

**Diplomarbeit**

**Effects of Human Milk Oligosaccharides on  
neonatal outcomes**

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## GLOSSARY AND ABBREVIATIONS

2'FL	2'-Fucosyllactose
3FL	3-Fucosyllactose
3'SL	3'-Sialyllactose
3'SLN	3-Sialyl-N-acetyllactosamine
6'SL	6-Sialyllactose
6'SLN	6-Sialyl-N-acetyllactosamine
BMI	body mass index
FUT2	$\alpha$ 1-2-fucosyltransferase
FUT3	$\alpha$ 1-3/4-fucosyltransferase
GlcNAc	N-Acetylglucosamine
HMO	Human Milk Oligosaccharides
LDFT	Lacto-di-fuco-tetraose
Le	Lewis
LNDFH	Lacto-n-di-fuco-hexaose
LNFP1	Lacto-n-fucopentaose 1
LNFP23	Lacto-n-fucopentaose 2,3
LNH	Lacto-n-hexaose
LNnT	Lacto-N-neotetraose
LNT	Lacto-N-tetraose
LSTa	Lactosialotetraose a
LSTb	Lactosialotetraose b

LSTc	Lactosialotetraose c
NEC	Necrotizing enterocolitis
Sia	Sialic acid
Se	Secretor
V1, V2, V3	Visit 1, 2, 3

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## ZUSAMMENFASSUNG

**Hintergrund:** Zu den Humanen Milcholigosacchariden (HMO) zählen zahlreiche hoch aktive Glykane, die einige positive Auswirkungen auf das gestillte Kind haben, aber auch schon während der Schwangerschaft im Kreislauf von Mutter und Kind nachweisbar sind.

**Hypothese:** Wir untersuchten einerseits, ob mütterliche und fetale HMO Konzentration und Zusammensetzung korrelieren und andererseits, ob mütterliche oder kindliche HMOs die neonatale Körperzusammensetzung beeinflussen.

**Material und Methoden:** Die Diplomarbeit war Teil einer longitudinalen Pilotstudie, welche am gynäkologischen Forschungslabor an der Medizinischen Universität Graz durchgeführt wurde. Insgesamt wurden 39 Frauen rekrutiert, die in die Analysen mit einbezogen wurden.

HMO Konzentration und Zusammensetzung wurden im mütterlichem Blut zwischen der 20-24 Woche und im Nabelschnurblut (sowohl arteriell als auch venös) direkt nach der Geburt bestimmt. Die neonatale Körperzusammensetzung wurde mittels Lipometer (subkutane Fettgewebsdicke) und PEAPOD Messgerät gemessen. Zusätzlich wurden Geburtsgewicht, Länge und Ponderal Index ( $\text{kg}/\text{cm}^3$ ) bestimmt.

**Resultate:** Zwischen mütterlichen und kindlichen HMO Konzentrationen und Zusammensetzungen ließ sich keine Korrelation eruieren. Weiters konnten auch keine Auswirkungen von mütterlichen HMOs auf das Körperfett des Neugeborenen nachgewiesen werden.

Allerdings waren kindliche HMOs mit einigen neonatale Messungen assoziiert. Eine statistisch signifikante Korrelation wurde zwischen der Lacto-di-fuco-tetraose (LDFT) Konzentration im arteriellen und venösen Nabelschnurblut und dem Geburtsgewicht gefunden. Außerdem wurde ein statistisch signifikanter Zusammenhang zwischen der 3`-Sialyllactose (3`SL) Konzentration im arteriellen sowie venösen Nabelschnurblut und der Dicke des subkutanen Fettgewebes nachgewiesen.

**Schlussfolgerung:** Diese Resultate könnten darauf hinweisen, dass fetale HMOs einen Einfluss auf die kindliche Körperzusammensetzung haben, oder dass sie von den gleichen Faktoren beeinflusst werden. Interessant wäre es, die mütterlichen HMOs zu einem späteren Zeitpunkt zu untersuchen, um zu sehen, ob sich dadurch an den Ergebnissen etwas ändert. Außerdem sollte die gefundene Assoziation zwischen fetalen HMOs und neonatalen Outcomes in einer größeren Studie überprüft werden.

## ABSTRACT

**Background:** Human Milk Oligosaccharides (HMOs) are a group of highly active glycans with numerous beneficial effects in the breastfed infant. HMOs were found in the urine of pregnant woman and recently, they have been discovered in serum of pregnant mothers and in cord blood.

We aimed to test the following hypotheses: 1) that maternal HMO concentration and composition is associated with fetal HMOs, 2) that maternal HMO concentration and composition have an influence on neonatal body composition, and 3) that fetal HMO concentration and composition are associated with neonatal body composition.

**Material and Methods:** This diploma thesis was part of a longitudinal pilot study. A total of 39 women were included in this study held at the department of obstetrics and gynaecology at the Medical University of Graz.

HMOs in maternal blood samples taken at midpregnancy (20-24 weeks) and fetal blood samples (arterial and venous cord blood) were analyzed by high performance liquid chromatography. Infant body composition was assessed by air displacement plethysmography (PEAPOD) and by measurement of subcutaneous adipose tissue (SAT) thickness using an optical device (lipometer). Furthermore birth weight, length and Ponderal Index were measured.

**Results:** No correlations were found between maternal and fetal HMOs. Furthermore, no correlations could be detected between maternal HMOs and neonatal outcomes.

However, fetal HMOs were associated with several neonatal outcomes. Lacto-di-fuco-tetraose (LDFT) concentration in arterial ( $r=-0.54$ ,  $p=0.014$ ) as well as venous cord blood ( $r=-0.58$ ,  $p=0.007$ ) significantly correlated with birth weight. A significant correlation was found between 3'-Sialyllactose (3'SL) concentration in arterial and venous cord blood and total and upper SAT thickness. Arterial 3'SL concentration was also correlated with lower SAT.

**Conclusion:** This might imply that fetal HMOs have affect neonatal body composition. Fetal HMOs and neonatal body composition could also be influenced by the same factors for example, maternal nutrition, maternal body composition or physical activity.

These results provide the foundation for further research. It would be interesting to investigate associations of maternal HMOs later in pregnancy with neonate body composition. Furthermore, the association between fetal HMOs and neonatal outcomes should be examined in a larger study including possible confounders.

## 1. BACKGROUND

Human Milk Oligosaccharides (HMOs) are a group of highly active glycans which have a number of beneficial effects on the nursing infant. These include for instance immune modulating and anti-inflammatory functions (Bode, 2012).

HMOs have been found in the urine of pregnant woman and recently, they have been discovered also in serum of pregnant mothers and in cord blood (Jantscher-Krenn *et al.* unpublished).

Therefore, we hypothesized that prenatal HMO concentration and composition in the maternal and fetal circulation may have an influence on the development of the fetus.

This study evaluates, for the first time, correlations between maternal and fetal HMO concentration and composition and neonatal body composition.

## 1.1 NEONATAL BODY COMPOSITION AND RELATED HEALTH ISSUES

Various studies show that overweight and obese children have an increased risk of becoming overweight and obese in adulthood. Consequently, they suffer more often from obesity related diseases such as type two diabetes and cardiovascular disorders (Li, et al., 2011). Birth weight is known as important determinant of health in later life (Donnelly, et al., 2015). Low birth weight contributes to the risk to develop diseases like diabetes mellitus type 2, osteoporosis, sarcopenia, coronary heart diseases and others in adulthood (Godfrey, et al., 2011) Furthermore, the incidence of cardiovascular diseases, metabolic syndrome and obesity in later life are also linked to a high birth weight (Donnelly, et al., 2015).

It is unclear whether the birth weight *per se* or the related fat mass differences cause these relations. A recent study suggests that a higher fat free mass at birth might go along with a positive effect on brain growth and development (Abera, et al., 2017).

To predict future risk for obesity and metabolic diseases, birth weight alone is inadequate. Numerous studies show that weight and length indexes cannot be equated with fat mass proxies (Perng, et al., 2017). Body composition measurements and knowledge about the neonate fat distribution may help to establish risk models for more precise predictions (Donnelly, et al., 2015).

Furthermore, the risk for childhood obesity is strongly associated with pre-natal and early post-natal determinants. Several factors including maternal obesity, excessive weight gain during pregnancy, cigarette smoking, breast milk composition as well as breastfeeding duration have been identified as modulators of offspring body composition and growth (Fields & Demerath, 2012).

## 1.2 BREAST FEEDING

Human breast milk is commonly considered the best method of infant feeding. Infant formula lacks many of the factors abundant in human milk important for regulating metabolism and development of the immune system (Oddy, 2002). Breast feeding is also associated with improved neurological development (Belfort, et al., 2016). Additionally, breast milk delivers components for the maturation of the gastrointestinal tract (Martin, et al., 2016). Recently, studies have suggested that breastfeeding is associated with a reduced risk of obesity, diabetes type 1 and 2, Sudden Infant Death Syndrome and Necrotizing Enterocolitis (Smilowitz, et al., 2014).

The World Health Organization recommends exclusive breast feeding for the first six months after birth. After this period infants should receive appropriate complementary foods (Martin, et al., 2016).

### 1.2.1 COMPOSITION OF BREAST MILK

Breast milk consists of about 87 percent water, 4 percent fat, 1 percent protein and 7 percent lactose.

In addition, it includes various other components such as vitamins, minerals, carbohydrates, hormones and digestive enzymes (Martin, et al., 2016).

Some of these constituents differ depending on maternal dietary intake, especially vitamins and fatty acids, others are genetically determined.

Human Milk Oligosaccharides (HMOs) make up one of the largest fractions of macromolecules. It is currently thought that the composition of HMOs is primarily modulated by genetics (Sprenger, et al., 2017).

## 1.3 HUMAN MILK OLIGOSACCHARIDES

Human milk oligosaccharides (HMOs) are a group of highly diverse, unconjugated glycans and comprise close to 200 different molecules. They constitute a significant part of breast milk, reaching from 7g/L up to 20g/L (Hennet, et al., 2014).

### 1.3.1 HMO CONCENTRATION AND STRUCTURE

The HMO concentration and composition differ over the course of lactation as well as between individuals. The highest concentrations can be found in colostrum, it contains up to 25g/L. During the course of lactation, HMO concentrations decline. The five monosaccharide's glucose, galactose, N-acetylglucosamine, fucose and sialic acid are the building blocks of HMOs. All HMO structures consist of a lactose core, which can be elongated by the addition of N-acetyllactosamine as well as  $\beta$ 1-3 or  $\beta$ 1-6 linked lacto-N-biose. These molecules can be fucosylated or sialylated (Bode, 2012).

It is thought that genetics as well as environmental factors have an impact on HMO synthesis. For example, it was shown, that underweight women with a BMI below 18kg/m<sup>2</sup> had significantly lower HMO concentrations in their milks (Bode & Jantscher-Krenn, 2012).

The influence of genetic disposition will be discussed in the next section.

### 1.3.2 SECRETOR STATUS AND LEWIS BLOOD GROUP STATUS

HMO composition is associated with blood group characteristics. Four milk groups can be distinguished depending on the activity of two gene loci encoding for two specific glucosyltransferases. The *Secretor (Se)* gene encodes for  $\alpha$ 1-2-fucosyltransferase (FUT2), the *Lewis (Le)* gene encodes for  $\alpha$ 1-3/4-fucosyltransferase (FUT3) (Thurl, et al., 2010; Bode & Jantscher-Krenn, 2012).

#### 1.3.2.1 Secretor Status

Corresponding to the activity of the *Secretor (Se)* locus, a classification into secretors and non-secretors can be made. If the *Se* gene is active, the woman is classified as a secretor. The milk of *Se* positive women contains high amounts of 2'-fucosyllactose (2'FL), lacto-N-fucopentaose 1 (LNFP1) and other  $\alpha$ 1-2-fucosylated HMOs. Milk of non-secretor women does not contain these HMO species because non-secretors lack FUT2, which is needed to modify the HMO backbone (Bode & Jantscher-Krenn, 2012).

#### 1.3.2.2 Lewis Blood Status

An active *Le* gene locus results in the expression of FUT3, which adds fucose onto a subterminal GLcNAc on type 1 chains. Lewis negative women lack an active FUT3 and thus,  $\alpha$ 1-4-fucosylated HMOs, for instance lacto-N-fucopentaose (LNFP2) (Bode, 2012).

Based on the expression of FUT2 and FUT3 breast milk can be assigned to one of four milk groups (Thurl, et al., 2010):

**Table 1. Secretor and Lewis blood group status**

Lewis positive Secretor ( $Le^+$ , $Se^+$ )	Lewis negative Secretor ( $Le^-$ , $Se^+$ )
Lewis positive Non-secretor ( $Le^+$ , $Se^-$ )	Lewis negative Non-secretor ( $Le^-$ , $Se^-$ )

The milk of Lewis negative non-secretor women still contains some fucosylated HMOs such as 3-fucosyllactose (3FL) or lacto-N-fucopentaose 3 (LNFP3). This suggests that other *Le-* and *Se-* gene locus independent factors also contribute to fucosylation. FUT1 seems to be involved in HMO fucosylation because  $\alpha$ 1-2-fucosylated HMO have also been detected in the milk of non-secretor women (Newburg, et al., 2005).

### 1.3.3 POSTULATED BENEFICIAL EFFECTS OF HMOS

HMOs are not digested in the infant's intestinal tract and therefore, seem to have no nutritional value for the breastfed infant. However, various beneficial local (in the gut), and systemic effects are postulated (Hennet, et al., 2014).

#### 1.3.3.1 Prebiotic Effects

Protective bacterial strains colonize the gut of breastfeed neonates. These beneficial microbes protect, feed and communicate with the intestine (Smilowitz, et al., 2014). Specifically, the occurrence of *Bifidobacterium* species is strongly associated with HMOs. Several strains were shown to grow on HMOs as sole carbon source. A good example is *Bifidobacterium longum subspecies infantis*. It utilizes HMOs completely, including the degradation products (Sela, et al., 2008). Different bacterial strains use different catalytic mechanisms to break down HMOs (Smilowitz, et al., 2014). *Bifidobacterium longum subspecies infantis* and other bacterial strains influence the intestinal flora and support the growth of commensal bacteria (Bode, 2012), positively affecting infant, and potentially adult health.

#### 1.3.3.2 Pathogen Deflection

The same glucosyltransferases that synthesize glycans displayed on cell surfaces are also used in HMO biosynthesis. Therefore, HMOs share common epitopes found on host cells, such as on intestinal epithelial cell surfaces (Smilowitz, et al., 2014).

Studies suggest that ingested HMOs behave like receptor decoys and can bind to pathogens (Newburg, et al., 2005). This way, HMOs reduce the binding of pathogens to intestinal mucosal surfaces. HMOs are able to bind to pathogens like rotavirus, HIV as well as *Streptococcus pneumonia* and many others (Hong, et al., 2009; Andersson, et al., 1986).

Based on the large diversity of HMO structures, specific HMOs can protect from different pathogens. For instance, the adhesion of *Escherichia coli* to intestinal cells is blocked by sialylated HMOs, whereas fucosylated HMOs prevent *Campylobacter jejuni* binding to intestinal cells (Smilowitz, et al., 2014).

#### 1.3.3.3 Immune Modulation

Various studies suggest that there is also a direct influence of HMOs on the infant's immune system. It is assumed that HMOs affect the macrophage stimulation and the lymphocyte cytokine production. The exact receptors and signalling pathways remain unknown (Bode, 2012).

#### 1.3.3.4 Prevention of Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC) is one of the most frequent diseases in very low birth weight preterm infants. It often goes along with very serious intestinal disorders. About one quarter of the infants dies, and the survivors often suffer from persistent neurological sequelae. In animal studies, specific HMOs were shown to prevent NEC (Bode & Jantscher-Krenn, 2012). If this finding can be translated to human infants, this would explain why various clinical studies have shown that breastfeeding significantly decreases risk for developing NEC (Bode & Jantscher-Krenn, 2012).

#### 1.3.3.5 Intestinal Barrier Function

Many factors work together supporting the intestinal barrier function. As mentioned above, the prebiotic function of HMOs leads to an increased number of *bifidobacteria*. This in turn, might enhance gut barrier function by a lower pH level through the production of short-chain fatty acids. Additionally, *Bifidobacterium longum subspecies infantis* grown on HMOs, showed increased binding to intestinal cells and elevated tight junction protein expression and immunomodulatory interleukin 10. Furthermore, studies suggest that *Bifidobacterium longum subspecies infantis* reduces interferon gamma and tumor necrosis factor alpha (Smilowitz, et al., 2014).

#### 1.3.3.6 Influence on brain development

Studies suggest that breastfed infants achieve slightly better results at intelligence quotient tests at the age of 7 than formula fed infants (Belfort, et al., 2016; Lucas, et al., 1990).

Cognition and brain development correlate with sialic acid concentration. Sialic acid is known as an essential nutrient with a significant impact on infant brain development. Human breast milk is an important source of sialic acid. Sialylated HMOs constitute the mayor part of sialic acid in human milk (Bode, 2012).

#### 1.3.3.7 Effects on the Mother

Infants might not be the only ones benefitting from HMOs. A recent study suggests that specific HMOs might protect nursing mothers from developing mastitis (Bode, 2012). HMOs can shape the composition of the milk microbiome in a similar manner as described before in the context of the infant gut microbiome.

Already early in pregnancy, HMOs can be detected in women`s urine (Hallgreen, et al., 1977a). Very recently, HMOs have also been detected directly in the serum of pregnant mothers (Jantscher- Krenn *et al.* manuscript submitted). This implies that there are potentially unknown systemic effects also in nursing mothers (Bode, 2012).

#### 1.3.3.8 HMO Effects on Metabolism

Few studies have investigated associations between HMOs in breast milk consumed by the infant and infant growth. Recently, a study suggests that HMOs in human milk are associated with infant body weight and infant fat mass in the first six months of life (Alderete, et al., 2015). LNFP1 in breast milk was positively correlated with infant fat mass, while LNFP2 was negatively correlated. In contrast, another study investigated whether high or low FUT2 associated HMO concentrations had an effect on infant growth in breast fed infants, and did not find any differences in the first 4 months of life (Sprenger, et al., 2017). However, the latter study did not assess infant body composition. Furthermore, a mouse study implies that a specific HMO, LNFP3 might have direct metabolic effects, influencing insulin sensitivity and lipogenesis (Bhargava, et al., 2012). Taken together, there are several observations suggesting that HMOs could affect metabolic functions and thus, body composition.

However, none of these studies takes the prenatal HMO concentration and composition into account. We here asked whether prenatal HMOs could influence body composition of the neonate.

HMOs are not only present in the maternal blood stream, but are also found in the fetal circulation (Jantscher-Krenn *et al.* unpublished). How differences in HMO concentrations or composition might affect neonatal outcomes is presently unknown. The question arises whether there is any link between the HMO concentration and composition during pregnancy and the neonatal outcomes such as length, body composition, weight, and ponderal index. These intriguing questions are sought to be answered in this thesis.

## 2. HYPOTHESIS AND AIMS

The goal of this thesis was to establish a link between maternal HMO concentration and composition during pregnancy, fetal HMOs and neonatal body composition. The aim of this thesis was to correlate maternal HMO concentrations at mid pregnancy as well as fetal HMO concentration in cord blood with the neonatal outcomes.

We aimed to test the hypotheses: 1) that maternal HMO concentration and composition is associated with the fetal HMOs, 2) that maternal HMO concentration and composition have an influence on neonatal body composition, and 3) that fetal HMO concentration and composition are associated with neonatal body composition.

If this proves to be true, it would have far reaching consequences for the understanding of different pregnancy outcomes and fetal health.

## 3.MATERIAL AND METHODS

### 3.1 STUDY DESIGN AND SETTING

This diploma thesis was part of a pilot study on the effects of physical activity in pregnancy on low grade inflammation (PAP study). The PAP study, a prospective observational study, was conducted at the department of obstetrics and gynaecology at the Medical University of Graz.

One of the objectives of the PAP study was to characterize HMO concentration and composition during pregnancy and to describe variability and temporal changes. Furthermore, correlation of maternal physical activity and body mass index with prenatal and perinatal HMO concentration and composition was studied.

Here, I used data from the PAP study to perform association studies, investigating HMO concentration and composition with infant outcomes.

Detailed definitions of the inclusion and exclusion criteria are provided in the appendix.

Briefly, women were eligible for the study if they had a healthy ongoing pregnancy and planned the delivery at the University Hospital Graz. We enrolled women with pregnancies between 10 to 14 weeks of gestation during their first visit at the clinic. Recruitment was from February to October 2013.

Exclusion criteria were multiple pregnancy, pre-pregnancy diabetes, pre-pregnancy hypertension and smoking. The women were recruited as soon as possible in their pregnancy, but not after the 14<sup>th</sup> week of gestation.

At enrolment, we obtained written informed consent from all women. The study protocol was approved by the ethical committee at the Medical University of Graz.

## 3.2 MEASUREMENTS

### 3.2.1 HMO ANALYSIS

At four times during pregnancy (V1, 10-14 weeks; V2, 20-24 weeks; V3, 35 weeks, V4, shortly before delivery), maternal blood samples and right after delivery, cord blood samples were collected. HMOs were analysed in serum samples from each visit and after delivery, in arterial as well as venous cord blood.

For this thesis, only the maternal HMO measurements of the second visit (V2) and the cord blood measurements (arterial and venous separately) were taken into account.

To investigate, whether maternal HMO concentration and composition could influence fetal growth and fat accretion, we used the time point at midpregnancy, V2, and analysed correlations with neonatal outcome measures. The time interval between V3 or V4 and delivery was too short, to draw conclusions about causality. Hence, we decided to only include HMO analysis at V2 in this thesis.

Human Milk Oligosaccharides were analysed by high performance liquid chromatography with fluorescence detection as described elsewhere (Bode & Jantscher-Krenn, 2012). The advantages of HPLC-FL include high sensitivity, repeatability as well as a high selectivity.

In brief, after raffinose was added to 25  $\mu$ L of serum, chloroform/methanol extraction followed by SPE with C18 columns was used for deproteination. Porous graphitic carbon columns were used in high throughput format for desalting. Isolated HMOs were then eluted in 96 deep well plates before they were dried and labelled with the fluorescence tag 2-aminobenzamide (2AB). HMOs were separated with an amide-80 column and monitored using a fluorescence detector.

### 3.2.2 NEONATAL OUTCOMES

All neonatal measurements were conducted prior to hospital discharge between one and three days *post partum*.

#### 3.2.2.1 Birth weight, length and ponderal index

Birth weight and length were measured right after birth in the delivery room by the midwife on duty.

The ponderal index (PI) was calculated as follows:  $PI = \text{weight}/\text{length}^3$  (Chen, et al., 2017).

#### 3.2.2.2 Neonatal Fat Mass

The measurements of the neonatal fat mass were performed using two different methods. On the one hand, we used an air displacement plethysmography technique via a device called PEA POD. On the other hand, we used an optical device called Lipometer to measure the subcutaneous adipose tissue (SAT) thickness.

The Pea Pod System as a device for air displacement plethysmography is validated for body composition measurements. In the newborn, the major part of the fat tissue is located in the subcutaneous layers. The proportion of visceral fat is comparatively low. Therefore, the measurement of the subcutaneous fat can be considered an accurate representative measure for fat mass (Eder, et al., 2015).

### 3.2.2.2 1 PEA POD

The PEA POD measurement is usually well tolerated by newborns. It is a non-invasive technique with little compliance issues. The measurement takes about five minutes while the infant is placed in the warm and quiet chamber. Before each testing the system was calibrated. A two kilogram weight was used to calibrate the scale and a calibration cylinder, with known volume, to calibrate the PEA POD chamber. For the measurement, the newborn was completely undressed. Any items that could not be removed, for example the umbilical clamp, were used to tare the scale for weight and the chamber for volume measurement. After weighing, the infant was placed inside the PEA POD chamber where the body volume was measured, which lasted about two minutes (Eriksson, et al., 2010)

Fat mass (FM), fat free mass (FFM), body volume (BV), body density (BD) as well as fat mass density (FMD) and fat free mass density (FFMD) were determined.

### 3.2.2.2.2 LIPOMETER

The Lipometer is a handheld optical device that measures SAT, not visceral fat. However, changes by environment or nutrition *in utero* occur especially in the subcutaneous fat depots (Eder, et al., 2015). Fifteen defined locations all over the body were measured. These fifteen recordings give a good overview of neonatal fat distribution.

The Lipometer uses light emitting diodes. Depending on the SAT thickness different light intensities are backscattered. These are measured by a photodiode (Jürimäe, et al., 2007).

All study members performing the measurements were trained and supervised by an experienced operator. All Lipometer measurements were conducted in triplicate by one operator. For further analyses, the means were used.

To estimate total SAT, the SAT thickness of all fifteen body sites was summed. In addition to total SAT, upper body SAT and lower body SAT were calculated by summing measurements of the respective body sites. Upper body SAT included the neck, biceps, triceps, upper back, front chest, lateral chest, upper abdomen, lower abdomen, lower back and hip. The lower body SAT included front thigh, rear thigh, lateral thigh, inner thigh and calf (Eder, et al., 2015).

For the statistical analysis only the total SAT, upper SAT and lower SAT was taken into account.

### 3.3 DATA MANAGEMENT

The study consists of two parts.

On the one hand the clinical part, which was mainly executed by a team of experienced study nurses led by Dr. Bence Csapo. This section comprised the recruitment, the scheduling of the visits, the collection of the various questionnaires, all clinical measurements for instance the Lipometer, PEA POD and fetal growth measurements, as well as the collection of maternal blood, and cord blood.

After successful recruitment, a random study identifier was used to encrypt the participant's identification. All gathered data and information were only labelled with the study identifier.

On the other hand, the analytical part consists of all analyses and sample handlings, which was carried out in the laboratory. The laboratory team only has access to pseudo anonymized samples and data for laboratory and statistical analysis.

All involved persons, which received any study related data have signed an appropriate confidentiality agreement.

### 3.4 DATA ANALYSIS

#### 3.4.1 SAMPLE SIZE

Because the study, as a pilot study, was aimed to test the feasibility of methods and techniques, there was no formal sample size calculation planned. Based on this study, appropriate sample sizes and methods for future larger studies should be calculated. Originally, the study was planned to recruit 60 women to get an idea of recruitment rate, drop out and feasibility of exposure.

### 3.5 STATISTICAL ANALYSIS

The data was analysed using descriptive statistics.

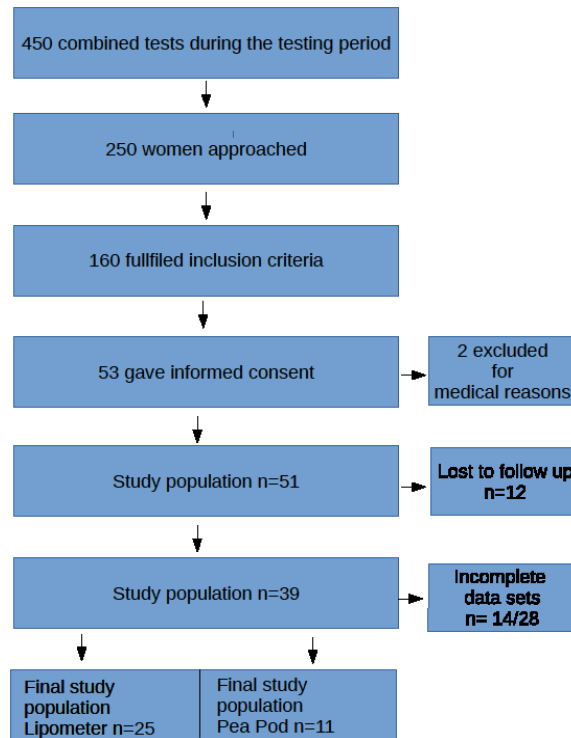
All statistic calculations were performed in IBM SPSS Statistics 23. The software used for figures and tables included SPSS Statistic 23 and Microsoft Excel 2016.

Prior to any further calculations, all our data was tested for normal distribution by Kolmogorov-Smirnov test as well as Shapiro-Wilk test.

If the data was distributed normally according to the test for normality, the Pearson correlation coefficient was used. For non-parametric distributed data, the Spearman correlation coefficient was used. Associations were considered statistically significant if p value was smaller than 0.05.

## 4 RESULTS

### 4.1 STUDY POPULATION



**Figure 1. Study Population and Recruiting.**

Figure 1 shows how the study population was achieved. Women were recruited when they came to the clinics for the combined test. During the test period, 450 combined tests took place. 250 women were approached but only 160 of these fulfilled the inclusion criteria.

A total of 53 women were enrolled between February and October 2013, two women were excluded for medical reasons. Of the 51 women, 12 women failed to provide blood samples on all relevant visits. For 39 women, we had measurements for maternal HMOs available. For 20 women-neonate pairs we had measurements for fetal HMO available.

In order to investigate associations between HMOs and the neonatal outcomes, maternal HMO data at V2 were used as described before.

Of the 39 mother-neonate pairs, PEA POD measurements from only 11 neonates were available for statistical analysis, whereas Lipometer measurements and birth weight as well as length from 25 mother-neonate pairs were available.

In conclusion, only 11 data sets remained for the statistical analysis of maternal HMOs and the Pea Pod data and 25 for the analysis of Lipometer data.

We did not analyse associations between fetal HMOs and PEA POD measurements because of too small sample sizes. Only 7 pairs remained for this analysis. For the analysis of fetal HMOs and Lipometer data 20 pairs remained.

## 4.2 HMO

In this study, 16 different HMOs were quantified in maternal and fetal blood. As mentioned before the concentration of the individual HMOs was determined by high performance liquid chromatography (HPLC). For the purpose of this diploma thesis, only the maternal HMO data of the second measurement (Visit 2) were used.

Table 2 provides a list of the sixteen different HMO molecules which were determined and the abbreviations used.

**Table 2. HMO molecules and abbreviations used**

Abbr.		Abbr.	
2`FL	2`-Fucosyllactose	LNnT	Lacto- N- neotetraose
DSLNT	Disialyl- lacto- N- tetraose	LSTa	Lactosialotetraose a
3`SL	3`-Sialyllactose	LSTb	Lactosialotetraose b
3`SLN	3`-Sialyl- N- acetyllactosamine	LSTc	Lactosialotetraose c
6`SL	6`-Sialyllactose	LNFP1	Lacto- N- fucopentaose 1
6`SLN	6`-Sialyl- N- acetyllactosamine	LNFP2	Lacto- N- fucopentaose 2
LDFT	Lacto- di- fuco- tetraose	LNFP	Lacto- N- fucopentaose 3
LNT	Lacto- N- tetraose	LNH	Lacto- N- hexaose

## 4.2.1 MATERNAL HMO

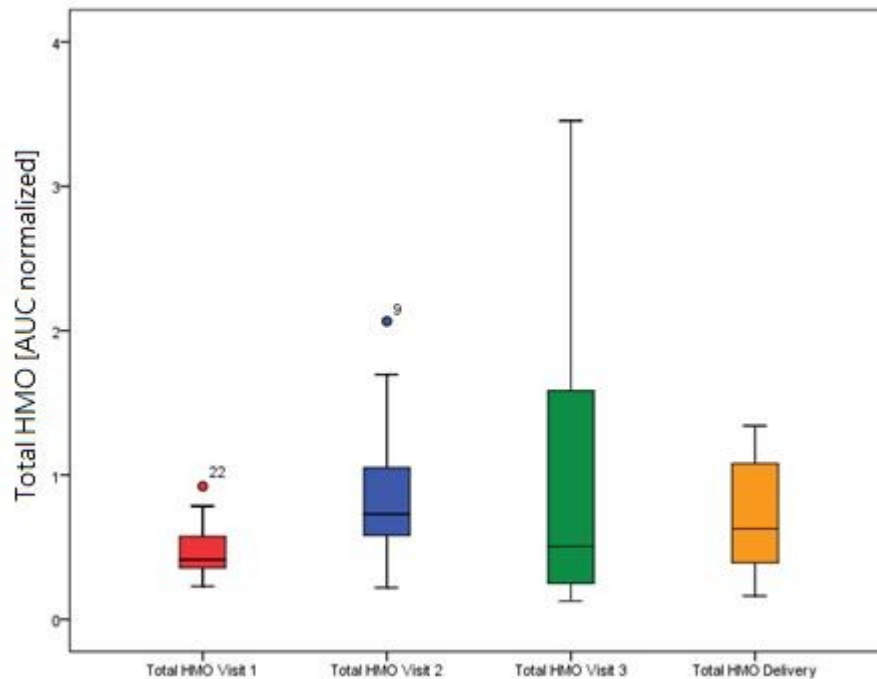
### 4.2.1.1 Total HMO concentration during pregnancy

In order to get an idea about the HMO concentration and changes during pregnancy in the subcohort of 25 mother-neonate pairs, the total HMO concentrations at each visit are shown in Table 3. For visit one (V1) and two (V2), 25 samples were available. For visit three (V3) and delivery only 23 whole data sets were accessible. For further analyses, only the data of V2 were used, as mentioned before.

**Table 3 Total HMO concentration at 3 visits during pregnancy**

Total HMO	N	Median	Min.	Max.
V1	25	.452	.229	.921
V2	25	.738	.220	2.064
V3	23	.459	.125	3.454
Delivery	23	.611	.162	1.340

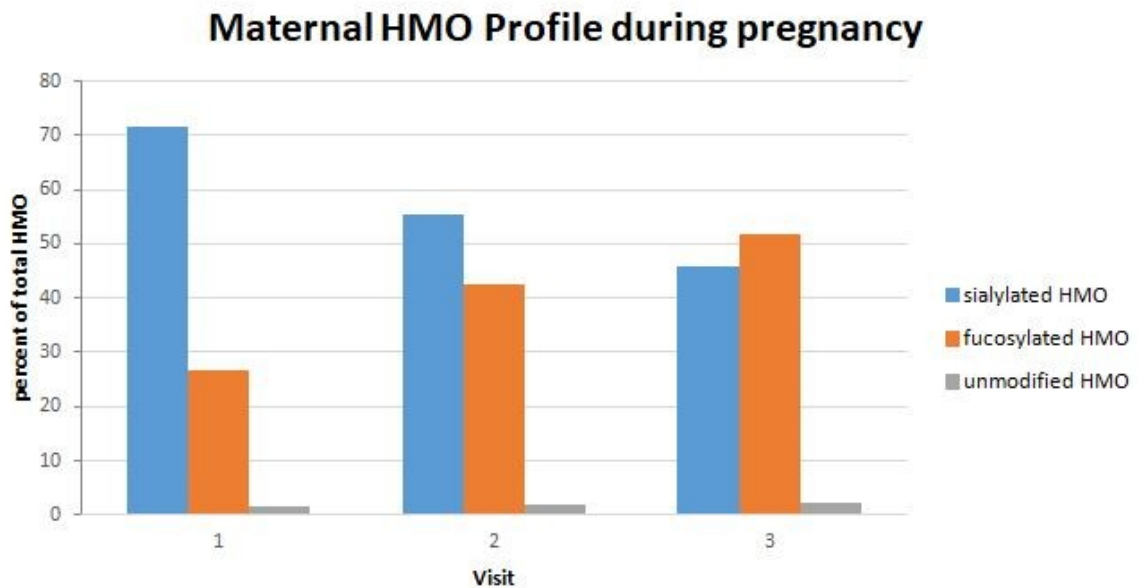
Figure 2 shows the total HMO concentration throughout pregnancy. The highest concentrations are measured at V3.



**Figure 2. Total HMO concentration during pregnancy. Box plot shows medians with whiskers (25<sup>th</sup> to 75<sup>th</sup> Percentiles) of AUC normalized to internal standard.**

#### 4.2.1.2 Maternal HMO profile throughout pregnancy

Figure 3 shows maternal HMO profiles at the three time points during pregnancy. The mean relative concentration of sialylated HMOs (blue) declines during pregnancy, whereas relative concentration of fucosylated HMO (orange) increases. The unmodified HMO group (grey) showed hardly any change.



**Figure 3. Maternal HMO Profile during pregnancy. Percent of sialylated, fucosylated and unmodified HMO in visit 1-3 are shown**

#### 4.2.1.3 Relative concentration of individual HMOs at V2

Figure 4 shows the mean relative concentration of individual HMOs at V2. 3'-Sialyllactose (3'SL) accounted for the largest portion, followed by 2'-Fucosyllactose (2'FL) and Lacto-difucotetraose (LDFT).

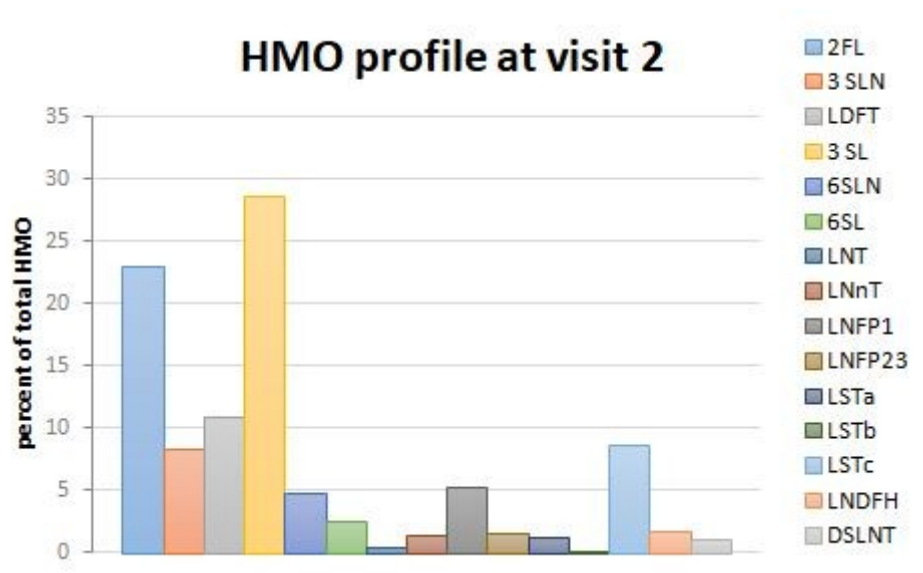


Figure 4. Relative concentration of individual HMOs at visit 2. data are shown as mean percentage of total HMO.

### 4.3. NEONATAL AND DELIVERY CHARACTERISTICS

In our cohort of 25 mother-neonate pairs, 12 (48 percent) of the measured neonates were male. The gestational age was between 36 and 41 weeks (277 +/- 8.8 days) and the mean birth weight was 3232.20 +/- 358.95 g. The measured mean length was 51 +/- 2.2 cm.

No significant difference could be detected between the sexes regarding to weight, length or ponderal index. Table 5 summarizes the baseline characteristics of the neonates.

**Table 4 Baseline Characteristics of the neonates. Birth weight, length, head circumference, gestational age at delivery**

	N	Min.	Max.	Mean	SD
length (cm)	25	47.0	55.0	51.0	2.2
weight (g)	25	2486	3830	3232	359
head circumference (cm)	25	20.0	36.5	34.1	3.17
gestational days at delivery	25	254	291	277	8.8

#### 4.4 ASSOCIATIONS BETWEEN MATERNAL HMO CONCENTRATION AT VISIT 2 AND BIRTH WEIGHT, LENGTH AND PONDERAL INDEX

Next to total HMOs, the 4 most abundant HMOs, Lacto-difucotetraose (LDFT), 2`Fucosyllactose (2`FL), 3`Sialyllactose (3`SL) and 3-Sialyl-N-acetylosamine (3`SLN) concentrations were used for correlation analysis.

Because the maternal HMOs were not normal distributed, the Spearman correlation coefficient was used.

Table 4 shows that no significant correlations between maternal total HMOs 3`SL, 3`SLN, 2`FL or LDFT concentrations at V2, with newborn length, weight or ponderal index were found.

**Table 5. Spearman correlations between maternal HMO at v2 and length, weight or ponderal index. n=25**

HMOs at V2		Length	Birth weight	Ponderal index
2`FL	r	-.040	-.063	-.012
	p	.85	.77	.95
LDFT	r	.093	-.162	-.261
	p	.66	.44	.21
3`SL	r	.303	.250	-.173
	p	.14	.23	.41
3`SLN	r	.147	.114	-.152
	p	.48	.59	.47
total HMO	r	.183	.097	-.168
	p	.38	.65	.42

Correlations analysis of relative HMO concentrations in percent with birth weight, length and PI revealed no significant correlations (data not shown).

#### 4.5 SAT THICKNESS/ LIPOMETER RESULTS

The SAT thickness of all 15 measurement points are shown in Table 6. The SAT thickness of all measurement points were significantly correlated to each other. No significant difference could be detected between the sexes regarding to the SAT at the different body sites, total SAT, upper or lower SAT.

To estimate total SAT, the SAT thickness of all fifteen body sites was summed. According to the anatomic location of the 15 measurement points a division into upper body SAT and lower body SAT was introduced. Upper body SAT includes summed measurements at the neck, biceps, triceps, upper back, front chest, lateral chest, upper abdomen, lower abdomen, lower back and hip were summarized. Whereas the lower body SAT includes front thigh, rear thigh, lateral thigh, inner thigh and calf (Eder, et al., 2015).

**Table 6. Anthropometric Characteristics. Abbreviations used: Coefficient of variation (CV), Subcutaneous Adipose Tissue (SAT)**

	N	Min.	Max.	Mean	SD	CV
Neck, mm	25	1.10	2.73	2.01	.43	.21
Triceps, mm	25	1.72	3.20	2.31	.38	.17
Biceps, mm	25	1.23	3.83	2.38	.68	.28
Upper back, mm	25	1.23	3.10	1.95	.42	.21
Front chest, mm	25	1.17	2.90	2.14	.46	.21
Lateral chest, mm	25	1.00	2.83	1.97	.46	.23
Upper abdomen, mm	25	1.00	3.23	1.97	.58	.29
Lower abdomen, mm	25	.80	4.33	2.40	.92	.38
Lower back, mm	25	1.77	3.80	2.82	.50	.18
Hip, mm	25	1.00	3.00	1.94	.52	.27
Front thigh, mm	25	1.63	4.17	2.64	.52	.20
Lateral thigh, mm	25	1.57	4.03	2.90	.64	.22
Rear thigh, mm	25	1.97	3.17	2.49	.35	.14
Inner thigh, mm	25	1.03	4.00	2.14	.62	.29
Calf, mm	25	1.03	5.13	2.86	.77	.27
SAT_total	25	27.80	42.21	34.93	3.59	.10
Upper_SAT	25	15.23	24.70	19.96	2.15	.11
Lower_SAT	25	11.07	18.10	14.97	1.81	.12

#### 4.5.1 ASSOCIATIONS BETWEEN MATERNAL HMO CONCENTRATION AND COMPOSITION AND NEWBORN SAT

Table 7 shows that no significant correlations were found between maternal total HMOs, 2`FL, LDFT, 3`SL or 3`SLN concentrations with total SAT thickness in the newborn. Apart from this, no significant correlation with the upper SAT thickness or lower SAT thickness was found for these parameters.

**Table 7. Spearman correlation between HMO concentrations at V2 and newborn SAT thickness (n=25).**

HMOs at V2		total SAT	upper SAT	lower SAT
2`FL	r	.008	-.049	.067
	p	.97	.82	.75
LDFT	r	.095	.042	.129
	p	.65	.84	.54
3`SL	r	.011	.088	-.007
	p	.96	.68	.97
3`SLN	r	-.002	.040	.019
	p	.99	.85	.93
total HMO	r	.048	.005	.116
	p	.82	.98	.58

Furthermore, associations of percentage of 2'FL, LDFT, 3'SL and 3'SLN with total SAT were analysed, and no correlations were observed. These results are shown in Table 8. Data was found to be normally distributed, thus the Pearson correlation coefficient was used.

**Table 8. Pearson Correlations between relative individual HMO concentration in percent and newborn SAT thickness. (n=25)**

HMOs at V2		total SAT	upper SAT	lower SAT
2'FL_perc	r	.009	-.065	.094
	p	.97	.76	.65
LDFT_perc	r	.224	.132	.286
	p	.28	.53	.17
3'SL_perc	r	.019	.094	-.074
	p	.93	.66	.73
3'SLN_perc	r	-.019	.000	-.037
	p	.93	.99	.86

To test whether associations were masked because of the differences between secretor positive and negative HMO profiles, we excluded secretor negative women and performed analyses with secretor positive only (n=20). Data was not normally distributed, and thus, the Spearman correlation coefficient was used. We did not find any significant correlations between maternal HMO concentrations at V2 and newborn SAT in the secretor positive group (Table 9).

**Table 9. Spearman correlation between HMO at V2 in secretor positive women and newborn SAT Thickness. (n=20)**

HMOs at V2		total SAT	upper SAT	lower SAT
2`FL	r	-.260	-.292	-.117
	p	.27	.21	.62
LDFT	r	-.152	-.182	-.068
	p	.52	.44	.77
3`SL	r	-.296	-.159	-.354
	p	.21	.50	.13
3`SLN	r	-.305	-.152	-.302
	p	.19	.52	.19
total HMO	r	-.215	-.226	-.111
	p	.36	.34	.64

Correlations analysis of relative HMO concentrations in percent with secretor positive data only revealed no significant correlations (data not shown).

## 4.6 PEA POD RESULTS

### 4.6.1 NEONATAL AND DELIVERY CHARACTERISTICS

Pea Pod Data were available for only 11 neonates. Of these children 5 (45%) were male.

The mean ( $\pm$ SD) birth weight was 3202.27  $\pm$ 336.53g and the gestational age was between 37 and 41 weeks (277  $\pm$  7.1 days). The average length was 50.73  $\pm$  2.00 cm.

The anthropometric characteristics of this study group are shown in the table 10 below.

**Table 10. Anthropometric characteristics of the PEA POD Study group**

	N	Min.	Max.	Mean	SD
Fat Mass (kg)	11	.06	.39	.26	.10
Fat Free Mass (kg)	11	2.22	3.14	2.73	.32
Body Mass (kg)	11	2.37	3.44	2.99	.33
Body Volume (L)	11	2.25	3.29	2.85	.31
Body Density (kg/L)	11	1.04	1.06	1.05	.01
Fat Mass Density	11	.90	.90	.90	.00
Fat Free Mass Density	11	1.07	1.07	1.07	.00

#### 4.6.2 CORRELATIONS BETWEEN MATERNAL HMO CONCENTRATION AT V2 AND FAT MASS MEASURED BY PEAPOD

A significant correlation was found between total HMOs at V2 and the fat mass ( $r=0.609$ ,  $p=0.05$ ) (Table 11). Significant correlations are marked with an asterisk.

**Table 11. Spearman correlations between HMO concentrations and Pea Pod data. N=11.**

HMOs at V2		Fat Mass	Fat Free Mass	Body Density	Fat Free Mass Density	Volume
2`FL	r	.445	-.118	-.427	.107	-.036
	p	.17	.73	.19	.76	.92
LDFT	r	.601	-.246	-.569	.012	-.150
	p	.05	.47	.07	.97	.66
3`SL	r	.418	.064	-.282	.060	.118
	p	.20	.85	.40	.86	.73
3`SLN	r	.409	-.345	-.427	.195	-.236
	p	.21	.30	.190	.57	.48
total HMO	r	<b>.609*</b>	-.109	-.555	.042	.027
	p	<b>.05</b>	.75	.08	.90	.94

Correlations analysis of relative HMO concentrations in percent with PeaPod data revealed no significant correlations (data not shown).

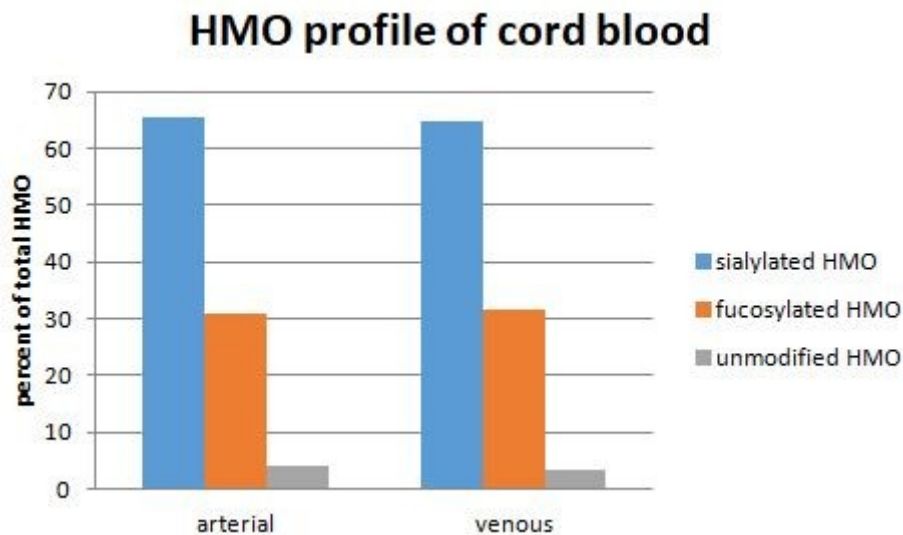
Exclusion of secretor negative mothers from the analysis did not alter results. No correlations could be detected (data not shown).

## 4.7 FETAL HMO

Additionally, HMO concentrations in arterial and venous cord blood of 20 neonates were investigated, as representing the fetal HMO concentrations.

### 4.7.1 FETAL HMO PROFILE IN ARTERIAL AND VENOUS CORD BLOOD

Figure 5 shows the distribution of sialylated, fucosylated and unmodified HMOs in percent in arterial and venous cord blood at birth. No significant differences were found between venous and arterial cord blood. The largest fraction was the sialylated HMOs (blue) with about 65%, followed by fucosylated HMOs (orange) with about 31%.



**Figure 5. HMO Profile of arterial and venous cord blood. Percent of sialylated, fucosylated and unmodified HMO are shown.**

Correlations between sialylated HMOs in arterial and venous cord blood are shown in Table 12. Correlations between fucosylated HMOs are described in Table 13. Statistically significant correlations are marked. For the analysis of the sialylated cord blood HMOs, the Pearson correlation coefficient was used because a normal distribution was found. For the analysis of cord blood fucosylated HMOs, the Spearman correlation coefficient was used because they were not normally distributed.

**Table 12. Pearson Correlations between sialylated HMOs in venous and arterial cord blood. (n=20)**

		ven_3'SLN	ven_3'SL	ven_total_HMO
art_3'SLN	r	<b>.694**</b>	<b>.525*</b>	.352
	p	<b>.00</b>	<b>.02</b>	.13
art_3'SL	r	<b>.476*</b>	<b>.711**</b>	<b>.537*</b>
	p	<b>.03</b>	<b>.00</b>	<b>.02</b>
art_total HMO	r	<b>.466*</b>	<b>.755**</b>	<b>.811**</b>
	p	<b>.04</b>	<b>.00</b>	<b>.00</b>

**Table 13 Spearman correlations of fucosylated HMO concentrations in venous and arterial cord blood. (n=20)**

		ven_2FL	ven_LDFT	ven_total_HMO
art_2'FL	r	<b>.864**</b>	<b>.828**</b>	<b>.661**</b>
	p	<b>.00</b>	<b>.00</b>	<b>.00</b>
art_LDFT	r	<b>.796**</b>	<b>.931**</b>	<b>.670**</b>
	p	<b>.00</b>	<b>.00</b>	<b>.00</b>
art_total HMO	r	<b>.744**</b>	<b>.709**</b>	<b>.693**</b>
	p	<b>.00</b>	<b>.00</b>	<b>.00</b>

#### 4.8 CORRELATIONS BETWEEN MATERNAL HMOs AT VISIT 2 AND FETAL HMO AT BIRTH

Table 14 shows the correlation between the sialylated HMOs in venous and arterial cord blood and the sialylated HMOs at V2. A significant correlation between the maternal 3'SL concentration at V2 and the venous 3'SLN concentration was found ( $r=-0.57$ ,  $p=0.01$ ).

**Table 14. Spearman Correlations between maternal and fetal HMO concentration and composition. (n=20)**

		V2_3SLN	V2_3SL	V2_total HMO
art_3'SLN	r	-.393	-.294	-.253
	p	.09	.21	.28
art_3'SL	r	-.083	.032	-.131
	p	.73	.90	.58
art_total HMO	r	-.030	-.110	-.041
	p	.90	.65	.87
ven_3'SL	r	-.280	-.284	.038
	p	.23	.23	.88
ven_3'SLN	r	-.419	<b>-.568**</b>	-.197
	p	.06	<b>.01</b>	.40
ven_total HMO	r	.006	-.395	-.215
	p	.98	.084	.36

Table 15 shows the correlation between the sialylated HMOs in cord blood in percent and the sialylated HMOs at V2 in percent. No significant correlation could be detected.

**Table 15. Spearman correlations between sialylated maternal and fetal percentage HMO.  
(n=20)**

		V2_3SLN_perc	V2_3SL_perc
art_3`SLN_perc	r	.317	.365
	p	.17	.11
art_3`SL_perc	r	.182	.331
	p	.44	.15
art_total HMO	r	-.075	-.174
	p	.75	.46
ven_3`SLN_perc	r	.257	.304
	p	.27	.19
ven_3`SL_perc	r	.041	.200
	p	.87	.40
ven_total HMO	r	.135	-.173
	p	.57	.47

Table 16 shows the correlation between the fucosylated HMOs in venous and arterial cord blood and the fucosylated HMOs at V2. A significant correlation between the arterial LDFT and the maternal LDFT concentration could be detected ( $r=0.511$ ,  $p=0.02$ ). No other significant correlation could be detected.

**Table 16. Spearman Correlations between maternal fucosylated HMOs and fucosylated HMO at birth. (n=20)**

		V2_2FL	V2_LDFT	V2_total HMO
art_2`FL	r	.338	.315	.086
	p	.14	.18	.72
art_LDFT	r	.429	<b>.511*</b>	.200
	p	.06	<b>.02</b>	.40
art_total HMO	r	.092	.175	-.041
	p	.70	.46	.87
ven_2`FL	r	.236	.164	-.080
	p	.32	.49	.74
ven_LDFT	r	.342	.417	.026
	p	.14	.07	.91
ven_total HMO	r	.060	.101	-.215
	p	.80	.67	.36

The fucosylated HMO concentrations are not normally distributed and thus, were analysed by Spearman rank correlation. Thereby, a significant correlation between percentage of 2`FL in cord blood and the maternal percentage of 2`FL V2 could be detected (arterial:  $r=0.56$ ,  $p=0.01$ ; venous:  $r=0.62$ ,  $p=0.00$ ). Furthermore, a significant correlation between percentage of LDFT in cord blood and the maternal percentage of LDFT concentration at V2 could be detected (arterial:  $r=0.53$ ,  $p=0.02$ ; venous:  $r=0.57$   $p=0.01$ ). Results are shown in table 17.

**Table 17. Spearman correlation between fetal and maternal fucosylated HMO. (n=20)**

		V2_2FL_perc	V2_LDFT_perc
art_2`FL_perc	r	<b>.564**</b>	.221
	p	<b>.01</b>	.35
art_LDFT_perc	r	<b>.508*</b>	<b>.531*</b>
	p	<b>.02</b>	<b>.02</b>
art_total HMO	r	.198	.182
	p	.40	.44
ven_2`FL_perc	r	<b>.617**</b>	.340
	p	<b>.00</b>	.14
ven_LDFT_perc	r	<b>.486*</b>	<b>.572**</b>
	p	<b>.03</b>	<b>.01</b>
ven_total HMO	r	.229	.047
	p	.33	.85

When we analysed the data in the secretor positive group only, no significant correlation could be found (Data not shown).

#### 4.9 ASSOCIATIONS OF FETAL HMOs WITH BIRTH WEIGHT, LENGTH AND PONDERAL INDEX

The correlations of fetal sialylated HMO concentration and composition with birth weight, length and ponderal index are shown in Table 18.

For this analysis, the same patient panel was used as for the SAT analysis.

A significant correlation between 3`SLN concentration in venous cord blood and newborn length was found ( $r=-0.56$ ,  $p=0.01$ ). No other correlations were found. Table 18 shows the correlation between sialylated HMOs and weight, length and PI. The Pearson correlation coefficient was used as a normal distribution was found for the sialylated HMOs. Significant correlations are marked with an asterisk.

**Table 18. Pearson Correlations between sialylated cord blood HMOs and birth weight, Length, ponderal index. n=20**

		weight	length	Ponderal Index
art_3`SLN	r	-.336	-.402	.245
	p	.15	.08	.30
art_3`SL	r	-.300	-.300	.138
	p	.20	.20	.56
art_total HMO	r	-.351	-.210	-.028
	p	.13	.37	.91
ven_3`SLN	r	-.424	<b>-.555*</b>	.366
	p	.06	<b>.01</b>	.11
ven_3`SL	r	-.309	-.362	.219
	p	.19	.12	.36
ven_total HMO	r	-.378	-.314	.083
	p	.10	.18	.73

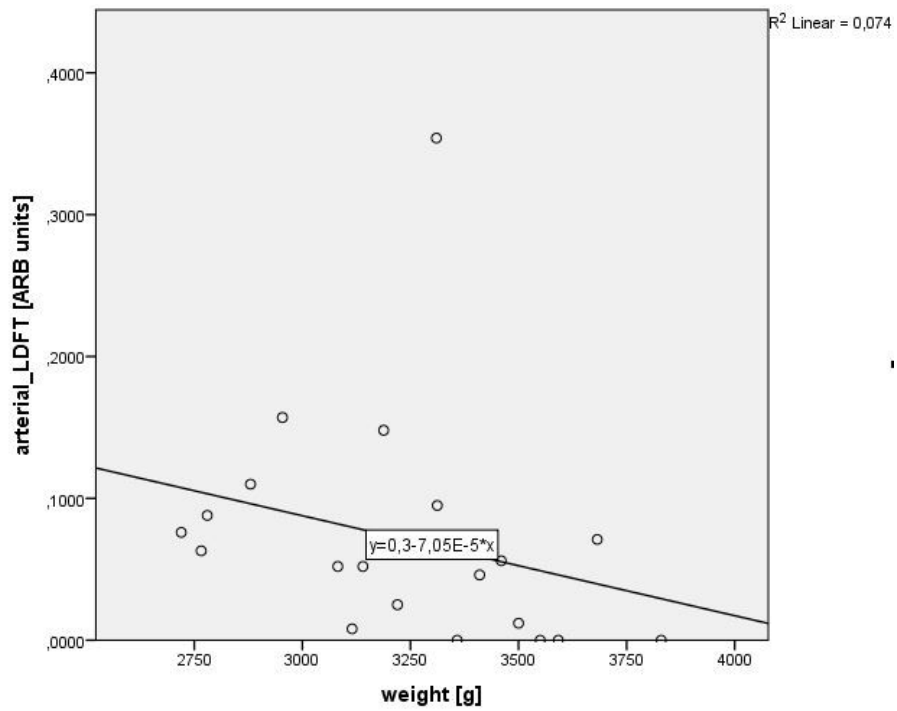
Table 19 shows the correlations between fucosylated HMOs and weight, length and PI. The Spearman correlation coefficient was used as fucosylated HMOs were not normally distributed. LDFT concentration in arterial cord blood was significantly correlated with birth weight ( $r=-0.54$ ,  $p=0.01$ ) (Figure 6). LDFT concentration in venous cord blood was also negatively correlated with birth weight ( $r=-0.58$ ,  $p=0.01$ ) (Figure 7). 2'FL concentrations in arterial or venous cord blood were negatively associated with birth weight ( $r=-.415$ ,  $p=0.07$  and  $r= -4.02$ ,  $p=0.08$ ), without reaching statistical significance. Venous 2'FL was significant correlated with length ( $r=-0.45$ ,  $p=0.05$ ).

**Table 19. Spearman Correlations between fucosylated cord blood HMOs and birth weight, Length, ponderal index. (n=20)**

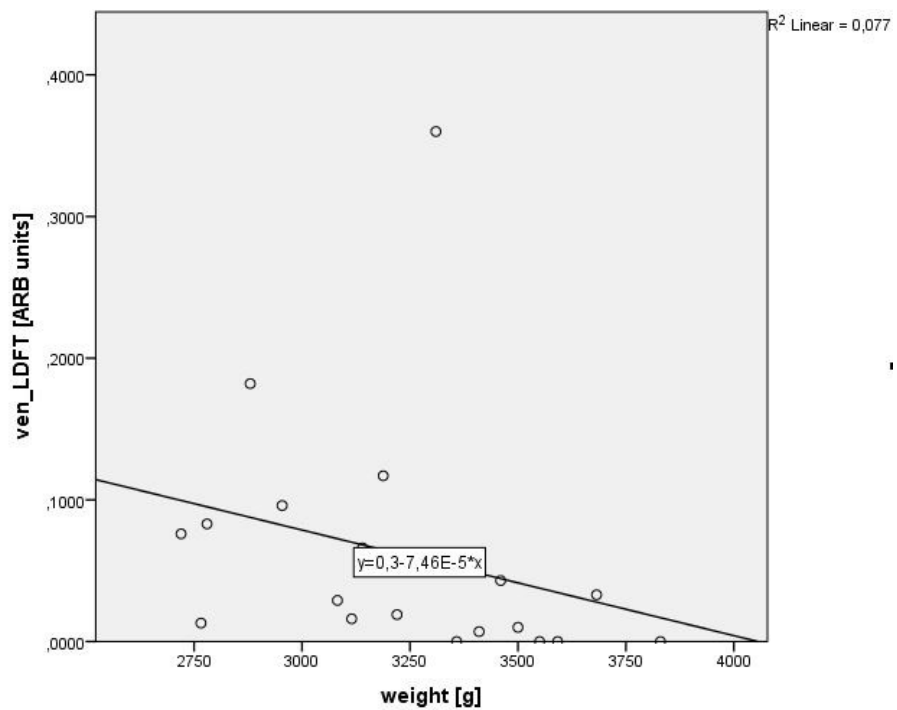
		Birth weight	Length	Ponderal Index
art_2'FL	r	-.415	-.308	-.013
	p	.07	.19	.96
art_LDFT	r	<b>-.539*</b>	-.283	-.171
	p	<b>.01</b>	.23	.47
ven_2'FL	r	-.402	<b>-.446*</b>	.235
	p	.08	<b>.05</b>	.32
ven_LDFT	r	<b>-.583**</b>	-.318	-.137
	p	<b>.01</b>	.17	.56

Correlations analysis of relative HMO concentrations in percent with birth weight, length and PI data revealed no significant correlations (data not shown).

Exclusion of secretor negative mothers from the analysis did not alter results. No correlations could be detected (data not shown).



**Figure 6. Association between arterial LDFT [arb units], and weight[g]. Spearman Correlation Coefficient  $r = -0.54$ ,  $p = 0.01$**



**Figure 7. Association between venous LDFT [arb units], and weight[g]. Spearman Correlation Coefficient  $r = -0.58$ ,  $p = 0.01$**

#### 4.10 ASSOCIATIONS BETWEEN FETAL HMO CONCENTRATION AND COMPOSITION WITH SAT THICKNESS

Arterial cord blood 3`SL was found significantly correlated with upper (r=-0.46 p= 0.04), lower (r=-0.46, p=0.04) as well as total SAT (r=-0.50, p=0.02) thickness (Table 20 and figures 9-11). For 2`FL, LDFT, and 3`SLN no correlations with SAT thickness could be detected (Table 20/21, Figure 8-10)

Venous cord blood 3`SL was also found significantly correlated with upper (r= -0.48, p= 0.03), as well as total SAT (r=-0.48, p=0.03) thickness. Associations between 3`SL and lower SAT thickness did not reach significance. For 2`FL, LDFT, 3`SLN no correlations with SAT thickness could be detected (Table 20/21)

**Table 20. Pearson Correlations between cord blood sialylated HMO concentrations and newborn SAT thickness. (n=20)**

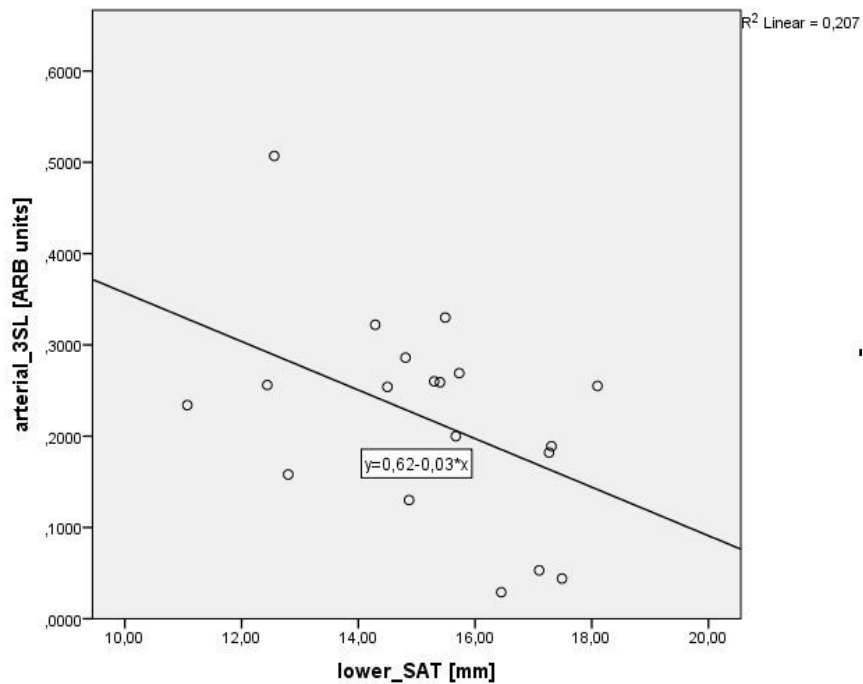
		total SAT	upper SAT	lower SAT
art_3`SL	r	<b>-.502*</b>	<b>-.464*</b>	<b>-.455*</b>
	p	<b>.02</b>	<b>.04</b>	<b>.04</b>
art_3`SLN	r	-.406	-.377	-.365
	p	.08	.10	.11
art_total HMO	r	-.271	-.252	-.246
	p	.25	.28	.30
ven_3`SL	r	<b>-.484*</b>	<b>-.481*</b>	-.397
	p	<b>.03</b>	<b>.03</b>	.08
ven_3`SLN	r	-.352	-.363	-.273
	p	.13	.12	.24
ven_total HMO	r	-.196	-.216	-.136
	p	.41	.36	.57

**Table 21. Spearman Correlations between cord blood fucosylated HMO concentrations and newborn SAT thickness. (n=20)**

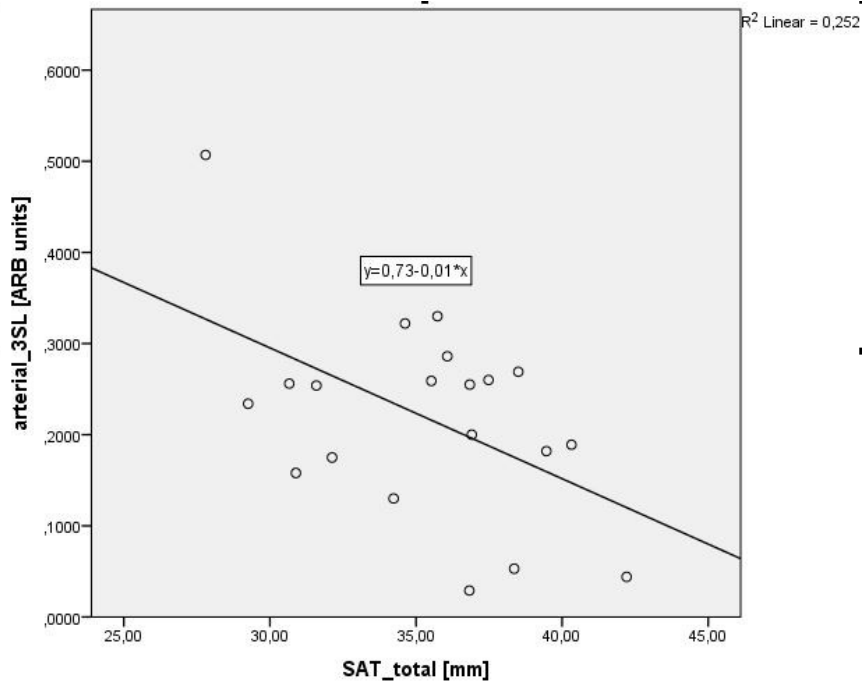
		total SAT	upper SAT	lower SAT
art_2`FL	r	-.099	-.059	-.187
	p	.68	.81	.43
art_LDFT	r	.050	.009	.014
	p	.84	.97	.96
ven_2`FL	r	-.177	-.206	-.199
	p	.46	.38	.40
ven_LDFT	r	.118	.046	.107
	p	.62	.85	.65

Furthermore, the associations between individual cord blood HMOs in percent and SAT thickness were analysed, and no correlations were found (data not shown).

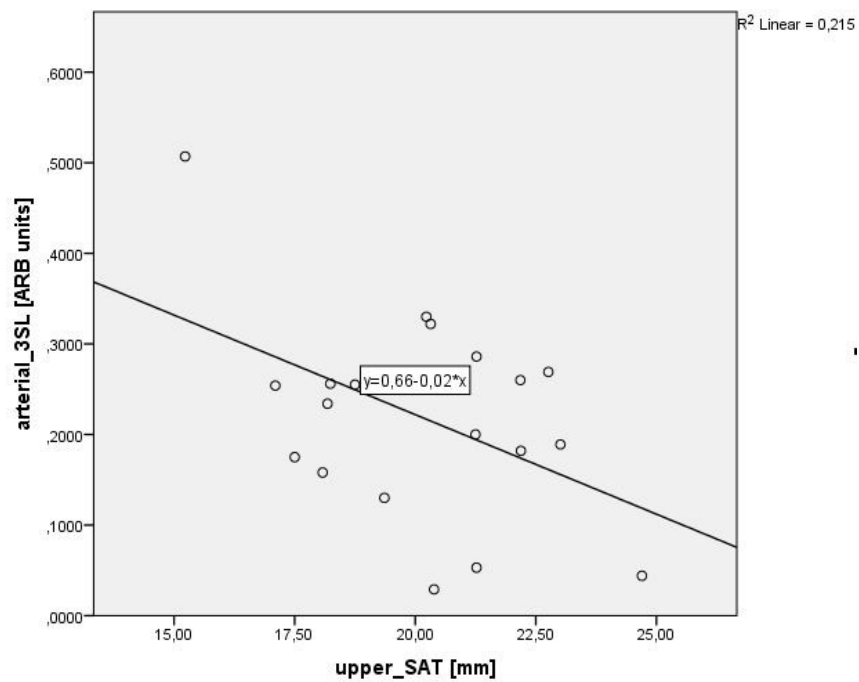
When we analysed correlations of relative HMO concentrations in secretor positive group only, we found similar results. No correlations were found (data not shown).



**Figure 8. Correlation between arterial 3'SL concentration [arb units] and Lower SAT Thickness [mm] . PEARSON Correlation Coefficient  $r=0.455$ ,  $p=0.04$**



**Figure 9. Association between arterial 3'SL [arb units], and total neonatal SAT [mm].  
PEARSON Correlation Coefficient  $r = -0.50$ ,  $p = 0.02$**



**Figure 10. Correlation between arterial 3'SL concentration [arb units] and Upper SAT Thickness [mm]. PEARSON Correlation Coefficient  $r = -0.46$ ,  $p = 0.04$**

## 5. DISCUSSION

The aim of this thesis was to investigate the influence of maternal HMO concentration and composition during pregnancy on fetal HMOs and on neonatal outcomes. In this study, we found that maternal HMO concentration between 20-24 weeks (visit 2, V2) were not significantly associated with fetal HMO concentration. Furthermore, we found that HMO concentration and composition at V2 were not significantly correlated with neonatal outcomes such as subcutaneous fat measured by Lipometer, birth weight, length and ponderal index. Referring to the PEA POD measurements, a significant positive correlation was found between total HMOs at V2 and the fat mass. All other parameters did not correlate.

However, a significant correlation was found between fetal HMOs in cord blood and neonatal body composition. The venous and arterial cord blood 3'SL concentration negatively correlated with the SAT thickness. Furthermore, we found a significant negative correlation between fetal LDFT concentrations and neonatal weight and a negative correlation between venous cord blood 2'FL and neonatal length.

Moreover, a significant correlation between venous cord blood 3'SLN concentration and length was found.

## 5.1 INTERPRETATION OF THE RESULTS

We found a correlation between fetal HMO and fetal outcomes but not between maternal HMO in mid pregnancy and fetal outcomes except the correlation between total HMO at V2 and the fat mass measured by PEA POD. Due to the small sample size this could have been a random effect.

The absence of other correlations between maternal HMO concentration and neonatal outcomes might be explained by the relative long time interval between midpregnancy and the fact that during the first half of gestation the accretion of fat and protein is slow. In the second half of the pregnancy, the fat accretion becomes faster (Toro-Ramos, et al., 2015). Thus, it would be interesting to investigate associations of maternal HMOs later in pregnancy with neonatal body fat composition. Maybe that would lead to different results.

Maternal HMOs at V2 and fetal HMOs at birth also did not correlate consistently. Similarly, this might be due to dynamic changes during pregnancy leading up to birth with many potential modulations of HMOs. The only statistically significant correlation that can be found between maternal 3`SL concentration at V2 and the venous 3`SLN concentration in cord blood might be a random effect. It would be interesting to correlate maternal and fetal HMOs concentration at the same time point, namely, at birth.

Although no correlations were found between absolute maternal and fetal HMO concentrations, a statistically significant correlation was found between maternal and fetal 2`FL in percent of total HMOs. This indicates that secretor phenotype of maternal and fetal blood match. Secretor positive women can produce 2`FL, and other  $\alpha$ 1-2-fucosylated HMO, whereas non-secretor women are unable to produce these HMO types (Bode & Jantscher-Krenn, 2012). The finding that the secretor phenotype seems to match between maternal and fetal blood suggest that in pregnancy, 2`FL and other HMOs are produced by the mother and cross the placenta to reach the fetal circulation.

When we excluded secretor negative women from the analysis no significant correlation were found. That might be due to the fact that the sample size was too small.

The observed associations between fetal HMOs and neonatal outcomes might indicate that prenatal HMOs in the fetal circulation have an effect on neonatal body composition. Of course, we cannot draw any conclusions about causality and these associations may imply that both, fetal HMOs and neonatal body composition are influenced by the same factors.

To our knowledge, this was the first study which investigated the relation of prenatal HMOs and neonatal anthropometrics. A recent study in breastfed infants found associations between specific HMOs in human milk and infant body composition. This study showed positive associations between LNFP2 and DSLNT with infant fat mass at 6 months (Alderete, et al., 2015). Furthermore, the authors found a negative association between LNFP1 and infant weight, lean and fat mass (Alderete, et al., 2015), but did not look at LDFT correlations with infant body composition. They suggested that HMOs might influence body weight and composition via the modulation of gut microbiome in the infant. However, the negative correlation of LDFT concentrations in cord blood with neonatal weight found in our study, indicates that HMOs might also have microbiome independent effects on fetal growth and fat accretion. We found 3'SL in cord blood to be negatively correlated with SAT at birth. It would be interesting to study 3'SL effects on lipogenesis in adipocytes *in vitro*. Again, we have to acknowledge that these findings are associations and do not allow conclusions about causality.

Furthermore, if maternal HMOs are able to cross the placenta and whether the placenta can modulate this process is presently unknown. However, if fetal HMOs at birth correlate with maternal HMOs at the same time, this could still speak for an influence of prenatal HMOs on neonatal body composition in the last trimester, a critical window of metabolic fate for the offspring. Future studies need to investigate correlation of maternal HMOs later in pregnancy with neonatal outcomes.

Although no correlations were found between absolute maternal and fetal HMO concentrations, a statistically significant correlation was found between maternal and fetal 2`FL in percent of total HMOs. This indicates that secretor phenotype of maternal and fetal blood match. Secretor positive women can produce 2`FL, and other  $\alpha$ 1-2-fucosylated HMO, whereas non-secretor women are unable to produce

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When we excluded secretor negative women from the analysis no significant correlation were found. That might be due to the fact that the sample size was too small.

## 5.2 LIMITATIONS OF THE STUDY

### 5.2.1 SAMPLE SIZE

The study was conducted as a pilot study to investigate for the first time, prenatal HMOs and their associations with fetal outcomes. Due to the small sample size, it is likely that we did not have enough power to detect effects of HMOs on neonates.

### 5.2.2 DROPOUT RATE AND INCOMPLETE DATA SETS

The high dropout rates and resulting incomplete data sets created a considerable limitation. Probable reasons for these high dropout rates include the study design as a longitudinal study itself. This design always bears the risk that study participants miss visits or decide to drop out. This was due to the fact that the participating women had no personal benefit through participation while contemporaneously the study requires a large amount of time (accelerometer, questionnaires).

### 5.2.3 HMOs DIFFER REGIONALLY

HMO concentration and composition may differ according to geographical regions (Erney, et al., 2000). Since the recruiting only took place in the department for gynaecology and obstetrics at the medical university of Graz, the study population is rather homogeneous and consequently not representative for a larger population. A coordinated multicentre study would be required to obtain optimal reproducible results.

#### 5.2.4 CONSIDERATION OF POSSIBLE CONFOUNDERS

It is not possible to describe causal effects because the possible confounders are not sufficiently considered in this work. These factors might include for example nutritional impacts on maternal and fetal HMO concentration and composition as well as physical activity related differences and their possible effects on HMOs as well as on the fetal outcomes. Maternal BMI and maternal body composition might be the largest confounders, potentially affecting HMOs and fetal body composition.

Studies suggest for instance that decreasing neonatal adiposity rates go along with increasing total energy exposure in late pregnancy (Harrod, et al., 2014). In this study, we did not control for physical activity.

But after all, the consideration of all these factors would go beyond the scope of this diploma thesis.

### 5.3 CONCLUSION

We hypothesised that maternal HMO concentration and composition in mid pregnancy and fetal HMO are associated. This could not be confirmed.

Furthermore, we hypothesized that maternal HMO concentration or composition has a significant influence on neonatal body composition. But this also could not be confirmed in this small cohort.

Nevertheless, we found a association between fetal HMO concentration and neonatal outcomes. This might imply that fetal HMOs have an effect on neonate body composition. Or it indicates that both are influenced by the same factors for example maternal nutrition, maternal body composition or maternal physical activity.

These results provide a basis for further research. It would be interesting to investigate associations of maternal HMOs later in pregnancy with neonate body composition. Furthermore, the association between fetal HMO and fetal outcomes should be examined in a larger study by involving possible confounders.

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# APPENDIX

## INCLUSION AND EXCLUSION CRITERIA

### INCLUSION CRITERIA

- ongoing pregnancy in the 10-14 gestational weeks
- giving informed consent

### EXCLUSION CRITERIA

- not wanting to give birth at the university hospital
- gestational age > 14<sup>th</sup> week of gestation
- multiple pregnancy
- 3 consecutive miscarriages
- increased risk > 1:100 after combined test and no non invasive Prenatal Testing or no invasive testing for chromosomal anomalies
- fetal anomalies which are associated with possible growth or genetic anomalies
- smoking
- pre-pregnancy diabetes type 1 or 2
- maternal metabolic risk factory (autoimmune conditions, increased risk for thromboembolic events needing anticoagulative therapy)
- pre-pregnancy hypertension