Correlation of Prenatal Human Milk Oligosaccharides with Maternal and Fetal Inflammatory Markers

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Abstract

Introduction Human Milk Oligosaccharides (HMOs) are complex glycans unique in structure and highly abundant in human milk. Their impact on the suckling infant include prebiotic, antiadhesive, cellmodulating and immunemodulating effects. This impact might be relevant for pregnant women, too, since circulating HMOs can be found in the mother's blood during pregnancy.

Hypothesis We hypothesise that the maternal inflammatory status in early and midpregnancy influences serum concentrations of HMOs in pregnancy as well as in fetal cord blood. To test this hypothesis we performed association studies with the maternal and fetal inflammatory markers CRP, sICAM-1, MCP-1, MIP-1α and MIP-1β and the maternal HMOs 2'FL and 3'SL.

Methods For this prospective pilot study, 53 healthy pregnant women were recruited. Blood samples were taken three times during pregnancy and at delivery and analysed using clinical chemistry methods, High Performance Liquid Chromatography (HPLC) with fluorescence detection and multiplex assay technology. Data was analysed using *IBM SPSS Statistics 23*.

Results Data sets of 23 pregnant women were analysed. We found numerous significant correlations between maternal HMOs and maternal inflammation markers: CRP, sICAM-1, MCP-1 and MIP-1β correlated significantly with either 2'FL or 3'SL at distinct points of time. Moreover, we detected that maternal HMOs in midpregnancy are related to the fetal inflammatory markers CRP and sICAM-1. We could not reveal any significant correlation between MIP-1α and HMOs.

Discussion The associations between 2'FL, 3'SL, CRP, sICAM-1 and MCP-1 possibly indicate a link with inflammation. It remains difficult to interpret their relationship. Since pregnancy pathologies are often induced by inflammatory processes it would be helpful to further investigate the functions and interactions of both HMOs and inflammatory markers. In the future, HMOs might serve as biological markers to detect inflammatory conditions in pregnancy or could function as prophylactic therapeutics in pregnancy related pathologies.

Zusammenfassung

Einleitung Humane Milch-Oligosaccharide (HMOs) sind komplexe und strukturell einzigartige Glykane, die reichlich in der Muttermilch vorkommen. Ihre Auswirkungen auf den Säugling umfassen präbiotische, anti-adhäsive, zellmodulierende und immunmodulierende Veränderungen. Ein ähnlicher Einfluss auf die Mutter ist wahrscheinlich, da bereits während der Schwangerschaft zirkulierende HMOs im mütterlichen Blut nachweisbar sind.

Hypothese Wir stellen die Hypothese auf, dass sich der Entzündungsstatus der Schwangeren auf die mütterlichen und fetalen Serumkonzentrationen von HMOs auswirkt. Um dies zu testen haben wir statistische Analysen mit den Entzündungsmarkern CRP, sICAM-1, MCP-1, MIP-1α und MIP-1β und den HMOs 2'FL und 3'SL durchgeführt.

Methoden Für diese prospektive, beobachtende Pilotstudie wurden 53 gesunde, schwangere Frauen rekrutiert. Blutproben wurden dreimal während der Schwangerschaft und zum Zeitpunkt der Geburt bei der Mutter sowie aus dem Nabelschnurblut entnommen und mittels klassischer Laboranalysen, Hochdruckflüssigkeitschromatographie und Fluoreszenz-Detektion, sowie der Multiplex Assay Technologie analysiert. Die Daten wurden mit der Software *IBM SPSS Statistics 23* ausgewertet.

Ergebnisse Wir haben die Datensätze von 23 schwangeren Studienteilnehmerinnen analysiert und dabei multiple signifikante Korrelationen zwischen HMOs und mütterlichen sowie fetalen Entzündungsmarkern gefunden: CRP, sICAM-1, MCP-1 und MIP-1β korrelierten entweder mit 2'FL und/oder mit 3'SL. Für MIP-1α und HMOs konnten wir keinen Zusammenhang nachweisen.

Diskussion Die Assoziationen zwischen 2'FL, 3'SL, CRP, sICAM-1 und MCP-1 stehen möglicherweise mit dem Entzündungsstatus in Verbindung. Die genauen kausalen Zusammenhänge bleiben bislang ungeklärt. Da Schwangerschaftspathologien häufig durch entzündliche Prozesse eingeleitet werden wäre es hilfreich, die Funktionen und Interaktionen von HMOs und Entzündungsmarkern genauer zu erforschen.

Zukünftig könnten HMOs als biologische Marker zur frühzeitigen Erkennung pathologischer, schwangerschaftsassoziierter Entzündungsvorgänge dienen und in diesem Zusammenhang gegebenenfalls prophylaktisch eingesetzt werden.

Abbreviations

2'FL 2'-Fucosyllactose

2AB 2-Aminobenzamide

3'SL 3'-Sialyllactose

3'SLN 3'-Sialyl-N-Acetyllactosamine

3FL 3-Fucosyllactose

6'SL 6'-Sialyllactose

ACN Acetonitrile

AUC Area under the Curve

C. jejuni Campylobacter jejuni

CB Cord Blood

CCL CC-Chemokine Ligand

CCR C-C Chemokine Receptor

CCR2 C-C Chemokine Receptor Type 2

COX2 Cyclooxygenase 2

CRP C-Reactive Protein

DC Dendritic Cells

DC-SIGN Dendritic Cell-Specific ICAM3-Grabbing Non-Integrin

DSLNT Disialyllacto-N-tetraose

E. coli Escherichia coli

E. hystolytica Entamoeba hystolytica

ENOS Endothelial Nitric Oxide Synthase

EPEC Enteropathogenic Escherichia coli

Fuc L-Fucose

FUT2 α-1-2-Fucosyltransferase

FUT3 α-1-3/4-Fucosyltransferase

Gal D-Galactose

Glc D-Glucose

GlcNAc N-Acetylglucosamine

Gp120 Glycoprotein 120

H. pylori Helicobacter pylori

HIV Human Immunodeficiency Virus

HMOs Human Milk Oligosaccharides

HPLC High Performance Liquid Chromatography

ICAM-1 Intercellular Adhesion Molecule 1

IFN Interferon

Ig Immunoglobulin

IL Interleukin

IUGR Intrauterine Growth Restriction

Lacto-N-Biose Galβ1-3GlcNAc

LDFT Lactodifucotetraose

Le Lewis

LFA-1 Lymphocyte Function-Associated Antigen 1

LNDFH Lacto-N-Difucohexaose

LNFP I Lacto-N–Fucopentaose I

LNFP II Lacto-N-Fucopentaose II

LNFP III Lacto-N-Fucopentaose III

LNH Lacto-N-Hexaose

LNnT Lacto-N-Neotetraose

LNT Lacto-N-Tetraose

LPS Lipopolysaccharides

LST c Lactosialotetraose c

MAC-1 Macrophage-1 Antigen

MCP-1 Monocyte Chemoattractant Protein 1

MIP-1 Macrophage Inflammatory Protein 1

MMP Matrix Metalloproteinase

NEC Necrotizing Enterocolitis

NK Natural Killer

PG Prostaglandin

ROS Reactive Oxygen Species

RSA Recurrent Spontaneous Abortion

RSV Respiratory Syncytial Virus

SCFA Short Chain Fatty Acids

Se Secretor

Sia Sialic Acid

sICAM-1 Soluble Intercellular Adhesion Molecule 1

Siglecs Sialic Acid Binding Ig-like Lectins

Th T Helper Cell

TLR Toll Like Receptor

TNF Tumor Necrosis Factor

Tregs Regulatory T Cells

V1 Visit 1

V2 Visit 2

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1 Introduction

1.1 Human Milk Oligosaccharides

Human Milk Oligosaccharides HMOs are a group of complex bioactive glycans present in human milk. Their amount and complex composition is unique to humans (Bode 2006). With the origin of HMOs research dating back over a century ago, other scientists blazed the trail and led to an increasing knowledge about HMOs today.

1.1.1 Structure

HMOs are composed of five monosaccharides: D-Glucose (Glc), D-Galactose (Gal), N-Acetylglucosamine (GlcNAc), L-Fucose (Fuc) and Sialic Acid (Sia) with N-acetylneuraminic acid being exclusively found in HMOs. Their reducing end is formed by lactose (Galβ1-4Glc), a sugar that is practically always the core structure of HMOs (Zivkovic et al. 2011, Sprenger et al. 2017). The lactose can be modified by addition of other disaccharides such as Galβ1-3GlcNAc or N-acetyllactosamine (Galβ1-4GlcNAc) via β1-3- or β1-6-linkages. Depending on the type of linkages, linear HMOs (*para-HMOs*, linkage type β1-3) or branched (*iso-HMOs*, linkage type β1-6) molecules arise (Bode 2015). Modification of the oligosaccharide backbone happens via sialylation and fucosylation. Responsable for sialylation are the so called sialyltransferases that bind sialic acid to lactose or other components of the oligosaccharide chain. α2-3 linkage generates 3'-Sialyllactose (3'SL) whereas α2-6 linkage generates 6'-Sialyllactose (6'SL) (Bode 2012).

α-1-2-Fucosyltransferase (FUT2) and α-1-3/4-Fucosyltransferase (FUT3) are enzymes that connect the monosaccharide fucose by either an α1-2, α1-3 or an α1-4 bondage to HMO molecules. This reaction is called fucosylation. Such fucosylated molecules are, for example, 2'-Fucosyllactose (2'FL), Lactodifucotetraose (LDFT), Lacto-N–Fucopentaose I (LNFP I) and Lacto-N-Difucohexaose (LNDFH). Whether and which of the two enzymes are more present in an individual depends on genectics. The Secretor (Se) gene encodes FUT2 whereas FUT3 is encoded by the Lewis (Le) gene. Although there is a huge variety of HMOs with more than 200 individual HMOs that have already been described, the Secretor and Lewis blood group system allows to classify HMO profiles

into four major milk groups (Bode 2012, Zivkovic et al. 2011). Figure 1 (modified based on Bode 2012) displays the Secretor and Lewis blood group system.

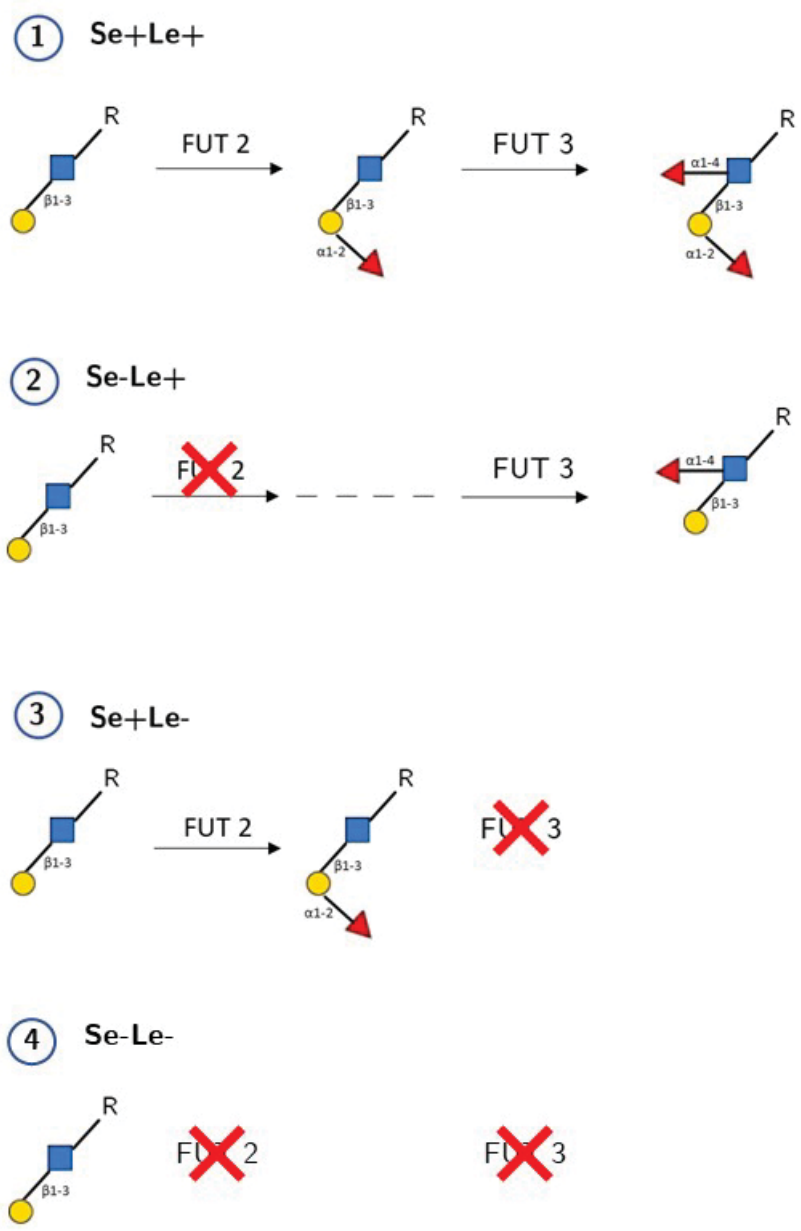

Figure 1: Secretor and Lewis Blood Group Status

In Se positive (Se $\oplus$) women who have an active Se gene and express FUT2, α1-2-fucosylated HMOs are most common (50-80%), whereas in Nonsecretors (Se $\ominus$) who lack an active FUT2 enzyme, α1-2-fucosylated are practically absent (Sprenger et al. 2017, Bode 2012). However, fucosylated oligosaccharides could be detected in Se $\ominus$ Le $\ominus$ women as well, a fact that led Newburg *et al.* assume that other pathways of enzyme activity might exist that have not been yet explored (Newburg et al. 2005). Moreover,

the variation of HMOs in women is to a large part determined by their activity or non-activity of FUT2. However, certain maternal factors and also environmental factors might influence HMO concentrations and composition in a mother's milk, although studies investigating this are still pending.

Figure 2 depicts selected HMO structures built by their five main monosaccharide units glucose, galactose, N-acetylglucosamine, fucose and sialic acid. Trisaccharides such as 2'FL, 3FL, 3'SL and 6'SL are structurally the simplest HMO (Bode & Jantscher-Krenn 2012). They can be elongated to more complex linear or branched HMO.

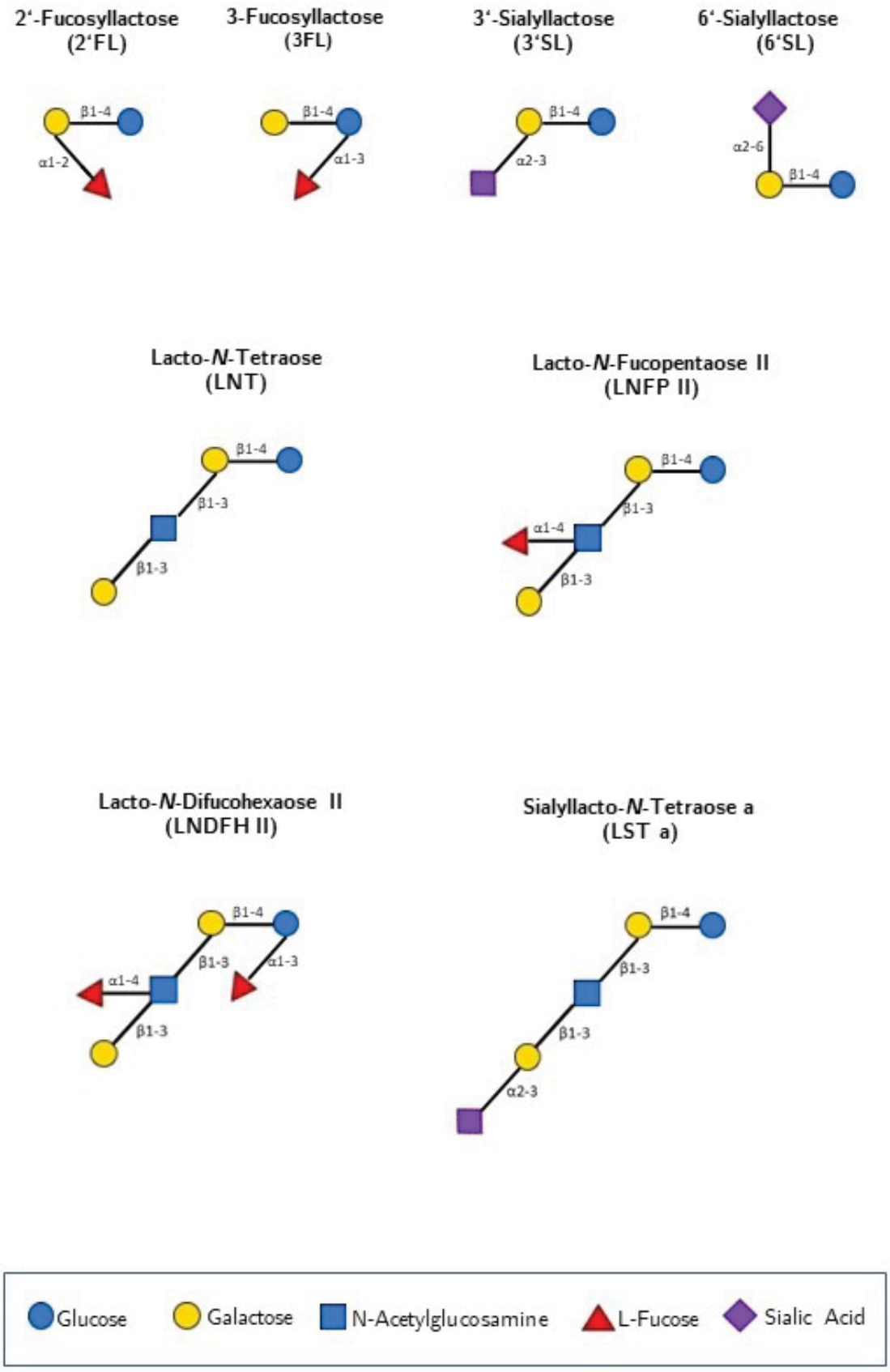

FIGURE 2: SELECTED HMO STRUCTURES

1.1.2 Biosynthesis and Sources of HMOs

The composition of HMOs is unique to humans and demonstrates that research on human milk oligosaccharides using animal models is not adequate. For instance, mouse milk only contains 3'SL and 6'SL and more complex fucosylated oligosaccharides as they occur in human milk cannot be detected at all. Interestingly, milk oligosaccharides of elephants are quite similar to human milk oligosaccharides (Bode 2012). The knowledge of natural biosynthesis pathways is still quite limited. It is known that the biosynthesis of HMOs takes place in the mammary gland where lactose is formed in the Golgi apparatus of lactocytes. Lactose seems to be a constant building block of almost all HMOs and may be modified and elongated afterwards by diverse enzymes. Yet, the mechanisms of HMO synthesis remain to be elucidated (Smilowitz et al. 2014, Bode 2012).

The production of HMOs has been quite challenging but some promising progresses have been made lately. By chemical and enzymatic processes as well as by whole-cell biotransformation with bacterial cells the synthesis of simple, short chain HMOs has been succeeded. Bacterial strains, capable of producing carbohydrate building blocks, are genetically modified to express specific enzymes of the HMO biosynthesis pathway. They are then used to produce specific HMO structures. 2'FL, 3'SL and other HMOs such as Lacto-N-Tetraose (LNT) are currently produced on a large scale. However, production of more more complex structures still faces significant obstacles (Sprenger et al. 2017, Soetaert 2016).

1.1.3 Appearance in Human Milk and Metabolism in the Breasted Infant

Appearance. The total amount of HMOs as well as their composition are individually different and change constantly during lactation (Sprenger et al. 2017, Bode 2012, Zivkovic et al. 2011). HMOs make up the third largest fraction of solid ingredients in human milk after lactose and lipids (Jantscher-Krenn & Bode 2012). The highest concentration of HMOs is found in colostrum, the milk that is produced in pregnancy and in the first three days after childbirth, where concentrations of 20-25g/l are reached. The milk of mothers who delivered preterm infants is richer in HMOs than the milk of

mothers who had a term baby. Throughout the course of lactation total amounts of HMOs are declining to 5-20g/L (Bode 2012).

Metabolism. Breast-fed infants on average ingest 50-150mg HMOs per nursing (Rudloff et al. 2012). HMOs resist – at least in large parts – the multiple mechanisms of digestion and are present in the neonate's feces. Additionally, HMOs have been detected in breast-fed infant's urine for the first time in 1996 (Rudloff et al. 1996). That suggests that at least some parts of them are intestinally absorbed. Consequently, HMOs circulate in the blood and could potentially act systemically. To further explore to which extend HMOs are renally excreted Rudloff *et al.* gave breast-feeding women ^{13}C-labelled galactose and sampled the infants urine for 36 hours. Shortly after the oral intake of the mother, the ^{13}C-molecules were present in the breast milk as well as in the infants' urine, either in their intact shape or slightly altered. This showed that Galactose has been directly brought to mammary epithelium and processed into HMOs. The detection of LNT, Lacto-N-Fucopentaose II (LNFP II) and the difference between HMOs ingested and excreted suggested that firstly, absorption must take place, and secondly, HMOs reach the circulation (Rudloff et al. 2012). This suggestion was consolidated by Goehring *et al.* who provided direct evidence of distinct HMOs (2'FL, 3-Fucosyllactose (3FL), Lacto-N-Neotetraose (LNnT)) in the circulation of breastfed infants for the first time (Goehring et al. 2014). At the same time, Ruhaak *et al.* distinguished different HMO profiles in the plasma of breastfed and bottle-fed infants (Ruhaak et al. 2014).

1.1.4 Effects on the Neonate

In the early days of HMO research was focused particularly on the prebiotic effect on the microbiota of the newborn. In the last decades there has been a large expansion of the potential effects of HMOs.

Background. The very first impact of breast feeding on the neonate that attracted the researchers interest in the late 19$^{\text{th}}$ century was the correlation between lower incidences of diseases in breastfed in contrast to bottle-fed infants, leading to a significant lower first-year mortality rate of the former. Moreover, human milk carbohydrates

were found to be structurally different than in bovine milk (Bode 2012).
In 1900, two investigators, Ernst Moro and Henry Tissier, revealed that the bacterial colonization of the neonate's bowel differs between breastfed and bottle-fed infants. Another three decades later the now called 'Bifidus factor' was discovered. Alongside, the additional carbohydrate fraction in human milk was analysed in greater detail and was then named 'gynolactose'. Finally, in 1954, Kuhn and György concluded that the 'Bifidus factor' was build up of oligosaccharides in human milk (Bode 2012). Ever since, more and more HMOs have been identified, facilitated by new analysing methods. Yet, many questions on structure-function relationships of HMOs remain unanswered until today.

Prebiotic Effects. Prebiotic characteristics describe the traits of being indigestible and to advance the composition, activity and/or growth of determined bacteria in the gut evoking health advantages (Bode 2012). The colonization of the intestine with bacteria influences our organism in many more ways than 'only' on a local level. A dysbalance of the desired microbiota is related to a higher risk of numerous conditions such as obesity, metabolic syndrome, chronic inflammatory bowel diseases, allergy, asthma, to only name a few of them (Zivkovic et al. 2013). Bifidobacteria belong to the predominant microbes in the infant's gut where they promote a healthy state, e.g. by producing Short Chain Fatty Acids (SCFA) and anti-microbial substances, rivaling pathogens for the restricted nutricient offer, diminishing inflammation and supporting gut impermeability - all in all making it harder for pathogens to survive (Zivkovic et al. 2013, Bode 2012, Wong et al. 2006, Gibson & Wang 1994). As shown in several studies, some bifidobacteria are able to survive with HMOs being the only food source, e.g. *B. longum* subsp *infantis* (B. infantis). *B. infantis'* genome expresses enzymes that provide transport and processing of HMOs. Sialidases, fucosidases and a lacto-N-biosidase are responsible for breaking down Galβ1-3GlcNAc (Lacto-N-Biose) so that the bacterium can then metabolise it (Smilowitz et al. 2014). Not all species of bifidobacteria are adapted to degradation of HMOs that well, which explains why *B. bifidum* grows slowlier on HMOs and is in contrast to *B.infantis* not able to digest all components of the oligosaccharides. *B. longum* subsp *longum* and *B. breve* can not proliferate on HMOs and digest only LNT (Galβ1-3GlcNAC-Lac, type I) (Bode

2012). Concluding, the prebiotic effect of HMOs on selected bacteria in the infant's bowel helps to provide a well-balanced microbiota and therefore promote the health of neonates in their first months or years of life.

Antiadhesive Effects. The antiadhesive characteristics of HMOs are based on their mimicry of glycans that are found on mucosal cell surfaces and needed by pathogens as a docking system to infect the organism. Therefore, HMOs serve as soluble ligand analogs or decoy receptors. Where HMOs are present, microorganisms bind less on mucosal cells and infections can be reduced (Sprenger et al. 2017, Bode 2012). The responsible lectin-glycan interactions are nevertheless quite specific, so that each pathogen has it's own 'preference' of binding molecule. *Escherichia coli (E. coli)* with type 1 fimbriae, for instance, adheres to mannose-containing glycans whereas *E. coli* with type S fimbriae connect to sialylated gylcans (Bode 2012). As reviewed by Lars Bode, clinically important bacteria, viruses and protozoas such as *Helicobacter pylori (H. pylori)*, *Campylobacter jejuni (C. jejuni)*, noro- and rotavirus, calicivirus, Human Immunodeficiency Virus (HIV) and *Entamoeba hystolytica (E. hystolytica)* interact via lectin-glycan or glycan-lectin binding and thus are influenced by the presence of HMOs. Diarrhea episodes caused by *C. jejuni* appear less often in children being breastfed by mothers with a high amount of 2'FL in their milk. 2'FL can interact with the pathogen which usually binds to α1-2-fucosylated epitopes on epithelial cells surfaces in the infant's intestine. HIV binds to Dendritic Cell-Specific ICAM3-Grabbing Non-Integrin (DC-SIGN) via Glycoprotein 120 (Gp120) to infect the organism. Apparently, DC-SIGN interacts with Lewis bloodtype antigens in HMOs *in vitro*. These observations might explain why the transmission rate from breastfeeding HIV-positive mothers to their offspring is only 10-20% (Bode 2012).

Lower incidences of ear and respiratory infections in breastfed than in bottle-fed infants have been observed (Abrahams & Labbok 2011, Downham et al. 1976). Common pathogens inducing those kind of infections are, for instance, *Streptococcus pneumoniae, Haemophilus influenzae* and/or Respiratory Syncytial Virus (RSV) (Abrahams & Labbok 2011, Downham et al. 1976). As those pathogens bind to epithelial surfaces via lectin-glycan interactions and HMOs have been shown to hinder those interactions

in vitro, it seems likely that not only maternal antibodies but also HMOs promote lower incidences of ear and respiratory infections (Bode 2012).

Cellmodulating Effects. It has been demonstrated that HMOs affect the composition of the glycocalix, induce apoptosis and differentiation and inhibit proliferation *in vitro* (Bode 2012, Kuntz et al. 2009). The composition of the glycocalix can be modified by specific HMOs which have been shown to inhibit the synthesis of sialyltransferases and lead to a lower amount of sialylated sugars on epithelial cells *in vitro*. Moreover, HMOs can inhibit attachment and infection of the microorganism *Enteropathogenic Escherichia coli (EPEC)* (Bode 2012). The exact underlying molecular mechanisms and the extent to which HMOs might modulate not only epithelial cells *in vitro* but also *in vivo* need to be further investigated.

Immunemodulating Effects. Local and systemic immune modulating effects of HMOs can be distinguished. Eiwegger *et al.* found that the amount of $CD3^+CD4^+$ lymphocytes synthesising the immune relevant cytokine Interferon (IFN)-γ as well as $CD3^+CD8^+$ lymphocytes producing Interleukin (IL)-13 was elevated when T-cells from cord blood are exposed to sialylated HMOs (Eiwegger et al. 2004). It has been further hypothesised that sialylated HMOs increase lymphocyte maturation and promote a state of a well-adjusted Th1/Th2-cytokine production (Bode 2012, Eiwegger et al. 2010). A condition of a balanced Th1/Th2-cytokine production is generally desirable to avoid not only excessive inflammatory periods induced by proinflammatory Th1-type cytokines but also anti-inflammatory states which eventually trigger allergic responses (Berger 2000). Chapter 1.2.1 will explore why and in how far this plays a decisive role in pregnancy as well. Acidic HMOs have been shown to reduce the synthesis of Th2-type cytokines by allergen specific T cells isolated from peanut allergic patients *in vitro*. Eiwegger *et al.* concluded that sialylated HMOs might therefore help to prevent allergies (Eiwegger et al. 2010).
Immunemodulating effects of the HMO Lacto-N-Fucopentaose III (LNFP III) have been observed. It has been shown to cause a peritoneal spread of distinct macrophages that suppress $CD4^+$ T cell proliferation in mice (Atochina et al. 2001). Additionally, LNFP III has been shown to stimulate the activity of macrophages and thus the secre-

tion of Prostaglandin (PG) E2, IL-10 and Tumor Necrosis Factor (TNF)-α (Atochina & Harn 2005).

The majority of mechanisms of how exactly HMOs modulate pathways and interact with the immune system remain unclear until today. However, it seems likely that these processes also depend on lectin-glycan interactions modified by HMOs. Sialic Acid Binding Ig-like Lectins (Siglecs) are immuneglobulins of the lectin family that bind to sialic acids. Sialylated HMOs and their Lewis blood group epitopes have a significant similarity to the structures Siglecs are docking to. Interactions between those immuneglobulins and milk oligosaccharides are possible and showed lowered effects of lectins in the presence of HMOs in *in vitro* studies (Bode 2012). Galectins (β-Galactoside Binding Lectins) alter immune responses and are able to bind on β1-3- or β1-4Gal that are found on non-reducing ends of HMOs (Bode 2012). Lectin-glycan interactions will be further explored in chapter 1.3.2.1.

Necrotizing Enterocolitis. Necrotizing Enterocolitis (NEC) is a disease that plays a major role in preterm neonates and is dreaded because of limited therapy options and fulminant devolutions with mortality rates up to 42% (Fitzgibbons et al. 2009). The HMO Disialyllacto-N-tetraose (DSLNT) but also 2'FL prevented the development of NEC and reduced mortality rates in rat models (Autran et al. 2016, Jantscher-Krenn et al. 2011, Bode 2012).

If the results explored above are reproducible under *in vivo* conditions this might be of importance in understanding and preventing diseases in infants or even in pregnant women.

1.1.5 Effects on the Mother

A great variety of potential effects of HMOs has been investigated for breastfed neonates. HMOs are detectable in maternal blood already during pregnancy (Jantscher-Krenn, Aigner, Reiter, Köfeler, Csapo, Desoye, Bode & Van Poppel 2018). This impact might already be relevant in pregnancy, affecting maternal and fetal health.

1.2 Inflammation

Inflammation is a reaction of the body to a damaged tissue caused by endogenic or exogenic factors and generally aims to reestablish the healthy status quo. The four characteristics of local inflammation, redness *(rubor)*, heat *(calor)*, swelling *(tumor)* and *(dolor)* have been firstly described in the 1st century and have been modified in 1870 by Rodulph Virchow who added 'loss of function' *(functio laesa)* to the already defined symptoms that 'reflect the effect of chemokines, cytokines and other inflammatory mediators' (Jabbour et al. 2009, e Silva 1978, Romero et al. 2007). But, contrary to what was described centuries ago and that what is still part of the daily clinical practices, absence of the described symptoms does not exclude an ongoing inflammation process. Romero states that histopathological inflammation is mostly subclinical (Romero et al. 2007). On a molecular biological level, cell modulations and higher concentrations of pattern recognition receptors, chemokines, cytokines and other markers are found as an indication of ongoing inflammatory processes, locally as well as systemically (Challis et al. 2009, Sacks et al. 1998).

In fertile women, inflammation regularly takes place: during ovulation, menstruation, nidation and at the introduction of parturition (Jabbour et al. 2009). Accordingly, it is also an indispensable process enabling reproduction. Without inflammation neither nidation nor expulsion of the mature fetus would be possible.

1.2.1 Inflammation in Pregnancy

In the past, embryo and placenta have been regarded as (semi-)allografts and pregnancy as an anti-inflammatory condition of immunosuppression. This point of view has been shown to be oversimplified. Whereas earlier pregnancy was considered as a phase of immunosuppression avoiding the rejection of the fetus, it is now considered a controlled state of inflammation: in early pregnancy at the implantation side and with proceeding of pregnancy also systemically. These modifications avoid the fetus' rejection while also supporting the physiologic adjustments of the women's body (Nayak et al. 2016, Szarka et al. 2010, Challis et al. 2009, Rusterholz et al. 2007, Sargent et al. 2006). Many altered mechanisms contribute to this exceptional immunomod-

ulated state. Central to these alterations is a shift towards a T Helper Cell (Th) 2 immunity, resulting in humoral mediated immune responses that are characterised as anti-inflammatory. Yet, this does not mean that proinflammatory factors are absent. On the contrary, Th1-mediated proinflammatory cytokines are detected in healthy pregnant women (Szarka et al. 2010) and may sometimes even lead to alterations similar to those of septic patients - with the difference that the pregnant population is not affected by it (Rusterholz et al. 2007, Sacks et al. 1998). Excessive or misadjusted Th1 response, however, was shown to be associated with pregnancy diseases including Recurrent Spontaneous Abortion (RSA), Intrauterine Growth Restriction (IUGR), premature labor and preeclampsia (Szarka et al. 2010, Challis et al. 2009, Romero et al. 2007, Makhseed et al. 2000).

It remains difficult to define if and when inflammation is sustaining a healthy status and when it might be menacing the well-being of mother and fetus. In the end, the net balance between Th1 and Th2 responses as well as their timing determine the immunologic state and the progression of the pregnancy.
Figure 3 provides an overview on possible immune conditions of healthy, non-pregnant individuals, healthy pregnant women and women with pregnancy pathologies.

Figure 3: Th1/Th2 Balance

Left: immune status of healthy pregnant women. *Center:* immune status of non-pregnant healthy women. *Right:* immune status of unhealthy pregnant women.

Local inflammatory processes during pregnancy cover the three trimesters of pregnancy roughly chronologically (Mor & Cardenas 2010):

1.2.2 First Trimester

The proinflammatory phase starts beginning with the ovulation, followed by conception, implantation and placentation. Therefore, communication between endometrium and embryo is necessary. On the one hand, proinflammatory cytokines are synthesised by the embryo, and on the other hand gene expression is increased by the endometrium. This enables the blastocyste to better adhere to the endometrial epithelium and allows syncytiotrophoblastic and cytotrophoblastic cells to invade the muscular layer. The interstitial trophoblast cells penetrate the uterine vasculature and access maternal spiral arteries, introducing the uteroplacental circulation (Mor & Cardenas 2010, Ulfig 2005, Staun-Ram & Shalev 2005). Moreover, it was shown that trophoblast cells induce immune cell differentiation and lead to an increase in IL-6, IL-8, Monocyte Chemoattractant Protein 1 (MCP-1) and Macrophage Inflammatory Protein 1 (MIP-1)β secretion *in vitro* (Fest et al. 2007). This might contribute to inflammatory processes in early pregnancy and promote trophoblast development and function (Mor et al. 2011, Fest et al. 2007).

Maternal macrophages, Natural Killer (NK) cells, Dendritic Cells (DC) and Regulatory T Cells (Tregs) physiologically invade the decidua and accrue around the embryonal cells. Several studies revealed that absence of already one cell type can be sufficient to cause termination of pregnancy. Depletion of NK cells for instance was shown to terminate pregnancy *in vivo* because trophoblast cells cannot reach maternal vessels (Mor et al. 2011). Absence of DC leads to delayed decidual formation and arrest of blastocyste implantation (Plaks et al. 2008). This shows that the immune system is highly active and plays an important role in physiological maintenance of pregnancy.

During all these tissue remodeling events, cells get damaged, cellular debris is generated and the implantation side resembles a wound requiring restoration by inflammatory modes. These inflammatory reactions together with hormonal changes cause the famous 'morning-sickness' (Mor et al. 2011, Mor & Cardenas 2010, Romero et al. 2007).

1.2.3 Second Trimester

The second trimester of pregnancy is locally marked as an anti-inflammatory phase. Initial reactions between the embryonal cells, placenta and maternal tissues are slowing down and mother, placenta and fetus have become a corresponding unit. The mother's condition often clearly improves in the second trimester (Mor et al. 2011, Mor & Cardenas 2010).

1.2.4 Third Trimester

During the third trimester the mother's body prepares the delivery. Parturition is induced by cytokines, released by myometrium, cervix and fetal membranes. They activate proinflammatory pathways and cause leukocytes, especially neutrophils and macrophages, to invade myometrium and cervix. Already during the last trimester of pregnancy inflammatory factors and their corresponding signal receiving receptors Toll Like Receptor (TLR)s are altered and increase dramatically during labour. Contractions might be promoted by cytokines, such as IL-1β. IL-1β triggers myometrial contractions by increasing intracellular Ca^{2+} concentrations *in vitro*. Furthermore, the expression of Cyclooxygenase 2 (COX2) and phosphodiesterase activity are elevated and give rise to myometrial contractions as well. Additionally, stimulated Matrix Metalloproteinase (MMP) expression leads to collagen breakdown and thereby ripening of the cervix. The latter is also caused by cervical invasion of nitric oxide releasing leukocytes (Mor et al. 2011, Jabbour et al. 2009).
However, why and how cytokines receive their activating signals shortly before parturition remains unclear. Similarly, detected rise in anti-inflammatory cytokines such as IL-10 during labour cannot fully be explained until now (Jabbour et al. 2009).

Summing up, pregnancy is a highly complex exceptional situation for the maternal as well as the feto-placental (immune) system, demanding for a high grade of adaptability of both organisms. Given that human implantation and pregnancy is unique and not comparable to those of any other animals, and ressources of human feto-placental structures are quite limited, research on this field still remains hindered. Further investigations will be needed to confirm the findings found *in vitro*, *in vivo* and *ex vivo*

(Staun-Ram & Shalev 2005).

1.3 Inflammation Markers

Since innumerable biochemical processes take place during inflammation, there exist a large number of inflammation markers. In this work we chose to focus on markers that have been less explored in the context of inflammation and that have provided interpretable results. We decided to analyse the following markers: C-Reactive Protein (CRP), Soluble Intercellular Adhesion Molecule 1 (sICAM-1), Monocyte Chemoattractant Protein 1 (MCP-1), MIP-1α and MIP-1β.

1.3.1 CRP

C-Reactive Protein is an acute phase plasma protein that belongs to the pentraxin family of proteins and provokes systemic immune responses in case of tissue injury or inflammation. Its name arised from its first explored function: it attaches to the C-polysaccharide of *Streptococcus pneumoniae* (Black et al. 2004, Hart et al. 2004, Du Clos 2000).
CRP is a common serum marker used in clinical contexts to detect and monitor ongoing inflammation. On the basis of its increase and decrease the development of the actual inflammation can be estimated. Secretion of CRP in hepatocytes is mainly triggered by the proinflammatory cytokine IL-6 and might be intensified by the presence of IL-1β (Du Clos 2000).

Comparable to Immunoglobulin (Ig) G, CRP may act as an opsonin, bind to pathogens and activate the complement system. As a pattern recognition molecule it interacts with ligands like phosphocholines, chromatine, small ribonucleoproteins and other cell particles that are released when cells are damaged or become apoptotic. Moreover, CRP binds to Fc receptors and subsequently leads to an antibody-mediated phagocytosis of pathogens and infected cells (Du Clos 2000).

Depending on the linkage to ligands either 'pro-' or 'anti-inflammatory' effects of CRP are unfolded (Black et al. 2004, Hart et al. 2004).

Activation of the complement system, promotion of phagocytosis, increased generation of IL-1, IL-6, IL-8, IL-18, TNF-α and up-regulation of adhesion molecules can be considered as 'proinflammatory' functions.
By contrast, increase of IL-10 concentration, production of IL-1-receptor antagonists and diminished IFN-γ production are regarded as the 'anti-inflammatory' reactions of CRP *in vivo*. Further functions of a slightly altered form of CRP *(neo-CRP, modified CRP)* include the augmented release of MCP-1 and enhancement of endothelial Intercellular Adhesion Molecule 1 (ICAM-1) expression. Further investigation on the diversified *in vitro* observations of the mode of action of CRP are necessary. The following overview summarises the pro- and anti-inflammatory effects of CRP:

Proinflammatory effects of CRP

- activation of the complement system
- promotion of phagocytosis
- increased generation of IL-1, IL-6, IL-8, IL-18, TNF-α
- up-regulation of adhesion molecules

Anti-inflammatory effects of CRP

- increase of IL-10 concentration
- production of IL-1 receptor antagonists
- diminished IFN-γ production

1.3.2 sICAM-1

ICAM-1, also known as CD54, is a transmembrane glycoprotein belonging to the Ig superfamily. It is structurally composed of five Ig-domains, a transmebrane domain and an intracellular tail. ICAM-1 can be available in a glycolysated or non-glycolysated form, resulting in different molecular weights. sICAM-1 is the circulating form of

ICAM-1 and is soluble and detectable in human serum. sICAM-1 does neither own a transmembrane nor an intracellular domain (Fig.4).

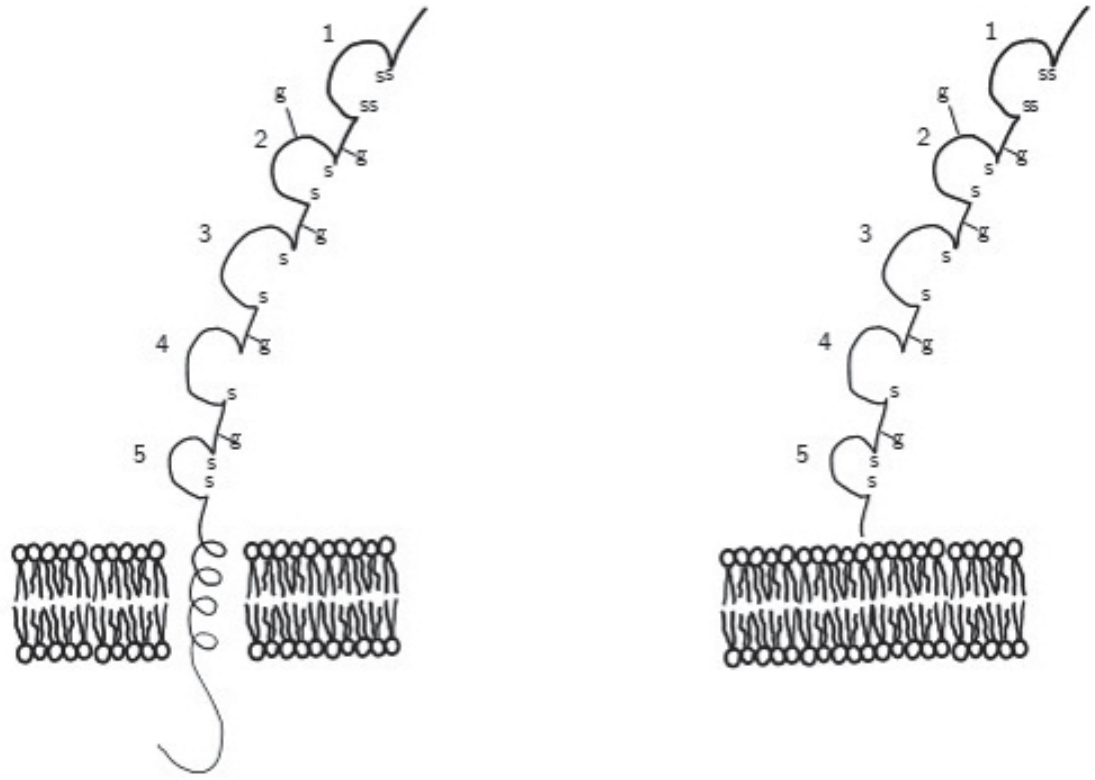

FIGURE 4: ICAM1 & sICAM1

The *left* chart displays the structure of ICAM-1 with its intracellular tail, the transmembrane domain and the Ig-domains. By contrast, sICAM-1 on the *right* consists of only five Ig-domains. Figure modified based on Witkowska and Borawska 2004 and Van de Stolpe & Van der Saag 1996.

1.3.2.1 Adhesion Molecules and Transmigration of Leukocytes

Cell adhesion molecules are central to reciprocation between leukocytes and endothelium during inflammation. Three groups of adhesion molecules are necessary for the organisation of leukocytes:

- Lectins are carbohydrate-binding proteins that deploy several processes based on their ability to detect and bind to specific glycoconjugates. Four major groups of lectins exist: 1. C-type lectins including selectins, 2. P-type lectins, 3. pentraxins and 4. galectins (Barondes et al. 1994). In the beginning of an inflammation event, cytokines signal the endothelial tissue to synthesise selectins, such as P- and E-selectin, which are membrane-bound cell adhesion molecules. They initiate extravasation by interacting between activated endothelium and sialylated

oligosaccharide ligands on leukocytes. Subsequently, leukocytes attach to the endothelium, decelerate and slowly roll over the endothelial cells.

- Adhesion molecules of the Ig superfamily, such as ICAMs, are able to grab the leukocytes and let the transmigration proceed into subendothelial tissue (Bode 2012, Murphy et al. n.d.).

- Integrines are the third group of molecules enabling extravasation and are found on leukocytes cell surfaces. Macrophage-1 Antigen (MAC-1) and Lymphocyte Function-Associated Antigen 1 (LFA-1) are important members of this group. As a counter-receptor, ICAM-1 can bind to MAC-1 and LFA-1 but also to fibrinogen, rhinoviruses and *Plasmodium falciparum*-infected erythrocytes (Lawson & Wolf 2009, Murphy et al. n.d., Witkowska & Borawska 2004, Hubbard & Rothlein 2000).

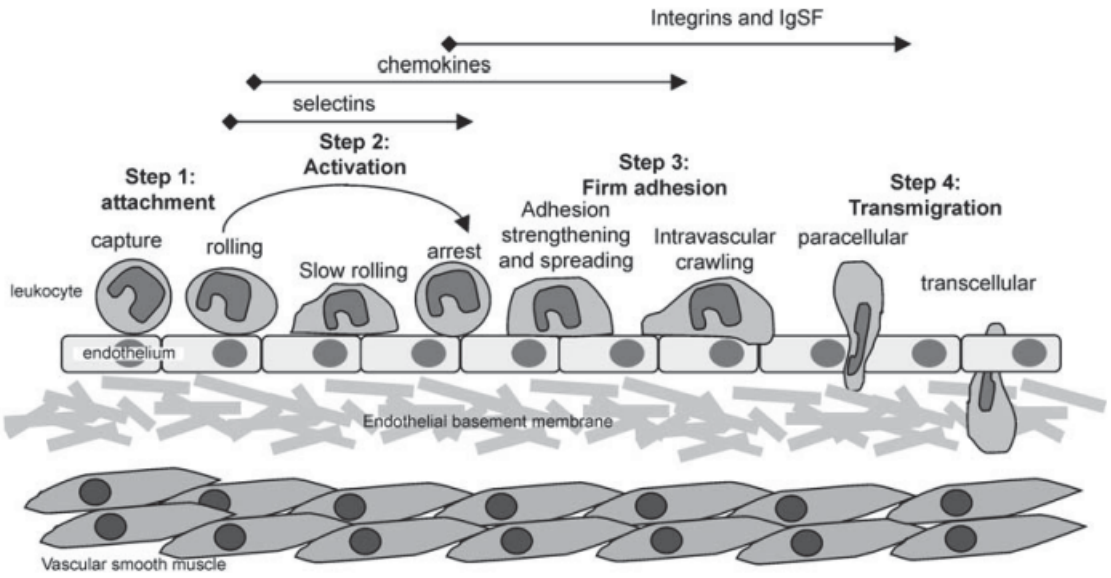

FIGURE 5: TRANSENDOTHELIAL MIGRATION OF LEUKOCYTES (LAWSON & WOLF 2009)

Step 1: Rolling and adhesion of leukocytes via interactions between the sialylated component of E- and P-selectin on endothelium and the carbohydrate structures closely related to sialyl lewis^x on leukocytes. **Step 2:** Chemokines induce a structural change and activate leukocyte-bound integrines. Adhesion to their ligands is enforced. **Step 3:** A stronger adhesion of the arrested leukocytes is reached by a LFA-1/ICAM-1 bondage, leukocytes advance and migrate. Intracellular Ca^{2+} release is promoted, wich assists the following migration processes through contraction of endothelial cells and weakened cell junctions. **Step 4:** Leukocytes transmigrate into sub-endothelium.

ICAM-1 is synthesised by endothelial and epithelial cells as well as by lymphocytes and monocytes. Expression increases in an proinflammatory environment under the influence of the cytokines IFN-γ, TNF-α, IL-1 and in the presence of Reactive Oxygen Species (ROS). As an adhesion molecule ICAM-1 enables cell-cell or cell-matrix interaction. It plays an important role in embyro-maternal communication in early pregnancy, embryonic development in general, in immunologic processes like leukocyte trafficking and transmigration, during antigen presentation to T lymphocytes, in microbial pathogenesis and in signal transduction. The latter for instance permits rearrangement of the actin cytoskeleton and by this way facilitates leukocyte transmigration as well as initiation of proinflammatory pathways. Activation of other pathways induces e.g. cytokine and ROS production, cell proliferation and augmented cell membrane protein expression (Lawson & Wolf 2009, Hubbard & Rothlein 2000, Van de Stolpe & Van der Saag 1996). This might explain why higher levels of ICAM-1 are found in sera of preeclamptic patients and additionally positively correlate with CRP and MCP-1, as well. They play a central role in preeclampsia, a severe and frequent pregnancy pathology that is accompanied by a systemic inflammatory response (Szarka et al. 2010).

sICAM-1 is the soluble form of ICAM-1. Its origin is not yet fully understood but two mechanisms might explain its appearance. Firstly, it is possible that sICAM-1 concentration mirrors ICAM-1 expression on endothelial cells. Secondly, it was hypothesised that specific genes for sICAM-1 do exist and enable its synthesis. By binding to LFA-1 on circulating leukocytes, sICAM-1 is able to inhibit lymphocyte attachment to endothelium and reduce the amount of transmigrating leukocytes. Being a competitor of ICAM-1, studies investigated sICAM-1 as a potenial therapeutic to inhibit leukocyte interaction with endothelium (Lawson & Wolf 2009). Nevertheless, sICAM-1 also triggers proinflammatory cascades by increasing the production of MIP-1α, TNF-α, IFN-γ and IL-6. sICAM-1 levels are higher after physical activity but also after meals with a high percentage of fat. Higher concentrations of sICAM-1 have also been shown to be associated with diverse pathologic conditions such as rheumatoid arthritis, rhinovirus upper respiratory tract infections, neurological disorders, atherosclerosis, coronary heart disease, cancer and transplantation graft failure. In the future, sICAM-1 might therefore be used as a biomarker for identification of patients with a risk for

cardiovascular diseases, graft failure or hematogenous metastases (Witkowska & Borawska 2004).

1.3.3 Chemokines

Chemokines are chemoattractant cytokines that take part in immune responses, the development of lymphoid organs, angiogenesis and inflammatory processes. As signalling proteins they regulate cell trafficking and selectively mobilise and stimulate leukocytes by chemotaxis (Bystry et al. 2001). Different cells release cytokines such as lymphocytes, NK cells, adipocytes and muscle cells (Nayak et al. 2016, Schmatz et al. 2010). During pregnancy, the placenta is an additional source of cytokine release (Nayak et al. 2016). More than 50 chemokines and 20 G-protein-coupled chemokine receptors have been identified. Defined by amount and position of the N-terminal cysteine residues, chemokines can be classified into 4 subfamilies: CC, CXC, C and CX_3C (Yadav et al. 2010, Semple et al. 2010, Deshmane et al. 2009, Jabbour et al. 2009, Schall et al. 1993). Homeostatic cytokines are produced constitutively and are important for immune surveillance. They manage lymphocyte trafficking to lymphoid tissues and may lead to cell differentiation and angiogenesis. Thus, they are indispensable for the maintenance of health. By comparison, inflammatory cytokines are only released when tissue is injured or infected and lead to an accumulation of leukocytes at the inflammatory side. Cells carrying the corresponding chemokine receptors are attracted as well and migrate following the chemokine gradient. These processes aim to reestablish the prevenient physiological health status of the tissue (Deshmane et al. 2009, Fernandez & Lolis 2002). Table 1 gives an overview of the chemokines MCP-1, MIP-1α and MIP-1β and their receptors and target cells.

1.3.3.1 MCP-1

CC-Chemokine Ligand (CCL) 2 or MCP-1 is the first detected human CC chemokine and is known for its ability to strongly attract monocytes and assist in their extravasation by arresting rolling monocytes on endothelial layers (Yadav et al. 2010). Monocytes and macrophages display the main source of CCL2 but endothelial, epithelial,

Table 1: Overview of Chemokines (Schütt & Bröker 2011)

Name	Nomenclature	Receptor	Target Cell
MCP-1	CCL2	CCR2	T, MNC, Baso, iDC
MIP-1α	CCL3	CCR1, CCR5	MNC, Mφ, T (Th1 >Th2), NK, Baso, iDC
MIP-1β	CCL4	CCR1, CCR5	MNC, Mφ, T (Th1 >Th2), NK, Baso, iDC, Eo, B stem cells

Baso: Basophiles; DC: Dendritic Cells; Eo: Eosinophiles; iDC: Immature Dendritic Cells; Mφ: Macrophages; MNC: Monocytes; NK: Natural Killer cells; T: T cells

smooth muscle, microglial cells, fibroblasts and astrocytes can express CCL2 as well. Although chemokines may bind to several chemokine receptors and vice versa, the classical corresponding receptor for CCL2 is C-C Chemokine Receptor Type 2 (CCR2) which is expressed by several types of leukocytes (Yadav et al. 2010, Deshmane et al. 2009). Whenever a contact is made, signal transduction pathways are activated that lead primarily to monocyte chemotaxis but also to chemotaxis of T lymphocytes, basophils and NK cells *in vitro* (Semple et al. 2010). Kuziel *et al.* showed that mice lacking CCR2 were impaired both in leukocyte endothelial adhesion and leukocyte extravasation to inflammatory sites (Kyriakides et al. 2004, Kuziel et al. 1997) which seemed to be a protection against sensory neuropathies in studies by Thacker *et al.* (Deshmane et al. 2009).

1.3.3.2 MIP-1α and MIP-1β

MIP-1α (CCL3) and MIP-1β (CCL4) are members of the CC chemokine group as well and are central to leukocyte trafficking. Cells expressing MIP-1α are, amongst others, monocytes, macrophages, T and B lymphocytes, neutrophils, DC, NK cells, granulocytes, astrocytes, fetal microglial cells, epithelial cells and vascular smooth muscle cells. MIP-1α inducing molecules include ICAM-1, Lipopolysaccharides (LPS), IL-12, IL-15, IL-1β, TNF-α, IFN-γ and RSV- and HIV-infections. Inhibitors are, for instance, IL-4, IL-10, IL-13, IL-18, nitric oxide synthase inhibitors (NOS) and IFN-γ (Menten et al. 2002).

MIP-1β is synthesised by fewer cell types, including monocytes, T and B lymphocytes, DC, NK, fetal microglial cells, vascular smooth muscle cells and brain microvessel endothelial cells. Similarly, their functions are manifold, may overlap but likewise

differ, depending on cell of origin and other influencing factors. Inducers of MIP-1β are ICAM-1, LPS, IL-7, IL-4, IL-10, (IL-4 and IL-10 both of vascular smooth muscle cells), -1β and TNF-1α. Inhibitors are monocyte produced IL-4 and neutrophil produced IL-10 (Menten et al. 2002).
Chemoattractant properties are central and affect cells expressing C-C Chemokine Receptor (CCR) 1,2,4,5. Those cells are, for instance, monocytes/macrophages, DC, activated Th1 and Th2 cells, NK cells and eosinophils. After their activation those cells expand and concentrate at the inflammation side. MIP-1α and MIP-1β expression is upregulated in activated B cells and induced in activated DC and macrophages. PGE2 can inhibit MIP-1α and MIP-1β expression *in vitro* and *in vivo* (Jing et al. 2003, Bystry et al. 2001). Recently it was revealed that elevated concentrations of MIP-1α and MIP-1β can be found in nongravid after physical activity, suggesting that the same might be the case in pregnant women (Nonn 2017, Pedersen 2011).

MIP-1α is regarded as a more efficient T lymphocyte attractant with a larger effective width than MIP-1β. Which lymphocytes get attracted by MIP-1α depends on available concentrations of this protein. Whereas higher concentrations (10 ng/ml) provoke chemotaxis of B cells and cytotoxic T cells, lower concentrations (100 pg/ml) support migration of $CD4^+$ *in vitro* (Schall et al. 1993). Moreover, MCP-1 and MIP-1α play a role in T cell maturation (Fernandez & Lolis 2002).

MIP-1β intensifies the effect of activated B cells attracting T cells. It was shown to be the most potent chemoattractant of $CD4^+CD25^+$ Tregs *in vitro*. Absence of either MIP-1β or Tregs caused perturbations of immune response and resulted in formation of autoantibodies *in vivo*, indicating that MIP-1β participates in regulating immune responses (Bystry et al. 2001).

Leukocyte trafficking to inflammation sides is an indispensable mechanism of host defense. Resolution of these pathways and inflammatory states are not less important than their existence since otherwise immune mediated chronic inflammatory diseases might be favoured.

2 Hypothesis and Aims

In the suckling neonate HMOs have prebiotic, anti-adhesive, cell and immune modulating effects. These functions might be relevant during pregnancy, too, affecting maternal and fetal health. We have previously detected circulating HMOs in the blood of pregnant women. In this study, HMO concentrations and concentrations of inflammatory markers throughout pregnancy are described. Associations of prenatal HMOs with selected maternal inflammatory markers have been investigated, to our knowledge, for the first time.

We hypothesised that the maternal inflammatory status in early and midpregnancy influences maternal serum HMO concentrations. To test this hypothesis we performed association studies with the inflammatory markers CRP, sICAM-1, MCP-1, MIP-1α and MIP-1β and the main HMOs in maternal serum, 2'FL and 3'SL. Additionally, associations between maternal HMOs in midpregnancy and fetal inflammatory markers in Cord Blood (CB), collected directly after parturition, were elucidated.

If our hypothesis holds true, HMOs might serve as biological markers to identify inflammatory conditions in pregnancy.

3 Material and Methods

3.1 Study Design

For this prospective, observational pilot study 53 healthy pregnant women were recruited. During their pregnancy, one target date per trimester was chosen for measurements: the first one between week 10 and 14 of gestation, the second one between week 19 and 24, the third one between week 32 and 36 of gestation. Venous blood samples as well as further information by questionnaires and examinations have been collected. The fourth maternal venous blood sample was obtained shortly before delivery. Immediately after the delivery of the placenta, arterial and venous blood samples of the umbilical cord were taken. Placental tissues were collected in the laboratory for later analysis.

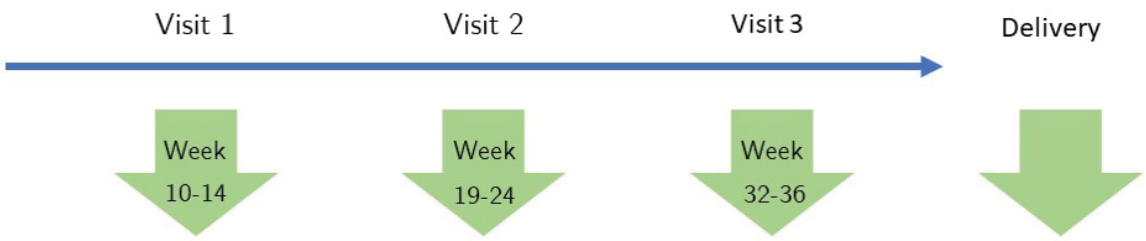

FIGURE 6: TIMETABLE OF ASSESSMENTS
Timetable displaying the planned time frames for the three assessments during pregnancy and at delivery.

3.2 Recruiting

From February to October 2013 pregnant women who came for the first-trimester ultrasound scan and combined test were recruited by the outpatient clinic of the Department of Obstetrics of the University Hospital of Graz. Written informed consent was obligatory to participate in the study.

Inclusion criteria were: [1] an ongoing pregnancy in the 10^{th} to 14^{th} week of gestation and [2] a given informed consent.
Exclusion criteria were: [1] gestational age was already more than 14th week, [2] deliv-

ery not planned at the University Hospital of Graz, [3] multiple pregnancies, [4] more than three consecutive miscarriages, [5] smoking [6] pre-pregnancy hypertension, [7] diagnosed diabetes mellitus type 1 or 2, [8] maternal metabolic risc factors such as autoimmune diseases or increased risc for thrombembolic events requiring anticoagulative therapy, [9] increased risk of chromosomal abnormalities after combined test (1:300 or lower), [10] fetal anomalies.

3.3 Data Collection

3.3.1 Blood samples

Data required for the study were collected by study nurses, medical students and members of the Perinatal Research Laboratory at the University Hospital of Graz. All involved persons were trained to take maternal and CB samples and process the placental tissue in the laboratory. Venous maternal blood samples and arterial and venous CB samples were collected (serum, EDTA- and sodium fluoride anticoagulated tubes). Afterwards, they were centrifuged at room temperature, pipettet and freezed at -80° degrees. Analysis was performed after sampling of all 4 sample dates was completed. Specific blood factors such as proteins, lipids, glucose and cytokines, were analysed by conventional clinical chemistry methods, ELISA or multiplex assay technology.

3.3.2 HMO Analysis

HMOs were analysed using HPLC, an analytical method to separate and quantify chemical substances. HMOs were separated by HPLC and monitored by fluorescence detection at the Center of Medical Research and the Department of Gynaecology and Obstetrics of the Medical University of Graz. Therefore, raffinose was added to venous serum as an internal standard, proteins were removed by Chloroform/Methanol extraction. Samples were further deproteinated by C18 use and finally desalted by porous graphitic carbon columns. Afterwards, HMOs were eluted, dried and labelled with 2-Aminobenzamide (2AB). Subsequently, labelled HMOs were separated in a linear gradient using an ammonium formiate and Acetonitrile (ACN) solvent system and

monitored by florescence detector (Em 360nm/Ex 425nm).

3.3.3 Cytokine Analysis

Levels of the inflammatory markers CRP, ICAM-1, MCP-1, MIP-1α and MIP-1β were measured by multiplex assay technology according to the manufacturer's instructions (*Aimplex Biosciences, Inc.*, Pomona, CA). This work was undertaken in collaboration with the Institute of Experimental and Clinical Pharmacology of the Medical University of Graz.

3.4 Data Management

Each study participant was assigned an electronically generated study ID to preserve anonymity. Anonymized data was collected in case report forms, transferred into Excel tables as well as into the *IBM SPSS Statistics* software.

3.5 Data Analysis

3.5.1 Statistical Analysis

Data was analysed using the software *IBM SPSS Statistics 23*. Descriptive statistics and selected tests were performed. Mean and standard deviation, median and quartiles were explored. Data was analysed for normal distribution by the Shapiro-Wilk test. Due to the fact that some variables could not be regarded as normally distributed, following analyses were performed by nonparametrical statistical methods. The level of significance was determined with $p < 0{,}05$ for all statistical analysis. The confidence interval was set at 95%.

The Spearman's rank correlation coefficient or Spearman's rho was applied to investigate bivariate correlations. A linear regression was used to scrutinize the relation of significantly correlating variables.

Table 2: Shapiro-Wilk Test of Normality

		Significance ($p < 0{,}05$)
V1	CRP	0,154
	sICAM-1	0,000
	MCP-1	0,391
	MIP-1α	0,000
	MIP-1β	0,042
V2	CRP	0,449
	sICAM-1	0,000
	MCP-1	0,344
	MIP-1α	0,000
	MIP-1β	0,145
V3	CRP	0,518
	sICAM-1	0,000
	MCP-1	0,256
	MIP-1α	0,004
	MIP-1β	0,147

As Table 2 shows, sICAM-1 and MIP-1α did not follow a normal distribution (significance level of $p < 0{,}05$). Data of MIP-1β were not normally distributed at Visit 1 (V1) either ($p=0{,}01$). For this reason, all the following statistical analyses were conducted with nonparametrical methods.

4 Results

We performed descriptive statistics and association studies to investigate possible associations between HMOs and selected inflammatory markers during pregnancy and in fetal cord blood.

4.1 Study Population

From the 53 participants enrolled in the study, only 23 were included in the following analyses. Except in the descriptive statistics illustrating the baseline demographics of the study population, all data sets featuring missing values were excluded. 14 of the study participants were lost, 2 due to medical exclusion criteria and 12 due to the incomplete consecutive control visits. Out of the 39 data sets 23 were regarded as complete and were used for further analysis. Incomplete data sets were caused by patients not showing up to one or more of the required visits, not delivering at the University Hospital of Graz and a by lack of communication of the midwives responsible for calling a study team member on duty for data acquisition.
Table 3 displays the main characteristics of the study population.

Table 3: Basic Maternal Characteristics

	n	Minimum	Maximum	Mean	SD
Age (years)	23	24	44	33,65	5,0
Height (cm)	23	160	177	167	4,9
BMI Pre-pregnancy	23	18,4	28,1	21,9	2,5
BMI Delivery	23	22,6	33,2	27,7	2,6

Characteristics of the study population including age, height and BMI. All participants were Caucasian. SD=Standard Deviation.

The actual assessment dates of the study participants slightly deviated from those planned in the study design (Fig.6). As depicted in Figure 7, V1 took place between week 11 and 13, V2 between week 17 and 25 and V3 between week 27 and 39 of pregnancy.

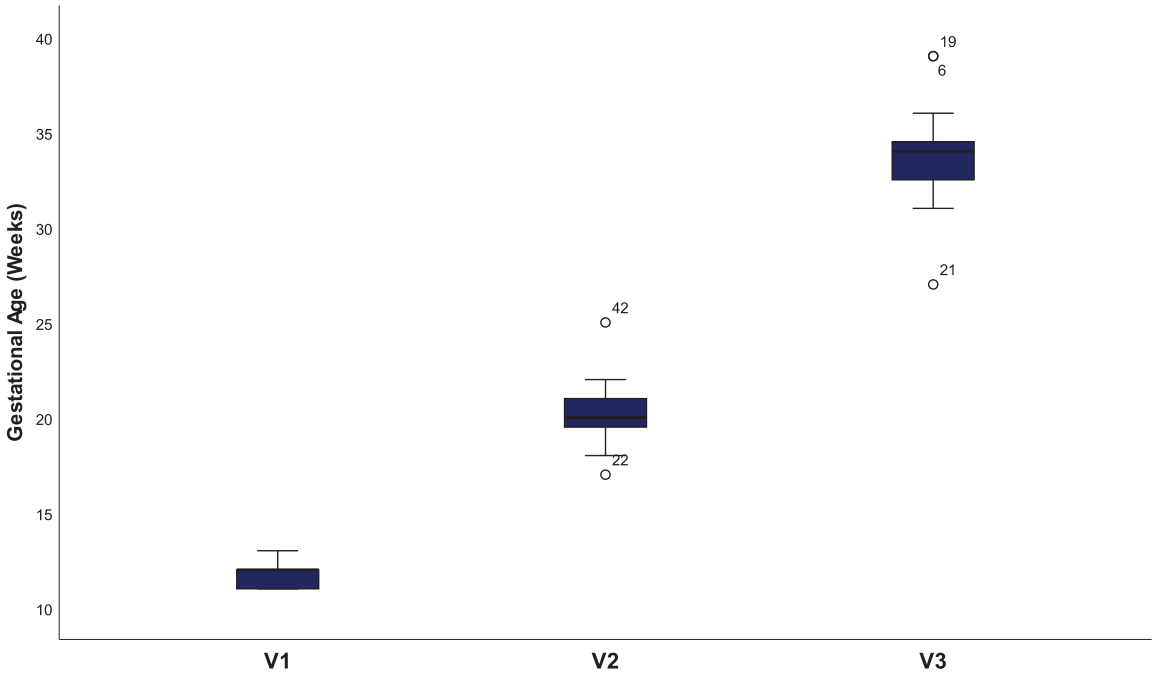

FIGURE 7: VISITS 1-3

Boxplot displaying the actual dates of assessment of the study population in their first, second and third trimester (n=23).

4.2 HMOs over the Course of Pregnancy

We decided to use 2'FL and 3'SL as the basis for our analyses as they provide the largest share of all HMOs beginning with the second trimester (Fig.10, 11 and 12). To investigate our hypothesis that inflammatory markers influence HMO synthesis, we wanted to find out whether inflammatory markers thoughout pregnancy are associated with HMOs.

We excluded the HMO 3FL from descriptive and statistical analysis since in HPLC with fluorescence detection 3FL was co-eluted with another, unidentified peak and data was therefore not reliable.

4.2.1 2'FL

Anti-inflammatory properties of 2'FL have been described recently. 2'FL was reported to inhibit LPS mediated inflammation by reducing CD14 expression and IL-8 synthesis, attenuating TLR4 signaling and depressing inflammatory cytokine production *in vitro* (He, Lawlor & Newburg 2016, He, Liu, Kling, Leone, Lawlor, Huang, Feinberg, Hill & Newburg 2016). Moreover, severity of NEC was reduced by intensified Endothelial Nitric Oxide Synthase (ENOS) synthesis and consequential augmented mesenteric perfusion in neonatal mice fed with formula added 2'FL (Good et al. 2016). Furthermore, Goehring *et al.* revealed that 2'FL regulates the shares of different T lymphocytes. By activating $CD8^+$ T cells their likelihood of apoptosis increases and cytokine synthesis decreases which could be the reason for a lower inflammatory immune response (Goehring et al. 2016).

4.2.2 3'SL

3'SL acts proinflammatory *in vivo* by activating mesenteric lymph node DC via TLR4. It induces cytokine synthesis that promotes Th1 and Th17 cell spread. Mice fed with 3'SL suffered from an aggravation of colitis whereas mice breastfed by milk lacking 3'SL experienced a relief of colitis (Kurakevich et al. 2013). 3'SL has antiadhesive and anti-infective effects, too. By modulation of cell surface molecules, binding of an *E.*

coli strain in uroepithelial cells was significantly diminished *in vitro* (Coppa et al. 1990).

4.2.3 HMOs in Maternal Serum throughout Pregnancy

HMO concentrations rose significantly from Visit 1 (V1) to Visit 3 (V3) (Figure 8 and 9). All HMO concentrations are Area under the Curve (AUC) values normalised to an internal standard.

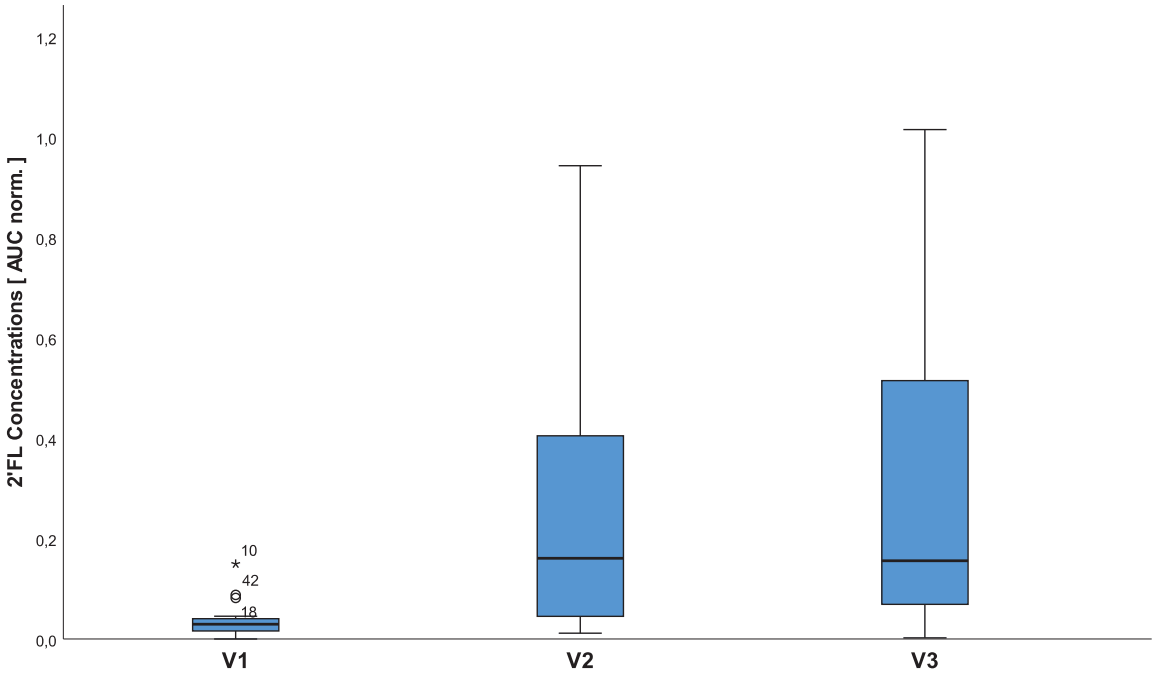

FIGURE 8: CONCENTRATIONS OF 2'FL AT V1, V2 AND V3

Boxplot displaying quartiles, median and interquartile range for 2'FL concentrations [AUC norm.] in the first and second trimester measured in maternal serum (n=23).

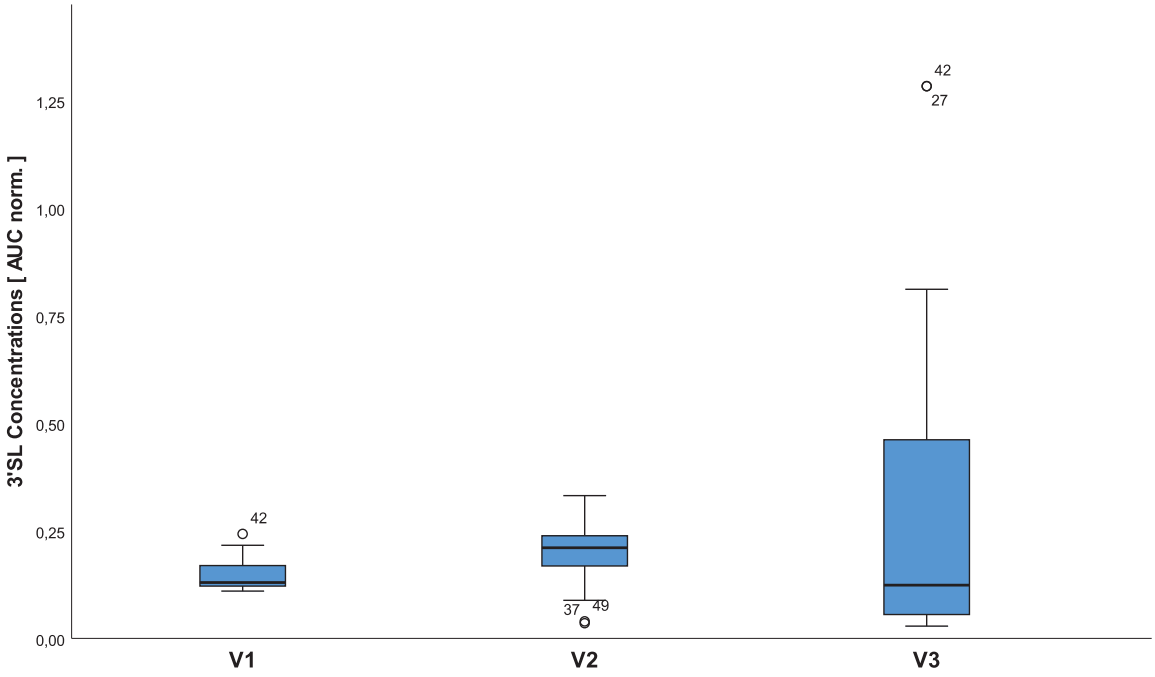

FIGURE 9: CONCENTRATIONS OF 3'SL AT V1, V2 AND V3

Boxplot displaying quartiles, median and interquartile range for 3'SL concentrations [AUC norm.] in the first and second trimester measured in maternal serum (n=23).

The following subchapters 4.2.4, 4.2.5 and 4.2.6 display the various HMOs found in maternal serum at different stages of pregnancy.

4.2.4 First Trimester

Figure 10 displays different HMOs found in serum at V1. In the first trimester, 3'SL makes up the largest share of HMOs, followed by Lacto-N-Hexaose (LNH), 3'-Sialyl-N-Acetyllactosamine (3'SLN) and Lactosialotetraose c (LST c).

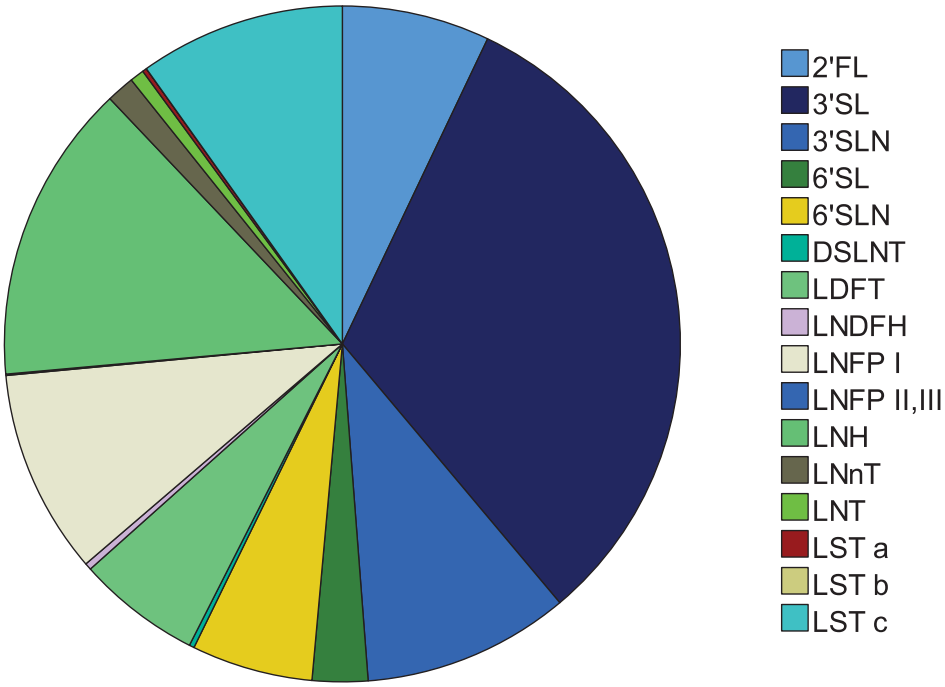

FIGURE 10: HMOS IN MATERNAL SERUM AT VISIT 1

Abbreviations 2'FL: 2'-Fucosyllactose; 3'SL: 3'-Sialyllactose; 3'SLN: 3-Sialyl-N-acetyllactosamine; 6'SL: 6-Sialyllactose; 6'SLN: 6-Sialyl-N-acetyllactosamine; DSLNT: Disialyl-lacto-N-tetraose; LDFT:Lacto-di-fuco-tetraose; LNDFH: Lacto-N-di-fuco-hexaose; LNFP I: Lacto-N-fucopentaose I; LNFP II, III: Lacto-N-fucopentaose II, III; LNH: Lacto-N-hexaose; LNnT: Lacto-N-neotetraose; LNT: Lacto-N-tetraose; LSTa: Lactosialotetraose a; LST b: Lactosialotetraose b; LST c: Lactosialotetraose c

4.2.5 Second Trimester

With the second trimester 2'FL percentage rises significantly. 2'FL and 3'SL together make up more than 50% of all HMOs.

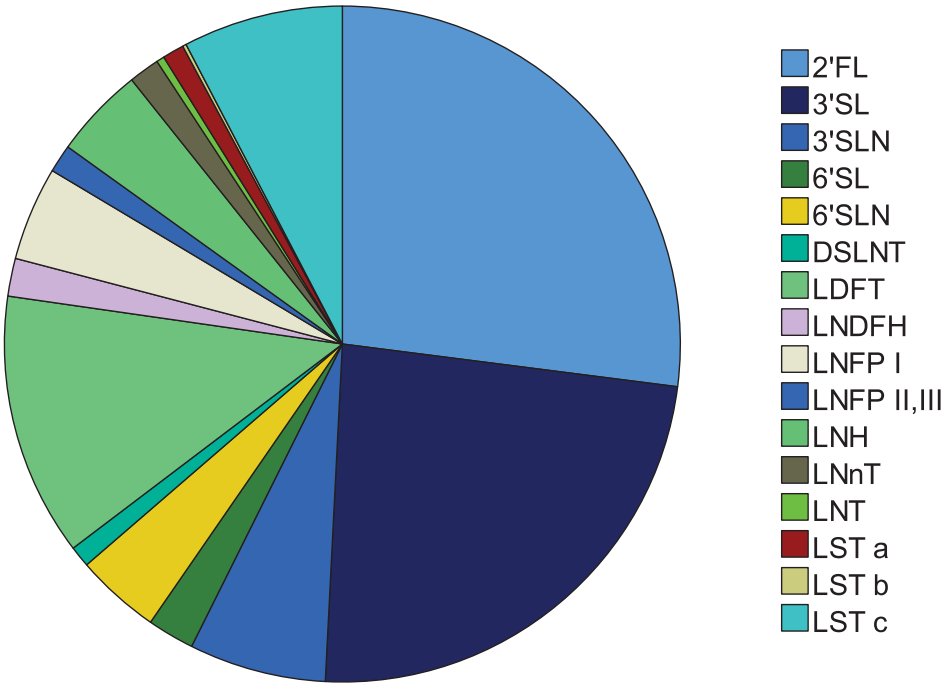

Figure 11: HMOs in Maternal Serum at Visit 2

Abbreviations 2'FL: 2'-Fucosyllactose; 3'SL: 3'-Sialyllactose; 3'SLN: 3-Sialyl-N-acetyllactosamine; 6'SL: 6-Sialyllactose; 6'SLN: 6-Sialyl-N-acetyllactosamine; DSLNT: Disialyl-lacto-N-tetraose; LDFT:Lacto-di-fuco-tetraose; LNDFH: Lacto-N-di-fuco-hexaose; LNFP I: Lacto-N-fucopentaose I; LNFP II, III: Lacto-N-fucopentaose II, III; LNH: Lacto-N-hexaose; LNnT: Lacto-N-neotetraose; LNT: Lacto-N-tetraose; LSTa: Lactosialotetraose a; LST b: Lactosialotetraose b; LST c: Lactosialotetraose c

4.2.6 Third Trimester

The ratios of 2'FL and 3'SL at V3 are the largest of all HMOs.

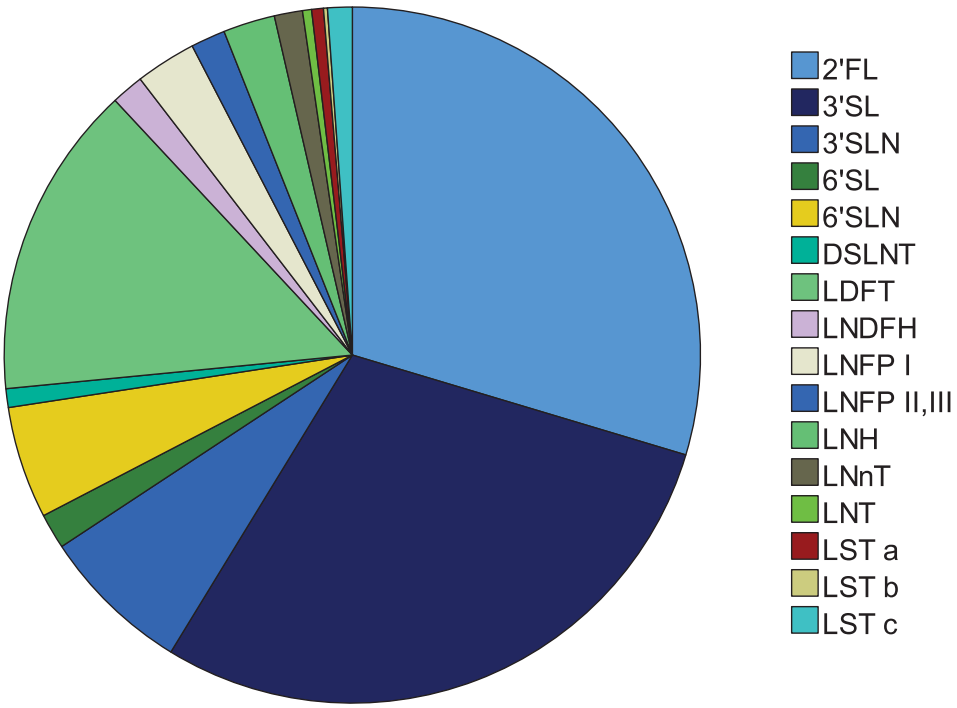

Figure 12: HMOs in Maternal Serum at Visit 3

Abbreviations 2'FL: 2'-Fucosyllactose; 3'SL: 3'-Sialyllactose; 3'SLN: 3-Sialyl-N-acetyllactosamine; 6'SL: 6-Sialyllactose; 6'SLN: 6-Sialyl-N-acetyllactosamine; DSLNT: Disialyl-lacto-N-tetraose; LDFT:Lacto-di-fuco-tetraose; LNDFH: Lacto-N-di-fuco-hexaose; LNFP I: Lacto-N-fucopentaose I; LNFP II, III: Lacto-N-fucopentaose II, III; LNH: Lacto-N-hexaose; LNnT: Lacto-N-neotetraose; LNT: Lacto-N-tetraose; LSTa: Lactosialotetraose a; LST b: Lactosialotetraose b; LST c: Lactosialotetraose c

4.3 Inflammation Markers over the Course of Pregnancy

4.3.1 CRP

With a sample size of n=23 the mean value of CRP was 1,33 ± 0,93 ng/ml at V1 and 1,27 ± 0,64 ng/ml at V2. At V3 the mean value of CRP was 1,21 ± 0,76 ng/ml (Table 4).

Table 4: Concentrations of CRP during Pregnancy

	n	Minimum	Maximum	Mean	SD
Visit 1	23	0,07	3,68	1,33	0,93
Visit 2	23	0,08	2,53	1,27	0,64
Visit 3	23	0,00	2,90	1,21	0,76

Concentrations are given in [ng/ml]. SD=Standard Deviation.

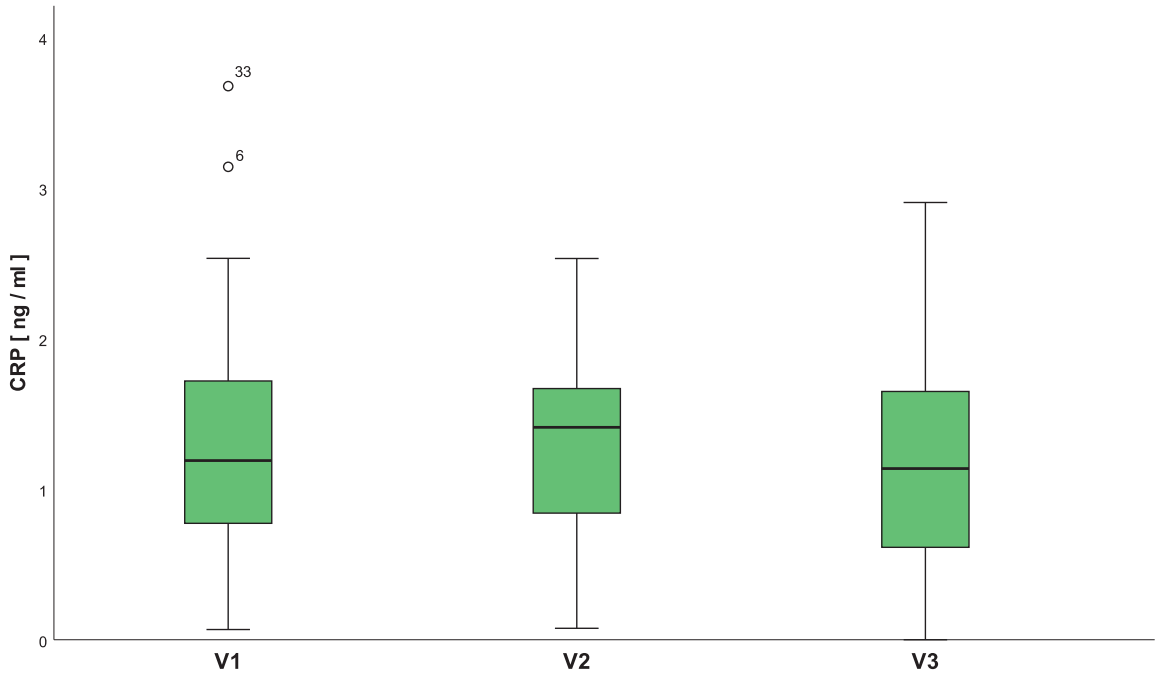

Figure 13: Concentrations of CRP during Pregnancy

Boxplot displaying quartiles, median and interquartile range for CRP concentrations [ng/ml] in the first and second trimester measured in maternal serum (n=23).

4.3.2 sICAM-1

As shown in Table 5 the mean values of sICAM-1 were 91,82 ± 78,34 ng/ml at V1 and 86,39 ± 66,51 ng/ml at V2, sample size n=23. At V3 the mean value was 82,80 ± 58,01 ng/ml.

Table 5: Concentrations of sICAM-1 during Pregnancy [ng/ml]

	n	Minimum	Maximum	Mean	SD
Visit 1	23	32,80	380,52	91,82	78,34
Visit 2	23	29,72	279,47	86,39	66,51
Visit 3	23	34,26	258,73	82,80	58,01

Concentrations are given in [ng/ml]. SD=Standard Deviation.

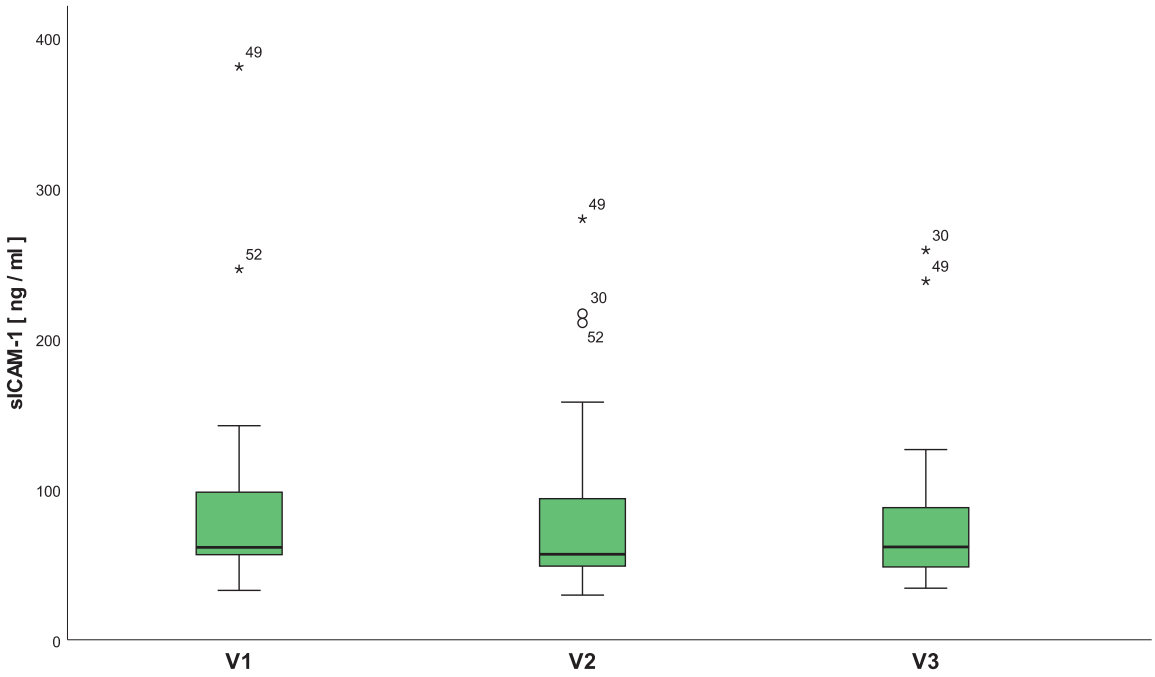

Figure 14: Concentrations of sICAM-1 during Pregnancy

Boxplot displaying quartiles, median and interquartile range for sICAM-1 concentrations [ng/ml] in the first and second trimester measured in maternal serum (n=23).

4.3.3 MCP-1

With a sample size of n=23 the mean value of MCP-1 was 39,75 ± 20,76 pg/ml at V1 and 44,60 ± 17,70 pg/ml at V2. At V3 the mean value of MCP-1 was 41,62 ± 11,57 pg/ml with a sample size of n=23 (Table 6).

Table 6: Concentrations of MCP-1 during Pregnancy

	n	Minimum	Maximum	Mean	SD
Visit 1	23	0,00	95,04	39,75	20,76
Visit 2	23	17,26	91,25	44,60	17,70
Visit 3	23	18,91	62,54	41,62	11,57

Concentrations are given in [pg/ml]. SD=Standard Deviation.

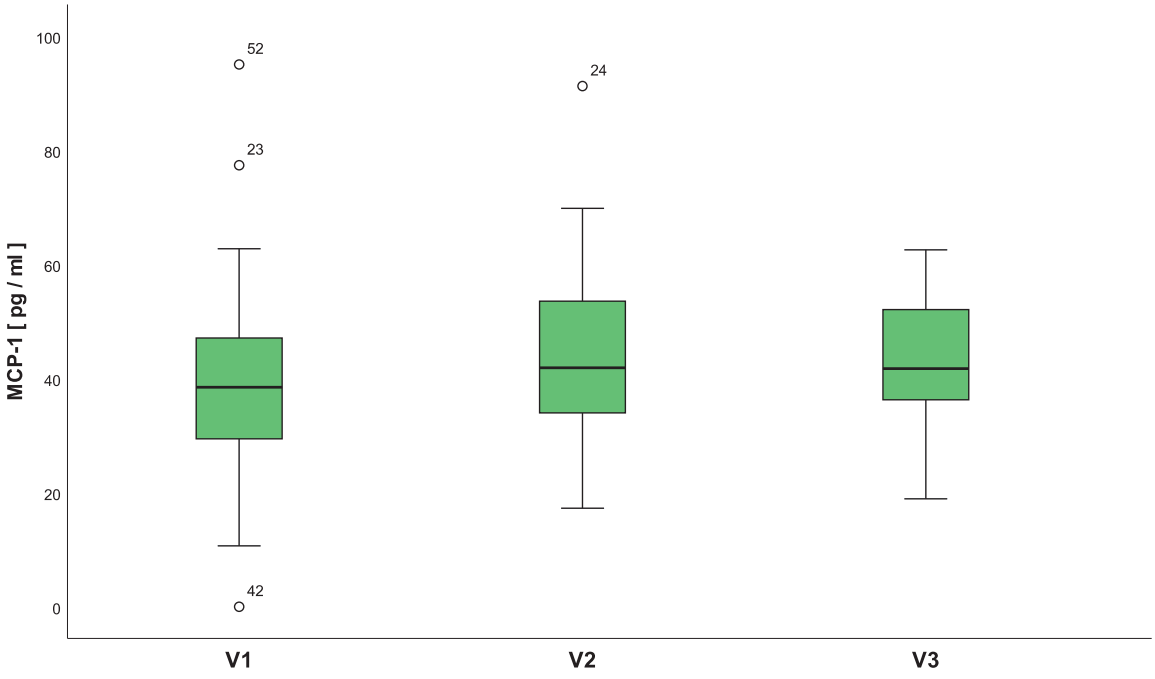

Figure 15: Concentrations of MCP-1 during Pregnancy

Boxplot displaying quartiles, median and interquartile range for MCP-1 concentrations [pg/ml] in the first and second trimester measured in maternal serum (n=23).

4.3.4 MIP-1α

With a sample size of n=23 MIP-1α serum levels have a mean value of 5,06 $\pm$ 5,52 pg/ml at V1 and 5,48 $\pm$ 5,30 pg/ml at V2. At V3 the mean value was 4,83 $\pm$ 2,04 pg/ml (Table 7).

Table 7: Concentrations of MIP-1α during Pregnancy

	n	Minimum	Maximum	Mean	SD
Visit 1	23	0,00	27,95	5,06	5,52
Visit 2	23	2,39	27,84	5,48	5,30
Visit 3	23	2,37	9,75	4,83	2,04

Concentrations are given in [pg/ml]. SD=Standard Deviation.

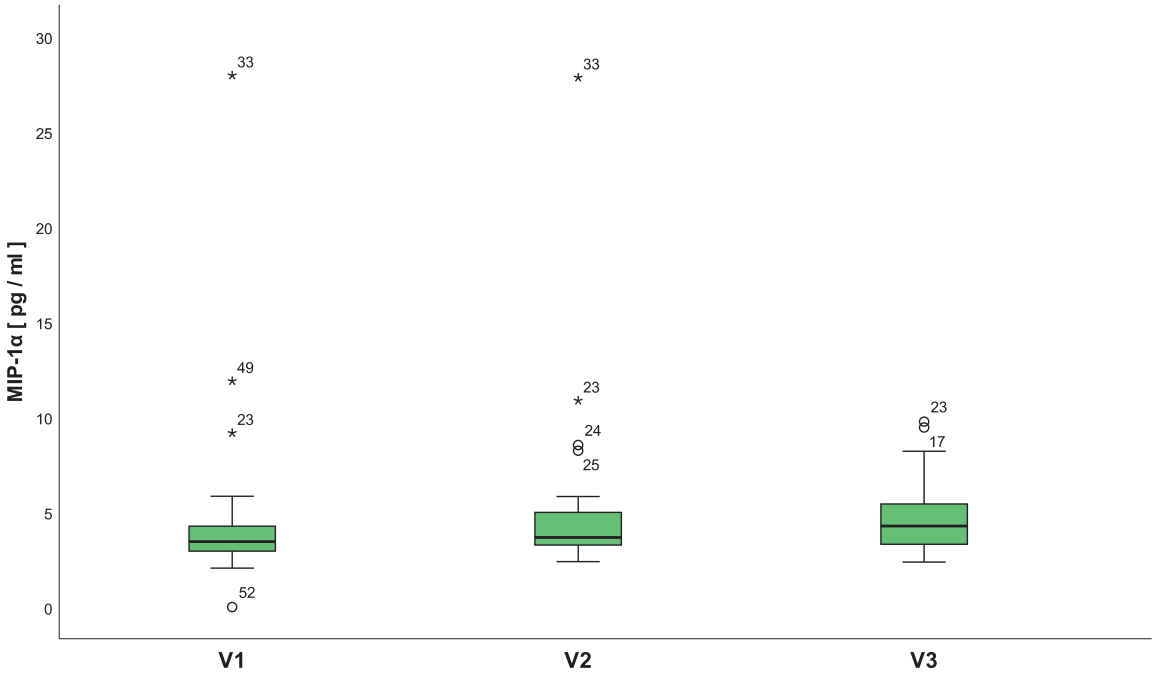

Figure 16: Concentrations of MIP-1α during Pregnancy

Boxplot displaying quartiles, median and interquartile range for MIP-1α concentrations [pg/ml] in the first and second trimester measured in maternal serum (n=23).

4.3.5 MIP-1β

With a sample size of n=23, MIP-1β serum levels have a mean value of 64,38 ± 26,31 pg/ml at V1 and 69,45 ± 19,43 pg/ml. At V3 the mean value was 77,42 ± 13,23 (Table 8).

Table 8: Concentrations of MIP-1β during Pregnancy

	n	Minimum	Maximum	Mean	SD
Visit 1	23	0,00	110,07	64,38	26,31
Visit 2	23	24,65	121,61	69,45	19,43
Visit 3	23	54,8	113,80	77,42	13,23

Concentrations are given in [pg/ml]. SD=Standard Deviation.

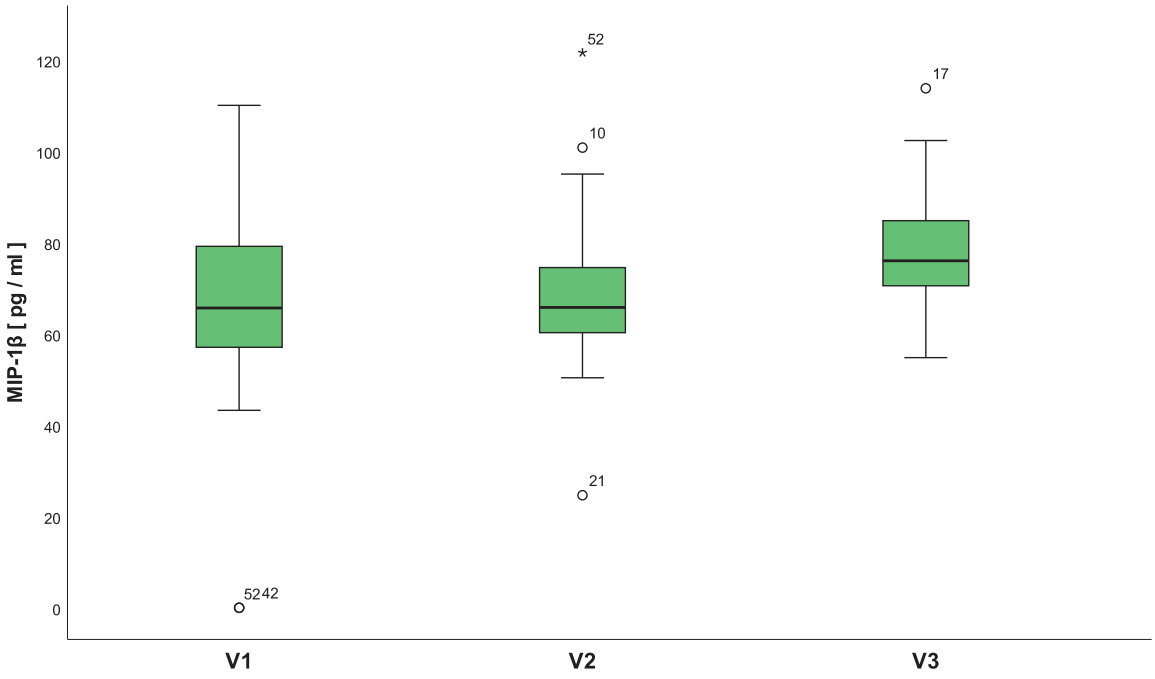

Figure 17: Concentrations of MIP-1β during Pregnancy

Boxplot displaying quartiles, median and interquartile range for MIP-1β concentrations [pg/ml] in the first and second trimester measured in maternal serum (n=23).

4.4 Association between Maternal Inflammatory Markers and Maternal HMO Concentrations in Midpregnancy and Late Pregnancy

Subsequently, our results of the bivariate correlation analyses which were tested using Spearman's rank correlation coefficient (Spearman's rho) are given. Simple linear regression models further explore the relationship of those variables that significantly correlate with each other.

4.4.1 CRP

As demonstratet in Table 9 we found that the inflammatory marker CRP was shown to correlate positively with 3'SL concentration at V1 and inversely with 2'FL concentration at V1. The significance level was $p < 0{,}05$.

Table 9: Spearman's rho of the association between CRP with 2'FL or 3'SL

	V1 CRP	V2 CRP	V3 CRP
V1 2'FL	−0,118 (p=0,593)	−0,430*(p=0,041)	−0,022 (p=0,921)
V1 3'SL	0,418*(p=0,047)	0,266 (p=0,220)	0,190 (p=0,386)
V2 2'FL	−0,084 (p=0,703)	−0,144 (p=0,511)	0,002 (p=0,993)
V2 3'SL	0,232 (p=0,286)	−0,006 (p=0,977)	0,252 (p=0,246)

* indicates a significance level of 5%.

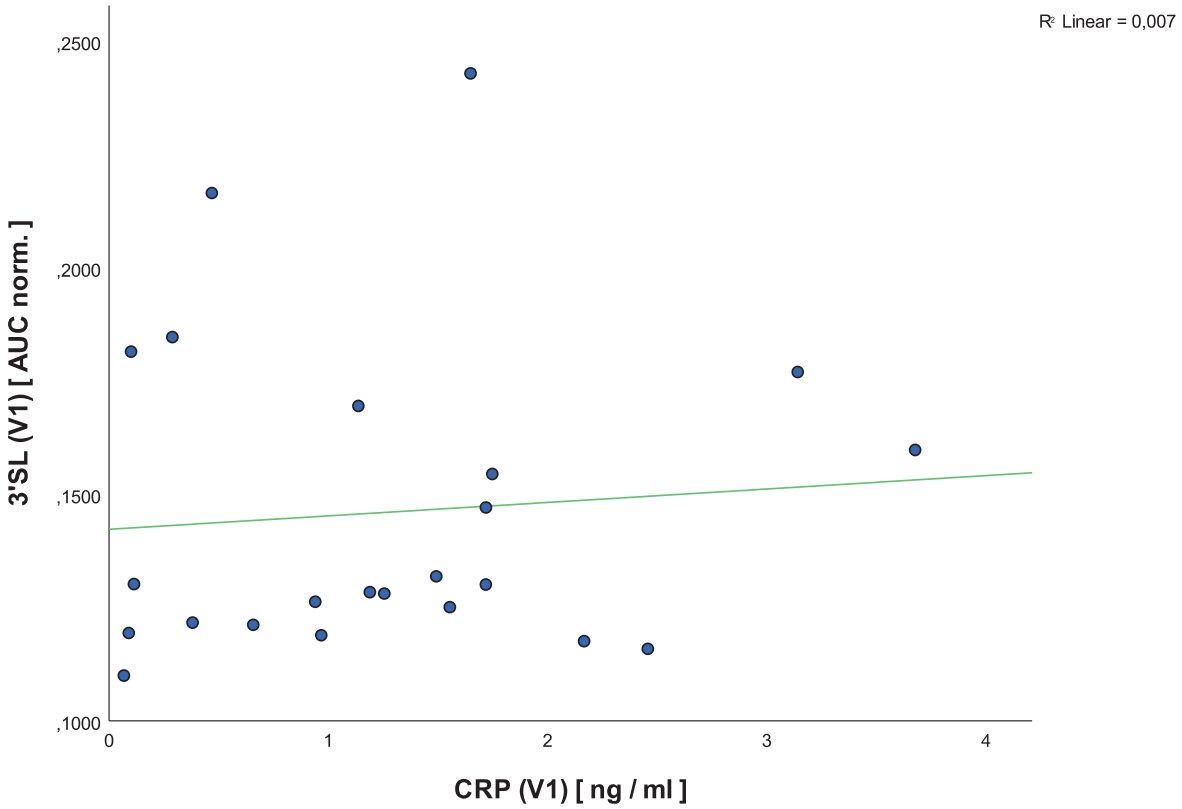

FIGURE 18: CORRELATION OF 3'SL (V1) AND CRP (V1)

Figure 18 displays the simple linear regression of 3'SL and CRP, both at V1 (n=23). Spearman's rho was 0,418 and p=0,047. The coefficient of determination is r^2=0,007 and implies a quite poor model fit since only 0,7% of the variance of 3'SL can be explained by CRP.

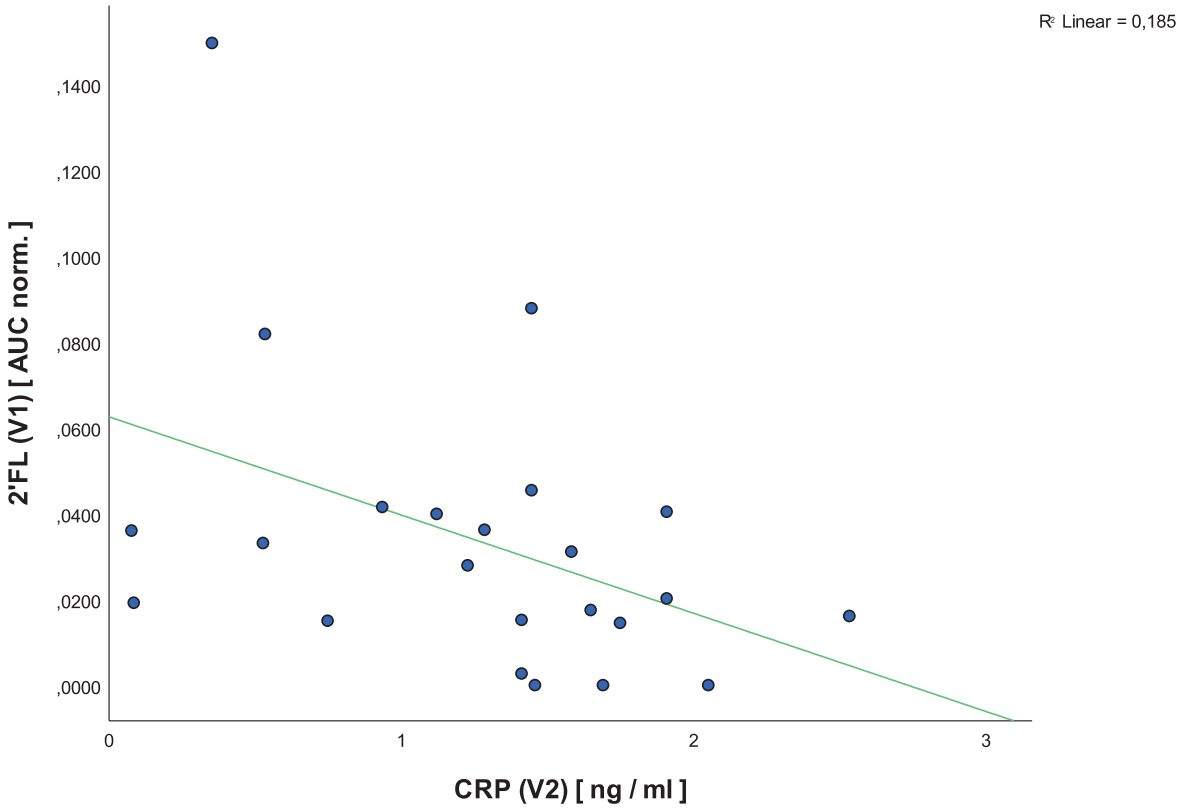

FIGURE 19: CORRELATION OF 2'FL (V1) AND CRP (V2)

Figure 19 displays the simple linear regression of 2'FL in early pregnancy and CRP in midpregnancy (n=23). Spearman's rho was -0,430 and p=0,041. In this case, the coefficient of determination r^2=0,185 implies that 18,5% of the variance of 2'FL can be explained by CRP.

To elucidate whether associations between inflammatory markers and HMOs might be different depending on the Secretor status we separated the study population in Se $\oplus$ and Se $\ominus$ women. The small size of the study population allowed reliable analysis for the group of Se $\oplus$ women, only.

In Se $\oplus$ women, no further significant correlations were found between CRP and 2'FL or 3'SL (Table 10).

Table 10: Spearman's rho of the association between CRP with 2'FL or 3'SL in Se ⊕ women

	V1 CRP	V2 CRP	V3 CRP
V1 2'FL	−0,111 (p=0,650)	−0,349 (p=0,143)	−0,030 (p=0,903)
V1 3'SL	0,314 (p=0,190)	0,253 (p=0,296)	0,156 (p=0,523)
V2 2'FL	−0,056 (p=0,819)	0,112 (p=0,647)	0,005 (p=0,983)
V2 3'SL	0,112 (p=0,647)	−0,030 (p=0,903)	0,219 (p=0,367)

4.4.2 sICAM-1

Analysis revealed four significant reciprocal correlations between sICAM-1 concentrations and both HMOs 2'FL and 3'SL (Table 11). The correlations were significant with a significance level of $p < 0{,}01$ and $p < 0{,}05$.

Furthermore it is noteworthy that the correlation between 3'SL at at Visit 2 (V2) and sICAM-1 (V3) is almost significant with rho=-0,410 and p=0,052.

Table 11: Spearman's rho of the association between sICAM-1 with 2'FL or 3'SL

	V1 sICAM-1	V2 sICAM-1	V3 sICAM-1
V1 2'FL	−0,223 (p=0,307)	−0,201 (p=0,357)	0,075 (p=0,733)
V1 3'SL	−0,328 (p=0,126)	−0,508*(p=0,013)	−0,239 (p=0,272)
V2 2'FL	−0,416*(p=0,048)	−0,372 (p=0,081)	−0,396 (p=0,061)
V2 3'SL	−0,418*(p=0,047)	−0,543**(p=0,007)	−0,410 (p=0,052)

* indicates a significance level of 5%.
** indicates a significance level of 1%.

Figure 20, Figure 21 and Figure 22 present the three negative correlations between sICAM-1 and 3'SL.

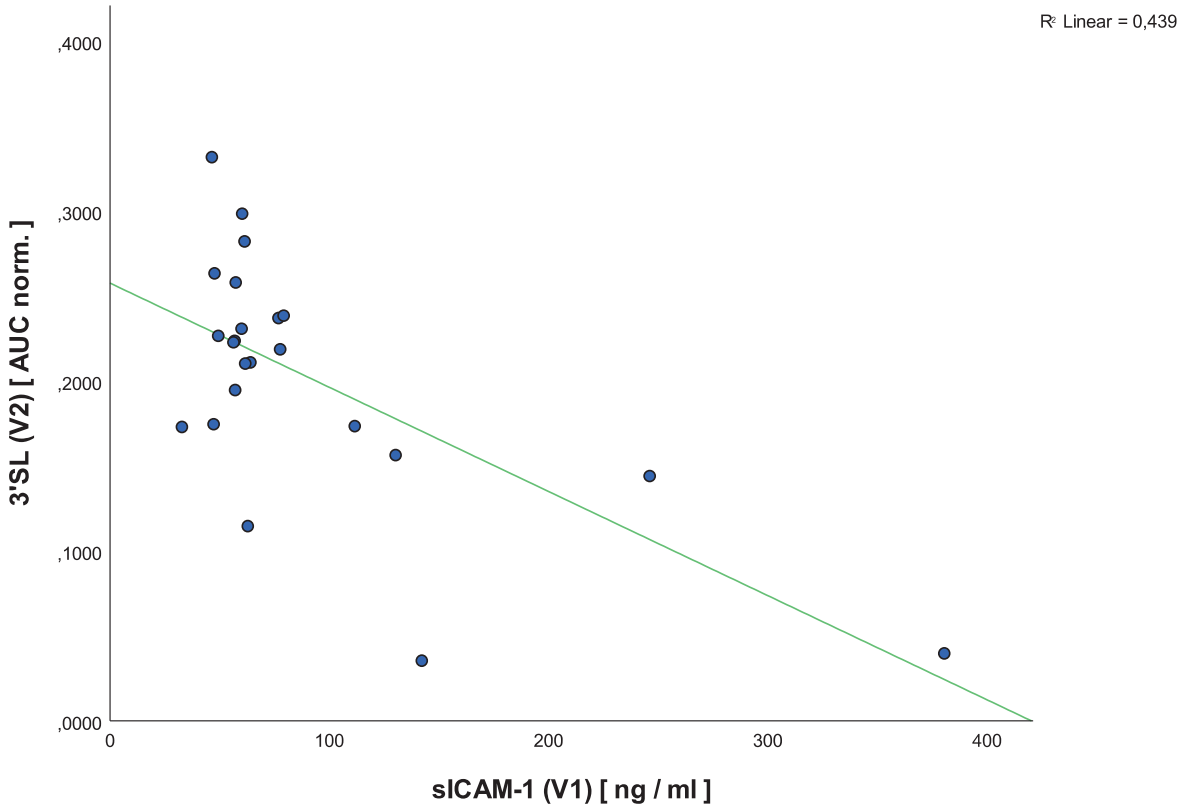

FIGURE 20: CORRELATION OF 3'SL (V2) AND SICAM-1 (V1)

The simple linear regression of 3'SL (V2) and sICAM-1 (V1) shows a negative correlation with a determination coefficient r^2=0,439 (Fig.20). Therefore, 43,9% of the HMO 3'SL (V2) variance can be explained by the inflammation marker sICAM-1 at V1.

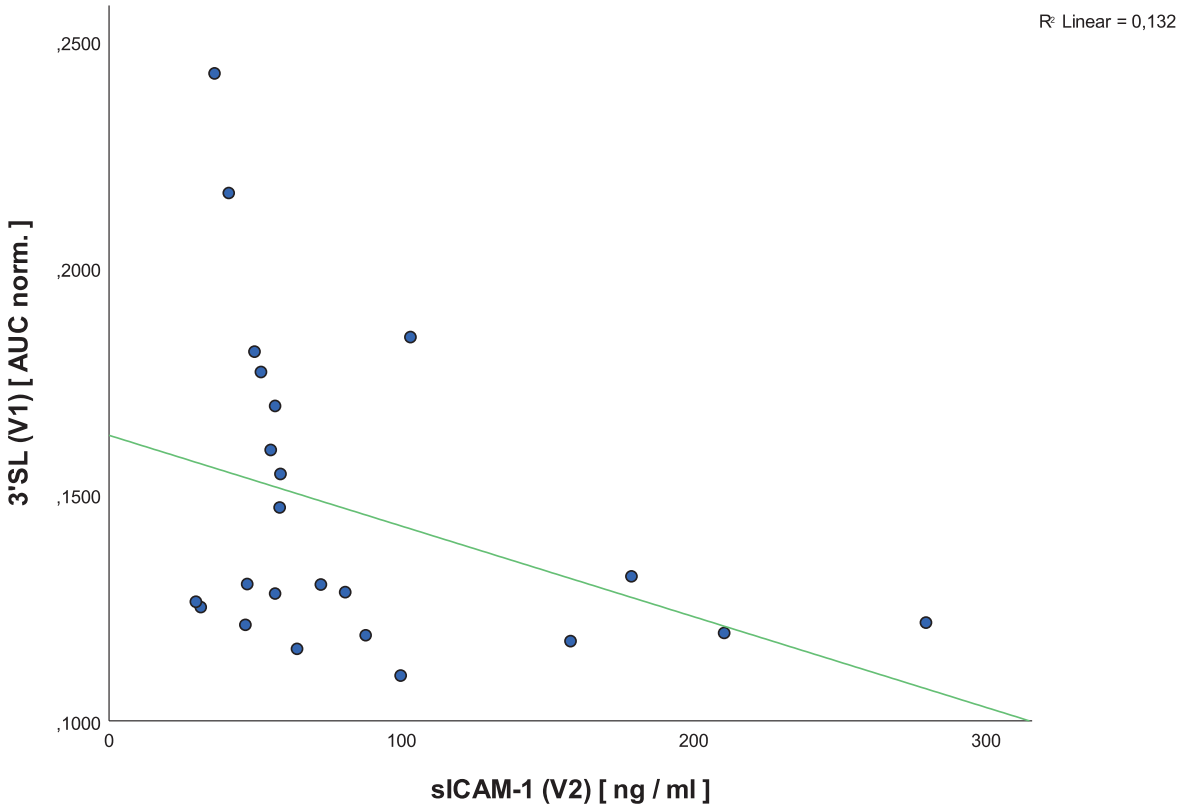

Figure 21: Correlation of 3'SL (V1) and sICAM-1 (V2)

With rho=-0,508 and p=0,013 (Figure 21) the coefficient of determination is r^2=0,132. In this linear regression model, 3'SL concentrations at V1 explain only 13,2% of the variance of sICAM-1 at V2.

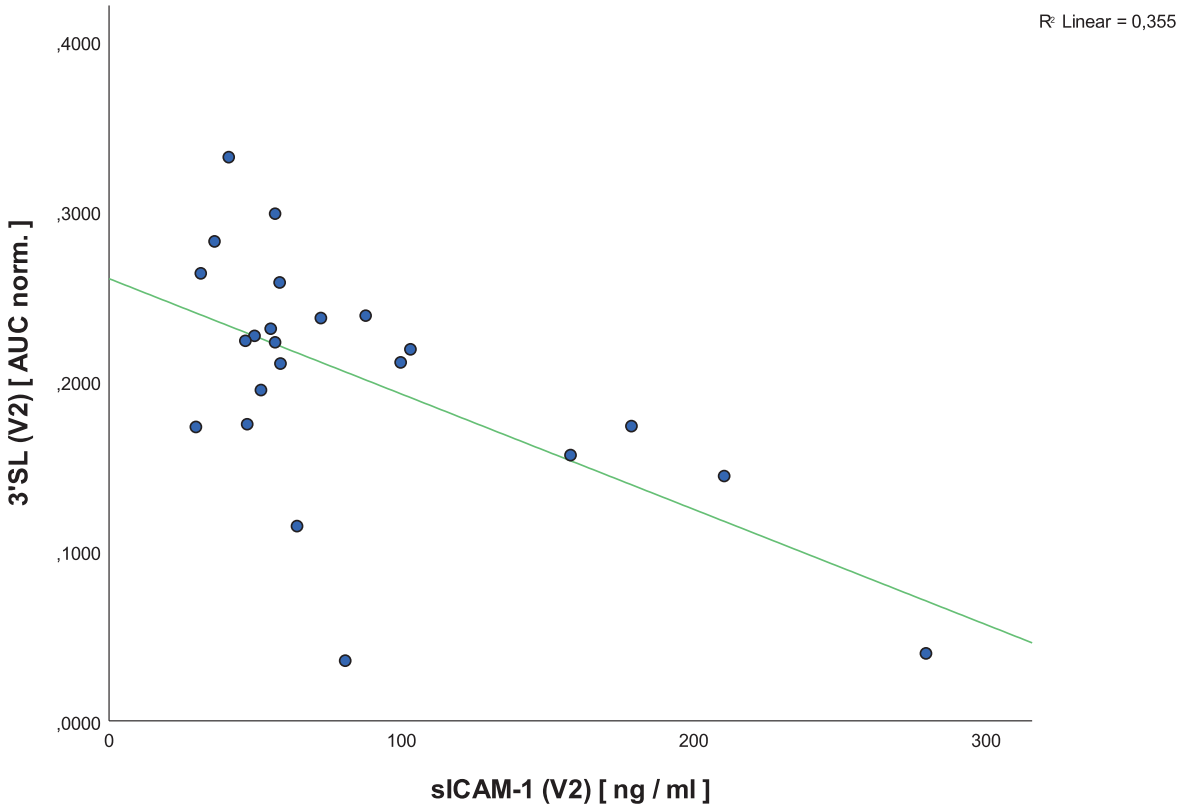

Figure 22: Correlation of 3'SL (V2) and sICAM-1 (V2)

For 3'SL and sICAM-1 in midpregnancy we found a stronger association: with Spearman's rho=-0,543 and p=0,007 the correlation was highly significant. The coefficient of determination r^2=0,355 (Fig.22) lets us assume that in comparison to the relationship between 3'SL at V1 and sICAM-1 at V2, nearly the twofold of 3'SL's variance, 35,5%, can be explained by the inflammation marker sICAM-1 in midpregnancy.

Another association was found between 2'FL (V2) and sICAM-1 (V1). With Spearman's rho=-0,416 and a significance level of p=0,048, the coefficient of determination is r^2=0,150 (Fig.23).

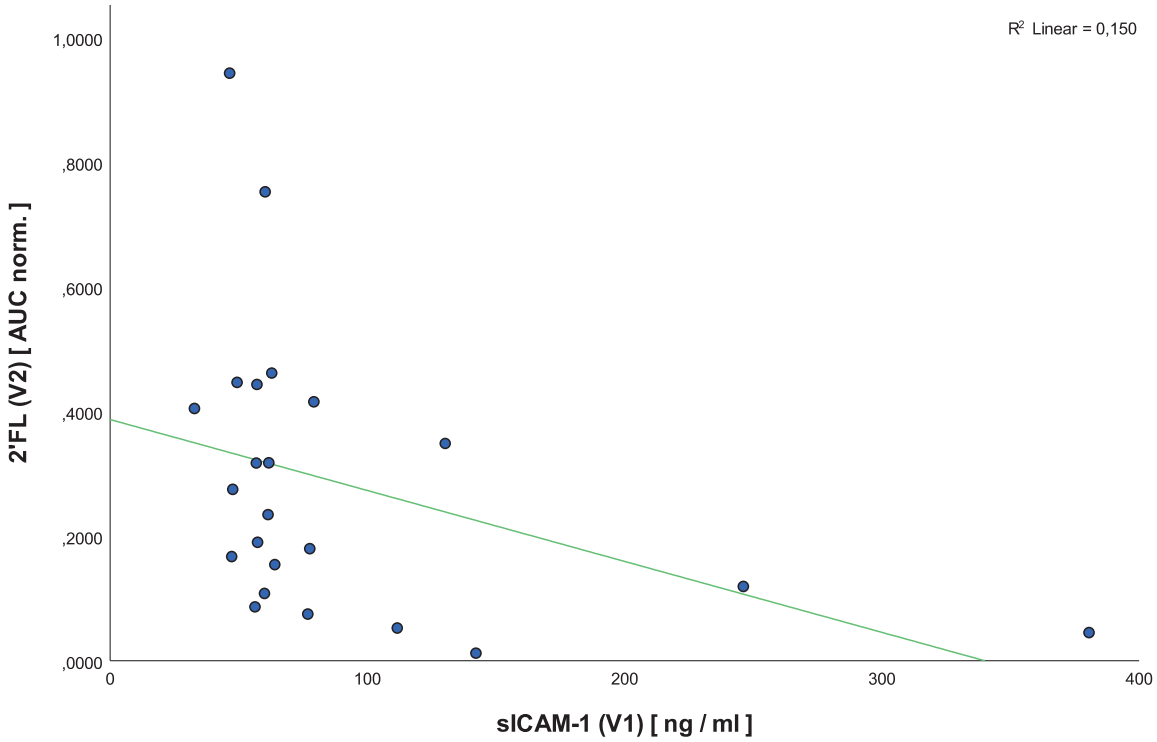

FIGURE 23: CORRELATION OF 2'FL (V2) AND SICAM-1 (V1)

In the group of Se ⊕ women only (Table 12, n=19), Spearman's rho showed a significant deviation for some associations in comparison to the whole study population (Table 11).

TABLE 12: SPEARMAN'S RHO OF THE ASSOCIATION BETWEEN SICAM-1 WITH 2'FL OR 3'SL IN SE ⊕ WOMEN

	V1 sICAM-1	V2 sICAM-1	V3 sICAM-1
V1 2'FL	−0,109 (p=0,657)	−0,162 (p=0,509)	0,183 (p=0,452)
V1 3'SL	−0,426 (p=0,069)	−0,558*(p=0,013)	−0,244 (p=0,314)
V2 2'FL	−0,511*(p=0,026)	−0,449 (p=0,054)	−0,275 (p=0,254)
V2 3'SL	−0,474*(p=0,040)	−0,519*(p=0,023)	−0,410 (p=0,052)

* indicates a significance level of 5%.

The simple linear regression of 2'FL (V2) and sICAM-1 (V1) shows a negative correlation with a coefficient of determination r^2=0,165 (Fig.24). Therefore, 16,5% of the variance of the HMO 2'FL (V2) can be explained by the inflammation marker sICAM-1 (V1) in Se ⊕ women.

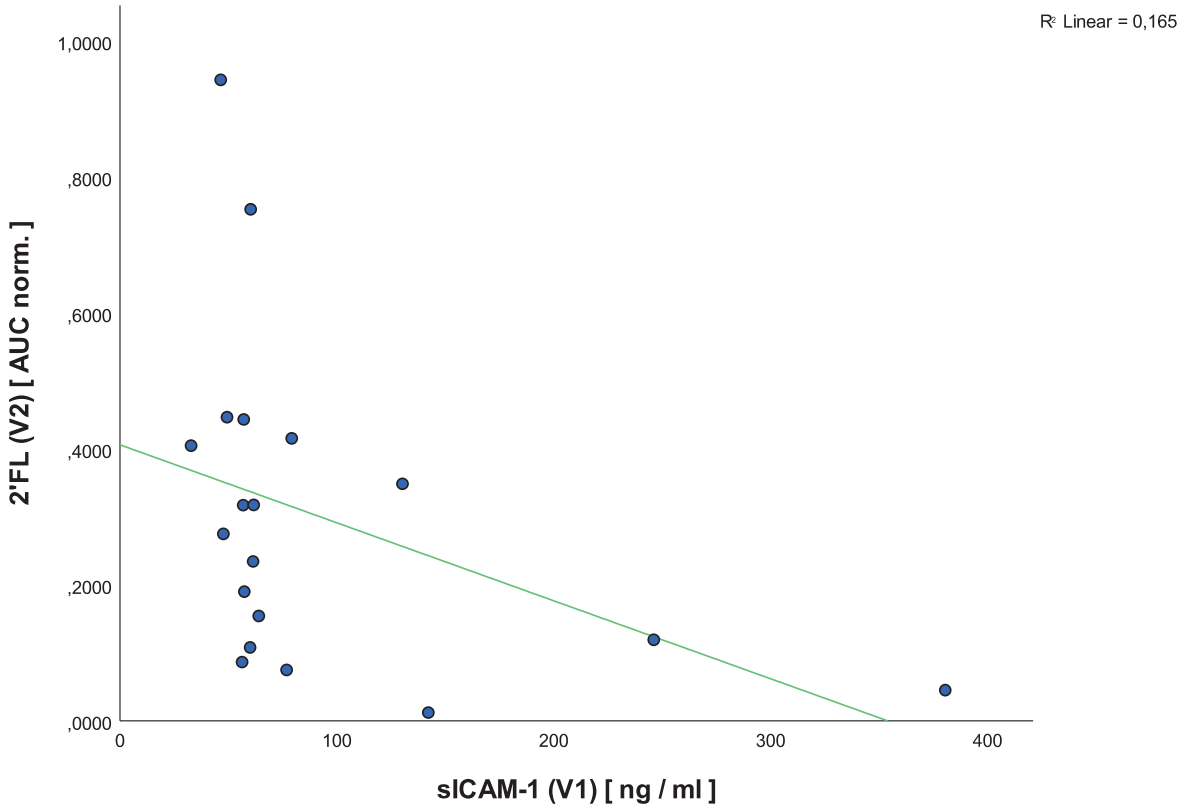

Figure 24: Correlation of 2'FL (V2) and sICAM-1 (V1) (Se ⊕ women)

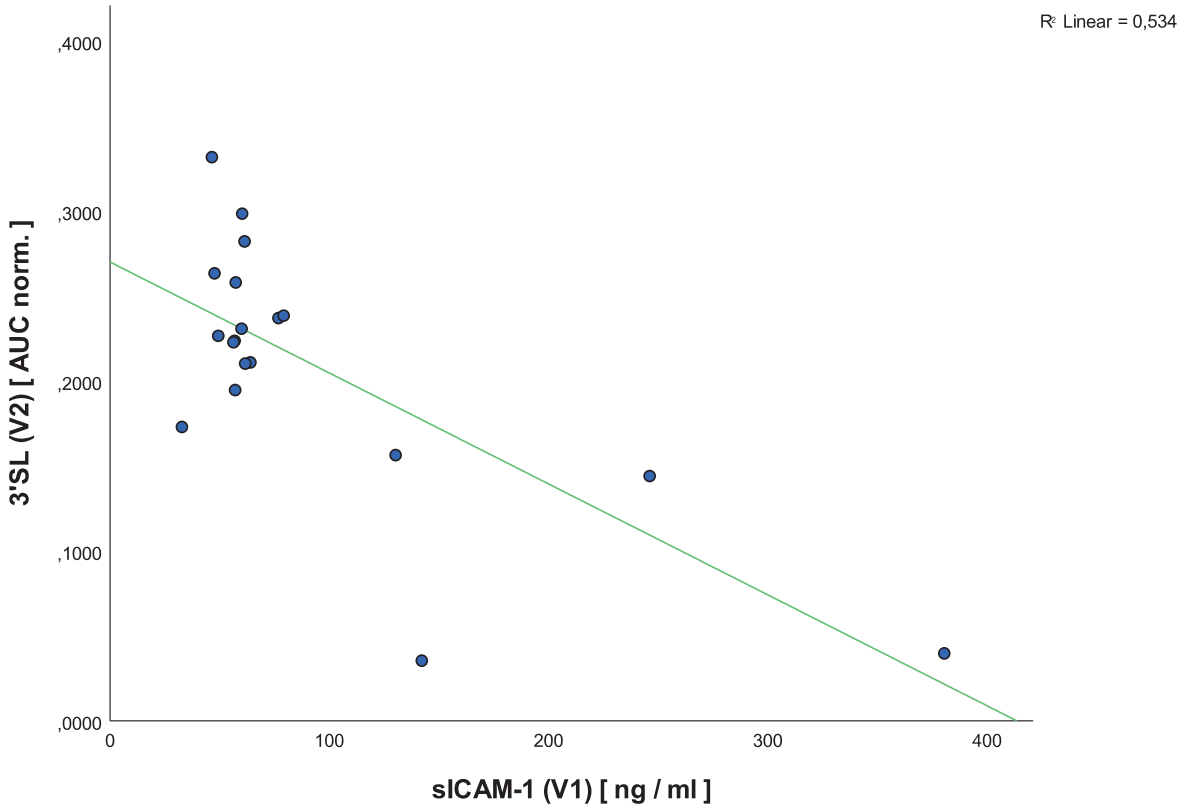

FIGURE 25: CORRELATION OF 3'SL (V2) AND SICAM-1 (V1) (SE ⊕ WOMEN)

As shown in Figure 25 the highest coefficient of determination of the linear regressions was r^2=0,534 and depicts the association between 3'SL (V2) and sICAM-1 (V1) (rho=-0,474; $p < 0,05$).

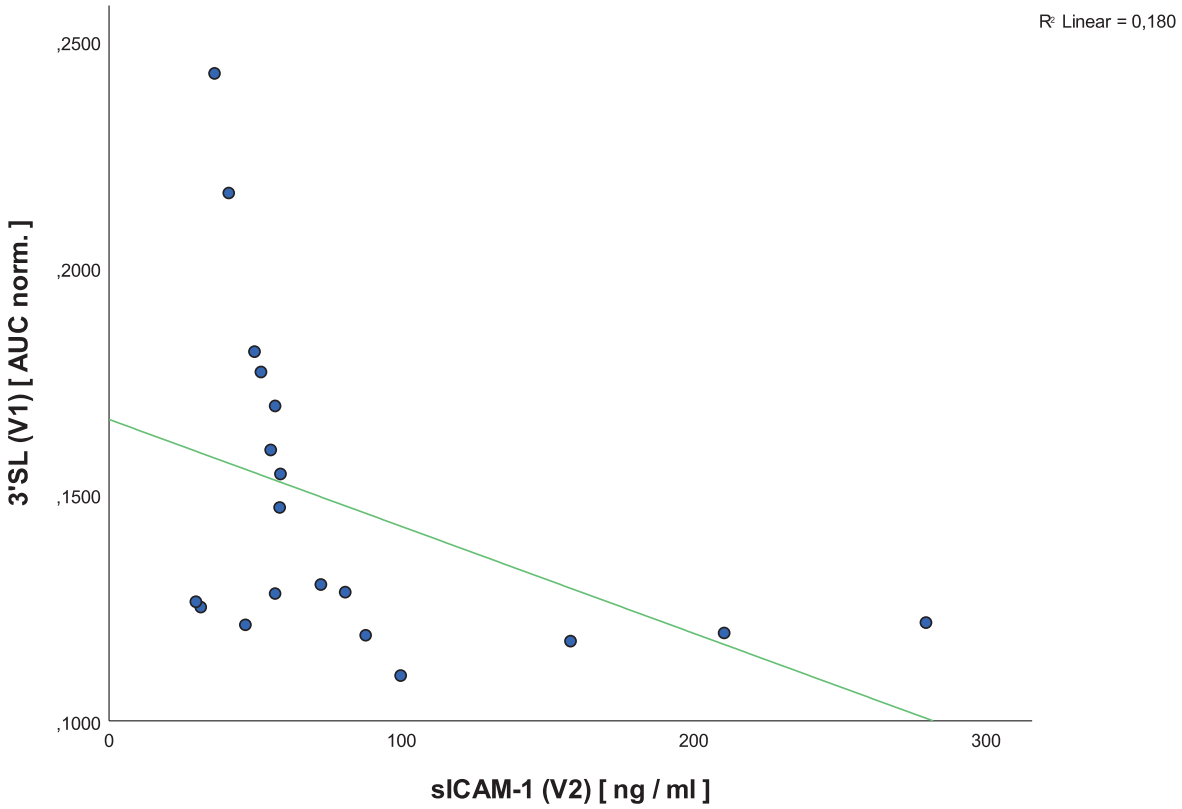

Figure 26: Correlation of 3'SL (V1) and sICAM-1 (V2)

The relationship between 3'SL (V2) and sICAM-1 (V1) in Se $\oplus$ women (rho -0,474; p=0,040) is displayed in Figure 26, where the coefficient of determination is r^2=0,180.

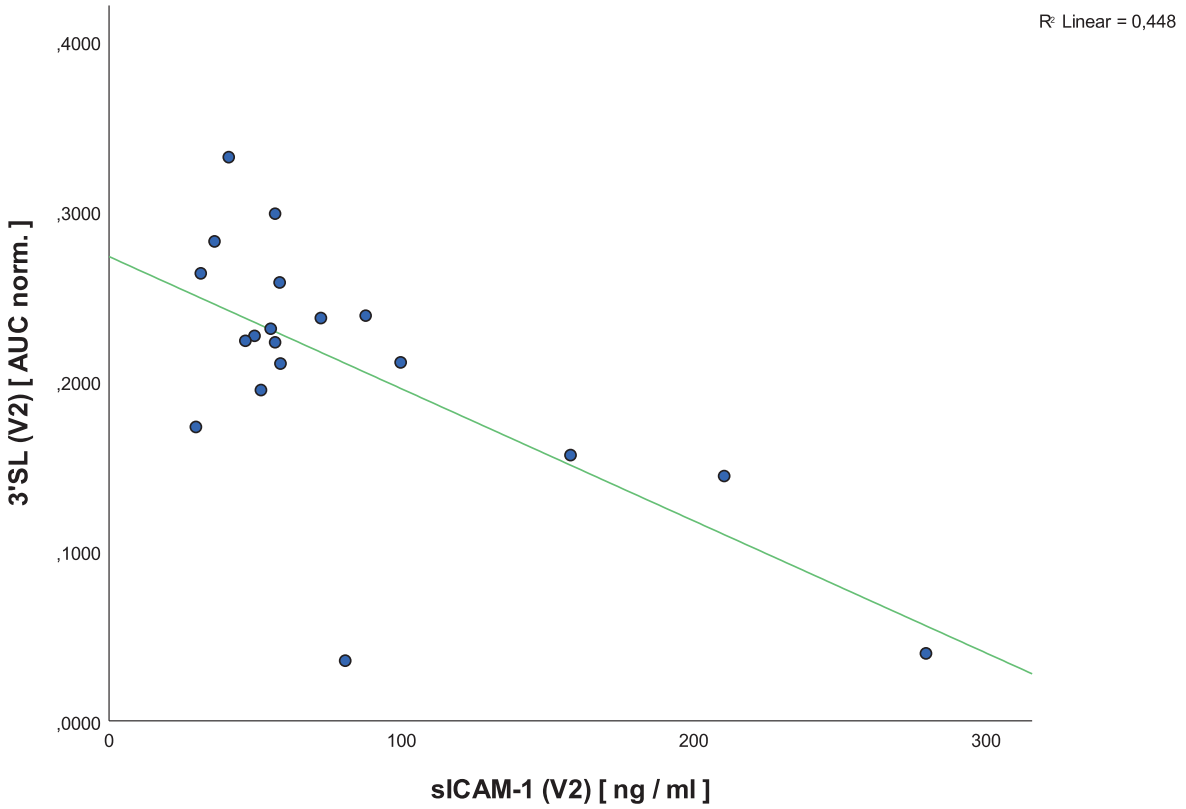

Figure 27: Correlation of 3'SL (V2) and sICAM-1 (V2)

For 3'SL and sICAM-1, both at V2, the correlation was even stronger: rho=-0,519 (p=0,023) and r^2=0,448 (Figure 27).

4.4.3 MCP-1

As shown in Table 13, no correlations between MCP-1 concentrations and the HMOs 2'FL and 3'SL were found in the total study population.

Table 13: Spearman's rho of the association between MCP-1 with 2'FL or 3'SL

	V1 MCP-1	V2 MCP-1	V3 MCP-1
V1 2'FL	-0,363 (p=0,089)	-0,021(p=0,925)	0,151 (p=0,491)
V1 3'SL	-0,373 (p=0,080)	-0,123(p=0,578)	-0,340 (p=0,113)
V2 2'FL	-0,002 (p=0,991)	0,066 (p=0,764)	0,313 (p=0,146)
V2 3'SL	-0,136 (p=0,535)	0,056 (p=0,799)	0,149 (p=0,497)

The group of Se $\oplus$ women showed a significant correlation between MCP-1 and 3'SL ($p < 0,05$; Table 14).

Table 14: Spearman's rho of the association between MCP-1 with 2'FL or 3'SL in Se ⊕ women

	V1 MCP-1	V2 MCP-1	V3 MCP-1
V1 2'FL	-0,302 (p=0,209)	0,004 (p=0,989)	0,167 (p=0,601)
V1 3'SL	-0,504* (p=0,028)	-0,267 (p=0,270)	-0,351 (p=0,141)
V2 2'FL	-0,055 (p=0,822)	0,096 (p=0,694)	0,372 (p=0,117)
V2 3'SL	-0,268 (p=0,268)	-0,042 (p=0,864)	0,128 (p=0,601)

* indicates a significance level of 5%.

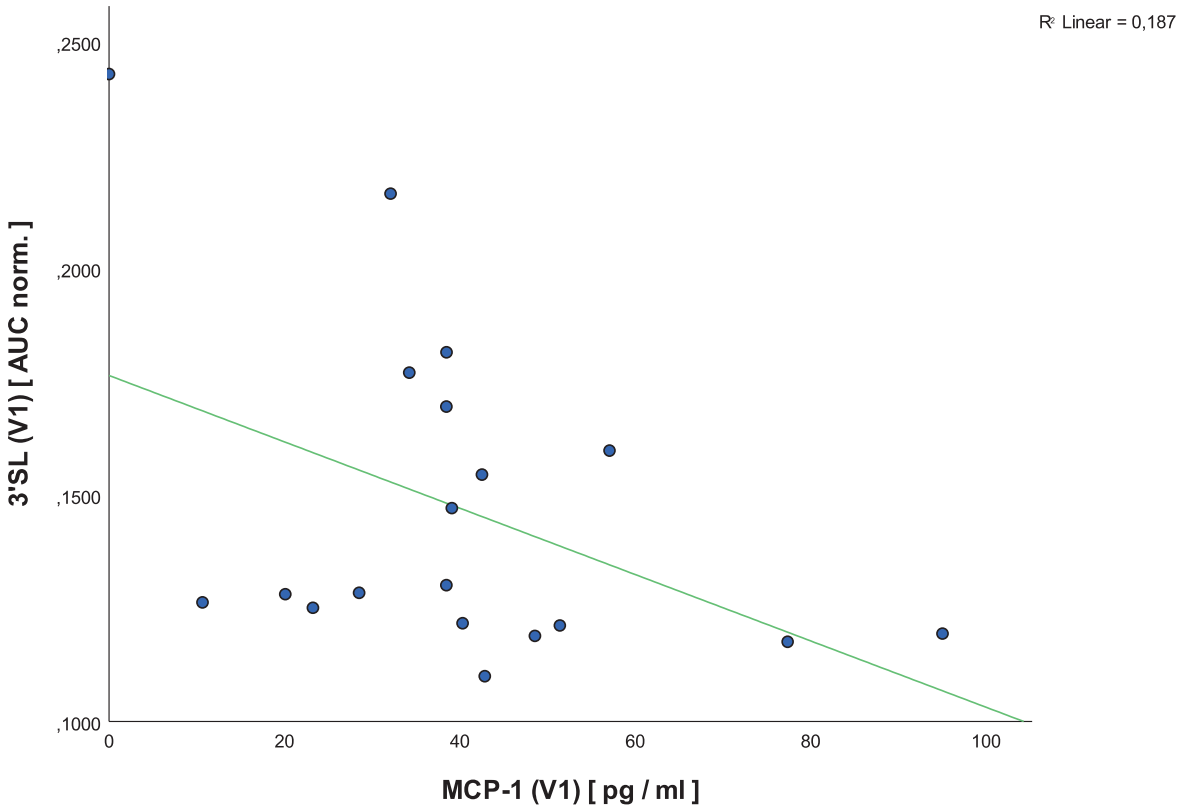

Figure 28: Correlation of 3'SL (V1) and MCP-1 (V1) (Se ⊕ women)

The simple linear regression in Figure 28 displays the negative correlation between 3'SL and MCP-1 at V1 (rho=-0,504; $p < 0,5$). R^2=0,187.

4.4.4 MIP-1α

Between MIP-1α concentrations and the HMOs 2'FL and 3'SL no correlations were found, neither in the total study population (Table 15) nor in Se ⊕ women (Table 16).

Table 15: Spearman's rho of the association between MIP-1α with 2'FL or 3'SL

	V1 MIP-1α	V2 MIP-1α	V3 MIP-1α
V1 2'FL	-0,242 (p=0,265)	0,104 (p=0,637)	-0,084 (p=0,703)
V1 3'SL	-0,266 (p=0,220)	0,081 (p=0,715)	-0,294 (p=0,173)
V2 2'FL	0,039 (p=0,861)	0,171 (p=0,434)	0,322 (p=0,134)
V2 3'SL	0,015 (p=0,946)	0,295 (p=0,172)	0,162 (p=0,460)

Table 16: Spearman's rho of the association between MIP-1α with 2'FL or 3'SL in Se $\oplus$ women (n=19)

	V1 MIP-1α	V2 MIP-1α	V3 MIP-1α
V1 2'FL	-0,128 (p=0,601)	0,085 (p=0,729)	-0,104 (p=0,673)
V1 3'SL	-0,304 (p=0,207)	-0,051 (p=0,836)	-0,414 (p=0,078)
V2 2'FL	0,156 (p=0,523)	0,062 (p=0,800)	0,209 (p=0,391)
V2 3'SL	0,082 (p=0,737)	0,190 (p=0,435)	0,040 (p=0,870)

4.4.5 MIP-1β

MIP-1β and 2'FL concentrations were negatively associated with each other at V1 (rho=-0,458; p=0,028). Correlations between the same were almost significant at V2 (rho=0,405; p=0,055). Between MIP-1β and 2'FL or 3'SL at other dates no further correlations were found (Table 17).

Table 17: Spearman's rho of the association between MIP-1β with 2'FL or 3'SL

	V1 MIP-1β	V2 MIP-1β	V3 MIP-1β
V1 2'FL	-0,458* (p=0,028)	0,375 (p=0,078)	0,148 (p=0,499)
V1 3'SL	-0,126 (p=0,567)	0,053 (p=0,809)	-0,369 (p=0,083)
V2 2'FL	0,054 (p=0,807)	0,405 (p=0,055)	0,376 (p=0,077)
V2 3'SL	0,104 (p=0,636)	0,327 (p=0,126)	0,247 (p=0,256)

* indicates a significance level of 5%.

The simple linear regression (Figure 29) shows a negative correlation of MIP-1β and 2'FL. Spearman's rho was -0,458 ($p < 0.05$) and r^2=0,016.

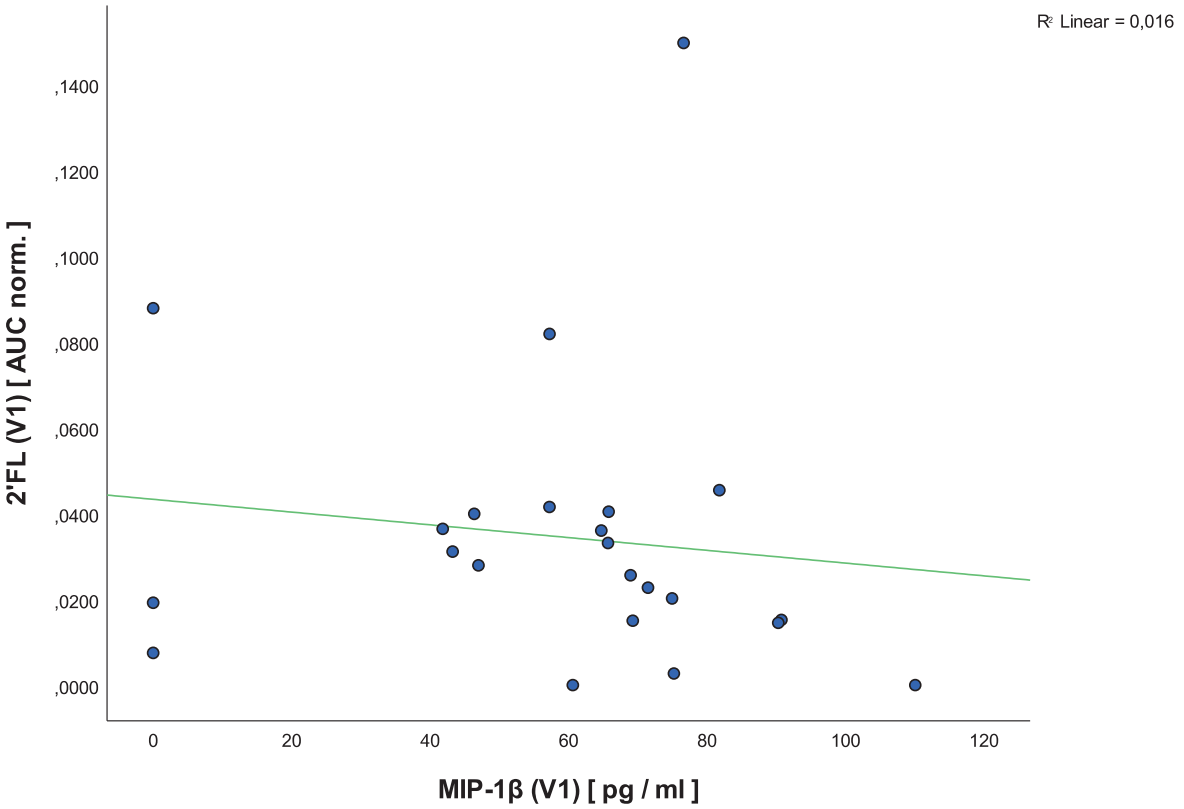

FIGURE 29: CORRELATION OF 2'FL (V1) AND MIP-1β (V1)

The analysis of Se $\oplus$ women revealed another significant correlation between 3'SL at V1 and MIP-1β at V3 (rho=-0,565; p=0,012; see Table 18).

TABLE 18: SPEARMAN'S RHO OF THE ASSOCIATION BETWEEN MIP-1β WITH 2'FL OR 3'SL IN SE $\oplus$ WOMEN

	V1 MIP-1β	V2 MIP-1β	V3 MIP-1β
V1 2'FL	-0,335 (p=0,160)	0,372 (p=0,117)	0,112 (p=0,650)
V1 3'SL	-0,120 (p=0,624)	-0,067 (p=0,786)	-0,565* (p=0,012)
V2 2'FL	0,176 (p=0,470)	0,246 (p=0,311)	0,263 (p=0,276)
V2 3'SL	0,195 (p=0,424)	0,196 (p=0,420)	0,100 (p=0,684)

* indicates a significance level of 5%.

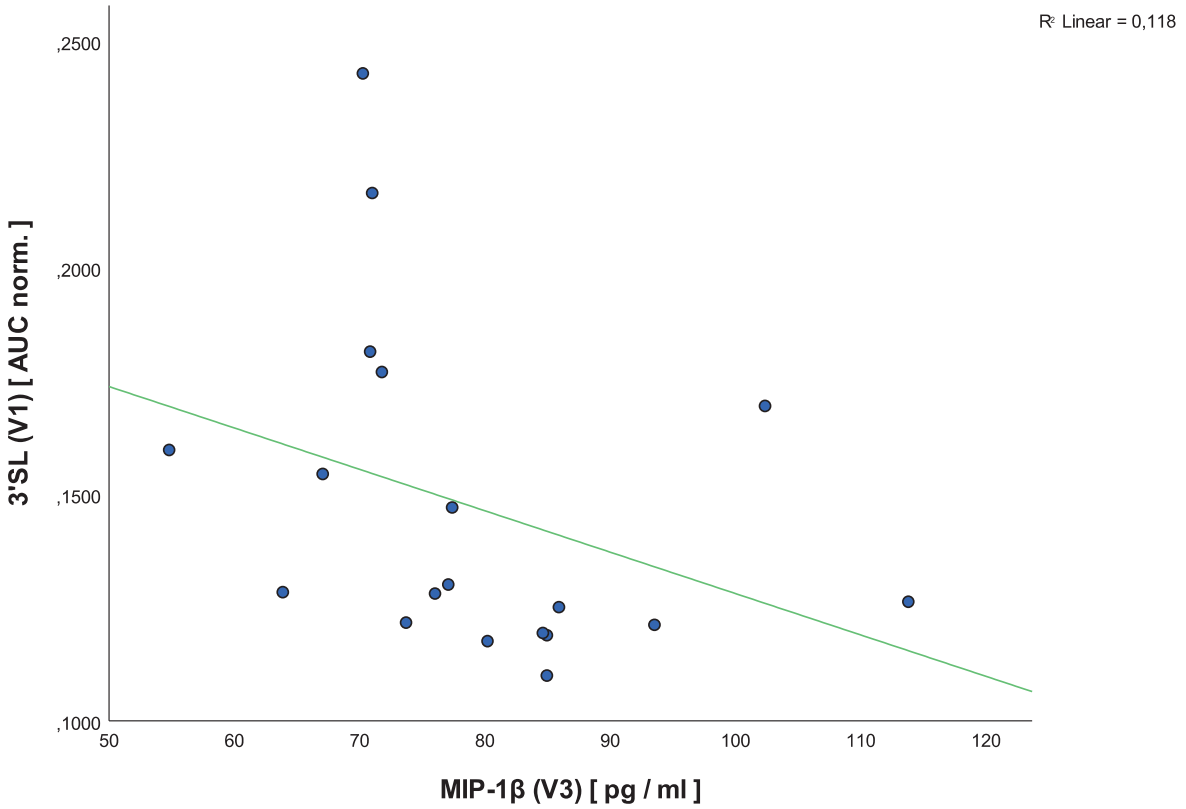

FIGURE 30: CORRELATION OF 3'SL (V1) AND MIP-1β (V3) (SE $\oplus$ WOMEN)

The significant, negative correlation between 3'SL at V1 and MIP-1β at V2 (rho=-0,565; p=0,012) is illustrated as a simple linear regression model in Figure 30 (r^2=0,118).

4.5 Association between Fetal Inflammatory Markers and Maternal HMO Concentrations

To investigate whether not only maternal but also fetal inflammation markers are associated with maternal HMOs in pregnancy we conducted correlation analyses for 2'FL and 3'SL (V2) and inflammatory markers in cord blood directly after delivery.

4.5.1 CRP

Table 19 contains Spearman's rank correlation coefficients for CRP in cord blood and HMOs in midpregnancy. A significant association was found between maternal 2'FL (V2) and fetal CRP (rho=0,452; p=0,030) in the total study population (n=23). Additionally, a significant, positive correlation in the same group was found between maternal 3'SL (V2) and fetal CRP (rho=0,531; p=0,009) in cord blood. No further significant correlations were found in children of Se ⊕ women only.

Table 19: Spearman's rho of the association between Fetal CRP with 2'FL or 3'SL

	CB CRP (n=23)	CB CRP (n=19)
V1 2'FL	0,073 (p=0,742)	−0,011 (p=0,963)
V1 3'SL	0,322 (p=0,134)	0,349 (p=0,143)
V2 2'FL	0,452*(p=0,030)	0,378 (p=0,110)
V2 3'SL	0,531**(p=0,009)	0,437 (p=0,061)

* indicates a significance level of 5%.
** indicates a significance level of 1%.
Left column: total study population.
Right column: Se ⊕ women.

Between 2'FL (V2) and CRP (CB) a significant correlation was found. With Spearman's rho=-0,452 and a significance level of p=0,030 the coefficient of determination is r^2=0,133 (Fig.31).

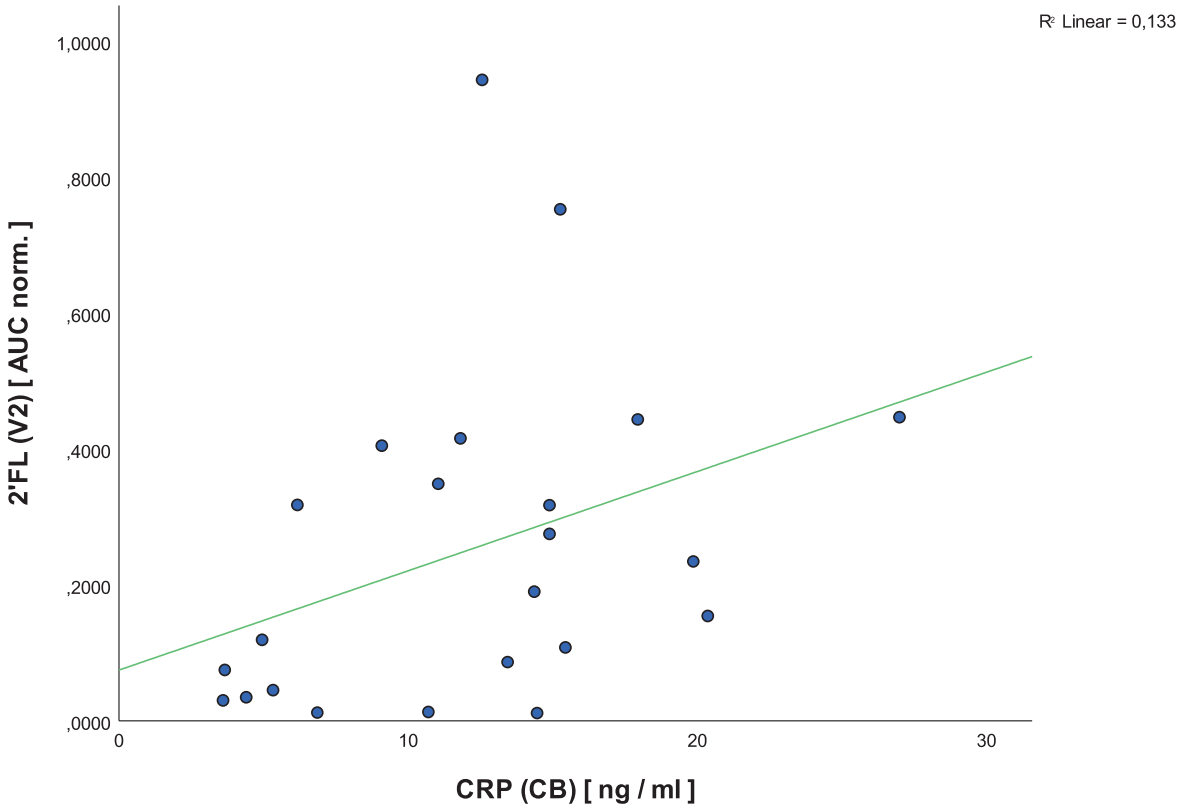

Figure 31: Correlation of 2'FL (V2) and CRP (CB)

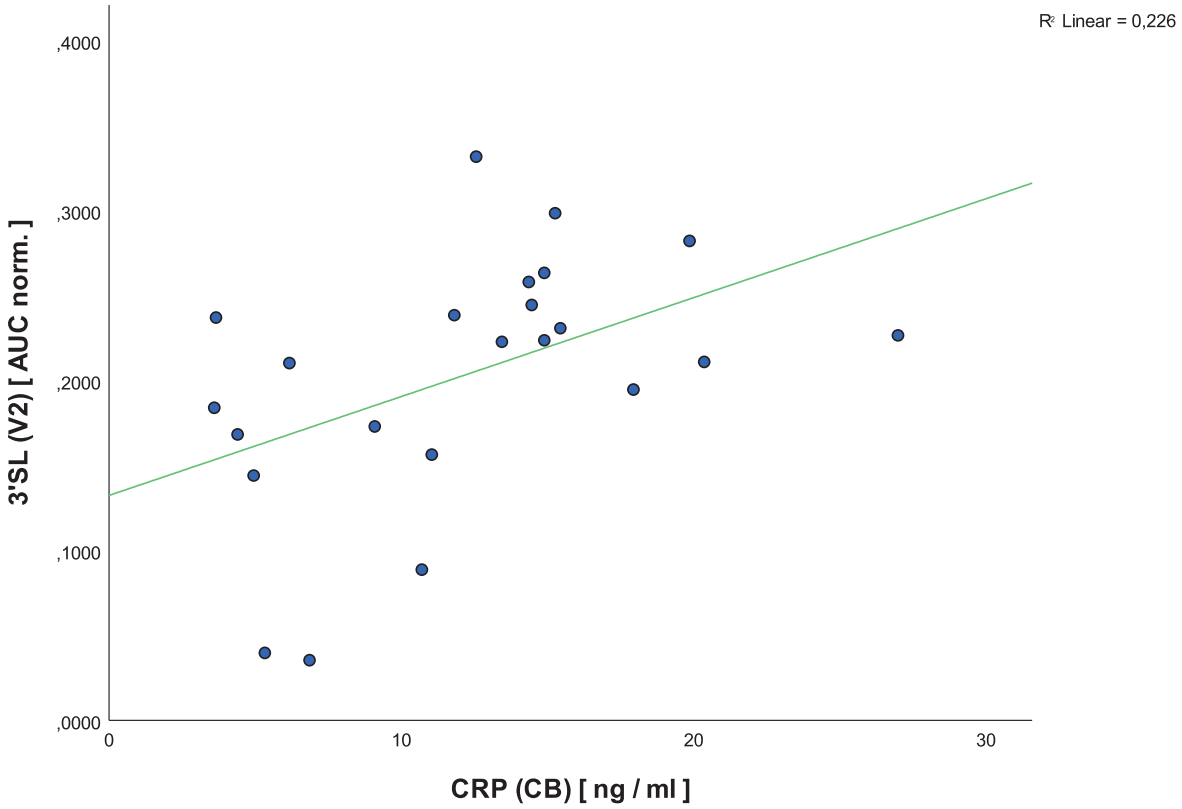

Figure 32: Correlation of 3'SL (V2) and CRP (CB)

Figure 32 presents the relationship between the maternal HMO 3'SL in midpregnancy of the total study population and the fetal inflammatory marker CRP measured in cord blood. Spearman's rho was highly significant (rho=0,531; p=0,009) and the determination coefficient r^2=0,226.

4.5.2 sICAM-1

We revealed two significant, negative associations of fetal sICAM-1 in cord blood and maternal HMOs in midpregnancy (Table 20): between 2'FL(V2) and sICAM-1 (rho=-0,431; p=0,040) and between 3'SL (V2) and sICAM-1 (rho=-0,493; p=0,017). In cord blood of Se $\oplus$ women only, no additional correlations were found.

Table 20: Spearman's rho of the association between Fetal sICAM-1 with 2'FL or 3'SL

	CB sICAM-1 (n=23)	CB sICAM-1 (n=19)
V1 2'FL	−0,207 (p=0,344)	−0,226 (p=0,353)
V1 3'SL	−0,037 (p=0,868)	0,133 (p=0,586)
V2 2'FL	−0,431*(p=0,040)	0,375 (p=0,113)
V2 3'SL	−0,493*(p=0,017)	−0,316 (p=0,188)

* indicates a significance level of 5%.
Left column: total study population.
Right column: Se $\oplus$ women.

The following two simple linear regressions (Fig.33, Fig.34) present the relation of HMOs in midpregnancy with the inflammation marker sICAM-1.
The linear regression of 2'FL (V2) and sICAM-1 (CB) displays the negative correlation with a coefficient of determination r^2=0,157 (Fig.33). 15,7% of the variance of the HMO 2'FL can be explained by the inflammation marker sICAM-1, both at V2.
Figure 34 shows a stronger association between maternal 3'SL and fetal sICAM-1. The coefficient of determination is r^2=0,277.

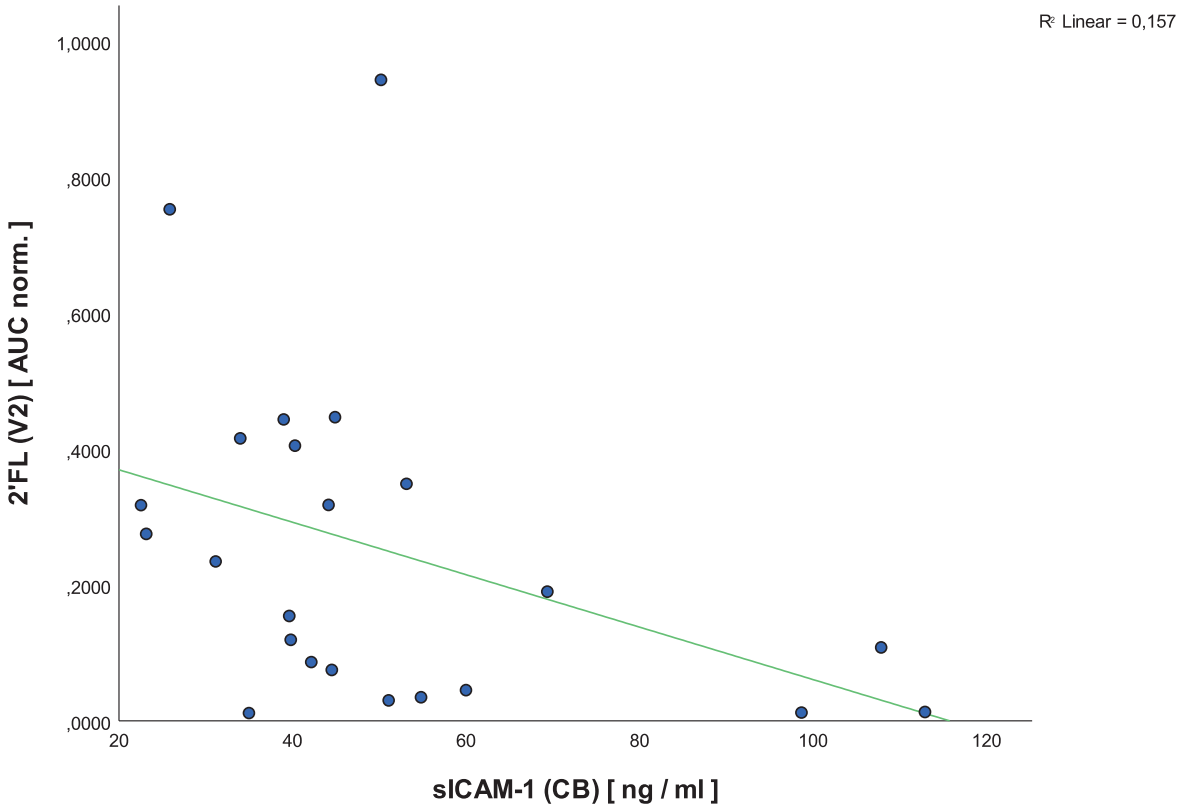

Figure 33: Correlation of 2'FL (V2) and sICAM-1 (CB)

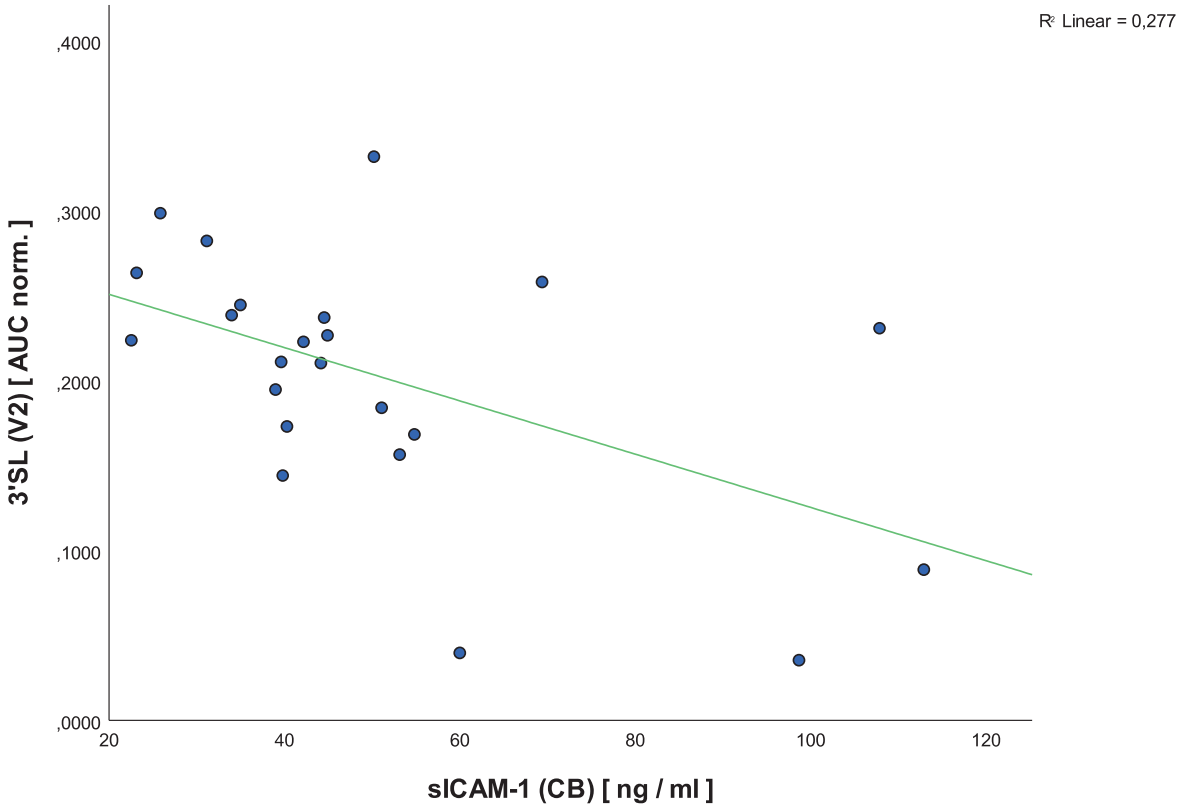

Figure 34: Correlation of 3'SL (V2) and sICAM-1 (CB)

4.5.3 MCP-1

We did not find any significant correlation between fetal MCP-1 concentrations and maternal 2'FL or 3'SL concentrations.

Table 21: Spearman's rho of the association between Fetal MCP-1 with 2'FL or 3'SL

	CB MCP-1 (n=23)	CB MCP-1 (n=19)
V1 2'FL	-0,233 (p=0,284)	-0,213 (p=0,381)
V1 3'SL	-0,038 (p=0,865)	-0,102 (p=0,679)
V2 2'FL	-0,120 (p=0,587)	0,082 (p=0,737)
V2 3'SL	0,068 (p=0,757)	0,044 (p=0,858)

Left column: total study population.
Right column: Se $\oplus$ women.

4.5.4 MIP-1α

No significant correlation was found between MIP-1α concentration in cord blood and maternal 2'FL or 3'SL concentrations.

Table 22: Spearman's rho of the association between Fetal MIP-1α with 2'FL or 3'SL

	CB MIP-1α (n=23)	CB MIP-1α (n=19)
V1 2'FL	-0,088 (p=0,696)	-0,226 (p=0,353)
V1 3'SL	-0,156 (p=0,487)	-0,194 (p=0,426)
V2 2'FL	0,212 (p=0,343)	0,085 (p=0,729)
V2 3'SL	0,172 (p=0,443)	0,061 (p=0,803)

Left column: total study population.
Right column: Se $\oplus$ women.

4.5.5 MIP-1β

Between MIP-1β concentrations in cord blood and maternal 2'FL or 3'SL concentrations, no significant correlation was found either.

Table 23: Spearman's rho of the association between Fetal MIP-1β with 2'FL or 3'SL

	CB MIP-1β (n=23)	CB MIP-1β (n=19)
V1 2'FL	-0,108 (p=0,624)	-0,272 (p=0,260)
V1 3'SL	-0,239 (p=0,272)	-0,154 (p=0,528)
V2 2'FL	0,154 (p=0,483)	0,091 (p=0,710)
V2 3'SL	-0,048 (p=0,826)	0,054 (p=0,825)

Left column: total study population.
Right column: Se $\oplus$ women.

5 Discussion

In this exploratory study we measured prenatal maternal HMOs levels at three different stages during pregnancy. HMO concentrations rose clearly with progress of pregnancy (Figure 8 and 9). None of the inflammatory markers showed a clear trend of increase or decrease that would underline specific pro- or anti-inflammatory phases across pregnancy (Figure 13 - 17).

For the first time, to our knowledge, serum concentrations of HMOs in pregnant women and their relationships with maternal and fetal inflammatory markers were investigated. As proposed in our hypothesis the concentrations of HMOs were associated with maternal and inflammatory markers.
We found multiple significant correlations: four out of five inflammatory markers correlated significantly with either 2'FL or 3'SL at distinct points of time. 3'SL was associated with CRP, sICAM-1, MCP-1 and MIP-1β whereas 2'FL was related with CRP, sICAM-1 and MIP-1β. No association between MIP-1α with any of the inflammation markers was revealed.

The fetal inflammatory markers CRP and sICAM-1 were also found to be associated with maternal HMOs at midpregnancy, indicating a potential influence of maternal HMOs on inflammatory conditions in the fetus.

5.1 Interpretation of the Results

5.1.1 Simple Correlations between HMO and Inflammatory Markers

Three of the associations we found were significant exclusively at one point of time. 3'SL and CRP correlated positively at V1 in the whole study population (rho=0,418; p=0,047). 3'SL and MCP-1 correlated negatively in Se $\oplus$ women at V1 (rho=-0,504; p=0,028). 2'FL and MIP-1β at V1 were negatively correlated in the whole study population (rho=-0,458; p=0,028). For those associations we can state that significant correlations were observed.

There are two possibilities of interpreting the other associations we found in a time-dependent manner. In the following two subchapters 5.1.2 and 5.1.3 those options will be discussed.

5.1.2 Inflammatory Markers alter HMO Concentrations

The first assumption, that inflammatory markers influence HMO production, goes along with our study hypothesis. Several of the associations we found support this theory.

sICAM-1 negatively correlated with 2'FL and 3'SL (Table 11): sICAM-1 at V1 correlated negatively with 2'FL at V2 (rho=-0,416; p=0,048), leading us to speculate that a proinflammatory state of the mother in early pregnancy with lower sICAM-1 levels leads to increased biosynthesis of 2'FL. 2'FL could then act anti-inflammatory.
An even more significant association was found between sICAM-1 at V1 and 3'SL at V2 (rho=-0,418; p=0,047), as the result is underlined by the highly significant negative association between both at V2 (rho=-0,543; p=0,007). The same conclusion from this result could be drawn: the inflammation marker seems to negatively impact the HMO.

Given that sICAM-1 is a marker for inflammatory conditions this could lead towards a lower production of certain HMOs in pregnancy. As HMO have different functions they might then have various effects on pregnancy in general and specifically the inflammation.

5.1.3 HMOs affect the Inflammatory Status of Mother and Child

Contrary to what we have discussed in 5.1.2 there are also associations indicating that HMOs influence inflammatory markers of mother and child:

3'SL at V1 correlated significantly with sICAM-1 at V2 (rho=-0,508; p=0,013) (Table 12), indicating that 3'SL impacts sICAM-1, as the result is underlined by the highly significant negative association between both at V2 (rho=-0,543; p=0,007). The nearly

significant correlation between 3'SL at V2 and sICAM-1 at V3 (rho=-0,410; p=0,052) additionally supports this interpretation as the same causality seems to occur at a later stage, too. Another support is the finding that 3'SL concentrations in midpregnancy correlate negatively with sICAM-1 in cord blood (rho=-0,493; p=0,017; see Table 20).

2'FL at V1 correlated negatively with CRP at V2 (rho=-0,430; p=0,041). Regarding the correlation between 2'FL and CRP one might hypothesise that higher concentrations of the (anti-inflammatory) HMO 2'FL in early pregnancy impact the inflammatory status of the mother and lead to corresponding lower CRP concentrations in midpregnancy. However, the positive correlation between maternal 2'FL and fetal CRP (rho=0,452; p=0,030; Table 19) opposes this explanation.

The correlations between maternal HMOs and fetal inflammatory markers raise the question whether HMOs might reach fetal circulation and either directly or indirectly influence inflammatory status of the infant.
To date, no published data concerning transplacental transfer of HMOs exists. However, there is evidence by Jantscher-Krenn (2018) (unpublished), indicating that 2'FL crosses the human placental barrier and reaches fetal circulation *ex vivo* (Jantscher-Krenn, Hirschmugl & Wadsack 2018). These findings should give rise to further investigations exploring the interactions of HMOs at the maternal- fetal interface and could help to better understand the connection between HMOs and fetal inflammation markers.

5.1.4 Conclusion

For different reasons it remains difficult to interpret our findings. Firstly, association studies in general are limited in exploring causalities. Secondly, none of the significant associations showed a time consistency across V1-V3 that would allow to draw a final conclusion regarding cause and effect of associations of maternal HMOs and maternal inflammatory markers. We found some associations that underline our hypothesis that inflammatory markers lead to altered HMO concentrations and others that support the assumption that HMOs have an impact on the inflammatory status.

Thirdly, most of the markers we looked at are not fully understood regarding their partly contradictory effects concerning inflammation. At this stage, we do not know when and how exactly both HMOs and inflammatory markers act either proinflammatory or anti-inflammatory.

5.2 Limitations

The predominant limitation of the study is the small sample size. Out of 53 participants 2 were lost due to medical exclusion criteria and 12 due to missing control visits. Unfortunately, another 16 women had to be excluded during statistical analysis due to incomplete data sets, leading to a final study population of n=23.

The extent of missing values could have been reduced by improved communication between midwives, study members and patients. A few data sets were incomplete because study members on duty to perform the data acquisition were informed either too late or not at all. All patients who agreed to be part of the study were informed in detail about the procedure of the measurements. Yet, some patients lacked compliance. This might be improved by better patient management and a more frequent communication between team members and patients.
Even though all study members were trained on how to obtain blood samples and process the placental tissue in the laboratory, variations in handling of the laboratory work could not be eliminated. This was mainly due to the fact that team members on duty for night shifts were unable to reach a routined handling of the laboratory processes during long periods of day deliveries only.

V1 took place between week 11 and 13, V2 between week 17 and 25 and V3 between week 27 and 39 of pregnancy.
A source for imprecision is the variability of the assessment dates. The actual range of dates of blood sampling was wider than intended and greatest for V3 (Fig. 7). The range for V1 was 2 weeks (min. week 11, max. week 13). For V2 the range was 9 weeks (min. week 17, max. week 25). V3 had the greatest range of 13 weeks, which is equivalent to the third assessment being conducted between pregnancy week 27 and

39. The large time range for assessment dates in midpregnancy and late pregnancy and the resulting inconsistent data in the third trimester could also explain why the correlation between 3'SL at V2 and sICAM-1 was significant for all time periods apart from V3 (rho=-0,410; p=0,052). It seems likely that we could have found a significant correlation if the time frame for V3 would have been stricter and the data more reliable. Future studies should be improved in this regard and enforce a stricter time frame for control visits.

We showed that the Secretor status influences the associations of the examined markers. Of the total study population 19 women were Se $\oplus$ but only 3 were Se $\ominus$. The statistical power of this analysis and the informational value was therefore limited by the small sample size. Therefore, future and larger studies should pay attention concerning the possible effect of the Se status too.

5.3 Outlook

Pregnancy is an exceptional phase of controlled inflammation. Disturbance of the Th1/Th2 balance may lead to exacerbated immune responses and thereby give rise to pregnancy pathologies like (recurrent spontaneous) abortion, IUGR, premature labor and preeclampsia.

For future studies the serum levels of HMOs of pregnant women with an active inflammation during pregnancy might be of interest. Conditions such as RSA, preeclampsia or other pregnancy related inflammatory states should be considered. This might help to better understand the role of HMOs under general inflammatory conditions and furthermore open grounds for the search of biomarkers for specific pregnancy pathologies. In this study we aimed to investigate whether and in how far HMOs associate with certain inflammatory markers. Although the exact bio-molecular mechanisms responsible for the relationships between CRP, sICAM-1, MCP-1, MIP-1β and 2'FL and 3'SL remain unclear so far and require further investigation, the present work indicates these relationships. Understanding these associations better might lead to new biomarkers based on HMOs for detection of pregnancy pathologies.

List of Figures

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