activities, which have a generally anti-inflammatory effect. 2'FL, for example, lowers the levels of the mRNA and the membrane-bound CD14, which is a co-receptor for bacterial lipopolisaccharides (LPS) and, therefore, leads to a reduced LPS-induced inflammation. In addition, fucosylated HMOs are supposed to lower the levels of pro-inflammatory macrophage-derived IL-12 and IFN-γ and, on the other hand, to increase IL-10, which promotes Th2-polarization in vitro (24). LNFP III stimulates macrophages, which lead to activation of natural killer-(NK) cells. The NK-cells in turn, are responsible for the lysis of malignant and virus-infected cells (24).

LDFT lowers vascular inflammation through depression of thrombin-induced release of platelet-derived pro-inflammatory proteins (24).

Sialylated HMOs also have an intestinal protective effect by preventing pathogens from adhering to the epithelium. In addition, in vitro experiments with human whole blood have shown the stimulation of Th2 cells. Downregulation of inflammation via the reduction of platelet-neutrophil complex (PNC) formation and neutrophil activation suggests that sialylated HMOs play a role in the prevention of Necrotizing Enterocolitis (NEC) (Section 1.2.1.4) (24).

Symptoms of food allergies could also be influenced by HMOs. In the mouse experiment, an increase in regulatory T cells (Tregs) in mesenteric lymph nodes (MLN) and Peyer's patches has been observed (24,26).

1.2.1.4 Necrotizing Enterocolitis

Necrotizing Enterocolitis (NEC) is a common and, in many cases, fatal intestinal disease in preterm infants. The incidence of NEC ranges from 5 to 10% in very-low birth weight infants, whilst the mortality rate corresponds up to 25% among the NEC cases (27,28). Surviving children often also suffer from longtime neurological complications (27).

NEC occurs 6-10 times less frequently in breastfed children compared to those fed on formula without HMOs (3). Thus, HMOs were thought to contribute to the protective effect of human milk.

One of the hallmarks for the disease is neutrophil infiltration of the mucosa. The initial hypothesis was that HMOs block neutrophil infiltration protecting the intestinal mucosa against inflammation through inhibition of leukocyte rolling, adhesion and activation. A study using a neonatal rat model showed protective effects of HMOs supplemented in formula whereas rats fed formula without HMOs developed NEC. The protective effect was found to be resulting from a single disialylated HMO, more specifically disialyllacto-N-tetraose (DSLNT). Both sialic acids of the molecule were needed for the protective effects (29). However, the exact mechanism of action to protect infants against NEC still needs to be investigated. (3,5).

1.2.2 Effects on the Mother

HMOs have numerous well-known health benefits for children, including protective effects against NEC and diarrhea, as well as a reduction in the risk of allergy in breastfed children. However, recent studies suggest that HMOs can also have effects on the lactating mother. Breastmilk is not sterile. In recent years a human milk microbiome has been described that not only affects the health of the child, but also the health of the lactating mother (5). It has long been known that HMOs are detectable in urine already in early stages of pregnancy (12). Radioimmunoassay was used to detect certain HMOs in the urine, an indirect evidence for presence of HMOs in the systemic circulation (12,30). The fact that HMOs can be detected in the mother's urine already in the 13th week of pregnancy, and therefore, systemically, suggests that HMOs can also have effects on the mother-to-be or on the pregnancy (12,13). An explanation for HMOs being found in the systemic circulation so early in pregnancy might be the generally increased epithelial permeability in the body of the pregnant woman (31,32). The main focus on HMO effects on the mother is on systemic effects, which also have been found in nursing infants. Both, in vitro and in vivo studies have shown immunomodulating effects. HMOs have an inhibitory effect on many receptors and cells in the immune system and can thus, weaken a strong immune response. On the one hand, this can inhibit an immune response of the mother's immune system against the fetus during pregnancy. On the other hand, pregnancy induces a lowlevel inflammation, which persists throughout pregnancy. HMOs can potentially balance this inflammation and keep it at a controlled level through their influence on immune cells (13).

The anti-adhesive and antimicrobial effects of HMOs might also be defending the mother's urogenital tract from infections during pregnancy. Indeed, several in vitro and animal studies have shown the protective effects of HMOs against UPEC or other pathogens causing UTIs. Since infections are a major risk factor for PTB, this would also limit the unborn's risk of being born prematurely (13,21,33,34).

Similar to effects in the infant, maternal HMOs might have an impact on the urinary or vaginal microbiome and thus, benefit the pregnancy. We speculate that there is a link between the serum and urine HMO profiles and the (healthy) microbiome, and thus between HMOs and individual risk factors for PTB (35).

1.3 Preterm Delivery

By definition preterm birth (PTB) is to be born alive before 37 completed gestational weeks, where 4 subcategories can be distinguished:

- extreme preterm (less than 28 weeks of gestation)
- very preterm (28 to 32 weeks of gestation)
- moderately preterm (32 to 33 weeks of gestation)
- late preterm (32 to 37 weeks of gestation) (36)

About 15 million babies are born preterm every year worldwide. This means that 1 out of 10 babies is born too soon and the number is rising in nearly every country with available data, while the causes remain unclear (37).

Approximately 1 million newborns die of complications from preterm delivery each year. This means preterm delivery is the leading cause of death in the under 5-year age group and also in the perinatal and neonatal period. The highest numbers occur in Southern Asia (25.3%) and in sub-Saharan Africa (12.1%) (38). Low income countries have massively higher death rates among under five-year olds. The reason for this is the lack of affordable and feasible medical care. Thus, more than half of the deaths could be prevented by cheap and simple treatments like “kangarooing” (skin-to-skin contact with a parent and frequent breastfeeding) or either by IM dexamethasone or IM betamethasone, which are recommended as the antenatal corticosteroid of choice when preterm birth is imminent (39).

The reasons for preterm birth are numerous and sometimes idiopathic (40). Yet, in about only half the cases, the causes are identifiable. The medical conditions leading to preterm birth include infections or inflammations, vascular diseases and uterine overstretching (40). In other cases, the pregnant woman has more or less direct influence via lifestyle factors (smoking, alcohol and drug use, high stress levels and long working hours, late or no prenatal care, lack of social support). Some demographic factors such as low socioeconomic status or age <17 and >45 are also discussed as risk factors (36).

According to recent studies, WES (whole exome sequencing) and whole genome sequencing are promising in terms of genetic influences on PTB in context of pathologic inflammation. Strauss et al. have shown evidence of genetic predisposition as a cause of preterm labor and PTB. Amongst other things, familial aggregation without other explanations, evidence of disease susceptibility genes and racial disparities in premature births support this evidence (40).

Despite all this, no precise explanation for the increasing rate of spontaneous premature births worldwide could be found. How these PTBs and their consequences can be prevented remains yet to be investigated.

1.3.1 Vaginal Microbiome and Preterm Delivery

During the past few years, many studies have taken a closer look at the vaginal microbiome and its impact on maternal health. The species composition is determined using 16S rRNA gene sequencing (41). Ravel et al. examined vaginal samples from nearly 400 asymptomatic women and determined 5 community state types (CSTs) according to community composition. A Lactobacillus species was dominant in 4 out of 5 of the CSTs. In the 5th group, the most abundant was a strictly anaerobic organism. However, common to all is the production of lactic acid. Median pH levels were different, depending on which Lactobacillus species was the dominant one. This either indicates that the individual taxa produce different amounts of lactic acid or that they show different buffering capabilities (41).

A core microbiome of the vagina could not be found. Although there was a predominant Lactobacillus species in 4 groups, the vast majority of women tested also showed other lactic acid bacteria (41). In a further analysis, the ethnic groups of the subjects were compared, and the results have shown significant differences between the population groups. The dominance of different Lactobacilli also led to differences in the pH values of women. For example, pH was significantly higher in Black and Hispanic (4.7 and 5.0) than in White and Asian women (4.2 and 4.4). It is known that production of lactic acid and a low pH in the vagina can be protective against bacterial vaginosis, but based on the results, it cannot be concluded that White and Asian women in the study population were healthier than Black and Hispanic women. However, it was very clear that the diverse CST (not dominated by Lactobacillus species) was more common in Hispanic and Black women. Other studies that compared the microbiome between ethnic groups came to the same conclusion (41–43). The difference in the vaginal microbiome and, thereby, the higher likelihood of bacterial vaginosis, may lead to a higher risk for PTBs in Black women.

Fox et al. investigated the differences in the microbiome of nongravid and gravid women and concluded that the microbiome is less variable and more stable during pregnancy than in non-pregnant women. The susceptibility to changes in vaginal microbial communities is most likely influenced by sexual activity, hormonal fluctuations, but also age. Although the microbiome of the pregnant woman is also subject to changes there is, above all, an increase in Lactobacillus spp. (*L. crispatus, L. jensenii, L. gasseri,* and *L. vaginalis*) and a decrease in anaerobic bacteria (43). Lactobacilli abnormalities and disorders can lead to dysbiosis and bacterial vaginosis during pregnancy, which in turn are strongly related to PTB (43,44). Pathogens that are known for their association with PTB are *Ureaplasma urealyticum, Mycoplasma hominis, Bacteroides spp., Gardnerella vaginalis*, and *Fusobacterium nucleatum*. Although none of these bacteria has a high level of virulence in their original habitat, they are very likely to lead to infections if they enter the intrauterine environment (43). Another American prospective cohort study on 88 women found a correlation between vaginal microbial diversity and PTB. In addition, the researchers also came to the conclusion that the ethnicity of the mother has an influence on the microbiome (43,45).

1.4 HMOs in Urine

The fact that HMOs can be detected in maternal urine and blood very early on in pregnancies suggests that they provide a benefit on pregnancy and maternal health during this time. Already in 1976, Hallgren et al. examined urine samples of pregnant women with combined gas-liquid chromatography-mass spectrometry for HMOs and quantified them by detecting Fucose. The authors found HMO excretion starting at the 13th week of pregnancy (12). They divided the HMOs into 3 groups: fucosyl- and sialyl-oligosaccharides and those that contained neither Fucose nor Sialic Acid. The secretor status and the Lewis blood group could be determined based on the Fucose in specific HMOs.

Other studies from the 1960s measured HMOs in urine over the duration of pregnancy and lactation and also came to the conclusion that HMOs peak during lactation (12). HMOs were detected in urine of all patients (12). Hallgren et al. compared the urine of a blood group A, secretor woman (46) with that of a blood group 0, non-secretor woman (47). Twice as many milk oligosaccharides (20 vs. 9 HMOs) could be characterized in the urine of the secretor woman. It is interesting to mention here that in the non-secretor woman, oligosaccharides were detectable at the earliest time point investigated during pregnancy, with Lactose and 3-Fucosyllactose present in the highest concentration. In addition to these two glycans, they found Lacto-N-tetraose, LNFP-I and LNFP-II as well as 2 Fucohexaoses increasing most significantly during pregnancy. The increase during lactation was even more significant (46).

In the analyzed blood group A, secretor woman, the oligosaccharides peaked at week 31 during pregnancy but decreased towards the end of pregnancy. After giving birth, there was again a sharp increase in lactose and all HMOs containing a lactose backbone. On day 2 of lactation, the HMO peak detected in week 31 could be reached again, but after that HMOs finally started decreasing. (12,47).

One woman in a small longitudinal study cohort had a miscarriage during the 24th week of gestation. Up to this point, her pregnancy had developed normally. However, gel chromatography analyses showed that her HMOs from gestational weeks 11, 15 and 19 were comparable to the oligosaccharide distribution of a healthy, non-pregnant patient with the same blood group (12). The authors thus, speculated about a potential importance of HMOs for a healthy pregnancy.

1.4.1 Urinary Microbiome in Pregnancy

The dogma, that in healthy people, urine was sterile persisted until the 1950s (33). Especially in urology, one assumed for a long time that bacteria would be present only in a pathological setting. The relationship between some intestinal bacteria and the appearance of kidney stones has been described before (48,49). Recent studies show both, positive and negative health effects on detected bacteria in the urethra and bladder (33).

The Human Microbiome Project (HMP) is responsible for the first large-scale mapping of the microbiome using culture-independent methods. Originally, the bladder was not examined, since it was still assumed that it was not colonized by bacteria in healthy people, which is why the research is a few years behind compared to other body regions (34,50,51). One problem was that the culture techniques were only able to detect fast growing aerobic bacteria such as the uropathogenic E. coli (UPEC). Another problem was the possible vulvo-vaginal contamination in the collection of samples through the urethra. Here, one made use of suprapubic aspiration (SPA). In comparison of bacterial DNA from SPA and TUC-(transurethral catheter) urine, profiles were very similar, but compared to vaginal swabs they differed greatly, which is why TUC is now the collection method of choice (50).

Next was to clarify whether the bacterial DNA in the urine was living bacteria or residues of dead cells being flushed out. By means of 16S sequencing different taxa could be identified. These were also cultivable, but not under normal conditions. The enhanced quantitative urine culture (EQUC) protocol has found ways to cultivate urinary organisms. This again confirmed that the DNA found in urine was coming from living bacteria (50,52).

Whether disturbances in the urinary microbiome are involved in a higher risk for PTB is not known. A study found a correlation between Serratia marcescens and PTB, with one out of four operational taxonomic units (OTU) of urinary microbes being heavily colonized by S. marcescens. This bacteria is known as an opportunistic pathogen, and can lead to urinary tract infections, urethral catheter infections, and is a common source of nosocomial infections (53,54).

In a case control study from 2016 with 97 patients, no significant difference in α-or β-diversity between cases and controls was found. However, OTUs of certain bacteria (*Prevotella, Sutterella, L. iners, Blautia, Kocuria, Lachnospiraceae,* and *S.marcescens*) were more common in patients who delivered before gestational week 37+0 and women who had higher counts of *S. marcescens* in their urine were more susceptible for PTB (53).

Prevotella, a potential uropathogen commonly found in the vagina, have been associated with UTIs and bacterial vaginosis in several previous studies and have also been found in mothers who gave birth prematurely. The situation is different, however, in a study on African American mothers in whom *Prevotella* (measured in the vagina) was not associated with premature birth (53,55).

Sutterella and *Blautia* are bacteria that are very commonly found in stool and could therefore have ended up in the urogenital tract due to transmission from the rectum (53).

A definitive statement about the direct influence of the urinary microbiome on PTB cannot yet be made. However, a connection of some pathogenic microbes in the genitourinary tract and premature birth has been confirmed in numerous studies (56). This is in line with the results of meta-analyzes, which state that UTIs during pregnancy, despite accounting for several confounders such as age and parity, frequently lead to IUGR, gestosis and PTB (57).

2 Hypothesis and Aims

In newborns and mothers, numerous health benefits of HMOs have been demonstrated. We suspect a relationship between the concentration levels of individual HMOs in the maternal circulation and the risk for premature labor and thus, also preterm birth (PTB). While in some cases the cause for PTB is known, we investigated cases that cannot be explained by UTIs, medical conditions or other factors associated with preterm labor.

We hypothesize that the **HMO profile in the mother's blood during pregnancy has an impact on the risk for premature labor**. In this thesis, we aimed to find potential differences in concentration of individual HMOs between groups of women, formed based on their high or low risk factors for PTB (short vs. normal cervix, preterm labor vs. no preterm labor) or the incidence of PTB vs. term birth. In a larger study, we also examined the microbiome in urine and the vagina and other parameters of the same patients to find how HMO concentrations might influence bacterial taxa, which are associated with PTB.

3 Material and Methods

3.1 Study Design and Setting

This diploma thesis was part of a larger case-control study on the associations of the urinary and vaginal microbiome with maternal prenatal HMOs and metabolites in idiopathic preterm labor. The main focus of this thesis was on the HMOs and their potential influence on the risk for preterm birth. Therefore, a total of 60 women were recruited at the Department of Obstetrics and Gynecology between January 2017 to May 2018. All women came to the hospital due to suspected preterm labor during their gestational weeks 23+0 to 34+0.

The separation into cases and controls was made after medical examination. To diagnose preterm labor, cardiotocography (CTG) examinations were carried out in order to objectively determine the uterine contractions. In addition, a transvaginal ultrasound was performed to measure the cervical length and to differentiate between shortened cervix (<25mm) and normal cervical length (>25mm).

Tocolysis was administered when 4 or more contractions occurred within 30 minutes, which were felt by the pregnant woman and recorded with the CTG. For tocolytic treatment, Atosiban, an Oxytocin-receptor antagonist, was used.

Our main criterion for separation between cases and controls was tocolytic treatment vs. no tocolysis. Since the medical decision for tocolysis cannot be reached completely objectively and is, to some extent, dependent on the physician in charge, we also analyzed groups formed between patients with shortened and normal cervix.

In the groups formed based on tocolysis treatment, 3 patients with a shortened cervix were grouped with the controls, and 2 of them delivered preterm a few days later. These patients received no tocolysis, because they were close to a gestational age of 34 weeks (33+5 and 33+6 weeks) at the time of admission. From 34 weeks onwards, the guidelines recommend only fetal lung maturation medication. In addition, one of the patients had PPROM (preterm premature rupture of the membranes) and tocolysis would be contraindicated in this case.

3.1.1 Inclusion and Exclusion Criteria

Inclusion Criteria

- Healthy pregnant women
- Hospital admission due to potential preterm labor
- 18 years or older
- Viable pregnancy in gestational weeks 23+0 to 34+0
- Giving informed consent to all aspects of the protocol

Exclusion Criteria

- Women previously diagnosed any genitourinary infections
- Women who received treatment against genitourinary infections
- Multiple pregnancy
- Any known fetal anomalies associated with possible growth or genetic anomalies
- Patients with pre-pregnancy diabetes type 1 or 2 or gestational diabetes (GDM)
- Pre-pregnancy hypertension
- Any antibiotic/prebiotic treatment in the last 6 months

3.1.2 Sample Collection

The blood samples (collected with Vacuette-System), urine samples and vaginal swabs were taken before carrying out any therapy or treatment. The collection of samples was done by nurses or trained personnel and all samples were frozen at -80°C within 4 hours.

3.1.3 Data Collection

From some patients, especially in the control group, not all the data could be collected at our gynecological department as many of them gave birth in other hospitals. In these cases, our team tried to determine the missing data in a follow-up. This was primarily data such as the actual gestational age at birth and infant data such as Apgar score and birth weight. Due to the follow-up, we were able to work with complete datasets from almost the entire study population.

3.2 HMO Analysis

3.2.1 HMO Isolation from Human Serum

For the separation and identification of the oligosaccharides, the serum samples were thawed on ice and then 50µl of the sample was added to 250µl H_2O and 100µl internal standard (Gal-Lac, linear B6 Trisaccharide, Dextra UK). The first step was to remove proteins through chloroform/methanol (2:1) extraction. Next, a deproteination by solid phase extraction (SPE) using HyperSep C18 columns (Thermo Fisher Scientific) was carried out, and HMOs were desalted with HyperSep Hypergraph SPE columns (Thermo Fischer Scientific).

The samples were then dried down before they were labeled with 2AB (2-Aminobenzamide). The excess 2AB was removed using HyperSep Silica columns (Thermo Fisher Scientific) and after another dry down the samples were taken up with 50µl HPLC buffer B for HPLC (High Performance Liquid Chromatography) separation.

3.2.2 HMO Analysis

The HMO samples were then analyzed using HPLC (High Performance Liquid Chromatography), a chromatographic method to separate substance mixtures, to identify, quantify and purify individual components. The separation is based on the interaction of the analyte with the solid phase (column material) and the liquid phase (solvent) (58). The HMO samples were separated using HPLC and monitored using fluorescence detection at the Center for Medical Research of the Medical University of Graz. The HMOs were separated with an ammonium formate – acetonitrile buffer system and monitored with a fluorescence detector at 360nm excitation and 425nm emission. The area under the curve (AUC) for the individual HMOs was calculated from the resulting chromatogram and normalized to the internal standard. The values obtained were then used for further analyses. The secretor status was determined on the basis of the 2’FL and LDFT concentrations.

3.3 Data Management

A study ID number was assigned to the patients to protect sensitive data. Only this anonymized study ID was used for the statistical evaluations.

3.4 Data Analysis

3.4.1 Statistical Analysis

The data was analyzed using descriptive statistics and selected tests. Shapiro-Wilk test and Kolmogorov-Smirnov test were used to test for normal (Gaussian) distribution. Some variables did not follow a normal distribution, so nonparametrical statistical methods were chosen for these analyzes. Spearman's rank correlation coefficient (Spearman's rho) was used to analyze bivariate correlations.

All statistical evaluations were carried out using GraphPad Prism Version 8.3.0. GraphPad Prism and Microsoft Excel software were also used to create figures and tables. The level of significance was set with $p < 0.05$ for all statistical analysis.

3.5 Ethics Approval

Approval by the Ethics Committee is required for any research with human material. Our study protocol has been approved by the ethical committee of the Medical University of Graz under the EK number 28-525 ex 15/16.

4 Results

We performed descriptive statistics and association analyses to find associations between the concentration of HMOs and the different indicators and risk factors for preterm birth (PTB).

4.1 Study Population

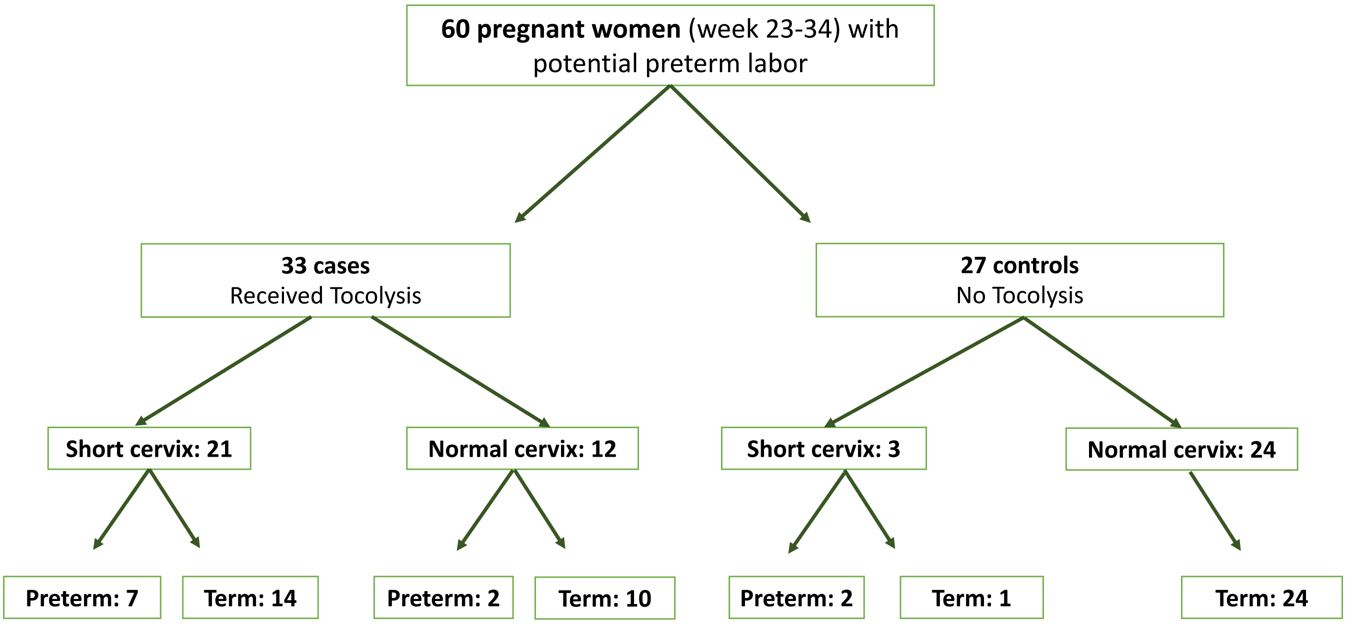


Figure 5. Study population. 33 cases and 27 controls fulfilled the inclusion criteria and were included in the study after giving informed consent.

For the study, 60 women were recruited at the hospital with potential preterm labor between the gestational age of 23+0 to 34+0 weeks. After medical examinations (CTG control and transvaginal ultrasound to measure cervix length) the patients were grouped into "cases" with confirmed preterm labor (and short cervix) and "controls" without confirmed preterm labor. The women in the case group received Atosiban for tocolysis.

In the control group, two patients delivered preterm shortly after admission (Fig.5), but did not receive tocolytic treatment, despite having contractions at the time of recruitment. In these two cases, it was decided not to give tocolysis, as the medication is recommended up to gestational age 34 + 0 (59), and these patients were one and two days before this gestational age on the day of admission. Excluding these patients from analysis did not change the results. HMO analyses were done for 52 patients (n=52). For calculations, we always used the largest number of complete data sets available.

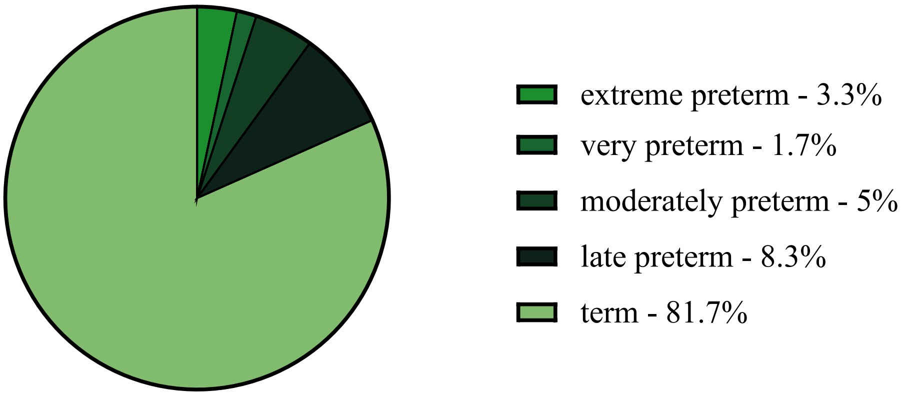


Figure 6. Proportion of preterm births in the study population. 82% of the babies in the study population were born after gestational week 37+0 (term); 3.3% were born before week 28+0 (extreme preterm); very preterm is 28 to 32 weeks of gestation (1.7%), moderately preterm is 32 to 33 weeks of gestation (5%), late preterm is 32 to 37 weeks of gestation (8.3%) (36).

Figure 6 shows the distribution of premature babies and term born babies in the total study population. Nearly 82% of the babies were born term (after gestational week 37+0). 8.3% or 5 babies were late preterm births, including the 2 from the control group, where the mothers received no tocolysis due to their late gestational age. The remaining preterm born babies were in the case-group. A total of 5%, 3 babies, were born at gestational age 32 to 33 weeks, with 1.7%, or 1 baby, belonging to the very preterm group, and 2 babies, or 3.3%, born extreme preterm, before gestational week 28+0. However, in the case group only 27% of the babies were born preterm and the majority was born on term.

4.1.1 Baseline Characteristics

Table 2. Baseline characteristics of cases and controls in the study population. Patients were grouped in cases who received tocolytic treatment and controls who did not receive tocolysis. Mann-Whitney-Test was used to test for differences between the groups. Level of significance $p < 0.05$;

		Cases			**Controls**		
	n	*Mean*	*SD*	*n*	*Mean*	*SD*	*p-value*
Maternal Age (years)	33	29.45	5.1	27	29.00	5.7	0.517
Gestational age at sampling (days)	33	201.5	23.53	27	199.6	22.53	0.771
Cervical length (mm)	33	22.45	9.5	27	34.92	9.9	**<0.0001**
Gravida	33	2.2	1.5	27	2.1	1.2	0.881
Parity	33	0.667	0.96	27	0.815	0.96	0.46
Miscarriages	33	0.55	1.1	27	0.33	0.6	0.876
BMI before pregnancy (kg/m^2)	33	22.27	3.6	27	24.34	4.6	0.106
CRP (mg/L)	33	7.43	7.59	27	9.76	17.14	0.845
Gestational age at birth (days)	33	261.4	30.7	27	273.6	12	0.453
Birth weight (grams) mv	32	2524	1289	25	2462	1464	0.991
Leukocytes (G/L)	33	13.77	9.1	27	10.80	3.3	0.061
Secretors (%)	33	87.9%		27	77.7%		0.488
Ethnicity: *Caucasian*	32	97%		23	85.2%		0.141
Asian	0	0%		3	11.1%		
African	1	3%		1	3.7%		

mv = missing values; SD = Standard Deviation; statistically significant values are in **bold** ($p < 0.05$).

In addition to medical data, socio-demographic characteristics and information on variables relevant to pregnancy were also queried during recruitment and thereafter. The characteristics were shown for cases and controls, according to whether the patient had received tocolysis or not.

Table 2 shows numbers of cases (n) as well as mean and standard deviation in the respective group. Using the Mann-Whitney test, the variables were checked for differences between the groups. The two groups did not significantly differ in the maternal age and gestational age at sampling, which speaks for a good distribution. The only variable that showed highly significant differences was cervical length. On average, cervical length was around 12mm shorter in cases, compared to controls.

The average number of miscarriages in the case group (0.55) was higher than in the controls group (0.33). One patient stated that she had 5 miscarriages. If this outlier was excluded from analyses, the range in both groups was 0 to 2 abortions, the mean in the cases was then 0.4. The BMI before pregnancy was slightly higher in the control group, but the p-value was below the level of significance. Interestingly, the CRP in the controls was initially higher than in the cases, but this was due to one patient with CRP 86 mg/L. When this patient was excluded from the analysis, the mean for CRP was 6.82 in the controls and thus, lower than in the cases group (7.43). However, there was no significant difference in CRP with or without the outlier.

As an additional inflammation marker, we used leukocyte counts at the time of sampling for analyses. Here, the counts in the case group were increased (13.8 G/L) compared to the controls (10.8 G/L). Testing for significance with Mann-Whitney test, results were near the significance limit (p= 0.06).

The gestational age at birth in the control group was 10 days higher than gestational age in the case group, which is due to the fact, that almost all patients in the control group gave birth after week 37+0. On average, babies of the control patients were born in the 40th week of pregnancy, babies in the case group in the 38th week of pregnancy.

It is interesting to note that the birth weight was higher in the cases than in the controls, despite the lower average birth age. However, there was no significant difference here either.

While the share of secretors in the controls roughly corresponded to the number in the total European population, the share of secretor positive women in the case group was slightly higher with 88%. However, the difference was below the level of significance. Finally, we compared the patients according to their ethnicity. Since the patients were recruited in Graz, the majority of the patients were Caucasians, with similar distribution of other ethnicities (Asian and African) in both groups without significant differences.

4.1.2 Distribution of Previous PTBs in the Study Population

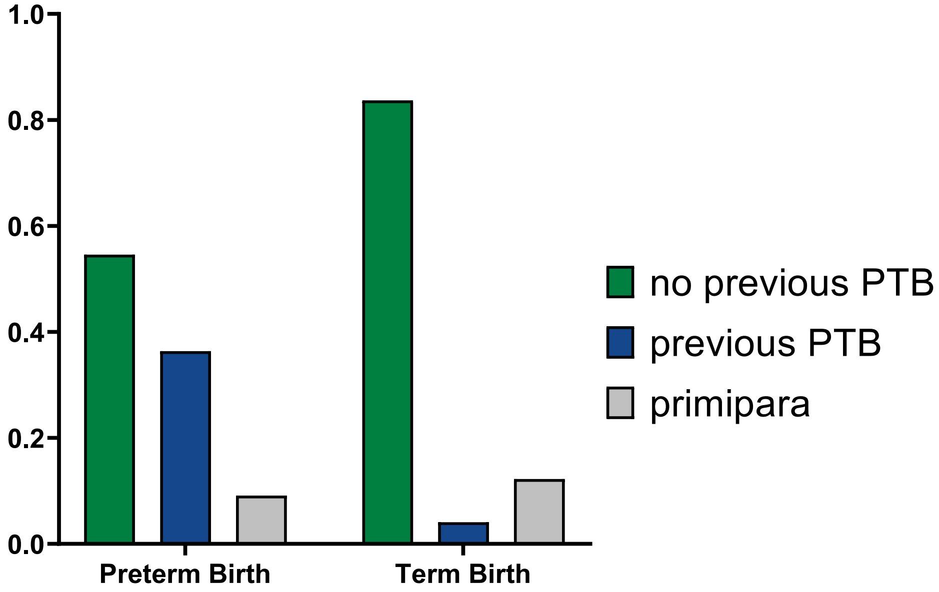


Figure 7. Proportion of women with previous preterm birth in the study group. Preterm Birth: no previous PTB (6 patients), previous PTB (4), primipara (1)**;** Term Birth: no previous PTB (41), previous PTB (2), primipara (6)

Table 3. Fisher's exact test of previous PTB and no previous PTB compared.

Data analyzed	**no previous PTB**	**previous PTB**	**Total**
Preterm Birth	6 (60%)	4 (40%)	10
Term Birth	41 (95.35%)	2 (4.65%)	43
Total	47	6	53

7 patients missing, these had no previous births; p= 0.0087; **p<0.01; Fisher's exact test was used because sample size was too small for Chi^2 test;

Figure 7 and Table 3 display the distribution of pregnant women who had given birth prematurely in the past. Figure 7 divides the study population into patients who gave birth preterm and term in the current pregnancy. In both groups, the proportion of women who had a PTB in a previous pregnancy (blue column) is shown. The green column shows the proportion of women who never gave birth preterm before. The primipara (grey column) were not included in Fisher's exact test (Tab. 3) to prevent bias.

Table 3 only shows the PTBs and term births in patients who had given birth before. In the PTB group 4 patients (40%) had a previous PTB and 2 patients in the term birth group (4.65%). In order to test the two groups for significant disparity in the occurrence of PTB, Fisher's exact test was carried out. Chi^2 test would be imprecise due to the small number of samples. The Fisher’s exact test shows a highly significant difference ($p = 0.0087$) when comparing the occurrence of PTB in the two study groups. Overall, we had 49 patients giving birth at term and 11 patients giving PTB.

4.2 HMO Analyses

4.2.1 HMO Concentrations in the Serum of Pregnant Women

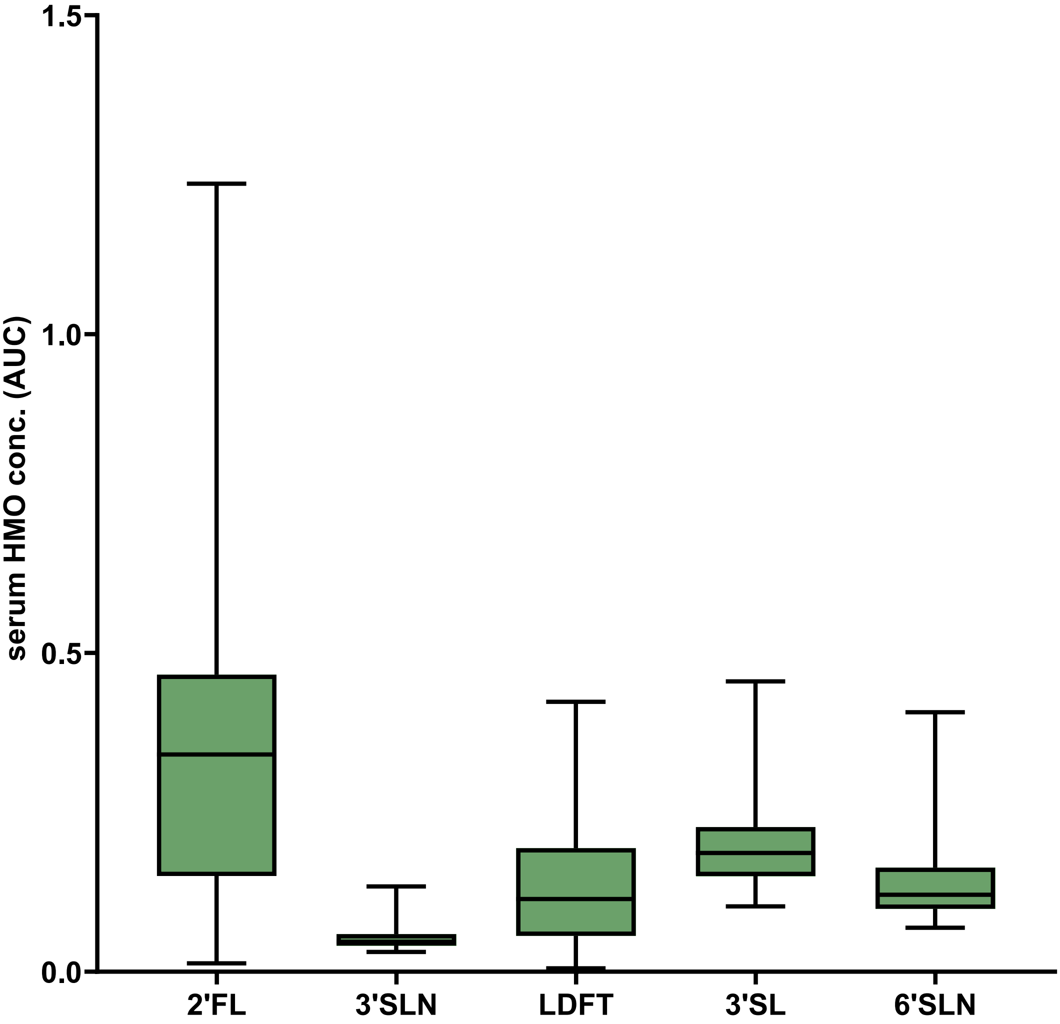


Figure 8. Serum HMO concentrations in pregnant women. Whiskers displaying min to max, boxplots displaying quartiles and median range for the 5 most abundant HMOs in blood serum of all women included in the study. 2’FL = 2’-Fucosyllactose; 3’SLN = 3’-Sialyllactosamine; LDFT = Lacto-N-difucotetraose; 3’SL = 3’-Sialyllactose; 6’SLN = 6’-Sialyllactosamine; n=52.

We analyzed the 5 most abundant oligosaccharides in maternal serum at the time of sampling (gestational age 23+0 to 34+0). Figure 8 shows boxplots and whiskers displaying the concentrations of 2’FL, 3’SLN, LDFT, 3’SL and 6’SLN of all patients in the study group at the time of sampling. The broadest range was found for 2’FL, as this oligosaccharide, as previously described, is highly dependent on the secretor status of the mother. However, due to the size of the study group, we analyzed secretors and non-secretors together. 3’SLN is the HMO with the lowest range in concentration.

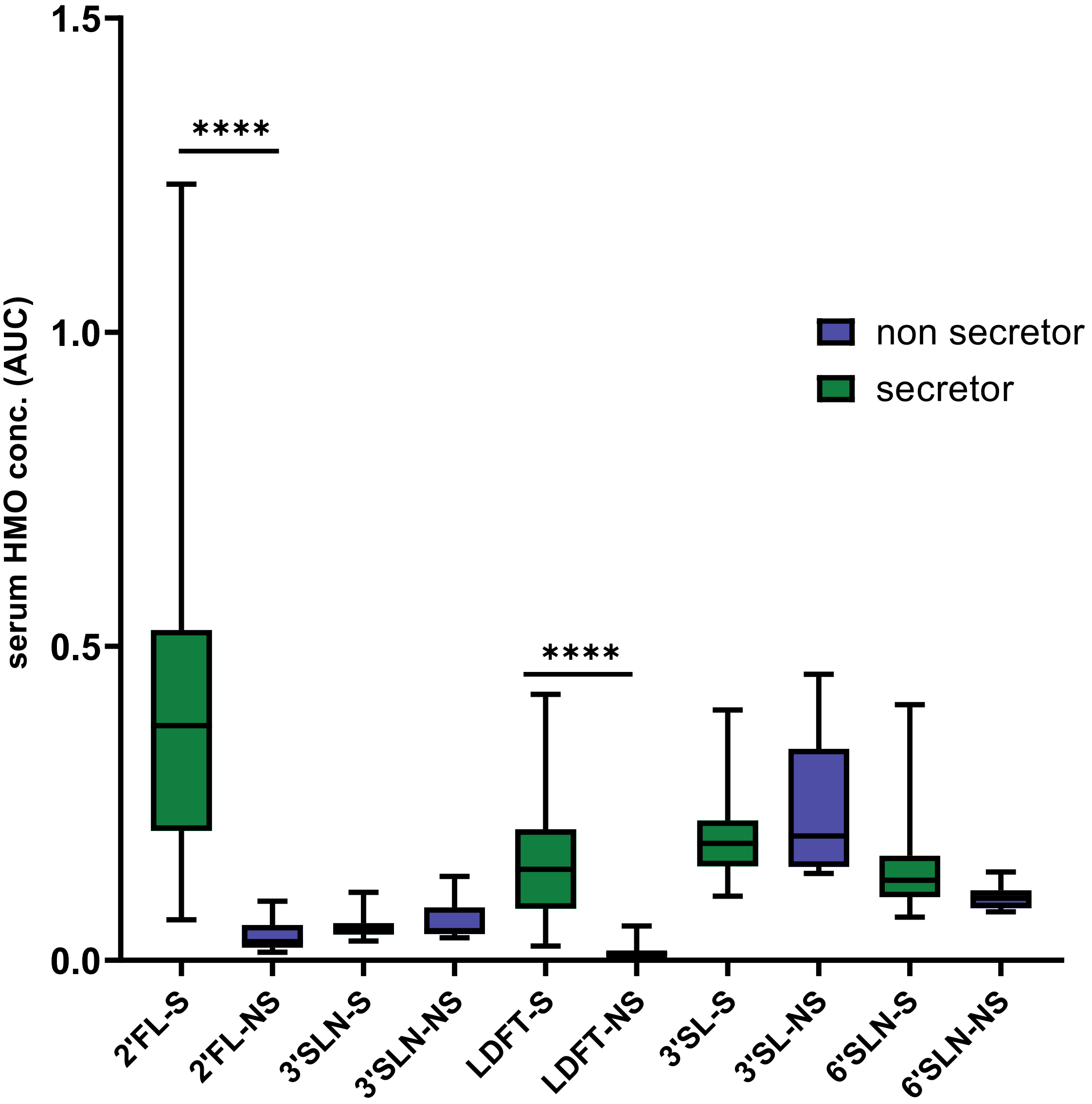


Figure 9. Serum HMO concentrations in pregnant women, comparison of secretors (green) and non-secretors (blue). Strong significance appears for 2’FL and LDFT concentrations; secretor status was assigned based on 2’FL abundance; ****$p < 0.0001$; S = secretors; NS = non-secretors; secretors n=44, non-secretors n=8.

In Figure 9, the HMO concentrations are shown for secretor (green) and non-secretor (blue) mothers separately. We assigned the secretor status based on the 2’FL concentration in serum. 2’FL is the most abundant HMO in most secretor mothers and is nearly absent in non-secretors. LDFT levels are also very low in non-secretor blood as one of the fucosylations in LDFT is an α1,2-fucosylation and thus, dependent on the Fucosyltransferase-2 (FUT2), which is not expressed in non-secretors. Table 4 shows minimum and maximum of HMO levels, as well as the mean and standard deviation for the data used for further analyses. All HMO concentrations are shown as area under the curve (AUC) values normalized to an internal standard. The sialylated HMOs show differences in their range, but their means were not different between secretors and non-secretors (Tab. 4). In the study population, we had 50 secretors (83.3%) and 10 non-secretors (16.6%), which corresponds to the average population in Europe (80% secretors vs. 20% non-secretors) (8,60).

Table 4. HMO concentrations in study group. Data used for analyses.

	n	Minimum	Maximum	Mean	SD
2'FL-S	44	0,0648	1,236	0,4003	0,2308
2'FL-NS	8	0,0131	0,0941	0,0383	0,0272
3'SLN-S	44	0,0309	0,1085	0,0502	0,0140
3'SLN-NS	8	0,0359	0,1337	0,0619	0,0343
LDFT-S	44	0,0227	0,4233	0,1538	0,0876
LDFT-NS	8	0,0053	0,0545	0,0146	0,0165
3'SL-S	44	0,1024	0,3988	0,1942	0,0660
3'SL-NS	8	0,1382	0,4554	0,2405	0,1146
6'SLN-S	44	0,069	0,4069	0,1399	0,0604
6'SLN-NS	8	0,0774	0,1406	0,1006	0,0202

HMO levels in [AUC norm]; SD = Standard Deviation; S =secretor; NS = non-secretor.

4.2.2 Serum Concentrations of HMOs – Cases vs. Controls

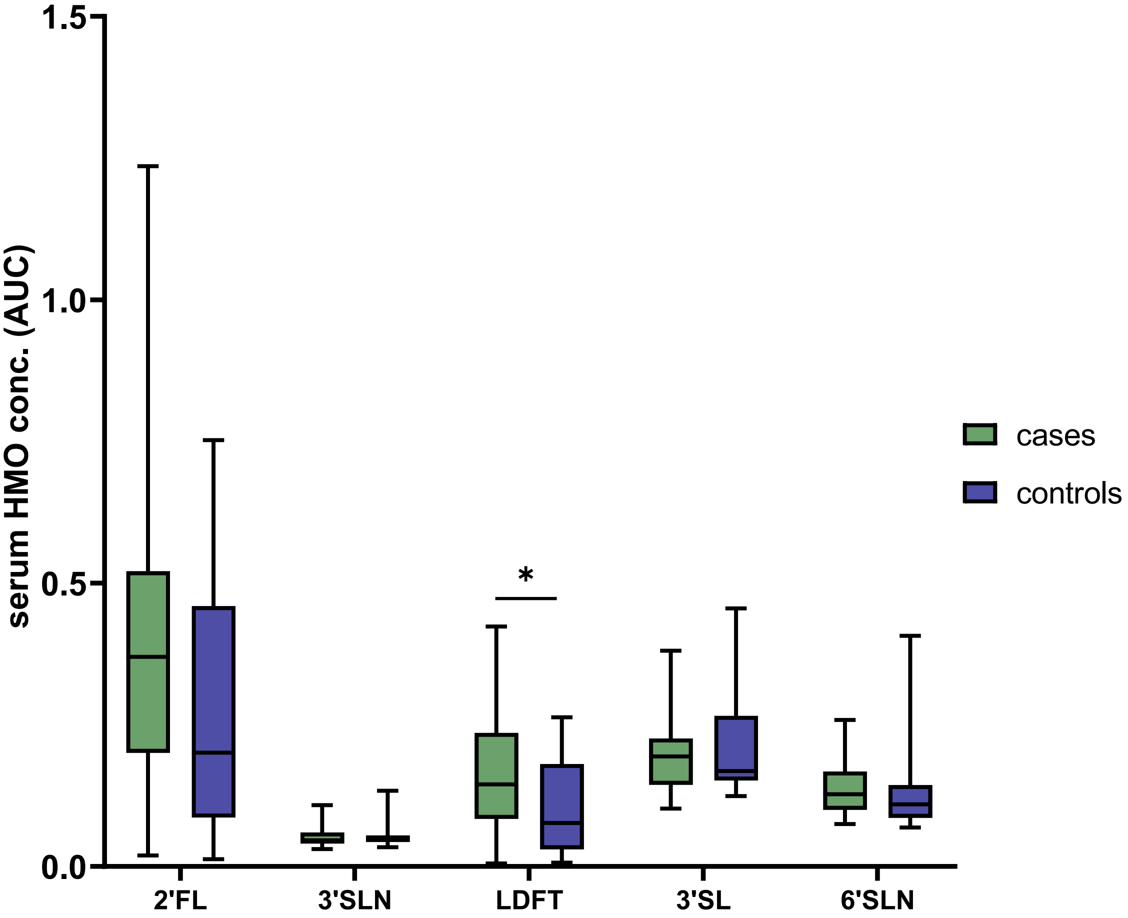


Figure 10. Differences in HMO concentrations in cases vs. controls. Boxplots showing differences in concentration of HMOs between cases (n=30) and controls (n=22) at the time of hospitalization (Mann-Whitney Test). There is a significant difference in concentration for LDFT. *p <0.05.

We investigated differences in HMO concentrations in women with and without confirmed preterm labor at the time of hospitalization (Fig. 10). LDFT was the only HMO with a significant difference in concentration (p= 0.0495; mean cases= 0,156; mean controls= 0,099).

4.2.3 Serum Concentrations of HMOs – Short Cervix vs. Normal Cervix

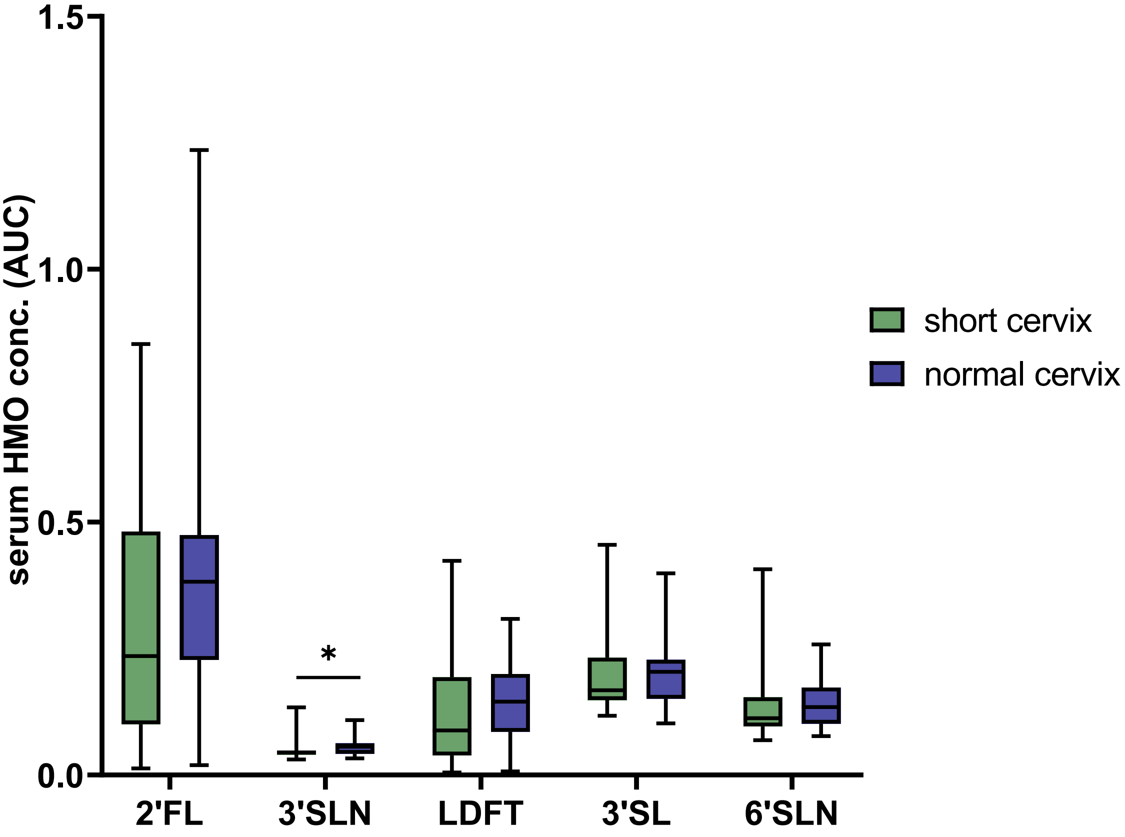


Figure 11. Differences in HMO concentrations in patients with short cervix vs. normal cervix. Boxplots showing differences in concentration of HMOs between patients with short cervix (green, n=30) and normal cervix (blue, n=22) at the time of hospitalization (Mann-Whitney Test). There is a significant difference in concentration for 3'-SLN. *p <0.05; **p <0.01.

We next analyzed differences in serum HMOs between two groups formed based on normal or short cervix length. In Figure 11, the concentrations of HMOs were compared between the groups. We found a strong significant difference for the 3'SLN concentration (p= 0.0153), which was slightly higher in patients with a cervix length of <25mm than in patients with normal cervical length (> 25mm).

We also performed the same analyses excluding the secretor negative women without changing the results. When secretors only were analyzed an even clearer significance for the 3'SLN levels was found (p= 0.0014).

4.2.4 Serum Concentrations of HMOs – Preterm vs. Term Birth

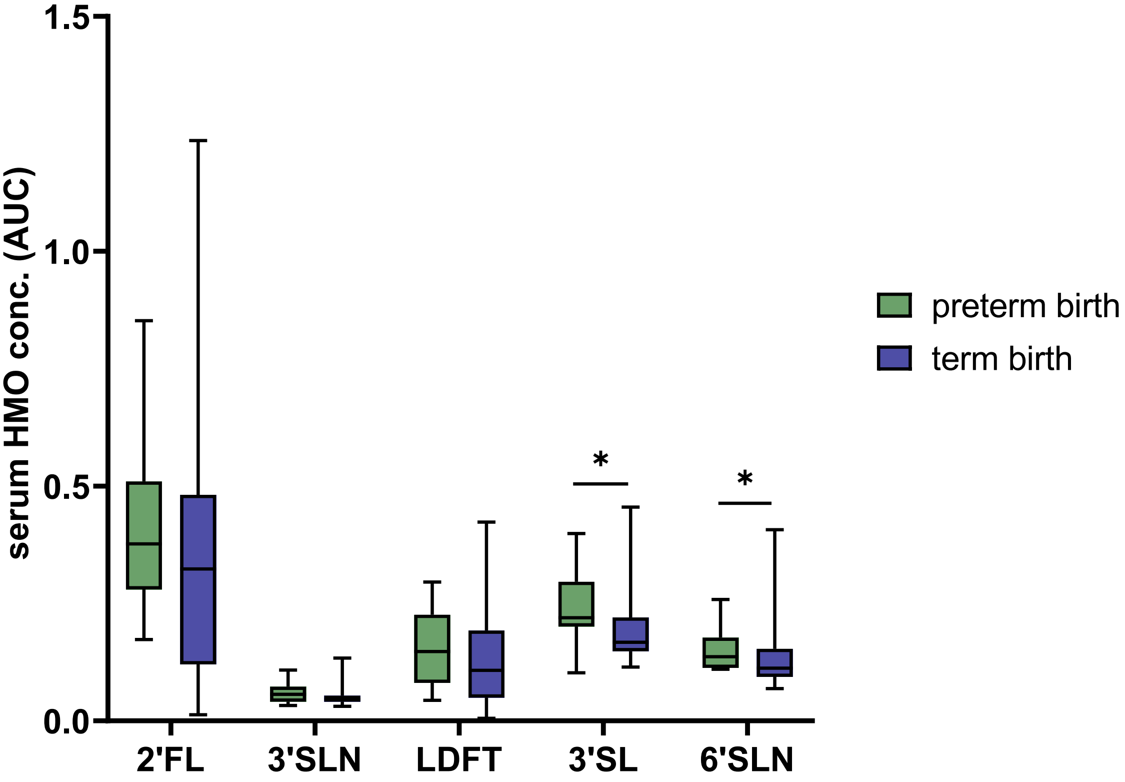


Figure 12. Differences in HMO concentrations in patients giving preterm vs. term birth. Boxplots showing differences in concentration of HMOs at the time of sampling between women who later gave birth preterm (green, n=10) versus women who delivered on term (blue, n=42) (Mann-Whitney Test). There is a significant difference in concentration for 3’SL and 6’SLN. *p <0.05.

Subsequently, we asked whether HMOs concentrations at blood sampling were different in women who later went on to deliver preterm compared to those having term delivery. Figure 12 displays the difference in HMO concentrations of the patients who gave birth preterm (before gestational week 37+0) compared to women with term deliveries. Here, we found significantly higher concentrations for the oligosaccharides 3’SL (p= 0.025) and 6’SLN (p= 0.043) in women who delivered preterm.

4.2.5 Serum Concentrations of HMOs – Preterm Birth vs. Term Birth in High Risk Patients

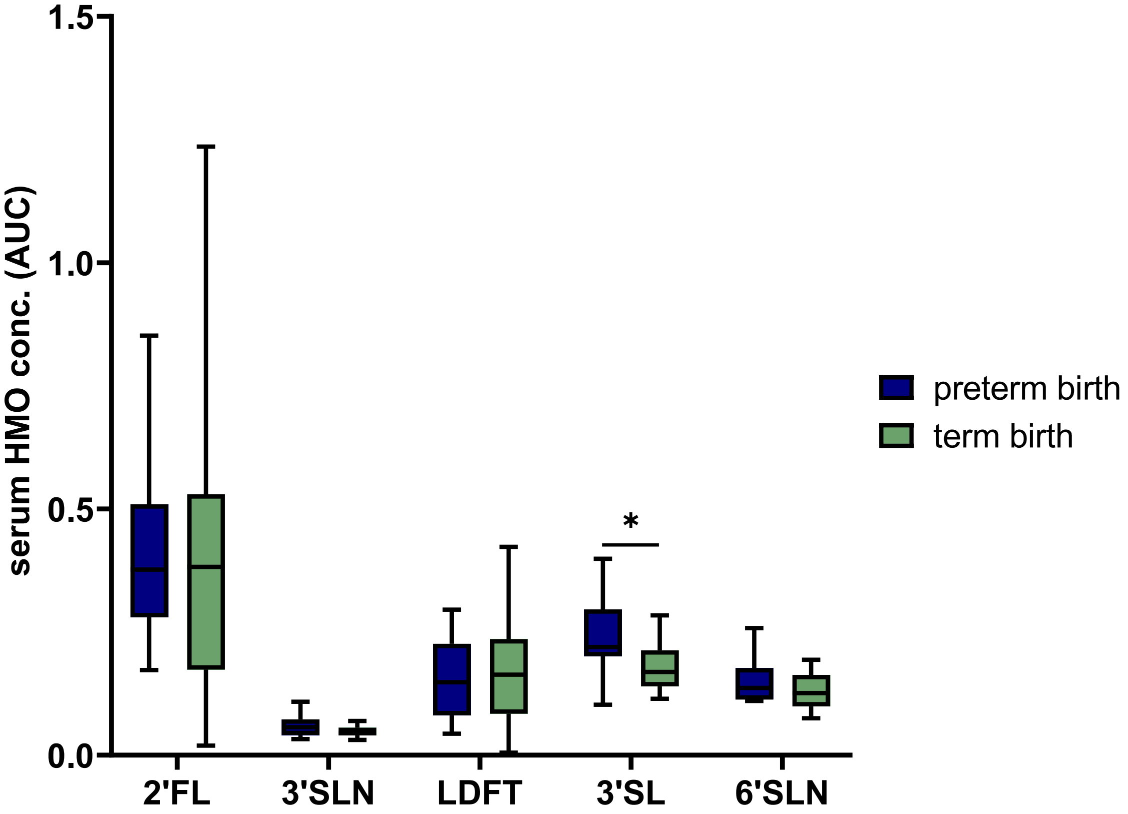


Figure 13. Differences in HMO concentrations in patients giving preterm vs. term birth in patients with confirmed labor at the time of admission. Boxplots showing differences in concentration of HMOs at the time of sampling between women who later gave birth preterm (blue, n=10) versus women who delivered on term (green, n=22) in the high-risk population (Mann-Whitney Test). There is a significant difference in concentration for 3'SL. p= 0.0222; *p <0.05;

In Figure 13, we compared the HMO concentrations of women who later delivered preterm with those who delivered on term in the high-risk group. Only patients who had confirmed contractions during admission were analyzed here. This included all patients from the "cases" group and 2 patients from the "controls". In these two specific cases, it was decided not to give tocolysis, as the medication is recommended up to gestational age 34 + 0, and these patients were one and two days before this gestational age on the day of admission. However, both of them gave birth in the preterm birth period and, therefore, they are analyzed as high-risk patients. Compared to Figure 12, the significance for 3'SL is slightly stronger (p= 0.022). This result also suggests that higher 3'SLN can predict greater risk for PTB in the high-risk group.

4.2.6 Differences in Cervical Length in Women Delivering Preterm or Term

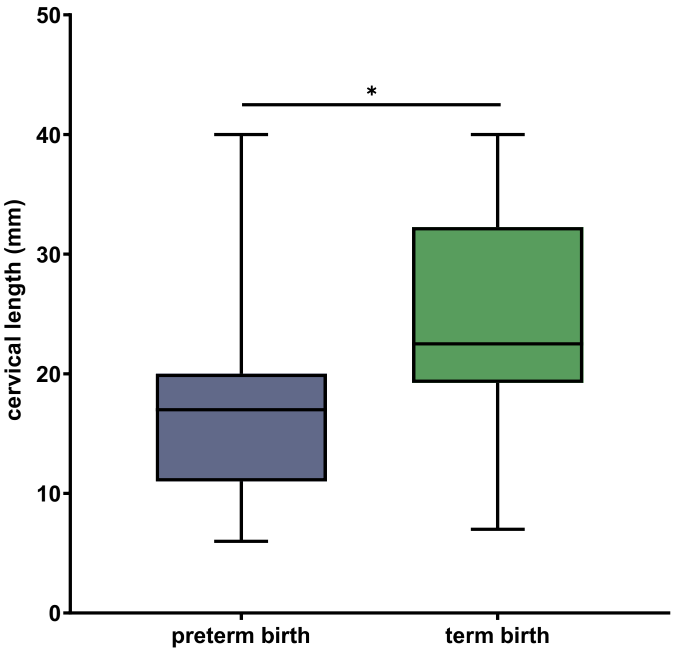


Figure 14. Difference in cervical length in patients with confirmed labor at the time of admission. Mann-Whitney test for differences in cervical length of patients with confirmed labor in women who went on to give PTB or term birth. Median cervical length of patients giving preterm birth (n=11) was 17mm. The median cervical length of patients giving term birth (n=24) was 22.5mm. p= 0.0167.

In the next step, we took a closer look at the cervical length of the patients. In Figure 14, we compared the measured cervical lengths of the high-risk patients in a Mann-Whitney test. Only patients who had confirmed contractions during admission were analyzed here. As expected, the PTB patients had shorter cervical lengths (17 mm) on average than the patients who gave birth after gestational week 37 + 0 (22.5 mm). The difference was significant with p = 0.0167.

4.2.7 Association between HMOs and Cervical Length

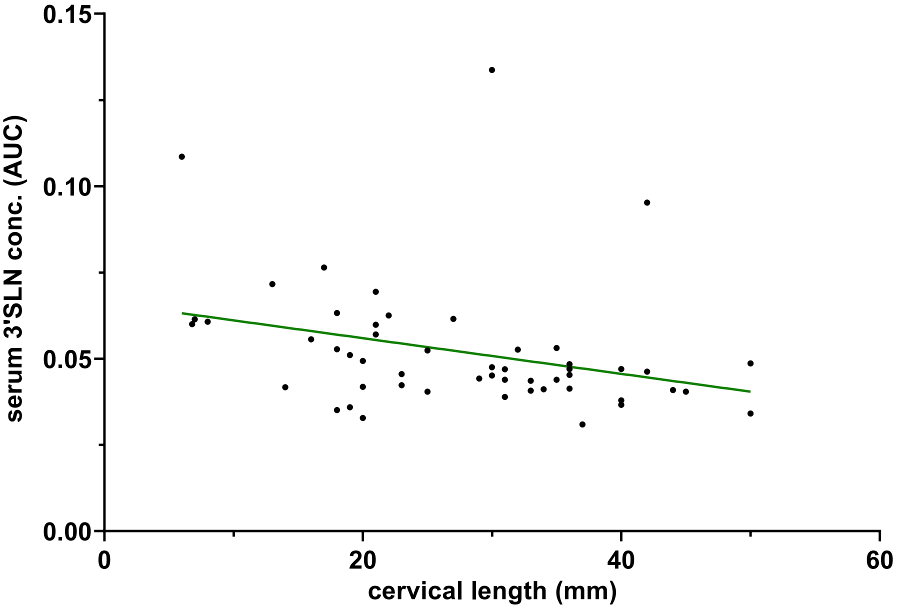


Figure 15. Association between 3'SLN concentrations and cervical length. Scatter plot showing associations between 3'SLN and cervical length. Data is not normally distributed; the regression line serves to illustrate the direction of the correlation. Spearman's r = -0.4223; p= 0.0018; **p <0.01; n=52.

In the following, we performed correlation analyses with HMOs and cervical length. The scatter plot in Figure 15 shows the association between 3'SLN and cervical length. The data was first tested for normal distribution, but both tests rejected Gaussian distribution, so Spearman Correlation was used to test for associations. The correlation was negative (Spearman's r= -0.42) and highly significant (p= 0.002). Other HMOs showed a weak to no association between the variables (data not shown).

4.2.8 Association between HMOs and Gestational Age at Birth

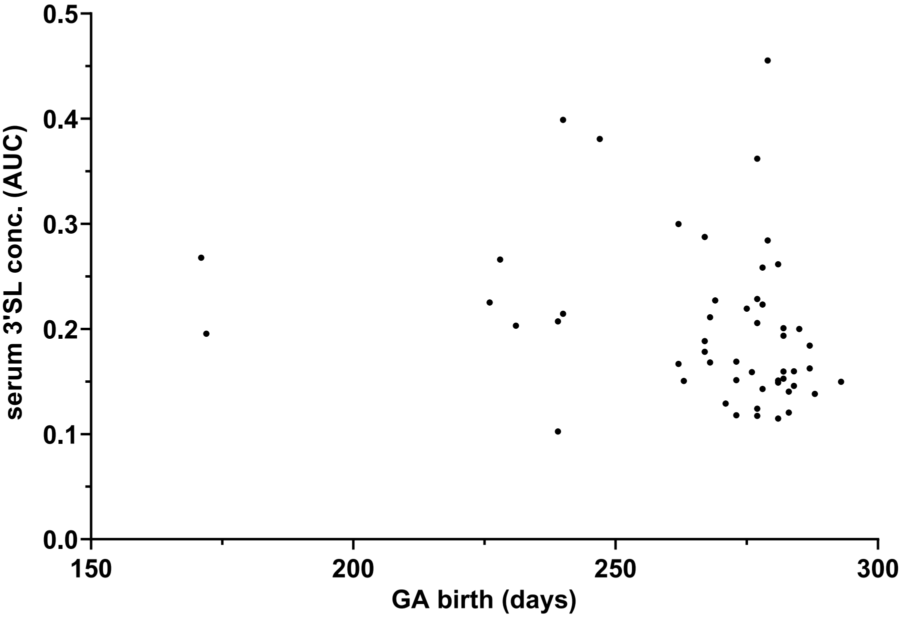


Figure 16. Association between 3'SL and gestational age at birth. Scatter plot showing associations between 3'SL and gestational age at birth; Spearman's r= -0.3664; p= 0.0075; n= 52.

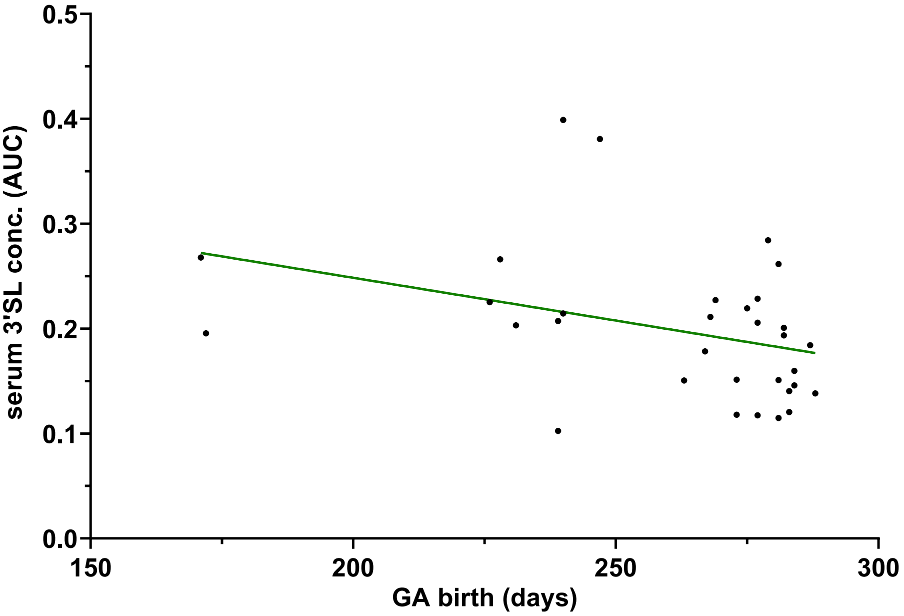


Figure 17. Association between 3'SL and gestational age at birth of patients with confirmed labor at the time of admission. Data is not normally distributed; the regression line serves to illustrate the direction of the correlation. Spearman's r= -0.4291; p= 0.0143; n=32.

We next asked whether HMOs at time of admission to the hospital with suspected or confirmed labor might be associated with length of the gestation. Figures 16 and 17 both show correlations between gestational age at birth and the oligosaccharide 3’SL. The scatter plot in Figure 16 shows the entire study population. The data is not normally distributed, so the Spearman analysis was used to calculate the correlation. Spearman's rho r= -0.366 showed a significant negative relationship between gestational age at birth and 3’SL (p= 0.0075). Figure 17 only shows the high-risk patients. Within the restricted study population, the negative correlation is slightly stronger (Spearman's r= -0.43, p= 0.0143).

For 6’SLN, a correlation can only be seen in the high-risk population. When the entire study population is analyzed, we see an insignificant, weak correlation between 6’SLN and gestational age at birth (Fig. 18). In the high-risk patients (Fig. 19), the 6’SLN values are normally distributed and Pearson's correlation shows a significant negative correlation with p= 0.04 and a Pearson's r of -0.36.

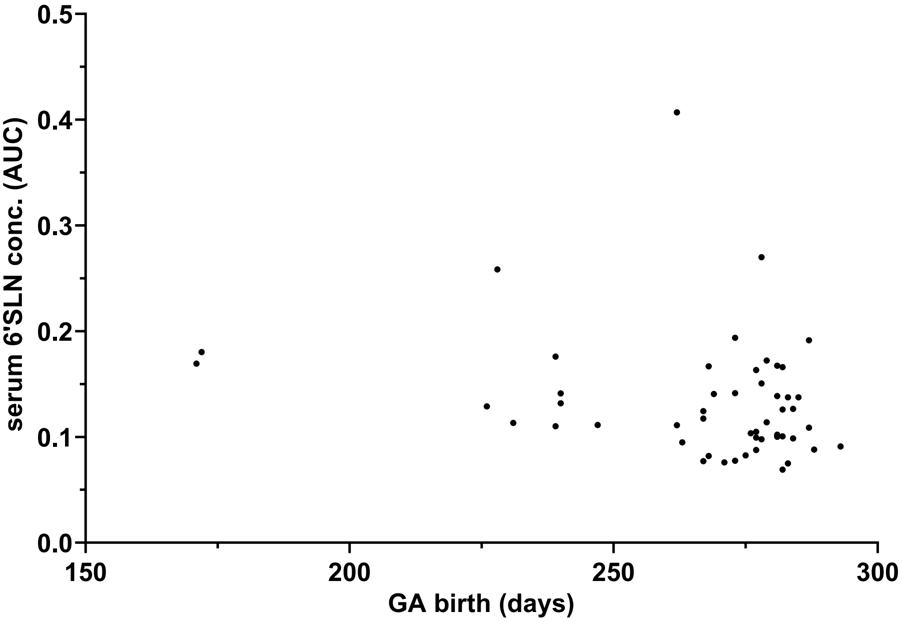


Figure 18. Association between 6’SLN and gestational age at birth. Data is not normally distributed; Spearman’s r= -0.2260; p= 0.1072; n= 52.

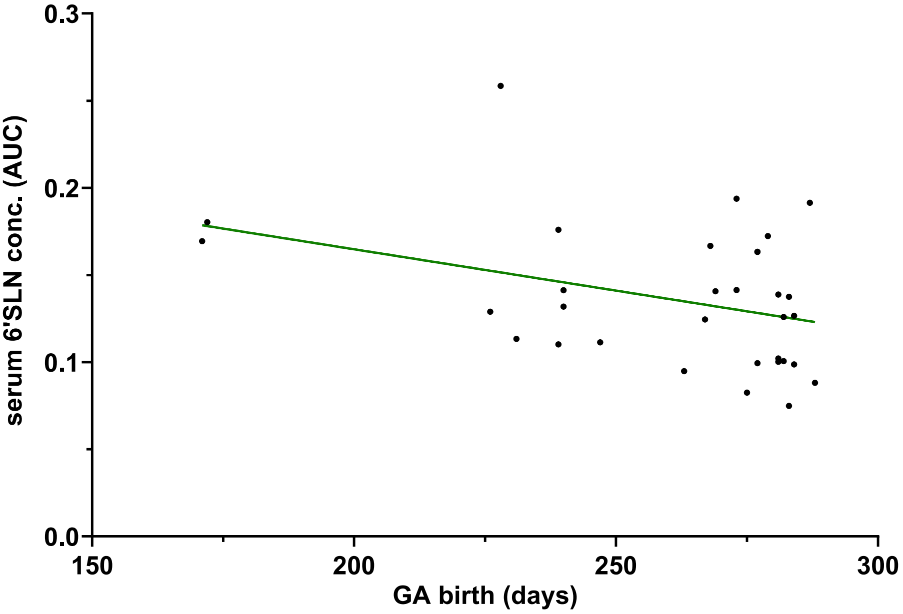


Figure 19. Simple linear regression between 6’SLN and gestational age at birth of patients with confirmed labor at the time of admission. Data is normally distributed. Pearson’s r= -0.3618; p= 0.0419; n= 32.

4.2.9 Association between HMOs and Gestational Age at Sampling

In a next step, we performed association analyses to investigate a link between HMOs and gestational age at sampling. Previous studies in pregnant women have shown that HMO levels are dependent on gestational age at sampling. Due to our sampling time window of 10 weeks we wanted to test whether the HMO levels are associated with gestational age at sampling and could account for the differences found, with regards to preterm indicators.

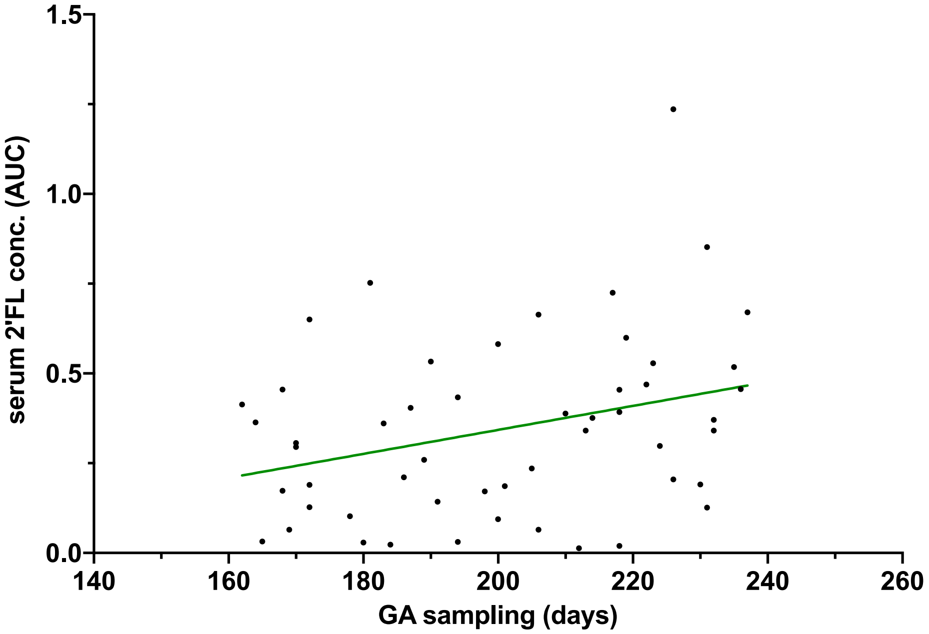


Figure 20. Association between 2’FL and gestational age at sampling. Scatter plot showing associations between 2’FL and gestational age in days; Data is not normally distributed; the regression line serves to illustrate the direction of the correlation. Spearman’s r= 0.3028; p= 0.029; n=52.

Figure 20 displays a positive correlation between 2’FL concentration and gestational age at sampling (Spearman’s r= 0.303). The data does not follow normal distribution, so the regression only serves to illustrate a direction of correlation.

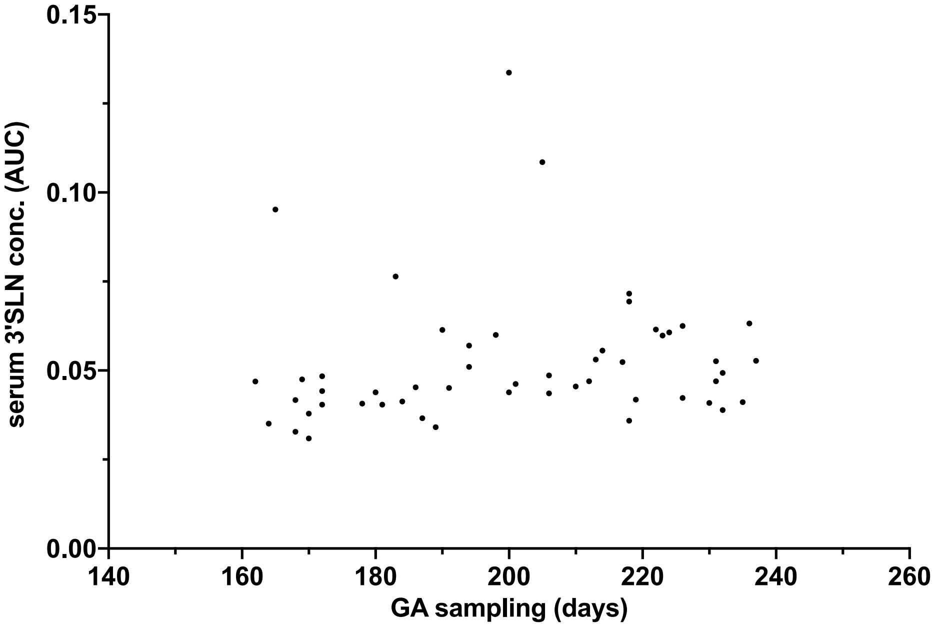


Figure 21. Association between 3’SLN and gestational age at sampling. Scatter plot showing associations between 3’SLN and gestational age in days; Spearman’s r= 0.3143; p= 0.0233; n= 52.

Figure 21 shows the association between 3’SLN and gestational age at sampling. With Spearman's r= 0.3143 and p= 0.023, there was a significantly positive association with gestational age.

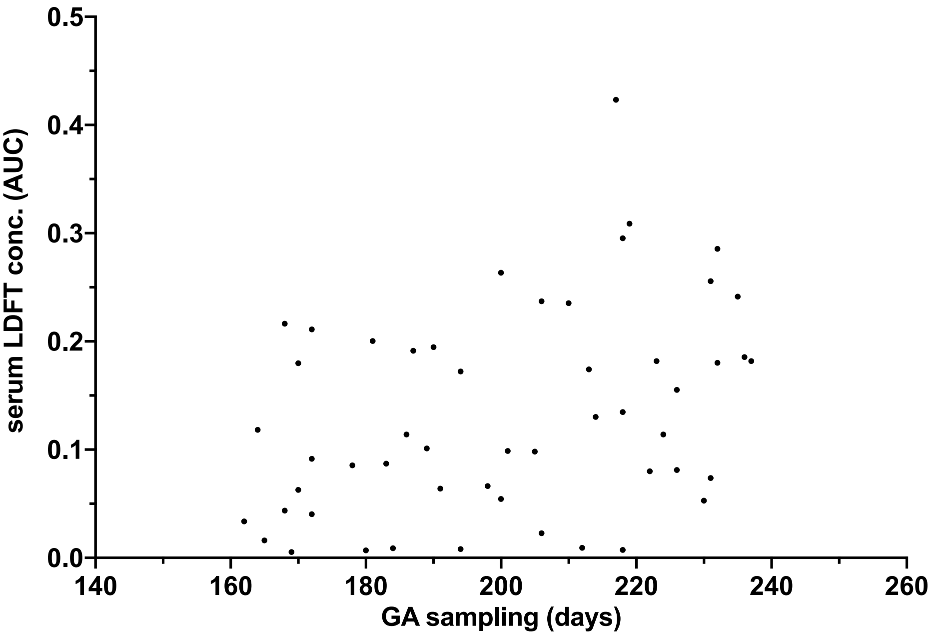


Figure 22. Association between LDFT and gestational age at sampling. Scatter plot showing associations between LDFT and gestational age in days; Spearman's r= 0.3623; **p= 0.0083; n= 52.

In Figure 22, the association between LDFT and gestational age at sampling for the study population is shown. The data was loosely distributed, but with Spearman's r= 0.36 and p= 0.008 there was a highly significant positive correlation. The data was tested for normal distribution and the Kolmogorov-Smirnov test confirmed a Gaussian distribution (but not Shapiro-Wilk test).

3'SL and 6'SLN did not show a significant correlation with gestational age at sampling (data not shown).

4.2.10 Association between HMOs and CRP

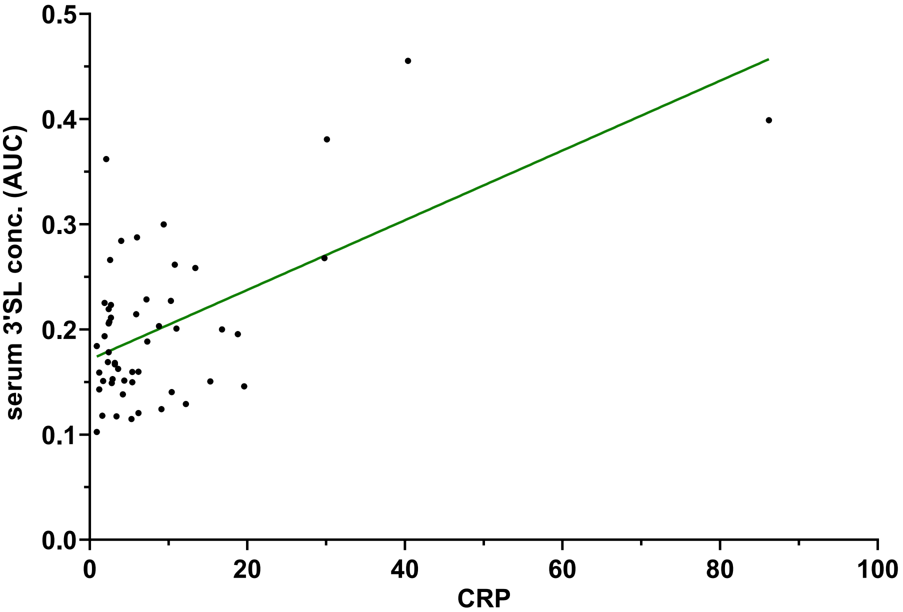


Figure 23. Association between 3’SL and CRP. Data is not normally distributed, the regression line serves to illustrate the direction of the correlation. CRP in [mg/L], standard value 0-8 mg/L; p= 0.064; Spearman’s r= 0.26; n=52.

In Figure 23, the association between 3’SL and CRP is shown. 3’SL is the only HMO showing correlation when analyzed with CRP. Since data was not positively tested for Gaussian distribution, the regression line is only used to visualize correlation of the data. Spearman correlation showed a slight positive trend (Spearman’s r= 0.2581), but without significance (p= 0.064). The standard value of 0-8 mg/L applies for the general population. A slightly elevated CRP is very common during pregnancy, although there is no exact guideline for treatment here. This can make antibiotic administration difficult since a decision has to be made individually. When an increase in CRP is due to pregnancy, it is unnecessary to treat. However, if it is due to inflammation, then antibiotic therapy must be given.

4.2.11 Association between HMOs and Leukocyte Counts

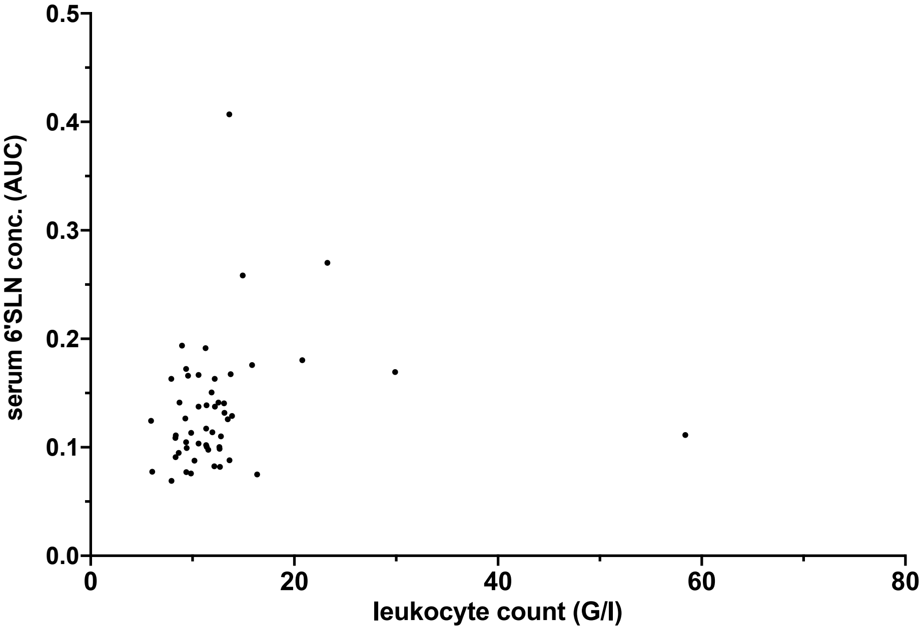
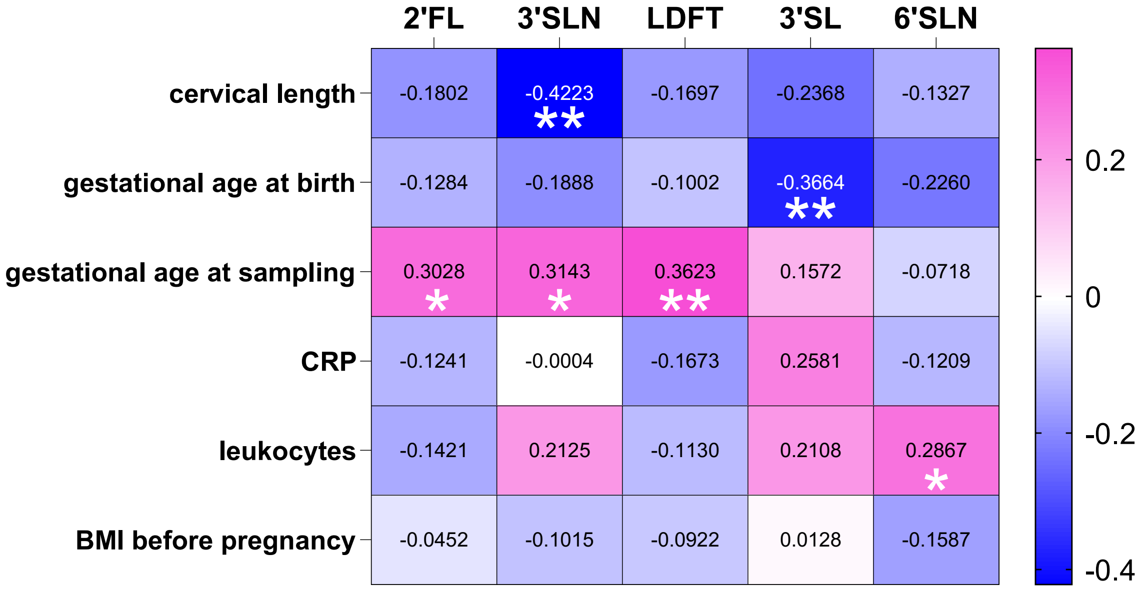


Figure 24. Association between 6'SLN and leukocytes. Standard value for Leukocytes: 4.4-11.3 G/L; Spearman's r= 0.29; p= 0.039; n=52.

In Figure 24, the correlation between 6'SLN and the leukocyte count in patients is shown. Leukocytes were significantly associated with 6'SLN (Spearman's r= 0.29; p= 0.039). All tests for normal distribution were negative.

4.2.12 Spearman Correlations between HMOs and Multiple Maternal Variables



	2'FL	3'SLN	LDFT	3'SL	6'SLN
cervical length	-0.1802	-0.4223 **	-0.1697	-0.2368	-0.1327
gestational age at birth	-0.1284	-0.1888	-0.1002	-0.3664 **	-0.2260
gestational age at sampling	0.3028 *	0.3143 *	0.3623 **	0.1572	-0.0718
CRP	-0.1241	-0.0004	-0.1673	0.2581	-0.1209
leukocytes	-0.1421	0.2125	-0.1130	0.2108	0.2867 *
BMI before pregnancy	-0.0452	-0.1015	-0.0922	0.0128	-0.1587

Figure 25. Heatmap of Spearman correlations between HMOs in serum and maternal variables. 2'FL = 2'-Fucosyllactose; 3'SLN = 3'-Sialyllactosamine; LDFT = Lacto-N-difucotetraose; 3'SL = 3'-Sialyllactose; 6'SLN = 6'-Sialyllactosamine; CRP = C-reactive Protein; level of significance *p <0.05; **p <0.01; ***p <0.001.

The heat map in Figure 25 displays positive (pink) and negative correlations (blue) between HMO concentrations in blood serum and some maternal factors.

3'SLN showed a strong negative and highly significant correlation with cervical length (Spearman's r= -0.4223; p= 0.002). Dividing the study population according to cervical length has indicated this trend in Mann-Whitney test (Fig. 11). Figure 15 is showing the same results in a scatter plot.

Gestational age at birth showed a strong correlation with 3'SL (Spearman's r= -0.3664). This is also the correlation with the strongest significance (p= 0.008). 6'SLN is showing a negative trend without significance. As displayed in Figure 18 and 19, the association became significant when only high-risk patients were analyzed.

Gestational age at sampling positively correlated with 2’FL (p= 0.029), 3’SLN (p= 0.023) and LDFT (p= 0.008), confirming that HMOs increase over the course of gestation. Thus, with higher gestational age at sampling, a higher HMO concentration can be expected.

CRP did not show strong associations with HMOs in Spearman correlation. 3’SL was positively associated to the inflammation marker, with a trend of being significant (p= 0.065). The other HMOs were negatively or not correlated with CRP.

6’SLN showed a significantly positive association with leukocytes (Spearman’s r= 0.29; p= 0.039). A scatter plot of the same data is shown in Figure 24.

For BMI before pregnancy, no significant correlations were found.

4.2.13 Secretors vs. Non-Secretors

There are striking differences in the HMO profiles depending on the secretor status of the mother. Thus, in a final step, we carried out analyzes depending on the secretor status. In almost all analyzes, the exclusion of the 8 secretor negative women did not change the results. The difference in 3'SLN concentration between women with short and normal cervix length was even more significant (Mann-Whitney Test, p= 0.0014) (Fig. 26).

Between women later having term and PTB, the 6'SLN concentration at sampling was no longer significantly different after excluding the non-secretors (Mann-Whitney Test).

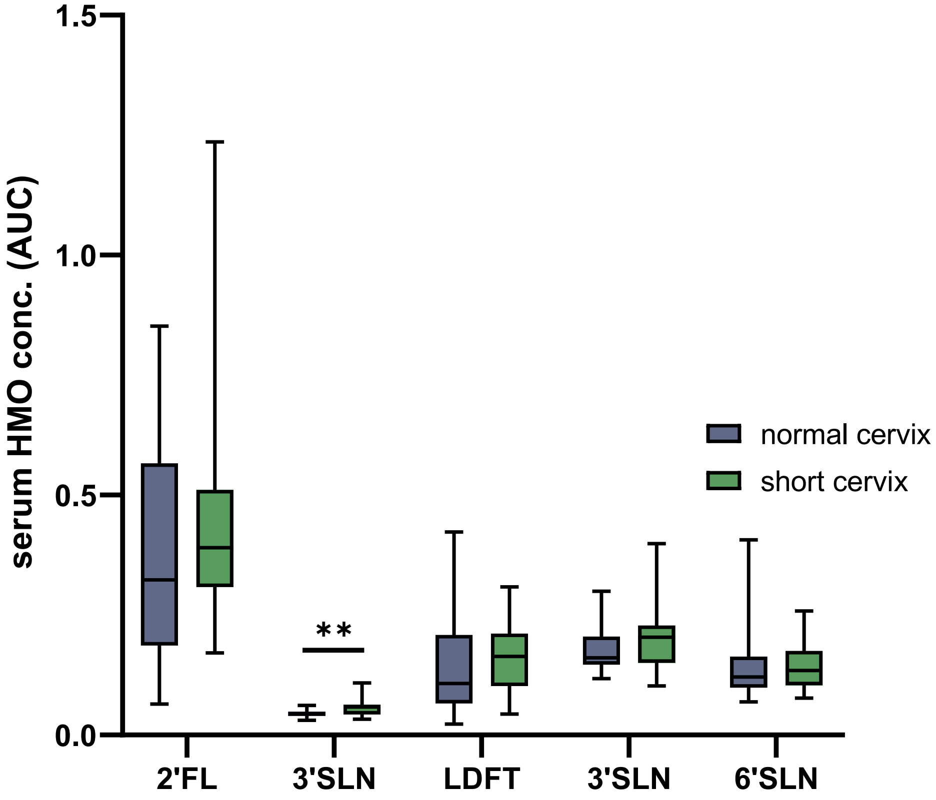


Figure 26. Differences in HMO concentrations in secretors only with short cervix vs. normal cervix. Compared to the whole study group, 3'SLN differences for secretor women show stronger significance. p=0.0014; n=44.

All correlations tested showed no noticeable differences when the non-secretors were not included in analyses.

5 Discussion

The aim of this thesis was to test whether HMOs in maternal serum are different in cases of idiopathic preterm labor compared to controls without labor, and whether HMOs are associated with preterm birth (PTB). We conducted a case control study in which numerous maternal variables were collected, and we analyzed these maternal factors with human milk oligosaccharide (HMO) concentrations in the serum. We hypothesized that the mothers' HMO profiles influence the risk for PTB. The results of our analyzes show that it is mainly the sialylated HMOs that show an association with the incidence of PTB (3'SL and 6'SLN), as well as with a shortened cervix (3'SLN). A certain concentration or composition of oligosaccharides predicting preterm labor or preterm birth could not be found in this study.

5.1 Main Findings of the Study

Sialylated HMOs. The main goal of the study was to find a potential impact of HMOs on the risk for PTB. A finding that is common in almost all analyzes is the association between higher concentration of sialylated HMOs and indicators of PTB. The comparison of HMO concentrations in patients with term birth versus PTB gave a first indication that sialylated HMOs, specifically 3'SL and 6'SLN (Fig. 12), are found to a greater extent in women who go on to give birth prematurely. The correlation between the HMOs and gestational age at birth supports this finding. With Spearman's r of -0.37, 3'SL shows a strong and highly significant correlation with gestational age at birth (Fig. 16). We then analyzed patients with confirmed labor only, which resulted in an even stronger correlation (Spearman's r= -0.43) (Fig. 17). Similar results were found for 6'SLN, however, the results were not significant across the whole study population. After restricting the sample size to the high-risk group, the results matched those of 3'SL (Pearson's r = -0.36 and *p <0.05). The high-risk group of patients were all patients from the "cases" group, that received tocolytic treatment, and two patients from the control group. For the two patients in the control group, tocolysis was not given, as Atosiban is recommended up to gestational week 35 and the patients were only one and two days before this gestational age on the day of admission.

The third oligosaccharide from the sialylated group is 3'SLN and it was found to correlate in several analyses with the patients' cervical length. First, we detected a significantly higher

3’SLN concentration in serum of women with a shortened cervix compared to women with normal cervix using Mann-Whitney test. 3’SLN was also significantly negatively correlated with cervical length.

Fucosylated HMOs. We also found two fucosylated HMOs to be associated with preterm indicators. When comparing HMO concentrations between cases and controls, LDFT serum concentration was significantly higher in cases. We could not derive a direct effect of LDFT on the incidence of preterm labor, but the oligosaccharide could have an indirect effect on the risk of PTB via another mechanism. One can speculate that LDFT influences the microbiome in the urogenital tract in a way that is associated with a dysbiosis and the onset of an infection. This indirect, microbiome dependent effect could then lead to contraction without necessarily leading to PTB, because the conditional preterm labor could be managed by medical treatment giving tocolysis and antibiotics.

2’FL and LDFT (and also 3’SLN) correlated positively with the gestational age at sampling. Due to the relatively long recruitment time period, this could speak for a possible confounder, since the concentration of fucosylated HMOs increases over the course of pregnancy and we did not correct HMO levels for sampling time.

Inflammation markers. Another interesting finding was the relationship between sialylated HMOs and inflammation markers. As in other studies we could find a link between sialylated HMOs and inflammation (61–63). The two sialylated HMOs, 3’SLN and 6’SLN, showed correlations with at least one of the inflammation markers, trending towards significance. 3’SLN showed a positive correlation with CRP, which was, however, significantly influenced by one outlier. Future analyses should be done in a larger study population, which may show a wider range of CRP values. Significant Spearman correlations with inflammation markers were only found for 6’SLN and leukocyte counts. For the majority of the study population, the leukocyte count was in the upper normal range or slightly increased. These slightly increased leukocyte counts did not speak for an unspecific inflammation, because pregnant women in general have an increased number of leukocytes. One could also speak of a low-grade inflammation through pregnancy, without this being a pathology or leading to premature birth in general.

Previous PTB vs. no previous PTB. We also took a closer look at the distribution of women with previous PTB and no previous PTB in our study population. Fisher's exact test confirmed a significant difference in the groups (Tab. 3), which shows that women with PTB in a previous pregnancy were more likely to give birth prematurely in this pregnancy. However, this result cannot estimate the actual risk for a PTB in current pregnancies. Furthermore, in our study population all women with confirmed labor have undergone tocolysis in order to prevent premature birth.

Secretors vs. Non-Secretors. We carried out analyzes depending on the patients' secretor status and excluded secretor negative subjects from the analyses. Except for slight differences in the significance for 3'SLN and 6'SLN in the analysis according to PTB vs. term birth and short cervix vs. normal cervix the same results were obtained in all analyses. We did not find significant differences for fucosylated HMOs in our study. It should be mentioned that we only carried out analyzes in the secretor group but not for the non-secretors alone, since the group size (n=8) was not large enough to perform statistically meaningful analyzes.

5.2 Strengths and Limitations of the Study

5.2.1 Strengths

We here, investigated for the first time, HMOs in maternal serum and their associations with several maternal variables during pregnancy with the aim of finding links to preterm labor. Due to the extensive follow-up after the initial consultation in the hospital, in addition to the clinical data, data on outcome of the pregnancy was available from the patients. These were later included in the analyses. Although our total study group was relatively small, based on the study design recruiting women with suspected labor in a defined gestational time window, we had collected biological samples and data from a high-risk group (cases) of whom 9 out of 30 women went on to deliver preterm (30%). This high preterm incidence allowed some additional valuable insights into the potential interplay of maternal HMOs and pregnancy outcome. Concluding from our study, the correlation of 3'SL with gestational age at birth seemed to be either causal or predictive as HMOs were elevated prior to preterm birth.

This diploma thesis project was part of a larger study also investigating the maternal microbiome. In addition to HMOs in the blood, the urinary oligosaccharides were also determined. In addition, associations of the HMOs with microbial species were examined, which in turn might have an influence on PTB. The paper on this study was posted as preprint, is currently being reviewed and will be published shortly (35). Results concerning the microbiome, however, are not included in this thesis.

5.2.2 Limitations

A sampling time point late in pregnancy (23 to 34 weeks of gestation) can also be seen as a limitation. This pilot study was designed as a cross sectional case control study to allow comparing HMOs in women with confirmed labor and gestational age matched controls. The obvious limitation is that we cannot draw conclusions about cause and effect of altered HMOs associated with indicators of PTB, preterm labor and short cervix. Future studies should be designed as larger prospective studies including longitudinal sampling starting early in pregnancy.

Another limitation of the study was the limited sample size with regards to the expected low numbers of the non-secretors. Although the total number of subjects in our study was relatively high, it was not enough to get a sufficiently high representation of non-secretors. Since non-secretors lack fucosylated HMOs, they have very different HMO profiles compared to secretor positive women, and HMO effects on preterm birth would have to be investigated separately. The number of non-secretors in the study population was too low to analyze them separately. However, excluding non-secretors in the analyzes did not significantly change the results. Future studies should be performed with a higher sample size to give better insight in HMO profiles of non-secretor women, since they make up about 20% of the European population (4).

Furthermore, the study did not consider ethnic groups other than Caucasians. Several studies show that maternal ethnicity can have an impact on the risk of PTB (36,45). In our study population recruited at the Department of Gynecology and Obstetrics at the Medical University of Graz, 55 out of 60 women (91,7%) were Caucasians. It can therefore not be determined whether our findings might apply to a general population or other ethnicities as well. The exclusion of different ethnicities from the analyzes did not make a difference in the results due to the small number of non-Caucasian women. There was only one African woman and no Asian women in the group of cases and three Asian women and one African woman in the controls. An analysis of these women separately would not have provided us with meaningful results.

5.2.3 Potential Confounders

Gestational age at sampling. Various studies suggest a change in the individual HMO concentrations over the duration of pregnancy (12,13). In secretor-positive women, especially 2’FL increases strongly from the first to the second trimester, and a difference in concentration can also be measured for LDFT (13). Sialylated HMOs, amongst them, 3’SL show less strong increase over time. Due to the recruitment time window of more than 10 weeks, time of sampling could have had an impact on the different HMO profiles in our patients. We found a positive correlation for 2’FL, 3’SLN and LDFT in the analyses with gestational age at sampling, suggesting that time point of sampling could be a possible confounder (Fig. 25).

BMI. A previous study on HMOs in pregnancy suggested associations of BMI more specifically with subcutaneous fat mass and HMO concentration in blood (13). 2'FL was negatively correlated with subcutaneous fat mass and might therefore, be associated with metabolic status of the mothers. In our study, we only had pre-pregnancy BMI which has limitations as marker for obesity. Since we did not see any significant difference in BMI between the cases and control group, nor associations of HMOs with BMI, we concluded that BMI was not a confounder in our study.

Subjective allocation to study groups. To prevent a possible bias in the study we analyzed patients not only according to the original classification into cases and controls, but also according to cervical length (normal cervix vs. short cervix) and term birth vs. PTB. We chose cervical length as an objectively measurable parameter for an additional stratification of the population. The classification into cases and controls is problematic, insofar as it always depends to a certain extent on the individual decision and the experience of the clinician in charge. Despite of a common routine treatment regime, decisions based on individual subjective considerations cannot be fully excluded. This factor also speaks for the analysis depending on cervical length, since it can be measured objectively. When we investigated HMO differences depending on cervix length (normal vs. short), we found significantly higher 3'SLN in the women with short cervix. The Spearman correlation showed similar results (Figures 15 and 25). 3'SLN showed a strongly negative correlation with the measured cervical length. This implies that patients with high 3'SLN concentrations had shorter cervical lengths.

Looking at the pregnancy outcome (term birth vs. PTB) added a third method of stratification of our study group. This allowed to investigate the influence of HMOs measured at time of admission to the hospital with suspected labor on the pregnancy outcome in a longitudinal manner. Since medical treatment (tocolysis, antibiotic treatment) might account for the largest impact on the outcome of the pregnancy, we also investigated the cases group separately for differences in HMOs between term and preterm birth (Fig. 13). All women in the cases group had received tocolysis.

Inflammation markers. We used both C-reactive Protein (CRP) and leukocyte counts as markers for inflammation. The leukocyte count is known to increase faster in case of inflammations in the body than CRP, which usually increases with a 12-24h delay. However, overall leukocyte counts are known to be slightly increased during pregnancy as a state of low-grade inflammation, compared to non-pregnant state. The findings that 3’SL in serum was positively associated with inflammation markers, and thus, with a probably unspecific inflammation suggest an increased risk for PTB.

5.3 Conclusion and Outlook

We hypothesized that the HMO profile in a woman's blood during pregnancy has an impact on the risk for premature labor. In this study, we looked at the relationships between the individual HMO concentrations and various maternal variables and observed different associations with indicators of PTB.

We found specific HMOs in serum related to an increased risk for PTB. This might point towards a potential for some HMOs as biomarkers for preterm labor. Further research is needed to find and understand the causes of preterm labor and birth. It would be interesting to study the maternal microbiome and whether it can be influenced by HMOs. Future studies with a focus on HMOs and their influence on microbial species associated with PTB might reveal a microbiome-dependent interaction.

It is well known that infections like UTIs are a risk factor for preterm labor. In this study, we had excluded women with symptomatic UTIs. We found a positive association between HMOs and inflammation markers in women not having obvious infections, speaking more for a sterile inflammation or an increased inflammatory status due to a beginning infection. How exactly this can affect premature birth is not yet well understood. Further studies in this area could answer these questions and help us to better understand the impact of inflammation on PTB.

In this pilot study, we investigated HMOs in serum at a relatively late time point in pregnancy in the acute situation of undergoing labor. While this study design allowed to identify a high-risk group for PTB, differences in subsequent medical treatments of the cases might also have complicated manners (more than one tocolysis treatment, antibiotic treatment). Thus, it would be interesting to monitor HMOs longitudinally in prospective studies, starting earlier in pregnancies to investigate whether alterations can predict risk for preterm delivery.

In conclusion, our results will help to guide the way in designing future larger studies for detection of biomarkers, improving the diagnosis and therapy of preterm labor.

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