Denise Hoch, BSc.

Effect of Human Milk Oligosaccharides on in vitro Angiogenesis in Feto-Placental Endothelial Cells

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Ass.-Prof. Dr.rer.nat. Ursula Hiden

Department of Obstetrics and Gynecology
Medical University of Graz

Mag. pharm. Dr.rer.nat. Evelyn Jantscher-Krenn

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Research is to see what everybody else has seen,
and to think what nobody else has thought.

Albert Szent-Gyorgyi

Abstract

Background. Human milk oligosaccharides (HMO) are highly bioactive factors in breast milk, also present in the maternal systemic circulation. New evidence showed that HMO are also found in the umbilical cord blood, hence in the fetal circulation, rendering the feto-placental vasculature a potential target tissue. Preliminary data showed that HMO influence network formation of feto-placental endothelial cells (fpEC) in a Matrigel Assay. Thus, we here aimed to investigate the effect of HMO on endothelial function, using functional assays for *in vitro* angiogenesis in fpEC.

Methods. To test whether potential HMO receptors were expressed in fpEC, we used real-time qPCR at baseline and stimulated with pooled HMO, or the individual HMO, 3'SL and 2'FL. Fibrin Angiogenesis Assay and Spheroid Sprouting Assay, both functional three-dimensional matrix assays, were used to observe HMO effects on *in vitro* aspects of angiogenesis in fpEC. HMO effect on proliferation was assessed using colorimetric MTT assay. To test effects on cytoskeleton architecture, F-actin staining with phalloidin was used. Furthermore, we assessed whether HMO treatment influences the secretion of paracrine factors associated with angiogenesis, performing ELISA of conditioned cell supernatants.

Results. We found the potential HMO receptors TLR-4, L-SIGN and E-Selectin expressed in fpEC, and expression was not significantly changed after HMO, 3'SL or 2'FL treatment. There was a trend of L-SIGN downregulation (p=0,058) after pooled HMO treatment and E-Selectin downregulation after treatment with 3'SL for 48 hours. Angiogenesis Assay and Spheroid Sprouting Assay confirmed the pro-angiogenic effects of HMO, measured as increase in total tube length. Pooled HMO, 3'SL and 2'FL increased cell number after 24, 48 and 72 hours significantly. Higher proliferation rate of fpEC caused by HMO treatment may contribute to the increased angiogenesis. Furthermore, treatment with pooled HMO, 3'SL and 2'FL showed profound cytoskeleton rearrangement in fpEC. Whereas serum starved cells showed membrane ruffles, HMO treated cells form well-ordered, parallel stress fibers, displaying a bright fluorescent staining. These changes in cytoskeleton are essential for proliferation and migration, both important processes in angiogenesis. HMO treatment caused a significant increase of IL-6 in cell supernatants. IL-6 is known to be both, a pro- and anti-inflammatory myokine, and also a potent pro-angiogenic factor promoting proliferation and migration *in vitro*. Increase of IL-6 can be one factor leading to an increase in proliferation and migration, both demonstrated in this thesis.

Conclusion. In summary, HMO treatment affects angiogenesis, by changing proliferation, cytoskeleton organization and altering expression of angiogenic factors. These results point towards a role for HMO in the modulation of placental vascular development.

Zusammenfassung

Hintergrund. Humanmilcholigosaccharide (HMO) sind stark bioaktive Faktoren. Sie kommen in Muttermilch vor, und wurden auch im mütterlichen Blutkreislauf nachgewiesen. Neue Studien legen dar, dass HMO auch im Nabelschnurblut auftreten, und somit auch in den fetalen Blutkreislauf gelangen. Dadurch stellt sich die Frage, ob HMO einen Einfluss auf feto-plazentare Endothelzellen (fpEC) ausüben und deren Funktionen bezüglich Angiogenese beeinflussen. Noch unveröffentlichte Studien zeigen, dass HMO die Ausbildung von Zell-Netzwerken in Matrigel Assays stimulieren.

Methoden. Mittels real-time qPCR wurde die Expression von TLR-4, L-SIGN und E-Selektin, mögliche HMO Rezeptoren, welche in anderen Zelltypen exprimiert werden, untersucht. Zwei unabhängige funktionelle 3D-Angiogenese Assays, Fibrin und Spheroid Sprouting Assay, wurden herangezogen, um die Bildung tubulärer Strukturen zu untersuchen. Proliferation wurde mittels MTT Assay untersucht. Eine F-Aktin Fluoreszenzfärbung wurde angewendet, um Umformungen des Zytoskeletts deutlich zu machen. Des Weiteren wurden spezifische ELISA herangezogen, um parakrine Faktoren in Zellüberständen zu identifizieren.

Resultate. Die Expression von TLR-4, L-SIGN und E-Selektin konnte bestätigt werden. Eine Veränderung der Genexpression dieser Rezeptoren nach der Behandlung mit HMO, 3'SL und 2'FL konnte nicht festgestellt werden. Wir fanden jedoch einen Trend hin zu niedriger L-SIGN Expression (p=0,058) nach HMO Behandlung und einer niedrigeren E-Selektin Expression nach der Behandlung mit 3'SL. Die funktionellen Angiogenese Assays bestätigten, dass die Behandlung von fpEC mit gepoolten HMO und 3'SL zu einer signifikanten Verlängerung tubulärer Strukturen führte, was auf gesteigerte Angiogenese hinweist. Zudem konnte eine signifikante Erhöhung der Proliferation, verursacht durch die Behandlung mit HMO, 3'SL und 2'FL, festgestellt werden. Die Konzentration an Interleukin-6, ein immunregulatorisches Myokin, welches zudem pro-angiogene Wirkung zeigt, konnte durch die Behandlung von fpEC mit gepoolten HMO signifikant erhöht werden. Diese Konzentrationserhöhung kann Auswirkungen auf Proliferation und Migration der fpEC haben, was eine gesteigerte Angiogenese nach sich zieht, und schließlich zu einer Regulierung der Entwicklung des plazentaren Gefäßsystems führt.

Zusammenfassung. Die Behandlung von fpEC mit HMO beeinflusst Proliferation, die Ordnung des Zytoskeletts, und verursacht eine veränderte Expression an pro-angiogenen Faktoren. Diese Resultate weisen auf eine tragende Rolle der HMO bezüglich der plazentaren Gefäßentwicklung hin.

Appendix

2'FL	2'-Fucosyllactose
3'SL	3'-Sialyllactose
3'SLN	3'-Sialyl-N-acetyllactosamine
6'SLN	6'-Sialyl-N-acetyllactosamine
AIDS	Acquired immune deficiency syndrome
ATP	adenosine triphosphate
BMI	Body mass index
BrdU	Bromdesoxyuridin
CBS	Cord blood serum
Ct	Threshold cycle
DAPI	4',6-Diamidin-2-phenylindol
EBM	Endothelial Basal Medium
ECA	Arterial Endothelial Cell
FCS	Fetal Calf Serum
FGF2	Fibroblast Growth Factor 2
FITC	Fluorescein isothiocyanate
fpEC	Feto-Placental Endothelial Cell
GDM	Gestational Diabetes Mellitus
HBSS	Hank's Balanced Salt Solution
HIV	Human immunodeficiency virus
HMO	Human Milk Oligosaccharides
HPRT-1	Hypoxanthine-guanine phosphoribosyltransferase
hPS	Human pregnant serum
IGF-1	Insulin like growth factor -1
IL-10	Interleukin 10
IL-6	Interleukin 6
IL-8	Interleukin 8
Lac	Lactose
L-SIGN	Liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
LST	Sialyllacto-N-tetraose
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
nbCS	New born calf serum
PBS	Phosphate-buffered saline
TLR-4	Toll like receptor-4
TNF-α	Tumor Necrosis Factor alpha
VEGF	Vascular Endothelial Growth Factor

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1. Introduction

1.1. Human milk oligosaccharides

1.1.1. Discovery of HMO

At the end of the 19th century pediatricians and microbiologists found a relationship between infant's intestinal bacteria and their physiology of digestion (Escherich 1886). Further, they noted that human milk contains a 'different' type of lactose than bovine milk. However, chemists observed that both types of lactose in human and bovine milk were identical, but human milk contains an additional, yet unknown carbohydrate fraction. More than 40 years later, this carbohydrate fraction was characterized and called "gynolactose". This was the starting point of research on human milk oligosaccharides (HMO). The gynolactose fraction was described as insoluble in methanol and consisting of various components. They also found that nitrogen is an essential component. By applying 2-dimensional paper chromatography, Polonowski and Lespagnol together with Montreuil identified 2'-fucosyllactose and 3'-fucosyllactose for the first time. When Kuhn and György confirmed that the bifidus factor, a growth-promoting factor for Lactobacillus bifidus, in human milk, consists of oligosaccharides, HMO research experienced a breakthrough (Gyorgy et al. 1954). Since then, research in the field of HMO has continued and led to the identification of lacto-N-tetraose, lacto-N-fucopentaose I and II, difucosyllactose, and many others. More than 150 different HMO structures have been identified so far (Kunz 2012).

1.1.2. Biological diversity and structure of HMO

Oligosaccharides are defined as carbohydrates containing between three and ten monosaccharide residues which are covalently linked through glycosidic bonds. In human milk, oligosaccharides represent the third largest component after lactose and fat. These oligosaccharides in human milk are very unique in structure and concentration (German et al. 2008). Compared to bovine milk, concentration of oligosaccharides in human milk is very high. Additionally, the chemical structures of the oligosaccharides in bovine milk are similar to those in human milk, but not identical (Urashima et al. 2001). Two phosphorylated sialyl oligosaccharides, sialyl-lactosamine-1-phosphate and sialyl-lactosamine-6-phosphate are found in bovine

milk, but not in human milk (Gopal & Gill 2000). Whereas some oligosaccharides appearing in human milk are not present in bovine milk (e.g. Lacto-N-fucopentaose I-III) (Kunz et al. 2000).

The structural diversity of milk oligosaccharides varies from mother to mother and even changes longitudinally during lactation (Chaturvedi et al. 2001). Colostrum, a fluid secreted by the mammary gland a few days before and after parturition, and early milk are richest in oligosaccharides with a concentration of 20-25 g/L of HMO. HMO concentration declines to 5-20 g/L, when milk production maturates (Coppa et al. 1993).

HMO are composed of the five monosaccharides glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (Sia). N-acetylneuraminic acid (Neu5Ac) is the predominant form of Sia. The common compound of all HMO is the disaccharide lactose at their reducing end, which can be fucosylated and/or sialylated. Multiple glycosyltransferase enzymes expressed in the mammary gland biochemically modify the lactose with additional monosaccharide units, resulting in the HMO's elongation and branching. Elongation with lacto-N-biose appears to terminate the chain, whereas N-acetyllactosamine can be further extended. Chain branching is introduced by β1-6 linkage of two disaccharides. Some HMO occur in several isomeric forms, e.g. sialyllacto-N-tetraose (LST) (Bode 2012).

1.1.3. Local and systemic functions of HMO

HMO are highly bioactive factors, complex glycans and thought to provide many benefits to the breast-fed newborn, exerting local and also systemic effects. About 1% of the ingested HMO are absorbed and cross the border membrane of the intestine suggesting that HMO can act systemically, and are not restricted to the intestinal mucosal environment (Bode 2015). Important aspects of HMO benefitting newborns are anti-inflammatory, immuno-modulating and prebiotic effects (Bode 2012). First, HMO are indigestible and reach the colon intact where they serve as prebiotics and promote healthy gut colonization. They serve as metabolic substrates for beneficial bacteria, providing a growth advantage over potential pathogens. Next, HMO are antiadhesive antimicrobials, blocking adhesion of microbial pathogens to mucosal surfaces of the gastrointestinal and respiratory tract by serving as soluble glycan receptor decoys. As HMO also reach the systemic circulation and are excreted intact with the infant's urine (Rudloff et al. 1996), they can also prevent pathogen attachment

in the urinary tract. Thus, HMO prevent infection of the respiratory, gastrointestinal and urogenital tract, locally and systemically (Bode 2015).

1.1.4. HMO as signaling molecules

HMO also act as signal molecules, and were shown to have direct effects on different cell types. Angeloni *et al.* demonstrated effects of HMO on glycosylation pattern on epithelial cells, proliferation, differentiation and apoptosis. 3'SL treatment led to a differential expression of glycosylation related genes and surface glycome changes in HT-29 cells (Angeloni et al. 2005). A variety of neutral and sialylated HMO have been shown to reduce proliferation of HT-29, Caco-2 cells and non transformed small intestinal epithelial crypt cells of fetal origin (HIEC). Effects on proliferation, differentiation, apoptosis or cell dynamics were dependent on the cell lines used (Kuntz et al. 2008). Other studies showed effects of pooled HMO on cell cycle regulation, potentially by signaling effects through EGF receptor and Ras/Raf/ERK pathway (Kuntz et al. 2009). The combined observations from these *in vitro* studies suggest that HMO can directly interact with the infant's intestinal epithelial cells and alter cell signaling pathways.

1.1.5. Putative HMO receptors

The available literature provides little information on potential receptors in various target cells. As described in 1.1.4., incubation of intestinal epithelial cells with HMO induces a dose-dependent phosphorylation of the epidermal growth factor receptor (EGFR), indicating that these oligosaccharides interact with this receptor (Kuntz et al. 2009). In this study, HMO in concentration physiological to human milk modulated intestinal epithelial cell apoptosis, proliferation and differentiation (Kuntz et al. 2008). In addition, dendritic and T-cells showed altered cell response after HMO treatment in concentrations expected in the blood circulation. Putative receptors are C-type lectins (e.g. L-SIGN) and selectins that bind specific carbohydrate epitopes (Yabe et al. 2010). Furthermore, Toll-like receptor 4 (TLR4) which is also expressed on endothelial cells, is a potential HMO receptor candidate (Kurakevich et al. 2013). Being present in the systemic circulation, HMO are discussed as potential signaling molecules, able to interfere with cell-cell interaction through binding to respective receptors.

1.1.6. HMO in cord blood

Our previous study provides the first evidence that HMO are also present in cord blood serum (Jantscher-Krenn et al. unpubl.). Thus, HMO are circulating in the feto-placental unit and are in direct contact with feto-placental endothelial cells (fpEC). This renders the feto-placental endothelium a target tissue for prenatal HMO circulating in the fetal blood. Expression of putative receptors in fpEC remains to be investigated.

1.2. Placenta morphology and function

The placenta is a vascular, villous organ which is essential for fetal nutrient uptake, waste elimination and gas exchange (Tong & Chamley 2015). To support growth and development of the fetus, the placenta is highly specialized while its functions are precisely regulated and coordinated. Additionally, the placenta functions as an immune barrier by protecting the growing fetus from the maternal system's antigen attack. Furthermore, the placenta secretes and releases various hormones that affect fetal growth as well as fetal and maternal metabolism (Gude et al. 2004).

Structurally, the placenta is a hemochorial organ derived from the fetal compartment and separates the fetal and the maternal blood. The umbilical cord connects fetus and placenta and contains two umbilical arteries and one umbilical vein. The umbilical cord inserts in the chorionic plate (Caruso et al. 2012) of the placenta, where the large umbilical vessels branch into the villous vessels.

The maternal faced surface of the placenta is called basal plate and consists of 10-40 irregularly shaped regions, called cotyledons (Figure 1). Between the chorionic plate and the basal plate, the intervillous space is located, an area filled with maternal blood derived from endometrial arteries (Gude et al. 2004).

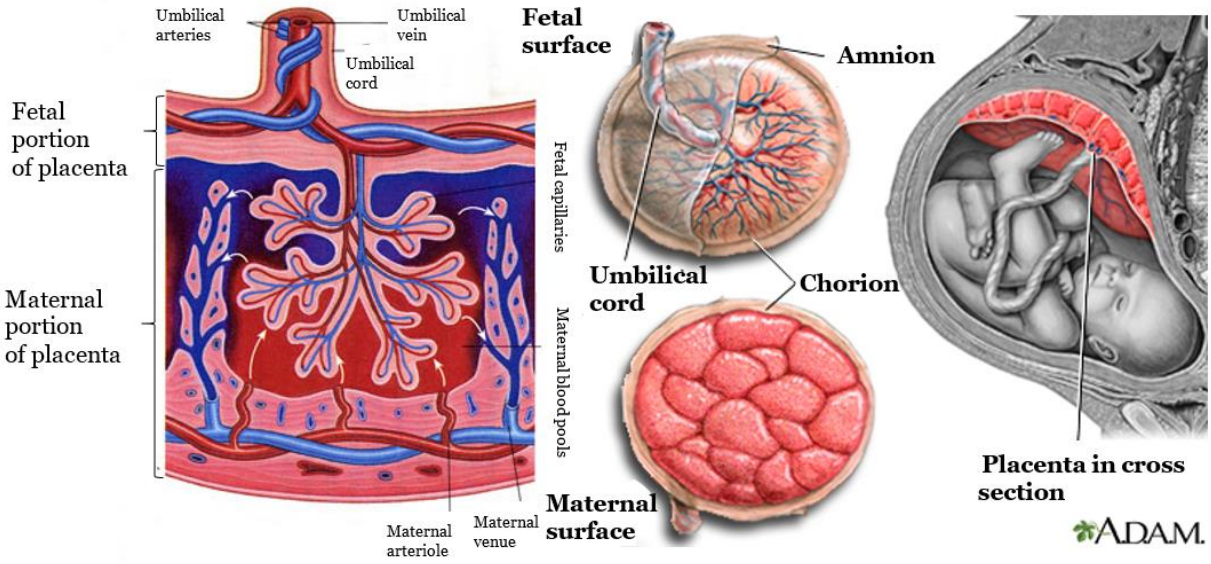

Figure 1: Schematic representation of the human placenta. The placenta is a fetal organ located between mother and fetus, and is responsible for sustaining fetal development by supplying nutrients and gases, exerting endocrine functions and delivering fetal waste products back to the maternal circulation. Adapted and modified from http://imueos.wordpress.com/2010/05/25/placenta-function/ and https://medlineplus.gov/ency/imagepages/17010.htm.

A mature placenta weighs about 500 – 600 grams and consists of 15 – 28 cotyledons. The placenta's diameters vary between 15 and 20 centimeter and the thickness ranges between two to three centimeters (Wang & Zhao 2010).

1.3. Placental Vasculo- and Angiogenesis

Placentation, the formation of the placenta, includes extensive angiogenesis in maternal and fetal placental tissues, accompanied by a marked increase in uterine and umbilical blood flows (Reynolds & Redmer 2001). The placenta is highly vascularized to provide the essential oxygen and nutrient transfer to the fetus. Vasculogenesis and angiogenesis are two distinct processes, involved in feto-placental vascular development in human pregnancy.

Angioblasts differentiate into endothelial cells which assemble to a primary vascular network. This *de novo* formation is called vasculogenesis. Angiogenesis is defined as the growth of blood vessels from already existing vasculature. Formation of vessel sprouts or intussusceptive growth are two modes of angiogenesis which lead to

maturation of this primary network. At day 21 after conception, a vascular plexus forms by differentiation of pluripotent mesenchymal progenitor cells into endothelial cells. This step is followed by connection of these first vessels and further angiogenesis mediating proliferation that continues until delivery (Leach et al. 2009).

Regulation and stimulation of angiogenesis in the placenta is related to various growth factors. A central determinant for angiogenesis is oxygen. Most types of cells respond to a hypoxic environment by secreting vascular endothelial growth factor (VEGF), a key proangiogenic growth factor, engaging kinase insert domain receptor (KDR), also known as vascular endothelial growth factor receptor 2 (VEGFR-2). Another well-known example is fibroblast growth factor 2 (FGF2). Angiogenic signals promote endothelial cell proliferation, changes in proteolytic balance, cytoskeletal reorganization, migration and differentiation, and the formation of new tubes (Munoz-Chápuli et al. 2004).

1.3.1. Proliferation

Proliferation of fpEC is an important process in angiogenesis preceding tube formation, needed for developing capillaries. In the developing placenta, vessel outgrowth occurs by proliferation of endothelial cells forming tubes and migration of endothelial tip cells in response to angiogenic stimulation (Ausprunk & Folkman 1977). Increase of cellular proliferation is a multi-step event, whereby quiescent endothelial cells are first activated to re-enter the cell cycle (Myers et al. 2002).

1.3.2. Cytoskeleton rearrangement

Angiogenesis includes coordinated events like cell motility, cell shape alteration, cell adhesion and differentiation. These extreme changes in cell morphology and polarity are critically dependent on cytoskeletal rearrangements. Based upon cellular demand, the highly dynamic actin cytoskeleton structure undergoes polymerization and depolymerization (Nemethova et al. 2008). G-Actin is the major cytoskeletal protein of endothelial cells and polymerizes to form helical actin filaments (F-actin). The F-actin assembly is highly dependent on the hydrolysis of ATP by its intrinsic ATPase activity. Actin filaments have a fast-growing barbed end (+ end) and a slow-growing pointed end (-end). Actin filament elongation or polymerization occurs mostly at the barbed end, and shortening or depolymerization at the pointed end (Welch & Mullins 2002).

The thin and flexible fibers are organized into higher-order structures, forming three-dimensional networks. These networks provide mechanical support, regulate cell shape and enable cell migration and division by allowing movement of the cell surface (Cooper 2000). Cell shape is determined by actin microfilaments, microtubules and intermediate filaments, which are in permanent communication with each other (Revenu et al. 2004). The constant remodeling of actin cytoskeleton into specialized cellular structures, such as filopodia, lamellipodia and stress fibers is crucial for cell migration. Filopodia contain long parallel actin filaments in tight bundles, whereas lamellipodia are cytoplasmic protrusions, forming the leading edge of migrating cells.

Stress fibers are actin filaments of inverted polarity distributed along contractile fibers (Lamalice et al. 2007). These stress fibers, also known as actomyosin fibers, are formed when a cell is stably connected to the matrix and arranged in parallel with myosin and actin binding protein to build long, straight, contractile fibers (Chrzanowska-Wodnicka & Burridge 1996). Anchored at focal adhesions, these structures are required for the traction of the rear of the cells toward the leading edge during migration. The dynamics of actin cytoskeleton and stress fiber formation are known to be regulated by the small GTPase protein RhoA. This regulation induces F-actin stress fibers formation and focal adhesions that are essential during cell migration (Buchsbaum 2007).

Actin stress fibers can be subcategorized into three distinct stress fiber types: dorsal and ventral stress fibers and transverse arcs. This categorization is based on their subcellular localization and association with focal adhesions and is expanded by classification according to the fibers connection to substratum as well as their dynamics and assembly mechanisms (Small et al. 1998; Hotulainen & Lappalainen 2006).

During angiogenesis, cells convert to an invasive form and tunnel through the basement membrane surrounding the vessel to form an angiogenic sprout. For vessel formation, endothelial cells migrate outwards as a cord of highly elongated cells (Carmeliet 2000). This vessel sprouting involves many morphogenic steps, during which endothelial cells migrate, polarize, establish cell-cell contacts and form vessel lumens (Potente et al. 2011).

1.3.3. Pro-angiogenic factors

Placental vascular development is tightly regulated by pro- and anti-angiogenic factors. Numerous growth factors have been shown to regulate and stimulate angiogenesis in the human placenta (Cvitic et al. 2014). The most prominent factors promoting placental angiogenesis are vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), leptin and the insulin/insulin like growth factor (INS/IGF) system.

The VEGF system consists of a family of growth factors and their receptors. Acting through a family of cognate receptor kinases in endothelial cells, VEGFs regulate blood-vessel formation. The most important member of the family is VEGFA (or VEGF). This family also includes placental growth factor (PlGF), VEGFB, VEGFC and VEGFD. Through a family of protein tyrosine kinase receptors (e.g. VEGFR1 (Flt1), VEGFR2 (KDR) and Flt4), VEGFs mediate their biological functions (Holmes & Zachary 2005). VEGF is known to be responsible for differentiation, growth and aggregation of the endothelial precursors. Thus, VEGF plays a key role in placental vascular development in early pregnancy (Shalaby et al. 1995; Fong et al. 1995).

Leptin is an adipokine, and its main function is to regulate energy balance by regulating satiety and food intake via negative feedback (Klok et al. 2007). Furthermore, it exerts growth factor and proangiogenic actions (Lipsey et al. 2016). Leptin is a non-classical pro-angiogenic factor playing an essential role in tumor angiogenesis by binding to the leptin receptor (OB-Ra/ OB-Rb). This leads to control of leptin ligand/receptor mediated pathways and consequently to an increase in the expression of vascular endothelial growth factor (VEGF), its receptor (VEGF-R2), and cyclin D1 (Gonzalez et al. 2006). Endothelial cells were shown to mediate their leptin-induced proliferation, through the activation of STAT-3 and extracellular signal-regulated kinases (ERK). Leptin also induced angiogenesis *in vivo* in the chick embryo chorioallantoic membrane (CAM) and in rat cornea assays (Bouloumié et al. 1998; Sierra-Honigmann et al. 1998). Ribatti *et al.* (2001) confirmed that leptin was able to specifically stimulate angiogenesis when applied onto the chick embryo CAM and showed that the angiogenic effect was similar to that obtained with FGF-2 (Ribatti et al. 2001). Feto-placental endothelial cells have been reported to express functionally active Ob-Ra and Ob-Rb (Gauster et al. 2011). Thus, feto-placental angiogenesis might be partly stimulated by leptin.

The insulin/insulin-like growth factor (INS/IGF) system constitutes a family of three ligands, insulin and the insulin like-growth factors 1 and 2 (IGF-1 and IGF-2). The cell surface receptors insulin receptor (IR) and the IGF-1 and IGF-2 receptors (IGF1R and IGF2R) mediate the biological effects of INS/IGF (Flier et al. 1997). Insulin and IGFs play a key role in regulation of fetal and placental growth and development by regulating cell invasion, migration and tissue remodeling (Hiden et al. 2009). Low expression of INS/IGF receptors on feto-placental endothelial cells in the first trimester suggests that insulin and IGFs contribute to placental angiogenesis rather than to placental vasculogenesis (Cvitic et al. 2014).

Inflammatory cytokines involved in the inflammation process lead to activation of endothelial cells. This activation leads to transcription of genes for proteins essential for the inflammation process. Interleukin-6 (IL-6) is a pleiotropic cytokine and directly associated with chronic inflammation (Fan et al. 2007). Pro-inflammatory cytokines promote neutrophil accumulation and the release of interleukin-6. In response to chemokines like IL-8, neutrophils shed their IL-6 receptors. Consequently, the regulation of chemokine production by endothelial cells is altered, promoting MCP-1 production and IL-8 decrease. This process affects monocyte accumulation and the expression of endothelial leukocyte adhesion molecules, which further promote leukocyte accumulation (Barnes et al. 2011). Next to immune-modulating effect, IL-6 is known as a potent pro-angiogenic cytokine which stimulates smooth muscle cell and cerebral endothelial cell proliferation and migration *in vitro* (Nilsson et al. 2005). In the third trimester placenta, IL-6 concentration is increased up to 4-fold, observed in trophoblasts and fetal vessels (Bennett et al. 1998). Motro *et al.* (1990) showed that IL-6 mRNA is expressed in several independent angiogenic processes in gonadotropin-primed hyperstimulated ovaries, suggesting an active role of IL-6 in angiogenesis (Motro et al. 1990).

Interleukin-10 (IL-10) is an anti-inflammatory cytokine, which inhibits the synthesis of pro-inflammatory cytokines by activated monocytes. IL-10 was shown to reduce the adhesiveness of monocytes to stimulated endothelial cells *in vitro* (Krakauer 1995).

As endothelial cells receive information from their environment leading them to progress the stages of new blood vessel formation, they play an important role in placental vasculo- and angiogenesis (Bach 2015).

1.4. *In vitro* feto-placental angiogenesis

1.4.1. Primary feto-placental endothelial cells as model to study endothelial function

Endothelial cells grow in a monolayer and line the interior surface of placental vasculature. They form an interface between circulating blood and the vessel wall, and function as a semi-permeable physical barrier. This semi-permeable character is a result of intercellular junctions mediating intercellular adhesion and communication. Endothelial cells participate in processes like control of vascular tone, developing and remodeling of the vasculature and blood flow, and trafficking of nutrients and gases (Becker et al. 2000; Deanfield et al. 2007). Isolated feto-placental endothelial cells have been extensively characterized and used as a model to study feto-placental functions (Lang et al. 2008). Confluent arterial endothelial cells, as shown in figure 2, present a typical growing pattern. They have a polygonal cell shape with a smooth surface and grow in a characteristic cobble-stone pattern (Lang et al. 2008).

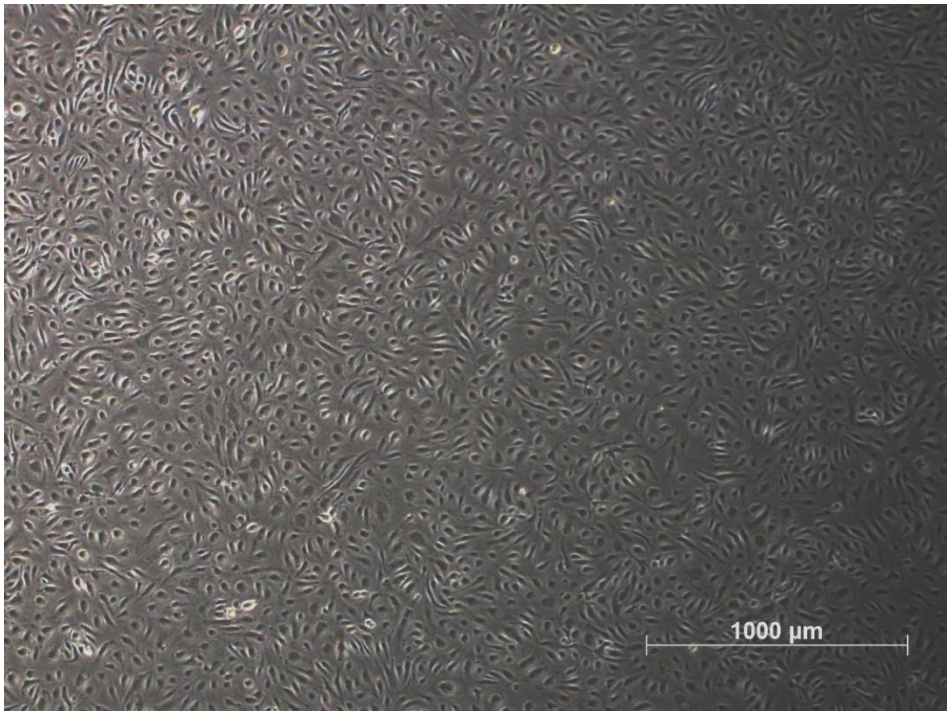

Figure 2: Morphology of feto-placental endothelial cells. The confluent cells show a polygonal shape with a smooth surface. Cultivated on gelatin in endothelial basal medium containing supplements.

In vitro cultivated primary feto-placental endothelial cells maintain their cell characteristics as expression of endothelial cell markers. Von-Willebrand factor, a

glycoprotein produced uniquely by endothelial cells and megakaryocytes, is routinely used to identify isolated endothelial cells.

1.4.2. *In vitro* angiogenesis assays

Aspects of feto-placental angiogenesis can be studied by *in vitro* assays with feto-placental endothelial cells cultured on two- or three-dimensional gels. The most widely used *in vitro* assay is the Network Formation Assay, where the tube formation takes place on a basement membrane matrix gel (e.g. Matrigel). Fibrin Angiogenesis Assay uses fibrin as a scaffold for invading endothelial cells. Spheroid Sprouting Assay is based on spheroids embedded in collagen gel (Heiss et al. 2015).

The **Network Formation Assay**, also called Matrigel Assay, measures the ability of endothelial cells to form capillary-like structures. The cells are plated at subconfluent densities on a gel derived from reconstituted basement membrane. This assay is mostly seen as a network formation assay and shows migration of cells (Di Blasio et al. 2014).

The principle of the **Spheroid Sprouting Assay** is based on sprouting and network formation from gel embedded aggregated endothelial cells (Blacher et al. 2014a). The cells are embedded in collagen gel and stimulated which leads to the formation of capillary-like sprouts originating from the spheroid (Ribatti 2014).

The **Fibrin Angiogenesis Assay** uses the invasion and tube formation of endothelial cells in a three-dimensional fibrin matrix to study the outgrowth of capillary-like tubular structures (van Hinsbergh et al. 2001).

Imaging and picture processing is used to analyze these three assays. Special software is used to detect structures and estimate values, such as tube length and number of branching points.

2. Hypothesis and objective

Recent data showed that human milk oligosaccharides (HMO) are present in umbilical cord blood serum (CBS). This is the first evidence for the presence of HMO also in the fetal circulation. Thus, they come in direct contact with the placental endothelium. As endothelial cells play a major role in feto-placental angiogenesis, the question was whether HMO also contribute to the regulation of angiogenic processes in the placenta.

In a preliminary study, feto-placental endothelial cells (fpEC) pre-incubated with HMO (125 µg/mL) showed increased 2D network formation in a Matrigel assay (Figure 3A).

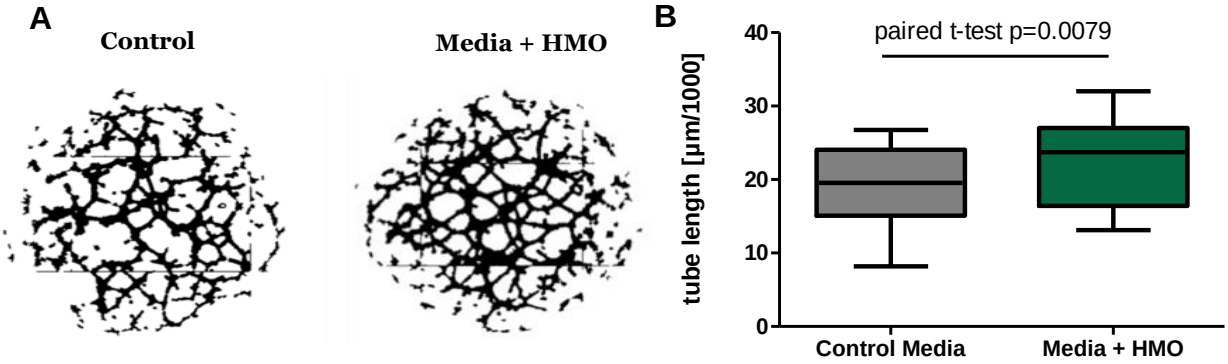

Figure 3: Pooled human milk oligosaccharides (HMO) increased network formation in primary fpEC. Placental endothelial cells from healthy pregnancies (n=7) were pre-incubated for 24 hours with EBM media + 5% FCS with or without pooled HMO prior to use in a 2D network formation assay. (A) Representative images of network formation with (right) or without (left) HMO treatment.(B) Box plot shows total tube length calcultated at 12 hour after seeding (median ± SEM, n=7)

The cells formed more cell-cell contacts and the network was more branched compared to cells without HMO treatment. Pretreatment with HMO increased total tube length after 12 hours to 120.3% ± 26.1% (Figure 3B).

Thus, I hypothesized that HMO affect *in vitro* angiogenesis in feto-placental endothelial cells.

Hence, in this thesis I aimed to

1. evaluate the gene expression of potential HMO receptors in feto-placental endothelial cells.
2. characterize the HMO effect on *in vitro* angiogenesis. Important aspects of angiogenesis, such as proliferation and cytoskeleton rearrangement after treatment with HMO were examined. The effect of HMO on tube formation was

evaluated using independent and novel functional angiogenesis assays that first had to be optimized.

3. test whether angiogenic factors are modulated by HMO exposure. I assessed if IL-6, IL-10, IGF-1 and Leptin concentrations are altered in conditioned media after HMO cell stimulation. Finally, gene expression of receptors of pro angiogenic factors, such as insulin receptor, insulin like growth factor receptor and VEGF-2 Receptor were analyzed.

3.Methods

Approval by the Ethics Committee of the Medical University of Graz was given and informed consent was obtained from all women who were recruited for this study. Only placentas of healthy pregnancies were used for this study. Consequently, placentas of pregnancies complicated by preeclampsia, gestational diabetes mellitus, human immunodeficiency virus infection (HIV) and acquired immune deficiency syndrome (AIDS), alcohol and/or drug abuse were excluded. The body mass index (BMI) of all mothers ranged between 18.5 and 24.9, so the mother's weight was regarded as normal.

3.1. Isolation of Feto-Placental Endothelial Cells

Before cutting two chorionic plate veins and arteries, the term placenta was disinfected with Betaisodona. Two veins and arteries were chosen and a minimum length of three centimeters was dissected. Arteries are crossing the veins and are more tailed. The blood vessels were transferred to a first HBSS (Gibco) washing tube followed by a second HBSS (Gibco) washing step. After transfer to a sterile glass plate, the vessels were smoothed out with a cannula to remove the blood inside. The vessel was cannulated and fixed on the cannula with a thread. The syringe was filled with 20ml of Collagenase/Dispase enzyme solution and connected to the cannula. After proper fixation cell digestion was started, with the first 3 drops going to waste. Subsequent cell digest solution was collected into the tube containing 10ml FCS. 20ml enzyme solution was rinsed through the vessel for 6-8 min (5ml/2min). The digested solution was mixed and centrifuged at 900 rpm for seven minutes. After supernatant aspiration the cell pellet was resuspended in 1 ml of EBM + Single Quots, containing 10% Pregnant Serum, and transferred into a well of a gelatin coated 12well plate. The cells were observed daily and media change was performed on the consecutive three days. Incubation settings were 37°C and 12% oxygen and 5% CO_2. When they reached up to 50% confluence, the cells were transferred in a 12 cm^2 flask.

3.2. Cultivation of Feto-Placental Endothelial Cells

Media change was performed every three days. The nutrient-deficient media was aspirated and ten milliliter of fresh EBM++ (37°C) was added.

When the cells reached up to 90% confluence, they were split. For splitting of endothelial cells, the media was aspirated from a 75cm^2 flask. Then, the cells were

washed with 10ml 1x HBSS and 1.5ml of 1x TrypLE Select (Gibco) was added and incubated for 3-5 min at 37°C. The cells were resuspended in 9ml of complete endothelial basal medium EBM++ to inactivate the enzyme. Cells were aliquoted into different flasks for cell expansion and filled up 75cm² flask to 12ml with EBM ++ media. Standard rate of splitting was 1:4, maximum rate up to 1:7 depending on growing rate of the respective isolation. The media was changed after 4-5 hours when cells had attached or on the following day to remove TrypLE (Gibco).

3.3. Endotoxin removal

Pooled HMO (kindly provided by Lars Bode, UCSD) were isolated according to the methods of Kunz et al. (Kunz et al. 1996). Since HMO isolations from breast milk are known to contain endotoxin, we took care to remove endotoxin by polymyxin B (Gnoth et al. 2000a). Endotoxins were removed by High Capacity Endotoxin Removal Resin (Thermo Scientific, Rockford, IL) according to manufacturer's instructions. The resin binds to reduce endotoxin levels in samples by ≥99% in 1 hour using a spin cup format. The modified polylysine affinity ligand eliminates the toxicity concerns using polymyxin B ligands and sodium deoxycholate buffers (de Oliveira Magalhães et al. 2007).

3.4. Angiogenesis assays

The concentration of compounds used for treatment in angiogenesis assay are shown in table 1. The positive control consisted of a combination of TNF-α, VEGF and FGF-2.

Table 1: Treatment compounds for angiogenesis assays

Compound	Company	Concentration stock solution	Concentration for treatment
TNF-α	Reliatech	10 µg/ml	10 ng/ml
VEGF	Sigma	650 nM	650 pM
FGF-2	Sigma	100 µg/ml	10 ng/ml
Pooled HMO	Lars Bode Lab, UCSD	5 mg/ml	100 µg/ml
3'Sialylactose	GLYCO	1 mg/ml	30 µg/ml
2'Fucoslylactose	Lars Bode Lab, UCSD	2.5 mg/ml	30 µg/ml

3.4.1. Fibrin angiogenesis assay

To investigate the effect of different substances on tube formation, a model for angiogenesis was used in which three-dimensional fibrin matrices were prepared. The appropriate amount of fibrinogen was weighed in a 50 mL Falcon tube, and preheated M199 w/o media was added. The fibrinogen was fully dissolved with only minimal mixing motions to avoid premature matrix coagulation. After 60 minutes of incubation at 37°C the solution was filtered through a 0.45 µm filter. The solution was divided into portions of 20 ml and kept on ice. The thrombin IIa stock was added to reach a final concentration of 0.03 U/ml. Only one tube at a time was prepared and the excess of fibrin matrix was used as coagulation control. The network formation immediately started after thrombin IIa addition. As a consequence, the wells had to be filled without delay by reverse pipetting to avoid air bubbles. The plates were incubated one hour at room temperature for fibrin network formation, followed by one hour at 37°C for polymerization. 100 µl per well CM199+ media was added and incubated for two hours at 37°C to inactivate thrombin IIa by FCS. The endothelial cells were seeded on the fibrin matrix with a cell number of $3.5x10^4$ cells per well. After overnight adhesion of endothelial cells, on the first day, the plate was tapped to loosen dead cells followed by aspiration of media and addition of 100 µl of prepared stimulation media. This step was repeated every two to three days until visual tube formation was observed. For fibrin assay termination, the washed cells were fixated at least two hours in warm 4% formaldehyde. After formaldehyde aspiration the wells were filled with 200 µl PBS, the plate was sealed with Parafilm and stored at 4°C prior to imaging.

3.4.2. Spheroid sprouting assay

The first step was cell seeding in hanging drops. The washed, detached and centrifuged cells were counted. 1000 cells per drop (25µl) were used for spheroids.

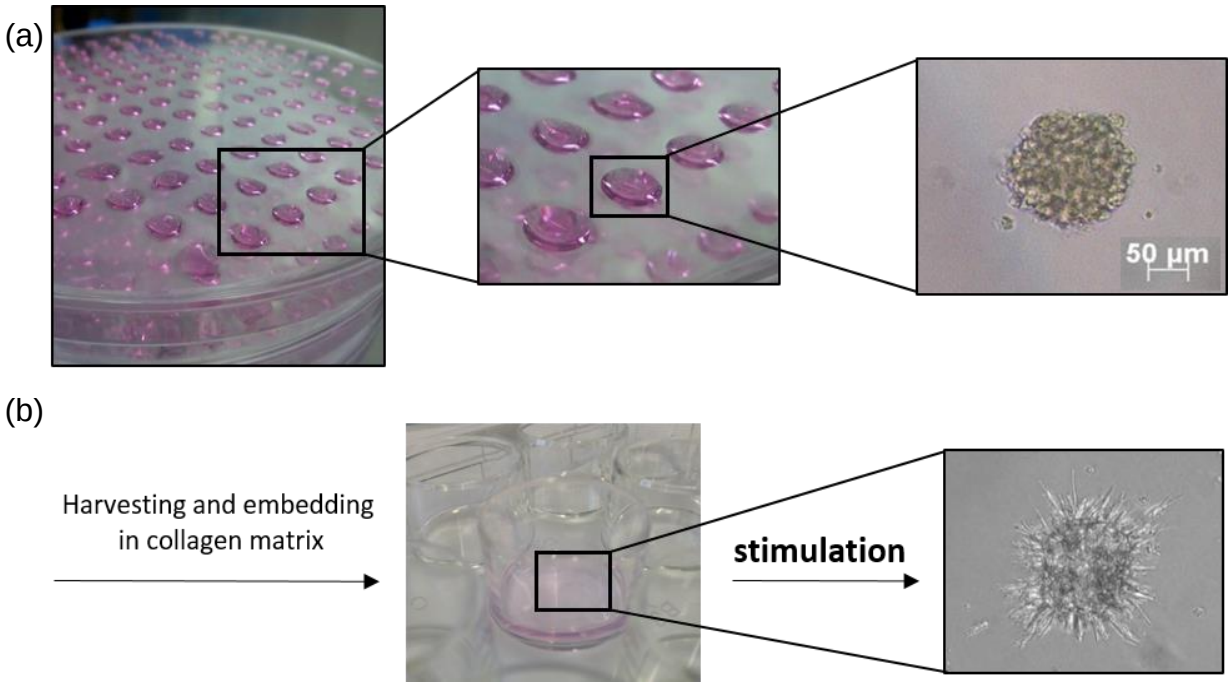

Figure 4: Generation of the spheroid sprouting model. (a) 1000 cells per 25 µl drop M199 medium were placed on the lid of a non-adhesive petri dish and incubated for 24 hours deriving endothelial cell spheroids. (b) The spheroids were collected, embedded in collagen I and stimulated after two hours of matrix polymerization.

Depending on the number of plates, appropriate volume of 20% methylcellulose working solution was diluted, and the cell suspension containing the appropriate number of cells was centrifuged and resuspended in methylcellulose working solution. By using a multichannel pipette, the cells were distributed in drops on the lid of a non-adherent plastic dish. The plates were inverted to generate spheroids in hanging drops and incubated for 24 hours at 21% O2 and 37°C in the incubator. The pictures in figure 4 show the steps of generating spheroids.

After checking the spheroid formation under the microscope, the spheroids were harvested by washing the spheroids off the plate with HBSS and transferring the suspension onto the next plate. 5-10 ml of HBSS was enough to wash approximately six plates. The collected suspension was transferred to a falcon tube and centrifuged for 5 minutes at 300xg and then 3 minutes at 500xg without brake. The supernatant and air bubbles were carefully aspired, and by running the tube over the grid of the bench the pellet was gently loosened. The spheroids were placed on ice and overlaid with 321.45 µl methylcellulose stock solution containing 40% FCS. 247.5 µl $NaHCO_3$ was added, and the solution was mixed with 1100.55 µl Type 1 Collagen stock (4mg/ml) gently, but thoroughly. To increase the pH for polymerization 16.5 µl NaOH was added and thoroughly mixed. After addition of NaOH, the collagen-spheroid solution was

immediately transferred into wells of a 96-well plate (500 µl/well). Everything was kept on ice, and extra care was taken working quickly to avoid premature collagen polymerization after addition of NaOH. The plate was incubated at 37°C, 21% O2 for two hours to allow collagen to polymerize. After two hours, stimulation media (compounds diluted in CM199) was added and incubated for a maximum of 16 hours. To terminate the experiment, the media was aspired and the spheroids were fixated for two hours with 500 µl of warm 4% formaldehyde. After the formaldehyde was aspirated, the wells were filled with 800 µl of PBS buffer. The plates were sealed with Parafilm and stored at 4°C prior to imaging (Di Blasio et al. 2014).

3.4.3. Imaging and analysis of angiogenesis assays

The plates were imaged by Cell IQ V2 MLF Cell Imaging and Analysis System, which is a continuous live cell imaging and analysis platform. The instrument uses Machine Vision Technology for the automatic identification, analysis and quantification of morphological features. The system can work with multiple plate formats and is specialized for live cell imaging.

The software includes Imagen System control, an imaging viewing software and Analyzer software for image processing, automatic stitching and easy conversion of single image files into high definition videos. This software also allows the automatic identification and quantification of cell tubes und branching points. Therefore, two separate classification protocols were used to increase the accuracy and to acquire proper background reduction.

The software delivers tube length in pixel, for conversion in µm the factor is multiplied by 0.7 or 1.8.

3.5. Immunocytochemistry staining for phalloidin

FPEC (50,000 cells/well) were seeded in 1% gelatin coated chamber slides, cultured for 24 hours and incubated with either 5% FCS supplemented M199 medium serving as control or with stimulation media containing a combination of TNF-α, VEGF and FGF-2; pooled HMO; 3'SL; 2'FL or sugar controls (lactose, fucose, galactose). The chamber slides were incubated at 21% oxygen and 37°C for 24 or 48 hours.

Monolayers were washed twice with HBSS 1x (Gibco), air dried and fixed with 3.7% formaldehyde solution in PBS for 10 minutes at room temperature. After washing 3 times with PBS (3 minutes each) the cells were permeabilized with Triton X-100 in

PBS for 10 minutes at room temperature. The slides were washed again 3 times with PBS followed by blocking with 2% BSA in PBS for 30 minutes at room temperature in a dark humid chamber.

After blocking, slides were incubated with Mouse Vinculin primary antibody (Neo Markers, Lab Vision) diluted 1:50 in 2% BSA-PBS for 90 minutes at room temperature. Stained cells were washed 3 times and simultaneously incubated with DL650 goat anti mouse (Thermo scientific) as secondary antibody for vinculin staining and Phalloidin-488 FITC (Alexa, Thermo scientific) for F-actin staining. DL650 goat anti mouse was diluted 1:100 and Phalloidin-488 FITC was diluted 1:20 in 2% BSA-PBS. After washing with PBS, Pro Long Gold Antifade DAPI (4′,6-Diamidin-2-phenylindol) mounting medium (Life technologies) was used to stain nuclei as DAPI binds strongly to A-T rich regions in DNA.

After overnight drying, the coverslips fluorescent staining was observed with the Zeiss LSM 510 META scanning laser confocal microscope. Integrated lasers are UV 405nm (for violet excitation of dyes such as DAPI), tuneable Argon 458/477/488/514nm (458,477, and 488 for blue excitation of dyes such as FITC) and Helium-Neon 633nm (for red excitation dyes, such as vinculin secondary antibody DL650 goat anti mouse). Zeiss LSM 510 software was used to observe F-actin organization.

3.6. Metabolic cell proliferation assay

To investigate if HMO treatment affects the proliferation rate of feto-placental endothelial cells, we analyzed the metabolic activity and cell viability by colorimetric MTT assay. Since for endothelial cell populations the total mitochondrial activity is constantly related to the number of viable cells, this assay is used to measure the *in vitro* effects of substances on proliferation. This assay is based on the cleavage of 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide molecule into strongly colored formazan crystals by mitochondrial dehydrogenases in viable cells (Van Meerloo J et al. 2011).

During proliferation the number of viable cells increases, thus mitochondrial activity increases, and the insoluble formazan adducts accumulate and can be solubilized for homogenous measurement. Thus, any increase or decrease in mitochondrial activity, which correlates with viable cell number, can be detected by measuring formazan

concentration reflected in optical density (OD) at 570 nm, using an Enzyme-Linked-Immunosorbent Assay (ELISA) microplate reader (Mosmann 1983).

In detail, metabolic cell proliferation was measured with *in vitro* Toxicology Assay Kit, MTT Based (Sigma Aldrich, Tox-1), according to manufacturer's instructions. $2.5x10^4$ and $5x10^4$ cells were seeded per well of a 96-well-plate. Triplicates were either untreated or treated with a combination of TNF-α, VEGF and FGF-2, pooled HMO, 3'SL or Lactose. The cells were incubated at 37°C and 12% oxygen for 24, 48 or 96 hours. After incubation time, we added reconstituted MTT in an amount equal to 10% of the culture medium volume to each well.

3.7. RNA isolation and cDNA synthesis

Total RNA from the pelleted cells was isolated and purified with miRNaesy mini Kit (Quiagen, Hilden, Germany) by affinity purification. RNA quality was evaluated by QIAxpert (Quiagen), a microfluidic UV/VIS spectrophotometer which profiles sample content to differentiate between RNA and DNA and sample impurities.

Complementary DNA, cDNA, was synthesized from 1 µg total RNA according to the manufacturer's instructions (SuperScript II Reverse Transcriptase protocol from Invitrogen, USA). Reverse transcriptase is an enzyme that translates RNA to DNA. This creates one DNA (cDNA) strand complementary to the RNA strand in a DNA-RNA hybrid. The RNA strand is then degraded by RNase H, leaving single stranded cDNA ready for PCR.

In this study random hexamers were used. The amount of each Superscript-II solution is 40 µl.

Primer-dNTP Master Mix:

1 µg total RNA in 20 µl DEPC treated water (Ambion Inc., Austin, TX, USA)

2 µl random hexamers (50ng/µl)

2 µl dNTP mix (10mM)

Incubated at 65°C for 5 min, and then on ice for 1 min

Master Mix:

8 µl 5X strand buffer

4 µl 0.1M DTT

2 µ Ribonuclease Inhibitor

Incubated at 25°C for 2 min.

After adding 2µl of SuperScript II RT and mixing by pipetting up and down, the tubes were incubated at 25°C for 2 minutes followed by an incubation at 42°C for 50 min. The reactions were terminated at 70°C 15 min and chilled on ice.

3.8. Quantitative real-time PCR

Real time qPCR was performed with TaqMan gene expression assays from Applied Biosystems (CA, USA) for the respective genes. The expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT1) gene was used as a housekeeping gene as its expression is not influenced by the treatment.

Table 2: Gene expression assays used for real-time qPCR

	Gene	Assay ID
Target genes	TLR-4	Hs00152939_m1
	CLEC4M (L-SIGN)	Hs_03805885_g1
	SELE	Hs00864392_m1
	KDR	Hs00911700_m1
	INS-R	Hs001873947_m1
	IGF1-R	Hs00609566_m1
Housekeeping gene	HPRT1	Hs01003267_m1

Every 20 µl reaction was performed in duplicates.

Master Mix:

10 µl TaqMan universal Master Mix (Applied Biosystems, New Jersey, USA)

5 µl DEPC treated water (Ambion Inc., Austin, TX, USA)

1 µl Gene expression assay (Applied Biosystems, Foster City, CA, USA)

4 µl cDNA [25 ng/µl]

PCR profile:

1.	UNG-Activity	50°C	2 minutes
2.	Initial denaturation	95°C	10 minutes
3.	Denaturation	95°C	15 seconds

4.	Annealing and Extension	60°C	1 minute
5.	Cycle from step 3 to 4		39 times
6.	Storage	4°C	hold

The first step avoids carry-over-contamination. Contaminating amplicons are digested before PCR by the enzyme Uracil-N-Glycosylase (UNG). The PCR reactions were run in a BioRad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA).

Data were analyzed according to the 2-ΔΔCt method (Schmittgen & Livak 2008). The cycle threshold value (Ct value) of each target gene was normalized to the Ct value of the housekeeping gene giving the ΔCt value. For the ΔΔCt quantification, the ΔCt values of the controls were subtracted from the ΔCt values of the treatments. To obtain the ratio between control and treatments, the $2^{-\Delta\Delta Ct}$ value was calculated.

3.9. Quantitative determination of cytokines, IGF-1 and Leptin in the conditioned media

Concentrations of IL-6, IL-10, IGF-1 and Leptin were determined by Quantikine ELISA Kit following manufacturer's instructions (R&D Systems Europe Ltd., Abingdon, UK). This assay employs the quantitative sandwich enzyme immunoassay technique. 100 µl of standard, control or sample are added to 100 µl of Assay Diluent RD1-19 in a well of a pre-coated microplate. The protein of interest present in the conditioned media was bound by the immobilized antibody. After washing away unbound substances, 200 µl of an enzyme-linked monoclonal antibody specific for the respective protein was added to the wells. After subsequent washing 100 µl of substrate solution was added to each well and color developed in proportion to the amount of the substrate of interest bound in the initial step. The color development was stopped and the intensity of the color was measured by an ELISA multi well plate reader (SpectraMax 250, Molecular Devices, CA, USA).

3.10. Statistical analysis

All statistical analyses were performed with GraphPad PRISM (GraphPad Software, Inc.). For all experiments mean values were expressed as mean ± SEM. A p value of less than 0.05 was considered significant and highly significant at $p < 0.01$. Statistical differences were assessed by Student's t-test unless otherwise stated.

4.Results

4.1. Potential receptors for HMO

For this study, Toll like receptor-4 (TLR-4), L-SIGN and E-Selectin were selected as potential HMO receptors, as they were previously found linked to HMO signaling in several cell types. In a first step, the expression of these receptors in feto-placental endothelial cells was examined. Real-time qPCR determined mRNA expression levels of untreated endothelial cells of 6 different fpEC isolations.

Results of real-time qPCR showed that TLR-4, L-SIGN and E-Selectin are expressed in placental arterial endothelial cells (Figure 5). The mean ΔCt values of each gene were normalized on the respective ΔCt value of the housekeeping gene.

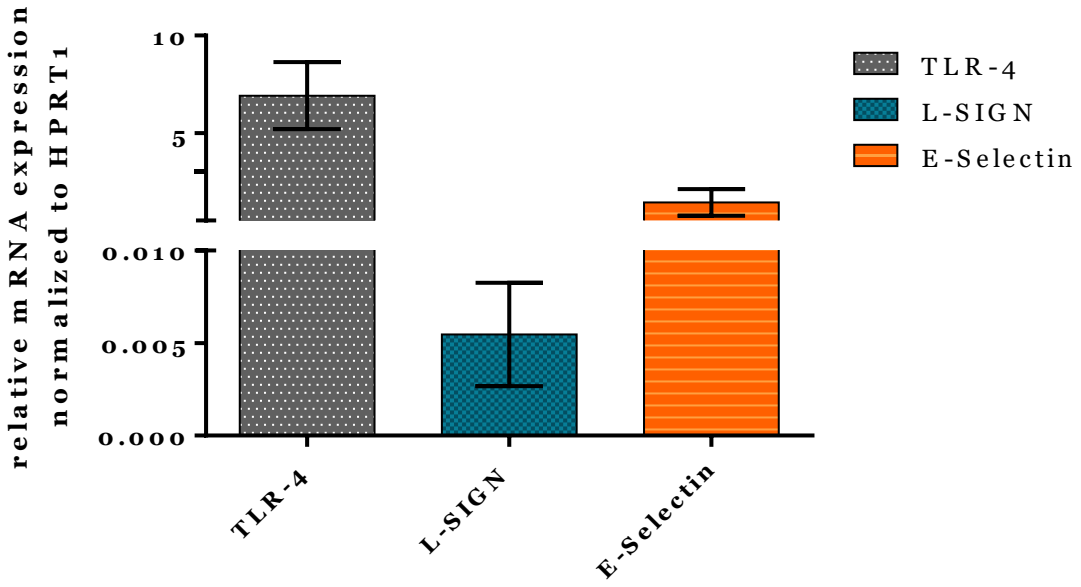

Figure 5: Gene expression of TLR-4, L-SIGN and E-Selectin normalized to the housekeeping gene HPRT1. Total RNA was isolated and real-time qPCR determined mRNA expression levels in untreated feto-placental endothelial cell isolations. Data are shown as mean of 6 fpEC isolations ±SEM.

To assess whether HMO can regulate gene expression of these potential receptors, we performed real-time qPCR upon treatment of the cells with pooled and individual HMO for 48 hours. As controls, we used a mix of TNFα+VEGF+ FGF2 as a known pro-angiogenic stimulant (positive control) and lactose as non-HMO sugar control.

4.1.1. Toll like receptor 4

Toll like receptor signaling is central to innate immunity. 2'Fucosyllactose is known to attenuate TLR-4 signaling by downregulation of CD14 (He et al. 2016). The effect of HMO, 3'SL and 2'FL on gene expression of TLR-4 on fpEC is shown in figure 6. All treatments, including the positive control and lactose showed a lower TLR-4 gene expression than the untreated negative control (Figure 6). Due to the control's high standard deviation, the differences were not statistically significant.

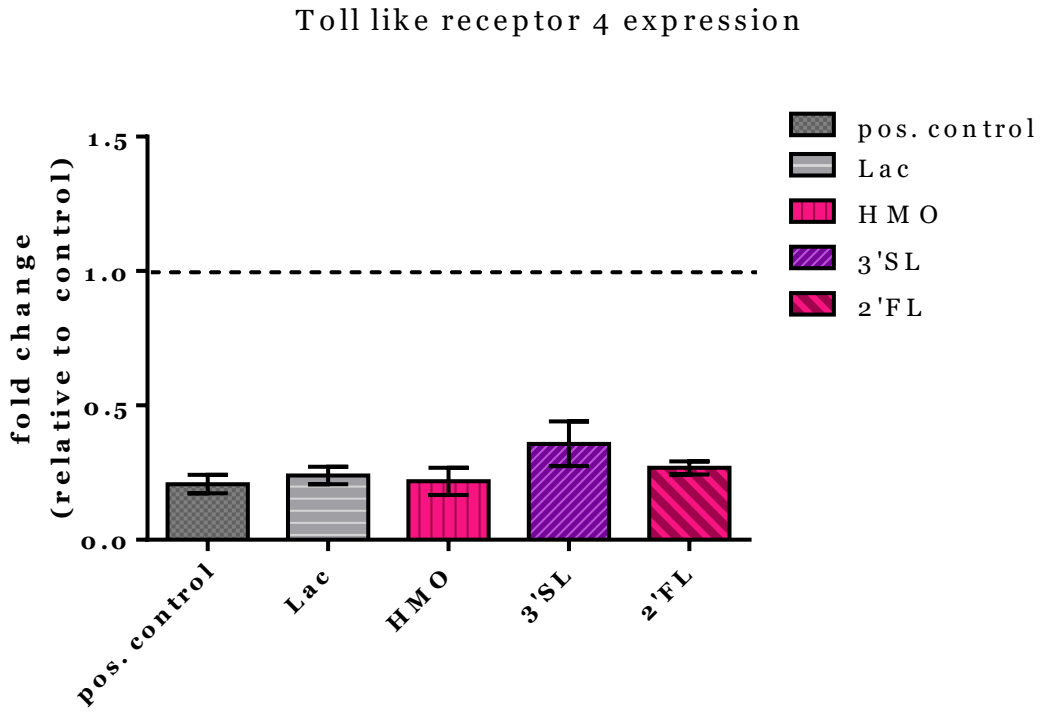

Figure 6: TLR-4 gene expression of treated placental endothelial cells normalized to the untreated control. Real-time qPCR determined mRNA expression levels of feto-placental endothelial cell isolations. Positive control, lactose, pooled HMO, 3'SL and 2'FL caused a lower TLR-4 gene expression compared to the untreated negative control. Data are shown as mean of 6 fpEC isolations ±SEM.

4.1.2. L-SIGN

Liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN/ DC-SIGN-R) is a calcium-dependent lectin expressed on endothelial cells of liver, lymph nodes and placenta. It is a C-type lectin and recognizes carbohydrate structures.

FpEC treated with pooled HMO showed a lower gene expression of L-SIGN compared to negative control, positive control and lactose. The results were not statistically different, but a p value of 0.058 showed a trend. However, 2'FL treatment reduced L-SIGN expression significantly to 85% of the expression level in untreated control cells. Cells treated with positive control, the mix of growth factors (VEGF, FGF-2) and TNF-α, and lactose as sugar control also showed reduced L-SIGN gene expression compared to the control, but without reaching significance (Figure 7).

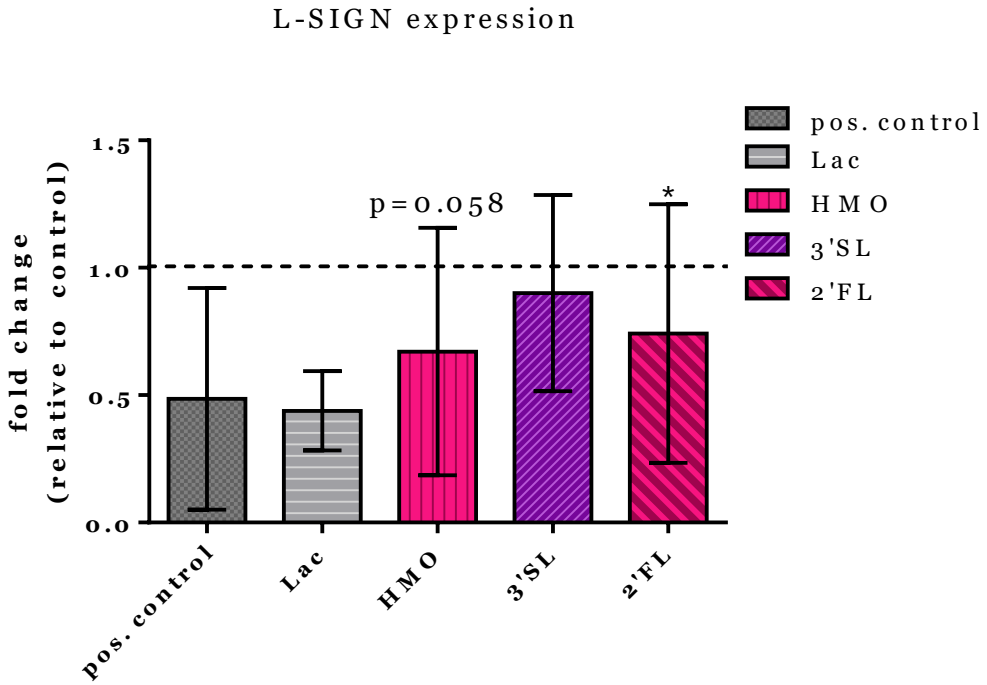

Figure 7: L-SIGN gene expression of treated feto-placental endothelial cells normalized to the untreated control. Real-time qPCR determined mRNA expression levels of feto-endothelial cell isolations. Cells treated with pooled HMO showed a trend of reduced L-SIGN gene expression (p= 0.058) compared to unstimulated control cells. 2'FL treatment led to a significantly lower L-SIGN gene expression compared to unstimulated control cells. Data are shown as mean of 6 fpEC isolations ±SEM.

4.1.3. E-Selectin

Selectins are a family of transmembrane adhesion molecules that mediate the inflammatory response and cancer metastasis cascade (Ley 2003). During inflammation, leukocytes are captured by the selectins lining blood vessels to facilitate exit from the bloodstream. E-selectin is upregulated in stimulated endothelial cells and binds to several ligands on the surface of leukocytes (Hanley et al. 2004). Selectins bind to glycans that carry sialylated Lewis blood group epitopes very similar to HMO (Bode et al. 2004). Hence, it is possible that E-Selectin expressed on fpEC serves as HMO receptor.

FpEC treated with HMO, 3'SL and 2'FL showed a lower E-Selectin gene expression. 3'SL treatment led to 68% lower E-Selectin gene expression, not reaching statistical significance (p value of 0.073). Positive control and lactose treated cells also showed a slightly lower E-Selectin gene expression (Figure 8).

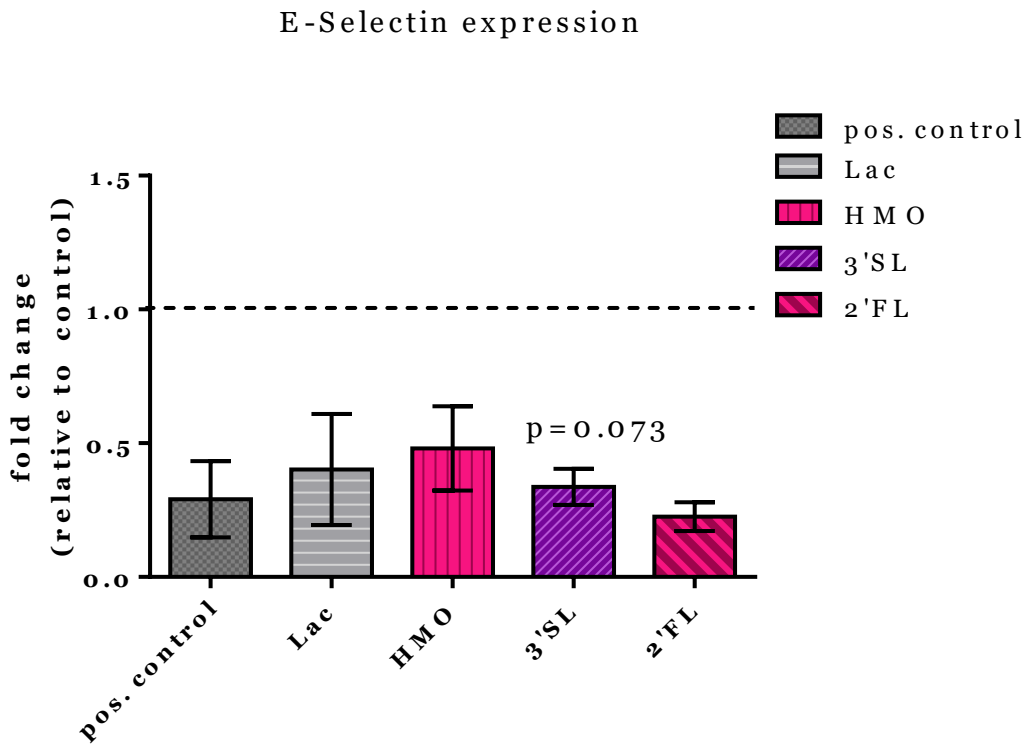

Figure 8: E-Selectin gene expression of treated feto-placental endothelial cells normalized to the untreated control. Real-time qPCR determined mRNA expression levels of feto-placental endothelial cell isolations. Treatment with pooled HMO and 2'FL lowered gene expression of E-Selectin. Cells treated with 3'SL showed a trend of reduced L-SIGN gene expression (p= 0.073) compared to unstimulated control cells. Data are shown as mean of 6 fpEC isolations ±SEM.

4.2. HMO stimulation increased tube formation in functional angiogenesis assays

Preliminary experiments showed that feto-placental endothelial cells stimulated with HMO show an increase of network formation in Matrigel assays. To address whether HMO also influence tube formation potential of feto-placental endothelial cells, two independent 3D angiogenesis assays were conducted.

4.2.1. Fibrin angiogenesis assay

The stimulated and fixed cells were imaged by Cell IQ. To determine tube length and branching points, the produced pictures were analyzed by image processing software. Figure 9a shows one quarter of the original picture, taken by Cell IQ. In figure 9b the red lines are depicting tubes between cells. The background on the left side of the picture was successfully excluded by the analyzing software, and other particles, like dead cells, were not classified as tubes. This optimized analysis was generated combining two different analysis protocols, which led to higher specificity in tube recognition and improved background reduction.

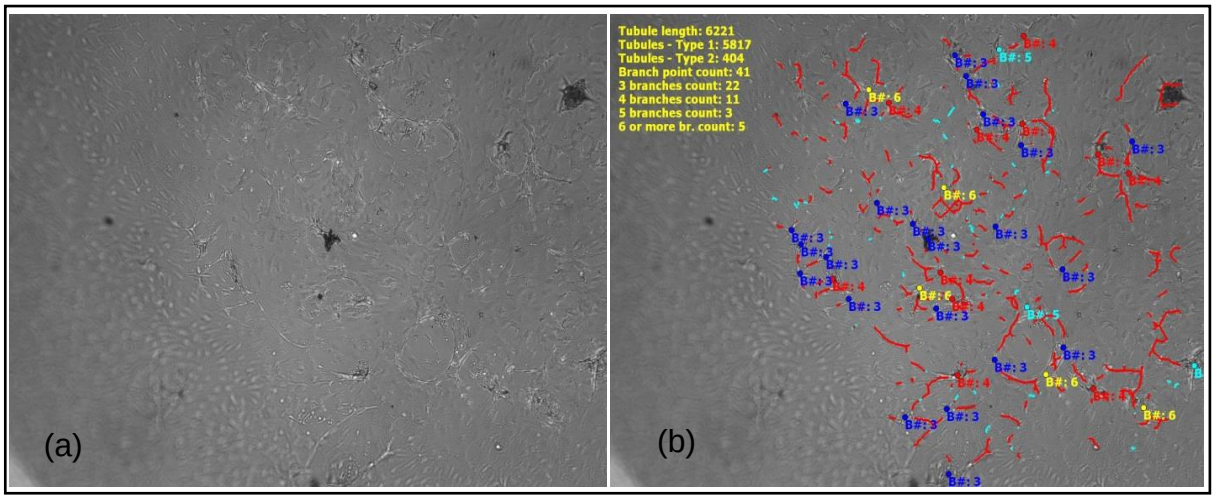

Figure 9: Image Analysis of fibrin angiogenesis assay. Each well is divided into four separate pictures. (a) The fourth (right down corner) picture shows excessive tube building. The four pictures are merged by the Analysis Software, and due to different brightness of pixels, tubes can be recognized and are represented as red lines in the analyzed picture (b).

To quantify tube formation after 48 hours, tube length between unstimulated control cells and HMO stimulated cells were compared.

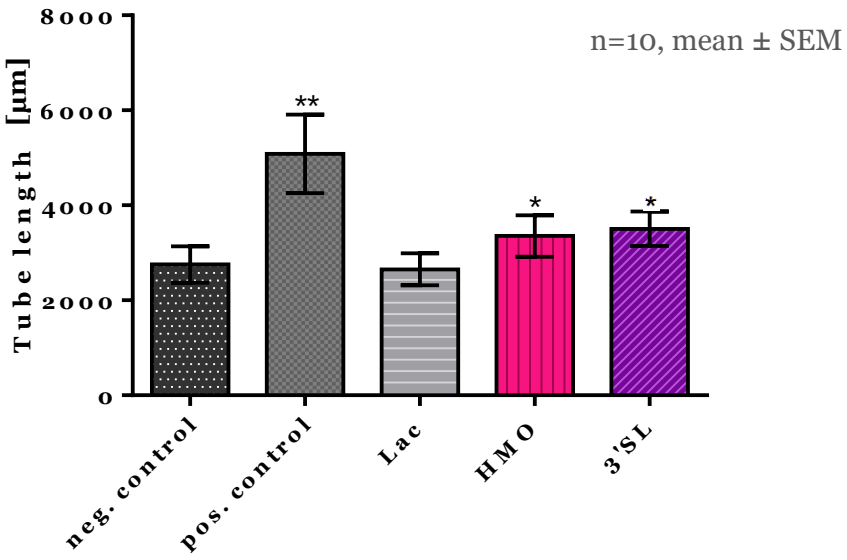

Figure 10: Fibrin angiogenesis assay. 3.5*10⁴ cells/well were seeded on a fibrin matrix. Pictures of the tube formation were taken by Cell IQ and analyzed by Imagen Analyzing software. Negative control and treatments with pooled HMO, a combination of TNF-α, VEGF and FGF-2, 3'-Sialyllactose, and lactose were compared. Positive control and treatment with pooled HMO increased tube length in fibrin angiogenesis assay significantly. Data are shown as mean ± SEM of 10 different fpECs * $p \leq 0.05$ ** $p \leq 0.01$

HMO treatment for 48 hours showed 24.3% increase in total tube length compared to untreated cells (neg. control). The 3'SL individually induced an increase of 25.3%. Stimulation with a combination of TNF-α, VEGF and FGF, as a positive control induced a significant increase of 69,3% in tube formation in fpEC. Treatment with lactose had no significant effect (5,1%) on the growth of vascular structures into the fibrin matrix.

4.2.2. Spheroid sprouting assay

Cells can adopt different modes of invasion. The outspread of tube-like structures was analyzed by image processing. The spheroid core and the background were effectively distinguished from the formed sprout leading to a precise recognition of tubular structures originating from the spheroid body into the collagen matrix. The image processing is shown in figure 11. The sprouts were correctly recognized as shown in figure 11b, whereas the spheroid body was successfully excluded from analysis.

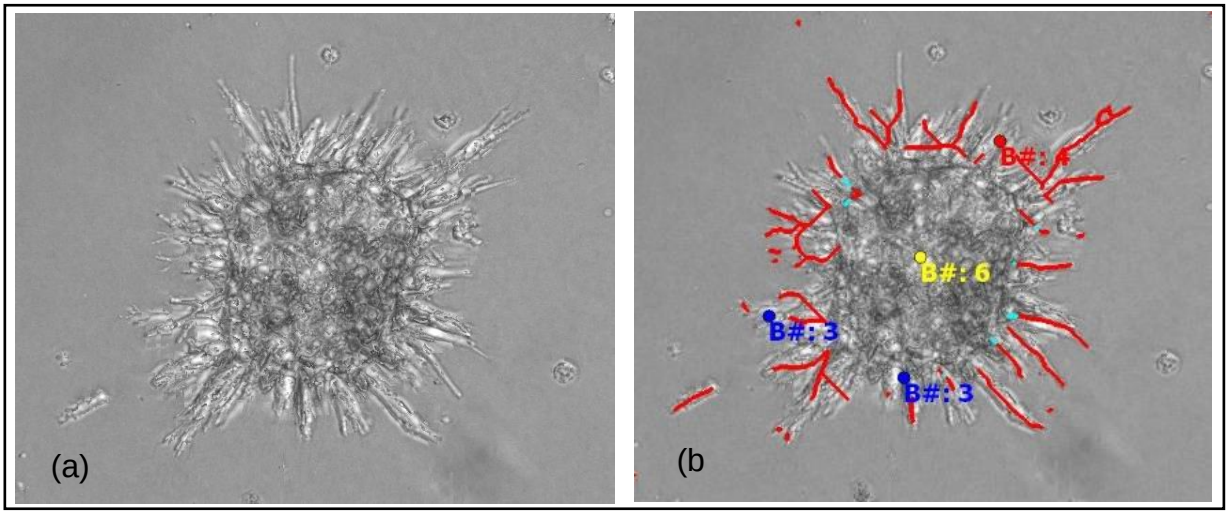

Figure 11: Image processing for spheroid assay. (a) Original photo of a sprout before image analysis. (b) After using morphological and logical operators the outgrowing sprouts were detected (red lines) and measured.

For analysis, tube length of treated cells was compared to the untreated control. Similar to the fibrin angiogenesis assay, cells treated with pooled HMO for 48 hours showed a significant increase of 32% in tube length compared to negative control. 3'SL alone increased tube length by 21.5%. Cells treated with TNF-α, VEGF and FGF-2 increased tube length by 83.5%. Lactose treatment did not influence sprouting of spheroid as the total tube length of lactose treated cells did not differ from untreated fpEC (figure 12).

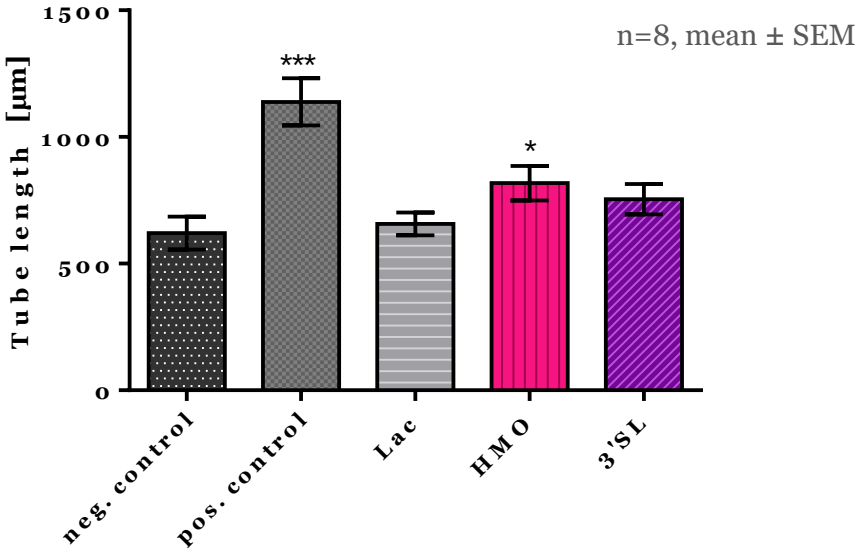

Figure 12: Quantitative analysis of spheroid sprouting after 16 hours of stimulation. FpEC were embedded as spheroids in a collagen matrix. Pictures were taken after fixation by Cell IQ followed by image analysis with Cell IQAnalyzer. Cells treated with positive control or HMO show a significant increase in tube length compared to untreated control cells. Data are shown as mean ± SEM of 8 different fpECs * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$.

4.3. HMO stimulation increased proliferation of fpEC

First, the cell proliferation assay was evaluated by seeding of cells in two different densities. To demonstrate the effect of a treatment, the MTT assay has to be completed during log phase to show accurate results.

Figure 13 summarizes proliferation, seen as increase in mitochondrial activity, of $5*10^4$ cells seeded in a well of a 96-well-plate. After 24 hours, all treatments except for the positive control (TNF-α, VEGF, FGF-2) ranged between an absorption of 0.14 to 0.19. Treatment with TNF-α, VEGF and FGF-2 led to an absorption of 0.25 indicating a stimulating effect on proliferation. At 48 hours of stimulation, control and lactose treated cells showed lower absorption than treatments with pooled HMO and 3'SL. Between 48 and 72 hours, cell growth was declining in the majority of treatments, and the cells were no longer growing exponentially. Absorption in negative control and lactose treated cells strongly decreased. 3'SL and HMO treated cells still showed an increase in cell growth, although, by a lower rate compared to the 48-hour measurement (Figure 13). Based to these observations, we used $2.5*10^4$ instead of $5*10^4$ cells per well of a 96-well-plate for the MTT assay to stay in the log phase for the entire time period of the MTT assay.

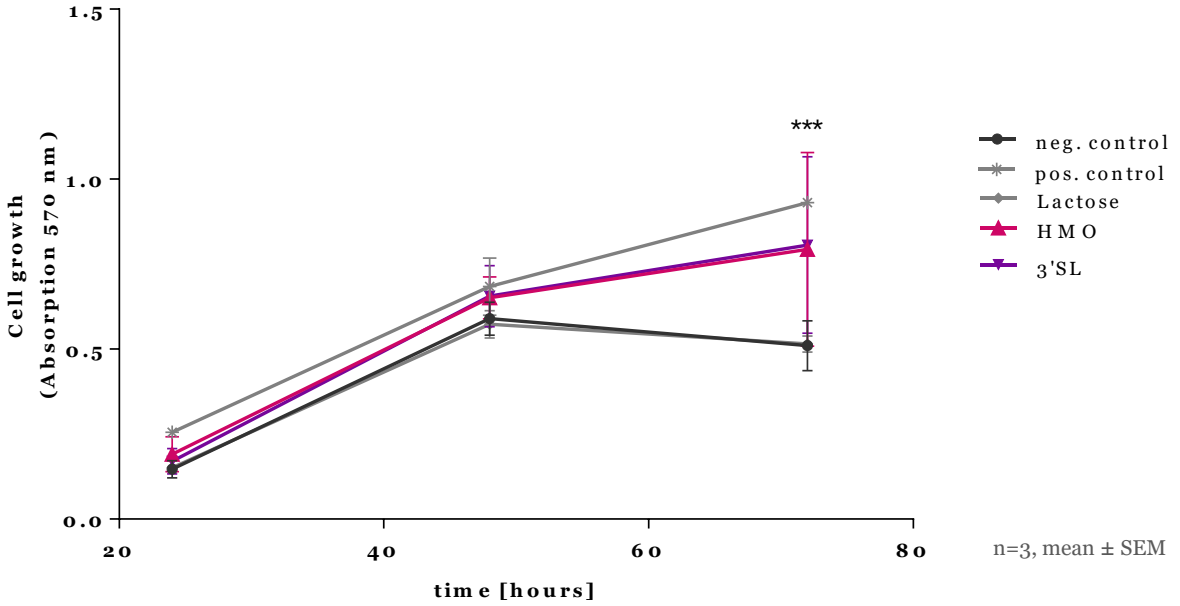

Figure 13: Proliferation assay performed with $5*10^4$ feto-placental endothelial cells per well. Comparison of treatments after 24, 48 and 72 hours, respectively. $5*10^4$ cells were resuspended with media containing pooled HMO, 3'SL or control compounds (positive control or lactose), or left untreated, and seeded into a well of a 96-well-plate. After each time point, MTT dye was

added, and the cells were incubated for another 4 hours before the reaction was terminated. Cell viability was determined by measuring the optical density (OD) at 570 nm in an Enzyme-Linked-Immunosorbent Assay (ELISA) microplate reader. Values shown are the mean ± SEM of three different fpEC isolations.

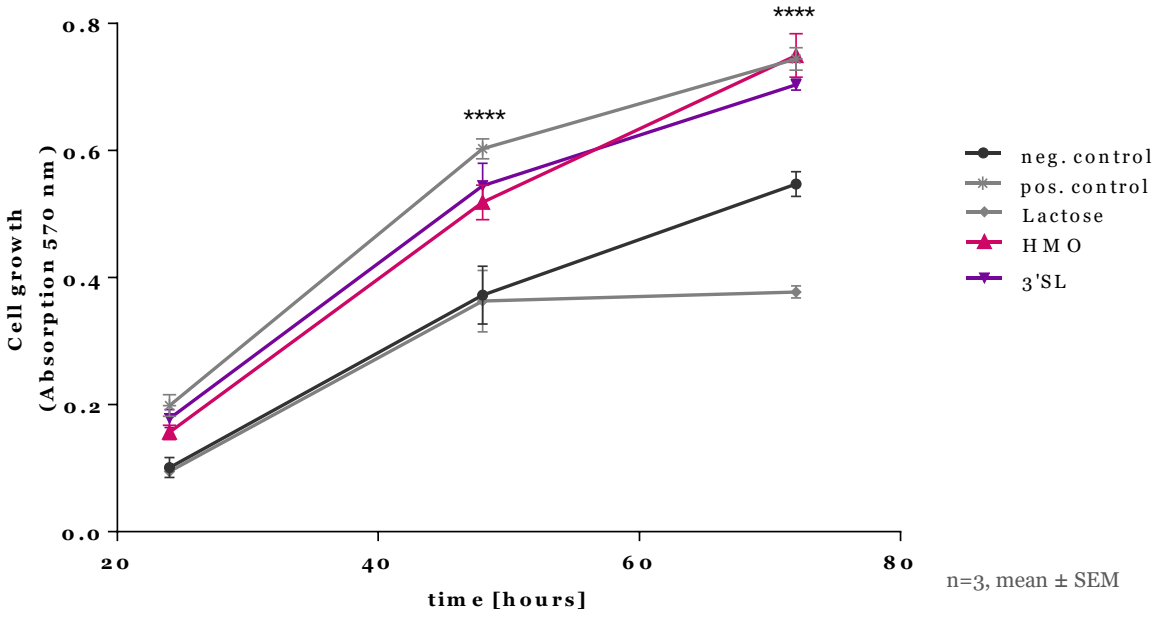

Figure 14: Proliferation assay performed with 2.5*10⁴ feto-placental endothelial cells per well. Comparison between treatments after 24, 48 and 72 hours, respectively. 2.5*104 cells were resuspended with media containing pooled HMO, 3'SL or control compounds (positive control or lactose), or left untreated, and seeded into a well of a 96-well-plate. After each time point, MTT dye was added, and the cells were incubated for another 4 hours before the reaction was terminated. Cell viability was determined by measuring the optical density (OD) at 570 nm in an Enzyme-Linked-Immunosorbent Assay (ELISA) microplate reader. Values shown are the mean ± standard deviation of three different fpEC isolations.

The second cell number tested was 2.5*10⁴ feto-placental endothelial cells per well. Using this concentration in the MTT assay provided reliable data, because all treatments except for Lactose remained in log phase until the end of the assay (Figure 14). At the 24-hour measurement, treatment with growth factors (pos. control), pooled HMO and 3'SL resulted in a higher absorbance at 570 nm than control and lactose treated cells (Figure 15A).

The stimulating effect of HMO on proliferation continued after 24 hours. At the 48-hour time point, treatments with HMO and 3'SL resulted in a significantly higher cell growth compared to control. The positive control showed a similar effect on proliferation, whereas lactose had no effect. The effect size on proliferation remained similar to the 24-hour time point (1.56% and 1.41% at 28 hours for HMO and 3'SL, respectively, and 1.80% and 1.48% at 48 hours for HMO and 3'SL, respectively) (Figure 15B).

After 72-hours, the difference in cell growth between control and treated cells remained remarkably high. Lactose treated cells plateaued after 48h, resulting in an even more pronounced gap in proliferation between HMO and lactose treatment (Figure 15C).

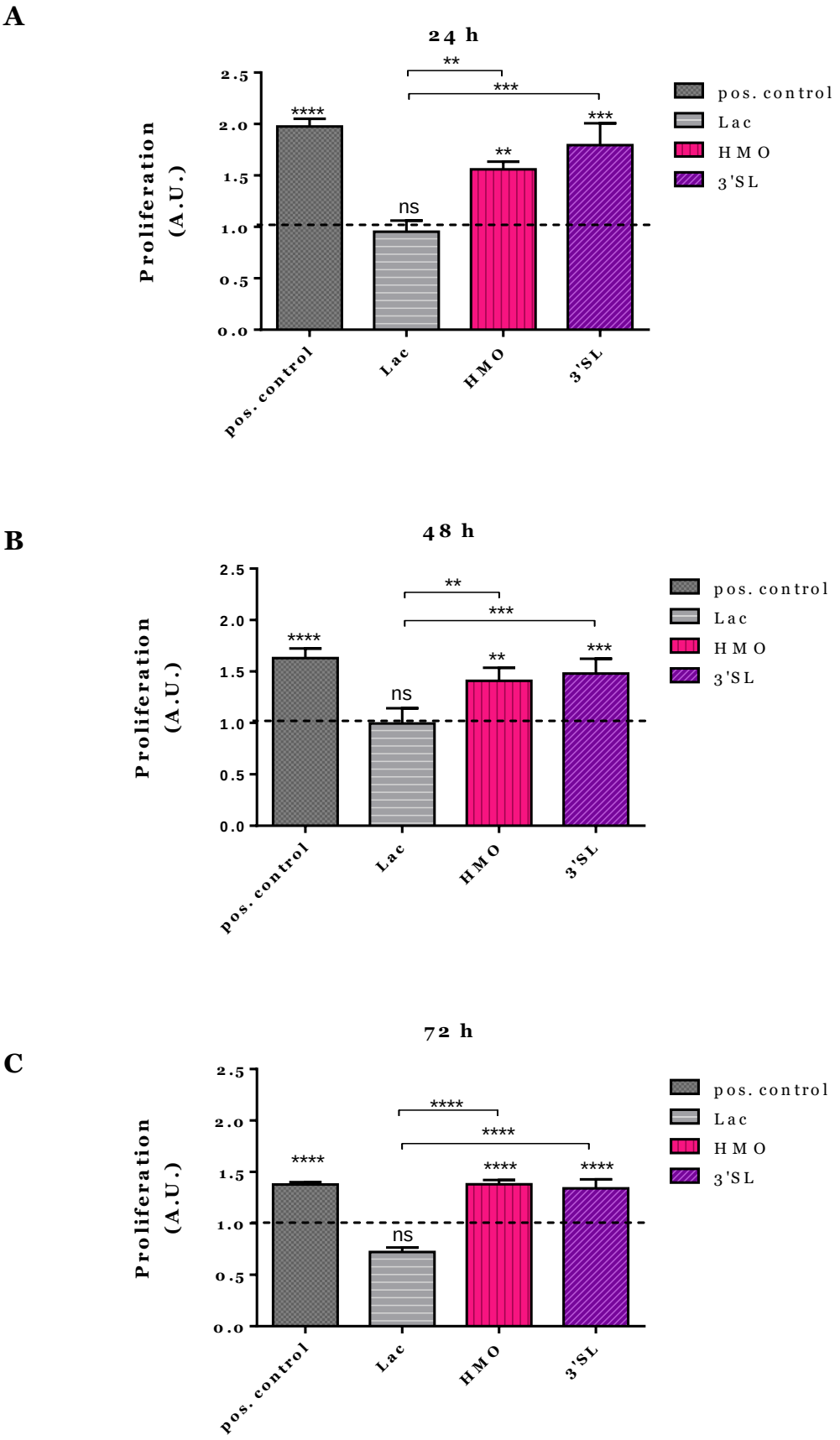

Figure 15: Effect of HMO on proliferation of feto-placental endothelial cells. $2.5*10^4$ cells were resuspended with media containing pooled HMO, 3'SL or control compounds (positive

control or lactose), or left untreated, and seeded into a well of a 96-well-plate. After each time point, MTT dye was added, and the cells were incubated for another 4 hours before the reaction was terminated. Cell viability was determined by measuring the optical density (OD) at 570 nm in an Enzyme-Linked-Immunosorbent Assay (ELISA) microplate reader. (A) After 24 hours, HMO or 3'SL treatment showed the highest relative promotion of cell growth compared to the untreated control which was similar to treatment with positive control. (B and C) At the 48 and 72-hour time points, HMO and 3'SL also significantly stimulated proliferation, as reflected by an increase in absorption at 570 nm. Data are shown as mean of three fpEC isolations ±SEM.

4.4. HMO stimulation caused cytoskeleton rearrangement

Actin cytoskeleton was visualized after 24 or 48 hours of treatment by scanning laser confocal microscopy (Zeiss LSM 510 META). The experiment was reproduced with five different fpEC isolations. Under control conditions, there was no difference in cytoskeleton organization observed between the 24- and 48-hour time point.

Untreated control cells, grown under starving conditions, in M199 media containing 5% FCS, showed membrane ruffling (big white arrows) and less intense F-actin fibers (Figure 16A). The cells look faint and showed a contracted, shrunken shape, instead of occurring in their original round shape. The cells were polygonal and had wispy actin filaments, a characteristic of serum-starved cells. Their actin filament bundles were much shorter and not longitudinally aligned with disorganized appearance. The cells' plasma membrane was only weakly stained with phalloidin.

Stimulation with TNF-α, VEGF and FGF-2 (10 ng/ml, 650 pM, 10 ng/ml) served as a positive control, inducing actin reorganization and migration (Morales-Ruiz et al. 2000). As expected, the treatment induced the formation of long, condensed stress fibers (thin white arrows, Figure 16B) that were aligned along the cell's longitudinal axis. Their plasma membrane was lined with bright phalloidin staining. The organized parallel fibers of fpEC treated with TNF-α, VEGF and FGF-2 are shown in Figure 16B.

fpEC treated with pooled HMO undergo profound changes in shape. Figure 16C shows representative images of actin/cytoskeleton organization induced by HMO treatment. F-actin stress fibers stretch through the cell body in a parallel and organized way (thin white arrows, Figure 16C). No membrane ruffles were noticeable, and the cells presented an intense F-actin fiber staining.

The treatment of fpEC with 3'SL showed similar actin reorganization as treatment with the positive control or HMO. 3'SL stimulated remodelling of the actin cytoskeleton into stress fibers. An intense F-actin staining and the parallel organized fibers is shown in figure 16D.

In addition, 2'FL alone (Figure 16E) also caused profound reorganization of F-actin. On the very right picture the intense phalloidin staining and stress fiber formation is visualized. Mostly, we observed ventral stress fibers and transverse arcs.

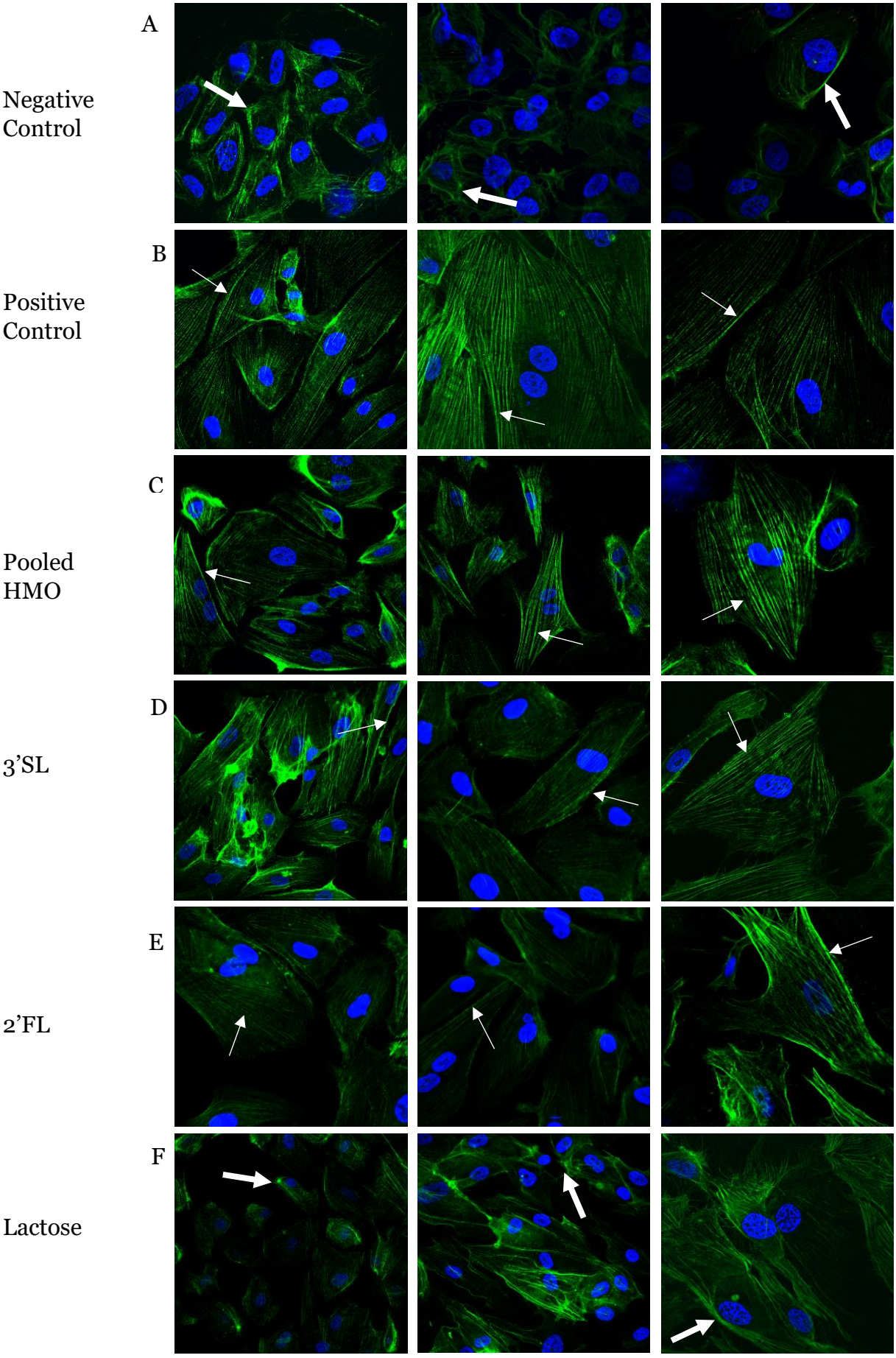
A
Negative Control
B
Positive Control
C
Pooled HMO
D
3'SL
E
2'FL
F
Lactose

Figure 16: Effect of different treatments on actin cytoskeleton organization of cultured feto-placental endothelial cells. 50.000 cells per well were seeded on gelatin coated chamber slides. fpEC were cultured for 24 hours in M199 media containing 10% hPS and 10% nBCS followed by a treatment of 24 with M199 media containing pooled HMO, 3'SL or control compounds (positive control or lactose), or left untreated. (A) Untreated control cells show membrane ruffle formation (big white arrows) and less intense phalloidin staining. (B) Cells treated with TNF-α, VEGF and FGF-2 served as a positive control. Their membrane was lined with bright phalloidin staining and F-actin stress fibers (thin white arrows) organized parallel and highly ordered. The treatment with (C) pooled HMO, (D) 3'SL, and (E) 2'FL was similar to the positive control, with parallel and strongly stained stress fibers. (F) The treatment with lactose, served as sugar control, and did not lead to an actin rearrangement to stress fibers. Membrane ruffle formation, as seen in the negative control, was observed. There was no difference between the 24- and 48-hour treatment (data not shown). Similar results were obtained in five individual experiments (n=5).

Lactose treatment led to actin disorganization and membrane ruffling (big white arrows). The lack of stress fibers and a less intense phalloidin staining resembled untreated control cells.

4.5. HMO effect on secretion of paracrine factors

To test the effect of HMO treatment on the secretion of potentially angio-active factors IL-10, IL-6, Leptin and IGF-1, we performed specific enzyme linked immunosorbent assays (ELISA) for the respective factors. HMO treatment did not significantly change IL-10, leptin or IGF-1 secretion of feto-placental endothelial cells (fpEC) (Figure 17).

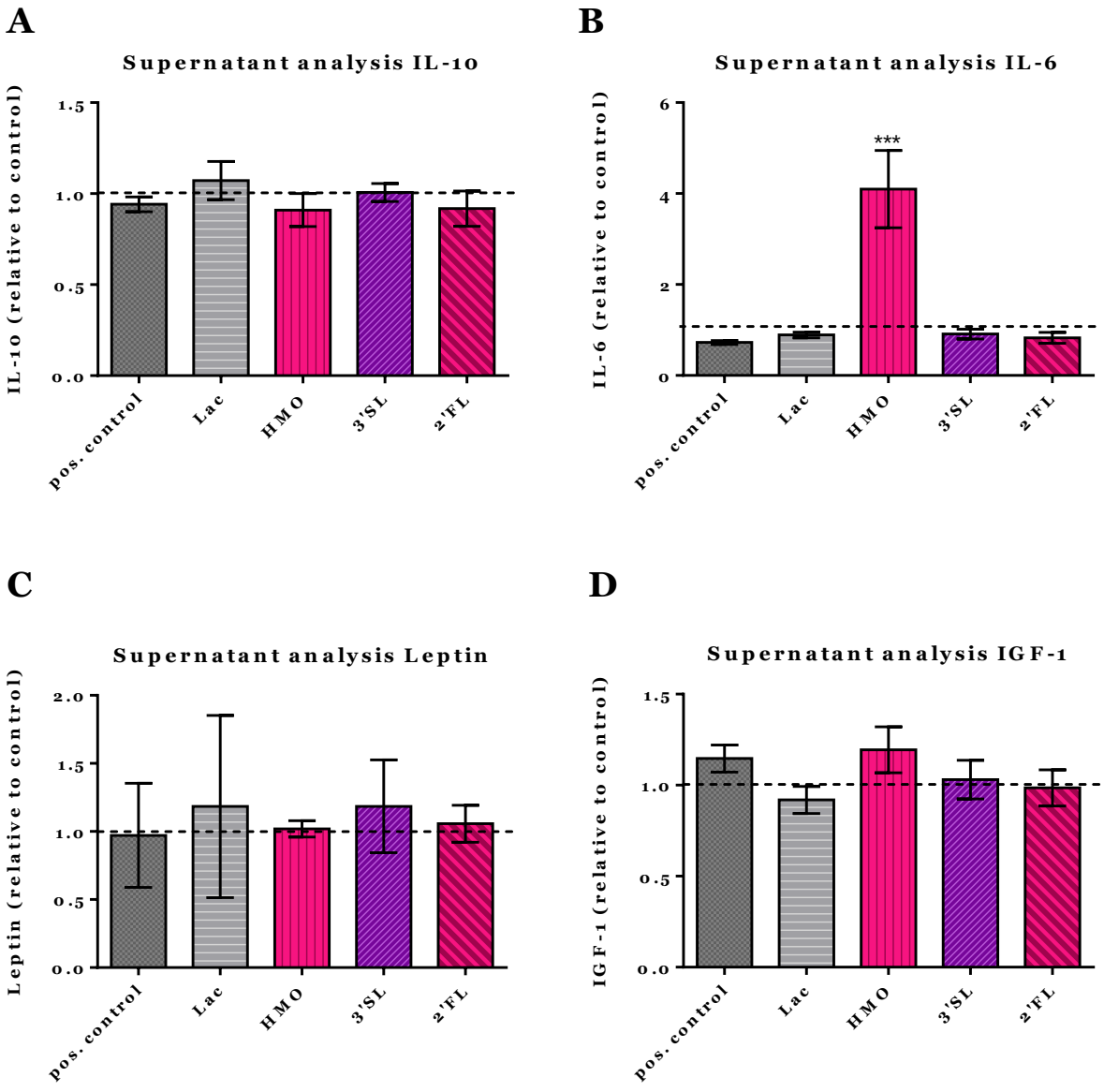

Figure 17: IL-10, IL-6, Leptin and IGF-1 levels in supernatants of HMO, 3'SL or 2'FL treated cells as assessed by ELISA after 48 hours. Supernatants of treated cells were analyzed by specific ELISA.IL-10 concentration was not significantly influenced by HMO treatment (A). IL-6 concentration in supernatants of fpEC treated with pooled HMO was significantly higher than concentrations in control supernatants (B). Leptin ELISA showed slightly increased concentrations in supernatants of lactose and 3'SL treated cells (C). IGF-1 concentrations in cell supernatants of cells treated with pooled HMO are slightly, but not significantly higher than supernatants of untreated cells

(D). Six different fpEC isolations were used in each assay. Values are expressed as mean ± standard error of the mean of three replicates. *** $p \leq 0.001$

IL-10 concentration slightly decreased in supernatants of cells treated with pooled HMO or 2'FL, whereas lactose treatment led to a non-significant IL-10 increase (Figure 17 A).

Interleukin-6 (IL-6) plays multiple functions in vascular remodelling and angiogenesis (Kamimura et al. 2003). Recent studies showed that IL-6 is a potent pro-angiogenic cytokine which stimulates smooth muscle cell (SMC) and cerebral endothelial cell (EC) proliferation and migration *in vitro* (Nilsson et al. 2005). The ELISA for IL-6 showed a drastic increase of IL-6 in supernatants of cells treated with pooled HMO. Treatment with 3'SL or 2'FL did not lead to changes in IL-6 concentrations in cell supernatants (Figure 17 B).

Leptin is an endocrine hormone regulating adipose tissue mass. Studies showed that leptin generates a growth signal relating a tyrosine kinase-dependent intracellular pathway and promotes angiogenic processes via activation of leptin receptor in endothelial cells (Bouloumié et al. 1998). Leptin ELISA revealed no significant changes in leptin concentrations upon treatment with HMO or positive control. Lactose or 3'SL treatment slightly increased leptin concentrations without statistical significance (Figure 17 C).

IGF-1 has also been shown to increase angiogenesis in numerous different models of tissue injury, potentially through an VEGF dependent mechanisms (Yadranji Aghdam et al. 2012). ELISA for IGF-1 of supernatants of HMO treated cells showed no significant changes compared to untreated cells. Pooled HMO, and the positive control increased IGF-1 concentration, compared to the untreated control, without reaching significance (by 19,4% and 14,6%, respectively).

4.6. HMO did not alter gene expression of KDR, INS-R, IGF1-R

Angiogenesis is influenced by pro- and anti-angiogenic factors. These factors bind to their specific receptors which is the starting point for signaling cascades. Subsequently, we asked whether HMO treatment affects angiogenesis by increasing cell responsiveness to pro-angiogenic factors, such as VEGF-2, insulin and insulin like growth factor (IGF). Thus, we assessed whether HMO alter gene expression of these pro-angiogenic receptors, using real-time qPCR.

3'SL induced VEGF-2 receptor (KDR) gene expression, but this finding was not significant. Pooled HMO and 2'FL treatment did not lead to changes in KDR gene expression (Figure 18).

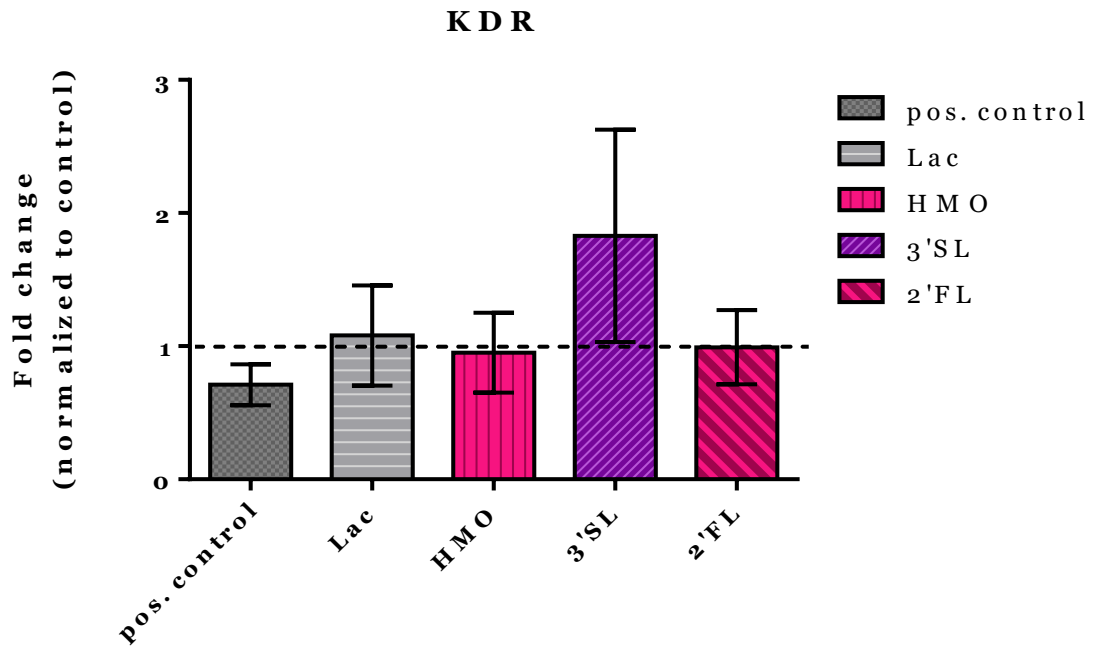

Figure 18: Graph showing the mean values of fold change for KDR relative to untreated control cells. Both control and stimulant values were relative to those of the internal control gene HPRT-1 with mutant values representing the fold change relative to that of controls, which was converted to 1. For each treatment, 6 different fpEC were used; data are shown as mean values, and the error bars show the SEM.

Treatment of fpEC did not lead to significant changes in insulin receptor expression compared to untreated cells, serving as a negative control (Figure 19).

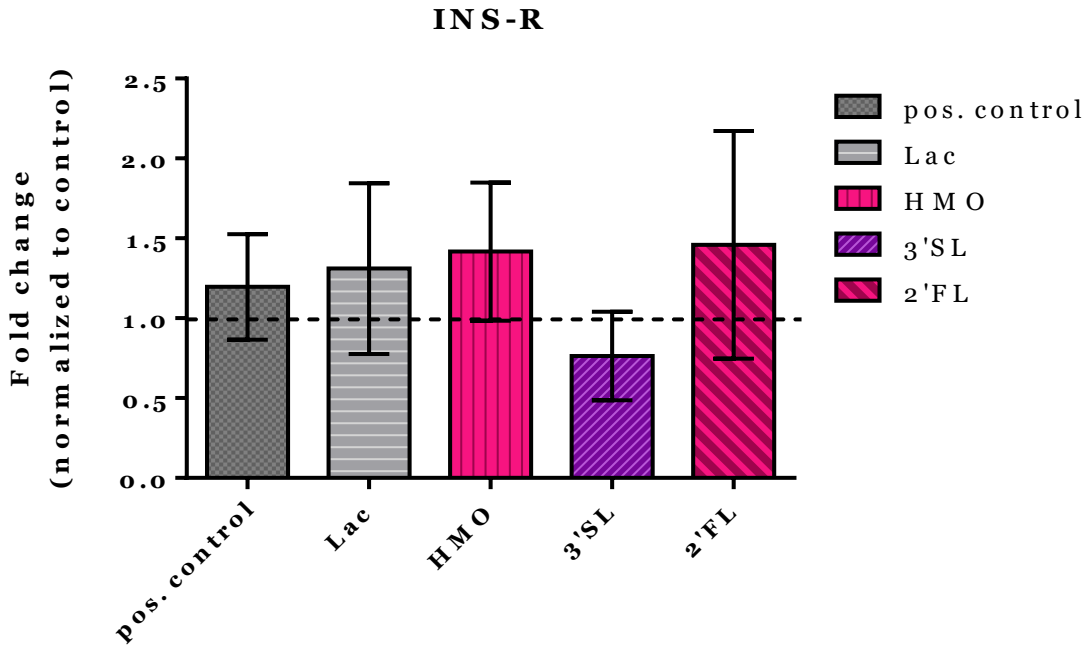

Figure 19: Graph showing the mean values of fold change for insulin receptor relative to untreated control cells. Both, control and stimulant values were relative to those of the internal control gene HPRT-1 with mutant values representing the fold change relative to that of controls, which was converted to 1. For each treatment 6 different fpEC were used; data are shown as mean values, and the error bars show the SEM.

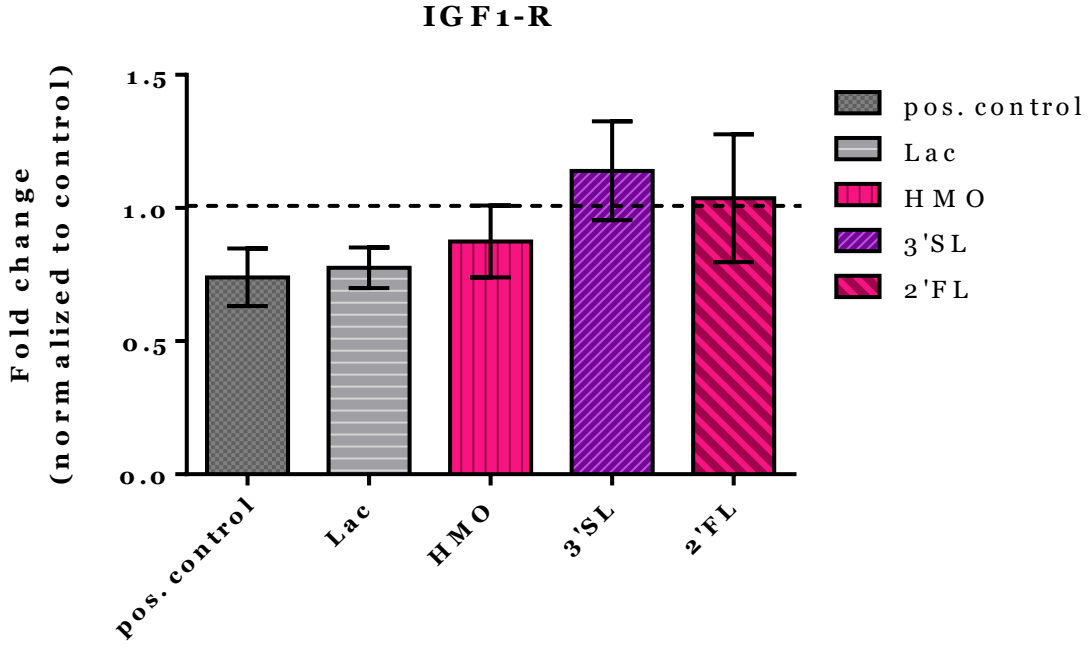

Figure 20: Graph showing the mean values of fold change for IGF1-R relative to untreated control cells. Both, control and stimulant values were relative to those of the internal control gene HPRT-1 with mutant values representing the fold change relative to that of controls, which was converted to 1. For each treatment 6 different FPEC were used, data are shown as mean values and the error bars show the SEM.

qPCR revealed no significant changes in gene expression of IGF-1 in cells treated as a positive control or with lactose, pooled HMO, 3'SL and 2'FL (Figure 20).

5. Discussion

As HMO are reported in cord blood (Jantscher-Krenn *et al.*, unpublished), they are in direct contact with the vascular endothelium. Hence, feto-placental endothelial cells were chosen as a cell model to study effects of HMO. Next to barrier function (Blundell et al. 2016), fpEC play a major role in placental vasculogenesis and angiogenesis. In this study, we assessed the novel effect of HMO on *in vitro* angiogenesis fpEC. We showed that potential HMO receptors, TLR-4, L-SIGN and E-Selectin are expressed on fpEC. Literature reported that HMO affect these receptors in other cell types, such as intestinal epithelial cell lines. In our study, pooled HMO and 3'SL induced downregulation of L-SIGN and E-Selectin by trend. The most important processes of feto-placental angiogenesis are proliferation, migration and tube formation. We here showed that HMO exposure increased tube length in fpEC. Both, fibrin angiogenesis and spheroid sprouting assay, showed that pooled HMO induced a significant increase in tube length (by 24.3% and 32% respectively). 3'SL independently induced an increase in tube length by 25.3% in the fibrin angiogenesis assay. Additionally, pooled HMO and 3'SL significantly increased proliferation of fpEC after 48 and 72 hours. For proliferation and migration, the cell undergoes drastic changes in cytoskeleton, that can be visualized by Phalloidin staining. We found that HMO and 3'SL induced stress fiber formation. As feto-placental angiogenesis is regulated by various pro- and antiangiogenic factors, we were interested in the influence of HMO in secretion of selected factors, such as IGF-1, leptin, IL-10 and IL-6. ELISA showed that pooled HMO significantly increased IL-6 concentration in cell supernatants. Using real-time qPCR, HMO did not alter gene expression of any pro-angiogenic receptors tested.

5.1. HMO

As HMO are found in cord blood, the question arises where these oligosaccharides are produced. The human mammary gland is thought to be the only organ where lactose and lactose-derived oligosaccharides are synthesized (Brew & Hill 1975). It would be interesting to see if HMO are present in cord blood of mothers who underwent full mastectomy. If the mammary gland is the only organ synthesizing HMO, they would not be found in cord blood in those women. Furthermore, changes in placental structure and vasculature due to absence of HMO could be investigated.

In pooled HMO, many different individual HMO structures are present, which potentially can affect fpEC in different ways. More of those individual structures have to be tested concerning an effect on angiogenesis in fpEC.

HMO used in this study, were purified from pooled human milk (Bode 2012). The composition of HMO in blood is similar, but not identical to the composition in human milk (Kunz et al. 2014). Most of the major HMO present in human milk, such as 2'FL, 3FL, lactodifucotetraose, 3'SL and 6'SL, are also present in cord blood. However, 3'Sialyllactosamine (3'SLN) and 6'Sialyllactosamine (6'SLN) were found in cord blood, but not in human milk. There is a higher percentage (63%) of negatively charged (sialylated) HMO in cord blood, compared to human milk, where fucosylated HMO are the predominant fraction (Jantscher-Krenn, unpublished). In this study, as a first step, pooled HMO from human milk was used as it was available and the composition is similar. In further studies, HMO purified from cord blood should be used to mimic the physiological concentrations.

5.2. Receptors for HMO

TLR-4, L-SIGN and E-Selectin expression in fpEC could be confirmed. A significant up- or downregulation induced by HMO treatment was not detectable. Pooled HMO and 3'SL induced downregulation of L-SIGN and E-Selectin, seen as a trend. Using primary cells, the results show a high biological variance. By increasing sample size, these trends might reach significance.

HMO might bind to TLR-4, expressed on fpEC, and thus activate downstream signaling. Interactions between TLRs and their ligands result into either the MyD88-dependent or MyD88-independent pathways (Lu et al. 2008). These pathways result in the activation of lymphocytes, expression of co-stimulatory signals, and release of cytokines. The activation of M1 macrophages contributes significantly in anti-microbial immune responses via the production of pro-inflammatory cytokines (e.g. IL-6, IL-8, IL-12), inducible nitric oxide synthase, and interferons (Pinhal-Enfield et al. 2003). The produced cytokines are not only pro-inflammatory, but also show pro-angiogenic effects. Thus, TLR4-mediated macrophages have been shown to be an important source of those pro-angiogenic factors (Murad 2014). Alternate activation of macrophages may lead to the M2 phenotype. This phenotype is reported to be involved in fibrosis and the wound repair by contributing to angiogenesis through VEGF production (Wu et al. 2010). TLR-4 signaling together with the growth factors

and cytokines such as IFN-γ, TGF-β, IL-1, and IL-6 have been implicated in a significant increase in VEGF levels. As VEGF is the most potent stimulator of angiogenesis, TLR-4 signaling might influence feto-placental angiogenesis. HMO binding to TLR-4 might induce pro-angiogenic cytokine release and lead to increased VEGF levels, thereby increasing feto-placental angiogenesis. If the observed HMO effect is indeed mediated by TLR4 remains to be determined in future studies, investigating downstream signaling.

5.3. Angiogenesis assays

Both, fibrin angiogenesis and spheroid sprouting assay could confirm the effect of HMO on angiogenesis, seen as an increase in tube length in fpEC. The effect of 3'-sialyllactose was similar to pooled HMO. Notably, the specific and complex structures of HMO seem to be essential for the effect. The disaccharide lactose, which is the minimal common part of all HMO, but not considered a HMO itself, was not able to influence angiogenesis.

Both *in vitro* assays in feto-placental endothelial cells cultured on three-dimensional gel recapitulated several aspects of vascular angiogenesis, each assay offering different advantages. The Fibrin Angiogenesis Assay allows for defined conditions without the addition of exogenous growth factors in the fibrin matrix. Cells grow into fibrin, leading to a three-dimensional construct. The stable fibrin network allowed longer incubation times, enabling observation over longer time periods. Due to this extended incubation time, this angiogenesis assay might show aspects of proliferation. It should also be mentioned that the Fibrin Angiogenesis Assay is based on Fibrin, which is a compound in wound healing. Moreover, the Spheroid Sprouting Assay provides a more representative model of *in vivo* angiogenesis than it can be achieved with monolayers. The aggregation of endothelial cells embedded into a three-dimensional matrix leads to sprouting and invasion into the surrounding matrix (Blacher et al. 2014b). Collagen used in the Spheroid Sprouting Assay, is not a compound of tissue repair, making it the more physiological model for feto-placental angiogenesis, not being based on pathogenic angiogenesis. However, due to the shorter incubation time compared to the fibrin angiogenesis assay, we expect the Spheroid Sprouting Assay to show migration rather than proliferation processes. Finding similar results in both assays, is in good accordance with our findings that both, proliferation as well as cytoskeleton rearrangement, were influenced by HMO treatment. The image

analysis is more time-consuming because of the objective change but the final processing and tube finding seemed more accurate.

5.4. Proliferation

We found pooled HMO and 3'SL induced increase in proliferation of fpEC after 48- and 72-hour incubation, which could indicate direct effects of HMO on fpEC. Proliferation was analyzed by metabolic MTT assay, which is based on the cleavage of 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide molecule into strongly colored formazan crystals by mitochondrial dehydrogenases in viable cells. As this assay represents mitochondrial activity, additional experiments, such as BrdU assay or CASY counting, remain to be conducted in future. These new results show that HMO may contribute to feto-placental angiogenesis via increased fpEC proliferation. These findings are in accordance to recent literature, where Kuntz et al. (2008) found that the HMO effect on proliferation is cell line dependent (Kuntz et al. 2008).

5.5. Cytoskeleton rearrangement

This study showed HMO induced actin cytoskeleton rearrangement in fpEC and enhanced formation of stress fibers. These stress fibers ended in pointed edges, indicating typical morphological features in migrating cells (Lamalice et al. 2007). These results indicated that pooled HMO, 3'SL and 2'FL stimulate fpEC migration by inducing stress fiber formation. The phalloidin staining was bright, suggesting that the actin cytoskeleton is a robust construct. As migration plays essential role in angiogenesis these results confirmed the pro-angiogenic effect of HMO on angiogenesis of fpEC, and suggests that is in part due to migration. Control cells showed less intense phalloidin staining, suggesting that the cytoskeleton is fragile and weak. Remodeling of cortical actin filaments, so called membrane ruffling, occurred and may result from adhesion to the ECM.

Endothelial cell proliferation and migration are essential processes in angiogenesis, which requires movement of cells in precise directions to specific locations. Cell migration is tightly associated with regulation, modification and organization of the actin cytoskeleton. These cytoskeletal changes are linked to the formation of focal adhesions and involve the development of stress fibers, lamellipodia and filopodia (Munoz-Chápuli et al. 2004).

5.6. Secretion of paracrine factors

Angiogenesis is a highly regulated and complex process where many different factors, secreted by the cells, regulate different phases. IL-6 concentration in supernatants of fpEC was highly increased due to treatment with pooled HMO. IL-6 is known as a pro-inflammatory cytokine, but it also significantly enhances endothelial cell migration, a key step in the process of angiogenesis, and further induces angiogenic responses. IL- 6 is also positively correlated with VEGF expression and promotes HUVEC proliferation and tube formation *in vitro* (Huang et al. 2004). Furthermore, IL-6 can directly induce vessel sprouting in the *ex vivo* aortic ring model (Gopinathan et al. 2015). In this study, we showed that HMO increased IL-6 concentrations in cell supernatants, suggesting that the observed pro-angiogenic effect might be partly mediated by a paracrine mechanism. IL-6 might contribute to increased cell migration and proliferation, as seen in cytoskeleton staining and MTT proliferation assay. It should be noted, that IL-6 gene expression is also known to be induced by lipopolysaccharide. During the HMO isolation from human milk, LPS can accumulate (Gnoth et al. 2000b). By performing endotoxin removal twice, we took great care reducing LPS content to a very minimum. However, since it is known that even minimal amounts can affect specific cells, we cannot rule out, that residual LPS in HMO preparations could account in part for the IL-6 stimulation. Future experiments with specific LPS inhibitors will be needed to confirm that the effect is mediated by HMO and not LPS.

Whether other paracrine factors might also contribute to the HMO mediated effects remains to be investigated in future studies. In addition to the factors tested in this study, selected based on the expression in fpEC, several angiogenesis promoting factors (e.g. fibroblast growth factor 2 (FGF2), matrix metalloproteinases (MMPs), angiogenin, IL-8) can be investigated in follow up studies.

5.7. Gene expression of pro-angiogenic receptors

Proangiogenic factors, influencing processes like proliferation, migration and tube formation, engage with their respective receptors, resulting in signaling cascades promoting these processes. We found that gene expression of KDR, insulin receptor and IGF-1 receptor was not significantly altered by HMO treatment. However, 3'SL treatment showed a KDR upregulation by trend. As primary cell isolations show a high

biological variance, increasing the sample size might lead to significance. Through an upregulation of KDR, present VEGF could be more effectively incorporated.

5.8. Conclusion

This is the first study, showing that HMO, present in cord blood, affect angiogenesis in fpEC in physiological concentrations. HMO might interact with receptors, expressed on fpEC, leading to altered cell proliferation, cytoskeleton rearrangement, tube formation and secretion of pro-angiogenic factors, all major aspects of placental angiogenesis. An efficient maternal-fetal exchange system is crucial for the growth and development of the placenta and the fetus (Boyd, J. D. Hamilton 1970). To perform the required exchange functions, the highly immature placental vasculature undergoes branching angiogenesis, which dramatically increases the number of villous blood vessels (Mayhew et al. 2004). The observed effect of HMO on angiogenesis might therefore be important for a healthy placental development.

6.References

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