Diplomarbeit

Correlations of prenatal Human Milk Oligosaccharides with maternal lipid profiles

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Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 25.6.2017 *Lukas E. Schönbacher eh*

Danksagungen

Ich danke Evelyn, für die gute Betreuung.
Und meiner Familie für die fortwährende Unterstützung.

Anmerkung

Diese Arbeit wurde am 2. September 2016 im Rahmen des in Graz stattfindenden Symposiums „HMOs in pregnancy – back to the future“ in Form eines Posters präsentiert.

Zusammenfassung

Einleitung: Humane Milcholigosaccharide (HMOs) sind komplexe, unkonjugierte Glykane, die in hoher Konzentration in menschlicher Muttermilch vorkommen und beim Säugling präbiotische, antimikrobielle und zellmodulierende Funktionen erfüllen. Als „pränatale HMOs“ tauchen sie bereits während der Schwangerschaft zu Beginn des zweiten Trimenons im Blut der Mutter auf, was auf potentielle systemische Funktionen schließen lässt. Umweltfaktoren, die die äußerst individuelle HMO-Komposition möglicherweise beeinflussen, wurden bis heute nicht untersucht. Für die vorliegende Diplomarbeit wurden Korrelationen zwischen pränatalen HMOs und Blutlipiden (als einem Teilaspekt des mütterlichen Stoffwechsels) untersucht.

Materialien und Methoden: Blutlipide (Triglyceride, Gesamtcholesterin, LDL, HDL) und 17 pränatale HMOs (gemessen in der 24. Schwangerschaftswoche mittels NP-HPLC) wurden in Serumproben von 94 niederländischen Frauen analysiert und anschließend die entsprechenden Rangkorrelationen nach Spearman berechnet.

Ergebnisse: Innerhalb der sekretor-positiven Untergruppe korrelieren relative Konzentrationen von 3’SLN positiv mit LDL ($r=0,42$; $p<0,001$) und Gesamtcholesterin ($r=0,39$; $p<0,001$). 2’FL hingegen korreliert signifikant negativ mit LDL und Gesamtcholesterin. Unter sekretor-negativen Frauen wurden andere Korrelationen gefunden.

Diskussion: Wir zeigen hier erstmals, dass erhöhte Blutfettwerte mit bestimmten Konfigurationen von pränatalen HMOs assoziiert sind. Genaue Zusammenhänge und detaillierte Mechanismen hinter einer potentiellen Wechselwirkung müssen noch näher untersucht werden.

Abstract

Introduction: Human milk oligosaccharides (HMOs) are complex, unconjugated glycans highly abundant in human milk and serve as prebiotics, antiadhesive antimicrobials and cell modulators. As "prenatal HMOs" they emerge in the mother's blood already during pregnancy from the early second trimester on, suggesting systemic functions. Potential environmental factors that influence the highly individual HMO composition have not been investigated so far. For this thesis, correlations between prenatal HMOs and blood lipids (mirroring one aspect of the mothers' "metabolic situation") have been analyzed.

Materials and Methods: Blood lipids (triglycerides, total cholesterol, LDL, HDL) and 17 prenatal HMOs (24^{th} week of gestation, measured by NP-HPLC) were analyzed in serum samples taken from 94 Dutch women, followed by a calculation of Spearman rank correlations.

Results: Within the subgroup of secretor-positive women relative concentrations of 3'SLN positively correlated with LDL ($r=0.42$, $p<0.001$) and total cholesterol ($r=0.39$, $p<0.001$). 2'FL on the contrary correlated significantly negative with LDL and total cholesterol. Within secretor-negative women other correlations were found.

Discussion: We here provide first evidence that elevated blood lipids are associated with specific configurations of prenatal HMOs. Detailed mechanisms of potential interactions remain to be investigated.

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Glossary and abbreviations

2'FL.....2'-fucosyllactose

2-AB 2-aminobenzamide

3'S3FL .....3'-sialyl-3-fucosyllactose

3'SL.....3'-sialyllactose

3'SLN .....3'-siallyllactosamine

6'SL.....6'-sialyllactose

6'SLN .....6'-sialyllactosamine

Acetyl-CoA .....acetyl coenzyme A

ACN .....acetonitrile

AUC .....area under the curve

BMI .....body mass index

COPD.....chronic obstructive pulmonary disease

DMSO .....dimethyl sulfoxide

DSLNT.....disialyl-lacto-N-tetraose

Gal.....galactose

GDM .....gestational diabetes mellitus

Glc .....glucose

GlcNAc.....N-acetylglucosamine

HDL .....high density lipoprotein

HMO .....human milk oligosaccharide

HPLC .....high-performance liquid chromatography

IL.....interleukin

LDFT .....lactodifucotetraose

LDL.....low density lipoprotein

Le.....lewis blood group system

LNFDH .....lacto-N-difucohexaose

LNFP 1/2/3 .....lacto-N-fucopentaose 1/2/3

LNnT.....lacto-N-neotetraose

LNT.....lacto-N-tetraose

LST a/b/c .....sialyl-lacto-N-tetraose a/b/c

MALT .....mucosa associated lymphoid tissue

Max .....maximum

Min ..... minimum

N ..... sample size

NEC ..... necrotizing enterocolitis

NP-HPLC ..... normal-phase-HPLC

r ..... spearman rank correlation coefficient

RCT ..... randomized controlled trial

RT ..... retention time

SD ..... standard deviation

Se ..... secretor blood group system

Se(-) ..... non-secretor, secretor-negative

Se(+) ..... secretor, secretor-positive

Sia ..... sialic acid (N-acetylneuraminic acid)

SPE ..... solid phase extraction

TFA ..... trifluoroacetic acid

TNF-α ..... tumor necrosis factor alpha

VLDL ..... very low density lipoprotein

Images

Tables

1. Introduction

1.1 What are human milk oligosaccharides?

It was in the last years of the 19th century, when infant first-year mortality reached up to 30% and breastfeeding was not considered a matter of importance, when pediatricians observed a much higher survival rate and lower incidence of infectious diarrhea in breast-fed infants compared to "bottle-fed" children. From that time on, interest and research started to focus on around 200 different sugars present in human milk (1).

The highly abundant and diverse carbohydrate fraction of human milk oligosaccharides (HMOs) was first found in the 1930s and called "gynolactose" from that time on. Some twenty years later, in the 1950s, gynolactose could be identified as the "bifidus factor", a substance presumed to be present in human milk (but not in cow's milk) promoting the growth of *Bifidobacterium bifidus*. Research soon revealed that gynolactose is not one single substance, but a conglomeration of dozens of different oligosaccharides, whose structures were discovered and characterized in the following decades (1).

Today up to 200 different HMOs are known, a diversity that is unique to human milk, although the main quantitative component is represented by just 20 to 25 highly abundant oligosaccharides. The detailed composition of the HMO profile varies between women and changes over the period of lactation.

Several specific functions of milk oligosaccharides are known today (see chapter 1.3), partly explaining the different outcome of breast- and formula-fed infants. Necrotizing enterocolitis (NEC) for instance, a severe disorder of the pre-term infant's bowel, affects formula-fed children five to ten times more often than breast-fed babies (2). Responsible therefore might be one single oligosaccharide, whose presence in human milk seems to prevent NEC development (3,4). Despite many formidable research findings concerning HMO functions during the last decades, the biological properties of HMOs are still not fully elucidated.

HMOs represent a large fraction in human milk (5). In comparison to cow's milk, which is most commonly used as the basis of infant formulas, human breast milk differs in several ways, some of which concern the carbohydrate fraction (6). Bovine milk contains about 40 g/l of lactose and just very little amounts of larger oligosaccharides. On the other hand, lactose amounts in breast milk reach up to 50-70 g/l, joined by a huge variety of oligosac-

charides in remarkable concentrations, usually exceeding the total quantity of milk proteins (6-8).

But not only milk contains HMOs. Since the 1960s it is known that oligosaccharides are to be found in urine of pregnant women (9-12). And indeed "prenatal HMOs" emerge in the mother's systemic circulation already from the early second trimester on, suggesting systemic functions during pregnancy (see chapter 1.4).

The present thesis focuses on these prenatal HMOs and examines the maternal lipid metabolism as one potential environmental factor influencing the composition of the individual HMO profile.

1.2 HMO structure and composition

So far, more than a hundred different HMOs have been identified in the human milk glycome (13). Despite this huge variety of different complex sugars, which is unique to human milk and cannot be found in most other species (14), all HMOs follow a basic blueprint and are built out of five different monosaccharides: glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (Sia), with N-acetylneuraminic acid (Neu5Ac) being the predominant form of Sia (see **Figure 1**). By linking three to twenty of these five monosaccharide building blocks to different linear or branched chains, the full range of HMOs is formed (14).

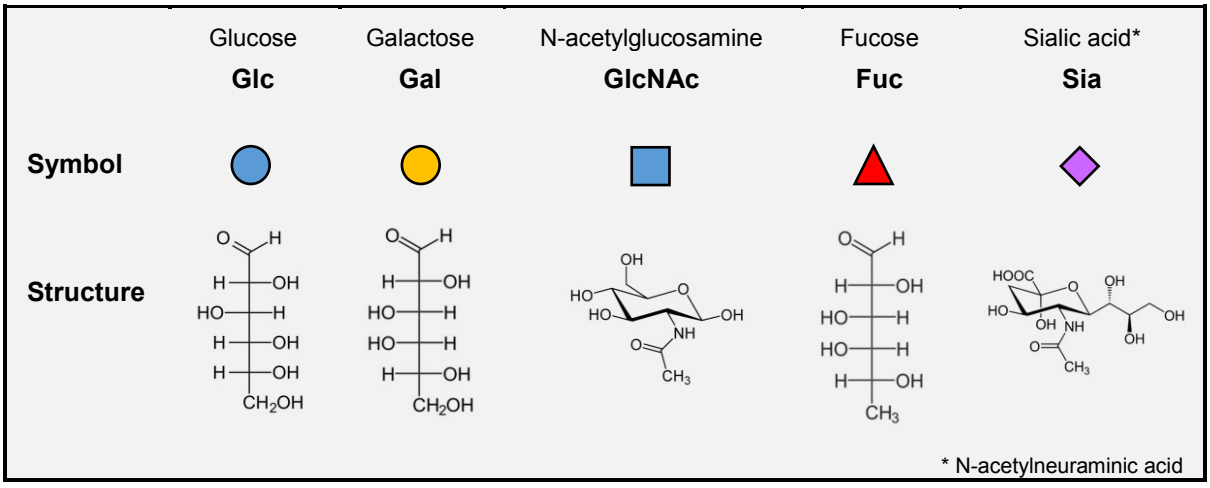

Figure 1: Five monosaccharide building blocks form all different HMOs.

Oligosaccharide core structure

Oligosaccharides are produced in the mammary gland and carry, with few exceptions, lactose at the reducing end (15). Lactose, a β1-4-linked disaccharide of glucose and galactose, is produced in the epithelial cells of the mammary gland by the lactose synthase complex, consisting of a β1-4 galactosyltransferase and α-lactalbumin that is expressed under the influence of lactation hormones (16). The synthesis of lactose occurs in the Golgi and is the start of HMO biosynthesis, since all HMOs are elongations of this lactose core (17).

Some small and simple HMOs are built by linking Fuc or Sia in different ways to the lactose core (e.g. 2'FL, 3'SL, 6'SL). Longer chains and more complex HMOs require an elongation of the lactose core, which can be reached by adding two different disaccharides, either Galβ1-3GlcNAc (type 1 chain) or Galβ1-4GlcNAc (type 2 chain) (18). Type 1 chain seems to terminate the elongation, whereas type 2 can be further extended by the linkage of another disaccharide. The linkage between these disaccharide units can either be β1-3 or β1-6; in the latter case, a chain branch is being introduced. Oligosaccharides with a branched structure are designated as iso-HMOs, whereas just linear structured glycans are called para-HMOs. **Figure 2** illustrates these principles of chain elongation.

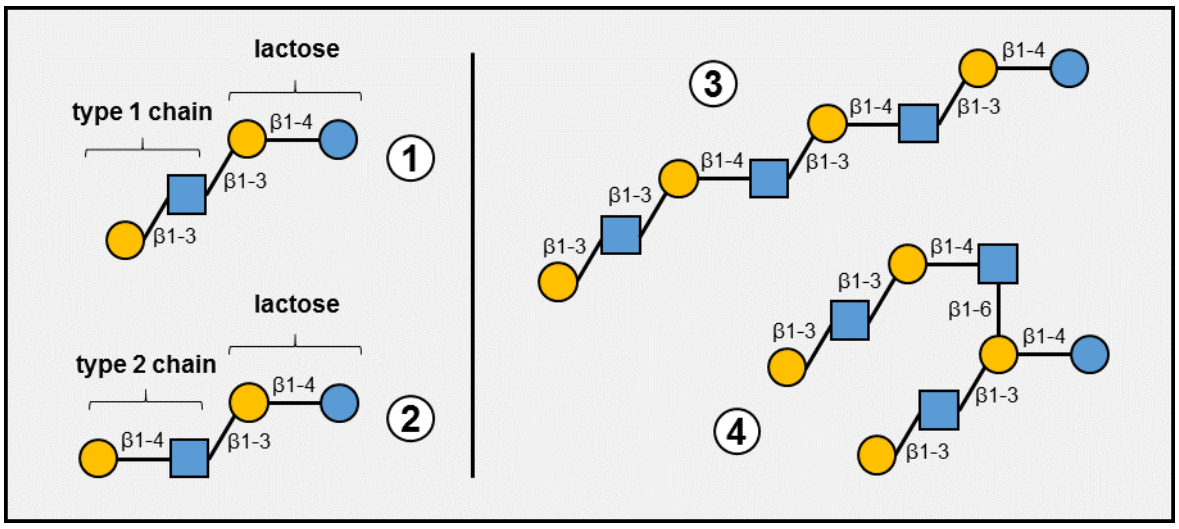

Figure 2: Principles of chain elongation.

All HMOs are built from a lactose core at the reducing end that might be elongated by either a type 1 chain or a type 2 chain. Four HMOs are shown exemplarily: 1 Lacto-N-tetraose (LNT); 2 Lacto-N-neotetraose (LNnT); 3 Para-Lacto-N-octaose; 4 Iso-Lacto-N-octaose.

Fucosylation and sialylation

HMOs which are built up of lactose and these various chains of Gal and GlcNAc are designated as core oligosaccharides; they represent the basic structures that can be further modified to gain the full range of different HMOs (14). Therefore, all of these core oligosaccharides can be fucosylated by α1-2, α1-3 or α1-4 linkage at various loci with one or more fucoses to obtain a fucosyl-oligosaccharide. Sialylation of a core or fucosyl-oligosaccharide with α2-3 or α2-6 linkage leads to a sialyl-oligosaccharide.

In average, 35-50% of all HMOs occurring in human milk are fucosylated, 12-14% are sialylated and 42-55% are core oligosaccharides (19).

Figure 3 depicts some exemplary structures of fucosylated and sialylated HMOs. It also shows that some oligosaccharides occur in several isomeric forms.

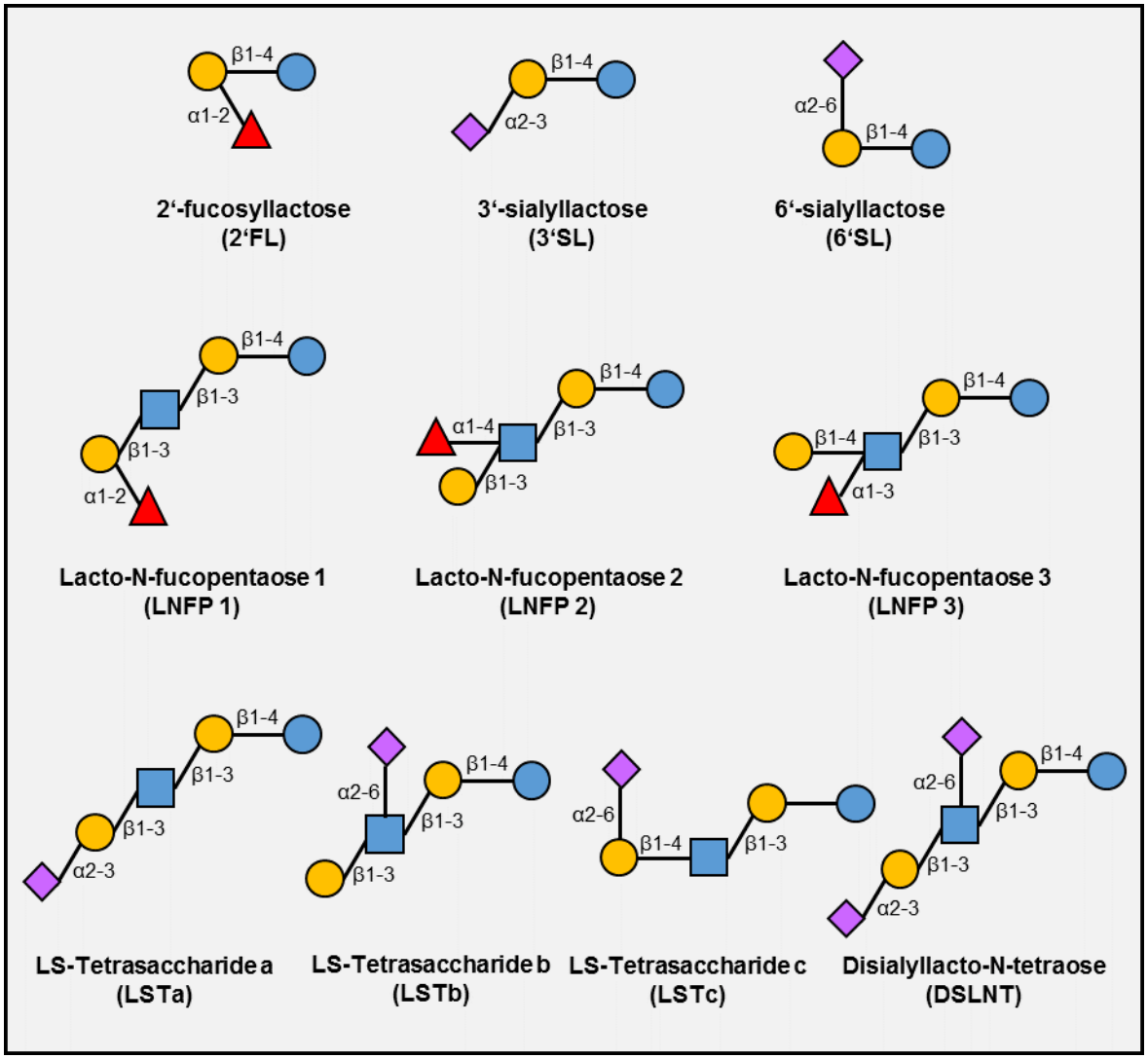

Figure 3: Structures of some exemplary HMOs.

Although hundreds of different oligosaccharides can be built according to this blueprint and occur in human milk (20), their distribution is rather unequal. The main quantitative component is represented by 20 to 25 highly abundant HMOs (most of which we used for analysis, see **Table 2**, page 19). The remaining oligosaccharides rarely occur and account just for minimal amounts.

Genetic factors – Secretor and Lewis genes

The individual composition of milk oligosaccharides as well as their quantity depends on the presence of several glycosyltransferases, which catalyze the elongation and branching of the Gal/GlcNAc core structure (20). Most of these enzymes, which are also responsible for building glycoconjugates at epithelial cell surfaces, are usually expressed within the mammary gland of all women.

More striking are the interindividual differences concerning the fucosylation, which is primarily done by two different fucosyltransferases, whose expression is genetically determined by two independent gen loci mirroring the Secretor (Se) and Lewis (Le) blood group systems: the α1-2-fucosyltransferase FUT2 (dependent on the Se gene) and the α1-3/4-fucosyltransferase FUT3 (dependent on the Le gene) (21-24). Both genes can independently either be active (Se(+), Le(+)) or inactive (Se(-), Le(-)), with the consequence of less fucosylated HMOs. Based on this, four different groups can be assigned (25,26), with the Se(+)/Le(+)-group showing the most complex HMO composition and the Se(-)/Le(-)-group the least complex composition. What is most apparent is that milk of secretor-negative women does not contain α1-2-fucosylated HMOs such as (the otherwise highly abundant) 2'FL and LDFT. Milk of lewis-negative women on the contrary lacks (much less abundant) α1-4-fucosylated HMOs, e.g. LNFP2.

However, when taking a closer look, the four-group system of fucosylation seems an oversimplification. To some extent, FUT2 and FUT3 compete for the same substrates, generating a continuum of different interindividual HMO compositions. Thus, the occurrence of some fucosylated HMOs within Se(-)/Le(-) women suggests the existence of yet unknown fucosyltransferases (27).

HMO sialylation does not follow this all-or-nothing principle of sialyltransferase expression. Interindividual differences in sialylation seem to be a much more complex interplay of variations in the expression of sialyltransferases or other enzymes and transporters, which is not fully understood yet (28).

Whether there are also environmental or nutritional factors that influence the HMO concentration and composition, has not been investigated so far (18).

Changes over period of lactation

The HMO composition varies not just due to interindividual (genetic) differences, but changes also over the course of lactation for each mother. Colostrum, the very first milk around parturition, shows the highest concentrations of oligosaccharides, containing as much as 20 - 25 g/l (7,8). Over the course of lactation, the HMO concentration decreases to 5 - 20 g/l (7,8,24,29-32). This, however, still exceeds the amount of total milk protein and is about 100-1000-fold higher than HMO-concentration in bovine milk, which is most commonly used as the basis for infant formula. Concentrations of lactose, in contrast, increase during the lactation period.

Milk of mothers with a preterm child is richer in oligosaccharides compared to the milk of mothers delivering a mature baby (8).

But there are also qualitative changes: structural analyses of oligosaccharides within the first weeks of lactation have shown a preponderance of short-structured HMOs (tri- to pentasaccharides), whereas longer structures emerge increasingly over time (14).

1.3 Postulated Functions

HMO metabolism

Once ingested, HMOs tolerate the low pH value of the breast-fed infant's stomach and are not digested by pancreatic and brush border enzymes (33,34). Therefore, HMOs don't have any nutritional value for the breast-fed child; most of them reach the distal small intestine and the colon intact and are excreted with the feces (35,36). Analyses of the excreted HMOs during the first two months of life revealed that they are similar, but not identical to the mother's HMO composition, suggesting a processing during the passage through the child's bowel. Processing and degradation increases in later months of breastfeeding; when other food than human milk gets introduced, HMOs completely disappear from the child's feces (37).

In-vivo studies with ^{13}C-labeled galactose showed that about 1% of the ingested HMOs is absorbed in the infant's intestine, reaches the systemic circulation and gets eliminated through the child's kidneys (17,38,39). This explained previous findings of intact HMOs in

the urine of breast-fed infants (40). The mechanisms of how exactly oligosaccharides are being absorbed as well as their functions in the systemic circulation remain unknown.
However, main functions of milk oligosaccharides seem to be located within the infant's intestinal tract. Mounting evidence suggests a leading role of HMOs as prebiotics, antimicrobials and modulators of epithelial and immune cell functions, which might explain some of the positive consequences associated to breastfeeding (28,41,42).

Prebiotic activity

At the time of birth, the child's gut is thought to be sterile. The comprehensive microflora of commensal bacteria strains, which are believed to play an important role in terms of protection, feeding and communication with the epithelial cells of the developing intestinal system, emerges within the first weeks of life (43). The gut's microbiota reacts very sensitive to environmental influences and hence gets influenced by specific nutritional components (44,45).
In-vitro fermentation studies showed that HMOs can be utilized by certain strains of *Bifidobacteriaceae* and *Bacteroidaceae* which possess an appropriate set of glycosidases to cleave the complex structure of HMOs. In an interplay with glycan binding proteins and sugar transporters, they are able to use the resulting monosaccharides as a nutritional source (46-48). The bacteria privileged by the presence of oligosaccharides may help to keep other, potentially harmful microbes in check by competing for the same nutritional sources. Besides, these bacteria produce metabolites (so-called post-biotics, such as short-chain fatty acids) that promote the growth of other, valuable commensals helping to suppress potential pathogens (49).

Antiadhesive antimicrobial effects

The role of HMOs is not just limited to their prebiotic functions. Serving as antiadhesive antimicrobials, they also directly deflect pathological microbes such as bacteria, viruses and protozoan parasites and thus, reduce the rate of microbial infections (15,27).
Before being able to proliferate and induce a disease, most microbes need to attach to an epithelial cell surface (such as the intestinal mucosa) as a first step of infection. This cell surface is covered by the glycocalyx, a layer of glycoproteins and glycolipids with complex sugar structures coating the membrane (50,51). Many pathogens are able to recognize specific glycan structures, including fucosylated and sialylated sugars, through highly spe-

cific receptors on their cell surface, called lectins. This lectin-glycan interaction usually initiates pathogen adhesion (52).

HMOs resemble some of the glycan structures of the glycocalyx and therefore work as soluble decoy receptors. Binding an HMO instead of cell-bound glycans prohibits pathogen adhesion and the microbe is washed out without inducing an infection (27). The large variety of different HMO structures suggests decoy functions for different pathogens, and indeed, a protective effect of specific HMO fractions against many relevant pathogens of the gastrointestinal, respiratory and urinary tract has been shown.

Core oligosaccharides, containing just Gal and GlcNAc, block the adhesion of *Vibrio cholaerae* (53), which causes severe diarrhea, and *Streptococcus pneumoniae* (54), responsible for infections of the respiratory tract and the middle ear. Incidental aspiration and nasal regurgitation of breast milk may protect the mucosa of the airways. Less incidence of otitis media has been shown within breast-fed infants compared to formula-fed infants (55).

Fucosylated HMOs deflect the binding of *Campylobacter jejuni*, the most common bacteria causing diarrhea, which is responsible for a considerable portion of infant mortality (56). A prospective study comprising almost hundred mother-infant dyads showed a significantly less occurrence of diarrhea caused by *Campylobacter jejuni* within children, whose mothers' milk was rich in 2'FL (57).

Sialylated HMOs inhibit the adhesion of *Escherichia coli* to erythrocytes (58); they deflect *Helicobacter pylori* (59), which is responsible for gastric diseases such as peptic ulcers, *Staphylococcus aureus* and *Clostridium botulinum* (52).

But not only bacteria are blocked by milk oligosaccharides. HMOs also bind to viruses such as rotavirus and norovirus (60) – the most common causes of (non-bacterial) diarrhea – and even the human immunodeficiency virus (HIV) (61). This might explain why HIV transmission through breast milk from infected mothers to their infants is rather inefficient. Around 80-90% of breast-fed infants do not acquire an infection, although their gut is continuously exposed to the virus over months.

Apart from combating bacteria and viruses, HMOs have a positive effect against certain protozoan parasites, such as *Entamoeba histolytica*, causing amoebic dysentery (62).

About 1% of all HMOs reach the systemic circulation and gets excreted with the urine. Therefore, the antiadhesive antimicrobial effects of oligosaccharides are not necessarily limited to the infant's gastrointestinal tract. There is some evidence that suggests a role of HMOs in preventing urinary infections (58).

In conclusion, antiadhesive antimicrobial effects of HMOs may be responsible for the reduced incidence of diverse infections observed in breast-fed infants compared to bottle-fed babies.

Epithelial cell modulation

In addition to the prebiotic and antiadhesive antimicrobial effects, there is also evidence that HMOs directly interact with epithelial cells of the hosts intestine. The incubation of human intestinal epithelial cells *in vitro* with the milk oligosaccharide 3'SL leads to a lowered expression of certain sialyltransferases and thus a reduction of α2-3- and α2-6-sialylated glycans within the glycocalyx on the cell surface (63). Since the enteropathogenetic *Escherichia coli* needs sialylated glycans on the cell surface to attach, binding of *E. coli* was significantly reduced.

More recent studies showed that HMOs also directly modulate cell responses of human intestinal cells. By altering growth-related cell cycle genes, HMOs were shown to diminish the growth of epithelial cells and to induct differentiation and apoptosis (64,65).

All the data concerning epithelial cell modulation was gained in cell culture models. Whether these findings can be translated to animal models or to the human intestine remains to be further investigated.

Immune modulation

It is conceivable that HMOs indirectly influence the infant's immune system through their prebiotic functions and by altering epithelial cell responses. Besides, there is also mounting evidence for a direct effect on specific immune functions. This impact of HMOs on cells of the immune system may either take place within the mucosa associated lymphoid tissue (MALT), which is closely adjacent to the intestine's mucosa, or on a systemic level (since about 1% of HMOs reach the systemic circulation (17,38,39)).

Exposing cord blood T cells to sialylated HMOs led to the finding that the number of interferon-γ-producing CD3+CD4+ and CD3+CD8+ lymphocytes as well as IL-13-producing CD3+CD8+ lymphocytes increased (66). The authors of this study speculate that sialylated milk oligosaccharides influence the maturation of lymphocytes and promote a more balanced Th1/Th2-cytokine production within T-cells.

Moreover, IL-4 production in a subset of lymphocytes from adults with peanut allergy was reduced by sialylated HMOs in an in-vitro model (67). This fueled some speculations that certain oligosaccharides may contribute to allergy prevention.

Two neutral HMOs, Lacto-N-fucopentaose 3 (LNFP 3) and Lacto-N-neotetraose (LNnT), have been shown to augment certain populations of peritoneal macrophages capable of suppressing CD4+ T-cell responses (68,69). LNFP 3 also enhances the secretion of prostaglandin E2, IL-10 and TNF-α of macrophages *in vitro* (70).

These studies suggest a specific role of HMOs in modulating immune functions and protecting the breast-fed infant. However, specific receptors or pathways transducing the HMO-mediated signals are currently unknown (18).

Further effects

In addition to the previously mentioned effects of HMOs, there are still some further aspects to be noted. It is known since the early 90s that formula-fed infants suffer a 6- to 10-fold higher risk of developing necrotizing enterocolitis (NEC) than their breast-fed counterparts (71-73). NEC is a common severe disorder of preterm infants with a mortality rate of more than a quarter (74-76). Etiology and pathogenesis of NEC are poorly understood yet, its treatment is limited and the surgical resection of necrotic parts of the intestine often represents the last remaining therapeutic option. Intervention studies in a rat model revealed that feeding HMOs indeed led to a lower incidence of NEC (3). Most intriguingly, one single HMO – DSLNT – seems to be responsible for the preventive effect. However, the specific mechanisms behind this seemingly structure-specific function need to be further elucidated. Human intervention studies are currently unfeasible because DSLNT and HMOs in general are not available in sufficient amounts.

Sialylated HMOs may also contribute significantly to the brain development. Preterm infants fed by breast milk show a faster brain development and reach higher intelligence scores at the age of 7 than formula-fed babies (77,78). Sia-containing gangliosides and glycoproteins seem to partly account for a sufficient brain development (79); the amount of Sia in the brain more than doubles during the first few years of life (80). Human milk is a rich source of Sia, mainly because of sialylated HMOs (81). As a carrier for the essential nutrient, HMOs may directly contribute to an adequate development of the brain and cognitive functions.

Finally, it is conceivable that the positive effects of HMOs as described previously are not only limited to the child, but also affect the mother. The prebiotic, antimicrobial and cell-modulating functions could also have an effect on the mother's mammary gland by preventing e.g. mastitis. However, there's currently no data confirming that. Nevertheless it is known for many decades that oligosaccharides appear in the urine of pregnant women

some time before parturition (9-12), suggesting a retrograde "leakage" into the maternal circulation even before lactation starts. This opens a new field of potential functions and effects of HMOs within the maternal circulation, becoming relevant already during pregnancy.

1.4 HMOs in pregnancy

Despite early findings of HMOs in the urine of pregnant women, several decades ago (9-12), which suggest a production of HMOs even before lactation starts, no focus of research has been put on these prenatal HMOs so far and no published evidence exists. A research group around E Jantscher-Krenn from the Medical University of Graz, Austria, recently dedicated its attention to this topic, investigating the role and functions of HMOs that systemically emerge in the mothers' circulation during pregnancy. This present thesis was developed within the framework of this research group. There is no data published so far; hence, this chapter is based on preliminary results and findings of this group.

Data by E Jantscher-Krenn provides first evidence that HMOs occur in the blood serum of healthy pregnant women already from the early second trimester on (demostrated for the 12^{th} week of gestation), but cannot be found in the serum of non-pregnant women. There are even some oligosaccharides found in serum which are usually not present in human milk (3'SLN, 6'SLN). The total HMO concentration increases over the course of gestation, but there are also changes in the composition of the HMO profile. Data of 30 sample sets of healthy pregnant women revealed a preponderance of sialylated HMOs at the 12^{th} week of gestation that changed to a more balanced sialylated/fucosylated HMO profile over the course of gestation, and led to a slight predominance of fucosylated HMOs at the end of pregnancy. This is mainly explained by a significant increase in the concentration of the highly abundant 2'FL, starting in the second trimester.

To proof whether the unborn child is exposed to HMOs, arterial and venous cord blood samples were collected and analyzed. And indeed 19 common oligosaccharides have been detected in cord blood so far. Their profile resembles the mothers' composition of systemic HMOs at the time of parturition, with small differences that need to be further investigated. So far, there is no evidence whether prenatal HMOs are just artifacts of a retrograde "leakage" from the developing mammary gland or if they fulfill any functions within the maternal or fetal organism. Further investigations on prenatal HMO concentrations and compositions in both, the maternal and fetal circulation, are necessary to elucidate potential con-

nections to certain functions for the mother or the child during the time of pregnancy, or to reveal relationships to specific disorders, such as gestational diseases.

1.5 Prenatal HMOs and blood lipids

So far, little is known about prenatal HMOs and their functions for the maternal or fetal organism. Studies on postnatal milk oligosaccharides showed highly structure-specific functions (see chapter 1.3), emphasizing the biological importance of the specific composition of the HMO profile for the breast-fed infant. The HMO composition is highly individual and depends partly on genetic factors (see chapter 1.2). Whether environmental factors, too, influence the individual HMO composition, is yet unknown.

Such an environmental influence could be, besides others, the woman's nutrition and life style, together leading to a specific metabolic situation. High caloric nutrition and a lack of physical activity, both of which characterize the western world's lifestyle, are a common cause for the metabolic syndrome, which is defined by the presence of four disorders: obesity, hypertension, dyslipidemia and insulin resistance. The prevalence of the metabolic syndrome increases and at the moment reaches about 20% of the whole population in Germany (82).

For this thesis, we chose to focus on blood lipids as one aspect of the woman's "metabolic situation" and investigated correlations that should reveal potential associations between HMOs and four types of blood lipids: triglycerides, total cholesterol, LDL and HDL. Thus, the human lipid metabolism shall be outlined shortly in the following chapters.

The human body contains several types of lipids, all of which are more or less lipophilic and highly diverse in their structure (83). Despite their heterogeneity, they can be assigned to five different groups of lipids: fatty acids, triglycerides, isoprenoids (such as cholesterol), phospholipids and glycolipids. The first two groups are primarily responsible for the energy supply of the body, lipids of the last three groups are important components of cell membranes and fulfill other, highly specific functions in the body (e.g. steroid hormones).

Triglycerides

Triglycerides (83) are esters of glycerol and three fatty acids that can either be saturated or unsaturated (see **Figure 4**). They are the main constituents of fat tissue and the main storage form of fatty acids. Fatty acids are important players of the energy metabolism. Broken down in the so-called beta-oxidation, which takes place in the mitochondria, they are im-

portant energy suppliers for the cell. Especially cells of the liver, heart and skeletal muscle use fatty acids as a main source of energy.

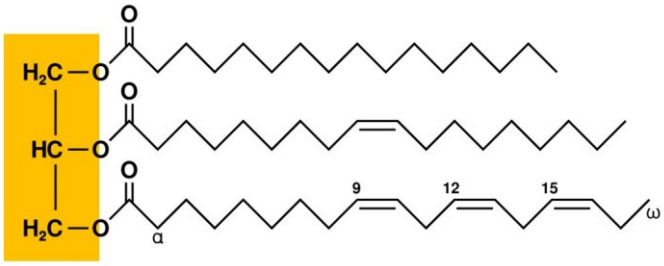

Figure 4: Structure of triglycerides.
Three fatty acids are linked to glycerol (marked orange) forming an ester. In this example, the three fatty acids are (from top to bottom): palmitic acid, oleic acid and alpha-linolenic acid.

The required amounts of triglycerides and fatty acids are partly absorbed from food. Furthermore, the body is capable of producing them by itself (exceptions are just two essential fatty acids: alpha-linolenic acid and linoleic acid). The biosynthesis of fatty acids starts with acetyl coenzyme A (acetyl-CoA), a molecule carrying an acetyl group that commonly originates from glucose metabolism. Several of these acetyl groups are linked to palmitic acid, a fatty acid with 16 carbon atoms and the starting point for the biosynthesis of other fatty acids. In times of energy and glucose excess, the body stores energy in this way. The responsible key enzymes are activated by the anabolic hormone insulin.
The main site for the biosynthesis of fatty acids is the liver. The subsequent synthesis of triglycerides enables the liver to send them via VLDLs (very low density lipoproteins) to peripheral organs for consumption or to fat tissue for storage.
On the contrary, during times of energy deficiency, triglycerides are being mobilized from fat tissue (lipolysis). Their fatty acids serve as a highly potent energy source for many other tissues (e.g. muscle, liver, kidney), whereas glycerol can be used for generating glucose in the liver (gluconeogenesis). This process of lipolysis is regulated by the hormones glucagon and adrenalin.
In the blood, lipophilic triglycerides are mainly transported in chylomicrons (after having been absorbed in the gut) and through VLDLs (when synthesized in the liver). The concentration in the blood serum depends on the uptake of triglycerides with nutrition, the meta-

bolic status (e.g. high vs. low caloric nutrition) and certain factors that influence the lipid metabolism (e.g. primary hypertriglyceridemia, cortisol, pregnancy). Pregnancy physiologically leads to an increase of serum triglycerides (84). After parturition, they are an important constituent of human milk and a central energy source for the breast-fed infant.

Cholesterol

In contrast to triglycerides, cholesterol (83) is not used as an energy source, but fulfills other important functions: It is a necessary component of cell membranes, modulating their fluidity, and the precursor for the biosynthesis of bile acids, steroid hormones and cardiac glycosides.

Due to a lack of enzymes, cholesterol cannot be broken down by the organism itself, but must leave the body via bile acids. An average adult loses around 1g cholesterol per day in this way. The same amount has to be replaced either through ingestion (accounts for usually less than half of the required amount) or through biosynthesis. Although all eukaryotic cells feature the required enzymes for cholesterol production, the liver is mainly in charge of it. Biosynthesis again starts with several Acetyl-CoAs and comprises around 50 different reactions to form the cholesterol structure, built by 27 carbon atoms (see **Figure 5**).

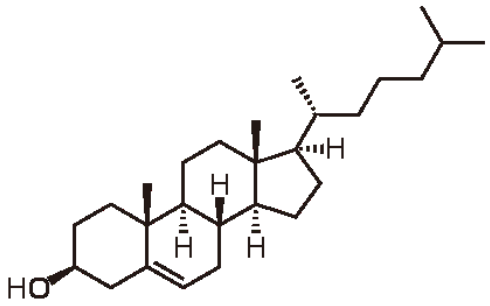

Figure 5: Structure of cholesterol.

Analogously to fatty acids, the biosynthesis of cholesterol is hormonally regulated by insulin (activating, representing a nutrient excess) and glucagon (inhibiting, representing a nutrient deficiency). In case of cholesterol overflow, cells are able to store little drops of it in their cytosol. Therefore, cholesterol esters need to be built to remove the polar hydroxy-group, making the molecule completely nonpolar. These cholesterol esters can also be sent through the body with the blood stream; again, lipoproteins such as VLDL, LDL and HDL are vehicles therefore.

Lipoproteins

Because of their lipophilicity, neither triglycerides nor cholesterol are soluble in blood and must therefore be packed together with special proteins (apoproteins) to so called lipoproteins (83), carrying lipophilic lipids in their core and amphiphilic lipids on their surface (see **Figure 6**).

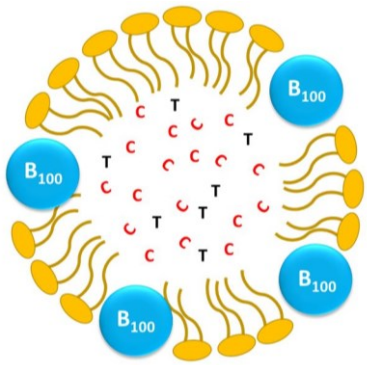

Figure 6: Structure of lipoproteins.
Schematic visualization of an LDL particle, proportions are not veritable. Triglycerides (T) and cholesterol esters (C) are surrounded by amphiphilic phospholipids (yellow) and apoproteins (ApoB-100 in case of LDL).

There are different types of lipoproteins, varying in size, composition, density and durability. Chylomicrons and very low density lipoproteins (VLDL) are the biggest lipoproteins, carrying primarily triglycerides. Chylomicrons are responsible for the transport of ingested triglycerides and cholesterol esters from the gut to their target organs (with the lowest durability of less than one hour), whereas VLDLs are built in the liver and carry triglycerides and lesser cholesterol to peripheral organs (persisting for a few hours). Responsible for targeting the aimed tissue are apoproteins that act as ligands for specific receptors, activate lipoprotein lipases (for breaking down triglycerides) or trigger endocytosis of the lipoprotein. By releasing particularly triglycerides to fat tissue, muscles or other organs, VLDLs become smaller and return as VLDL remnants to the liver again, where they are reassembled.

About 30% of all VLDL remnants do not get absorbed by hepatocytes, but are left over as low density lipoproteins (LDLs), characterized by a far higher percentage of cholesterol esters (about 40%). They carry apolipoprotein B-100 (ApoB-100) on their surface, which acts as a ligand for LDL receptors on almost all body cells.

LDLs are the cholesterol reservoirs of the body, enabling cells to easily gather cholesterol by LDL endocytosis and subsequently hydrolyze their cholesterol esters. The level of floating LDLs is registered by the liver, which regulates cholesterol biosynthesis based on this information.

While LDLs are responsible for transferring cholesterol to peripheral cells, the reverse cholesterol transport is done by HDLs (high density lipoproteins), the smallest of all lipoproteins. They collect excessive cholesterol from peripheral cells and transfer it to cells, which demand cholesterol, or to the liver, where cholesterol gets eliminated via bile acids.

LDL and HDL particles persist for several days in the blood circulation and represent two different aspects of the cholesterol metabolism. Both are important clinical markers for assessing the risk of arteriosclerosis. High levels of LDL lead to LDL deposits within the intima of arteries, where lipids are partly oxidized and subsequently phagocytized by macrophages, leading to the pathogenesis of arteriosclerosis. HDLs, on the other hand, are able to remove cholesterol from the arterial wall and from macrophages and have therefore preventive effects.

2. Materials and Methods

2.1 Study design

FitFor2-Study

To investigate correlations between special HMO configurations of pregnant women and their lipid profiles, we used serum samples of 94 women that were collected as a part of the FitFor2-study in the Netherlands (85,86). This study, carried out between 2007 and 2011 at the VU University Medical Centre in Amsterdam, was designed as a randomized controlled trial (RCT) and aimed to assess whether insulin sensitivity and fasting plasma glucose levels of pregnant women, who were at high risk for gestational diabetes, could be improved by a continuously carried out exercise program. Relating to the primary question, there were no significant differences found between the control and the intervention group (with the exercise program being the only intervention). Nevertheless, the collected samples and biometrical data turned out to be useful for further investigations.

Study population

The main target group of the FitFor2-study were pregnant women at increased risk for gestational diabetes (GDM). All participants were therefore either obese (BMI $\geq$ 30) OR overweight (BMI $\geq$ 25) with at least one of the three following additional risk factors for GDM (85):

- history of macrosomia (infant with a birth weight above the 97th percentile of gestational age)
- history of abnormal glucose tolerance during previous pregnancies
- first grade relative with diabetes mellitus type 2

Further inclusion criteria were: pregnancy between 14th and 20th week of gestation; age over 18 years; fluency in spoken Dutch; able to perform the exercise program; and written informed consent.

Exclusion criteria for the RCT were: diagnosis of (gestational) diabetes mellitus before randomization; hypertension (systolic pressure > 160 mmHg or diastolic pressure > 100 mmHg); serious cardiac, hepatic, pulmonary (COPD, asthma) or renal (serum creatinine < 150 µmol/l) disorders; malignant tumors; serious mental or physical impairment; alcohol

abuse (i.e. two glasses alcohol or more per day); drug abuse (except for incidental analgesic drugs); use of medication affecting insulin secretion or sensitivity (e.g. antiviral or antihypertensive drugs, corticosteroids, all concomitant medication was being discussed).

Participants, including both Caucasian and Non-caucasian women, were recruited and examined in ten midwifery practices and three large hospitals in Amsterdam, The Netherlands. **Table 1** shows some characteristics of the study population we used for our HMO analysis (n = 94):

Table 1: Characteristics of study population

		statistics (n = 94)
age (years)	mean	30.3
	standard deviation	5.00
	minimum	19
	maximum	42
body height (cm)	mean	165.4
	standard deviation	8.05
	minimum	150
	maximum	186
weight (kg) before pregnancy	mean	91.3
	standard deviation	15.79
	minimum	61
	maximum	130
BMI before pregnancy	mean	33.3
	standard deviation	4.92
	minimum	25.3
	maximum	51.3

Setting

To investigate potential correlations between blood lipids (LDL, HDL, total cholesterol, triglycerides) and specific HMO structures, we used data of lipid measurements carried out within the FitFor2-study. All data (including biometrical measurements such as weight and BMI) was collected at three time points during pregnancy (approx. 15^{th}, 24^{th} and 32^{nd} week of gestation) (85).

We analyzed HMO in maternal serum samples from visit 2 (24th week of gestation). The following HMOs were measured in relative concentrations:

Table 2: HMOs measured in serum samples

Abbr.	HMO
2'FL	2'-fucosyllactose
3'SLN	3'-siallyllactosamine
LDFT	Lactodifucotetraose
3'SL	3'-sialyllactose
6'SLN	6'-sialyllactosamine
6'SL	6'-sialyllactose
LNT	Lacto-N-tetraose
LNnT	Lacto-N-neotetraose
3'S3FL	3'sialyl-3-fucosyllactose
LNFP 1	Lacto-N-fucopentaose 1
LNFP 2+3	Lacto-N-fucopentaose 2+3
LSTa	Sialyl-lacto-N-tetraose a
LSTb	Sialyl-lacto-N-tetraose b
LSTc	Sialyl-lacto-N-tetraose c
LNFDH	Lacto-N-difucohexaose
DSLNT	Disialyl-lacto-N-tetraose

The secretor status was defined by the presence or absence of 2'FL and LDFT in visit 2. For a subset of 30 women, HMOs were analyzed at all three time points to show the development of HMO composition during pregnancy, and to determine whether the secretor status can be identified in the 24th week of gestation.

Finally, Spearman correlations were calculated for LDL, HDL, total cholesterol and triglycerides of all three visits on the one hand side and relative HMO concentrations (in percentage of total HMO concentration) on the other hand side.

2.2 Laboratory measurements

Lipid analysis

Lipid measurements (LDL, HDL, total cholesterol and triglycerides) have already been performed within the FitFor2-study in standardized routine laboratories in Amsterdam. Data was transmitted in mmol/l.

Sample preparation and HMO isolation for HPLC

HMOs were isolated from the venous serum as previously described by E Jantscher-Krenn and L Bode (3,62):

Chloroform-methanol extraction

50 µl of the serum sample, diluted in 350 µl H_2O were subjected to chloroform-methanol extraction in order to get rid of lipids and proteins. A 2:1 chloroform-methanol solution was therefore added twice (1 ml resp. 800 µl) to the sample and centrifuged for 15 minutes (1300 x g at 4 °C). Only the aqueous phase (upper layer) was further used. Adding 300 µl H_2O to the methanol phase after the second round led to precipitation of residual proteins. A third centrifugation formed a protein pellet; the supernatant (800 µl) was subjected to solid phase extraction (SPE).

SPE C18

SPE with C18 columns (C18 SPE Hypersep 50 mg/ml - Thermo Scientific #60108-390) on a vacuum manifold rack was further used to remove nonpolar molecules. After conditioning the columns with 1 ml methanol and 1 ml H_2O, the sample was washed through the column together with 1 ml H_2O and collected again.

SPE Carbograph

Carbograph columns (Hypercarb Hypersep SPE Columns PGC – Thermo Scientific #60302-602) were used to remove inorganic substances (desalting). After conditioning with 400 µl of 80% acetonitrile solution with trifluoroacetic acid (80% ACN/H_2O + 0.1% TFA) and 400 µl H_2O, the sample was loaded onto the column and washed through with 1 ml H_2O. Oligosaccharides were retained due to their organic structure by the carbonaceous adsorption media and finally washed out with 400 µl of a 40% acetonitrile solution (40% ACN/H_2O + 0.05% TFA).

2-AB labeling

After drying down the eluent, the HMOs (resp. their reducing lactose end) were labeled with the aromatic tag 2-aminobenzamid (2-AB) in order to detect and quantify them by an HPLC system with fluorescence detection (87). The 2-AB reagent was prepared as follows: 130 µl dimethyl sulfoxide (DMSO) and 70 µl glacial acetic acid were mixed (65:35); 13 mg of 2-AB were weight and dissolved; and finally, 13 mg of sodium cyanoborohydride ($NaBH_3CN$) were added and dissolved by sonication. 5 µl of this 2-AB reagent were put on each dried sample and incubated for two hours at 65 °C labelled all reducing sugars.

SPE Silica

The 2-AB-labeling was followed by a last SPE using silica columns (HyperSEP Silica Columns 25 mg – Fisher #60300-482). Columns were equilibrated with 200 µl H_2O and 200 µl of pure acetonitrile (ACN). Then, samples including the 2-AB reagent were taken up in 195 µl pure acetonitrile and transferred to the columns. After washing with another 800 µl pure ACN and 800 µl 96% ACN/H_2O, the adsorbed HMOs were eluted with 400 µl H_2O and dried down again.

HMO identification by HPLC with fluorescence detection

HMOs were separated on a normal-phase (NP)-HPLC system using an Amide-80 column (TSKgel Amide-80; 3 µm 3 mm x 15 cm) and a linear gradient. Their 2-AB tag was detected by a fluorescence detector.

Buffer preparation

Buffer A (50 mM ammonium formate solution, NH_4COO^-, pH 4.4): 2.6 ml formic acid were added to 950 ml H_2O. The pH was adjusted to 4.4 by adding approximately 6 ml ammonium hydroxide. The solvent was filled up with water to a total volume of 1 liter.
Buffer B (20% buffer A, 80% acetonitrile): 200 ml buffer A were added to 800 ml acetonitrile and degassed by sonication.

HPLC system

Dried samples were taken up in 50 µl buffer B. For peak identification (through comparing retention times), a standard mix of 17 HMOs (see **Table 2**, page 19) was prepared and dissolved in buffer B.
The HPLC system was set up with the following parameters:

- t=0 min, 100% buffer B; t=115 min, 0% buffer B; flow 0.18 ml/min;
- t=115.1 min, 0% buffer B; t=120 min, 100% buffer B; flow 0.45 ml/min;
- t=120.1 min 100% buffer B; flow 0.18 ml/min; total runtime is 125 min

The fluorescence detector settings were adjusted as follows: excitation wavelength 360 nm, emission wavelength 425 nm, bandwidth 13 nm.
25 µl of the sample were injected at each run. After five sample-runs, a run with the standard mix was set. Occasionally, a washing gradient without sample injection was used to clean the column.

Peak identification and integration

HMO peaks were identified by comparing the retention times to a standard mix of known HMOs (see **Figure 7** and **Figure 8**). **Table 3** shows the retention time of each analyzed HMO. After drawing a continuous baseline from the very beginning to the end of each chromatogram, integration marks were set manually for each peak. The resulting areas under the curve (AUCs) were summed up to calculate the relative concentrations for each HMO (in percentage of total HMOs).

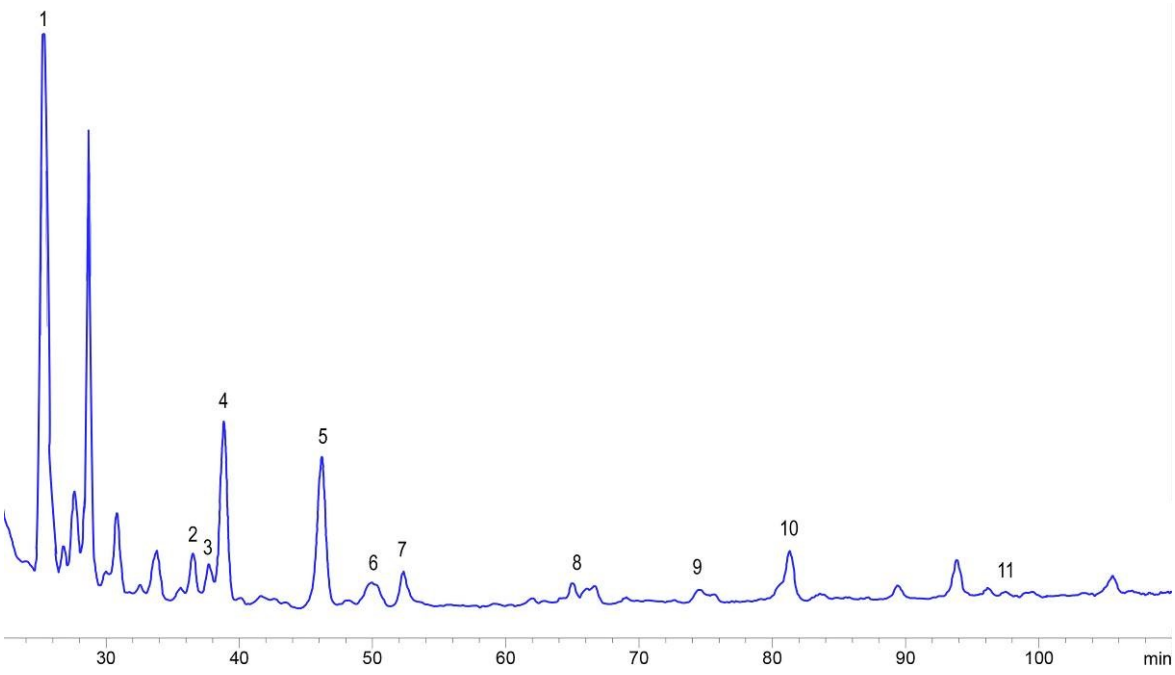

Figure 7: Typical chromatogram of 2-AB labeled HMOs separated by NP-HPLC.
Complete time-range of one exemplary sample. Peaks were identified by comparing retention times to an HMO standard mix (see **Figure 8**). The first relevant HMO-peak (2'FL) appears at 25.3 min, the last at 97.3 min (DSLNT). Not all examined HMOs were found in each sample. The following HMO-peaks are labelled in this example: 1 2'FL, 2 3'SLN, 3 LDFT, 4 3'SL, 5 6'SLN, 6 6'SL, 7 LNnT, 8 LNFP1, 9 LSTa, 10 LSTc, 11 DSLNT.

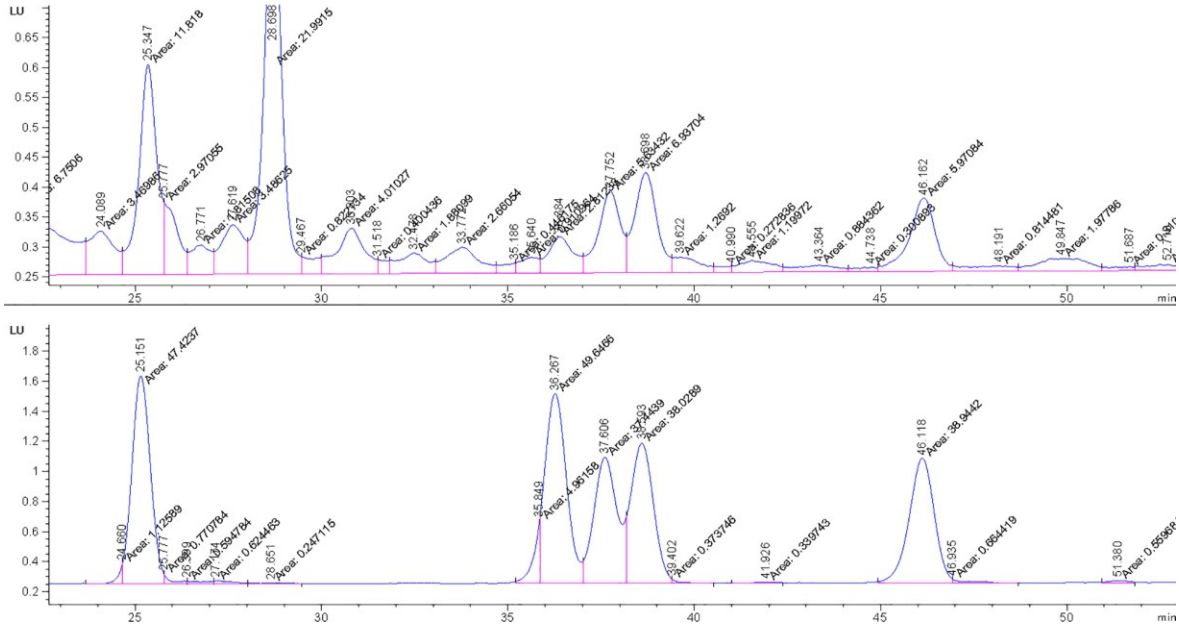

Figure 8: Peak identification and integration.

A time range between 25 min and 50 min is shown. The upper chromatogram represents the sample run, the lower one the HMO standard mix. The baseline is drawn pink in the upper chromatogram. Vertical pink lines indicate the integration marks used to calculate the area under the curve (AUC) for each peak. Retention times and integration marks fit well to the standard mix. Each peak is labeled with the retention time in minutes and the calculated AUC.

Table 3: Retention times of analyzed HMOs

HMO	RT (min)
2'FL	25.3
3'SLN	36.5
LDFT	37.7
3'SL	38.8
6'SLN	46.2
6'SL	50.3
LNT	51.4
LNnT	52.6
3'S3FL	53.6
LNFP 1	65.9
LNFP 2+3	70.7 (double peak)
LSTa	74.6
LSTb	78.3
LSTc	81.8
LNFDH	85.3
DSLNT	97.3

2.3 Statistical methods

After computing relative concentrations of each HMO (in percentage of total HMOs), all data was loaded into IBM SPSS Statistics 23.

Statistical analysis comprised descriptive statistics of the lipid and the HMO profile of our study population and calculation of Spearman's rank correlation coefficients (Spearman's rho). Spearman correlations were used (in contrast to Pearson product-moment correlation coefficients), because HMO data were not normally distributed.

Spearman's rho was calculated for all detected HMOs and total cholesterol, LDL, HDL and triglycerides of all three visits. Lipid data was used of all three visits in order to confirm and strengthen significant results: A significant correlation between an HMO and just one lipid measurement is more likely stochastical than significant results found during all visits. Due to already expected high differences in the percentage of HMO distribution between secretor-positive and -negative women, both subgroups were evaluated separately. Furthermore, additional parameters such as the BMI were taken into account for a more detailed and specific evaluation.

3. Results

3.1 Secretor status

Since the HMO distribution, which we compare in relation to maternal lipids, depends vastly on the secretor status (see chapter 1.2), we had to evaluate secretor-positive and negative women separately. The validity of our results therefore essentially depends on the correct definition of the secretor status. Women lacking 2'FL and LDFT in their serum sample of visit 2 were defined as secretor-negative.

To ensure that this approach is valid and the secretor status can reliably be identified in the 24th week of gestation, we tested whether there is a major change in the secretion pattern of 2'FL and LDFT later on during the course of pregnancy. We therefore analyzed HMOs at all three points in time (approx. 15th, 24th and 32nd week of gestation) in a subset of 30 women (see **Figure 9**). And indeed, all women who were classified as non-secretors based on visit 2 didn't show any secretion of 2'FL and LDFT later in pregnancy. On the contrary, there were no secretor-negative women missed in visit 2.

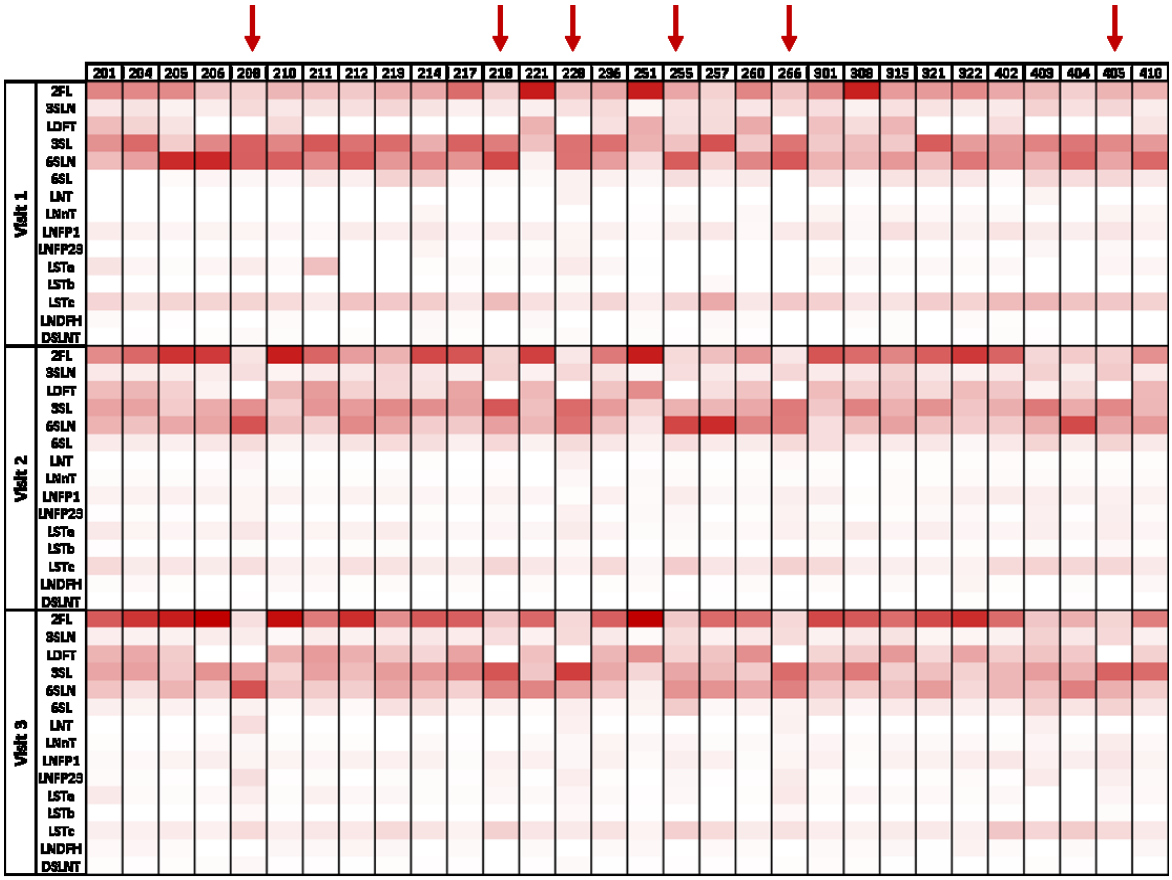

Figure 9: Heatmap of HMO distribution throughout the course of pregnancy.
Each column represents the HMO profile of one woman during all three visits (15th, 24th and 32nd week of gestation). The dark red color indicates a high relative concentration of the HMO. Women classified as non-secretors are marked by a red arrow at the top.

3.2 Descriptive statistics

Lipid profiles of study population

All lipid data presented here is quantified in mmol/l. **Table 10** (supplemented on page 49) may be used to find the equivalent values of mg/dl, which is often used in clinics.

Table 4 shows the lipid characteristics of the study population in total as well as broken down for secretor positive and negative women separately. The physiological increase in lipid concentrations (total cholesterol, triglycerides, LDL) through the course of gestation is due to altered nutritional requirements during pregnancy (84). There are no apparent differences between the subgroups of secretor positive and negative women. This has also been shown by an independent sample t-test (results not shown) that revealed no significant differences.

Table 4: Lipid characteristics of study population

		Week of gestation	N	Min	Max	Range	Mean	SD
				(mmol/l)				
Total population	Total cholesterol	15	92	2.90	7.15	4.25	5.03	0.90
		24	92	3.30	8.00	4.70	5.46	1.00
		32	77	3.50	8.40	4.90	5.90	1.08
	Triglycerides	15	92	0.56	2.81	2.25	1.54	0.51
		24	92	0.80	3.30	2.50	1.81	0.58
		32	77	1.01	3.80	2.79	2.25	0.65
	HDL	15	92	1.00	2.87	1.87	1.75	0.38
		24	92	1.00	2.73	1.73	1.83	0.41
		32	77	1.20	2.67	1.47	1.75	0.37
	LDL	15	92	1.07	4.92	3.85	2.59	0.81
		24	92	0.17	5.79	5.63	2.82	0.93
		32	77	1.21	5.69	4.48	3.14	1.03
Secretor-positive	Total cholesterol	15	64	3.20	7.15	3.95	5.01	0.84
		24	65	3.40	7.70	4.30	5.51	0.88
		32	54	4.00	8.40	4.40	5.90	1.01
	Triglycerides	15	64	0.67	2.81	2.14	1.53	0.48
		24	65	0.80	3.30	2.50	1.81	0.52
		32	54	1.10	3.80	2.70	2.27	0.63
	HDL	15	64	1.00	2.87	1.87	1.78	0.39
		24	65	1.00	2.72	1.72	1.84	0.42
		32	54	1.20	2.67	1.47	1.80	0.40
	LDL	15	64	1.07	4.36	3.29	2.54	0.74
		24	65	1.12	4.62	3.50	2.85	0.79
		32	54	1.21	5.03	3.82	3.08	0.97
Secretor-negative	Total cholesterol	15	28	2.90	7.10	4.20	5.06	1.03
		24	27	3.30	8.00	4.70	5.34	1.25
		32	23	3.50	8.20	4.70	5.89	1.25
	Triglycerides	15	28	0.56	2.70	2.14	1.56	0.58
		24	27	0.90	3.26	2.36	1.82	0.72
		32	23	1.01	3.60	2.59	2.19	0.70
	HDL	15	28	1.20	2.30	1.10	1.67	0.33
		24	27	1.10	2.73	1.63	1.79	0.40
		32	23	1.20	2.10	0.90	1.62	0.28
	LDL	15	28	1.11	4.92	3.81	2.69	0.96
		24	27	0.17	5.79	5.63	2.74	1.21
		32	23	1.21	5.69	4.48	3.29	1.16

Figure 10 shows that lipid profiles are widespread and almost normally distributed. Since both, pathological and non-pathological data, is represented, the samples suit well for investigations concerning the impact of blood lipids on HMO distribution.

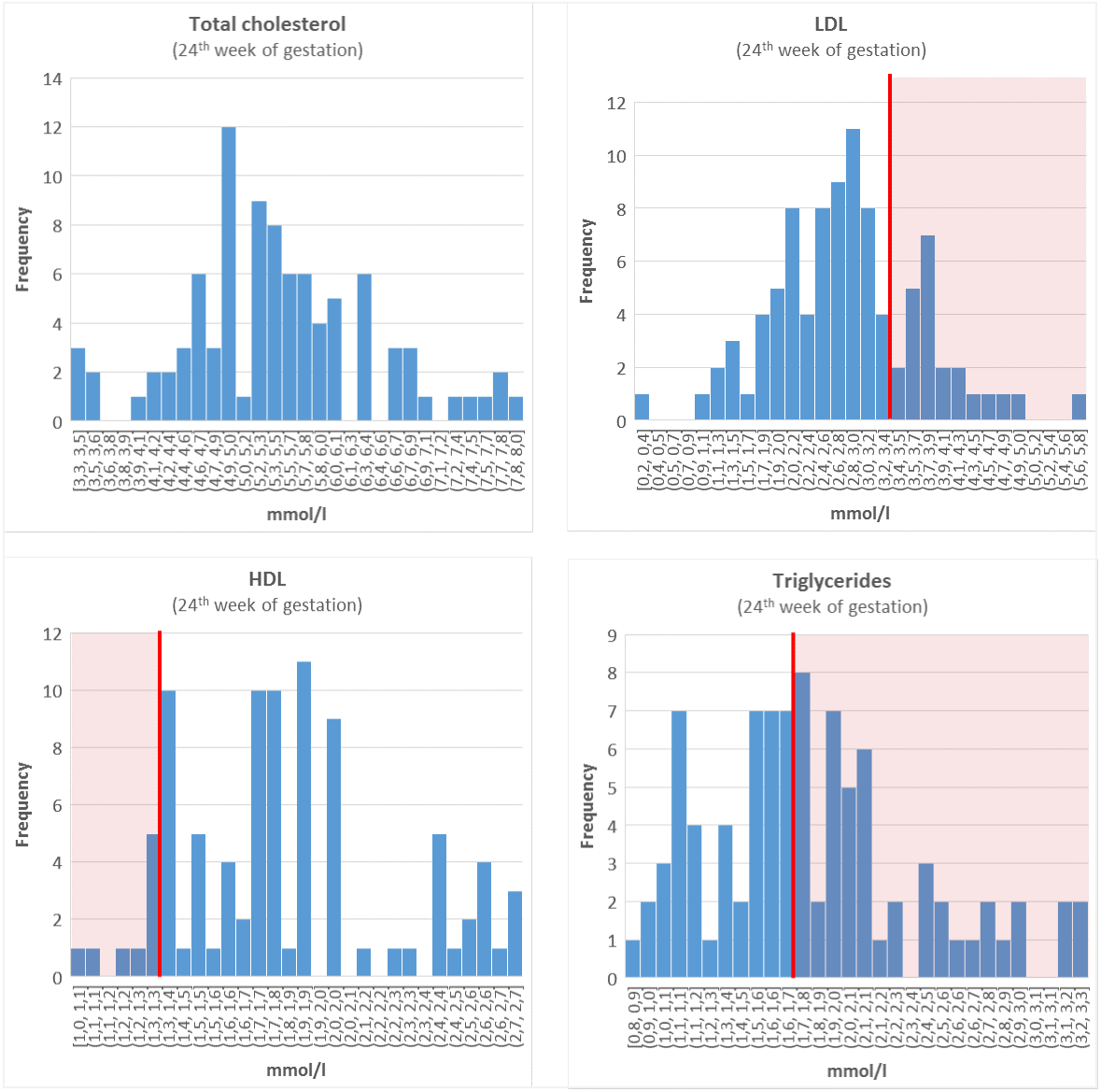

Figure 10: Distribution of blood lipids

Histograms of abundance of blood lipids in the study population (n=94), 24th week of gestation. Cholesterol, LDL, HDL and triglycerides are almost normally distributed, including pathological and non-pathological values. The threshold values for LDL and triglycerides, distinguishing the normal range from hyperlipidemia, are marked red. For HDL a minimal value of 1.3 mmol/l is recommended. Total cholesterol itself is currently not relevant for the diagnosis of hyperlipidemia. All recommended threshold values are based on the Austrian lipid consensus (88). Besides, it has to be taken into account that levels of blood lipids are elevated physiologically during pregnancy (84).

HMO distribution

The 17 HMOs analyzed in maternal serum from the 24th week of pregnancy were distributed as shown in **Table 5** and **Figure 11**. 28 out of 94 women were secretor-negative (29.2%). As expected, HMO distribution vastly depends on the secretor status which influences the presence of the two highly abundant HMOs 2'FL and LDFT. In general, the HMO chromatogram is dominated by the first six HMOs appearing after separation on the HPLC-column: 2'FL, 3'SLN, LDFT, 3'SL, 6'SLN, 6'SL. These HMOs partly account for 10% or much more of the total HMO distribution. LSTc, another HMO appearing later in the chromatogram, is also extensively represented and reaches up to 10% in the Se(+)-Group and 13% in the Se(-)-Group.

All the other, much less frequent HMOs constantly account for less than 10% of the total HMO distribution.

Table 5: Descriptive statistics of HMO distribution

		N	Min (%)	Max (%)	Mean (%)	SD (%)
Secretor-positive	2'FL	66	8.38	57.36	31.33	11.13
	3'SLN	66	1.94	9.63	4.91	1.34
	LDFT	66	0.00	23.85	11.20	4.05
	3'SL	66	9.19	33.08	18.88	5.43
	6'SLN	66	5.58	44.06	15.17	7.91
	6'SL	66	1.91	9.08	4.63	1.36
	LNT	66	0.08	1.71	0.36	0.58
	LNnT	66	0.00	3.61	1.06	0.80
	3'S3FL	66	0.00	1.93	0.66	0.11
	LNFP1	66	0.36	4.66	2.61	0.08
	LNFP2/3	66	0.00	3.41	0.52	0.99
	LSTa	66	0.00	4.47	2.09	0.30
	LSTb	66	0.00	1.01	0.10	0.53
	LSTc	66	1.96	9.66	5.09	1.50
	LNFDH	66	0.00	3.27	1.11	0.20
	DSLNT	66	0.00	1.05	0.27	0.40
Secretor-negative	2'FL	28	0.00	12.82	5.76	3.09
	3'SLN	28	4.39	11.14	8.12	1.74
	LDFT	28	0.00	0.00	0.00	0.00
	3'SL	28	16.30	41.77	30.69	6.18
	6'SLN	28	11.79	48.86	24.73	7.90
	6'SL	28	5.40	10.15	7.71	1.99
	LNT	28	0.00	6.06	2.00	1.50
	LNnT	28	0.73	3.54	1.67	0.28
	3'S3FL	28	0.98	3.31	1.94	0.42
	LNFP1	28	0.79	6.10	2.87	1.15
	LNFP2/3	28	0.00	6.99	3.18	1.03
	LSTa	28	0.00	5.12	2.90	1.41
	LSTb	28	0.00	1.70	0.35	0.92
	LSTc	28	2.96	12.64	7.29	2.82
	LNFDH	28	0.00	0.00	0.00	0.00
	DSLNT	28	0.00	1.88	0.78	0.16

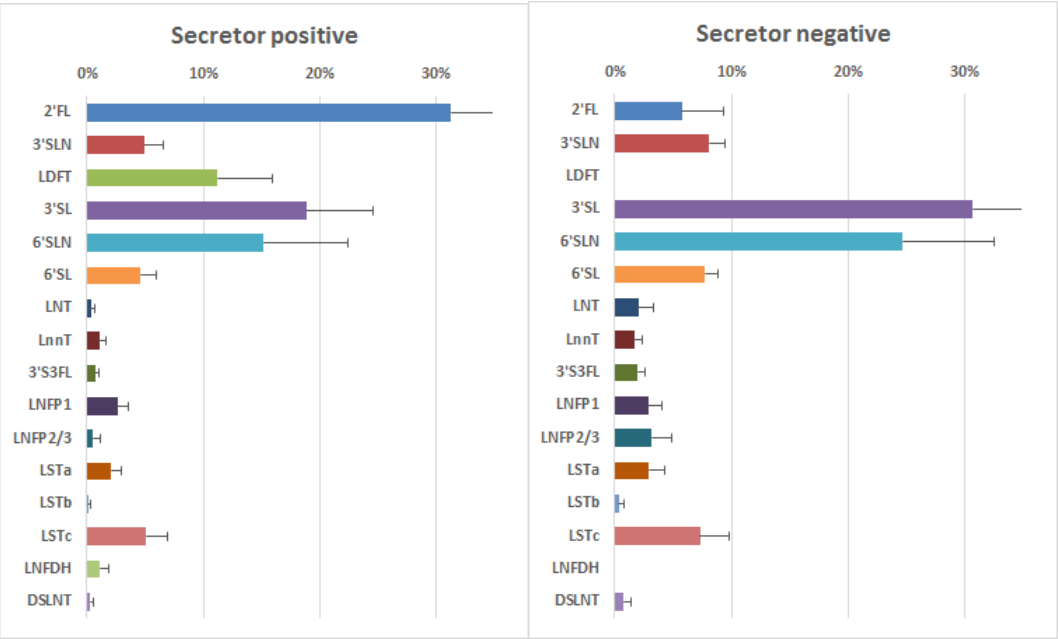

Figure 11: Mean relative frequency of HMOs

Separately shown for secretor-positive and -negative women, in percentage of total HMO concentration. The error bars depict the standard deviation.

3.3 Spearman correlations

Due to elementary differences in the percentage of HMO distribution of secretor-positive and -negative women (see **Figure 11**), both groups had to be evaluated separately for potential correlations. Because lipid data was not available for all women during all visits, the sample size, on which the calculation is based, varies between the visits. The reason, why lipid data of all three points in time were used for correlations, is to confirm and strengthen significant results: A significant correlation between an HMO and just one lipid measurement is more likely stochastical than significant results found during all visits.

In the following tables, r always represents the Spearman rank correlation coefficient, P the test for significance, and N the sample size of the corresponding calculation. Significant correlations ($P \leq 0.05$) are highlighted in bold characters; correlations at a very high level of significance ($P \leq 0.01$) are indicated in red.

The following chapters show just strikingly significant correlations. For an overview of all calculated Spearman rank correlations, see **Table 11**, page 50, and **Table 12**, page 51, in the appendix.

Secretor-positive group

Within the secretor-positive group, two HMOs were noticeable associated to total cholesterol and LDL **(Table 6**). There was a significantly negative correlation between 2'FL and both lipids in visit 2 and 3, with LDL being the slightly stronger and more significant correlation.

Analogously, relative concentrations of 3'SLN correlate positively with LDL and total cholesterol at a very high level of significance. The correlation was found during all visits, with visit 2 showing the strongest association in case of LDL.

Table 6: Spearman rank correlations between total cholesterol / LDL and 2'FL / 3'SLN within secretor-positive women

		Total cholesterol			LDL		
		Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3
2'FL (Visit 2)	Spearman r	-0.065	**-0.257**	**-0.308**	-0.193	**-0.294**	**-0.348**
	P value	0.609	**0.039**	**0.023**	0.126	**0.018**	**0.010**
	N	64	**65**	**54**	64	**65**	**54**
3'SLN (Visit 2)	Spearman r	**0.304**	**0.389**	**0.427**	**0.365**	**0.418**	**0.395**
	P value	**0.014**	**0.001**	**0.001**	**0.003**	**0.001**	**0.003**
	N	**64**	**65**	**54**	**64**	**65**	**54**

These correlations (with lipid data of visit 2) are visualized in the following scatterplots (**Figure 12**, **Figure 13**, **Figure 14**, **Figure 15**). The color of dots additionally depicts the BMI category of the women, with blue representing a BMI between 25 and 30, green indicating a BMI between 30 and 35 and orange a BMI higher than 35. All BMI data was measured pregestational. Separate calculation of the Spearman rank correlations for each BMI category led to much less significant results due to a lower sample size and is therefore not shown. As can be seen in the scatterplots, there are also no striking differences in the relative HMO frequencies between different BMI categories. This has also been shown by independent sample t-tests that revealed no significant results.

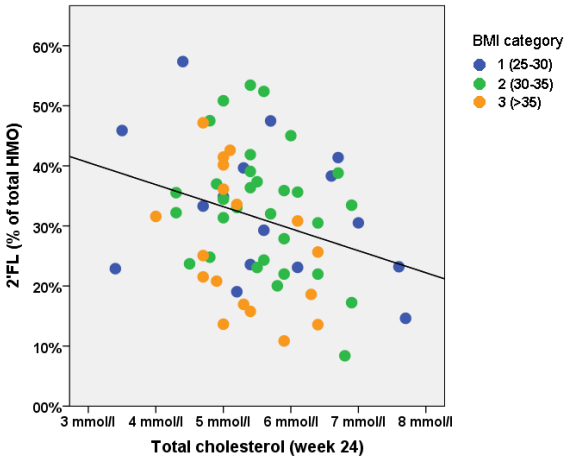

Figure 12: Correlation of total cholesterol and 2'FL

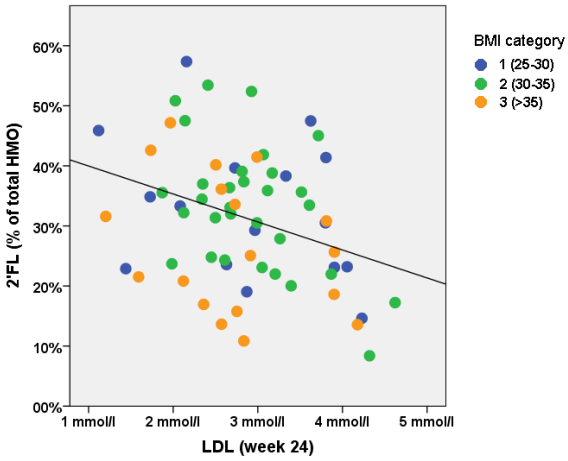

Figure 13: Correlation of LDL and 2'FL

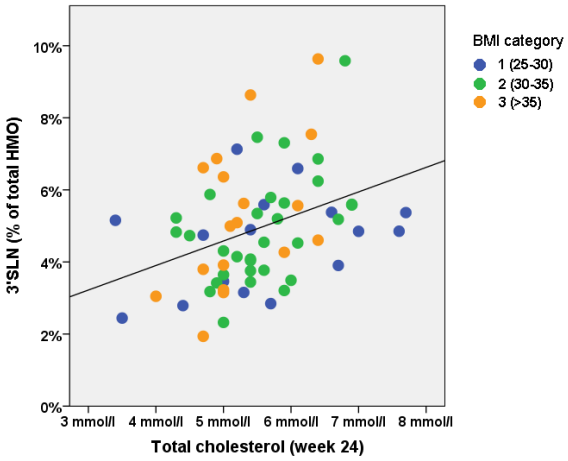

Figure 14: Correlation of total cholesterol and 3'SLN

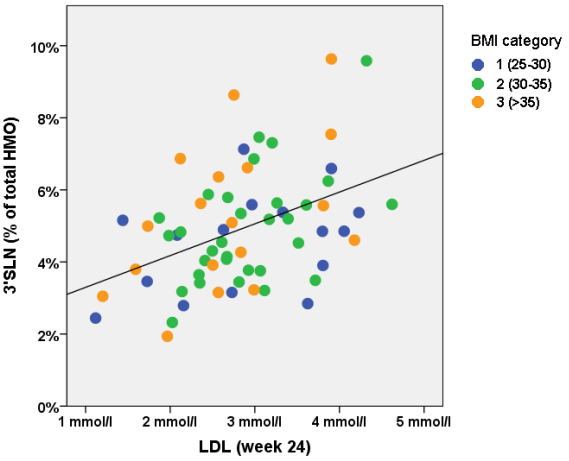

Figure 15: Correlation of LDL and 3'SLN

Secretor-negative group

Interestingly, the correlations found within the secretor-positive group were not found within the non-secretor group (especially concerning 3'SLN, 2'FL lacks naturally). On the contrary, some new correlations appeared. The total cholesterol correlated positively with relative concentrations of 3'S3FL and LSTb (see **Table 7**). The correlation with LSTb was highly significant during all visits and stronger than the correlation with 3'S3FL, which reached the level of significance just in visit 1 and 2. However, it was close to significance in visit 3; a higher sample size would probably lead to significant results.

Figure 16 and **Figure 17** again show the correlations of visit 2 for different BMI categories.

Table 7: Spearman rank correlations between total cholesterol and 3'S3FL / LSTb within secretor-negative women

		Total cholesterol		
		Visit 1	Visit 2	Visit 3
3'S3FL (Visit 2)	Spearman r	**0.430**	**0.407**	0.400
	P value	**0.022**	**0.035**	0.058
	N	**28**	**27**	23
LSTb (Visit 2)	Spearman r	**0.531**	**0.587**	**0.624**
	P value	**0.004**	**0.001**	**0.001**
	N	**28**	**27**	**23**

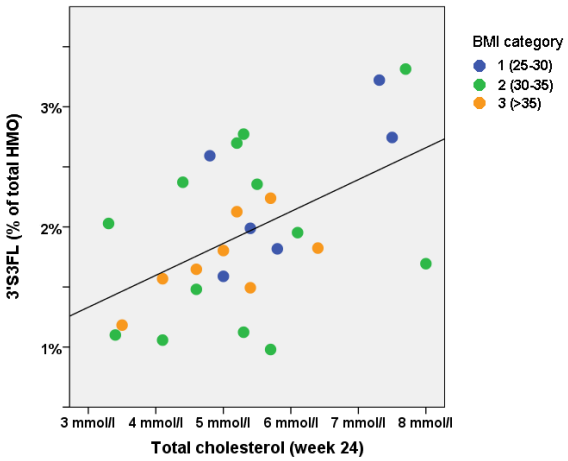

Figure 16: Correlation of total cholesterol and 3'S3FL

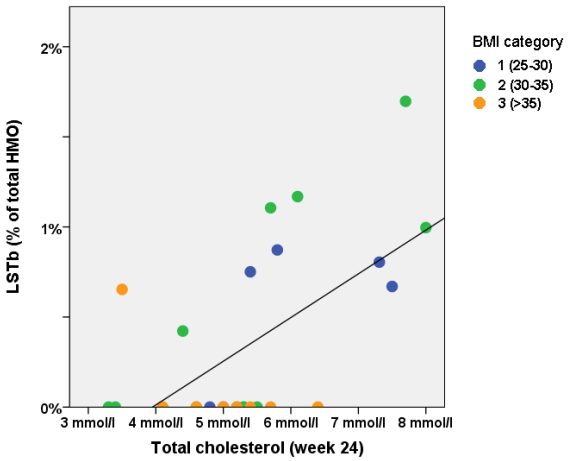

Figure 17: Correlation of total cholesterol and LSTb

3'S3FL and LSTb also correlated with LDL (see **Table 8**). Concerning 3'S3FL, correlation coefficients of LDL were similar to those of total cholesterol, with visit 3 being significant. LSTb again showed highly significant correlations that were little less strong compared to total cholesterol. LNFP2 and LNFP3, whose peaks could not be separated by HPLC, showed significant correlations with LDL at visit 1 and 2.

Table 8: Spearman rank correlations between LDL and 3'S3FL / LNFP2/3 / LSTb within secretor-negative women

		LDL		
		Visit 1	Visit 2	Visit 3
3'S3FL (Visit 2)	Spearman r	**0.455**	**0.402**	**0.453**
	P value	**0.015**	**0.037**	**0.030**
	N	**28**	**27**	**23**
LNFP2/3 (Visit 2)	Spearman r	**0.417**	**0.394**	0.339
	P value	**0.027**	**0.042**	0.114
	N	**28**	**27**	23
LSTb (Visit 2)	Spearman r	**0.436**	**0.484**	**0.556**
	P value	**0.020**	**0.010**	**0.006**
	N	**28**	**27**	**23**

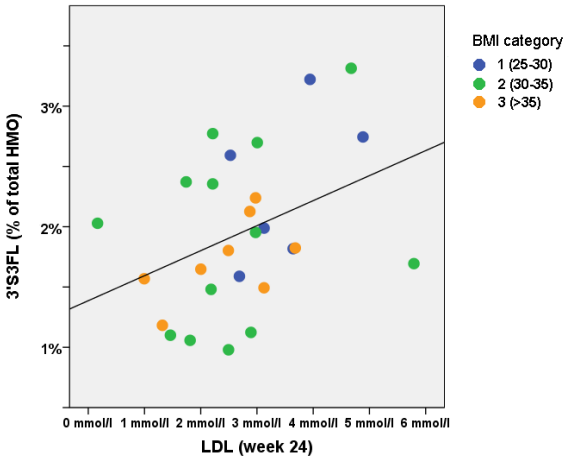

Figure 18: Correlation of LDL and 3'S3FL

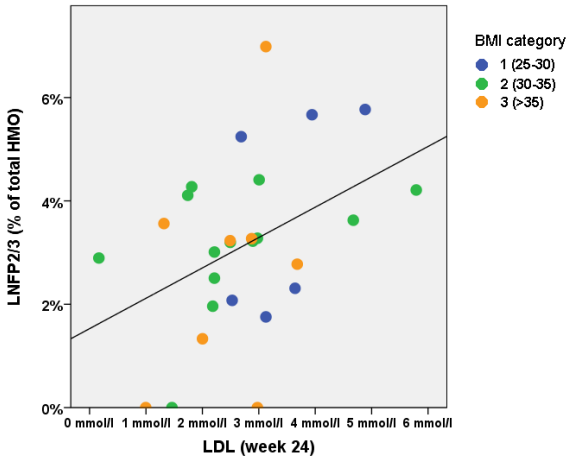

Figure 19: Correlation of LDL and LNFP2/3

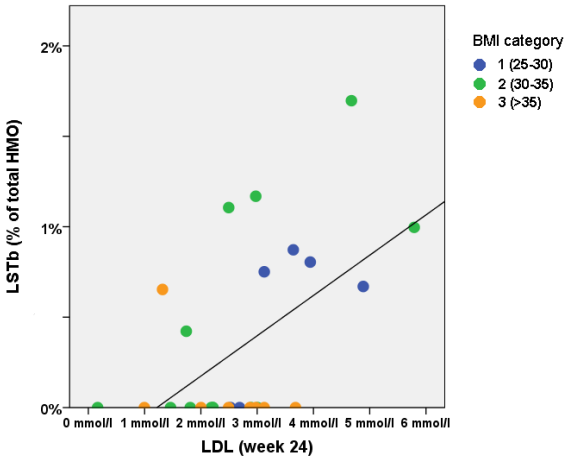

Figure 20: Correlation of LDL and LSTb

As far as triglycerides are concerned (see **Table 9**, **Figure 21** and **Figure 22**), 3'SL (the most common HMO within non-secretors) showed a significant and rather strong negative correlation in visit 2 and 3. LSTb (a rather rare HMO accounting for 1.7% at maximum) correlated again positively, with visit 2 showing the strongest and most significant correlation.

Table 9: Spearman rank correlations between triglycerides and 3'SL / LSTb within secretor-negative women

		Triglycerides		
		Visit 1	Visit 2	Visit 3
3'SL (Visit 2)	Spearman r	-0.314	**-0.423**	**-0.578**
	P value	0.104	**0.028**	**0.004**
	N	28	**27**	**23**
LSTb (Visit 2)	Spearman r	**0.390**	**0.536**	**0.427**
	P value	**0.040**	**0.004**	**0.042**
	N	**28**	**27**	**23**

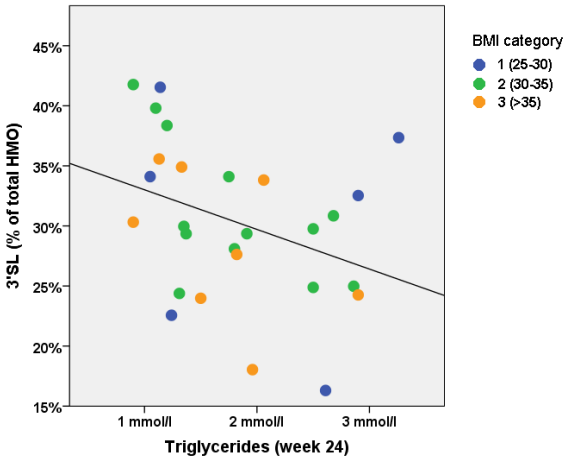

Figure 21: Correlation of triglycerides and 3'SL

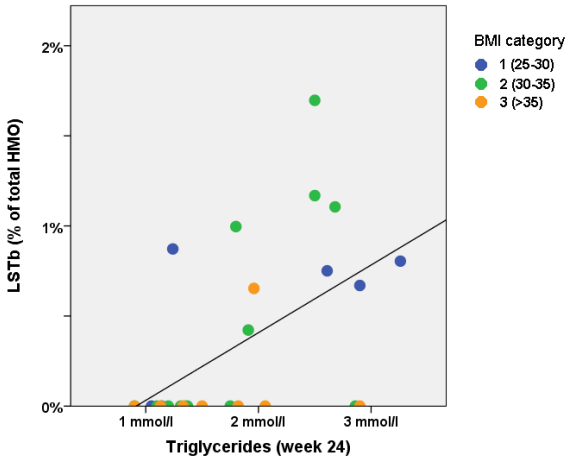

Figure 22: Correlation of triglycerides and LSTb

4. Discussion

So far, there has been no research on environmental factors influencing the profile of neither pre-, nor postnatal HMOs. We provide first evidence that elevated blood lipids are associated with specific prenatal HMO structures.

Comparing the results of both, the secretor-positive and -negative subgroup, we found different associations. Within the secretor-positive group, total cholesterol and particularly LDL correlated significantly negatively with 2'FL and positively with 3'SLN, two highly abundant prenatal HMOs. 2'FL accounts for 8-57% of all HMOs in the secretor-positive group, 3'SLN accounts for 2-10% and usually does not occur in human milk.

Within the secretor-negative group, naturally, there is no correlation with 2'FL, since non-secretor women lack the enzyme responsible for 2'FL synthesis. More surprisingly, correlations with 3'SLN were not to be found too. This may be due to altered relative concentrations caused by the absence of the highly abundant 2'FL, that makes comparisons between secretor-positive and negative women difficult.
In the secretor-negative group, we found correlations of total cholesterol and LDL with 3'S3FL, LSTb and LNFP2/3, all of which are much less abundant (usually less than 5% of all prenatal HMOs). For this reason, potential inaccuracies in the measurements may have a greater impact on the results, making them less reliable.

The physiological mechanisms behind the revealed associations remain to be investigated. Looking at the positive correlation of LDL with 3'SLN, it is conceivable that both are increased by the same cause, for instance high caloric nutrition rich in cholesterol. Many ways are imaginable of how cholesterol, or a general metabolic situation characterized by high levels of LDL may increase either 3'SLN production or 3'SLN bioavailability in blood. Vice versa, the inverse association of elevated cholesterol or LDL with lower relative 2'FL may point towards a deficiency in production/bioavailability of a secretor active HMO in secretor positive mothers with a certain metabolic profile. However, revealing detailed mechanisms underlying such an interaction is certainly a difficult task due to the complexity of HMO synthesis and lipid metabolism and was not within the scope of this study.

The present study is just a first step towards elucidating environmental factors influencing the HMO profile. Further studies will be needed to proof and strengthen these results. A larger sized and more representative study population would be sensible (analyzing just obese and overweight women is one limitation of the present work). Furthermore, it would be interesting to calculate the Spearman correlations between lipids and absolute instead of relative quantities of HMOs, which is methodically more challenging. If the observed associations can be confirmed, it would be interesting to investigate the outcome of metabolically altered HMO profiles for the pregnancy and the newborn.

Finally, the associations found could be interpreted more boldly the other way around: 3'SLN and other systemic prenatal HMOs might contribute to adjusting the mothers' lipid metabolism to pregnancy and breastfeeding, during which higher amounts of cholesterol and triglycerides are needed (84). This would suggest an important role for systemic HMOs, beyond a simple retrograde "leakage". However, at present, all of this remains speculation.

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Supplements

Table 10: Lipid conversion table

Lipid conversion table			
Total cholesterol / LDL / HDL		**Triglycerides**	
mmol/l	mg/dl	mmol/l	mg/dl
1	38.7	1	87.5
2	77.3	2	175
3	116.0	3	262.5
4	154.6	4	350
5	193.3		
6	232.0		
7	270.6		
8	309.3		

Table 11: Spearman rank correlations of secretor-positive women

		Total cholesterol			Triglycerides			HDL			LDL		
		Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3
2'FL	r	-.065	**-.257**	**-.308**	.119	-.140	-.202	.053	.061	.187	-.193	**-.294**	**-.348**
	P	.609	**.039**	**.023**	.350	.265	.144	.677	.627	.176	.126	**.018**	**.010**
	N	64	**65**	**54**	64	65	54	64	65	54	64	**65**	**54**
3'SLN	r	**.304**	**.389**	**.427**	-.092	.141	.241	.001	-.015	-.094	**.365**	**.418**	**.395**
	P	**.014**	**.001**	**.001**	.468	.262	.079	.995	.907	.499	**.003**	**.001**	**.003**
	N	**64**	**65**	**54**	64	65	54	64	65	54	**64**	**65**	**54**
LDFT	r	-.081	-.146	-.177	.060	-.029	.126	.109	.015	-.022	-.154	-.176	-.196
	P	.527	.247	.200	.640	.819	.363	.392	.907	.872	.223	.162	.156
	N	64	65	54	64	65	54	64	65	54	64	65	54
3'SL	r	.167	.147	.105	.070	.234	.182	-.128	-.084	-.136	.238	.182	.153
	P	.188	.243	.448	.585	.061	.187	.315	.506	.326	.058	.146	.270
	N	64	65	54	64	65	54	64	65	54	64	65	54
6'SLN	r	-.027	.213	.221	-.076	.054	.089	-.004	.069	-.073	.073	.203	.240
	P	.834	.088	.108	.550	.668	.522	.975	.586	.598	.566	.104	.080
	N	64	65	54	64	65	54	64	65	54	64	65	54
6'SL	r	.153	.173	.257	-.148	.135	.149	-.070	-.130	-.164	.234	.218	**.277**
	P	.228	.169	.061	.242	.284	.281	.582	.303	.236	.063	.082	**.042**
	N	64	65	54	64	65	54	64	65	54	64	65	**54**
LNT	r	**.274**	.182	.106	.050	.012	-.092	.122	.002	-.071	.208	.188	.137
	P	**.029**	.147	.445	.693	.925	.510	.338	.989	.612	.099	.134	.322
	N	**64**	65	54	64	65	54	64	65	54	64	65	54
LNnT	r	.192	.037	-.002	-.042	.013	-.102	**.296**	.217	.168	.084	-.034	-.057
	P	.129	.770	.991	.743	.920	.464	**.017**	.083	.224	.509	.786	.682
	N	64	65	54	64	65	54	**64**	65	54	64	65	54
3'S3FL	r	**.342**	.190	.177	-.029	.118	.008	**.259**	.199	.120	**.261**	.095	.144
	P	**.006**	.130	.200	.818	.350	.954	**.039**	.111	.388	**.037**	.450	.298
	N	**64**	65	54	64	65	54	**64**	65	54	**64**	65	54
LNFP1	r	.006	.125	.076	-.039	-.070	-.045	-.087	-.112	-.121	.077	.188	.113
	P	.962	.323	.584	.762	.580	.746	.495	.374	.382	.547	.133	.415
	N	64	65	54	64	65	54	64	65	54	64	65	54
LNFP2/3	r	**.295**	.135	.035	.206	.033	-.056	.177	.090	.049	.181	.122	.041
	P	**.018**	.284	.800	.103	.792	.689	.161	.478	.727	.151	.334	.771
	N	**64**	65	54	64	65	54	64	65	54	64	65	54
LSTa	r	.029	.018	.047	-.214	-.172	-.064	-.100	-.098	-.136	.096	.082	.114
	P	.817	.884	.734	.090	.170	.645	.433	.437	.327	.451	.517	.413
	N	64	65	54	64	65	54	64	65	54	64	65	54
LSTb	r	.037	.002	-.042	-.028	-.015	.033	.033	.000	-.101	.068	.070	-.011
	P	.772	.985	.764	.827	.904	.815	.793	.998	.470	.593	.581	.935
	N	64	65	54	64	65	54	64	65	54	64	65	54
LSTc	r	-.053	.185	.222	-.190	-.052	-.061	-.030	.037	-.046	.064	.183	.236
	P	.679	.139	.107	.132	.683	.664	.815	.770	.743	.615	.144	.085
	N	64	65	54	64	65	54	64	65	54	64	65	54
LNFDH	r	-.010	-.046	-.094	.116	-.036	.065	.028	-.085	-.147	-.025	-.014	-.056
	P	.940	.716	.499	.361	.779	.640	.828	.503	.289	.845	.913	.688
	N	64	65	54	64	65	54	64	65	54	64	65	54
DSLNT	r	-.069	-.081	-.170	-.057	-.177	-,340	.019	.127	.136	-.105	-.135	-.145
	P	.590	.519	.218	.657	.158	.012	.884	.315	.326	.410	.284	.296
	N	64	65	54	64	65	54	64	65	54	64	65	54

Table 12: Spearman rank correlations of secretor-negative women

		Total cholesterol			Triglycerides			HDL			LDL		
		Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3
2'FL	r	-.126	-.106	-.101	-.075	-.098	.114	-.100	.053	.016	-.080	-.113	-.078
	P	.524	.598	.647	.705	.627	.604	.614	.795	.942	.687	.575	.722
	N	28	27	23	28	27	23	28	27	23	28	27	23
3'SLN	r	.201	.205	.257	.059	.164	.037	.171	.196	-.149	.148	.158	.266
	P	.304	.305	.236	.764	.415	.867	.383	.327	.498	.451	.433	.220
	N	28	27	23	28	27	23	28	27	23	28	27	23
LDFT	r												
	P												
	N	28	27	23	28	27	23	28	27	23	28	27	23
3'SL	r	-.145	-.272	-.127	-.314	**-.423**	**-.578**	.252	**.442**	.160	-.197	-.242	-.065
	P	.461	.170	.565	.104	**.028**	**.004**	.195	**.021**	.467	.315	.224	.768
	N	28	27	23	28	**27**	**23**	28	**27**	23	28	27	23
6'SLN	r	-.191	-.041	-.097	-.038	.127	-.065	-.117	-.317	-.123	-.193	-.064	-.088
	P	.331	.839	.660	.846	.527	.769	.553	.107	.577	.326	.753	.690
	N	28	27	23	28	27	23	28	27	23	28	27	23
6'SL	r	-.013	.010	-.145	-.010	.022	-.207	.077	.181	.046	-.026	-.055	-.196
	P	.947	.961	.508	.959	.913	.344	.697	.366	.834	.894	.785	.371
	N	28	27	23	28	27	23	28	27	23	28	27	23
LNT	r	.146	.109	.119	.064	.016	.112	-.100	-.188	-.157	.216	.180	.115
	P	.459	.590	.590	.745	.938	.610	.611	.348	.474	.270	.369	.601
	N	28	27	23	28	27	23	28	27	23	28	27	23
LNnT	r	.077	-.006	.064	.148	-.011	.156	-.081	.145	-.041	.103	-.020	.057
	P	.698	.978	.771	.453	.958	.478	.683	.471	.853	.600	.923	.795
	N	28	27	23	28	27	23	28	27	23	28	27	23
3'S3FL	r	**.430**	**.407**	.400	.334	.342	.253	-.091	.054	-.093	**.455**	**.402**	**.453**
	P	**.022**	**.035**	.058	.082	.081	.245	.646	.788	.673	**.015**	**.037**	**.030**
	N	**28**	**27**	23	28	27	23	28	27	23	**28**	**27**	**23**
LNFP1	r	.234	.273	.014	**.392**	.308	.345	-.066	.082	.149	.245	.221	.002
	P	.232	.168	.950	**.039**	.118	.107	.739	.684	.497	.210	.268	.993
	N	28	27	23	**28**	27	23	28	27	23	28	27	23
LNFP2/3	r	.374	.295	.407	.211	.211	.300	-.075	-.177	-.140	**.417**	**.394**	.339
	P	.050	.135	.054	.282	.290	.165	.703	.376	.525	**.027**	**.042**	.114
	N	28	27	23	28	27	23	28	27	23	**28**	**27**	23
LSTa	r	.227	.368	.257	.123	.081	**.421**	-.022	.139	-.099	.267	.272	.197
	P	.244	.059	.237	.533	.688	**.046**	.911	.491	.654	.170	.170	.368
	N	28	27	23	28	27	**23**	28	27	23	28	27	23
LSTb	r	**.531**	**.587**	**.624**	**.390**	**.536**	**.427**	-.044	-.120	-.061	**.436**	**.484**	**.556**
	P	**.004**	**.001**	**.001**	**.040**	**.004**	**.042**	.823	.551	.782	**.020**	**.010**	**.006**
	N	**28**	**27**	**23**	**28**	**27**	**23**	28	27	23	**28**	**27**	**23**
LSTc	r	.294	.240	-.093	.234	.337	.310	.005	-.166	.045	.280	.235	-.079
	P	.129	.228	.671	.231	.085	.149	.980	.408	.839	.149	.238	.720
	N	28	27	23	28	27	23	28	27	23	28	27	23
LNFDH	r												
	P												
	N	28	27	23	28	27	23	28	27	23	28	27	23
DSLNT	r	.016	-.074	.109	-.127	-.141	-.066	-.158	-.327	-.197	.162	.192	.125
	P	.937	.713	.620	.519	.484	.765	.421	.096	.368	.409	.338	.570
	N	28	27	23	28	27	23	28	27	23	28	27	23