Diplomarbeit

Effects of Physical Activity in Pregnancy on Human Milk Oligosaccharides in Maternal Serum

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Alles Wissen und alle Vermehrung unseres Wissens endet nicht mit einem Schlusspunkt, sondern mit Fragezeichen.

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Zusammenfassung

Einleitung: Humane Milcholigosaccharide (HMO) sind bioaktive Glykane, die in der Muttermilch, aber auch während der Schwangerschaft im Kreislauf von Mutter und Fötus, vorkommen. Einige HMO Strukturen haben bekannte anti-inflammatorische und/oder immunomodulierende Effekte. Ebenso sind für körperliche Aktivität den Inflammationsgrad senkende Auswirkungen in nicht-schwangeren Individuen bekannt. Für Schwangere, die physiologisch einen Zustand einer leichten Inflammation aufweisen, werden von der WHO aktuell 150 Minuten körperliche Aktivität (PA) pro Woche empfohlen. Die Konzentrationen und Zusammensetzung von HMOs werden von genetischen Faktoren beeinflusst, ob aber Umweltfaktoren wie körperliche Aktivität einen Einfluss haben, ist nicht bekannt.

Hypothese: Wir haben angenommen, dass körperliche Aktivität während der Schwangerschaft den HMO Spiegel und deren Zusammensetzungen im maternalen Serum beeinflusst.

Material und Methoden: Gesunde Schwangere in ihrer 10-14. Schwangerschaftswoche wurden zwischen Februar und Oktober 2013 im Universitätsklinikum für Geburtshilfe, LKH Univ.-Klinikum Graz, rekrutiert. Ausschlußkriterien waren mehrfache Fehlgeburten, erhöhtes Risiko nach dem Combined Test, fötale Anomalien, Rauchen, mütterliche metabolische Risikofaktoren, pregravider Diabetes Mellitus und Hypertension. Studienteilnehmerinnen unterzogen sich vier Untersuchungen während der Schwangerschaft und einer bei Entbindung. Körperliche Aktivität (PA) wurde objektiv per Accelerometer (ActiGraph) dreimal während der Schwangerschaft für eine Woche gemessen. HMO Werte wurden aus maternalen Blutproben gewonnen, die bei drei Untersuchungen während der Schwangerschaft und bei Entbindung abgenommen wurden. HMOs wurden durch HPLC mit Fluoreszenz Detektion analysiert.

Ergebnisse: Von 12 der rekrutierten gesunden schwangeren Frauen, im Alter von 34.5 ± 5.5 Jahren und mit einem pregraviden BMI von 22 ± 2.6 kg/m^2, hatten wir komplette Datensätze zu PA Messungen und HMO Werten. Unsere statistische Analyse zeigte, dass PA negativ mit den konsekutiv gemessenen totalen bzw. individuellen HMO Konzentrationen korrelierte. PA der ersten Untersuchung korrelierte signifikant mit HMO Konzentrationen nachfolgender Untersuchungen (Untersuchung 2, 3, und bei Entbindung). Die HMO Zusammensetzung während der Schwangerschaft schien nicht durch PA beeinflusst zu sein. Keine Beziehungen von BMI, Gewicht, und subkutanen Fett mit HMO konnten gefunden werden.

Diskussion: Zusammenfassend fanden wir eine Beziehung von PA im ersten Trimester und späteren HMO Konzentrationen im maternalen Serum, aber nicht mit deren Zusammensetzung. Um diese Ergebnisse zu bestätigen, würden größere Studien, evtl. mit interventionellem Aufbau bezüglich PA und Life Style, benötigt werden.

Abstract

Background: Human Milk Oligosaccharides (HMO) are bioactive glycans found not only in human milk, but already in the systemic circulation of both mother and foetus. Some HMO structures are known to have anti-inflammatory and/or immunomodulatory effects. HMO are known to be influenced by genetic factors, which amongst others are determining HMO biosynthesis. Moderate-to-vigorous physical activity (MVPA) was postulated to have anti-inflammatory effects in non-pregnant individuals. For pregnant individuals, who are in a physiological state of low-grade inflammation, a minimum of 150 minutes MVPA per week is recommended by the WHO. However, whether environmental factors such as regular physical activity may influence the prenatal HMO concentrations and composition, is not known.

Hypothesis: We hypothesised that physical activity during pregnancy influences HMO levels and composition in maternal serum.

Subjects and Methods: Healthy women with a singleton pregnancy in their 10-14th week of gestation were recruited at the Department of Obstetrics, University Hospital Graz, between February and October 2013. Exclusion criteria were multiple miscarriages, increased risk after combined test, foetal anomalies, smoking, maternal metabolic risk factors, pre-pregnancy Diabetes mellitus and pregravid hypertension. Women participating had three study visits during pregnancy and one at delivery. Physical activity (PA) was objectively measured by accelerometer (ActiGraph) over one week, at three time points during pregnancy (visits V1, V2, V3). HMO were determined from maternal blood samples, which were collected at V1, V2, V3, delivery, and were analysed by HPLC with fluorescence detection.

Results: We obtained complete data sets on PA measurements and HMO samples from 12 healthy pregnant women. Their mean age was 34.5 ± 5.5 years, pre-pregnancy BMI was 22 ± 2.6 kg/m^2 with an overall BMI increase during pregnancy of 6.3 ± 1.6 kg/m^2. In unadjusted analysis we found that early pregnancy PA correlated negatively with total or individual HMO concentrations, measured at the visit following the respective PA-recording. PA at the first visit correlated significantly to HMO concentrations at visits 2, 3 and at delivery. PA, as measured objectively in early pregnancy, did not have any effect on HMO composition during the course of gestation. No association was found of HMO and various weight-related outcome measures.

Conclusion: In summary, we showed that PA, as measured objectively in early pregnancy, seems to influence the concentration but not composition of maternal HMO in serum.

Further studies, e.g. with PA as life-style intervention, are needed to confirm this finding, as well as larger sample size to allow statistical adjusting for confounding factors

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Glossary and Abbreviations

(IL12) p40	IL12 40 kDa subunit
(IL12) p70	IL12 70kDa (total cytokine)
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
B. (infantis, longum, breve)	*Bifidobacterium*
BDNF	brain derived neurotropic factor
β3-GalT	β 3 Galactosyltransferase
β-3-GlcNAcT	β 3 N-acetylglucosaminyltransferases
β4-GalT	β 4 Galactosyltransferase
β-6-GlcNAcT	β 6 N-acetylglucosaminyltransferases
β-HCG	β human choriogonadotropin
BMI	body mass index
C13	carbon 13 isotope
CaCo2 monolayer	Colon carcinoma derived epithelial cell line
CCLL22	gene on chromosome 16q13 that encodes a CC-type cytokine
CD	cluster of differentiation
CDC	Centre for Disease Control
CPM	counts per minute
CRD	carbohydrate recognition domain
DC	dendritic cell
DC-SIGN	dendritic cell specific ICAM3-grabbing Non-Integrin
Del	Delivery
DSLNT	Disialyl-lacto-n-tetraose
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxic *E. coli*
FFA	free fatty acids
Fuc	Fucose
FUT	Fucosyltransferase
Gal	Galactose
GalT	Galactosyltransferase
GDM	Gestational Diabetes Mellitus

Glc	Glucose
GlcNAc	N-Acetylglucosamine
GlcNAcT	N-acetylglucosaminyltransferase
HDL	high density lipoprotein
HMO	Human Milk Oligosaccharides
HPL	human placental lactogen
IGF	insulin like growth factor
iGnT, IGnT	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme (I blood group)
IL	interleukin
INGFBP	insulin like growth factor binding protein
LDFT	Lacto-di-fuco-tetraose
LDL	low density lipoprotein
Le	Lewis
LIF	leukaemia inhibitory factor
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
MCP	monocyte chemotactic protein
MEMS	micro-electro-mechanical system
MIP1α	macrophage inflammatory protein 1 α
MVPA	moderate to vigorous physical activity
NK	natural killer cells
PA	physical activity
PAMP	pathogen-associated molecular patterns
PGH	placental growth hormone
PNC	platelet neutrophil complex
R (as in sIL1R)	Receptor
R (as in sIL-2Rα)	Receptor
R (as in s-IL6R)	Receptor
ra (as in IL1ra)	receptor antagonist

ROS	reactive oxygen species
s (as in sIL1R)	soluble
s (as in sIL-2Rα)	soluble
s (as in s-IL6R)	soluble
Se	Secretor
Sia	Sialic acid
Siglec	sialic-acid binding Ig-like lectins
sIL1R	soluble interleukin-1 receptor
sIL-2Rα	soluble interleukin-2 receptor α
s-IL6R	soluble interleukin-6 receptor
ST3Gal	sialyltransferase
sTNF-R	soluble TNF receptor
Th	T helper
TLR	Toll-like receptor
TNF-α	tumour necrosis factor α
UDP	Uridine-diphosphate
UPEC	uropathogenic *E. coli*
UTI	urinary tract infection
V1, V2, V3	Visit 1, 2, 3

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1 Introduction

Pregnancy in humans, and in mammals in general, is an intriguing mechanism of supporting maturation of the offspring and of protection whilst it is happening.

With an array of protective mechanisms interacting with the maternal immune system, the mammalian foetus is nurtured in utero, until it is mature enough for delivery. Thus, mother and offspring are connected biologically for a longer period than any other class in their reproductive time. Postpartum, by providing a perfectly suited nutrition, namely breast milk comprising many bioactive components, the infant is further supported in its development.

Human breast milk, as a complex and highly variable biofluid has evolved over millions of years and its composition is believed to be specifically adjusted to meet the infant's needs concerning nutrition and immunisation. A variety of antimicrobial and immunomodulatory components are thought to compensate deficiencies in the neonate's immune system. Evidence supporting this is for instance that breastfed infants are less susceptible to gastrointestinal or respiratory infections than formula fed infants (1).

Human milk composition is highly variable inter-personally and also intra-personally, as for example it is depending on the stages of lactation (2).

One of the largest fractions in breast milk is composed of human milk oligosaccharides (HMO), bioactive glycans that have a wide array of functions that are not all elucidated yet. With over 150 structures identified, most of the cellular pathways leading to known functions are still unclear (3). Although their name suggests otherwise, HMO are not only found in human breast milk, but also in maternal serum during pregnancy. HMO are thought to be biosynthesised in the mammary gland (2), with an onset potentially following conception. HMO concentration gradually increase in maternal serum during pregnancy (Jantscher-Krenn *et al.* unpublished (4)). Newest unpublished studies found HMO also in foetal cord blood (4). All these findings suggest that HMO do not only play a role during lactation but already prior to delivery. Their occurrence in systemic circulation indicates further important functional roles to the pregnant women as well as to the offspring in utero.

When looking at some of the immunomodulatory functions of HMO, we may infer important roles also for the pregnant mother, potentially using HMO to protect the foeto-maternal unit from various stressors, and so ultimately protecting both mother and foetus

during a vulnerable time of altered maternal immunity in pregnancy, which, although physiological, may also have adverse effects (5).

A low-grade inflammatory environment in pregnancy, as for instance induced by elevated pro-inflammatory cytokine levels that positively correlate with the mother's subcutaneous adipose tissue for instance, might lead to adverse effects on maternal and neonatal pregnancy outcomes. While HMO are attributed certain biological effects, such as anti-inflammatory and immunomodulatory functions, little is known about which maternal factors, e.g. environmental factors such as physical activity may influence HMO concentration and composition.

2 Background

Breast milk can be differentiated into colostrum, the early stage breast milk, transitional milk and mature milk by the stages of lactation. Major components in breast milk can be divided into groups of different macromolecules, lipids, proteins, immunoglobulins and human milk oligosaccharides (1,2,6).

Immunoglobulins represent a high fraction of human milk components especially in early stage lactation. Proteins, contributing to a fraction of human milk, can, for instance, alter enzymatic activities, act as metabolic mediators, influence development, maturation and repair of the gastrointestinal tract. They can also affect the microbiota development and immune functions, and are known to stimulate nutrient absorption and possess antimicrobial and immunomodulatory properties. 80–90% of milk proteins are synthesised by lactocytes, the remainder is for the most part taken up from maternal circulation by transcytosis and then secreted into the lumen. Lipids are the largest source of energy in breast milk, contributing to 40–55% of the total energy (6). However, corresponding to the human brain's high energy requirement, human milk has the highest lactose concentration amongst mammals.

In addition, human milk has a great variety of diverse and complex carbohydrates (2,6).

Among these carbohydrates, human milk oligosaccharides make up the largest part and in total, the third largest fraction in human milk. HMO are indigestible (7) and their functions, both local and systemic appear to be highly structure dependent (3). Some genetic factors determining the individual HMO composition were identified. While their specific functions, metabolism and influencing factors of HMO are being heavily researched, epidemiologic data in human cohorts is emerging and backing the beneficial effects of human milk, and with it, effects of the HMO fraction (1–3).

2.1 Human Milk Oligosaccharides

Human milk oligosaccharides (HMO) are unconjugated glycans highly abundant in human milk, whereas infant formula hardly contains any HMO. Bovine milk, which is used as basis for most infant formula, has a concentration of oligosaccharides 10^{2-3} times lower than human milk (3,8,9). While there is evidence of beneficial effects of human milk due to its HMO content, there is little evidence that structurally different oligosaccharides, which are currently added to infant formula, have similar effects on neonates, mimicking the broad variety of functions of HMO. Among these beneficial HMO effects we find a lower incidence of different intestinal infections in breastfed children (1,6).

One litre of mature human milk contains 10–15g HMO and concentrations in colostrum may even be higher at approximately 20–25 g/l (10). During the first three days of lactation, sialyloligosaccharide compositions and concentrations in colostrum change most significantly (11). Total HMO concentration reflect a change over the course of lactation. While colostrum has the highest HMO concentration, as milk matures, total concentration is gradually decreasing to a concentration level of 5–20g/l in mature human milk, however still exceeding the overall protein concentration (10).

2.1.1 Brief History

At the end of the 19th century scientists first observed that while infant mortality was as high as 30%, breastfed infants still had a significantly higher chance of survival than formula-fed infants. Thus having had their attention called to human milk, two paths subsequently lead to the discovery of human milk oligosaccharides, namely the chemists' path, trying to characterize the abundant carbohydrate fraction of human milk, and the paediatricians' and microbiologists' path, seeking to understand the observed beneficial effects of breastfeeding on the neonates (8).

Before the two parties found the link and identified HMO, a few decades with important milestones passed by (8).

In 1886, Escherich, an Austrian microbiologist and paediatrician, had discovered a correlation between intestinal bacteria and the physiology of neonatal digestion. His former student Moro observed the relation of infant health with both intestinal bacteria and breastfeeding and found a difference in the faecal bacterial composition of breastfed and bottle-fed infants, as did Tissier in an independently run research. Meanwhile, chemists

discovered a “different type of lactose” in human milk than in bovine milk in 1888. Over forty years later, after thorough characterisation, it was called gynolactose (8).

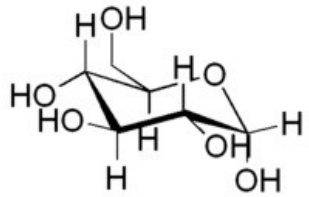
Only in 1954 the collaborating chemist Kuhn and paediatrician György hypothesised that there was a connection between “gynolactose” and the faecal bacteria composition in breastfed infants. Indeed, Kuhn and György could confirm that the long sought “bifidus factor” consisted of oligosaccharides, finally linking the two paths (8).

From this time on, several individual HMO were characterized (Kuhn 1956–1962) and in some of the structures, activities of blood group determinants could be shown, leading to the discovery of Lewis and H blood group determinants (8). The concept of HMO was expanded to what is known today.

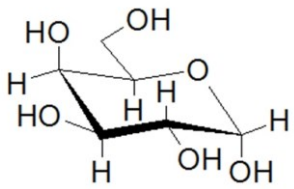
2.1.2 Structure

Over a 150 structurally specific HMO have been identified since, all of which follow a basic blueprint in their synthesis. HMO have 5 monosaccharides as elementary units, forming then the subcategories of trisaccharides, the simplest HMO, and the longer, more complex HMO. All HMO have the disaccharide lactose at their reducing end (3,8). These basic monosaccharide units are glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (Sia) with N-acetylneuraminic acid being its only form occurring in HMO (3).

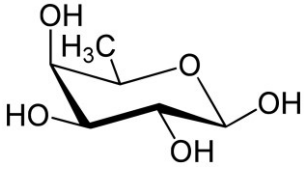
TABLE 1. THE FIVE BASIC MONOSACCHARIDE UNITS OF HMO. Glucose, Galactose, Fucose, N-acetyl-glucosamine, and N-acetyl-neuraminic acid are shown in chair conformation.



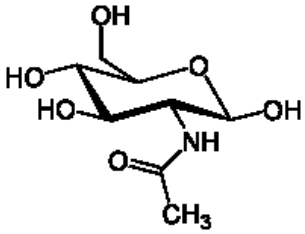
Glucose
Glc
(α-D-glucopyranose)



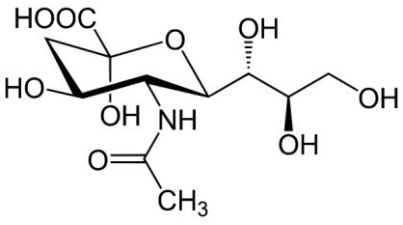
Galactose
Gal
(α-D-galactopyranose)



Fucose
Fuc
(β-D-fucopyranose)



N-Acetyl-glucosamine
GlcNAc
(N-acetyl-D-glucosamine)



Sialic acid
Sia
(N-acetylneuraminic acid)

2.1.2.1 Trisaccharides

When the disaccharide lactose is fucosylated or sialylated without further addition of disaccharides, it forms the trisaccharides 2'- and 3-fucosyllactose (2'FL – α1-2 linkage with fucose; 3FL – α1-3 with fucose) or 3'- and 6'-sialyllactose (3'SL – α2-3 linkage with Sia and 6'SL – α2-6 linkage with Sia), respectively. These are structurally the simplest HMO (3,8,9).

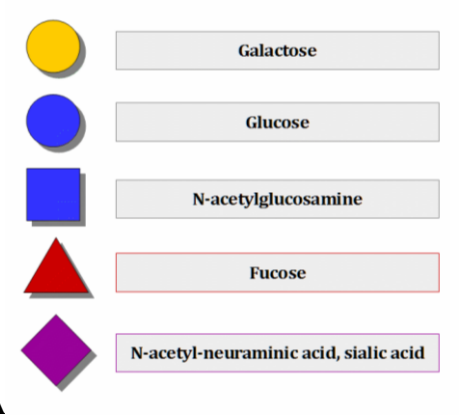
2.1.2.2 Complex HMO

HMO can also form more complex structures, when the basic lactose at the reducing end is further elongated with disaccharides units. They are added to the non-reducing end of lactose, forming a backbone which then may be subject to supplementary modifications such as fucosylation and/or sialylation (3,8).

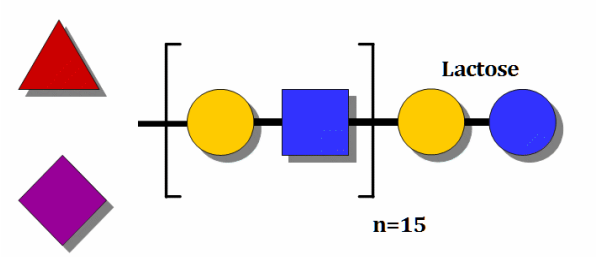
The HMO backbone is synthesised by addition of disaccharide repeats, consisting of the two monosaccharides shown above, Galactose and N-acetyl-glucosamine (3,8).

- *Iso-HMO:* By introducing a β1-6 linkage between two disaccharide building units, the HMO chain is branched and so called Iso-HMO are synthesised.
- *Para-HMO:* linear HMO are designated as para-HMO, when their chain is being elongated by further β1-3 linkages.
- *Type 1 chains:* The predominance of type 1 chains is a characteristic specific for human milk (12). When elongated in β1-4 or β1-6 linkages by the addition of lacto-N-biose (= Galβ1-*3*GlcNAc), a type 1 chain is synthesised, contrary to:
- *Type 2 chains:* which are synthesised by adding β1-3 or β1-6 linked N-acetyllactosamine (= Galβ1-*4*GlcNAc) to the non-reducing end of the chain.

- *Elongation:* N-acetyllactosamine (Galβ1-4GlcNAc) can be further extended by one or two disaccharides
- *Chain Termination:* the addition of lacto-N-biose (Galβ1-3GlcNAc) seems to terminate the chain.



A



B

FIGURE 1. (A) MONOSACCHARIDE COMPONENTS AND (B) BASIC STRUCTURE OF HMO. (A) HMO use five different monosaccharides for formation of HMO structures, Galactose (yellow sphere), Glucose (blue sphere), N-acetylglucosamine (blue square), Fucose (red triangle), and sialic acid (purple rhomb). **(B)** HMO form more complex structures, when lactose at the reducing end is further linked to disaccharides units. The backbone is made up of by disaccharide repeats, consisting of Galactose (yellow sphere) and N-acetylglucosamine (blue square). Fucose and sialic acid can be added and thus modify the backbone.

2.1.2.3 Sialylation and Fucosylation

Both modifications result in a modification of structure–function relationship leading to HMO having different functions whether they are sialylated, fucosylated or both (3).

Sialylation is the process when a sialic acid, represented predominantly by N-acetyl-neuraminic acid, is linked to lactose or, in complex HMO, to the backbone in α2-3 or α2-6 linkages. Sialic acid, containing a carboxylic group, introduce a negative charge to the

HMO they are linked to. Sialic acid can be added either to terminal Galactose or to internal GlcNAc. The reaction is catalysed by different sialyltransferases, resulting in a high graduation of the final sialyl residues linked to the HMO backbone due to slight interindividual changes of sialyltransferase expression, and accordingly, variations of enzyme and transporter expression in sialylation pathways as well (3,8).

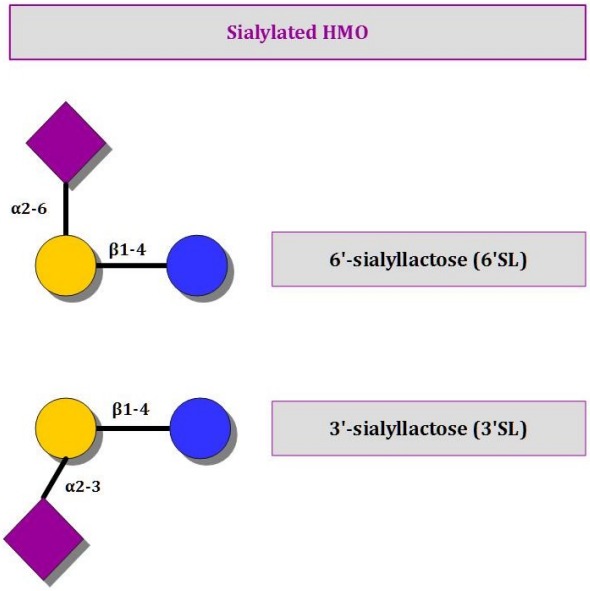


FIGURE 2. SIALYLATION OF HMO. N-acetyl-neuraminic acid may be linked to the backbone in complex HMO, or to lactose, in α2-3 (see 3'SL) or α2-6 linkages (see 6'SL). Binding may occur to either terminal Galactose or internal N-acetylglucosamine. Sialic acid contains a carboxylic group and introduces a negative charge to the HMO they are linked to.

Fucosylation on the other hand is the modification occurring to HMO when α1-2, α1-3 or α1-4 linkages with Fucose are made (see Figure 3). Responsible for catalysing this reaction are mainly the fucosyltransferase 2 and 3 (FUT2 and FUT3). In contrast to sialylation, the fucosylation process is almost following an all-or-none-law with less graduation. If one of the two fucosyltransferases involved in HMO fucosylation processes is not expressed, the result is a completely different set of HMO being produced. Based on the expression of these fucosyltransferases, human milk can be categorized into 4 groups (Table 2). These groups are the most distinct interpersonal variations known, resulting from genetically determined differences in glycosyltransferase expression with effects on the fucosylation of HMO (3).

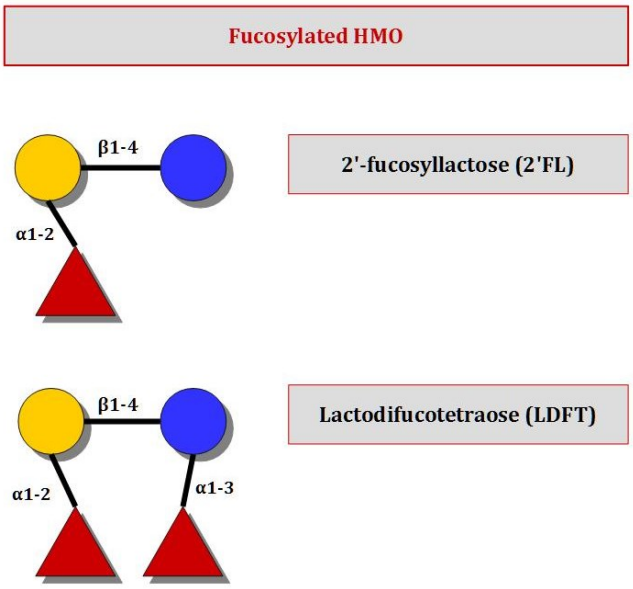


FIGURE 3. FUCOSYLATION OF HMO. Modification of HMO occurs when Fucose is linked in α1-2, α1-3 or α1-4 to HMO. Responsible for catalysing this reaction are mainly the fucosyltransferases 2 and 3 (FUT2 and FUT3).

2.1.2.4 Interpersonal Variations

Over 150 HMO are determined thus far, and each woman produces an individual set of HMO. While each set remains highly variable, they can be roughly categorised into four distinct groups accounting for differences in the fucosylation status of the present HMO. Each class depends on which glycosyltransferases the mother expresses, based on the activity of two gene loci encoding for the α1-2-fucosyltransferase, FUT2, and α1-3/4-fucosyltransferase, FUT3 (1,3,8,13).

TABLE 2. FOUR HMO GROUPS (2). FUT = Fucosyltransferase; Secretor positive Lewis positive (Se+Le+) women express both FUT2 and FUT3; Nonsecretor Lewis positive women (Se- Le+) have FUT3 only. Secretor Lewis negative women (Se+Le-) only have an active FUT2; Nonsecretor Lewis negative women (Se-Le-) do not express either of these Fucosyltransferases.

	Secretor positive (FUT2)	***Secretor negative (no FUT2)***
Lewis positive (FUT3)	Se+ / Lewis b antigen (a-b+)	Se - / Lewis a antigen (a+b-)
Lewis negative (no FUT3)	Se + / Lewis – (a-b-)	Se - / Lewis (a-b-)

Regarding fucosylation, Secretor Lewis positive (Se+ Le+) women have the most complex set of HMO, yet Nonsecretor Lewis negative women still present a considerable amount of HMO (concentration of several g/L) (3,8).

2.1.2.4.1 Secretor Status

Se gene encodes for α1-2-fucosyltransferase, FUT2. With an active Se locus the individuals are assigned to the Secretor positive group, with an inactive gene locus they are classified as Nonsecretors. Secretor women abundantly synthesise 2'-fucosyllactose and lacto-N-fucopentaose 1 (LNFP1) and other α1-2-fucosylated HMO such as Lactodifucotetraose (LDFT) and Lacto-N-difucohexaose (LNDFH), whereas Nonsecretor women lack these types of HMO completely due to the absence of the α1-2-fucosyltransferase as the only enzyme able to modify the HMO backbone accordingly (3,9,14).

2.1.2.4.2 Lewis Blood Group Status

The Le gene encodes for α1-3/4-fucosyltransferase, FUT3, the expression of which is entirely independent of FUT2 expression. The enzyme FUT3 links fucose in an α1-4-binding onto a subterminal GlcNAc in a type 1 chain, as a consequence a Lewis a antigen is expressed in Nonsecretors and a Lewis b antigen in Secretor women. In Lewis negative women who do not express FUT3 these epitopes are absent (Lewis a-b-) and α1-4-fucosylated HMO such as LNFP2 are not being synthesised (2,3,13).

The Le gene does not only encode for the fucosylation of HMO but also for blood group epitopes (see Figure 4).

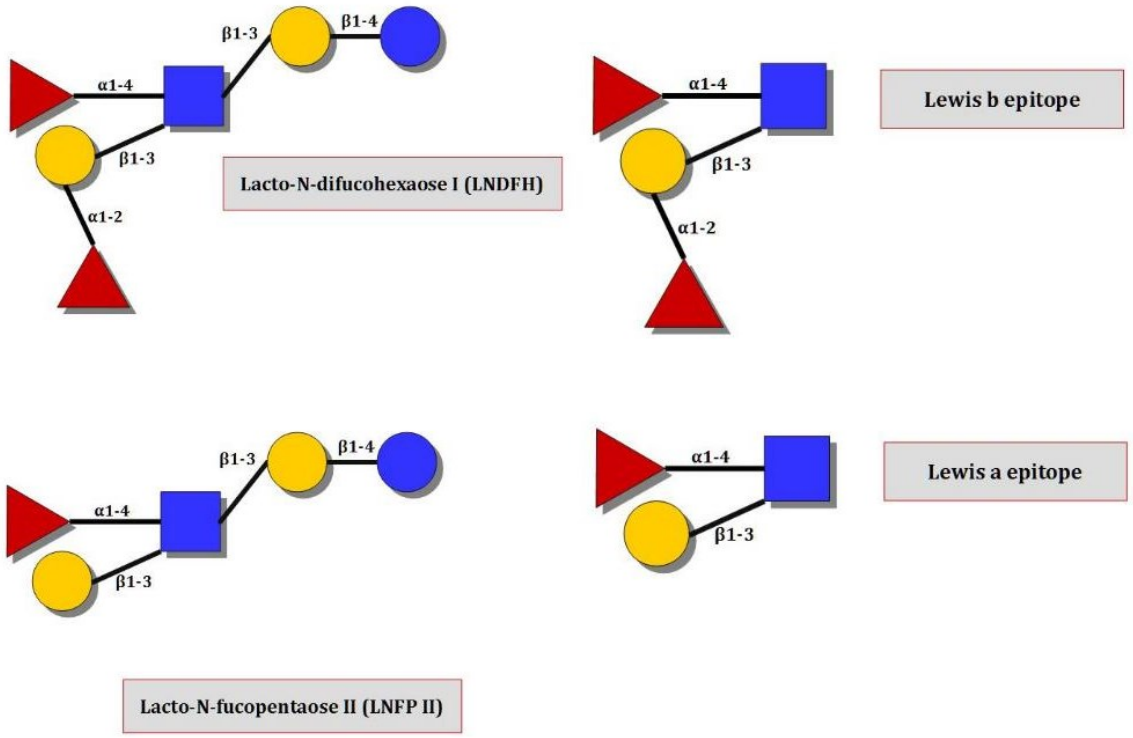


FIGURE 4. LEWIS BLOOD GROUP EPITOPES AND HMO COMPOUNDS. On the right hand side Lewis a and Lewis b epitopes are shown, on the left hand side LNDFH and LNFP2 as examples for HMO compounds with Lewis epitopes. LNDFH contains the Lewis (a-b+) epitope, LNFP2 contains the Lewis (a+b-) epitope.

However, these four groups are rather an oversimplification, considering that FUT2 and FUT3 occasionally compete for the same substrate, that Le-Se- women synthesise 3FL and LNFP3 regardless of the presence of FUT2 or FUT3, and that α1-2-fucosylated HMO are being found in Nonsecretors in terminal lactation stages, suggesting the existence of alternative, Se-Le-independent pathways of HMO fucosylation. A range of enzyme expression levels generates a continuous spectrum of HMO profiles in pregnant and lactating women (2,3,13).

2.1.3 Biosynthesis

HMO biosynthesis is not wholly clear but since all HMO carry lactose at their reducing end, it is hypothesised that it generally follows the lactose biosynthesis pathway beginning in the Golgi of lactocytes. The first molecule required is Glc, whereupon cytosolic Glc is activated to UDP-Glc. It is then converted into UDP-Gal, Gal being an epimeric form of Glc (different position of hydroxyl groups on anomeric carbon atoms) (8).

Following this step the UDP-Gal is linked to Glc in the Golgi by a lactose synthase

complex, consisting of two components. One part, the A protein, is a β-1,4-galactosyltransferase transferring UDP-Gal to a terminal GlcNAc during glycoconjugate synthesis. The second part is the lactation hormone dependent B protein, α-lactalbumin, which in combination with the A protein shifts its substrate specificity from GlcNAc to Glc in order to finally synthesise lactose (8).

However, with C13-labelled Gal taken up orally, the direct use of exogenous Gal to yield lactose was shown, a pathway where the detour of Gal being converted into Glc, following a UDP activation and finally a reconversion of Glc to Gal, is unnecessary (8).

It remains a challenging task in research to elucidate the exact biosynthesis of HMO, also due to the fact that milk oligosaccharide concentration and composition is extremely variable in different species and thus, animal models do only insufficiently function as means to understand the complex processes involved in HMO elongation and branching (8).

One hypothesis states that HMO elongation and branching is completed by lactose being extended by the alternating actions of N-acetylglucosaminyltransferases (GlcNAcT) and galactosyl-transferases (GalT) alternating actions, adhering to the scheme of poly-N-acetyllactosamine synthesis on glycoconjugates. Linear chain elongation might be initiated by iGnT (β-3-GlcNAcT) corresponding to the “i” blood group antigen, an antigen found on blood cells and widely distributed in human tissues. Chain branching might occur by IgnT (β6-GlcNAc-T), corresponding to the “I” blood group antigen. Type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc) disaccharide units might be produced by β3GalT and β4GalT, respectively. Nevertheless, however convincing this hypothesis might seem with the current knowledge of glycan synthesis, there is no evidence supporting it yet (15,16).

Now, whereas the basic biosynthesis of the various HMO backbones is not yet clarified, the modifications such as fucosylation and sialylation are fairly well understood. Fucosylation involves blood group synthesising fucosyltransferases that are genetically encoded and basically follow an all-or-none-law with some alternative ways around it, which however do not yield the same amount of fucosylation. Sialylation, on the other hand, knows much more degrees of modification (see above) (3,8).

2.1.4 Metabolism of HMO

For an HMO to be bioactive and exert certain physiological functions in the infant's gut – in contrast to being solely a nutritive carbohydrate –, there are some essential conditions to be met, such as indigestibility.

In studies in the late 80s and 90s, undigested HMO were isolated from the infant's faeces, showing evidence that, in fact, some HMO are to some extent resistant to degradation in the gastrointestinal tract and that nutrition of the infant is not their sole purpose. Later on, Engfer *et al.* showed indeed in an *in vitro* study the insusceptibility of HMO to hydrolysis in the upper intestinal tract, by using enzyme preparations of pancreas enzymes and brush border membranes, thus explaining why intact HMO can be found in faeces (7).

More recently established techniques helped separating and identifying even more HMO structures in breast-fed infants' faeces. When monitoring excreted oligosaccharides in faeces of the first 6 postnatal months, Albrecht *et al.* found that the oligosaccharide profile was subjected to constant changes. Shortly after birth acidic and neutral oligosaccharides identified in faeces were similar but not identical to the respective HMO profile of the mother's breast milk. In older infants the oligosaccharides found were closer to blood group determinants, the composition in faeces following a gradual change over time. The authors also discovered that these oligosaccharides completely disappeared as soon as solid food was introduced (17–19).

But intact and only slightly modified HMO can not only be found in faeces but also in urine of breastfed infants, in contrast to formula fed infants where no HMO can be found in urine. First demonstrated by Rudloff *et al*. in preterm infants, this suggests the absorption of HMO through the intestinal barrier into the systemic circulation, followed by renal excretion. This was proven in an *in vivo* C13-labelling study where C13-labelled Gal was orally administered to mothers. Approximately 10% of the isotopic carbon was found incorporated in breast milk lactose as well as HMO. After breastfeeding the infant, around 1% was found enriched in the infant's urine in compounds like fucosyl-lactose and lacto-N-tetraose (LNT) and Lacto-N-difucohexaose and LNFP (20). Hence, the argument that orally taken exogenous Gal is being integrated in HMO, which then reach the infant’s circulation with only minimal changes and rearrangements to it, was consolidated. The origin of HMO is supposedly the mammary gland, since lactocytes are the only known cell type capable of synthesising lactose. Thus, there are two possible hypotheses explaining the origin of structurally modified HMO found in infant urine. They may either derive

from human milk and be modified on their way, or, they might be synthesised completely anew from small precursors. These modifications and degradation products may be made by intestinal bacteria (21).

Since not all HMO are subject to intestinal degradation and some appear either unmodified or changed in urine, it was inferred that some HMO serve a functional purpose specifically based on their structural layout and the integrity thereof. In combination with *in vitro* studies showing effects of specific HMO, some of the following functions have been found. However, most of them are not verified *in vivo* (3,7,14).

2.1.5 Transcytosis and Permeability

Another question was to elucidate by which means the HMO in question were transferred, to urine for instance, without transformation. A most interesting finding was made by Gnoth *et al*. in an experiment with Caco2 monolayers. CaCo-2 cell lines are derived from colon carcinoma and are similar to small intestinal enterocytes when cultured under specific circumstances, showing a typical epithelial polarity (apical – basal) (22). The authors showed that both neutral and acidic HMO may cross epithelial barriers *in vitro*, results correlating very well to the *in vivo* study of Obermeier *et al*. (14,20,23).

Acidic HMO were shown to traverse epithelium, at least intestinal barriers, via paracellular ways. Remarkably, neutral HMO used paracellular pathways only to an estimate of 60% – the remainder of the neutral fraction was shown to use transcytotic pathways. The net flux of neutral HMO decreased when intracellular vesicle trafficking was inhibited artificially, and there was a satiable, stereospecific, apical uptake of HMO. Both indicate that neutral HMO most likely use receptor-mediated endocytotic transport, which was also found to have a sidedness, having the highest uptake on the apical side. Additionally, neutral HMO were not degraded intracellularly. For LNFP1 and LNT, the two major neutral HMO, the absorption followed Michaelis-Menten-kinetics, the model for enzyme kinetics. Acidic HMO seem to exclusively use paracytotic way of transport (14,20,22,23).

2.1.6 Function

One of the first effects of HMO discovered was their prebiotic effects on intestinal microbiota (2). But along with the discovery of structurally different HMO, more and more of their potential functions were unveiled. Many of the functions are a result of the specific biochemical structures of HMO (3).

2.1.6.1 Local effects

The most prominent site in the human body related to local effects of HMO is the gastrointestinal tract. As the infant ingests its mother's milk, HMO rinse the neonate's mouth, larynx, oesophagus, stomach, small intestine until they reach the colon as prebiotics for the gut microflora. These prebiotic effects were the first to be discovered and are still heavily investigated. So are the local antiadhesive effects, that HMO bring about to pathogenic intruders, blocking pathogen adhesion to epithelial surfaces by ligand mimicry in form of soluble decoys receptors (2,3,13,24). There is a number of local effects worth noting, since they open grounds for further hypotheses of how HMO might function on a systemic level in the mother.

Important for the hypothetical transition of the well-researched effects of HMO on infant gut microbiota to maternal effects, is the knowledge about the human microbiome. While over a hundred years ago the idea of humans being born sterile was widespread, in recent years the notion was solidified that various sites in the body possess distinctly different and thus site-specific microbiota. Even further, environmental influences and the pathways of cross-talk between microbiota and human host were discovered. While factors such as age, nutrition, morbidities, etc. influence the different organ-specific microbiota, hormonal changes, short- and long-term, were also found to alter the composition. Not only did “long-term” lasting changes such as puberty and menopause alter the commensal flora, but it was found that the microbiome was also subject to changes during pregnancy (5).

2.1.6.1.1 HMO as Prebiotics

2.1.6.1.1.1 Known effects on the infant gut Microbiota

The intestinal microbiota is believed to be central in a child's intestinal and immune development. Since the infant microbiota was discovered, its importance for the maturation of the infant's immune system has increasingly been appreciated.

The highly variable human intestinal microbiota consists of 10^{14} bacteria, as well as a plethora of other microorganisms such as viruses, fungi and archaea. While a foetus supposedly does not have any microbiota, the inoculation of the infant with these important, physiological intestinal bacteria starts at the very moment of birth, since already the mode of delivery, e.g. vaginal or caesarean, determines the pattern of colonisation the neonate acquires in the following. In the postnatal phase, the infant microbiota develops in contact with the environment until the child's approximately second year of life.

However, according to recent findings the infant's inoculation begins even before, and the maternal microbiota might play an important role already during pregnancy (25).

The importance of physiological functions of the microbiota is best seen in situations when there is a lack thereof. In axenic mice, i.e. germ-free mice, Macpherson *et al*. found hypoplastic Peyer plaques, a decreased number of intra-epithelial lymphocytes, deficient T-lymphocyte populations, a decreased intestinal IgA secretion as well as a low cytokine secretion, compared to their microbiota harbouring peers. Additionally, evidence for the beneficial effects for the immune system's maturation by microbiota was given, when this phenotype was rescued by inoculating the mice with the microbiota of the control mice population (26). Certain bacterial strains can influence intestinal homeostasis, based on the balance of T-effector and T-regulatory lymphocytes. Moreover, microbiota also influence the epithelial tight junctions, thus hindering pathogen invasion, and induce the production of antimicrobial peptides. Furthermore, the commensal gut flora can bind to epithelial receptors and compete for nutrients with pathogenic micro-organisms, thus suppressing their growth (26). Different microbiota species degrade distinct types of nutrients due to their specialised enzymatic tools. *Ruminococcus* and *Bifidobacterium* for instance use glycosides, Desulfovibrio uses hydrogen gas as nutrient and *Propionibacterium* and *Clostridium* for example degrade proteins from the intestinal lumen.

So, in consequence to their selectivity for nutrients, besides an important role played by our genetic background, food can form our commensal flora along with other exogenous factors described earlier such as delivery mode, pre- and probiotic consumption, degree of exposure to microbial and antibiotic treatments.

The most efficient composition of natural prebiotics, able to shape the intestinal microbiome, is found in human milk (27). Among the prebiotics in human milk, human milk oligosaccharides are some of the most important contributors forming the infant's intestinal flora. By being resistant to hydrolysis, enzymatic degradation in the upper GI tract, and gastrointestinal absorption, they meet basic criteria for functioning as prebiotics.
In the first months, the infant shows a milk-oriented gut microbiota that later transition to a more adult-like profile. In neonates, especially the species *Bifidobacterium* is observed. Already early in the history of HMO research, the then named "*Lactobacillus bifidus*" was discovered to uniquely grow on a HMO fraction. Humans do not possess the enzymes required for breaking down these specific oligosaccharide structures and so the free HMO in the intestinal lumen can be used up by *Bifidobacteria* species. When sequencing the genome of the *B. infantis*, an entire array of specific glycosidases, sugar transporters and glycan binding proteins has been discovered – and all of them for the purpose of HMO utilisation. In contrast, *B. bifidum* grow slower on an HMO substrate and instead of consuming HMO they leave behind degradation products. However, there are some *Bifidobacterium* subspecies that are even pickier: *B. longum subsp longum* and *B. breve* metabolise only LNT but not LNnT (Gal-β1-3-GlcNAc-Lac type 1 vs. Gal-β1-4-GlcNAc-Lac type 2; see Figure 5). *B. infantis* preferentially consumes smaller fucosylated and sialylated HMO (1).

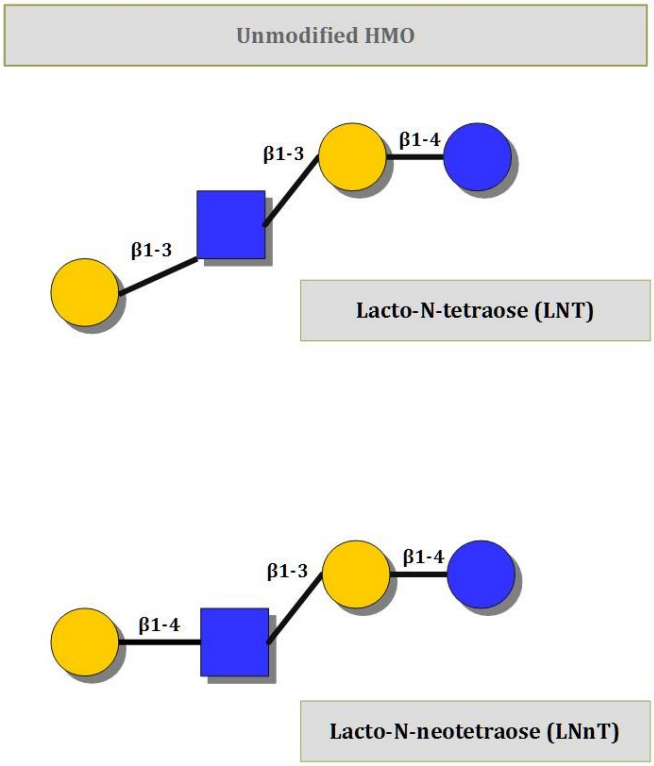


FIGURE 5. UNMODIFIED HMO. LNT and LNnT (Gal-β1-3-GlcNAc-Lac type 1 vs. Gal-β1-4-GlcNAc-Lac type 2. *B. longum subsp longum* and *B. breve* metabolise only LNT but not LNnT. *B. infantis* preferentially consumes smaller fucosylated and sialylated HMO.

There are some beneficial and anti-inflammatory functions of microbiota that are HMO-mediated, meaning HMO are influencing the infant's cells indirectly.

But not only the gut microbiome interacts with HMO, microbial commensal flora at many other sites in the human body are likely to be influenced by the prebiotic functions of HMO. A number of organs have been found to accommodate bacterial communities that are in this case organ-specific, opening even more possibilities of HMO – microbiome interaction on a systemic level (28,29).

2.1.6.1.1.2 Possible Analogous Pathways of HMO as Prebiotics Influencing the Maternal Microbiome of Genitourinary Sites, Placenta and Mammary Gland

Some of these sites harbouring beneficial microbes that may be influenced by circulating HMO, are for instance the genitourinary sites, since HMO are found intact in the urine. Other sites could be the placenta and mammary gland, where HMO may interact with the microbiota while present in systemic circulation in maternal serum.

2.1.6.1.1.2.1 Urinary Microbiome

Whereas bacteria were considered immanently pathogenic in the urinary tract which was long regarded as essentially sterile, researchers have recently identified the urinary microbiota to consist of slowly growing bacteria. They were found to be contrary to the fast growing, aerobic bacteria for which urine is commonly screened, explaining why the urinary microbiota was long undetected. The bacteria identified in multiple studies were all found to be distinctly different from the vaginal or intestinal flora, meaning that the urinary microbiota is organ-specific and not a contamination of the neighbouring colonies. Possible functions attributed to the urinary microbiota are bacteria-produced neurotransmitters, regulation and maintenance of epithelial junctions, production of antimicrobial compounds, priming epithelial defences, as well as developmental and immunological functions (29).

Different studies suggest the existence of a ‘core microbiota’ consisting of various genera found across different age groups. The genetic determinant of microbiota is supported by clinical evidence that children of women with recurrent UTIs are themselves target to a history of UTIs and have altered microbiota compared to healthy individuals. Hosts may be expressing some receptors that enable better adhesion of specific bacteria (28–30).

However, not only genetic factors may influence the urinary microbiota, but also other factors could be identified. For instance, significant differences between the microbiota of women and men could be found, as well as sexual activity seems to be a determinant for the composition of the individual’s urinary microbiota. When comparing the genera of different age groups, significant changes in the composition could be found. In women especially, this also means the influence of hormonal changes as occurring during puberty and after menopause. The fact that UTIs are more common in postmenopausal women, is

another indicator of the susceptibility of the urinary microbiota to hormonal changes. This may also apply for the hormonal changes occurring during pregnancy (28–30).

Dietary factors may also influence the urinary microbiota – bacteria are capable of interacting with exogenous organic and anorganic compounds, altering thus the risk for diseases (29). Along this line, a possible influence of HMO on the urinary commensal flora, which have been shown to interact with intestinal microbiota, seems likely.

Intestinal microbiota has been shown to influence urolithiasis, and although urolithiasis does not occur more often in pregnant women, it is the most common reason for nonobstetrical abdominal pain. Up to eighty percent of the stones pass spontaneously with conservative treatment, however a persistent calculus may initiate premature labour and interfere with the progression of normal labour, and cause urosepsis on grounds of an UTI (31).

HMO are known to interact with gut microbiota, thus interaction with urinary microbiota is conceivable, possibly protecting the pregnant woman from urinary infections and urolithiasis by enhancing urinary microbiota colonies. *Bifidobacterium* species were characterised as part of the urinary microbiome by different studies (31). At the same time extensive data about the influence of HMO on *Bifidobacterium infantis* and other strains exist, for instance increased adhesion of HMO-grown *B. infantis* to the host tissue (32).

2.1.6.1.1.2.2 Vaginal Microbiome

As the urinary microbiome appears to be site-specific and not a product of contamination by adjacent sites as the vagina and rectum, so is the vaginal microbiome unique and different from gut and urinary tract. The vaginal microbiome is very diverse within individual women, but is also highly variable among different healthy women. *Lactobacillus* is one of the core species occurring in the vaginal flora. It metabolises glycogen to lactic acid, thus creating a physiological acidic environment preventing pathogen colonisation.

One of the conditions that may result from a shift in the vaginal microbial communities, is bacterial vaginosis (BV). BV affects 10-15% of women of reproductive age and is also associated with pregnancy complications, including preterm birth, chorioamnionitis, and intrauterine growth restriction. (33) In BV, an overgrowth of other non-commensal bacteria produces noxae like polyamines, that trigger pro-inflammatory cytokines such as IL-1β and IL-8 to be released (34). However, probiotics administered vaginally but not orally reduced the recurrence of BV (35,36). A Cochrane systematic review showed efficacy of various probiotic treatments to reduce the risk of vaginal infection (37).

But the "vaginal microbiome" is not a stationary state of a certain number of bacteria; they too, are subject to changes throughout a lifetime, influenced by both exogenous and endogenous factors. There is some evidence that vaginal microbiota is different in various ethnicities and that the host genotype influences composition of vaginal microbiota. Other environmental factors such as age, hormone status, are not yet investigated to have an influence. However, the vaginal microbiota composition is known to change significantly during pregnancy (5,38).

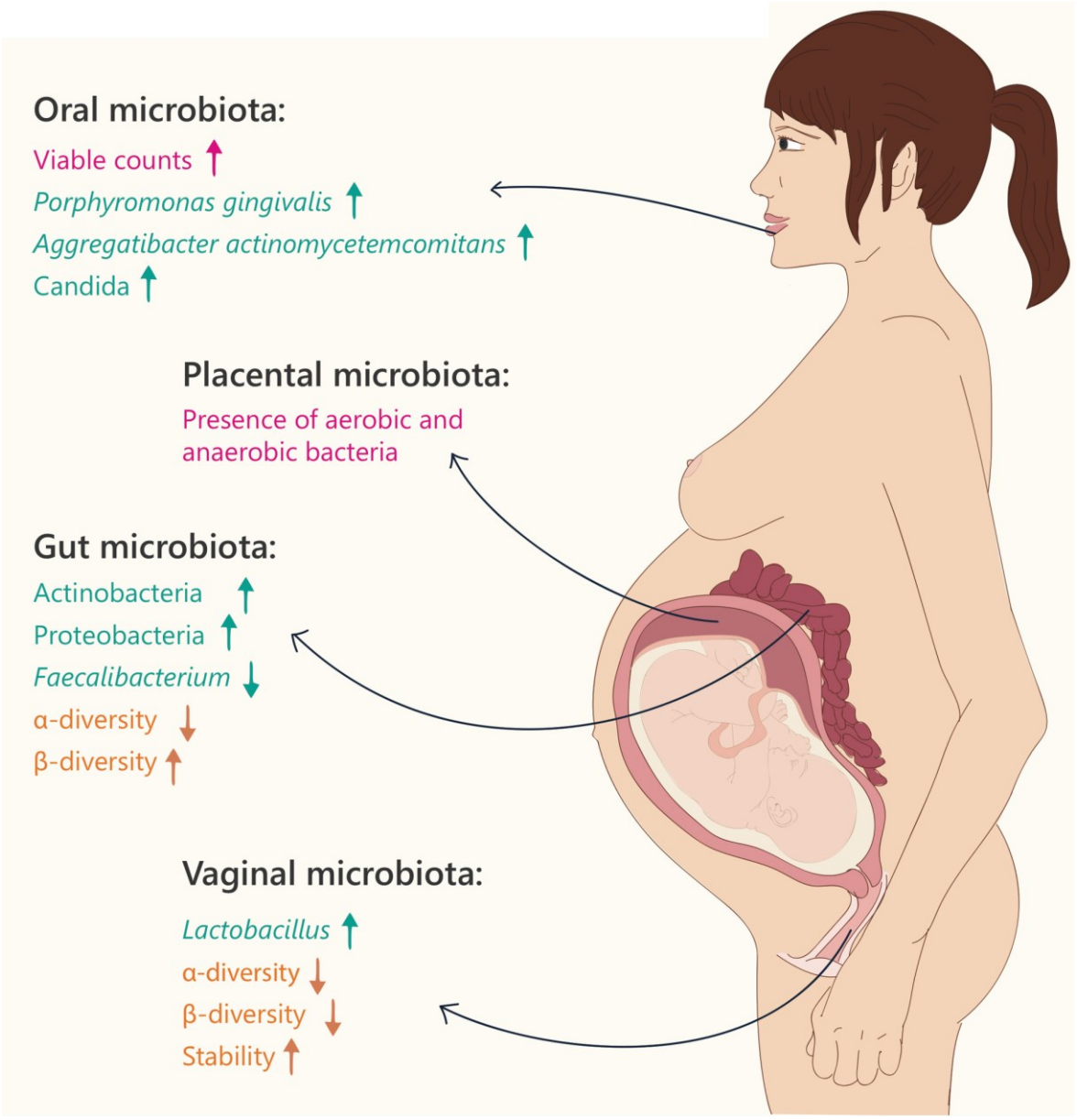


FIGURE 6. CHANGES IN THE MATERNAL MICROBIOME DURING PREGNANCY. General changes (pink), changes in specific taxonomy (green) and community diversity (orange). α-diversity refers to diversity within an individual, β-diversity to diversity between individuals. (Figure from Nuriel-Ohayon *et al*. 2016 (5)).

The vaginal microbiome, comprehensively investigated in pregnancy across subsites and gestational age, is uniquely structured compared to non-pregnant controls. Overall, it is less rich and less diverse. Changes could not be accounted for by other factors (38), and thus these changes may be an additional protecting factor against ascending infections originating from bacterial vaginosis or trichomonas infections and resulting in cervical inflammation, a risk factor for preterm labour (39). Haahr *et al*. reported further evidence that vaginal microbiome plays a more important role in pregnancy, suggesting that an abnormal vaginal microbiome may limit IVF success (40). Considering hence the significance of the integrity of the microbiome, in this case, too, a protective factor specific for pregnancy seems plausible and therefore, the influence of HMO, perhaps via vaginal secretion or by being stored in the uppermost mucosal layer which is then shed and metabolised by bacteria, is well thinkable.

2.1.6.1.1.2.3 Placental Microbiome

As these current paradigms suggest that most intrauterine infections associated with preterm birth originate from urogenital bacteria contaminating the sterile intrauterine environment, it was also found that there is a link between women with periodontitis and preterm delivery. Several species of the oral microbiome were detected in the placenta, indicating a possible haematogenous transfer of bacteria to the placenta. Agaard *et al.* discovered then, that the placenta has a unique microbiome, distinctly different from the urogenital commensal flora. Some of the oral microbes found in the placenta, are capable of binding to vascular endothelium and alter permeability, possibly functioning as a prerequisite for facilitating further haematogenous transmission of other commensals to the placenta (25). The placental microbiome is a low-abundance microbiome, nonetheless having a significant microbial metabolical output. As for the possible effects, it was demonstrated that placental microbiome is associated with preterm birth <37 weeks, and it was also linked to be proportionately associated with the interval week of delivery (25). A link of placental microbiota to antenatal infection was made as well. A significant alteration was found in women with chorioamnionitis, having a higher abundance of urogenital and oral commensal bacteria, accompanied by a variation of microbial metabolic pathways. Affected metabolic pathways, comprised amongst amino and nucleotide sugar metabolism, also the butanoate metabolism. This is particularly interesting since butyrate has been shown to reduce intestinal inflammation (41). Moreover, the placental microbiome showed a significantly lower diversity in low-birth weight infants compared normal-birth weight infants (42).

2.1.6.1.1.2.4 Mammary Gland Microbiome

The human mammary gland microbiome, similarly to the placental microbiome, is potentially created by migration of the microbes from other sites and partly via contamination from the skin (43). It is also highly interesting in terms of HMO interaction. Here, not only HMO may influence the microbiota directly, but, in turn, the microbiota might influence the cells in the mammary gland and thereby the hypothesised local synthesis of HMO in the lactocytes.

In a study of Cabrera-Rubio *et al*., who investigated the human milk microbiome, a range of interesting findings were shown. The human milk microbiome changes over lactation; in colostrum, a different spectrum of species is found which is more site-specific than later when the microbial flora is made up of an increasing number of microbes typically found in the oral cavity. The authors found that the microbiome is changing over time, but also that maternal factors are influencing the composition of this commensal flora. Obese mothers tend to have a less diverse bacterial community in milk compared to normal-weight mothers, as well as women undergoing electively a caesarean delivery. These women, showing some risk factors during pregnancy such as macrosomia of the child, had different colonisations than women who underwent non-elective C-sections or vaginal deliveries. The latter is suggesting that not the operative intervention per se influenced the microbial composition in milk, but rather the absence of physiological stress or hormonal signals, as found at delivery and during labour (44).

Urbaniak *et al*. found microbiome in breast tissue samples, ranging from the nipple to the chest wall, pointing towards a more expansive concept of breast microbiome not only restricted to the mammary gland. (43,45)

2.1.6.1.2 HMO Acting as Antiadhesives

Contrary to these beneficial and commensal bacteria, pathogens can be detrimental to the host. Pathogenic microbes achieve their virulence and cause impairment to the host by using strategies such as colonisation in a bio-niche in the host, immunoevasion, immunosuppression, adhesion, invasion, and obtaining or rather withdrawing nutrition from the host. The mechanisms beneath the entities of virulence depend to a great part on glycan patterns and glycan-binding receptors found in the pathogen and in the host cell (46).

Mucosal epithelial surfaces are covered with glycans, glycoproteins, glycolipids, glycosaminoglycans, mucins and others – called the glycocalyx. Intestinal mucosa, for instance, is one of the most glycosylated tissue in the human organism. Besides multiple other biological functions, these glycans also serve as the first barrier towards the outside. Most interestingly, HMO and human epithelial glycans are synthesised by similar glycosyltransferases and thus, have similar epitopes.

A copious amount of viral, bacterial and protozoan pathogens exploits these host cell-surface glycans as a possibility for cell attachment and subsequent tissue colonisation via glycan-binding proteins (GBPs) they express. These glycan-binding proteins expressed by pathogenic microorganisms, can be pathogen cell-surface bound (adhesins, haemagglutinins) or secreted proteins (heat-labile toxins). Adhesins for instance, bind to a host cell surface ligand, designated as receptor, that is often a terminal or internal carbohydrate motif inherent to some glycocalyx components (47). Many adhesins contain carbohydrate-recognition domains (CRDs) using the same domains as mammalian lectins, and thus their structural homology creates overlapping in their ligand selectivity. Like animal cell lectins, some microbial adhesins bind to terminal sugar residues, whereas others bind to internal sequences found in linear or branched oligosaccharide chains (47,48).

This lectin interaction is structure specific, i.e. specific lectins can only bind to structurally suitable host ligands (13,47). Some lectins require their ligands to be sialylated, such as *E. coli* with S fimbriae as well as *H. pylori*. Other lectin-glycan-interactions require fucosylated host ligands. For example, *E. coli* with type 1 fimbriae binds to mannose-containing glycans. Different pathogens use different lectins that each adhere to specific cell-surface glycans (47).

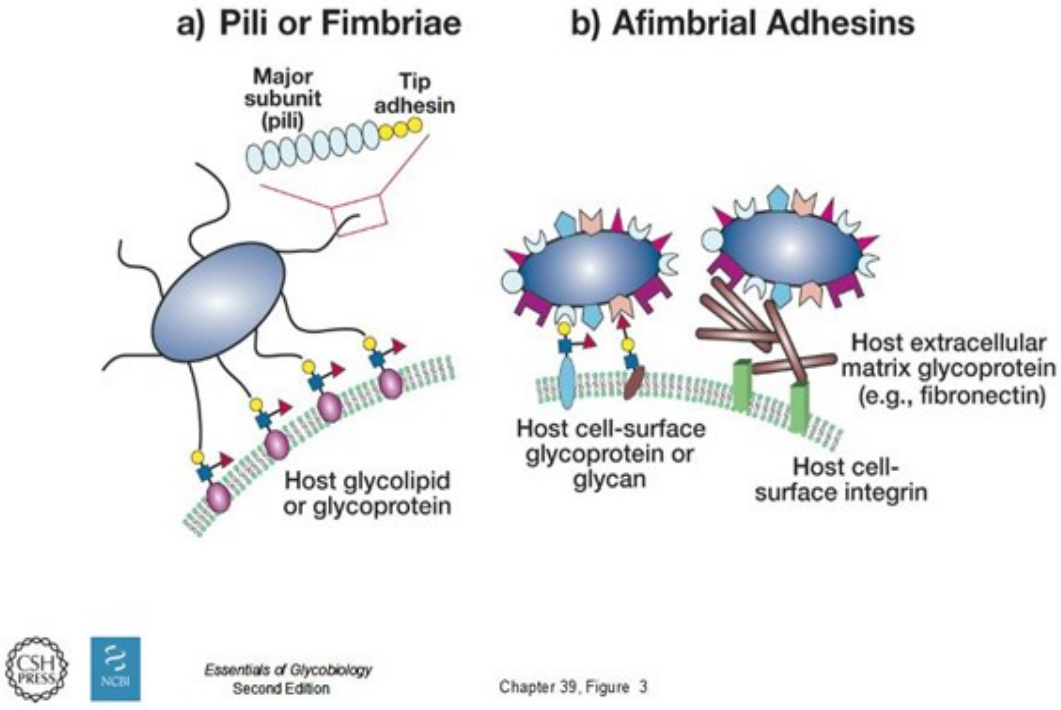


FIGURE 7. EXAMPLES OF MECHANISMS OF BACTERIAL ADHERENCE TO HOST-CELL SURFACES. (**A)** Adhesion mechanisms mediated by pili and fimbriae are shown. (**B)** Pathogen adhesion by afimbrial adhesins. Saccharide structures schematically shown: N-acetylglucosamine (blue square), Fucose (Red Triangle), Galactose (Yellow circle). (Figure from *Essentials of Glycobiology, Second Edition* (47))

When an organ-specific glycan epitope, which a pathogen can bind via its lectins, is mimicked by a soluble glycan, the soluble glycan can competitively inhibit the pathogen lectin and consequently make it unavailable for further binding to host cells.

This way HMO can act as a soluble decoy receptor for various microbes, hence blocking adhesion and subsequently reducing infection and disease in the host. A single HMO structure cannot block many pathogen lectins at once, and this structure-specific way of the lectin-glycan-interaction might explain the wide variety of different HMO, possibly selected throughout evolution while keeping pathogenic invaders and their toxins at bay. Epidemiological data display distinct beneficial effect of breastfeeding in infants in terms of infectious diseases, backing the hypothesis of HMO being antiadhesives *in vivo*.

2.1.6.1.2.1 The role of different HMO modifications in antiadhesive effects

Fucosylation

Some of the most consistent data on antimicrobial HMO in relation to bacterial diarrhoeal episodes is available for *Campylobacter jejuni* infections in infants, being the major cause for diarrhoea in infants in developing countries. The risk of campylobacter diarrhoea was found to be inversely related to the concentration of 2'FL in obtained milk samples, indicating a protective effect of this specific HMO. *C. jejuni* normally would bind to the H-2 epitope expressed on the surface of human intestinal epithelium, but 2'FL also contains this H-2 moiety and thus inhibits binding to the regular target epitope of *C. jejuni*. The structure in question is an α-1,2-linked oligosaccharide, and could also be found to be responsible for the absence of symptoms in an enteropathogenic *E. coli* infection in children who consumed high concentrations of 2'FL or 2'-fucosylated HMO with breastmilk. Also, Calicivirus diarrhoea showed a decreased incidence when milk contained high levels of lacto-N-difucohexaose (LNDFH), another α-1,2-fucosylated HMO. Such glycan-mediated attachment mechanisms are also used by Norovirus and Rotavirus, the most common cause of viral diarrhoea in children. There is *in vitro* evidence that LNDFH-1, a fucosylated HMO containing a Lewis b moiety, binds to norovirus. The level of LNDFH-1 is inversely linked to the risk of norovirus-associated diarrhoea in breastfed infants (49).

The total concentration of α-1,2-linked fucosyloligosaccharides in milk samples were inversely related to a risk of diarrhoea in breast-fed neonates of a variety of causes (50).

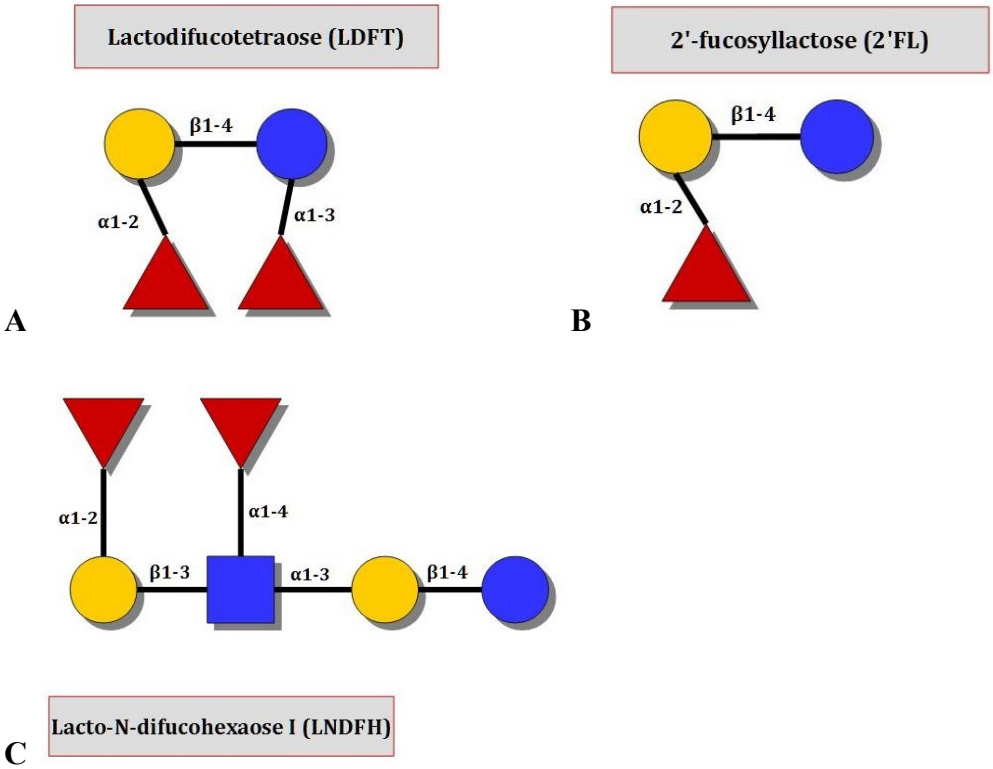


FIGURE 8. α-1,2-FUCOSYLATION IN HMO IS BLOCKING PATHOGENS ASSOCIATED WITH DIARRHOEA IN INFANTS. *Campylobacter pylori*, *Escherichia coli*, and Calicivirus are blocked by HMO containing an α-1,2-fucosylation, such as **(A)** LDFT, **(B)** 2'FL, **(C)** LNDFH.

However, α-1,2-fucosylation is not the key to inhibit all of the pathogens common in neonatal infections.

For some specific pathogens the fucosylation even masks this beneficial effect of HMO, as the protozoan pathogen *Entamoeba histolytica* for instance which is the third leading cause of deaths from parasitic infections. *E. histolytica* employs a glycan-binding protein as a major virulence factor. This lectin is also implicated in the subsequent killing and phagocytosis of the infested host cells. The Gal/GlcNAc lectin used by *E. histolytica* to attach to the host's epithelium uses Gal, GlcNAc and lactose as binding sites – and consequently these oligosaccharides were shown to block the pathogen attachment *in vitro*. A study by Jantscher-Krenn *et al*. found that HMO had a protective effect on intestinal cells against *E. histolytica*. Unmodified lacto-N-tetraose (LNT) displayed inhibiting effects on attachment and cytotoxicity. LNFP-1 had no effect, but most interestingly, LNFP 2 and 3, which are also monofucosylated LN(n)T isomers such as LNFP-1, had an effect. The crucial difference, highlighting the importance of structural properties in antiadhesive

effects of HMO, was that LNFP-1 carries an α-1,2-fucosylation at the terminal Gal and hence masked the imperative terminal Gal needed for E. histolytica to bind (see Figure 9). This is in contrast to LNFP2,3 carrying fucose residues in an α1-4 and α1-3 linkage, respectively, at the subterminal GlcNAc. Thus, LNFP 2 and 3 with their intact terminal Gal can act as soluble decoy receptors. This is another example of how specific the effects of the different HMO can be regarding antiadhesives functions.

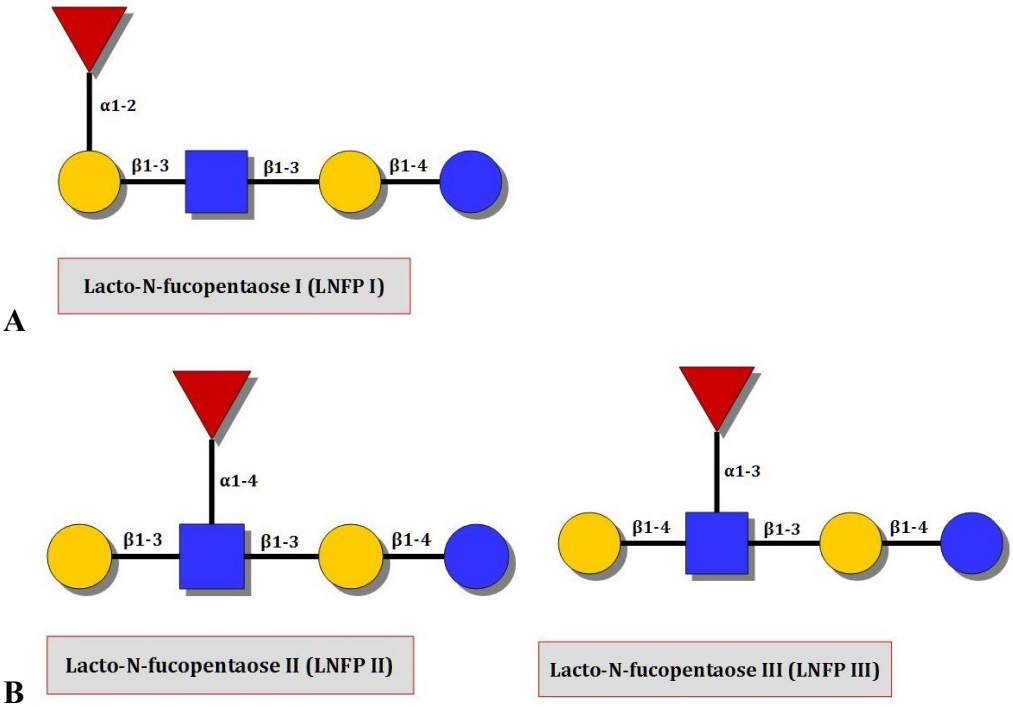


FIGURE 9. LNFP II AND III WITH UNMASKED TERMINAL GALACTOSE ACT AS SOLUBLE DECOY RECEPTORS. Structural properties are crucial in antiadhesive function of HMO; **(A)** LNFPI carries an α-1,2-fucosylation at the terminal Galactose, hence masking the terminal galactose for *E. histolytica* to bind. **(B)** LNFP II and III have fucose residues in α1-4 and α1-3 linkage from the subterminal N-acetylglucosamine, leaving the structure imperative for pathogen-binding intact.

Sialylation

Sialylation is another modification of the HMO backbone, altering the basic structure in various places. Sialylated HMO for instance can inhibit enterotoxic *E. coli* attachment, as well as uropathogenic *E. coli* attachment. Martin-Sosa *et al*. found that the specific adhesins of uropathogenic *E. coli*, UPEC, and enterotoxic *E. coli*, ETEC, agglutinating erythrocytes, could be inhibited by HMO. However, when the HMO fraction was desialylated, higher quantities of neutral HMO were needed for the same effect of inhibition. 3'SL, 6'SL, LSTa, LSTb, LSTc and DSLNT showed a significant effect on the inhibition of *E. coli* strains commonly found in humans (51).

Unmodified HMO fraction as antiadhesives

Infection with enterotoxic *E. coli* is a dominant aetiology for diarrhoea, its pathogenesis is associated with two toxins, the labile and (heat) stable toxin. In an *in vivo* experiment, suckling mice were fed the stable toxin with or without HMO, respectively. In the mice fed HMO, the lethality of the secretory diarrhoea induced by the *E. coli* toxin was significantly lower than in those without HMO, concluding that neutral HMO had an inhibitory effect on stable toxin (13).

2.1.6.2 Systemic Effects – HMO and Inflammation

2.1.6.2.1 HMO-Grown Probiotics – Indirect Immune Modulation

HMO rinse the neonatal mucosal epithelia of the intestine and laryngopharyngeal regions and contribute to immunity of the still immature and immune incompetent infant (1).

HMO may affect characteristics of certain probiotics and thereby indirectly modulate the host cells. When certain probiotics were grown on HMO, they showed different characteristics and induced changes in host's gene expression, other than bacteria not grown on HMO as substrate.

One of the mechanisms which involve HMO, is the substrate-driven selection of bacterial commensals on mucosal tissue. HMO-grown *B. infantis*, for example, showed significantly increased adhesion to intestinal epithelium than *B. infantis* grown on other substrates; These HMO-grown *B. infantis* strains showed changes in the transcriptome in comparison to other non-HMO-grown strains (52).

In turn, again depending on which substrate they were grown on, *B. infantis* also differentially affected the intestinal epithelial cells attached to, changing the interaction of microbiota and their host, and the host's immune responses. These commensal bacteria strains can induce the anti-inflammatory capacities of the epithelial cells, reduce markers of inflammation as well as the production of proinflammatory IL-17 and interferon-γ (53). *Bifidobacteria* altered gene expression of intestinal epithelial cells and increased the level IL-10, an anti-inflammatory interleukin while further decreasing TNFα levels, as well as tightened cell-junctions, and enhanced the epithelial barrier further. HMO-grown microbiota also attenuated lipopolysaccharide- and IL-1β-induced IL-8 and IL-6 expression and decreased TLR 2 and 4 signalling (1). They also decreased ICAM1 expression, an intracellular adhesion molecule and cell surface glycoprotein which is typically expressed on endothelial cells and immune cells (54). ICAM1 is known to be highly expressed in inflammatory bowel diseases such as Crohn's disease and Ulcerative Colitis.

In summary, the expression of an array of inflammation related genes was downregulated in epithelial cells via HMO-grown *Bifidobacteria*, linking them to a modulatory role in inflammation (53).

HMO may not only increase the adhesion capacity of beneficial bacteria, while reducing those of pathogenic intruders, but also modulate bacterial functions on the host, indirectly affecting host immunity.

In UPEC (uropathogenic *E. coli*), the primary cause for urinary tract infections (UTIs), HMO alters gene expression of the pathogen leading to less adhesive capacity (55).

2.1.6.2.2 HMO Modulate the Cell Directly

After realising that HMO not only cross the epithelial barrier but also enter into epithelial cells, it was not long before it was discovered that they can also directly interact with intestinal epithelial cell transcriptomes.

HMO have been identified as agents to alter gene expression of epithelial cells, modulating cell apoptosis, proliferation and differentiation and changing the glycocalyx (53,56,57). Local mucosal-associated lymphoid tissues, MALT, may be one of the targets of HMO-immunomodulation, but systemic lymphoid tissues and other cells may be subject to direct modulation already in pregnancy, since unpublished data shows HMO circulating in maternal serum and foetal cord blood. Postnatally, 1% of intestinal HMO is absorbed and circulating systemically in the infant (23). HMO can also modulate cell responses, such as reducing cell growth and inducing differentiation and apoptosis via modification of cell cycle genes that are growth-related. EGFR and Ras/Raf/ERK signalling might play a role (57).

2.1.6.2.2.1 Possible Pathways Transducing HMO-mediated effects

At this point, the signalling pathways and receptors by which HMO-mediated effects may be transduced are unknown. However, due to their distinct structural similarities to receptors/ligands present in human cells, it is inferred that HMO possibly interfere with these immuno-modulatory processes via their similar structure (8). Possible receptors or interaction partners involved in HMO-induced cell responses are the following:

Siglecs: Siglecs are sialic-acid binding Ig-like lectins, cell surface receptors involved in the immune system. Siglecs recognise sialic acids and bind to terminal α2-3 and/or α2-6 bound sialic acid, as present on HMO, and have also been shown to bind sialylated HMO (58,59).

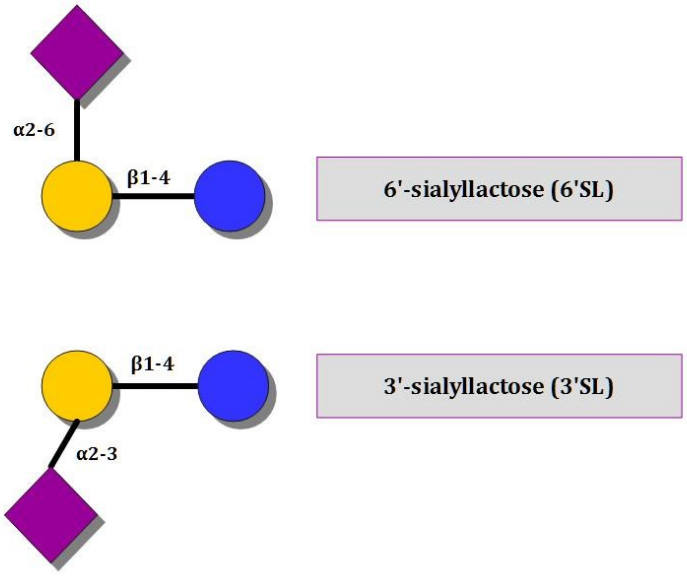


FIGURE 10. SIGLECS RECOGNISE AND BIND TO TERMINAL α2-3 AND/OR α2-6 BOUND SIALIC ACID. Examples of HMO with the respective binding of sialic acid are for instance 3'SL, which has α2-3 bound sialic acid on its terminal galactose, and 6'SL with an α2-6 bound sialic acid.

Galectins: are galactoside-binding lectins, that bind β-galactoside and poly-N-acetyllactosamine-enriched glycans (46). The HMO backbone is formed by these poly-N-acetyllactosamine chains. HMO are β-galactosides often containing β-1,3- or β-1,4-linked Galactose at their non-reducing end, thus being potentially an agent in galectin-mediated processes. Galectins function in cell growth regulation, proliferation, and apoptosis, as well as cell-cell and cell-matrix interactions (8,59). They also modulate immune responses.

Selectins: Selectins are C-type lectins and cell adhesion molecules that mediate leukocyte trafficking and platelet-neutrophil complex formation. Selectins bind to glycans carrying the sialyl Lewis blood group epitope. These sialyl Lewis epitopes are sialylated and fucosylated lacto-N-biose or N-acetyllactosamine, a characteristic also shown by some HMO and encoded for genetically in the mother. The fact that HMO possess Lewis blood group antigens makes them eligible for reducing selectin-mediated cell-cell interactions, such as leukocyte extravasation or leukocytes binding platelets – both selectin-type mediated actions, important for inflammation responses (46,60).

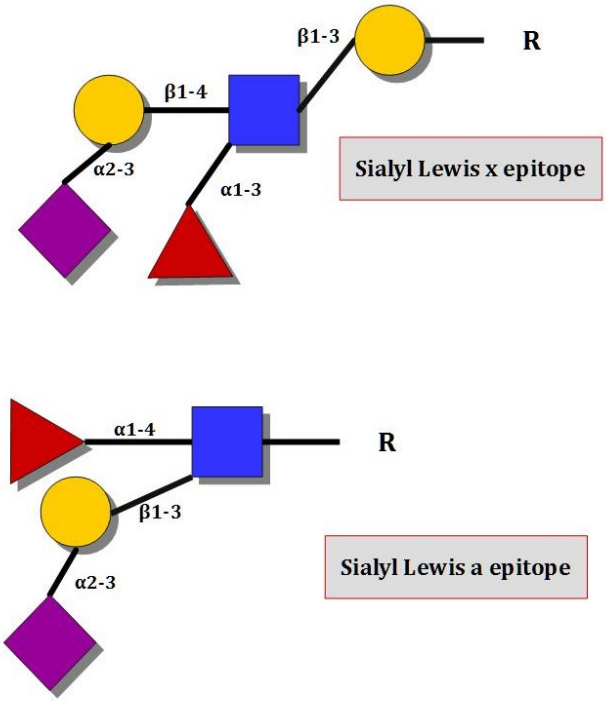


FIGURE 11. SIALYL LEWIS EPITOPES. Selectins bind to glycans carrying the sialyl Lewis blood group epitope, which are sialylated and fucosylated lacto-N-biose or N-acetyllactosamine. Some HMO possess Lewis blood group antigens, rendering them eligible for reducing selectin-mediated cell-cell interactions, such as leukocyte extravasation or leukocytes binding platelets. (R= Residue).

2.1.6.2.2.2 HMO decreases sialyltransferase gene expression – UPEC and 3'SL

3'SL treatment of urothelial cells diminished the gene expression of sialyltransferases (ST3Gal1, -2, -4). Thereupon, the cell surface sialylation of glycans is altered, impeding the binding of pathogens such as UPEC which requires sialic acid and lactosamine as key ligands. HMO-treated urothelial cells showed a modest decrease in UPEC attachment and the internalisation of the pathogen was significantly diminished. HMO suppressed apoptotic cell signals, making them unsusceptible to the cytotoxic efforts of UPEC to kill invaded cells (55). Since UTIs are more common in pregnant women, being among the most common bacterial infections in pregnancy and *E. coli* accounting for 80–90% of the UTI causes, the protective effect of excreted HMO against UPEC seems highly plausible. Nowadays, with a simple but effective antibiotic treatment at hand maternal UTIs very rarely affect the child directly and prognosis is excellent. Nonetheless, UTIs may have severe complications with a risk of maternal septic shock, respiratory failure, hypotensive hypoxia and subsequently a risk for the foetus' cerebral hypoperfusion via uterine hypoperfusion due to maternal dehydration and anaemia, and direct bacterial endotoxin damage. Long-term risks for the child are low birth weight, prematurity, pre-eclampsia and

amnionitis (61). When considering HMO-driven protection in the light of human evolution, it seems evident to establish self-defensive mechanisms against such severe risks for the mother and foetus.

2.1.6.2.2.3 Dendritic cells and HMO interaction – 3'SL is sensed by TLR4 and interacts with DC-SIGN

Dendritic cells (DC) and macrophages sense luminal antigens and then communicate their finding: they can either signal for tolerance, when for instance commensal bacteria are recognised, or they can induce inflammatory responses.

Intestinal DCs for example can be characterised by their expression of a specific integrin (αE, a ligand for E-Cadherin on epithelial cells) and a chemokine receptor (for leukocyte adhesion and migration). DCs expressing this integrin are the main population of migratory intestinal DCs influencing regulatory T cells. Those DCs expressing the chemokine receptor are the ones resident and initiating local immune responses, when sensing pathogen-associated molecular patterns via their Toll-like or Nod-like-receptors (TLR, Nod) for instance.

Most interestingly, a specific Toll-like receptor, TLR4 – which is normally scanning for pathogen associated molecular patterns, PAMPs, such as lipopolysaccharides – was shown to sense 3'SL, a trisaccharide HMO. When investigating the role of 3'SL interacting with dendritic cells, a number of findings were made in experiments involving knock out mice, all strongly arguing for a pro-inflammatory role of 3'SL (62). Mesenteric lymph node dendritic cells (CD11c+) were therein stimulated with 3'SL, which induced the production of cytokines required for Th1 and TH17 T cell induction.

Th1 cells are T helper cells controlling bacterial and protozoan threats, whereas Th17 cells produce IL-17, a pro-inflammatory cytokine recruiting monocytes and neutrophils to sites of inflammation, maintaining mucosal barrier. However, IL-17 is also implicated in autoimmune and inflammatory diseases.

An increase in pro-inflammatory monocytes, granulocytes, T cells and leukocyte infiltration was observed upon 3'SL exposure. Yet, 3'SL and 6'SL had no direct impact on leukocyte populations and IgA secretion (63).

Upon further investigation of the inflammatory profiles after 3'SL exposure of mice, increased levels of IFN-γ, and increased levels of TNF-α, IL-17 and IL-12 were observed, indicating that 3'SL exposure increased multitude of pro-inflammatory factors. The cytokines elevated are part of a strong inflammatory response. IFN-γ is a cytokine usually

released by Th1 cells after contact to antigen-presenting macrophages or secreted by dendritic and NK cells in order to stimulate natural cellular defence mechanisms. IL-12 is mainly produced by antigen activated DCs (62).

In another study, 3'SL was shown to raise susceptibility of mice to acute colitis, and to increase severity of the dextran sulphate sodium induced colitis (DSS is used to experimentally induce colitis) (63). The pattern of inflammation markers implied that the intestinal inflammation induced by 3'SL was Th1/Th17 driven, a T helper innate immune response that generally occurs in response to intracellular pathogens (64). But an increased IL-4 expression also indicated a Th2-mediated control of the inflammation, being the chronic response to Th1-inducing pathogens. In all this, the structurally very similar 6'SL was not observed to be causative for any immune response recorded. In conclusion, 3'SL was found to be a very immunoactive pro-inflammatory agent, possibly having a long-lasting effect on mucosal immunity (63). When trying to sort these findings into a comprehensive concept why a molecule, that is part of the natural nutrition and prior to that even courses through the maternal-foetal circulation, could have such extensive pro-inflammatory effects, one of the explanations may lie in the structural similarity of 3'SL and sialic acid. Sialic acid is a common PAMP, found on *C. jejuni*, *H. influenzae, N. gonorrhoeae*, etc. *Campylobacter jejuni* derived oligosaccharides containing α-2,3-sialic acid (as in 3'SL) induce for instance Th2 responses in Siglec-expressing dendritic cells, and 3'SL also bind *H. pylori*, indicating that 3'SL is a ligand for the microbial lectin present on this specific ulcerogenic bacterium (62,63). With such close resemblance for PAMPs, 3'SL may be priming the immature innate immune system via human milk, and perhaps even *in utero* via serum HMO, as though staging a practice for the real emergency ensuing once the foetus is born (62).

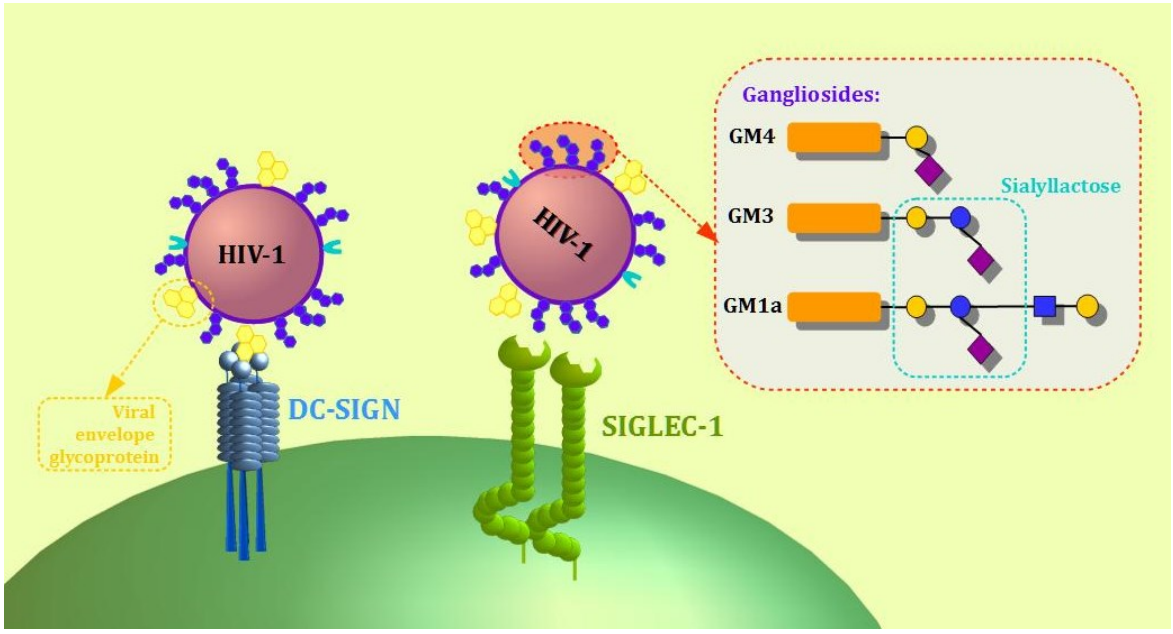


FIGURE 12. DC-SIGN AND SIGLEC-1 INTERACT WITH HIV-1. (A) HIV-1 can bind to DC-SIGN via the viral envelope glycoprotein. DC-SIGN has a high affinity to Lewis blood group antigens, and some HMO possess Lewis epitopes. Thus the interaction of HMO/DC-SIGN might explain the lower rate of HIV transmission in breastfeeding women. **(B)** The HIV-1 lipid membrane has gangliosides which expose a Sialyllactose moiety (GM3, GM1a). SIGLEC-1 can bind to Sialyllactose and can thus interact with HIV-1 (65), but may also interact with HMO.

Dendritic cells and HMO have further noteworthy interactions. DC-SIGN (dendritic cell specific ICAM3-grabbing Non-Integrin) is found on dendritic cells in mucosal surfaces, screening the lumen for pathogens and then presenting the caught antigen to other immunocompetent cells. Some of the pathogens that bind to DC-SIGN are hepatitis C, Ebola, CMV, Dengue virus, Mycobacterium, *Candida albicans* and HIV-1. HIV-1 for example uses glycoprotein gp120 to bind to DC-SIGN (see Figure 12a), a receptor with a high affinity for Lewis blood group antigens. This trisaccharides, Lewis x, is part of some HMO in women who are Lewis positive (encoding for the Lewis blood group epitope). The structural similarity of the ligands brings to mind that there, in fact, may be an HMO/DC-SIGN interaction (66), aside from TLR4 interacting with 3'SL. This might explain why breast-feeding by HIV-positive mothers is rather ineffectively transmitting the virus (80–90% of the breast-fed children do not acquire the virus (67)), and there is evidence for HMO being the cause for this (8,59). In a study breast-feeding HIV-positive mothers were observed, and those with a total HMO concentration above the median, associated with higher proportions of 3FL and LNnT, and lower proportions of LNFP1 and 2'FL. Moreover, mothers with higher concentrations of LNnT in their milk were less likely to transmit the virus to their children. 3'SL was higher in transmitting women (67,68).

2.1.6.2.2.4 Leukocyte Rolling and PNC – Sialylated HMO reduce pro-inflammatory immune responses

Circulating leukocytes migrate to inflammation sites through the endothelium and subendothelial regions along an increasing cytokine/chemokine gradient. These pro-inflammatory cytokines induce P- and E-Selectin expression in endothelium. Subsequently, these selectins reach out and bind glycoconjugates on the cell surface of circulating leukocytes. By this initial binding, leukocytes in the blood flow decelerate and finally roll over the endothelial cells, before they are stopped by additional cell adhesion molecules. Thus, the leukocytes can transmigrate the cell layer and extravasate towards the site of inflammation, infiltrating the mucosa. P-Selectin is also used by activated platelets, in order to bind ligands on neutrophil surfaces. Binding leads to an increased expression of adhesion molecules, induction of phagocytosis, neutrophil extravasation and production of ROS, initiating an immune response of the platelet-neutrophil complex (PNC) (60,69). The formation of PNC is necessary for the recruitment and activation of neutrophils, since platelets prime neutrophils for efficient adhesion to endothelium via upregulation of integrins and enhanced responsiveness to chemokines (70).

In an *in vitro* experiment with TNF-α activated endothelial cells it was shown that sialylated HMO reduce leukocyte rolling and adhesion. Furthermore, HMO inhibited PNC formation and the ensuing neutrophil activation. The increased efficiency of pooled HMO suggested the importance of multivalent HMO, rather than only one sialylated blood group epitope being responsible on its own (60). However, even if selectins do bind sialylated lacto-N-bioses or N-acetyllactosamines, 3'SL and 6'SL had no effect on leukocyte rolling. This indicates that a sole sialyl HMO is not sufficient, and fucosylation was also essential for the selectin ligand interaction.

The sialylated fraction of HMO reduced PNC formation and leukocyte rolling in a dose-dependent manner and in the range of physiological HMO levels, indicating that sialylated HMO may contribute to anti-inflammatory processes in the immature immune system.

2.1.6.2.2.5 Necrotising Enterocolitis – DSLNT is a protective factor

Another example of HMO's protective effects was shown in necrotising enterocolitis, one of the most lethal diseases in preterm infants, with a 5% incidence amongst very-low-birth-weight preterm neonates. Breast-feeding was long known to be a protective factor, and, indeed, a single specific HMO could be identified to contribute for this benefit of milk nutrition: disialyl-lacto-n-tetraose, DSLNT, was found to protect in a neonate rat model.

The mechanisms involved are not yet clear. However, since there was no difference of leukocyte infiltration in formula-fed and HMO-fed rats' intestinal mucosa, and DSLNT has no fucosylation, it was deemed unlikely that it was acting by reducing leukocyte rolling and PNC via selectin-mediated pathways. Another finding was, that, in fact, not only one, but the two sialyl residues were required for this effect, making it a highly structure specific function. However, whether these sialylations are necessary for DSLNT to interact with host cells via Siglecs or whether its functions may be bacteria-mediated, is unclear (3,69).

2.1.6.2.2.6 HMO influencing immune cells and cytokine profiles

In foetal cord blood derived lymphocytes, exposure with sialyl-HMO altered the count of interferon producing lymphocytes (CD3+, CD4+ and CD3+, CD8+ subpopulations) as well as IL-13 producing lymphocytes (CD3+, CD8+). These findings lead the authors, Eiwegger *et al*., to the hypothesis that HMO might influence lymphocyte maturation and shift T-cell responses towards a more balanced Th1/Th2 cytokine production and low-level immunity. Another indication for HMO influence on immune cells is that sialylated HMO reduced IL-4 production in lymphocytes in adult patients with peanut allergy (71,72).

Other studies showed that LNFP3 and LNnT can expand peritoneal macrophage populations and suppress naive CD4+ T cell responses, and that LNFP3 stimulates macrophage activity and increases prostaglandin E2, IL-10 and TNF-α secretion (73,74).

2.1.7 Environmental Factors Influencing the Milk Components

There are some environmental factors known to influence breastfeeding and lactation, possibly influencing HMOs as well.

Generally, the pregnant metabolism in normal weight and in obese individuals is relevant for post-partal health of women and lifelong health for their offspring. For instance, obese women breastfeed less than their leaner age control group (75) while breastfeeding is a protective factor against childhood obesity.

Obese women are prone to commence breastfeeding later due to a late onset of lactation. A meta-analysis conducted by Amir and Donath concluded that obese women breastfeed for a shorter period. Amongst the reviewed studies, they found that 75% of studies, that examined onset of lactation, reported also a significant relationship between obesity and delayed lactogenesis. One third of obese women compared to only one sixth of normal weight women were likely to have a delayed onset of lactation. One study found that obese women had a lower prolactin response to breastfeeding than lean women (75). Most intriguingly, it was found on the other hand, that physical activity increases prolactin levels (76).

As for offspring health benefits, breastfed children later in life were found to be less likely obese, to have a lower incidence of type-2 diabetes, hypertension, and a higher IQ than formula-fed children. One of the hypothesis explaining these findings is that breast milk contains bioactive compounds, missing in infant formula (77).

Amongst the major factors affecting prevalence and duration of breastfeeding are maternal ethnicity, education, employment, inadequate breast milk production and breast diseases, but also socioeconomic factors as well as the presence of supportive means for breastfeeding (guidance from healthcare professionals, etc.) (77).

Preterm delivery might also be a factor affecting lactation and milk components. One study found that mothers delivering preterm infants had a higher concentration of HMO in their milk than those with on-term deliveries. Moreover, women delivering preterm infants had a higher abundance and variability of LNT and their HMO fucosylation seemed less well regulated, resulting in a higher inter-individual variation of HMO (78). Lactation-stage specific gene transcripts were found, and a strong modulations of key genes involved in lactose synthesis and insulin signalling was observed. Lactation specific genes are associated with making, modifying, transporting, and packaging milk proteins (79) and gene expression may be altered by cytokine-mediated environmental influences.

Another interesting finding is that HMO compositions are geographically and regionally variable. Milk samples from 435 women residing in 10 countries were analysed, and 2'FL was found, for instance, in 100% of the samples from Mexico (n = 156), whereas only 46% of the samples from the Philippines (n = 22) contained this structure (80). This suggests a regionally distributed genetic component, but may also reflect regional lifestyles influencing HMO synthesis.

Environmental factors such as medication, maternal age, diet, comorbidities, etc. are yet unknown to alter HMO biosynthesis. Determining factors have been the focus of recent research. Dietary vitamin A intake was positively related to concentrations of sialic acid in human milk. Around 82% sialic acid found in human milk is bound to free HMO (81). A study showed a late-onset lactation and morphological alterations of the mammary gland of rats fed with a high fat diet (82).

It has been suggested that maternal factors such as diet have a larger influence on the composition of human milk in late lactation stages than in the early phases (83). Another factor known to change milk components is pre-eclampsia, the pathogenesis of which is thought to be driven by excessive maternal inflammatory responses. Milk of formerly preeclamptic women had higher cytokine levels, notably of IL-8 and TNF-α. Whereas in healthy women overall cytokine levels decreased over the course of lactation, in the preeclamptic women the levels remained high, consistent with a chronic high inflammatory state (13). This may suggest that other comorbidities based on raised inflammatory states could influence the maternal milk composition in other ways, possibly affecting HMO composition and concentration as well.

Another example of a common environmental influence on human milk is mastitis, changing some of the normal characteristics of milk. Inasmuch as anti-inflammatory components stay the same as in healthy women's milk, some pro-inflammatory cytokines (IL-6, IL-1β) and soluble receptors (sTNFR1, s-IL6R, sIL1R) are secreted by polymorphonuclear cells. This is thought to protect the child from residual bacteria (13), however it is another indication that maternal inflammation may alter milk components.

However, there is also evidence suggesting that the milk metabolome of healthy individuals is relatively insensitive to environmental changes. Smilowitz *et al.* investigated environmental factors such as maternal age, diet, physical activity, lifestyle and phenotype and their relation to milk metabolites. They analysed metabolites such as amino acids and its derivatives, energy metabolites, fatty acids, vitamins, nucleotides, and found that

maternal BMI at 60 days postpartum was inversely correlated with acetone concentrations in milk. However, no other relations could be found.

Besides these metabolites, 2'FL, 3FL, LNFP I-III, 6'SL, 3'SL, LNT, LNnT, LDFT and fucose, glucose, galactose, lactose were analysed. The cohort included Secretor and Nonsecretor women with different concentrations of FUT-dependent HMO. They seemed insensitive to the investigated environmental factors, however, the extensive correlations between the sugars coupled with the conserved total oligosaccharide concentration lead Smilowitz *et al.* to suggest a regulation of HMO concentration by diverse maternal phenotypes (84).

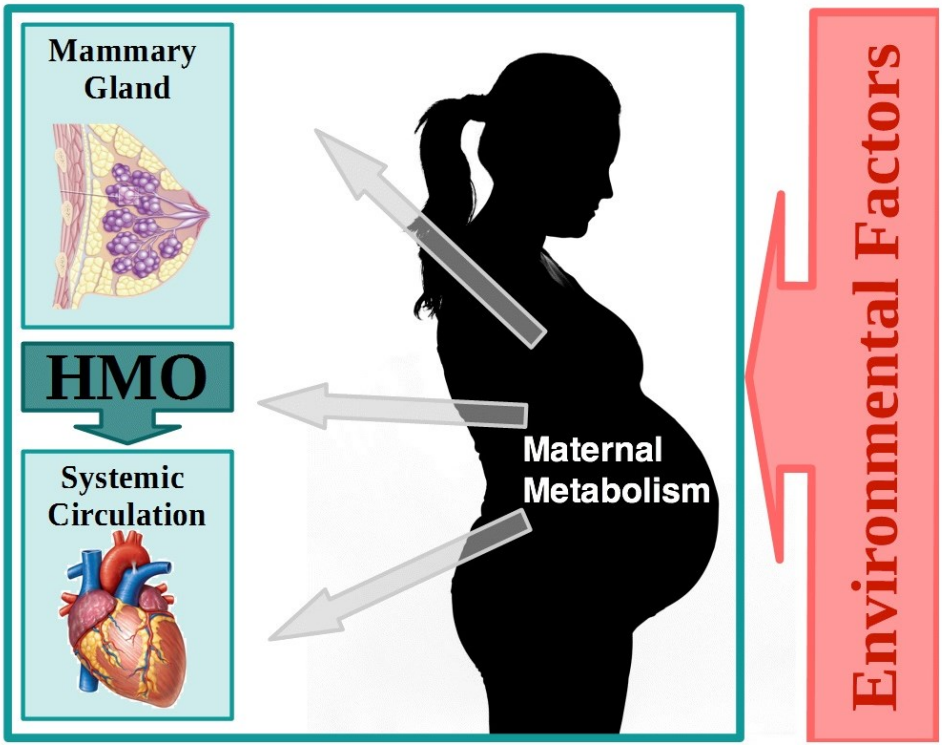


FIGURE 13. ENVIRONMENTAL FACTORS MAY ALTER HMO. Environmental factors are known to alter the maternal metabolism during pregnancy, as well as lactation later on. Environmental factors may influence HMO in the systemic circulation during pregnancy.

2.2 Physical Activity in Pregnancy

2.2.1 Physical Activity Maintains Health

Physical activity (PA) has long been in the focus of research, for its ability to decrease the risk for various metabolic diseases. In recent years, the skeletal muscle as a secretory organ has been discovered, with its ability to secrete an array of cytokines, called myokines. Linked to this new systemic role of the muscle were a number of diseases of civilisation which are thought to be prevented by exercise. For these diseases linked to PA, the term "diseasome of physical inactivity" coined by Pedersen seems fitting (85).

However, not every type of exercise is enough to avert metabolic diseases. The *2008 Physical Activity Guidelines for Americans,* published by the U.S. Government's Centre for Disease Control (CDC), advocated that for maintaining health, the activity type has to be at least moderate to vigorous. For this type of activity, a minimum of 150 minutes of moderate intensity aerobic activity was recommended for obtaining substantial health benefits. Furthermore, it was stated by the CDC, that a higher level of activity is necessary for greater health benefits (300 min of moderate activity) (86). Moderate activity, as described by the CDC, is such exercise that "a person doing moderate-intensity aerobic activity can talk, but not sing, during the activity", for instance brisk walking (<3 mph), gardening, riding a bike at <10mph, ballroom dancing or water aerobics. Vigorous activity would include running, riding a bike at >10mph, aerobic dancing, jumping rope, hiking uphill with a heavy backpack, etc. all of which should result in the "person not being able to speak more than a few words without having to pause for a breath" (86).

Activity may be categorised by different means. Absolute intensity of PA is the amount of energy expended per minute, meaning that moderate-intensity activity expends 3–5.9 times the energy than when resting, vigorous-intensity activity expends 6 times the energy of our normal resting energy expenditure. However, this is not taking into account how much effort it costs different individuals to do the same activity, therefore a relative intensity of PA is determined as well. Here, the cardiorespiratory effort is scaled from 0 to 10, 0 being the effort needed to sit and 10 being the highest possible cardiorespiratory effort the individual can make. By this classification moderate activity would be a 5 or 6, vigorous activity a 7 to 8 (86).

These energy expenditure categories are then used to objectively measure the amount PA by an accelerometer. Normally, accelerometers for activity measurement use acceleration data, recorded in three axes, and sum them up over a certain time period, creating units

such as counts per minute. Empirically, these recorded counts per minute are compared to the energy expenditure the individual was needing and thus, with the above mentioned cardiorespiratory effort recorded the categories for different types of physical exercise are set. Hence, PA can be measured objectively and it is possible to determine whether the recorded acceleration data can be classified as “moderate to vigorous PA” (MVPA) or not, and if it was therefore beneficial for health or not.

In some conducted studies, PA of pregnant women was observed by accelerometer, using these standardised categories based on the CDC’s recommendation to determine their activity level. The CDC issued general PA recommendations for pregnant and post-partum women, wherein the same 150 minutes of moderate-intensity PA, as recommended for nonpregnant adults, are also recommended for healthy pregnant women. For women who already did vigorous-intensity activity before the pregnancy the CDC and WHO recommend continuation of their habitual activity regime until objections regarding the general health may be advanced (86), (87).

Arguably, pregnant women show a different cardiovascular effort for the same standardised activity as their nonpregnant peers, due to various physiological changes in pregnancy.

2.2.2 Physical Activity and its Relationships to Metabolism in Pregnancy

Obesity, first and foremost associated with the "diseasome of physical inactivity", is one of the most commonly occurring risk factors in pregnancy. In the past decades, the prevalence of obesity in pregnant women has continuously increased, from 9% in the 1990s to around 16–19% in the 2000s, with rising tendencies (88).

The risks for various adverse outcomes on maternal and neonatal side are elevated in obese women. Adverse pregnancy outcomes associated with a BMI of over 30 kg/m^2 include miscarriage, foetal congenital anomalies, thromboembolism, gestational diabetes mellitus (GDM), pre-eclampsia, postpartum haemorrhage, wound infections, stillbirth, and neonatal death. The rate for caesarean sections in obese women is higher. Obese women are breastfeeding less than women with a healthy BMI (88).

Even maternal death may be associated with obesity. The *Confidential Enquiry into Maternal and Child Health's* report on maternal deaths in the years 2003–2005 found that 28% of occurring deaths were in obese women – at that point the general prevalence of obesity in the pregnancy was 16–19% (88).

Along this line of epidemiological evidence, it is hardly surprising that PA in pregnancy is a prevailing topic in obstetric research, since PA in non-pregnant individuals is, along with dietary behaviour changes, the most effective way of weight reduction and changing the metabolic state towards a healthy one.

For a long time however, the misconception that PA during pregnancy was harmful, was pervasive throughout the population. Amongst the hypotheses leading to this, was the assumption that the redistribution from the splanchnic system to the musculoskeletal system could cause to transitory foetal hypoxia, consistent with a reduction of up to 35% in uteroplacental blood flow (89). The assumed protective response was thought to be the redistribution blood flow to the placenta instead of the myometrium, resulting in haemoconcentration and a greater affinity of foetal blood for oxygen. However, Melo *et al*. showed that moderate-intensity physical exercise had no lasting effect on the uteroplacental blood flow throughout pregnancy and no case of foetal centralisation was found (89). In other studies, physiological foetal responses such as tachycardia reactive to bouts of exercise have been frequently documented but no long-term harm could be found to date (90).

Other studies investigated birth weight and lean body mass of children born to physically active mothers, and largely found no impact of exercise on the foetus birth weight, nor on foetal growth and gestational age (89,91,92).

Findings on maternal benefits from PA also remain controversial. Ruifrok *et al*. found that neither objectively measured exercise nor sedentary behaviour had an effect on the gestational weight gain of women. After finding that only 12% women in late pregnancy maintained their exercise level of >30min per day in comparison to 32% in early pregnancy, the authors concluded that exercise in pregnancy needs to be promoted amongst women, especially since no adverse effects in association with moderate to vigorous pregnancy-adapted PA are recorded.

PA did not have an effect on pre-eclampsia, for which medical condition obesity is a risk factor. Gestational Diabetes Mellitus (GDM), defined as glucose intolerance first manifested in pregnancy and associated with pre-eclampsia, hypertension, premature birth, higher rates of C-sections and later type 2 DM in the mother (93), is another condition highly expected to be influenced by PA. However, a study by Van der Wijden *et al*. concluded that PA was not related to any metabolic outcome, except in early pregnancy. Stafne *et al*. showed in a larger cohort that a 12-week intervention program in pregnancy had no effect on the development of gestational diabetes in healthy women with a normal BMI (94,95). Accordingly, a meta-analysis found that exercise and dietary changes are not sufficient to fully prevent GDM (96). The UPBEAT study, a large cohort study (UK Pregnancies Better Eating and Activity Trial), which enrolled women to a guided class of exercise once per week for 8 weeks, similarly concluded that dietary interventions and exercise alone are insufficient to reverse the insulin resistance leading up to a full blown clinical GDM (96). However, data is still controversial – according to another meta-analysis of randomised controlled trials, moderate exercise reduced the risk of GDM by 31% as well as maternal weight gain (97).

In the light of unclear evidence about the beneficial effects of PA but the definite absence of data on adverse effects on mother and foetus, PA in pregnancy is strongly promoted, also on the basis of available data on the effects of exercise on non-pregnant individuals.

In non-pregnant individuals, during exercise, the threefold of basal energy expenditure is used. PA leads to an increased blood flow through the adipose tissue, thus releasing more free fatty acids as energy source for the muscle. However, if sufficient stores are available, glycogen is the primary source of energy. After the depletion of glycogen, the metabolism changes to using fat as source, thus releasing the FFA induced by lipase activation via

hormones such as epinephrine, norepinephrine, glucagon and growth hormone. Regular exercise has a wide range of long term effects, such as ameliorating insulin sensitivity, LDL/HDL ratio and other metabolic changes, but also hormonal changes at rest, such as the pulsatile release of growth hormone leading to increased cell growth and increased lipolysis preventing muscle loss.

On the whole, the very same is applicable for pregnant women. But there are some substantial changes in the pregnant woman's metabolism, which are important to be taken into account. Especially the glucose metabolism along with a physiologically increased insulin resistance is noteworthy. All these changes are owed to the fact that the maternal metabolism is providing not only for its own but also for the foetus.

Postprandially, a complex cascade of hormonal actions takes place in the pregnant woman, including a surge in blood glucose and secondary secretion of pancreatic insulin, glucagon, somatomedins, and adrenal catecholamines – all these ensuring that sufficient but not excessive supply of glucose is available to both mother and foetus (98).

Pregnant women tend to develop hypoglycaemia between meals and during sleep, compared with nonpregnant subjects. Cause is a glucose steal phenomenon, where the foetus continuously draws glucose across the placenta from the maternal circulation even during fasting periods. These interprandial hypoglycaemic episodes are more distinct as the foetus evolves and its demands increase (98).

A continuous linear rise in levels of placental steroid and peptide hormones (such as oestrogens, progesterone, and chorionic somatomammotropin) takes place in the second and third trimesters. This induces an increasing tissue insulin resistance as their levels rise, and due to this insulin resistance the demand for increased prandial insulin secretion ever grows during pregnancy. By the end of pregnancy, 24-hour mean insulin levels are 50% higher than in the nonpregnant state (98).

On top of this, in pregnancy there are two placenta-derived modifiers of the maternal energy homoeostasis. Human placental lactogen (HPL) increases maternal food intake, to cover the additional calories needed, and promotes maternal insulin synthesis. The other hormone, placental growth hormone (PGH), may induce maternal insulin resistance, thus facilitating the foetal nutrition via maternal glucose mobilisation (94). It is hypothesised that, responding to maternal blood glucose changes, the syncytiotrophoblast, which is in direct contact with maternal blood, modifies the PGH levels secreted. PGH may also account for an increase in insulin-like growth factor 1 and 2 (IGF-1 and -2), which is in turn an important positive factor for placental and foetal growth and relates to maternal

BMI and gestational weight gain (94).

But not only peripheral changes occur, the pregnant woman also develops a central leptin resistance, meaning that leptin, considered a major food intake inhibitor and modifier of glucose metabolism and insulin sensitivity, is not adequately operating.

However, with all the physiological metabolic changes in pregnancy, the CDC included pregnant women in the general recommendation for PA of adults, with adaptations only to the type of PA recommended. The sports recommended without limitations include jogging, hiking, cycling, gymnastics, swimming. The type of sports only recommended with controlled intensity are sports such as squash, tennis, sailing. Sports with danger of high impact trauma and injury or an excessive physical stress are altogether deemed unfit for pregnancy, such as team sports, contact sports, marathon/triathlon (87).

Even with the best compliance, moderate and vigorous intensity exercise decreases due to physiological changes in pregnancy, such as weight gain, the relaxing effect on the ligamentous apparatus of relaxin and oestrogen, restrictive ventilation disorder by the extra thoracic displacement of lung volume paired with an increased basal oxygen demand, faster hypoglycaemia, etc. Van der Wijden *et al*. reported a drop of moderate to vigorous intensity PA in healthy non-obese women from a median of 6.5 minutes per day, objectively measured at week 15 in pregnancy, to 2.1 minutes at week 35 (94). In another study with the objective recording of PA in obese women conducted by van Poppel *et al*., the amount of time spent in moderate and vigorous activity decreased by 17% from early to late pregnancy (99).

2.2.3 Physical Activity – its Effects and Regulatory Mechanisms

Associations between physical inactivity and low-grade systemic inflammation in healthy young individuals have long been established. Moreover, evidence also exists that visceral fat, related to being accumulated due to physical inactivity, is more inflamed than subcutaneous fat, which may be therefore a relevant origin of systemic inflammations. Chronic inflammations are promoters of the development of insulin resistance, neurodegenerations and tumour growth.

Pregnancy already represents a physiological state of systemic low-grade inflammations, i.e. circulating pro-inflammatory markers are elevated 2- to 4-fold, as well as anti-inflammatory cytokines and acute-phase proteins, together with neutrophil and natural killer cell counts being increased. Due to this natural inflammatory state during pregnancy, the potential anti-inflammatory effects of PA are desirable as possible means of cheap, safe and effective health intervention (100,101).

The exact cytokine profile induced by PA is yet unknown, but studies reported a multitude of cytokine interactions during and following exercise (101). During PA, cytokines act in a hormone-like manner, mediating metabolism in the liver and in adipose tissue, in the working skeletal muscle and even influencing angiogenesis and neurobiology (102). PA stimulates large increases in serum levels of IL-6, IL-8, IL-10, IL-1ra (receptor antagonist), granulocyte-colony stimulating factor, as well as smaller increases in TNF-α, monocyte chemotactic protein-1 (MCP-1), IL-1β, brain-derived neurotropic factor (BDNF), and IL-15 (102).

Cytokines involved are all expressed in skeletal muscle, but not all are released into the circulation from the skeletal muscle during exercise. Contrarily, some cytokines are not expressed in the muscle but are found circulating. However, the reasons for these discrepant cytokine responses and this dissociation of local gene expression in the muscle and systemic concentration are yet unclear (102).

Increased gene expression in the skeletal muscle could be found for some cytokines. For instance, TNF-α and IL-1β expression in skeletal muscle after exercise was increased, but their circulating levels increased only slightly. Other cytokines of which the gene expression was upregulated were: IL-6, IL-10, IL-8, MCP-1, IL-15, leukaemia inhibitory factor (LIF), TGF-β. In the studies conducted to determine the local and systemic response to PA, different protein expressions could be found for different types of exercise. In endurance activity BDNF and IL-6 protein expression was increased, whereas in resistance exercise protein expression of IL-6, IL-8, and MCP-1 was increased (102).

Serum concentration, however, was different; serum LIF and TGF-β, both upregulated in local gene expression, were unchanged after exercise. Slight increases in serum, as opposed to distinctly increased gene expression, could be found in TNF-α and IL-1β, as well as in BDNF and IL-15. The most marked increase was seen in IL-6, IL-10, IL-1ra, IL-8, MCP-1 (102). After strenuous exercise mainly IL-8 and macrophage inflammatory protein (MIP-1α) as well as MCP-1β were elevated (101).

Interestingly, at first glance, the cytokine profile found after exercise differs from that of a sepsis only by the lack of a preceding TNF-α rise, which in response to PA increases only moderately (101).

However, most of the evidence gathered for cytokine expression is derived from the analysis of homogenised muscle tissue – not allowing a distinction between intracellular, sequestered and interstitial sources.

The best researched muscle cytokine, i.e. myokine, is probably IL-6. The post-exercise increase of IL-6 has been a consistent finding in various studies. An IL-6 increase is followed by the appearance of IL-1ra and IL-10, an anti-inflammatory cytokine. In summary, IL-6 is produced and released in response to muscle contractions, where muscle fibres express the myokine IL-6. This myokine has both local and systemic effects. Peripherally it acts hormone-like; one of these effects is its increasing hepatic glucose production and lipolysis in adipose tissue during activity.

Van Poppel *et al*. conducted a trial involving the objective measurement of exercise in obese and overweight pregnant women, wherein they found that higher IL-6 was found in more active women. Moreover, they found high IL-6 levels to be linked to a reduced first-phase insulin response. The myokine IL-6 is known to be related to glucose and insulin parameters in early gestation. Further findings of the study were the association of IL-10, an anti-inflammatory cytokine, with MVPA, as well as associations of exercise with the cytokines TNF-α and IL-1β. These cytokines showed different effects, depending on whether women were active or inactive (99). However, all of these women were overweight and obese, and due to their altered metabolism, findings have to be transferred with caution onto normal weight women. A study in healthy lean women, investigated the relationship between MVPA and insulin resistance, the IGF-1-system, leptin and weight change and found no significant differences in IGF-1 and insulin-like growth factor / binding protein (INGFBP-3) between active and inactive women (94).

3 Hypothesis and Aims

PA is thought to reduce inflammatory status in nonpregnant individuals and has been shown to have effects on pregnant women's metabolism. Environmental factors, i.e. PA, might have an effect on HMO concentration and composition.

We therefore hypothesised that PA during pregnancy influences HMO levels and composition in maternal serum.

The aim of this diploma thesis was to describe PA and HMO profiles, as well as HMO concentrations, during pregnancy.

Furthermore, we aimed to investigate whether **physical activity** (PA), as one of the environmental factors influencing maternal and neonatal outcomes in pregnancy and beyond, **had an effect on the concentration and composition of human milk oligosaccharides in maternal serum**. We therefore investigated the associations of HMO and PA and compared HMO concentrations and composition in groups of active and inactive pregnant women.

4 Material and Methods

4.1 Study Design

The study is a longitudinal, observational prospective study in which 53 healthy pregnant women were recruited and aimed to be divided into two groups by their pre-pregnant levels of PA. These women were followed throughout their pregnancy, overall on four assessment dates during pregnancy and at the time of their delivery. For this specific scientific question, serum samples were collected at three study visits during pregnancy and one at delivery for HMO analysis, whereas PA was measured objectively at the three visits during pregnancy.

4.2 Recruiting

Eligible women were recruited from February to October 2013 at an as early stage as possible in their pregnancy, and no later than their 14th week of gestation, through the outpatient clinic of the Department of Obstetrics, Medical University of Graz. Initially, when filing for the ethics committee vote, three groups of women were targeted. Two groups should be regularly active or inactive, with 20 women in each group, and a third group of pregnant women with a history of preeclampsia or positive history for metabolic conditions increasing the risk of hypertensive disorders in pregnancy, also set to have 20 women, was targeted. However, recruitment target of the third group of women, with a past medical history of preeclampsia or metabolic conditions which increase gestational hypertension, was not met. It was then proceeded to recruit for two groups of active and inactive pregnant women.

After giving their written informed consent, the women participating in the study were initially assorted to one of the groups by a short questionnaire about their PA behaviour.

It was sought to ensure the overall recruitment target of 20 women per physical active/inactive group and to overcome thus a likely bias in recruitment numbers of physically inactive women.

Inclusion criteria were

1) an ongoing pregnancy in the 10–14th gestational week and

2) their giving informed consent.

Exclusion criteria were

1) if women did not want to give birth at the University Hospital,

2) if the gestational age surpassed 14th week,

3) women having a multiple pregnancy,

4) three or more consecutive miscarriages,

5) an increased risk of >1:1000 after combined test (minimum parameters were: maternal age, foetal CRL, nuchal translucency, nasal bone and maternal biochemistry, i.e. free-ß-hCG and PAPP-A) and no NIPT (non-invasive Prenatal Testing) or no invasive testing for chromosomal anomalies,

6) foetal anomalies which are associated with possible growth or genetic anomalies,

7) smoking, pre-pregnancy diabetes type 1 or 2 (T1D, T2D),

8) maternal metabolic risk factors such as autoimmune conditions or an increased risk for thromboembolic events requiring anticoagulative therapy

9) pre-pregnancy hypertension

Screening questionnaire

A screening questionnaire was used at recruitment, consisting of nine questions, screening for age, smoking status, and PA levels of the last seven days, categorizing into moderate PA when breathing frequency is moderately elevated compared to normal frequencies and vigorous activity when breathing is significantly heavier than normal.

- On how many days was a moderate/vigorous PA performed for at least 10 minutes without interruption
- How much time (in minutes and hours) spent on one of these days performing moderate/vigorous PA
- On how many days was the participant walking for at least 10 minutes, how much time spent on one of these days walking (hours/minutes)
- On how many days was the participant riding a bike for at least 10 minutes without interruption, and how much time spent on one of these days cycling?

4.3 Study Population

The groups were planned to be formed by evaluating a short screening questionnaire completed by eligible participants, in which the women reported their self-assessed PA levels of early pregnancy and their pre-gravid activity levels.

One group was set to be composed by a number of women who regularly performed PA before and in early pregnancy, meaning that they exercised at least 150 min per week regardless as to whether these 150 min of moderate to rigorous PA were completed on one or on several occasions. All physically active women were asked to maintain their routine of exercise as long as no contraindications arose and they felt comfortable with it.

The second group comprised women who were performing less than 150 minutes of exercise per week and were assorted to the physically inactive group.

However, the women's activity was measured objectively and no interventional changes were made due to their initial activity level.

4.4 Assessments

The overall number of observations throughout the study, relevant for this thesis, was carried out at four time points of assessments, three time points during pregnancy and one at delivery.

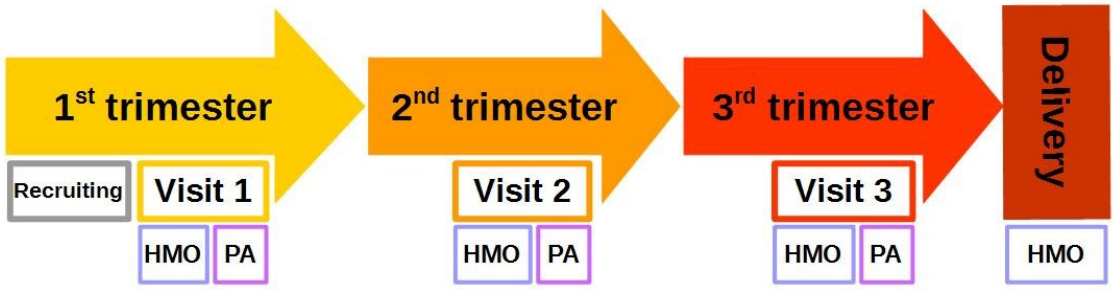


FIGURE 14. STUDY DESIGN FOR HMO AND PA MEASUREMENT. After giving informed consent and being recruited to the study before their 14th week of pregnancy, women underwent four assessment points. During pregnancy on three occasions HMO and PA measurements were realised, serum samples for HMO determination were additionally collected at delivery in the University Hospital.

During the pregnancy following points in time were chosen:

1) first trimester (week 10–14)

2) second trimester (week 20–24)

3) third trimester (week 32-36)
followed by a fourth assessment at birth.

4.4.1.1 Samples Taken at Assessment Points for HMO Analysis

At each of these points, blood samples were taken (20 ml each time, Serum and EDTA Vacuette blood collection system). HMO were determined from these blood samples by High Performance Liquid Chromatography (HPLC).

4.4.1.2 Physical Activity Measurement

The women were asked to complete a questionnaire in order to pre-evaluate their PA behaviour before enrolment.
To objectify the data gathered, they were asked to wear an accelerometer (ActiGraph) for 7 days after the study visit to the outpatient clinic. For the period of time the device was worn, the women were also asked to write a logbook. Because the ActiGraph is not water resistant and cannot be worn during activities in the water, e.g. swimming, aquatic sports were only recorded by specifications made in the log. This way, combining data from the ActiGraph with additional written information enabled us to analyse the data with regard to the type of PA (weight-bearing versus non-weight bearing) and the context in which it was performed, but also correcting wrongly perceived activity levels.

4.4.1.3 Further Maternal Outcomes Measured

Maternal demographic, anthropometric and clinical data were recorded such as: maternal age, ethnicity, marital status, parity, gravity, maternal height, weight and weight gain, fat distribution (measured by a Lipometer, for description see Chapter 4.7.1 – subcutaneous adipose tissue measurement), fasting glucose, lipid profile (triglycerides, phospholipids, free fatty acids, HDL/LDL/total cholesterol), cytokines. Fasting glucose was measured at an additional study visit, at the time of the oral glucose tolerance test (oGTT) at 24–28 weeks.

4.5 HMO Analysis

HMO in maternal serum had been previously measured using HPLC using fluorescence detection, at the Research Laboratory of the Department of Obstetrics and Gynaecology at the Medical University Graz and the ZMF, Central Research Utilities of the Medical University Graz.

In brief, samples were worked up as following: To 25 µl maternal venous serum, raffinose was added as internal standard, subjected to Chloroform/Methanol extraction, followed by deproteination using C18 and desalting with porous graphitic carbon in a high throughput format (96-well SPE columns). Isolated HMO were then eluted in 96-deep well plates, dried and labelled with the fluorescence tag 2-aminobenzamide (2AB).

Following the 2AB labelling, samples were subjected to HPLC using an amide-80 column. Separation was monitored by fluorescence detection.

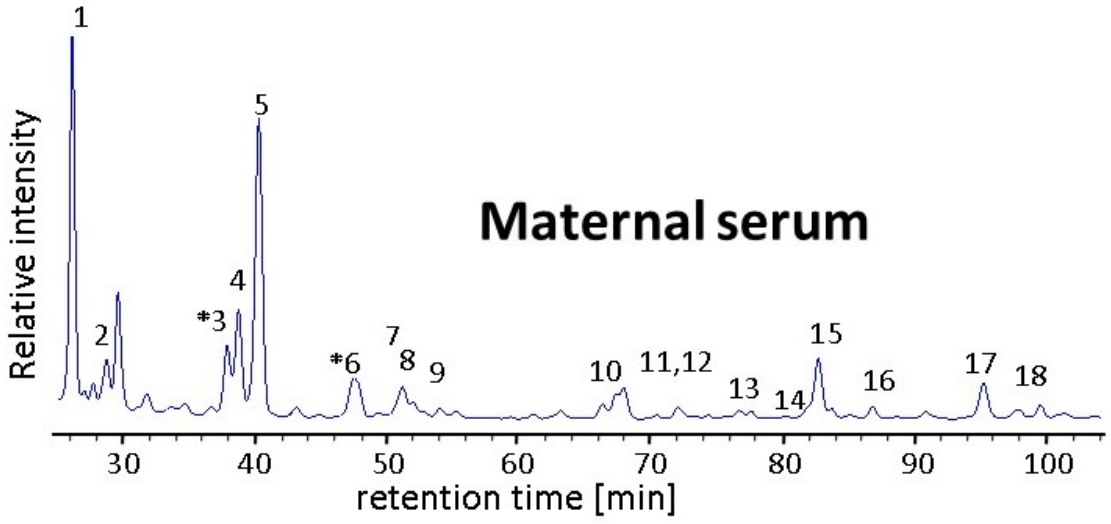


FIGURE 15. AN EXAMPLE FOR HPLC – PEAKS OF OLIGOSACCHARIDES. HPLC-FL chromatogram of Human Milk Oligosaccharides (HMO) isolated from maternal serum. Separation was monitored by fluorescence detection, the peaks analysed can be identified when they coincide with retention time of known HMO standards. (1) 2'-Fucosyllactose, 2'FL; (2) 3-Fucosyllactose, 3FL; (3) 3'-Siallyllactosamine, 3'SLN; (4) Lactodifucotetraose, LDFT; (5) 3'Sialyllactose, 3'SL; (6) 6'-Sialyllactosamine, 6'SLN; (7) 6'-Sialyllactose; 6'SL; (8) Lacto-N-tetraose, LNT; (9) Lacto-N-neotetraose, LNnT; (10) Lacto-N-fucopentaose 1, LNFP1; (11) Lacto-N-fucopentaose 2, LNFP2; (12) Lacto-N-fucopentaose 3, LNFP3; (13) Sialyl-lacto-N-tetraose a, LSTa; (14) Sialyl-lacto-N-tetraose b, LSTb; (15) Sialyl-lacto-N-tetraose c, LSTc; (16) Lacto-N-difucohexaose 1, LNDFH1; (17) Lacto-N-hexaose, LNH; (18) Disialyl-lacto-N-tetraose, DSLNT. *Asterisks mark structures not found in human milk.

4.6 Physical Activity Measurement

For objective measurement of PA, the women were asked to wear an accelerometer for a week at each assessment point. The accelerometer device used in this study was ActiGraph.

4.6.1 Technical Basics

ActiGraph is an accelerometer system quantifying activity by different motion-quantifying algorithms. Generally, accelerometers use one of the three following algorithms: zero crossing, digital integration or time above threshold. It is also possible to simultaneously quantify movement in several modes. The movement is quantified following horizontal, vertical and perpendicular axes, thus, permitting to classify different activity types by using data from an inclinometer feature that indicates whether a subject is sitting, standing, or resting. The device can also indicate non-wearing time. Accelerometer data were collected from a group of test persons while they remained in a fixed body posture (standing, sitting, lying), to obtain control data of these postures and to calibrate the system accordingly.

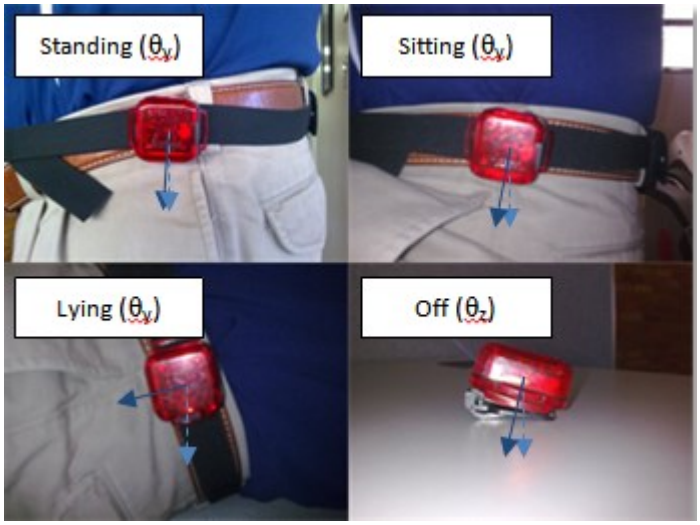


FIGURE 16. INCLINOMETER FEATURE PERMITTING TO CLASSIFY DIFFERENT ACTIVITY TYPES. Examples of this y-axis offset angle in the standing (top-left), sitting (top-right), lying (bottom-left), and z-offset angle in non-wearing (bottom-right) positions. When no activity is recorded, the accelerometer senses the acceleration due to gravity. Depending on which axis and with what degree of an offset angle the gravitational acceleration is recorded, the data is categorized into standing, sitting, lying postures or non-wearing time.

The ActiGraph includes both a micro-electro-mechanical system (MEMS) based accelerometer and an ambient light sensor.

The data recorded thus includes activity acceleration data from the vertical axis, as well as from the horizontal and perpendicular axis (axes 1–3, units: mG/LSB – milli G per least significant bit) and ambient light data (units: lux). Recording such ambient light data would enable the user later to identify indoor and outdoor activity by classifying data collected into discrete intervals with threshold values in lux, ranging from twilight to direct sunlight. Ambient light data was not evaluated in the thesis, which concentrated purely on motion data collected from study participants.

The data were collected in raw format in the selected sample rate (Hz) and not accumulated into so called epochs, as previously commonly used in other accelerometers types. The raw data were then post-processed with the affiliated software, ActiLife, where the user can generate files containing any desired combination of parametric data at a later time. It is thus possible to compare data to studies which used different filter techniques and accumulation sizes, e.g. 60 second epochs, that are finite time intervals measured mostly in seconds, facilitating retrospective compatibility.

4.6.1.1 Post-Processing of Data

In order to evaluate the data saved on the accelerometers by using the ActiLife software, data had to be imported from the devices and was then processed. The ActiGraph was set to collect acceleration data with a frequency of 30 Hz, i.e. 30 times every second, which is then shown in the raw data. However, the raw data can be summed up across a period of time, referred to as an “epoch”. The resulting value is referred to as a “count”. In this study, 60s epochs were applied after data collection, meaning that over a period of 60s the data was averaged and then saved on the device. However, raw data was kept in any case for further post-processing e.g. pattern recognition.

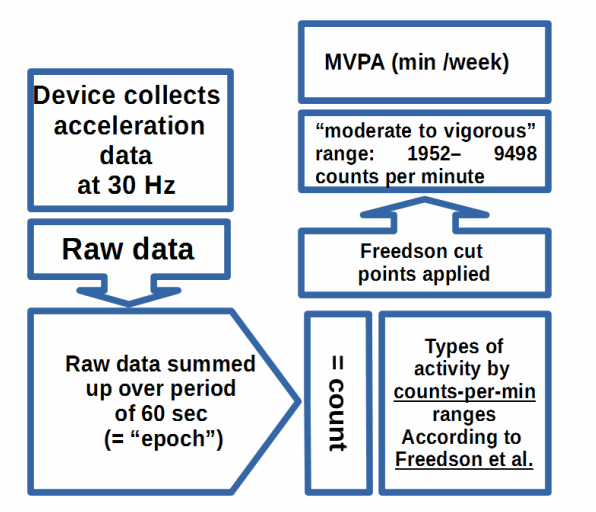


FIGURE 17. RAW DATA COLLECTION AND PROCESSING WITH ACTIGRAPH ACCELEROMETER. Data collection occurs in raw format at a selected sample rate (30 Hz), and is later summed over a period (60 sec) into epochs. Thus counts per minute (CPM) are calculated, which can be used to classify recorded activity into categories defined by CPM Ranges in literature. We applied Freedson cut points to have the final MVPA (= moderate to vigorous PA) category used for analysis.

For classification purposes, the overall measurement of different axes acceleration can be broken down into categories, depending on how many counts per minute the average epoch had. Freedson *et al*. established a set of cut points for different categories of activity, starting from sedentary, light, lifestyle, moderate, vigorous and very vigorous activity. At the time when collecting raw ActiGraph data for this study, the category "Lifestyle" still existed, in 2014 however the cut points provided in the ActiLife software changed slightly, by the authors' request.

All cut points use 60 second epochs, so if the epochs are less than 60 seconds long the software would scale the count levels up to a 60-sec-epoch equivalent and then use the cut points provided – e.g. 10-sec-epoch with 200 counts would become 200x6=1200 in a 60-sec-epoch, then these 1200 counts are used to categorise the original 10-sec-epoch.

Freedson Adult cut points (1998) (104):

Sedentary: 0 – 99 CPM (counts per minute)

Light: 100 – 759 CPM

Lifestyle: 760 – 1951 CPM

Moderate: 1952 – 5724 CPM

Vigorous: 5725 – 9498 CPM

Very Vigorous: 9499 – ∞ CPM

In adults using a 60s epoch is generally accepted and is consistent with the calibration studies that most cut points for activity behaviour patterns are derived from. However, there are studies suggesting that using shorter epochs is more valuable since it is easy to combine epochs to one bigger cumulative epoch whereas it is impossible to reduce it down when once summed up to a larger epoch. Moreover, newer algorithms tend to use shorter epochs.

4.6.2 Objective Physical Activity Measurement – Methodology

Study participants wore the accelerometer three times during pregnancy, after each study visit at the obstetrics outpatient department, University Hospital Graz. Wear-time was set to be one week for each assessment point.

4.6.2.1 Wear Time

The ActiGraph is normally worn all day in order to record PA and the day-to-day energy expenditure of the study patients, i.e. put on in the morning before rising from the bed and put away in the evening before sleeping.

To receive the most accurate results when collecting data, the ActiGraph had to be fixed to the body's centre-of-mass (around the hip). The participant was given instructions how to wear the ActiGraph, usually on an elastic belt either over or under clothing. To obtain usable data, the device had to be worn tightly around the hip in order to prevent it from swinging around and influence thus the acceleration data. The participants were explicitly told not to wear it in any pockets of their clothing, nor in a backpack or in a handbag, etc.

Women were advised to wear the accelerometer for 7 full days (~10 hrs/d). However, once the accelerometers were returned the wear-time evaluation revealed that participants wore it for different amount of days, so in fact all data were averaged per day in post-processing. Later PA per day was summed up to 5 days, in order to be comparable to WHO/CDC guidelines, recommending 30 minutes of PA per day on 5 days a week, thus resulting in 150 minutes per week of PA.

4.6.2.2 Mail Time

The women carried the accelerometer home from the visit in the hospital, started the measurement the next day and after the assessment time they sent the accelerometer back via mail. So, basically the accelerometer had two definite and predictable non-wear-days,

non-wear-time due to non-compliance excluded. In a previous study, Keadle *et al*. found that algorithms used to find such "mail-days" were in fact not as effective as anticipated. This study found a large proportion of participants (27.2% and 78.7%, respectively, when using two different algorithms for wear time detection) having a longer wear-time than originally instructed, while the log the participants kept resulted in only 0.2% additional wear time (105). In our study, the participants were also asked to keep a wear-time log and when post-processing raw data from the ActiGraph this provided a possibility to recheck the actual wear-time, and when needed, to restrict it to the log-wear-time.

Battery life of the device was sufficient for the wear-time period and a possibly prolonged mail time period, minimizing the risk of data loss. To give an example about the battery life and memory expectancy of the ActiGraph when used for a seven-day-data-collection period: when the sample rate is set to 30 Hz, the battery life is expected to be around 31 days and the memory capacity lasts up to 42 days, so when charged properly, there should be no unexpected failure and data loss.

4.6.2.3 Non-Wear Time and Wear Logs

Once the mail-days were eliminated from the raw data, the actual activity data without nights could be assessed. In order to have some structured insight into the activity patterns of the study patients, we used Freedson cut points. After the whole raw data set of activity data, collected during the three assessment points (V1 – V3), had been broken down into these various activity categories, it was possible to evaluate activity patterns.

To separate valid from invalid data, the returned ActiGraph data was manually screened and whenever it was necessary, manually adjusted or a time filter used (i.e. when the device was worn over night).

Intermittent non-wear time could be identified as well, and for a full day to count the study participants were required to wear the accelerometer for at least 10 hours per day. The participants started wearing the device the day after their visit to the clinic, and thus, the device was programmed to start recording the following day at 8 am.

However, the ActiGraph model used in our study was not water-resistant and thus, had to be removed when any activity involving water contact was planned. The participants were given a form where they could note time periods of ActiGraph-removal and also specify what their activity during the non-ActiGraph-monitored period was (swimming, showering, etc.).

4.7 Further Study Outcomes

Besides outcomes relevant for the specific question of this thesis, the study also gathered further outcome measured, here planned for use of integration in linear regression model. It was to be used for statistical control of possible confounding factors and for descriptive analysis in this thesis.

4.7.1 Maternal Outcomes

4.7.1.1 Blood Samples

The blood samples obtained had been analysed by conventional clinical chemistry methods, for CRP, glucose, lipids, and ELISA for C-peptide. The following circulating cytokines were taken into account: IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-9, IL-12 p40, IL-12 p70, MCP-1, TNFα, sIL-2Rα, MDC (CCL22), RANTES, MIP1α. These were determined by using LuminexxMAP multiplexing arrays.

Initially the values were intended to be used in linear regression models of this thesis.

4.7.1.2 Subcutaneous Adipose Tissue Measurement

The women's subcutaneous adipose tissue (SAT) was measured at each visit by a device called Lipometer. In this thesis SAT values were set to be used as possible confounding factor and was planned to be integrated in calculation of linear regression models.

The Lipometer is an optical device designed to perform non-invasive and instantaneous measurements of subcutaneous adipose tissue (SAT) layer thicknesses located anywhere on the human body, without subjecting the patient to any risk of radiation or other. Computer tomography has been used to validate the Lipometer measurements, which, according to the manufacturer, are exact up to a 50mm thick SAT layer (106,107).

To measure the thickness of a subcutaneous adipose tissue layer, one part of the Lipometer head, a source of photo-emitting diodes, flashes red light (λ = 660 nm, light intensity 3.000 mcd) perpendicularly onto the skin and into underlying tissue layers at the measuring site. From the back scattered light in the SAT the corresponding light intensities are measured in a photo-detector, which makes up the second part of the Lipometer sensor head. Based on these data, the absolute values of SAT layer thickness in mm are computed. Calibration and evaluation of the backscattered light pattern values were fitted to absolute values provided by computer tomography as a reference method, using a nonlinear regression analysis (108) .

The determination of subcutaneous fat distribution among individuals is granted by specification of a set of fifteen anatomically clearly defined body sites, from neck to calf [4].

In a study from Möller *et al*. the Lipometer measurements were used to derive equations estimating the total body fat percentage (TBF%) and comparing it to a reference method, i.e. the total body electrical conductivity (TOBEC). The Lipometer results showed a good concordance with TOBEC data (r = 0.96) and furthermore the Lipometer output allowed additionally an individual subcutaneous fatty tissue distribution profile to be calculated, which are found for instance when looking at fat distribution patterns in women and men (107,110).

4.7.1.3 Questionnaires

Besides the screening questionnaire, we used a range of questionnaires to have additional information about nutritional behaviour in the time of PA assessment by an accelerometer and about general wellbeing of participants.

WHO General Wellbeing Survey

is assessing the wellbeing and psychological health of participants throughout the two-week period prior to the appointment, by asking whether and how much patients agree to following statements: “I was happy and in a good mood”, “I was feeling relaxed”, “I felt energetic and active”, “I felt good and rested when waking up”, “My everyday life was full of things that interested me”.

Nutritional Behaviour Survey

Women were asked to write a nutritional diary over seven days, in which they were asked record every meal, as well as any dietary and supplement intakes.

4.7.2 Foetal/Neonatal Outcomes

Following outcomes were measured and used in the thesis:

Birth weight, foetal sex, gestational age at delivery, length, head circumference.

4.8 Data Management

The study was composed of a clinical and an analytical/processing part. Upon study enrolment, a study ID was created by a random study identifier with regard to encoding the study participant's ID. All further written or electronic study data and biological samples retrieved were assorted exclusively to this study ID, a computer-generated code, from which the study participant's identity cannot be deducted. This identifier was connected to the personal data such as name, date of birth and contact information only in a password-protected database accessible to the study principal investigator and team of study nurses, on account of the personal data being essential for scheduling the study's multiple visits in order to ensure the longitudinal data collection.

The clinical part of the study was performed by a team led by the study PI and study nurses, who were responsible for recruiting participating, consenting (exclusively by PI), scheduling of visits, collection of questionnaires, medical activities such as blood pressure and weight measurements, management of accelerometers at each visit, Lipometer and PEAPOD measurements, foetal growth measurements by ultrasound and the collection of biological material as urine, blood, placenta, cord blood during the visits.

The analytical/processing part of the study took place in the laboratory where all sample handling and analysis was performed, and for this the laboratory team only had access to pseudo-anonymised samples and data for the laboratory and statistical analysis. The technical resources of the hospital, LKH Klinikum Graz, were used to resolve the encoding of any person specific data, data protection and storage of data and biological material. All persons participating in the study, i.e. medical staff and researchers, signed a confidentiality statement.

4.9 Data Analysis

4.9.1 Sample Size

There was no formal sample size calculation planned, since the study as a whole was intended as a pilot study. The purpose of this pilot study was to test for feasibility of methods and techniques in order to calculate an appropriate sample size for future larger studies using the data gathered in the pilot study.

With 60 women were initially targeted to be recruited for the pilot study, a good picture could be expected to be obtained of the feasibility of exposure and outcome assessments, recruitment rate, and drop out.

4.9.2 Statistical Analysis

Although differences in outcomes between the two groups with different activity levels and were to be tested for statistical significance, the statistical analysis was not set to be the main focus of the pilot study, since there was not enough power to detect relevant differences between the groups. The analysis was planned to be mostly descriptive.

Whenever possible or relevant, linear or logistic (depending on the outcome measure) multivariate regression analyses were planned to be performed. By entering the PA level – active or non-active before and in early pregnancy – in the model, the coefficient for the PA variable would indicate the difference between the two groups for that particular outcome measure, adjusted for relevant confounders. With longitudinal multilevel analyses, differences between active and non-active women can be studied over time, taking into account that repeated measures within individuals are not independent from each other.

Software used for statistical analysis was *IBM SPSS Statistics 23*, software used for figures and tables were *GraphPad Prism, IBM SPSS Statistics 23*, and *Microsoft Office Excel 2016.*

4.9.3 Covariables

Other factors influencing neonatal growth and body composition were planned to be taken into account in the analyses, e.g. parity, maternal weight.

5 Results

5.1 Study Population

Out of the 53 women recruited at the out-patient clinic of the Gynaecology & Obstetrics Department of the Medical University Graz between February and October 2013, only 39 could finally be included in the study. Two out of the 14 had to be excluded for medical reasons, one even resulting in a termination of pregnancy. One patient who had to be excluded for medical reasons had a preterm delivery.

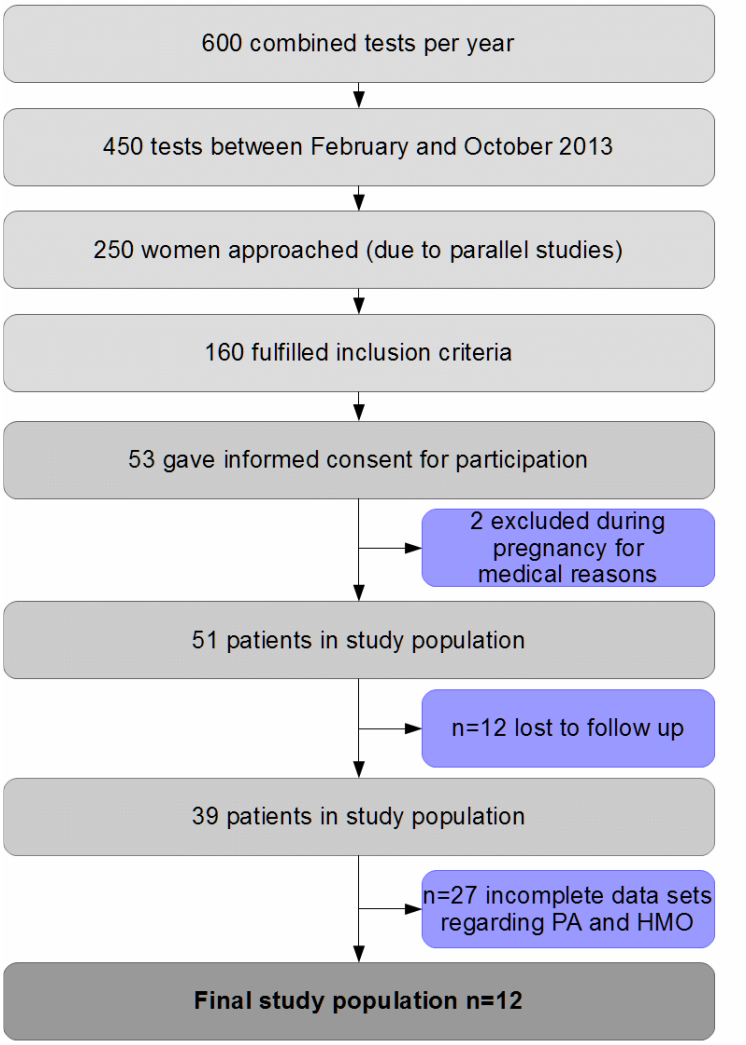


FIGURE 18. PATIENT RECRUITMENT AND FINAL STUDY POPULATION. From 53 women giving informed consent and participating in the study, 12 were lost to follow up and two had to be excluded for medical reasons. For analysis of this specific question, another 27 participants had to be excluded due to incomplete data sets. Final number of study participants analysed in this thesis were 12 women.

For this specific aim, of these 39 remaining patients we chose to exclude another 27 due to incomplete data sets regarding the objectively measured activity and the serum HMO levels. 12 participants remained in our study population, eligible for statistical analysis of this scientific question.

Due to a higher drop-out rate than initially calculated and missing data sets, we used modified statistical analysis matching sample size. For all our data, we first tested for normal distribution by Shapiro-Wilk test, and then proceeded using non-parametric statistical analyses for further data assessment. Due to small sample size, linear regression models were not appropriate, instead, data underwent an explorative analysis.

Divided into two groups based on their activity levels, the women's data were analysed by Mann Whitney U Test (with a significance level of $p<0.05$ for rejection of the null hypothesis), for differences in baseline characteristics of both groups. However, with small sample size and insufficient power, we proceeded to analyse women's PA profiles, HMO profiles, and their relationship descriptively. For bivariate correlations we used Spearman's Rho, with a confidence interval set at 95% and significance level at $\alpha=0.05$. Since our data was reasonably uniform regarding baseline demographics, further stratification was not needed.

Regarding PA data gathered from the initial questionnaires, we decided to deem them as an unreliable source for further analysis, since already at the first visit data from the questionnaire regarding activity and accelerometer data drastically diverged. Because of this inconsistency in questionnaire-based data, we did not include data reporting on water-borne sports which, for technical reasons, could not be objectively measured by accelerometer.

5.1.1 Baseline Demographics

The study patients completed questionnaires at their recruitment and demographic data was collected.

The 12 women ultimately being included, had a mean maternal age of 34.5 years, ranging from 26 to 44 years. Subjects were asked to complete a questionnaire when being recruited, from which we gathered information about their current employment situation, highest education they received, the household they live in, and their civil status, as well as other demographic data.

We found that from the 12 answers completed, it was equally distributed amongst the three possible levels of education: one third had a compulsory school or secondary school

education, the second group, consisting of 33.3% of participants graduated from a university and the remaining third held a diploma in a vocational school.

TABLE 3. BASELINE MATERNAL DEMOGRAPHICS. Education, Occupation, Household and Civil Status.

	n	%
Education of mother		
compulsory school / secondary school	4	33.3
diploma / vocational school	4	33.3
university	4	33.3
Occupation of mother		
house wife / unemployed	1	8.3
part time job / student	3	25.0
full time employment	8	66.7
Household		
living alone	2	16.7
with partner/husband	7	58.3
with partner/husband/own children	3	25.0
Civil Status		
in a relationship but living separately	2	16.7
in relationship/married	10	83.3

Of the 12 answers we were given about the civil status of our participants, approximately 17 percent were in a relationship, but living separately, and roughly 83% were married or in a relationship.

When asked about their domestic situation, 2 said to live alone, 7 were living with their partner / husband, and 3 lived with their partner and own children – this translates to roughly 17% living alone, 58% living with their partner and 25% with their partner and children.

Over 66% of the participants were full time employed before taking their maternity leave, 25% had a part time job or were students and only about 8% were unemployed or a housewife.

5.1.2 Maternal Weight and BMI

The study participants showed a mean maternal weight of 63.4 kg ± 3.5 before the pregnancy. With a mean overall weight gain of 17.9 ± 1.63 kg in the whole study population, 50% remained still in the range for the recommended weight gain of 15kg during pregnancy whereas the other half did not meet the criteria. The mean weight of the women measured at delivery was 81.33 ± 3.33 kg.

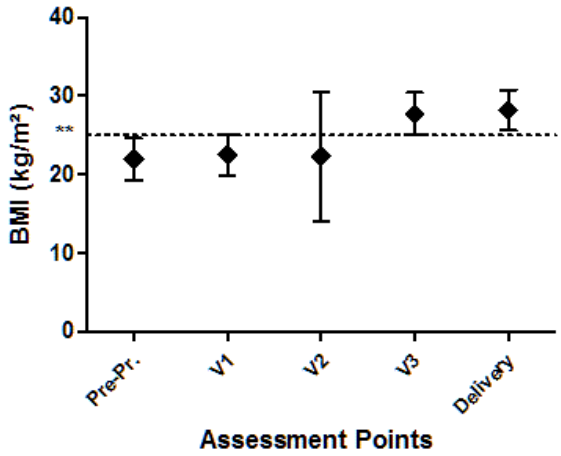


FIGURE 19. BMI CHANGES OVER THE COURSE OF PREGNANCY (PRE-PREGNANCY TO DELIVERY). Mean BMI values and SD are shown. The dotted line indicates the cut-off for an overweight BMI (** = 25 kg/m^2); only one of the women was overweight before pregnancy. After Visit 3 all the women were classified as overweight (V3: n=11, Delivery: n=12), after an overall gestational weight gain of 17,9 ± 1,63 kg. 50% of the women remained within the 15kg recommended gestational weight gain.

The women started with a mean BMI of 22.48 ± 2.64 kg/m^2 before their pregnancy and their BMI was calculated at every visit thereafter. From the 12 women participating only one woman was overweight by means of her BMI (BMI > 25). At delivery, the study participants showed a mean BMI of 29.1 ± 0.8 kg/m^2 with an overall BMI change of 6.45 ± 0.6 kg/m^2, the biggest changes in their body mass index was recorded from their BMI at visit 2 to visit 3 (mean 5.45 ± 2.54 kg/m^2).

TABLE 4. WEIGHT AND BMI IN STUDY POPULATION. Recommended weight gain during pregnancy is 15 kg (111).

	Range	Mean	SD
BMI Pre-Pregnancy (kg/m^2)	9.20	21.97	2.64
BMI at V1 (kg/m^2)	9.16	22.48	2.64
BMI at V2 (kg/m^2)	7.50	24.76	2.66
BMI at V3 (kg/m^2)	7.80	27.75	2.68
BMI at Delivery (kg/m^2)	8.00	28.22	2.56
BMI Increase from Pre-Pregnancy BMI to V1 (kg/m^2)	2.24	0.52	0.69
BMI Increase from V1 to V2 (kg/m^2)	3.05	1.78	0.88
BMI Increase from V2 to V3 (kg/m^2)	5.44	2.96	1.72
BMI increase from V3 to delivery (kg/m^2)	3.46	1.35	0.98
BMI Change Overall (kg/m^2)	5.07	6.26	1.65
Weight Gain Overall (kg)	5.07	17.18	4.52
Weight Pre-pregnancy (kg)	37.7	63.4	3.5
Weight Delivery (kg)	37	81.3	3.3

5.1.3 Delivery and Neonatal Characteristics

Out of the 12 women, 9 were nulliparous and 3 primiparae at the time point of enrolment in the study. 66.7% were primigravid, and for 16.7% of the women, it was their second gravidity, and for the same percentage their third gravidity.

TABLE 5. OBSTETRICAL BASELINE CHARACTERISTICS. Gravidity, parity, delivery mode, neonatal sex

	n	%
Number of Gravidities		
1	8	66.7
2	2	16.7
3	2	16.7
Parity		
Nulliparae	9	75.0
Primiparae	3	25.0
Neonatal Sex		
male	7	58.3
female	5	41.7
Delivery Mode		
Spontaneous Vortex Delivery	2	16.7
Primary C-Section	2	16.7
Secondary C-Section	5	41.7
Vacuum Delivery	3	25.0

Around 16.7% had a spontaneous delivery, 16.7% had a primary C-section and 41.7% a secondary caesarean section, while 25% had to undergo vacuum extraction (i.e. vacuum-assisted vaginal delivery). The mean gestational age at delivery was 275.4 ± 2.7 days, ranging from 254 to 289 days.

TABLE 6. BASELINE NEONATAL DEMOGRAPHICS. Birth weight, length, head circumference, gestational age at delivery.

	Min.	Max.	Mean	SD
Birth Weight (g)	2780	3720	3233.75	336.81
Neonatal Length (cm)	48.0	55.0	51.25	2.38
Head Circumference (cm)	33.0	36.5	34.71	1.37
Gestational Age (days) at Delivery	254	289	275.42	9.34

Out of the 12 neonates 5 were female (41.7%) and 7 were male (58.3%), with a mean neonatal weight of 3255.42 ± 92.355 g (16.3% to 73.6% percentile range), a mean length of 51.25 ± 5.4 cm (25.3% to 98.2% percentile range) and the mean head circumference of 34.83 ± 0.38 cm (12.1% to 92.3% percentile range).

5.1.4 Gestational Age at Visits

The women had three visits during their pregnancy and the delivery at the university clinic. Because there was a frame of a few weeks set for each visit, the exact gestational age was varying at these study visits.

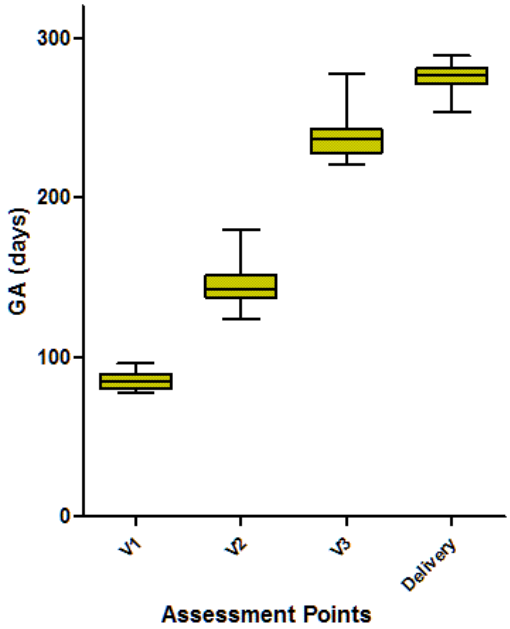


FIGURE 20. VARIABLE GESTATIONAL AGES (IN DAYS) AT V1 – 3 AND AT DELIVERY. First visit mean gestational age was 83.43 ± 3.12 days (~ 11 weeks), mean GA at V2 was 147.57 ± 5.8 days (~21 weeks), mean GA at V3 was 240.14 ± 7 days (~34 weeks). Gestational age at delivery was 275.42 ± 9 days (~39 weeks). Box plots show median, whiskers show minimum and maximum values.

Figure 6 shows the median gestational age (GA) in days, at the point of each assessment visit during pregnancy.

At the first visit the mean GA was 83.43 ± 3.12 days (~ 11 weeks), mean GA at V2 was 147.57 ± 5.8 days (~21 weeks), and mean GA recorded at the third visit were 240.14 ± 7 days (~34 weeks). GA at delivery was 275.42 ± 9 days (~39 weeks).

5.2 Physical Activity (PA)

Objectively measured ActiGraph data was post-processed, and corrected for a standardised time span. Overall activity was expressed as average minutes of PA per week (5 days).

5.2.1 PA Profiles in Pregnancy

Descriptive statistical analysis was done for all the PA categories, based on the Freedson Cutpoints. We analysed sedentary behaviour, light activity, lifestyle, moderate, vigorous, and very vigorous activity.

5.2.1.1 Sedentary Behaviour (1)

Sedentary behaviour is typically described in the contexts of TV viewing, computer and game-console use, workplace sitting, and time spent in automobiles. Sedentary behaviour represented the largest portion of the recorded data.

TABLE 7. SEDENTARY BEHAVIOUR V1 – V3.

	min.	max.	mean	SD
Sedentary Behaviour V1 (min/week)	7591.83	9230.33	8513.19	446.99
Sedentary Behaviour V2 (min/week)	7170.67	10474.00	8880.96	961.28
Sedentary Behaviour V3 (min/week)	7059.17	9629.00	8545.04	927.93

The values had quite a large range (V1: 1638.5 min; V2: 3303.33 min; V3: 2569.83 min) and the distribution of sedentary behaviour at the three visits remained practically constant over the course of the trimesters (means V1-V3: 8513.2 ± 447 min, 8881 ± 961.3 min and 8545 ± 928 min). One-Way ANOVA analysis of the medians of each visit's sedentary activity did not show a significant difference (p=0.4198).

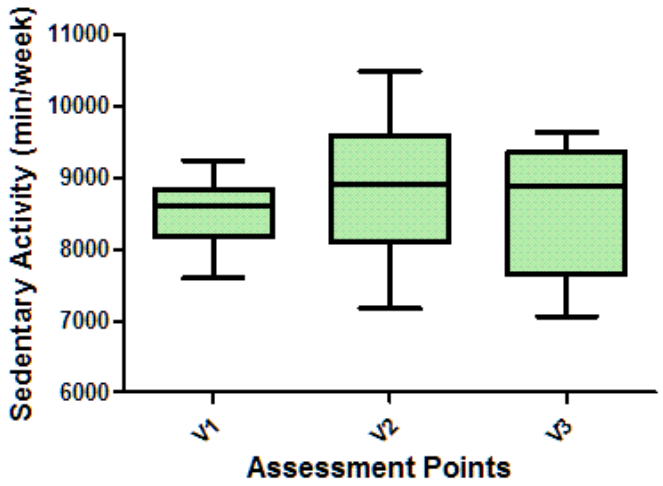


FIGURE 21. SEDENTARY BEHAVIOUR DURING PREGNANCY (V1 – V3). One-Way ANOVA did not show a significant difference of the three visits median (p=0.4198). Whiskers within upper and lower 1.5 IQR and outliers are shown. The median (IQR) of Sedentary behaviour at V1 were 8602.0 min/week (8168 – 8834 IQR), at V2 8912.0 min/week (8096 – 9579), and at V3 8868.0 min/week

5.2.1.2 Light Activity (2)

The mean levels of light activity during the three assessment periods were 710.6 ± 324.8 min at V1, 646.5 ± 333.2 min at V2 and 658 ± 427.5 min.

TABLE 8. LIGHT ACTIVITY AT V1 – V3.

	min.	max.	mean	SD
Light Activity V1 (min/week)	261.50	1443.00	710.63	324.85
Light Activity V2 (min/week)	67.67	1091.00	646.51	333.17
Light Activity V3 (min/week)	191.33	1346.83	657.99	427.47

This represents around 7% in the first assessment, around 6.4% in the second and approximately 6.6% in the third assessment of the total recorded acceleration data. One-way ANOVA of the three datasets showed no significant difference between the level of light activity throughout pregnancy (p=0.873).

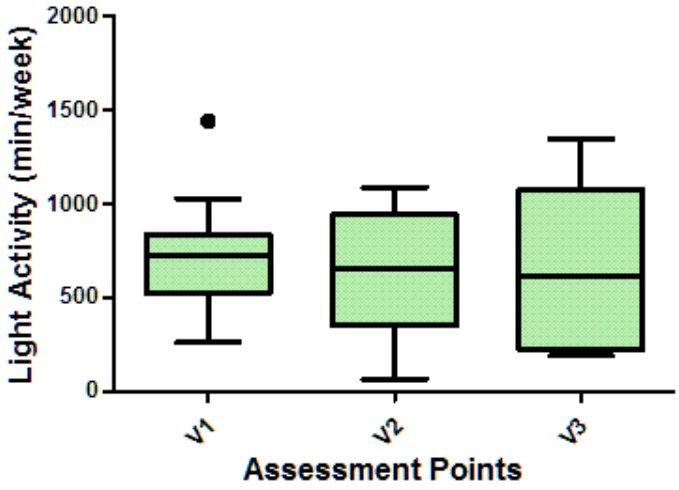


FIGURE 22. LIGHT ACTIVITY DURING PREGANCY (V1 – V3). Median (IQR) of measurements were 721.7 min/week (526.7 – 832.5) at V1, 652.3 min/week (351.8 – 943.2) at V2 and 618.0 min/week (224.0 – 1078.0) at V3. Whiskers within upper and lower 1.5 IQR and outliers are shown. Kruskal-Wallis Test showed no significant differences between the three measurements (p=0.873).

5.2.1.3 Lifestyle Activity (3)

The proportion of lifestyle activity of the women's activity represented approximately 4% at the first visit, 3.2% at the second and 3.9% at the third visit.

TABLE 9. LIFESTYLE ACTIVITY AT V1 – V3.

	min.	max.	mean	SD
Lifestyle Activity V1 (min/week)	138.50	1058.33	408.47	266.01
Lifestyle Activity V2 (min/week)	31.00	732.17	320.40	196.72
Lifestyle Activity V3 (min/week)	105.33	1133.17	386.04	324.61

One-way ANOVA showed no significant differences between the activity levels at the three time points (p= 0.6388), meaning that the amount of lifestyle type activity remained similar throughout pregnancy.

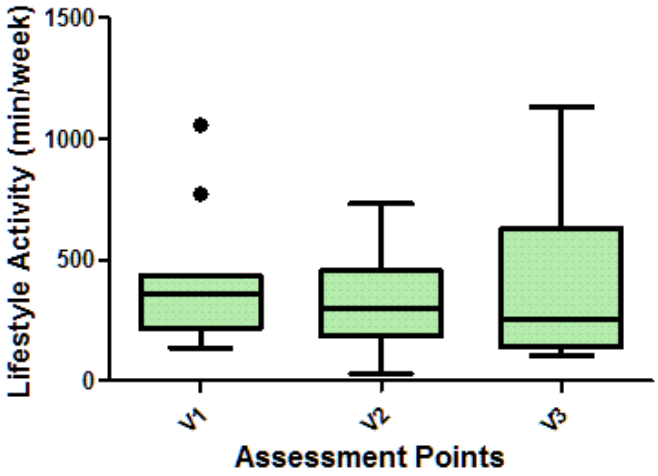


FIGURE 23. LIFESTYLE ACTIVITY DURING PREGANCY (V1 – V3). One-way ANOVA showed no significant differences (p= 0.6388) of lifestyle type activity throughout pregnancy. Whiskers within upper and lower 1.5 IQR and outliers are shown. Median (IQR) at V1 is 358.1 min/week (220.5-437.1), at V2 297.8 min/week (188.0 – 456.4), and at v3 258.4 min/week (140.3 – 629.4).

5.2.1.4 Moderate Activity (4)

The proportion of moderate activity was approximately 3% (V1), 2.3% (V2), 2.2% (V3) of the total recorded data at each assessment period. Mean activity levels of moderate activity were 302 ± 184.5 min (V1), 222 ± 168.84 min (V2) and 212.8 ± 162.6 min (V3).

TABLE 10. MODERATE ACTIVITY AT V1 – V3.

	min.	max.	mean	SD
Moderate Activity V1 (min/week)	66.17	613.50	301.97	184.50
Moderate Activity V2 (min/week)	25.67	477.00	221.96	168.84
Moderate Activity V3 (min/week)	25.83	532.33	212.75	162.62

The one-way ANOVA showed no significant differences between the three groups (significance level 0.05).

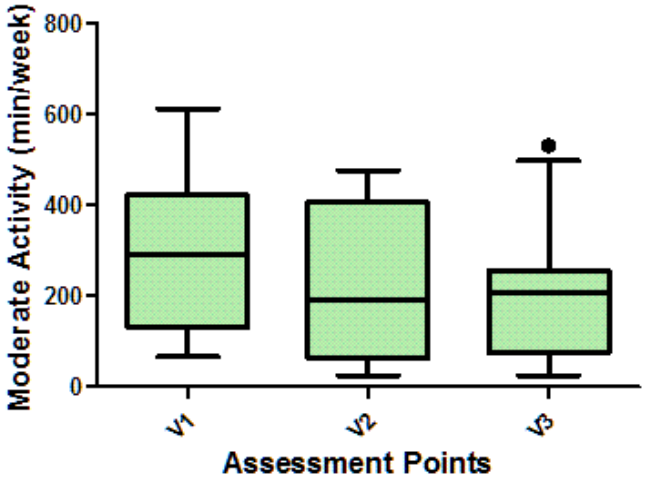


FIGURE 24. MODERATE ACTIVITY DURING PREGANCY (V1 – V3). Median levels were 292 (131-422 IQR) min/week (V1), 193 (65.3 – 406 IQR) min/week (V2) and 206 (76.6 – 257 IQR) min/week (V3). One-way ANOVA did not show significant differences between groups (p= 0.319). Tukey whiskers and outliers are shown.

5.2.1.5 Vigorous Activity (5)

The proportion of vigorous activity equalled roughly 0.3% (V1), 0.1% (V2), and 0.1% (V3) of the total recorded data at the respective assessment periods in each trimester. Mean activity levels of vigorous activity were 27 ± 66.36 min (V1), 9.56 ± 17.1 min (V2) and 7.2 ± 18.16 min (V3).

TABLE 11. VIGOROUS ACTIVITY AT V1 – V3.

	min.	max.	mean	SD
Vigorous Activity V1 (min/week)	1.17	232.00	27.00	66.36
Vigorous Activity V2(min/week)	0.17	59.33	9.56	17.14
Vigorous Activity V3(min/week)	0.50	64.67	7.17	18.16

The one-way ANOVA showed no difference of the three datasets on a significant level (p= 0.2087).

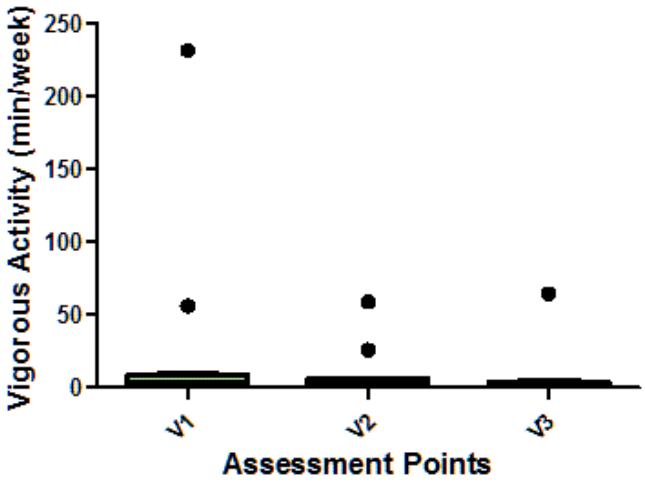


FIGURE 25. VIGOROUS ACTIVITY DURING PREGANCY (V1 – V3). Tukey whiskers and outliers are shown; Kruskal-Wallis test showed no significant difference in groups (p= 0.2087). Mean activity levels were low throughout pregnancy, from 1.5 ± 4.7 min at V1, 0.6 ± 1.7 min at V2 up to 5.3 ± 18.3 min at V3.

5.2.1.6 Very Vigorous Activity (6)

Mean activity levels of very vigorous activity were rather low, ranging from 1.5 ± 4.7 min at V1, 0.6 ± 1.7 min at V2 up to 5.3 ± 18.3 min at V3 – over the 5-day-assessment period as total sum.

TABLE 12. VERY VIGOROUS ACTIVITY AT V1 – V3.

	min.	max.	mean	SD
Very Vigorous Activity V1(min/week)	0.00	16.33	1.53	4.67
Very Vigorous Activity V2(min/week)	0.00	5.83	0.60	1.66
Very Vigorous Activity V3(min/week)	0.00	63.33	5.33	18.27

In this category of activity, Kruskal-Wallis test showed no significant difference of the three groups (p= 0.5038).

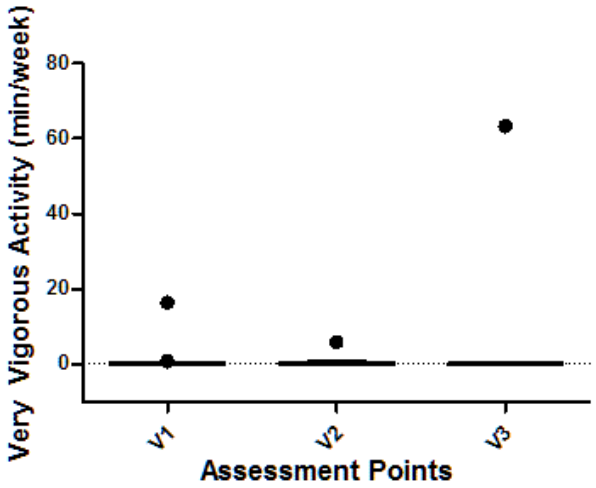
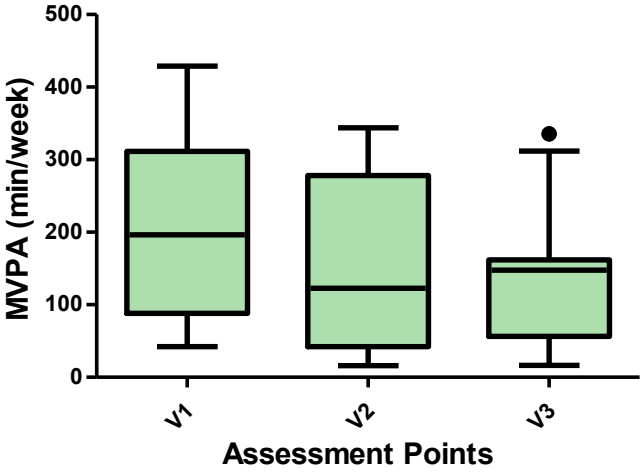


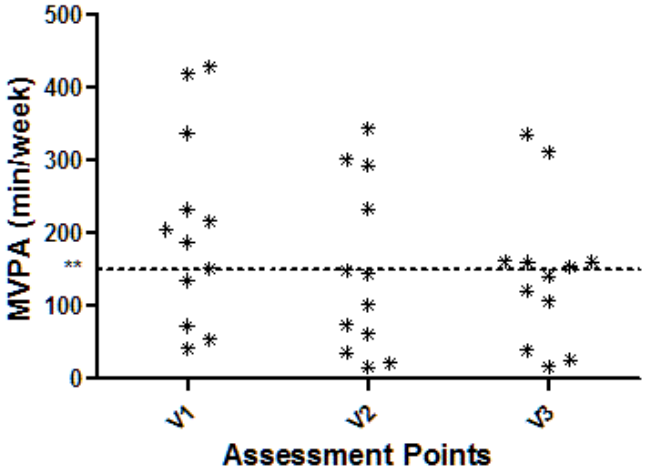
FIGURE 26. VERY VIGOROUS ACTIVITY DURING PREGANCY (V1 – V3). Tukey whiskers and outliers are shown. One-way ANOVA shows no difference between groups (p=0.5038). Median at V1 is 0.17 min/week, at V2 0.085 min/week and at V3 0 min/week.

5.2.1.7 Moderate to vigorous Physical Activity (7)

The term MVPA refers to “moderate to vigorous PA”. This category of activity is formed by summing up the previously described three activity types (moderate, vigorous, and very vigorous), as defined by the Freedson Cut Points, thus resulting in a broader category. Hence MVPA is the total amount of activity a person spent above the “moderate” cut point level, indicating that, in means of health benefits, a “significant” level of PA has occurred.
Mean amount of MVPA at visit 1 was 207 ± 131 min (minimum 42 min, maximum 428 min). At visit 2 the mean of MVPA was at 148 ± 117 min (minimum 16, max. 344 min.) which decreased with a mean of 144 ± 99 min of MVPA at visit 3.
5% trimmed median of V1 was 203.8 min, of V2 144.3 min and for V3 141.2 min.
When comparing objectively measured MVPA at different time points, we noticed a decrease of total MVPA by advancing gestational age in days (Figure 27).
One-way ANOVA showed no significant difference between the different total amounts of moderate-vigorous activity ($p= 0.3502$). The decrease from the first measurement in the first trimester to the third trimester measurement was approximately 30%. Additionally, this decrease in MVPA implicates that by the third trimester of their pregnancy, 50% of women fulfilled the WHO activity criteria of 150 min of MVPA (see Figure 27b).



A



B

FIGURE 27. (A) TOTAL MVPA DURING PREGANCY (V1 – V3); Over the course of pregnancy, a decrease in mean MVPA of approximately 30% from the first visit between week 10-14 and the third visit in the third trimester was seen. Median (IQR) are 196 min/week (88.3 – 311.0) at V1, 123 min/week (42.1 – 278) at V2, and 148 min/week (56.5 – 162) at V3; Tukey whiskers and outliers are shown. **(B) SCATTERPLOT OF MVPA AT V1 – V3.** A continuous decrease of total MVPA during pregnancy is shown, dropping below the recommended 150 minutes of MVPA (** = 150 min/week MVPA; horizontal dotted line). At V1 n=8 met WHO guidelines, at V2 n=4 and n=6 at V3 met the guidelines.

5.3 HMO

In this study, 17 different HMOs were determined by high performance liquid chromatography (HPLC).

HMO were isolated using SPE (solid phase extraction) to deproteinate and desalt 50 µl of maternal serum and were, after 2-AB labelling, analysed by HPLC with fluorescence detection. Peaks recorded were then identified using HMO standards.

TABLE 13. ABBREVIATIONS OF HMO. 17 different HMO were determined.

Abbr.		Abbr.	
2'FL	2'-Fucosyllactose	**LNnT**	Lacto-N-neotetraose
3FL	3-Fucosyllactose	**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	**LNFP23**	Lacto-N-fucopentaose 2,3
LDFT	Lacto-di-fuco-tetraose	**LNDFH**	Lacto-N-di-fuco-hexaose
LNT	Lacto-N-tetraose	**LNH**	Lacto-N-hexaose
		DSLNT	Disialyl-lacto-N-tetraose

Since the peak of the retention time of 3FL was not discrete, coinciding with an unidentified peak, we excluded the 3FL fraction from further analysis.

5.3.1 Total HMO Concentration During Pregnancy

The overall Human Milk Oligosaccharides concentration increased during pregnancy. In our cohort of pregnant women, we found an increase of the total HMO concentration in maternal serum from the first visit up until a maximum concentration at delivery. One-way ANOVA showed a significant difference between the total concentrations of HMO from V1 to delivery, p= 0.0017.

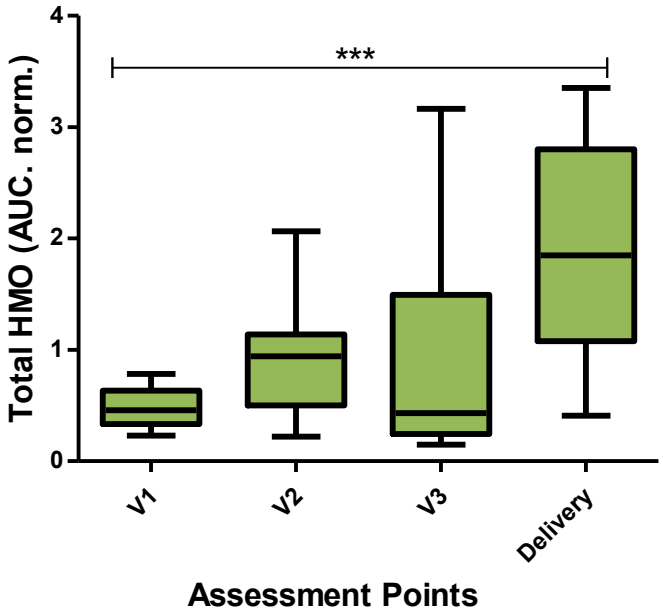


FIGURE 28. TOTAL HMO CONCENTRATION AT V1 – V3 DURING PREGNANCY AND AT DELIVERY. Significant differences (***) between concentrations were found when testing the concentrations of V1 – delivery with one-way ANOVA (p= 0.0017). Concentrations are area-under-the-curve (AUC) values normalised to a raffinose standard; Median (IQR) values are: 0.46 (0.33 – 0.63) for visit 1, 0.94 (0.50 – 1.14) for V2, 0.43 (0.24 – 1.49) for V3, and 1.85 (1.08 – 2.80). Whiskers are within 1.5 upper and lower IQR (Tukey).

TABLE 14. TOTAL HMO CONCENTRATION THROUGHOUT PREGNANCY. Values of HMO are AUC normalised to a raffinose standard; assessment points were V1 – Delivery.

	Min.	Max.	Median (IQR)
Total HMO (V1) (AUC norm.)	0.23	0.78	0.46 (0.33 – 0.63)
Total HMO (V2) (AUC norm.)	0.22	2.06	0.94 (0.50 – 1.14)
Total HMO (V3) (AUC norm.)	0.15	3.16	0.43 (0.24 – 1.49)
Total HMO (delivery) (AUC norm.)	0.41	3.35	1.85 (1.08 – 2.80

5.3.2 HMO Profiles

Analysis of HMO profiles revealed a significant variability throughout pregnancy. The individual HMO changed as percentage of total HMO concentration over time, and also the proportions of fucosylated, sialylated, and unmodified of subgroups varied between visits.

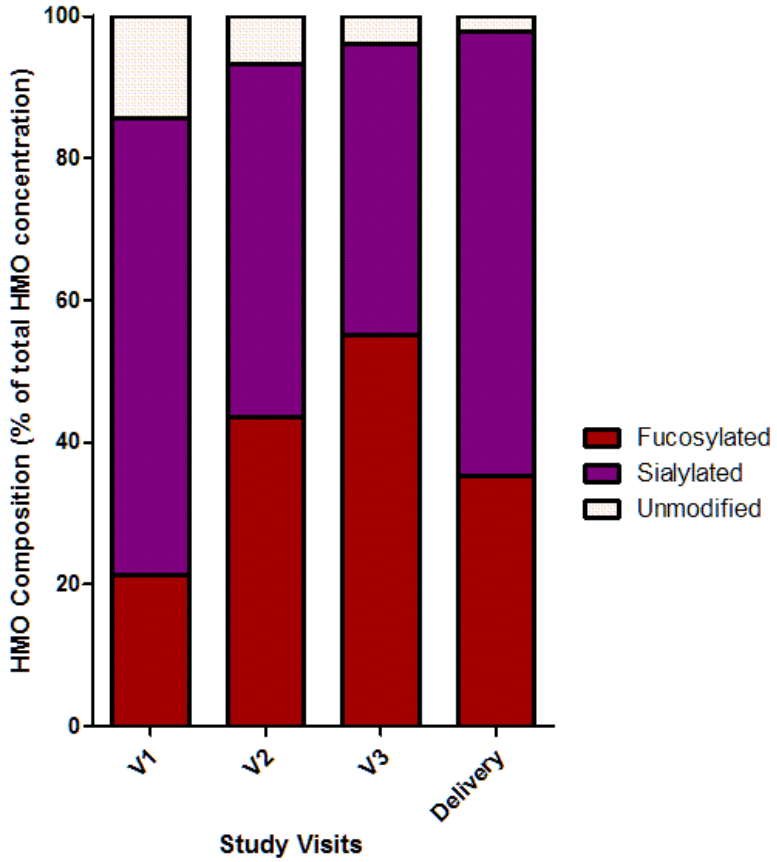


FIGURE 29. HMO SUBGROUPS COMPOSITION (% FUCOSYLATED, SIALYLATED, UNMODIFIED HMO OF TOTAL HMO CONCENTRATION) THROUGHOUT PREGNANCY (V1 TO DELIVERY). Sialylated HMO proportion decreased from the first to the third trimester, whereas fucosylated HMO increased. At delivery the ratio of sialylated to fucosylated was increased. Two women were Non-Secretors.

Early in pregnancy, the composition in our cohort was mainly composed of sialylated HMO (approximately 64%) and fucosylated HMO (around 21%). At this visit, unmodified HMO had their highest share (~14%) in overall HMO concentration compared to the following three visits, where their proportion decreased to a level at around 2% at delivery.

The amount of fucosylation increased from the first visit to the last visit during

pregnancy, visit 3 in the third trimester, when fucosylated HMO made up more than half of all individual HMO with 55%. Sialylation of HMO decreased from the first to the third visit, from 64% to 40% sialylated HMO.

At the last sampling point, at delivery, the ratio of fucosylated to sialylated HMO decreased again (fucosylated HMO: 35%; sialylated. HMO: 62%).

From the participating women two were Non-Secretor.

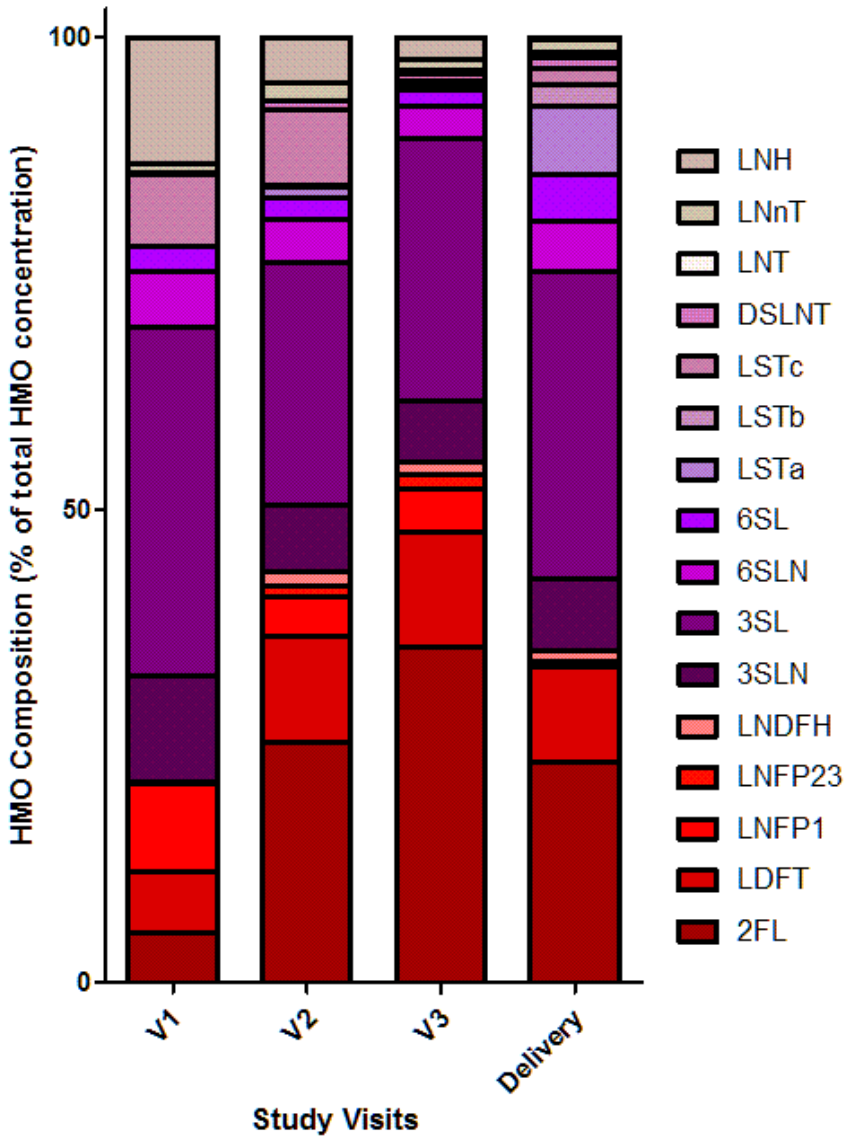


FIGURE 30. INDIVIDUAL HMO COMPOSITION (% OF TOTAL HMO) THROUGHOUT PREGNANCY (V1 TO DELIVERY). In early pregnancy, the set of individual HMO lacks several HMO (LNFP23, LSTa and LSTb). The third trimester and peripartum HMO are more diverse, with composition changes throughout pregnancy. The different HMO are colour-coded according to their chemical modifications; Fucosylation (red shading), Sialylation (shades of purple), Unmodified HMO (beige).

Some HMOs were present at higher concentrations (see Figure 30) than others, with some not being present at all at different points during pregnancy. For visit 1, for instance, LNFP2/3, LSTa and LSTb were missing from the overall composition of HMOs found during that period. In visit 2, 3 and delivery HMO compositions, all of the tested HMOs were detected.

5.4 Physical Activity and Human Milk Oligosaccharides

Next, we analysed PA assessment and HMO concentration and composition for correlations, using different approaches regarding the time points for data collection. One set of analyses was done using HMO and PA data from the same visit, the other set of analyses was performed using HMO values from the visit subsequent to the visit of PA data collection, considering that there may be a delayed influence.

In our cohort of pregnant women, we found an increase of the total HMO concentration from the first visit up until a maximum concentration at the point of delivery.

Mean amount of moderate to vigorous PA (MVPA) at visit 1 was 207 ± 131 min (minimum 42 min, maximum 428 min). At visit 2 the mean of PA was at 148 ± 117 min (minimum 16 min, maximum 344 min) which decreased to visit 3 with a mean of 144 ± 99 min of MVPA.

The collected data of PA and HMO were then used for further statistical analysis testing for correlations.

Initially we intended to assort women into two groups, based on whether they met WHO guidelines or not. However, the number of women performing more than 150 min/week of MVPA was inconstant throughout pregnancy (Fig. 31). We therefore divided women into groups based on the visit's median of MVPA (Fig. 31).

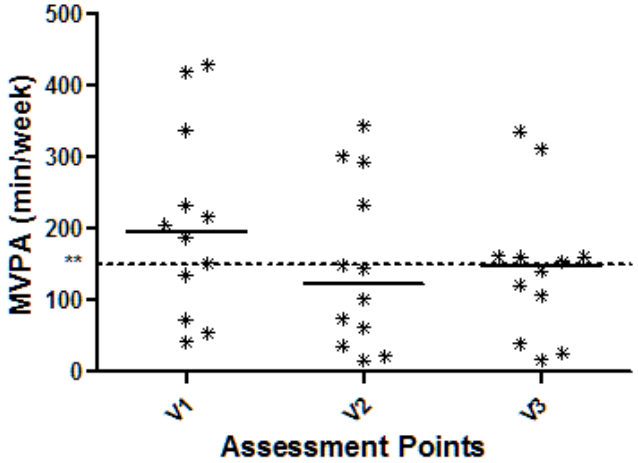


FIGURE 31. MEDIAN OF MVPA TO GROUP ACTIVE AND INACTIVE WOMEN. Active women were above the respective visit's median, inactive were below. The median was used to have uniform group sizes as opposed to activity-classification based on the WHO guidelines (** = 150 min/week MVPA). 5% trimmed median (continuous line) of V1 was 203.8 min, of V2 144.3 min and for V3 141.2 min.

5.4.1 Active and Inactive Women with Groups Based on Early Pregnancy MVPA (V1)

Women were classified once, based on their first visit's activity measurement, i.e. they were not re-categorised based on each of the following visit's PA measurement. This was chosen assuming that PA levels in early pregnancy may be most similar to pre-pregnancy PA levels and thus to women's general lifestyle. Furthermore, we considered the possibility that PA early in pregnancy could influence factors during ongoing pregnancy.

For each group of active (n=6) and inactive women (n=6), we analysed for differences in basic characteristics and did not find any significant differences (tested for by Mann Whitney U Test, see table 15).

The groups were analysed for differences in:

Maternal characteristics: weight before and in pregnancy, weight gain overall and between visits, BMI pre-pregnancy – delivery, BMI changes between visits and overall change, gestation age at V1 – delivery.

Newborn characteristics: sex, length, weight, head circumference, neonatal growth percentile.

TABLE 15. BASELINE CHARACTERISTICS OF ACTIVE AND INACTIVE WOMEN (V1). Classification into the groups is based on whether they were below or above the median of MVPA measured at V1. Mann Whitney U test was used to test for differences in groups. Significance level was $p<0.05$. m.v. = missing values. Values are expressed as median and IQR.

			Active (V1)		***Inactive (V1)***	
		n	*Median (IQR)*	*n*	*Median (IQR)*	*p*
Birth Weight (g)		6	3385.0 (3050.0 - 3691.5)	6	3212.5 (2911.0 - 3359.0)	0.394
Neonatal Growth Percentile (%)		6	41.0 (29.8 - 66.3)	6	22.5 (12.8 - 42.3)	0.132
Neonatal Length (cm)		6	51.0 (49.0 - 53.5)	6	52.0 (48.0 - 53.3)	0.818
Neonatal Head Circumference (cm)		6	35.5 (34.5- 36.5)	6	34.5 (33.0 - 35.3)	0.180
Maternal Age (years)		6	35.0 (32.8 - 43.3)	6	32.5 (28.3 - 36.0)	0.240
Weight (kg)	*Pre-pregnancy*	6	58.8 (54.3 - 73.8)	6	60.5 (50.8 - 63.0)	0.589
	V1	6	60.0 (56.0 - 73.8)	5	61.5 (51.6 - 66.1)	0.699
	V2	2	78.0 (m.v.)	4	65.0 (56.5 - 71.5)	0.381
	V3	3	86.0 (m.v.)	6	73.0 (66.9 - 80.5)	0.400
	Delivery	6	78.50 (68.3- 90.3)	6	74.5 (72.8 - 78.8)	0.937
Weight Gain (kg)	*Pre-pregnancy to V1*	6	1.0 (0.0 - 2.13)	5	2.0 (-0.5 - 3.5)	0.818
	V1 to V2	2	2.50 (m.v.)	4	6.0 (4.4 - 7.5)	0.381
	V2 to V3	2	15.50 (m.v.)	4	8.5 (5.0 - 9.6)	0.133
	V3 to Delivery	3	2.0 (m.v.)	6	3.5 (2.3 - 7.9)	0.400
	Overall (Pre-pregnancy to V4)	6	15.5 (13.8 - 18.9)	6	16.5 (14.0 - 23.8)	0.818
BMI (kg/m²)	*pre-pregnancy*	6	20.50 (19.88 - 24.50)	6	22.90 (19.28 - 23.55)	1.000
	V1	6	21.10 (20.70 - 24.50)	6	23.41 (19.59 - 24.55)	0.937
	V2	4	23.48 (5.49 - 27.32)	5	25.52 (21.75 - 26.33)	0.476
	V3	4	27.23 (25.64 - 31.12)	6	27.14 (25.23 - 30.07)	1.000
	Delivery	6	27.27 (25.30 - 29.77)	6	28.05 (26.96 - 30.26)	0.394
BMI increase (kg/m²)	*Pre-pregnancy to V1*	6	0.36 (-0.01 - 0.71)	6	0.78 (-0.16 - 1.31)	0.818
	V1 to V2	4	0.81 (-15.62 - 1.69)	5	2.03 (1.46 - 2.81)	0.114
	V2 to V3	4	4.98 (2.31 - 4.42)	5	3.13 (0.93 - 3.56)	0.111
	V3 to delivery	4	1.06 (0.16 - 1.90)	6	1.12 (0.94 - 2.49)	0.556
	Overall (Pre-pregnancy to V4)	6	5.39 (4.77 - 6.74)	6	6.39 (5.19 - 9.00)	0.310

5.4.1.1 Comparison of HMO Concentration in Active and Inactive Women

We further investigated, whether there was a difference in HMO concentration between active and inactive women. We divided women into these two groups, choosing the median of the first visit's activity level as cut off point for assorting them into the active group when above and into the inactive group when below the median.

For the concentration of sialylated HMO at the second time point, we did not find differences between active and inactive participants. Active women had significantly lower concentration 3'SL (Mann Whitney U, p=0.026; see Figure 32), as well as significantly lower LDFT concentrations (Mann Whitney U, p=0.026; see Figure 33).

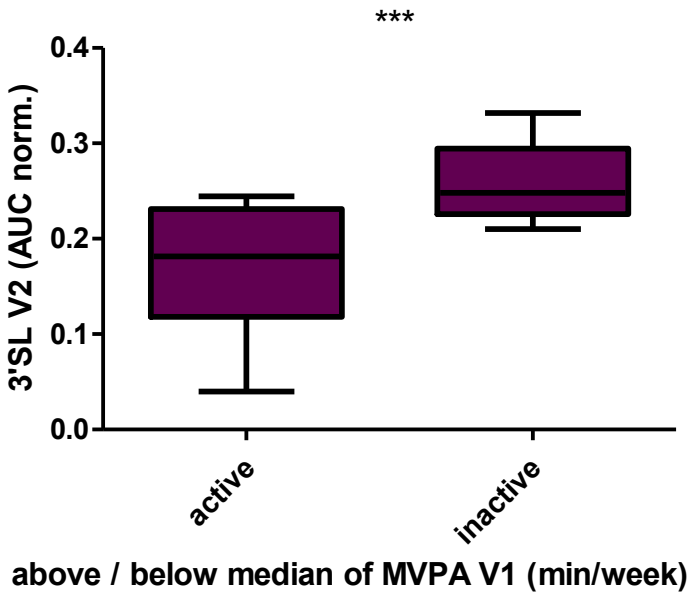


FIGURE 32. SIGNIFICANT DIFFERENCES IN 3'SL CONCENTRATION AT V2 IN ACTIVE AND INACTIVE WOMEN BASED ON THE MVPA V1 MEDIAN. When grouping the women based on the median of MVPA measured at V1, a significant difference (***) in levels of 3'SL measured at V2 can be seen (Mann Whitney U, p=0.026). The graph shows median and IQR, whiskers min to max.

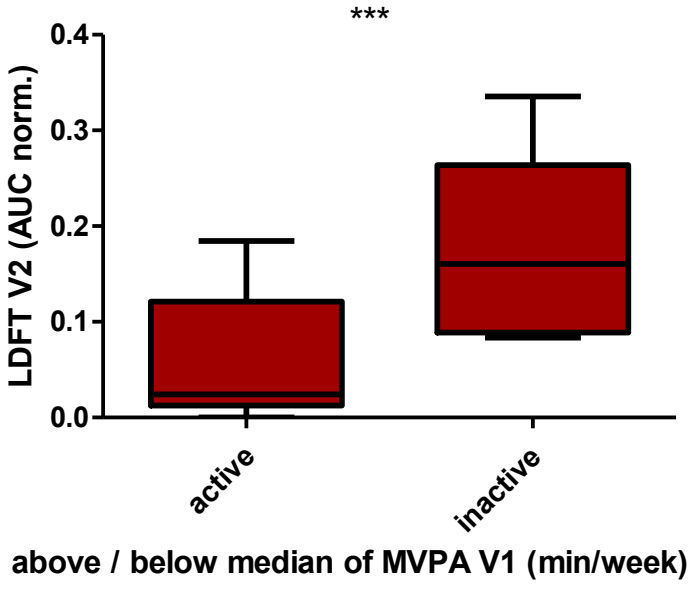


FIGURE 33. SIGNIFICANT DIFFERENCES IN LDFT CONCENTRATION AT V2 IN ACTIVE AND INACTIVE WOMEN BASED ON THE MVPA V1 MEDIAN. When grouping the women based on the median of MVPA measured at V1, a significant difference (***) in levels of LDFT measured at V2 can be seen (Mann Whitney U, p=0.041). The graph shows median and IQR, whiskers min to max.

5.4.1.2 Comparison of HMO Composition Active and Inactive Women

The percentage of each individual HMO of the total concentration was analysed for significantly different distributions when women were assorted into two groups, using the nonparametric Mann Whitney U Test.

No statistically significant differences between the two groups were found in HMO composition at either of the three visits and at delivery (Fig. 35).

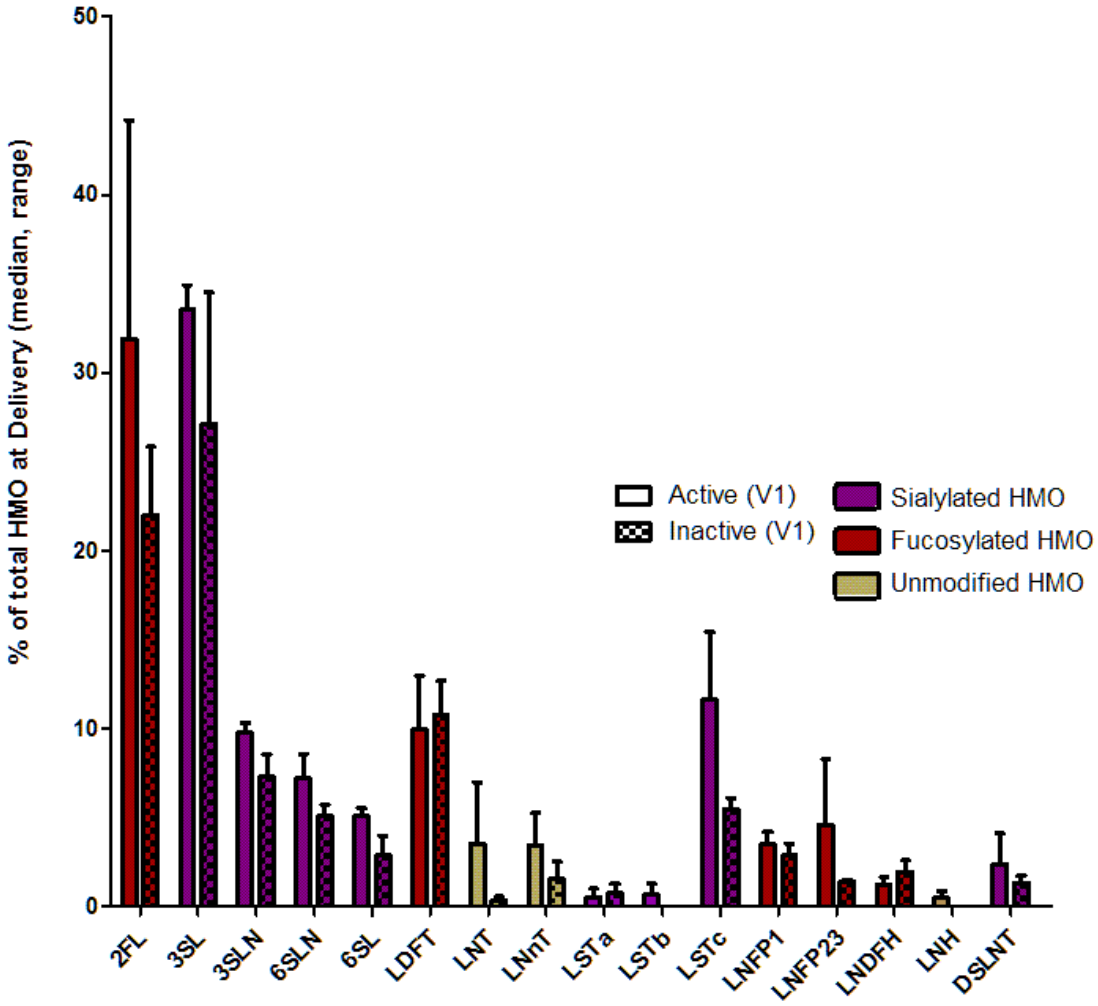


FIGURE 34. NO SIGNIFICANT DIFFERENCES IN HMO COMPOSITION AT DELIVERY IN ACTIVE AND INACTIVE WOMEN BASED ON THE MVPA V1 MEDIAN. Nonsecretor study participants are included. Mann Whitney U test found no significant differences across the two groups. Median % of total HMO and range in error bars shown. Values of active group are shown in bars on the left (plain), values of inactive women shown on the right (checked). Individual HMO are additionally colour-coded for their chemical modification: fucosylation (red), sialylation (purple), unmodified HMO (beige).

One exception was the distribution of the 6'SL percentage determined at delivery, which was significantly different across these two groups ($p=0.041$, significance level of 0.05). Arguably, the Secretor status, and therefore the women's ability to synthesise high levels of fucosylated HMO (mainly 2'FL), could be a confounder when evaluating HMO composition.

We therefore excluded the Nonsecretor women from further analysis and no differences in HMO proportions were found when testing for differences of HMO percentages in active versus inactive women, categorised using their first trimester MVPA measurement.

No significant findings were made.

5.4.2 Active and Inactive Groups of Women, Based on Late Pregnancy MVPA (V2, V3)

However, when analysing the individual study participants' activity profiles over time, discontinuous activity levels could be observed in most of the women (Fig. 35).

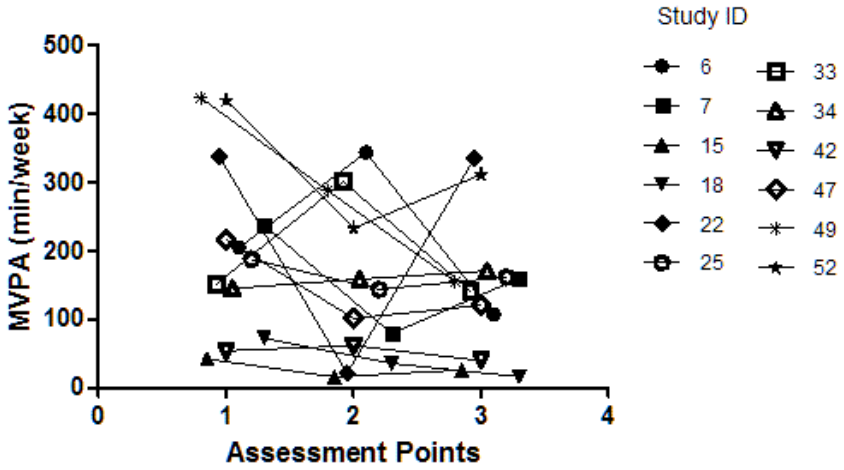


FIGURE 35. MVPA AT V1 – V3 FOR EACH OF THE STUDY PARTICIPANTS. Their moderate to vigorous PA (MVPA) levels measured at each assessment point in pregnancy is connected by a line. Symbols on the right show individual participants. Most participants did not show constant PA behaviour throughout pregnancy.

Therefore, separate explorative subanalyses were performed additionally, where women were re-categorised into the active and inactive group at each of the three visits, based on each of the respective visit's median. No differences in baseline characteristics were found (Table 16). The groups were then analysed for differences in all consecutive HMO data.

TABLE 16. BASELINE CHARACTERISTICS OF BOTH ACTIVE AND INACTIVE WOMEN (V2, V3). Classification into the groups is based on whether they were below or above the median of MVPA measured at V2 and V3. Mann Whitney U test was used to test for differences in groups. Significance level $p<0.05$. m.v. = missing values. Values are expressed as median and IQR.

		Active (V2)		*Inactive (V2)*			*Active (V3)*		*Inactive (V3)*		
		n	*Median (IQR)*	*n*	*Median (IQR)*	*p*	*n*	*Median (IQR)*	*n*	*Median (IQR)*	*p*
Birth Weight (g)		6	3225.0 (3031.3 - 3404.5)	6	3360.0 (2911.0 - 3592.5)	0.699	6	3180.0 (3031.3 - 3414.0)	6	3405.0 (2911.0 - 3583.0)	0.373
Neonatal Growth Percentile (%)		6	38.0 (22.8 - 53.5)	6	31.0 (13.5 - 51.0)	0.589	6	33.5 (22.8 - 53.3)	6	35.5 (13.5 - 51.3)	0.064
Neonatal Length (cm)		6	51.0 (48.8 - 54.3)	6	52.0 (49.5 - 52.3)	0.818	6	49.5 (48.8 - 53.0)	6	52.0 (51.0 - 54.3)	0.482
Neonatal Head Circumference (cm)		6	35.0 (33.0 - 35.4)	6	35.5 (33.8 - 36.1)	0.589	6	35.0 (33.0 - 35.4)	6	35.5 (33.8 - 36.1)	0.482
Maternal Age (years)		6	34.5 (28.3 - 36.0)	6	34.5 (30.5 - 43.3)	0.589	6	35.0 (32.0 - 43.3)	6	32.5 (29.0 - 36.0)	0.373
Weight (kg)	*Pre-pregnancy*	6	58.0 (51.8 - 61.13)	6	63.0 (53.8 - 73.8)	0.240	6	58.0 (54.3 - 67.8)	6	62.3 (50.8 - 64.5)	1.000
	V1	6	57.5 (55.0 - 63.5)	6	66.15 (54.6 - 73.8)	0.240	6	57.5 (56.0 - 70.8)	6	64.5 (51.6 – 67.0)	1.000
	V3	3	73.0 (m.v.)	4	75.5 (65.6 - 96.5)	1.000	4	73.0 (69.3 - 94.0)	3	83.0 (m.v.)	0.571
	Delivery	6	72.0 (68.3 - 78.5)	6	79.5 (73.5 - 91.0)	0.240	6	73.5 (68.3 - 82.8)	6	79.5 (72.8 - 86.3)	0.727
Weight Gain (kg)	*Pre-pregnancy to V1*	6	1.3 (0.3 - 4.0)	6	0.5 (0.0 - 3.08)	0.485	6	1.0 (-0.5 - 4.0)	6	1.3 (0.0 - 3.08)	0.727
	V1 to V2	4	5.5 (4.3 - 6.8)	3	4.70 (m.v.)	1.000	3	4.0 (m.v.)	4	5.5 (4.8 - 7.5)	0.571
	V2 to V3	3	8.0 (m.v.)	3	9.80 (m.v.)	0.700	3	8.0 (m.v.)	3	9.80 (m.v.)	1.000
	V3 to Delivery	3	2.0 (m.v.)	4	4.0 (2.5 - 7.90)	0.114	4	2.5 (2.0 - 3.8)	3	4.0 (m.v.)	0.095
	Overall (Pre-pregnancy to V4)	6	15.0 (13.8 - 19.6)	6	16.5 (14.0 - 23.8)	0.699	6	15.0 (13.8 - 15.5)	6	20.9 (14.8 - 24.1)	0.727
BMI (kg/m²)	*pre-pregnancy*	6	20.3 (19.70 - 22.88)	6	23.20 (20.03 - 3.03)	0.310	6	21.65 (20.3 - 24.58)	6	21.50 (19.28 - 23.48)	1.000
	V1	6	21.10 (20.21 - 23.03)	6	23.83 (20.35 - 25.88)	0.310	6	21.98 (20.88 - 25.15)	6	21.82 (19.59 - 24.55)	0.727
	V2	4	23.8 (21.99 - 25.59)	6	25.36 (15.53 - 28.12)	0.762	4	25.52 (26.35 - 27.48)	6	23.56 (21.64 - 26.33)	0.517
	V3	3	27.76 (m.v.)	6	26.39 (25.06 - 31.78)	0.548	4	27.83 (25.8 - 31.31)	5	26.70 (25.23 - 29.69)	0.548
	Delivery	6	27.03 (25.30 - 28.52)	6	28.29 (27.08 - 32.95)	0.180	6	27.51 (25.30 - 30.19)	6	27.81 (26.96 - 29.84)	0.482
BMI increase (kg/m²)	*Pre-pregnancy to V1*	6	0.43 (0.04 - 1.48)	6	0.2 (0.00 - 1.17)	0.589	6	0.36 (-0.19 - 1.48)	6	0.43 (0.02 - 1.17)	0.600
	V1 to V2	4	1.95 (1.52 - 2.62)	6	1.54 (-5.21 - 2.09)	0.476	4	0.74 (-15.62 - 2.42)	6	1.74 (1.55 - 2.48)	0.517
	V2 to V3	3	3.13 (m.v.)	6	3.56 (1.36 - 9.43)	0.905	4	3.64 (1.90 - 20.00)	5	3.43 (1.03 - 4.8)	0.548
	V3 to delivery	3	0.78 (m.v.)	6	1.51 (0.99 - 2.39)	0.167	4	0.95 (0.67 - 1.39)	5	1.52 (0.55 - 2.8)	0.262
	Overall (Pre-pregnancy to V4)	6	5.73 (5.20 - 7.13)	6	5.90 (4.60 - 9.00)	0.937	6	5.45 (4.77 - 6.02)	6	7.38 (5.09 - 9.00)	0.482

5.4.2.1 Comparison of HMO Concentration between Active and Inactive Women

Later in pregnancy, no difference between HMO concentrations in the two groups sorted by the activity level at the third visit could be found. Exception is LNFP23 at delivery, which was found to be significantly lower (p=0.004; see Figure 36) in active women based on the median of V3 MVPA. LNT at delivery was different across the same groups (Mann Whitney U; p=0.015), being lower in active women.

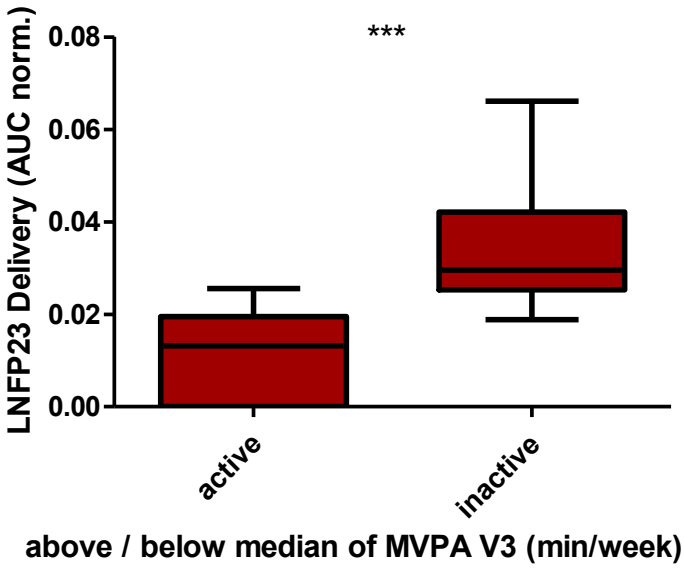


FIGURE 36. CORRELATION BETWEEN MVPA AT V3 AND LNFP23 CONCENTRATION OF TOTAL HMO AT DELIVERY. LNFP23 was found to be significantly lower (Mann Whitney U; p=0.004 - ***) in active women.

5.4.2.2 Comparison of HMO Composition between Active and Inactive Women

We again excluded the Nonsecretor women from further analysis and found, when testing for differences of HMO percentages in active versus inactive women, that the LNnT proportion calculated for groups based on the median of V3 MVPA was higher in inactive women than in active (p=0.033). We also found that peripartum LNT proportions were significantly higher in inactive women (p=0.038).

These findings were only present when groups were divided based on previous visit MVPA, the differences in HMO proportions were not found when categorising women using their first trimester MVPA measurement.

5.4.3 Descriptive Analysis of the Relationship of Physical Activity and HMO Composition and Concentration

Since the sample size was small and was further decreased with division into two groups, we analysed data with descriptive statistical analysis.

5.4.3.1 Physical Activity Had No Influence on HMO Composition

Analysing the data for a correlation of MVPA and HMO composition, with calculation of Spearman's Rho, we could not consistently find any significant correlations.

Firstly, we analysed for significant correlations of individual HMO percentage at different time points in pregnancy in relation to PA recorded at the first visit between the 10^{th}-14^{th} week of pregnancy.

Therein, PA in early pregnancy (MVPA at V1) showed a correlation with late-pregnancy HMO composition (V3, delivery; see Table 17). LSTa and LNH percentage from the third visit showed strong positive correlations (LSTa: rho=0.674, p=0.016; LNH: rho=0.737, p=0.006). The LNH percentage of total HMO was also positively correlated with exercise in early pregnancy when measured at delivery (LNH: rho=0.615, p=0.033), as well as 6'SL (rho=0.615, p=0.033).

TABLE 17. CORRELATION OF MVPA EARLY IN PREGNANCY AND HMO COMPOSITION IN EARLY TO LATE PREGNANCY HMO. Significant positive correlations were found for LSTa and LNH in third trimester HMO composition, and 6'SL and LNH at delivery. Spearman's rho was calculated, significance level was * p<0.05 and ** p<0.01 (2-tailed). n.c. = not calculable.

	Total MVPA at V1							
	HMO % at V1		HMO % at V2		HMO % at V3		HMO % at Delivery	
	rho	p-value	rho	p-value	rho	p-value	rho	p-value
2'FL	-.235	.463	-.217	.499	-.245	.443	-.385	.217
3'SLN	-.063	.846	.259	.417	.077	.812	.538	.071
3'SL	-.245	.443	.189	.557	-.133	.681	.189	.557
6'SL	.035	.914	.042	.897	.182	.572	**.615***	**.033**
6'SLN	.028	.931	-.070	.829	.140	.665	.021	.948
LDFT	-.441	.152	-.552	.063	-.224	.484	-.566	.055
LNT	.218	.495	-.422	.172	-.312	.323	-.470	.123
LNnT	.473	.120	.134	.678	-.408	.187	.110	.733
LNFP1	-.147	.649	.294	.354	.308	.331	-.133	.681
LNFP23	n.c.		-.056	.862	-.007	.982	.134	.677
LSTa	n.c.		.032	.923	**.674***	**.016**	.175	.587
LSTb	n.c.		-.167	.605	.131	.685	.088	.787
LSTc	.239	.454	.378	.226	-.226	.480	-.452	.140
LNDFH	-.393	.206	-.168	.601	-.224	.484	-.235	.463
LNH	.301	.342	.399	.199	**.737****	**.006**	**.615***	**.033**
DSLNT	0.000	1.000	-.228	.477	.209	.514	-.296	.351

Secondly, we analysed correlations of same-visit data, as well as correlations of HMO data subsequent to PA measurement. Neither HMO and PA data from the same visit, nor HMO data from the subsequent visit to later trimester PA-measurement, yielded any significant correlations.

Exception was the LNT fraction of the total HMO measured at delivery, which was found to be strongly and significantly negatively correlated with the time spent in MVPA around visit 3 (rho = - 0.836; p=0.001; significance level p=0.01; see figure 36).

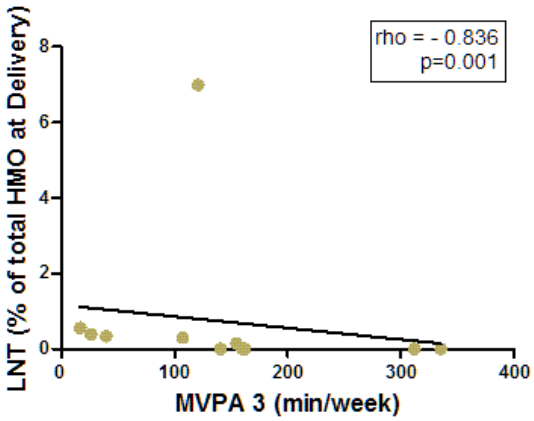


FIGURE 37. CORRELATION BETWEEN MVPA AT V3 AND LNT PERCENTAGE OF TOTAL HMO AT DELIVERY. Spearman's rho was calculated and showed a strong negative correlation (- 0.836) that was statistically significant (p=0.001, significance level p<0.01).

5.4.3.2 PA was Negatively Associated with HMO Concentration

We found that the MVPA correlated negatively with total HMO concentrations, measured at the visit following the respective PA-recording. Significant correlations were calculated for PA at V1 and HMO at V2. We found a trend but no statistically significant correlation of MVPA at V2 with the HMO concentration at V3, but no correlation for PA at V3 and HMO concentrations at delivery.

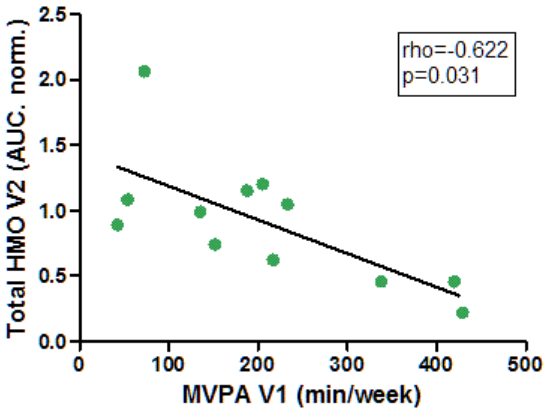


FIGURE 38. CORRELATION BETWEEN MVPA AT V1 WITH TOTAL HMO CONCENTRATION AT V2. Spearman correlations were calculated, showing a strong negative correlation (rho=-0.622; p=0.031).

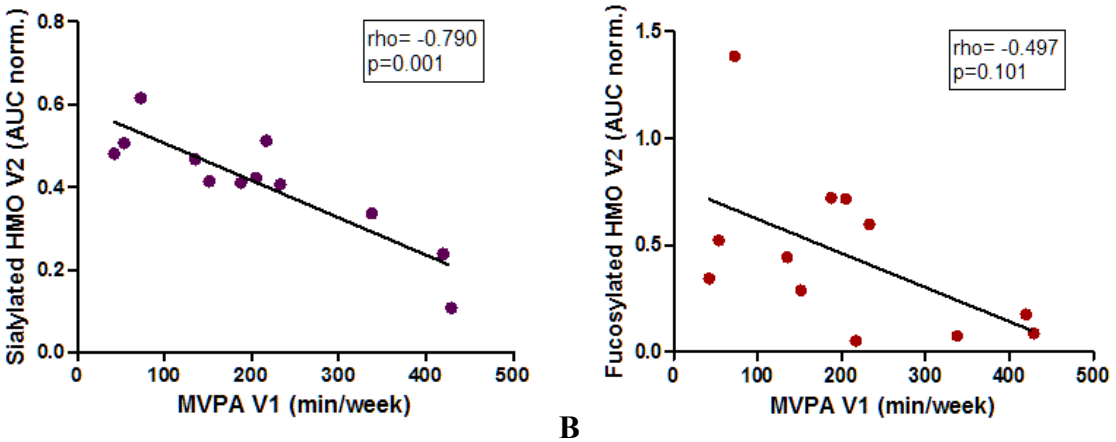
5.4.3.2.1 Subgroups of HMO Showed Different Correlations

We also investigated whether subfractions of HMO correlated with MVPA. To this end, we summed up the individual HMO into subgroups, based on their chemical modification, whether they were sialylated, fucosylated or unmodified.

TABLE 18. CORRELATIONS BETWEEN CONCENTRATIONS OF HMO SUBGROUPS AND PRIOR MVPA. * – significance level p=0.05; ** – significance level p=0.01. Sialylated and unmodified HMO from V2 are negatively correlated with prior MVPA from V1, fucosylated peripartum HMO (Delivery) are negatively correlated with prior MVPA (V3). HMO concentration values are AUC norm. to raffinose.

	MVPA (V1) / HMO (V2)		**MVPA (V2) / HMO (V3)**		**MVPA (V3) / HMO (Delivery)**	
	rho	p-value	rho	p-value	rho	p-value
Sialylated HMO	**-.790****	**.002**	-.264	.433	-.140	.665
Fucosylated HMO	-.497	.101	-.009	.979	**-.615***	**.033**
Unmodified HMO	**-.580***	**.048**	-.160	.639	-.416	.179

Analyses showed that the concentration of sialylated and unmodified HMO at V2 were significantly and negatively correlated with MVPA from the V1, fucosylated HMO showed a trend towards a negative correlation. No correlations were found in the second set (HMO from V3 and MVPA from V2). In the last set, fucosylated HMO in maternal serum at delivery were significantly correlated with MVPA at V3 (rho= -0.615, p=0.033).



A **B**

FIGURE 39. CORRELATIONS OF CONCENTRATIONS OF HMO SUBGROUPS AND MVPA AT V1. MVPA was measured at V1 and expressed as total min per week. Significant negative correlations (Spearman) were found for summed unmodified HMO (rho= 0.58 p=0.048, not shown), and **(A)** summed sialylated HMO (purple; rho=-0.790, p=0.001), whereas **(B)** summed fucosylated HMO (red; rho=0.497, p=0.101) were not significantly correlated.

5.4.3.2.2 Individual HMO were Differentially Associated with MVPA Concordantly

We next asked which individual HMO contributed most to the associations found between MVPA and concentration of HMO subgroups. Spearman's rho was calculated for individual HMO in correlation to MVPA of both the same visit and the previous visit.

We found a number of individual HMO showing negative correlations with previous exercise, as well as with exercise in early pregnancy.

Some individual HMO with a negative correlation with MVPA, were found: sialylated HMO (3'SL and 3'SLN - see Table 19; Fig. 39), and fucosylated individual HMO (LDFT – Fig. 40) were significantly negatively correlated with previous MVPA.

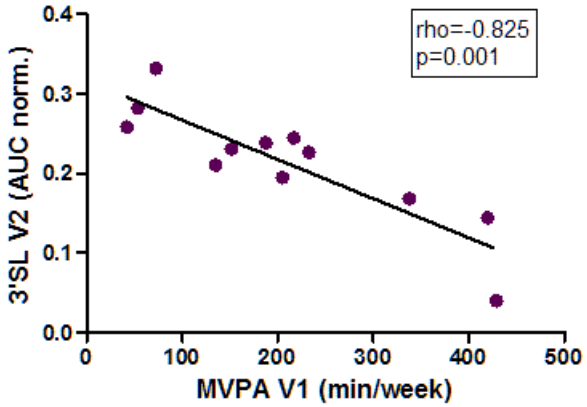
5.4.3.2.2.1 Sialylated HMO

When we analysed individual sialylated HMO, we found a number of negative correlations with MVPA. Physical exercise measured in the first trimester showed statistically significant negative correlations with 3'SLN, 3'SL, 6'SLN, and LSTc in the second trimester (Table 19).

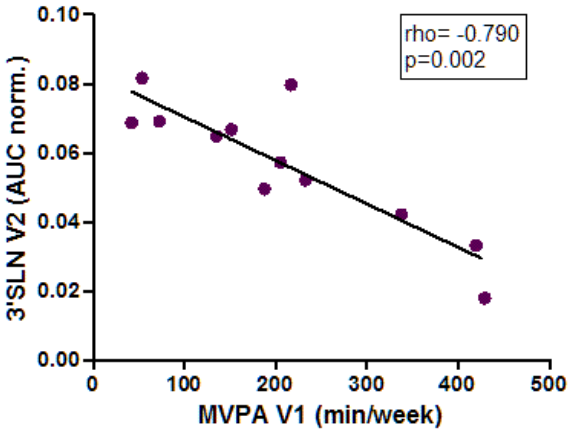
TABLE 19. CORRELATION OF SIALYLATED HMO AT V2 AND MVPA MEASURED AT V1. Spearman's correlation was calculated with: ** significance level was 0.01; * significance Level was 0.05 (two-tailed).

HMO (V2)	Total MVPA (V1)	
	rho	p-value
3'SLN	-.790**	.002
3'SL	-.825**	.001
6'SLN	-.587*	.045
LSTc	-.587*	.045

Although the finding was not statistically significant, DSLNT at delivery was found to have a trend towards negative correlation with exercise levels from the previous visit 3 (rho= -0.563; p=0.056).



A



B

FIGURE 40. CORRELATIONS OF 3'SL AND 3'SLN (V2) AND EARLY PREGNANCY MVPA (V1). Scatterplots of 3'SL **(A)** and 3'SLN **(B)** in the second trimester showed a correlation to early pregnancy MVPA levels. 3'SL (rho=-0.825, p=0.001) and 3'SLN (rho=-0.790, p=0.002) both showed significant negative correlations with MVPA from the previous assessment point.

5.4.3.2.2.2 Fucosylated HMO

LDFT was both at V1 and V2, negatively correlated with previous MVPA (LDFT at V2 and MVPA at V1: rho= - 0.587, p=0.045; and LDFT at delivery correlated with MVPA at V3: rho= - 0.678; p=0.15; sign. level 0.05). Peripartum serum levels of LNFP23 were also found to be negatively correlated with late pregnancy MVPA levels (rho= - 0.683; p=0.014).

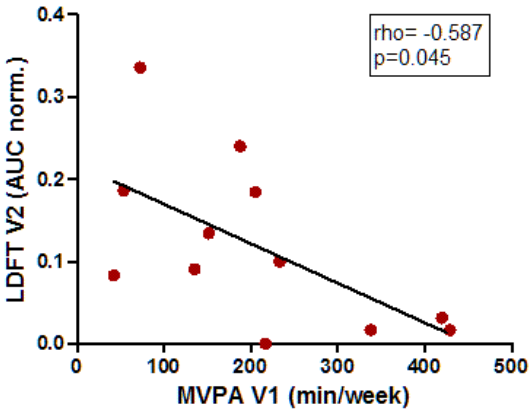


FIGURE 41. CORRELATION OF LDFT CONCENTRATION (V2) AND MVPA (V2). A moderate negative correlation of LDFT at V2 to MVPA at V1 can be seen (rho=-0.587, p=0.045). The negative association of LDFT at delivery with late pregnancy activity levels is not shown (rho=-0.678, p=0.15).

5.4.3.2.2.3 Unmodified HMO

Summed unmodified HMO were associated with previous MVPA in early pregnancy (V1). Out of this group of unmodified HMO, the only individual unmodified HMO found to correlate with previous MVPA was LNT at delivery (with MVPA at V3; rho= - 0.806; p=0.002; Fig. 41).

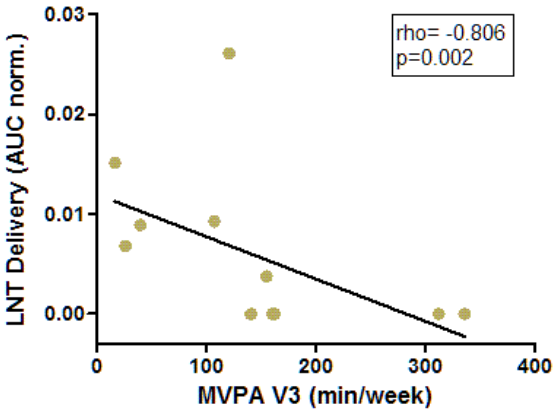


FIGURE 42. CORRELATION OF LNT CONCENTRATION AT DELIVERY AND MVPA (V3). LNT was negatively correlated with previous PA in late pregnancy; rho= - 0.806; p=0.002.

6 Discussion

In this thesis, we investigated the association of objectively measured MVPA and human milk oligosaccharide (HMO) concentration and composition in maternal serum during pregnancy. We found that MVPA in pregnancy did not have a significant effect on overall HMO composition in maternal serum. However, MVPA early in pregnancy correlated negatively with HMO concentration in maternal serum. No associations of maternal BMI and gestational weight gain in the same group could be found.

Strengths and limitations of the study

This study was the first pilot study to investigate HMO in maternal serum and their associations with maternal and environmental factors. The longitudinal observational design allowed investigating temporal changes of MVPA and serum HMO and their relationships at different time points in pregnancy and at delivery. With additional data from questionnaires, data prior recruitment at 10–14 weeks, i.e. pre-pregnancy data, were gained. Although some of the questionnaire-based data had to be excluded due to inaccuracies and unreliability by the participants, weight-related data was complete and could be included for further analysis. The numerous outcome variables were very diverse; they covered a multitude of possible confounders for later statistical adjustment, and comprised cytokines, lipid serum profiles, etc., which enabled synoptically analysing interactions and associations. They were, however, not included in this thesis.

The study, being observational, would have an evidence level of 2–3. With a well-designed longitudinal observational study, anticipating possible confounding by rigorous research design and later by statistical techniques to adjust for their effects, it is possible to see temporal associations and infer about causality of the investigated relationships. Here, however, the sample size was too small for statistical adjusting. Therefore, as a major limitation of this thesis, we cannot rule out a random bias in the results.

A major advantage of our study was the objective measurement of PA. This helped to overcome reporting bias in study subjects and provided comparable outcomes that were valid inter-individually. A possible factor distorting measured PA time, may be that participants were more attentive of their activity patterns once wearing the device and hence, tried to spend more time exercising and reaching recommended PA levels; Another limitation of this study might be, that we assorted to the active and inactive group based on

their first trimester measured activity level, without including water-borne activities. This could lead to potentially misrepresenting actual time spent exercising in women who were enrolled in pregnancy aqua gym classes. Water-borne activity was not quantifiable by the non-waterproof device we used, and questionnaires used to detect water-borne sports proved to be a rather unreliable source of information on PA and therefore, were not included in final analysis.

However, when analysing the overall PA profiles in pregnant women, we found a decrease of around 30% in MVPA throughout pregnancy, as well as constant sedentary behaviour. These findings were well in line with other studies using accelerometer data in pregnancy (91,94,99).

Van der Wijden *et al*. reported an overall 17% decrease in PA in a cohort of obese and overweight women. However, women already started with a low median of 6.5 min/day (range: 1.0–25.7). This corresponds to a median of 32.5 min/week, whereas in the present study, women started off with nearly the sixfold amount of time spent in significant PA, i.e. the 5% trimmed median for visit 1 MVPA was 203,8 min per week (minimum 42 min, maximum 428 min) (94).

The decrease of MVPA in pregnancy, a causal factor may be the continuously advancing pregnancy, and hence, increasing physiological limitations concerning mobility. Ruifrok *et al*. conducted a study to assess the relationship of objectively measured PA and sedentary behaviour with gestational weight gain and birth weight. They found that while 31% of women in the first trimester met the ACOG guidelines, spending more than 150 min/week with MVPA, in the last trimester, only 12% of all women did. Their MVPA decreased from 120 ± 80 min/week to 90 ± 110 min/week, whereas sedentary behaviour remained stable during pregnancy (91). These findings were confirmed in our sub-analysis for PA, where MVPA was making up 2–3% of total recording time, in accordance with Ruifrok *et al*.

Strengths of our study design were the extensive data collection and the frequent assessment points during pregnancy and at delivery. Moreover, the first assessment point for our "physical activity in pregnancy" (PAP) study was earlier than in comparable studies in the literature (91,94,99). However, the variability of gestational age at respective visits was relatively high. At the first visit the range was only 14 days, with a minimum GA of 78 days and a maximum gestational age of 92 days (mean 83.43 ± 3.12 days), ranging from 11–13 weeks where the visit was set to be between weeks 10–14. Later on, during the visits 2 and 3, the range was much wider. Gestational ages at V2 varied from a

minimum of 18 weeks to a maximum of 25 weeks (131 days – 180 days), where the second visit was set to be between 20 and 24 weeks. This resulted in a 7 week range (mean GA at V2: 147.57 ± 5.8 days). The range of gestational days labelled as the same visit even increased at V3, resulting in a range of 8 weeks for the gestational age at the third visit. The minimum was 31weeks and the maximum was 39 weeks (221 days and 278 days respectively; mean 240.14 ± 7 days). This leaves the interval between maximal GA at V2 and the minimal GA at V3 smaller (41 days) than the range within V2 and V3 (49 days and 57 days, respectively). It also leads to an overlapping between the total range of gestational ages at V3 and the gestational ages of the neonates at the delivery (V3: 221–278 days, Delivery: 265–289 days). For future studies, it would be advantageous to tighter monitor the scheduling of visits to the outpatient clinic.

In our study, we noticed that the BMI, in the whole study population as well as in the subgroup of women analysed for PA, was not as diverse as in comparable other studies in healthy women found in literature (91,94,99). In the subset of 12 women analysed, all women were lean with a pre-pregnancy BMI of <25 kg/m², with only one woman having a BMI of 25,15 kg/m² later at the first visit. Arguably, the women interested in participating were already more aware of a healthy lifestyle and might have been more compliant with the PA measurements and other assessments. This is indicated by the small number of overweight women in our study. Out of the initial group (n=52), from which the women for analysis of this thesis were drawn, 20.8% had a BMI over 25 kg/m²; the mean BMI there was only slightly higher with 22.4 ± 2.7 kg/m² as compared to the sub-group of 12 women with complete data sets with 21 ± 2.6 kg/m². This apparent bias due to study design, leading to a small set of solely lean women completing the study, can also be seen as an advantage. This way we were able to investigate the effect of PA independent of BMI, since the group studied was very homogeneous with regards to BMI. However, other factors such as gestational weight gain (GWG) might still be a possible confounder. Notably, the mean GWG surpassed the recommended limits of 15 kg (mean overall GWG = 17.18 ± 4.52 kg). For statistical reasons this could not appropriately be addressed by our analysis, since this study lacked statistical power for linear regression models to be calculated. Other studies have looked at the influence of PA for instance on inflammation markers in overweight and obese women only (99). Mean baseline MVPA early in pregnancy are likely different in normal weight versus overweight and obese women. Therefore, our results in lean women may not be transferred to overweight and obese women. Further studies are needed to investigate the influence of MVPA on maternal

HMO.

Moreover, since recruitment took place in the University hospital's outpatient clinic and no registered doctors were involved in recruitment, the sample is not representative for a larger population. We know that HMO composition is regionally different (80). The recruitment was, since it was a small pilot study, local. Ideally a multi-centric study would have to be conducted to overcome methodological issues in subject selection and replicate findings.

On the other hand, this uniformity of baseline demographics is advantageous for small sample size of the sub-analysis conducted. The longitudinal observational study was intended as a pilot study in order to investigate feasibility of methods and techniques. A sample size calculation beforehand was therefore not applicable, and with a loss to follow up and missing data the final size was reduced to 22% of the initially recruited women. Attrition bias should normally not exceed 20% of initial sample size.

The group of participants lost to follow-up, together with those excluded for not having complete data-sets for this specific question, was compared to the final group of study participants included in analysis (n=12). We analysed baseline characteristics, PA profiles and amounts, HMO concentrations and compositions with Mann Whitney U Test. We could only find a significant difference in weight gain from V2 to V3 between the two groups (p=0.033) in baseline characteristics. No differences in PA were found. Some differences in HMO concentrations could be found, 3'SL at visit 1 was higher in our subgroup (p=0.032) and LNT at visit 2 was higher in excluded participants (p=0.01). The two groups were relatively similar.

However, for the small sample size, we could not follow our initial approach for statistical analysis, where we planned to assort women to a group of active women, who spent more than 150 min per week in significant PA, and a group of inactive women spending less than 150min/week exercising. The initially defined cut-off for assorting based on the women's exposure to PA was not applicable since not enough women met this guideline. We decided to employ the median of time spent in MVPA in this subset of women. Still, the method was applicable only in limited ways. Therefore, we proceeded to analyse the women as a case series.

However, the results found regarding correlations of HMO concentrations and compositions were consistent. We compared them to results found for those data entities in the larger study group, and where analyses were possible, they corresponded in the majority of cases.

PA and HMO concentration – possible mechanisms

Although no conclusion on causality can be drawn from these correlation studies, possible mechanisms leading to a negative correlation of HMO concentrations and MVPA may be i) an altered HMO synthesis in the mammary gland, ii) a potential shift of HMO in compartments, or iii) an altered HMO clearance. These changes in maternal HMO levels could be due to PA induced physiological cardiovascular effects, such as blood flow redistribution, to PA mediated metabolic effects, such as an elevated glucose metabolism, or due to systemic cytokine responses as well as myokine responses of the muscle, triggered by physical exercise, and many more.

The energy sources may be diverted away from the HMO synthesis, due to a higher priority of keeping the muscle sufficiently provided with energy sources. To keep such a sophisticated biosynthesis going, may not be possible due to energy homoeostasis. Other maternal factors, such as diet, could also play a role in modulating maternal HMO. In animal studies, lactation was found to be influenced by high fat diet, delaying onset of lactation and showing a disrupted morphology of the mammary gland in rats fed a high fat diet. Consistently, the authors found a significant decrease in mRNA expression of milk protein genes and glucose transporter 1 (GLUT1) (82). The mammary epithelial cells use facilitative glucose transporters (GLUT), expressing mainly GLUT1 and GLUT8 (113). Glucose is the main precursor for lactose, which is synthesised in Golgi vesicles of the mammary gland, as well as one of the HMO building blocks. Therefore, high fat diet may influence the synthesis of HMO, which is thought to take place in the lactocytes using glucose and galactose as building blocks.

Furthermore, during PA the consumption of saccharides in order to provide an effective energy source, may lower the availability of HMO building blocks, needed for synthesis. In a study analysing substrates in exercising pregnant women, it was discovered that levels of glucose decreased post-exercise (112). Stating that 68% of galactose in milk is derived from plasma glucose during maternal postprandial state as opposed to 50% in a fasting state, Smilowitz *et al*. found that overall galactose levels in human milk were higher when comparing postprandial with fasted states (84). Galactose is likely transported via GLUT1 into the Golgi of lactocytes (113), where HMO synthesis is thought to take place. During physical exercise, plasma glucose is usually used by skeletal muscle as an energy source. This higher cellular uptake of glucose, and other possible saccharide substrates for HMO synthesis, may lower HMO production via lacking precursors.

Furthermore, in pregnant women higher levels of free fatty acids were found during exercise (112). PA may indirectly influence HMO synthesis by interfering with fat levels. This would be consistent with the negative correlation of HMO concentrations and PA we found.

The increased GLUT expression during lactogenesis is not stimulated by the lactogenic hormones, new evidence however suggests that low oxygen tension may play a role in GLUT1 expression in mammary epithelial cells. Low oxygen tension may occur by increased oxygen consumption due to an increased metabolic base rate in pregnancy (113), but may also be the result of increased oxygen consumption during exercise. In women undergoing regular aerobic exercise during pregnancy, higher cardiorespiratory fitness and higher flow-mediated dilatation was measured, meaning an improved endothelium-dependent vasodilation in pregnancy (114). Also, the British UPBEAT study suggests a better glucose control in active pregnant women (115). These may be pathways influencing HMO synthesis via substrate availability.

Exercise was also shown to alter gene expression in placental tissue (116,117). The expression of various transport proteins in the placenta was altered, among them GLUT1 showing a high correlation with sugar intake. This ability of physical exercise to impact tissues crucial to pregnancy, is possibly contrary to the insensitivity of pregnancy-associated metabolism postulated by some (96). In further consequence, this may implicate a direct effect of PA on gene expression of enzymes involved in HMO synthesis, perhaps also via alteration of gene expression of human placental lactogen secretion in the placenta and other hormonal factors influencing the pregnant hormonal state.

Expression of a gene, that is regulating mammary gland involution for lactogenesis, was also altered by high fat diet, suggesting a pathway through an increase in serotonin (5-HT) production within the mammary gland, ultimately leading to an inflammatory process (82). If a serotonergic pathway indeed alters mammary gland morphology and lactation outcomes, this may implicate a connection to physical exercise – in non-pregnant individuals, serum serotonin levels are linearly associated with PA (118). This pathway could also be consistent with the inverse correlation of HMO and PA we found.

Neutral HMO were suggested to use receptor-mediated transcytosis (22). A possible mechanism of how PA could influence HMO concentration, may be PA-mediated modulation of this receptor-mediated uptake of neutral HMO, altering the serum concentration. The question remains, how lasting this effect would be. Acidic HMO use

paracytotic ways (22), their uptake possibly being influenced by a different serum glucose gradient during exercise.

Concerning the direct influence of PA, it seems plausible that synthesis is positively influenced by a long-term and short-term increased blood flow to the mammary gland, where lactose, and most likely HMO, are synthesised already during pregnancy, i.e. increasing flow of simple substrates for HMO synthesis and enhancing the cell metabolism in the mammary gland. The mammary gland is vascularised by branches from the internal mammary artery (i.e. A. thoracica interna, branch from subclavian artery), which also supplies the pectoral muscles.
Isometric exercise of the upper extremity results in a short-term increased blood flow to the internal mammary artery. For amino acids for instance it is known that their abundance in milk is regulated not only by hormones, but also by blood flow through the mammary gland (119). The long-term effect regarding vascularisation and improved blood flow is mediated by relative hypoxia and various growth factors, such as VEGF (120).
On the other hand, especially with aerobic exercise such as cycling, the redistribution of the blood flow would mean blood flow increases in muscles used as well as in coronary and pulmonary arteries. Normally, this would mean a direct “steal phenomenon” from the internal mammary artery, i.e. away from any organs unimportant when engaging in exercise (121). Thus, relative decrease blood flow to the mammary gland may result in decrease of energy and building blocks for HMO transported to the site of synthesis.

In non-pregnant individuals PA increases serum levels of different pro-and anti-inflammatory cytokines (101,102). If this holds up in pregnant women, PA-induced cytokines might be capable of affecting the function of lactocytes.
Prolactin, known for its important role as regulator of cellular proliferation, was most intriguingly found to be increased by PA. Studies showed that depending on intensity and duration of PA, prolactin levels increase (76,122). It would be interesting to test, whether prolactin levels are influenced by PA in pregnancy, and whether prolactin levels are associated with HMO.
It was also found, that women exercising in pregnancy may have a higher TNF-α level due to a low-grade basal inflammation, as well as generally increasing after exercise (102). TNF-α may be a possible pathway to influence HMO concentration, since TNF-α was

shown *in vitro* to be a key inhibitor of lactogenesis and is involved in mammary gland development as a multifunctional regulator (99,123,124).
PA also stimulates a large increase of serum levels of different pro- and anti-inflammatory cytokines, which are also capable of stimulating the lactocytes. Another known effect of PA is its positive influence on insulin sensitivity, which is also known to have an effect on lactation (125).

MVPA in early pregnancy was strongly negatively associated with sialylated HMO, i.e. 3'SL at the following visit. If these findings can be confirmed in larger studies, one could speculate about a modulation of specific types of HMO, with potentially specific regulatory roles in pregnancy. 3'SL, for example, has been discussed to have pro-inflammatory effects (62,126). Thus, future studies are needed to test, whether 3'SL is also associated with other inflammatory factors.

Furthermore, there is a wide range of factors known to influence lactogenesis, and therefore, possibly also influencing HMO synthesis. In obese women a late onset of lactogenesis was found, together with a lower prolactin response rate to breastfeeding (127). In our study, the association of MVPA and HMO seems to be independent of other maternal factors, such as pre-pregnancy BMI and gestational weight gain, which were not correlated with PA, nor with HMO.

Since, due to the small sample size, incomplete data, and a likely attrition bias, no causal relation between HMO and exercise can be made yet, all these hypothesised mechanisms remain to be elucidated and verified in further research.
Our results showing an association of PA and HMO concentration in pregnant women warrant future investigations.

7 References

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Effects of Physical Activity during Pregnancy on Human Milk Oligosaccharides in Maternal Serum

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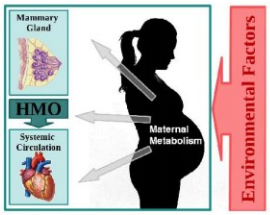
Background

Human Milk Oligosaccharides (HMO) in Pregnancy

- HMOs are bioactive glycans found not only in human milk, but already in pregnancy, in the systemic circulation of both mother and foetus.
- Some HMO structures are known to have anti-inflammatory and/or immunomodulatory effects.
- Pregnancy is a physiological state of low-grade inflammation.
- Physical activity is thought to reduce the inflammatory status in non-pregnant individuals. Currently, *WHO* recommends a minimum of 150 minutes per week of moderate-to-vigorous activity for adults to maintain health.
- Whether environmental factors such as nutritional status or physical activity influence prenatal HMO concentrations and composition is not known.



Hypothesis

We hypothesised that physical activity during pregnancy influences HMO levels and composition in maternal serum.

Subjects and Study Design

A - Study Design: Assessments during Pregnancy and at Delivery

Physical activity (PA) was measured three times during pregnancy (V1, V2, V3).
HMO were determined from maternal blood samples, which were collected at V1, V2, V3, delivery

B - Study Population Characteristics

12 healthy pregnant women, recruited at the Department of Gyn-Ob, University Clinic Graz, had a complete data set on PA measurements and HMO samples. Their age was 34.5 ± 5.5 years, gestational age (GA) at delivery was 275 ± 9 days. Their pre-pregnancy BMI was 22 ± 2.6 kg/m² with an overall BMI increase during pregnancy of 6.3 ± 1.6 kg/m².

Methods A – Physical activity

ActiGraph accelerometer

- Objectively measured by accelerometer.
- Daily logs of activity kept by participants for control purposes.
- To receive the most accurate results when collecting data, the ActiGraph had to be fixed to the body's centre-of-mass (around the hip).

Methods B – HMO Analysis

HMOs were determined from maternal serum samples collected:

- HMO were isolated using SPE to deproteinate and desalt 50µl serum samples, and after 2AB labelling, were analysed by HPLC with fluorescence detection. Peaks were defined by HMO standards.
- Retention time of 3FL coincided with an unidentified peak and values were thus omitted.
- Values are AUC normalised to raffinose.

Results A – HMO Concentration

A1. Physical activity correlates negatively with total HMO concentrations.

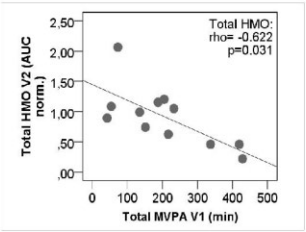


Fig 1. **Correlation between moderate-to-vigorous physical activity at visit 1 with total HMO concentration at visit 2.** Spearman correlations were calculated.

A2. Physical activity correlates negatively with sialylated HMO.

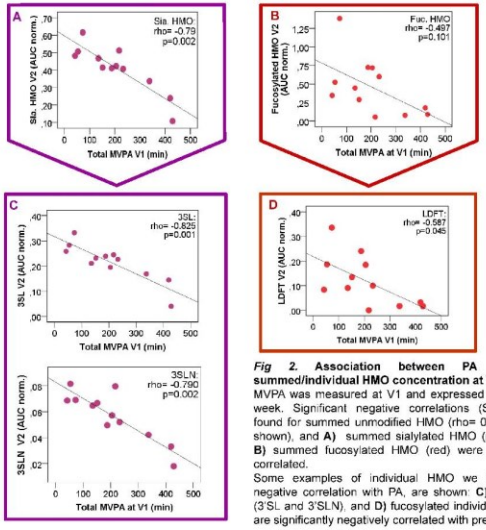


Fig 2. **Association between PA at V1 and summed/individual HMO concentration at V2.**
MVPA was measured at V1 and expressed as total min per week. Significant negative correlations (Spearman) were found for summed unmodified HMO (rho= 0.58 p=0.048, not shown), and **A)** summed sialylated HMO (purple), whereas **B)** summed fucosylated HMO (red) were not significantly correlated.
Some examples of individual HMO we found having a negative correlation with PA, are shown: **C)** sialylated HMO (3'SL and 3'SLN), and **D)** fucosylated individual HMO (LDFT) are significantly negatively correlated with previous MVPA.

Abbreviations & Colour Code

2AB	2-Aminobenzamide	LNT	Lacto-N-tetraose
2FL	2'-Fucosyllactose	LNnT	Lacto-N-neotetraose
3FL	3-Fucosyllactose	LSTa,b,c	Sialyl-lacto-N-tetraose a, b, c
3SL	3'-Sialyllactose	LNFP1	Lacto-N-fucopentaose1
3SLN	3'-Sialyllactosamine	LNFP23	Lacto-N-fucopentaose 2+3
6SLN	6'-Sialyllactosamine	LNDFH	Lacto-N-difucohexaose
6SL	6'Sialyllactose	LNH	Lacto-N-hexaose
HPLC	High Performance Liquid Chromatography	DSLNT	Disialyl-lacto-N-tetraose
LDFT	Lactodifucotetraose	SPE	Solid Phase Extraction

MVPA	moderate to vigorous physical activity
PA	physical activity
AUC	area under the curve
GA	gestational age
PURPLE	sialylated HMO
RED	fucosylated HMO
BEIGE	unmodified HMO

Conclusions

- In this small pilot study, we **did not find** that physical activity (PA), as measured objectively in early pregnancy, had **any effect on HMO composition** during the course of gestation.
- However, we found that **PA** correlated negatively with total or individual **HMO concentrations,** measured at the visit following the respective PA-recording (PA at V1 and HMO at V2, PA at V3 and HMO concentrations at delivery).
- PA at the first visit correlated significantly to HMO concentrations at visit 2, 3 and at delivery.
- No association was found of HMO and various BMI values (BMI pre-pregnancy, BMI at V1-Delivery, changes in BMI, etc.).

- In summary, we showed that PA, as measured objectively in early pregnancy, seems to influence the concentration of maternal HMO in serum.
- Larger studies are needed to confirm this finding, e.g. studies with physical activity as life style intervention.

Results B – HMO Composition

B1. The composition of HMO in groups of active and inactive women is not different.

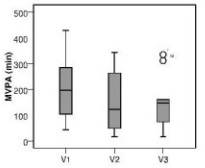


Fig 3. **Moderate-to-vigorous physical activity (min) during pregnancy (V1-3).**
5% trimmed median of V1 was 203.8 minutes, of V2 144.3 minutes and for V3 141.2 minutes.

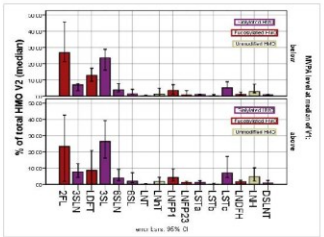


Fig 4. **HMO composition and MVPA of previous visit.**
Women were assigned into two groups: MVPA values above and below the median, calculated for each visit.
The groups were analysed for differences in maternal characteristics (weight gain, BMI pre-pregnancy, BMI at V1, V2, V3, Delivery, BMI changes, placenta weight, gestation age at V1-Delivery) and fetal characteristics (sex, length, weight, head circumference, percentile). The characteristics were evenly distributed.
The percentage of each individual HMO of the total concentration was analysed for significantly different distributions (Mann Whitney U) between the two groups.
No difference was found in HMO composition between women with MVPA below or above the median at any time point. In Figure 2, HMOs at visit 2 is shown in relation to MVPA level at visit 1.