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To my family

ABSTRACT

KATI MOKKALA

Intestinal permeability in pregnancy: dietary and microbial determinants and metabolic consequences

University of Turku, Faculty of Medicine, Institute of Biomedicine, Medical Microbiology and Immunology, Turku Doctoral Programme of Molecular Medicine (TuDMM)

Annales Universitatis Turkuensis, Medica- Odontologia, Painosalama, Turku, Finland, 2017

Metabolic disorders during pregnancy may challenge the mother and child to long-term health complications. Emerging data indicates a link between increased intestinal permeability and metabolic risk markers in a non-pregnant population. Whether intestinal permeability changes in pregnancy and is related to metabolic risk markers is poorly known.

The aim of the present study was to investigate the impact of gut microbiota and diet on serum zonulin concentration, a marker of intestinal permeability, and the relationship between serum zonulin concentration and metabolic risk markers during early pregnancy (n=100). The study also investigated the impact of supplemental probiotics and n-3 long chain polyunsaturated fatty acids (LC-PUFA) on intestinal epithelial integrity *in vitro* as well as changes in serum zonulin concentration from early to late pregnancy (n=200). The study population consisted of overweight and obese pregnant women (BMI>25), who were participating in a larger randomized double-blind placebo-controlled clinical trial.

The results show that a richer gut microbiota composition, a higher abundance of *Faecalibacterium prausnitzii*, a higher dietary intake of fibre and n-3 LC-PUFA as well as multiple vitamins and minerals were associated with lower serum zonulin concentration, ie. lower intestinal permeability. Serum zonulin concentration correlated with serum endotoxin activity, markers of low grade inflammation and glucose metabolism in early pregnancy, suggesting that intestinal permeability may be of importance in regulating maternal metabolic health. Serum zonulin concentration increased with pregnancy progress. While *Bifidobacterium animalis* ssp. *lactis* 420 and n-3 LC-PUFA enhanced intestinal epithelium *in vitro*, they had no effect on serum zonulin levels during pregnancy.

Keywords: intestinal permeability, serum zonulin, overweight and obese pregnant women, LPS, probiotics, LC-PUFA, metabolic risk factors

TIIVISTELMÄ

KATI MOKKALA

Suoliston läpäisevyys raskauden aikana: ruokavalion ja mikrobiston vaikutukset sekä aineenvaihdunnalliset seuraukset

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen mikrobiologia ja immunologia, Turun yliopiston molekyylilääketieteen tohtoriohjelma (TuDMM)

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Raskausaika saattaa vaikuttaa pitkälle sekä äidin että lapsen terveyteen. Viimeaikaiset tutkimukset osoittavat, että suoliston lisääntynyt läpäisevyys voi altistaa aineenvaihdunnan häiriöille. Toistaiseksi ei tiedetä, muuttuuko suoliston läpäisevyys raskauden aikana ja missä määrin muutokset vaikuttavat äidin aineenvaihdunnan häiriöihin liittyviin riskitekijöihin.

Väitöskirjan tavoite oli tutkia miten äidin suolistomikrobisto ja ravinnonsaanti vaikuttavat suoliston läpäisevyyttä kuvastavaan tekijään, seerumin zonuliiniin. Tavoitteena oli myös selvittää seerumin zonuliinipitoisuuden yhteyttä aineenvaihdunnan riskitekijöihin raskauden alkuvaiheessa (n=100). Lisäksi tutkittiin, miten ravintolisien, n-3 pitkäketjuisten monityydyttymättömien rasvahappojen ja probioottien, saanti vaikuttaa suoliston seinämän kuntoon solumallissa ja seerumin zonuliinipitoisuuden muutoksiin alku- ja loppuraskauden välillä (n=200). Aineisto koostui ylipainoisista ja lihavista (BMI>25) äideistä, jotka osallistuivat laajempaan satunnaistettuun kaksoissokko lumekontrolloituun kliiniseen tutkimukseen.

Tulokset osoittivat, että rikkaampi suolistomikrobisto ja suurempi *Faecalibacterium prausnitzii*-bakteerin osuus, suurempi kuidun, monityydyttymättömien rasvahappojen ja useiden vitamiinien ja kivennäisaineiden päivittäinen saanti liittyi pienempään seerumin zonuliinipitoisuuteen eli matalampaan suoliston läpäisevyyteen. Seerumin zonuliinipitoisuus korreloi seerumin LPS aktiivisuuden, matala-asteisen tulehduksen ja sokeriaineenvaihduntaa kuvaavien tekijöiden kanssa raskauden alkuvaiheessa, osoittaen, että suoliston läpäisevyydellä saattaa olla tärkeä rooli äidin aineenvaihdunnan säätelyssä. Zonuliinipitoisuus nousi raskauden myötä, probiootit ja n-3 pitkäketjuiset rasvahapot eivät vaikuttaneet seerumin pitoisuuteen, vaikka solumallissa *Bifidobacterium animalis* ssp. *lactis* 420 ja n-3 pitkäketjuiset monityydyttymättömät rasvahapot lisäsivät suoliston seinämän vahvuutta.

Avainsanat: suoliston läpäisevyys, seerumin zonuliini, raskaudenaikainen ylipaino, LPS, probiootti, monityydyttymättömät rasvahapot, aineenvaihdunnan riskitekijät

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ABBREVIATIONS

AA	Arachidonic acid
BMI	Body mass index
CaCo-2	Colon carcinoma cell line
CI	Confidence interval
CFS	Cell-free supernatant
CGM	Cell growth medium
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle Medium
DOHaD	Developmental origins of health and disease
E%	Proportion of energy intake
EGFR	Epidermal growth factors receptor
EPA	Eicosapentaenoic acid
F-actin	Filamentous actin
FITC	Fluorescein isothiocyanate
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GDM	Gestational diabetes
GlycA	Glycoprotein acetylation
HDL	High-density lipoprotein
HOMA2-IR	Homeostatic model assessment-2 insulin resistance
HP	Haptoglobin
hs-CRP	High sensitive C-reactive protein
IFN	Interferon
IL	Interleukin
IQR	Interquartile range
IRF	interferon regulatory factor
JAM	Junctional adhesion molecule
LBP	LPS binding protein
LC-PUFA	Long chain polyunsaturated fatty acid
LDL	Low density lipoprotein
L/M	Lactulose/mannitol
LPS	Lipopolysaccharide
LT	Leucotriene
MAPK	Mitogen-activated protein kinase

M cell	Microfold cell
MLC	Myosin II regulatory light chain
MLCK	Myosin light chain kinase
MUFA	Monounsaturated fatty acid
NAFLD	Non-alcoholic fatty liver disease
NF-Kb	Nuclear factor kappa-light –chain –enhancer of activated B-cells
NMR	Nuclear magnetic resonance
PAR	Proteinase-activating reaction
PCR	Polymerase chain reaction
PD	Phylogenetic diversity
PG	Prostaglandin
PKC	Protein kinase C
PRR	Pattern recognition receptor
PCOS	Polycystic Ovary Syndrome
QUICKI	Qualitative Insulin Sensitivity Check Index
RT qPCR	Real time quantitative PCR
SD	Standard deviation
SCFA	Short chain fatty acids
SFA	Saturated fatty acid
TEER	Transepithelial electrical resistance
Th	T helper
TJ	Tight junction
TLR	Toll like receptor
TNF	Tumor necrosis factors
ZO	Zonula occludens

LIST OF ORIGINAL PUBLICATIONS

I Mokkala K, Röytiö H, Munukka E, Pietilä S, Ekblad U, Rönnemaa T, Eerola E, Laiho A, Laitinen K. Gut Microbiota Richness and Composition and Dietary Intake of Overweight Pregnant Women Are Related to Serum Zonulin Concentration, a Marker for Intestinal Permeability. J Nutr. 2016; 146(9): 1694–700.

II Mokkala K, Pellonperä O, Röytiö H, Pussinen P, Rönnemaa T, Laitinen K. Increased intestinal permeability, measured by serum zonulin, is associated with metabolic risk markers in overweight pregnant women. Metabolism. 2017; 69: 43–50.

III Mokkala K, Pussinen P, Houttu N, Koivuniemi E, Vahlberg T, Laitinen K. Changes in intestinal permeability during pregnancy: the potential impact of probiotics and n-3 long-chain polyunsaturated fatty acids in a randomized clinical trial in overweight and obese women. Beneficial microbes. In press.

IV Mokkala K, Laitinen K, Röytiö H. Bifidobacterium lactis 420 and fish oil enhance intestinal epithelial integrity in Caco-2 cells. Nutr Res. 2016; 36(3): 246–52.

1 INTRODUCTION

During pregnancy, multiple changes occur in maternal glucose and lipid metabolism as well as in the inflammatory status in order to support the development of the child. These physiological changes are tightly regulated, but aberrancies, due to e.g. obesity may predispose the mother and child to health complications. These complications, such as gestational diabetes, with a current prevalence of 16% in Finland (THL 2015), may predispose the mother to metabolic disorders including type 2 diabetes in later life (Chen et al. 2015, Damm 2009, Lain and Catalano 2009). One of the prevalent modern day risk factors is maternal overweight and obesity. Recent estimates for the incidence of overweight show that it affects about half of the women of reproductive age in Finland (Männistö et al. 2012), suggesting that more women are entering pregnancy when they are overweight or obese.

Maternal overweight and obesity not only complicates maternal health during pregnancy, but also the health of the child. The most common manifestation includes an increased risk for macrosomia (Bautista-Castano et al. 2013). In addition, the long-term health of the child may be affected, as proposed by the developmental origins of health and disease (DOHaD) hypothesis (Baird et al. 2017). As a fetus adapts to the environment by altering its physiology and metabolism (Barker 2001, Hales and Barker 2001), the similarity between the environment in utero and after birth is critical; for example, in poor nutritional conditions, the fetus prepares for an optimal energy harvest which may induce metabolic challenge in well-nourished conditions, such as increased risk for type 2 diabetes and cardiovascular disease in later life (Portha et al. 2014). This theory has expanded to include perinatal exposure to other than compromised nutritional environments, such as increased maternal intake of fat and phenotypic alterations in the offspring (Zhou and Pan 2015).

In addition to overweight and obesity, identifying other potentially modifiable risk factors and mechanisms influencing maternal health is of importance. This thesis investigated the contribution of intestinal permeability, serum zonulin as a marker, to maternal metabolic risk factors including low grade inflammation. The study also evaluated whether pregnancy is accompanied by alterations in intestinal permeability as well as the relationship between maternal gut microbiota and diet with intestinal permeability.

2 REVIEW OF THE LITERATURE

2.1 The gut's role as a gatekeeper between environment and host

The intestinal epithelium forms an important interface between the environment and the host. The intestinal barrier is responsible for both absorption of nutrients as well as preventing the entry of potentially harmful gut components into the circulation. Disruption of intestinal epithelium with a subsequent increase in intestinal permeability has been related to multiple chronic inflammatory conditions, such as inflammatory bowel diseases, obesity, and metabolic diseases in a non-pregnant population (Bichoff et al. 2014, König et al. 2016, Wells et al. 2017). This highlights the importance of tight regulation of intestinal epithelial integrity.

2.1.1 Structure of intestinal epithelium

Intestinal epithelium consists of a single layer of rapidly renewing (every 3-5 days) epithelial cells, which are covered by a mucus layer. Most (80%) of the epithelial cells are absorptive enterocytes. Goblet cells produce mucus, a physical barrier for gut microbiota, and antimicrobial peptides together with Paneth cells (Turner 2009, Suzuki 2013, Wells et al. 2017). Bioactive molecules, such as hormones, are produced by various enteroendocrine and microfold cells (M cells). Dendritic cells, the immune cells central to immune regulation, deliver antigens to other immune cells. Below the epithelial cells, is the lamina propria, which contains immune cells, such as macrophages, dendritic cells, plasma cells and lymphocytes (Turner 2009, Suzuki 2013, Ulluwischewa et al. 2011).

The adjacent epithelial cells are joined together by an intercellular junction (Turner 2009, Suzuki 2013, Wells et al. 2017). The most apical junction is a protein complex, a tight junction (TJ), which is responsible for the regulation of paracellular transport. A TJ consists of multiple proteins including claudins, zonula occludens (ZO), occludin, junctional adhesion molecules (JAMs) and filamentous-actin (F-actin) (Turner 2009, Suzuki 2013). Other important components participating in the sealing of intercellular junctions are adherent junctions, desmosomes and gap junctions, and myosin light chain kinase (MLCK) (Turner 2009, Suzuki 2013, Wells et al. 2017).

Intestinal epithelium is constantly exposed to various dietary and bacterial components. These act as stimuli for modification of the epithelial barrier, either with enhancing or adverse effects on the epithelial integrity and intestinal permeability, e.g. acting through TJs.

2.1.2 Means of measurement of intestinal permeability

Methods used in the measurements of intestinal permeability include *in vitro* assays, mostly utilizing colon carcinoma cell lines (Caco-2), an *ex vivo* Ussing chamber with tissue specimens and *in vivo* assays measuring permeability of different size probes (De Santis et al. 2015, Bischoff et al. 2014, König et al 2016).

Permeability in *in vitro* models may be measured by transepithelial electrical resistance, TEER, between the two sites of the intestinal cell layer. In this method, cells, mostly Caco-2 cells, are placed on filters with different sized pores. When cultured, Caco-2 forms a monolayer and TJ complexes characteristic of enterocyte (Sambuy et al. 2005). TEER is thus a measure of the integrity of the intestinal barrier formed and hence used to follow the paracellular permeability of the barrier. In addition to the TEER measurement, the flux of labelled molecules, such as different size fluorescein isothiocyanate (FITC) -conjugated dextran can be measured (De Santis et al 2015).

The commonly used *in vivo* method is a non-invasive approach measuring the ratio of urinary excreted sugars with different sizes in the lactulose/mannitol (L/M) – test. The larger size lactulose is a marker of paracellular permeability while the small size mannitol can freely pass through the epithelium. If the intestinal epithelium is compromised, the lactulose can cross the epithelium, resulting in an increased L/M-ratio in the urine. Other methods include the use of various biomarkers reflecting the increased passage of the intestinal components, the release of TJ proteins and indigestible probes such as ^{51}Cr-EDTA (Bischoff et al. 2014, König et al 2015).

2.1.2.1 Serum zonulin, as a marker for intestinal permeability

Recently, serum zonulin has been used as a marker for intestinal permeability in human trials (Zak-Golab et al. 2013, Jayashree et al. 2014, Moreno-Navarrete et al. 2012, Moreira et al. 2012, Stenman et al. 2016, Zhang et al. 2014, 2015). Decreased concentration of serum zonulin has been shown to associate with decreased L/M-ratio in healthy individuals (Russo et al. 2012) and in colorectal cancer patients (Liu et al. 2013), and to correlate with L/M-test in type 1 diabetic patients (Sapone et al. 2006).

Zonulin, a ~47-kDa protein, is synthetized in the intestine and also in other tissues, such as the liver, heart, lungs and adipose tissue (Wang et al. 2000, Vanuytsel et al. 2013). It is eukaryotic counterpart of *Vibrio cholera* produced toxin called zonula occludens and regulates intestinal permeability by modulating TJs (Fasano et al. 2000, Wang et al. 2000). Zonulin is (Tripathi et al. 2009) precursor of haptoglobin-2 (HP2) (Figure 1). Haptoglobin, Hp, a cleaved pre-haptoglobin, is an

acute phase protein, the primary function being prevention of oxidative stress by binding to free hemoglobin (Tripathi et al. 2009, Sturgeon and Fasano 2016, Carter and Worwood 2007). Hp exists as two genetic variants, HP1, consisting of 5 exons and 4 introns and HP2, which contains additional 2 exons and intron as a result of duplication of exons 3 and 4 of HP1. HP1 encodes α1- protein (9 kDa) and HP2 encodes α2- protein (18 kDa) and when combined with constant β-chain (36 kDa), they form HP1-1, HP1-2, or HP2-2 (Tripathi et al. 2009, Sturgeon and Fasano 2016). After cleaved in endoplastic reticulum into a 2-chain form, it acts as an active, hemoglobin binding haptoglobin (Tripathi et al. 2009, Sturgeon and Fasano 2016) (Figure 1).

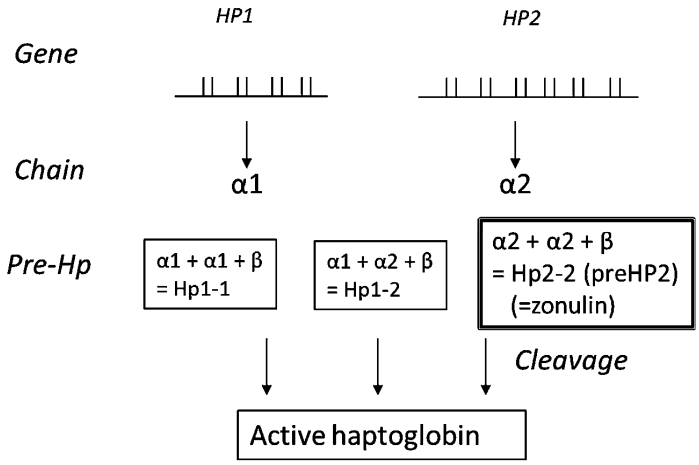

Figure 1. Serum zonulin and different haplotypes of haptoglobin. (Fasano et al. 2012, Sturgeon and Fasano 2016).

Zonulin (preHP2) increases intestinal permeability by transactivating the epidermal growth factors receptor (EGFR) via activation of the proteinase-activating receptor 2 (PAR2) (Figure 2) leading to a protein kinase C (PKC)-dependent TJ disassembly (Strugeon and Fasano 2016).

Bacteria and gliadin have been shown to trigger the release of zonulin from enterocytes (Fasano 2012). The suggested rationale for the pathogen driven zonulin release in the small intestine may be related to an innate immune response: elevated zonulin levels increase the paracellular efflux which flushes the pathogens and hence protects the host from pathogen colonization in the small intestine (Fasano 2011). Gliadin has been shown to increase the release of zonulin through the CXCR3 receptor mediated, MyD88-dependent mechanism (Lammers et al. 2008) (Figure 2). Gliadin is a wheat storage protein triggering intestinal inflammation (Ciccocioppo et al. 2005) and in genetically predisposed individuals,

this may trigger the autoimmune response resulting in celiac disease (Fasano et al. 2011).

The expression of the pre-haptoglobin gene is under IL-6 control (Oliviero and Cortese 1989), and thus zonulin may also be synthesized as a response to inflammatory stimuli and used as a marker for inflammation (Ohlsson et al. 2017a).

2.1.3 Regulation of intestinal permeability

Transport from gut lumen occurs through two different pathways that are regulated by different mechanism: paracellular diffusion of small molecules or transcellular transport of larger molecules (De Santis et al. 2015). Paracellular transport is regulated by altering the expression and localization of TJs. In inflammatory gastrointestinal diseases, inflammatory factors, such as interferon (IFN)γ, tumor necrosis factor (TNF)α, interleukin (IL)-1β and IL-17 have been shown to induce alterations in TJ and MLCK expression (Wells et al. 2017). MLCK, by phosphorylation of the myosin II regulatory light chain (MLC) increases intestinal permeability by altering the TJ structure (Shen at al 2006). Stimulation of the toll like receptor 2 (TLR2) and subsequent activation of protein kinase C (PKC), phosphorylation of TJ proteins, activation of mitogen-activated protein kinase (MAPK) pathways and activation of small GTPase RhoA are further mechanisms participating in the regulation of TJ complexes (Sturgeon and Fasano 2017, Bron et al 2017, DeSantis 2015, Ulluwishewa et al. 2011, Suzuki) (Figure 2). Induction of TLR2 by agonists, such as gram-positive bacteria peptidoglycan and lipoteichoid acid may be one mechanism how commensal bacteria enhances intestinal barrier function.

2.1.4 Dietary factors regulating intestinal permeability

Dietary nutrients may influence the intestinal epithelium directly or indirectly. Nutrients provide energy for epithelial cells and regulate intestinal epithelial integrity. The indirect effect is mediated through gut microbiota: the nutrients provide energy for specific bacteria thus modulating the microbiota composition and the metabolite production. The metabolites produced by gut microbiota may in turn promote the intestinal barrier integrity by acting as an energy source or as regulators (De Santis et al. 2015).

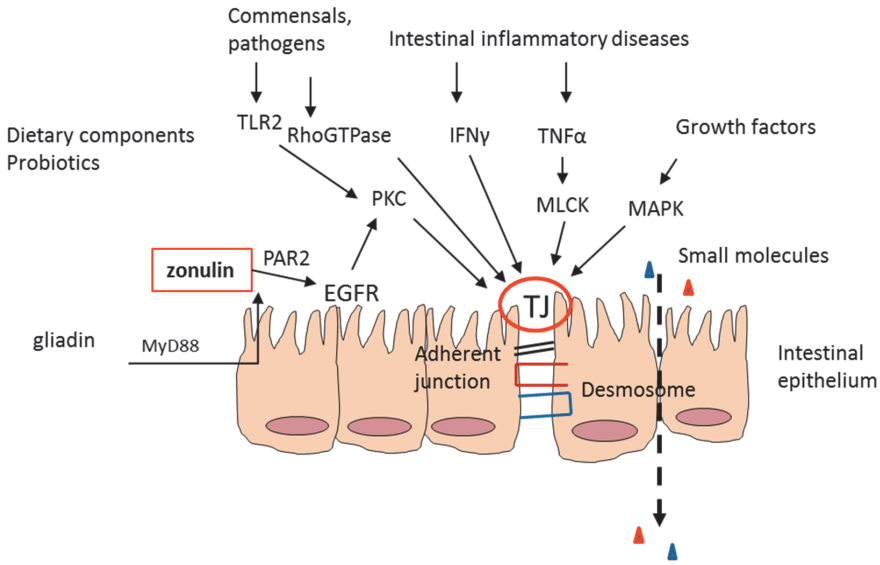

Figure 2. Mechanism by which zonulin impacts intestinal epithelium**.** Other mechanisms related to alterations in the intestinal permeability shown are protein kinase C (PKC) activation through toll like receptor 2 (TLR2) stimulation, myosin light chain kinase (MLCK) activation mediated by inflammatory factors and proteinase-activating receptor 2 (PAR2) mediated redistribution of tight junction (TJ), activation of mitogen-activated proteinase (MAPK) pathways and small GTPase RhoA (Bischoff et al. 2014, DeSantis et al. 2015, Sturgeon and Fasano 2016); all of which are also mechanism for the impacts of dietary components and probiotics.

Dietary glutamine is an important energy source for intestinal epithelial cells (Suzuki 2013). Dietary enteral formulas with glutamine supplementation have been shown to decrease intestinal permeability, indicated by decreased L/M-ratio in healthy and in malnourished children (Lima et al. 2005). Another study with premature neonates, showed decrease in L/M-ratio after oral supplementation with glutamine (Sevatsidou et al. 2011), while in other study glutamine enriched enteral nutrition had no impact on L/M-ratio in very-low-birth-weight infants (van den Berg et al. 2006). Beneficial effects with glutamine on intestinal permeability (L/M-ratio) has been detected in severely burned patients (Peng et al. 2004) and in patients with Crohn's disease (Benjamin et al. 2011). However, no effect on intestinal permeability (L/M-test) was found with glutamine enriched total parenteral nutrition in nutrition depleted patients (Hulsewe et al. 2004) or critically ill patients (Velasco et al. 2001).

Vitamins and minerals, either deficiency or supplementation have been reported to impact intestinal epithelium (De Santis et al. 2015, Bischoff et al. 2014). Vitamin D has been shown to protect intestinal epithelium by multiple TJ regulating pathways *in vitro* (Du et al. 2015, Chen et al. 2015a, Chen at al. 2015b) and in a mouse model of acute colitis (Zhao et al. 2012, Kong et al. 2007). Zinc has been

demonstrated to reduce lactulose permeability in children with diarrhea (Roy et al. 1992), and a zinc-fortified oral rehydration solution to improve intestinal permeability, measured by L/M-test, in children with gastroenteritis (Tran et al. 2015). In contrast, an increase in the L/M-ratio in children has been observed after supplementation with iron (Nchito et al. 2006).

Dietary fibre is fermented by gut microbiota, short chain fatty acids, SCFAs, such as acetate, propionate and butyrate as the end products. Butyrate is among the primary energy sources for epithelial cells (Carvalho and Saad 2013) and shown to enhance intestinal barrier integrity by regulating TJs in Caco-2 cells (Valenzano et al. 2015). In type 2 diabetic patients, galacto-oligosaccharide had no significant effect on intestinal permeability, measured by urinary recovery of ^{51}Cr-EDTA (Pedersen et al. 2016). In burn patients, oligofructose ingestion did not improve gastrointestinal barrier function (Olguin et al. 2005). As gut microbiota is involved in the indirect effects of fibre, the composition of gut microbiota may have an important role in determining the effect of fibre.

The impact of diet on serum zonulin has been investigated in only a few studies. Positive correlation has been detected between serum zonulin and energy intake, which was associated with higher fat intake in overweight and normal-weight study subjects (Zak-Golab et al. 2013). In the same study, an inverse correlation was detected between serum zonulin and protein intake as a proportion of energy intake. Dietary fiber in the form of inulin enriched pasta has been shown to decrease both serum zonulin and L/M-ratio in healthy young participants (Russo et al. 2012).

2.1.4.1 LC-PUFA and intestinal permeability

Consumption of a high fat diet has been linked to increased intestinal permeability in animal studies (Moreira et al. 2012, Cani et al. 2008). The direct impact of dietary fatty acids, such as saturated and polyunsaturated fatty acids, on intestinal epithelium have mainly been studied *in vitro*. Of the LC-PUFAs, eicosapentaenoic acid (EPA) enhanced the heat stress-impaired intestinal epithelial barrier in Caco-2 cells by elevating the expression of occludin and ZO-1, while docosahexaenoic acid (DHA) was less effective and arachidonic acid (AA, a n-6 PUFA) had no effect (Xiao et al. 2013). In other studies, an opposite effects of n-3 LC-PUFA on permeability have been detected (Aspenström-Fagerlund et al. 2007, Roig-Pérez et al. 2004, and 2010 and Usami et al. 2001).

LC-PUFA are substrates for eicosanoids, signaling lipids that regulate various inflammatory processes. Eicosanoids include prostaglandins (PGs), thromboxanes and leukotrienes (LTs). In immune cells, AA is usually the major component of the membrane phospholipids. The AA-derived eicosanoids, such as PGE_2,

thromboxane B_2, LTB_4, 5-hydroxyeicosatetraenoic acid and LTE_4 are strong promoters of inflammation. Replacement of n-6-LC-PUFA with n-3-LC-PUFA in membranes results in lower availability of AA for AA-derived eicosanoids and synthesis. Further, the n-3-LC-PUFA is substrate for alternative and less potent eicosanoids, such as LTB_5, LTE_5, and 5-hydroxyeicosapentaenoic acid (Calder 2006). PUFA has been shown to accumulate in phospholipid fraction of Caco-2 cells (Beguin et al. 2013) and into gut mucosal tissue of patients with inflammatory bowel disease (Hillier et al. 1991). The anti-inflammatory effect and enhancement of intestinal barriers integrity of n-3-LC-PUFA on intestinal epithelium may thus origin form the replacement of n-6-LC-PUFA in the cell membranes of mucosal tissues.

2.1.4.2 Probiotics and intestinal permeability

Gut microbiota and gut microbiota metabolites and components are important mediators in gut health. SCFAs, bile acids, various vitamins and bacterial LPS have been suggested as the factors impacting the intestinal epithelium (Nicholson et al. 2012). Further, gut pathogens directly or toxins produced by pathogens, may interact with epithelial cells disrupting the TJs (Suzuki 2013), one mechanism involving the release of zonulin (Fasano 2000). The effect of specific bacteria on intestinal epithelium have mainly been studied in animal models and *in vitro*, except the studies using probiotics. Probiotics are "live microorganisms that when administered in adequate amounts confer a health benefit to the host" (Hill et al. 2014).

In humans, studies investigating the impact of probiotics on intestinal permeability have shown variable outcomes. Both the L/M-test and serum zonulin have been used to measure intestinal permeability (Table 1). The beneficial impact of probiotics on intestinal permeability has been detected in one study in patients with inflammatory bowel diseases, IBS, in one study with patients with obstructive jaundice undergoing biliary drainage and in three studies with colorectal cancer patients undergoing colorectal surgery. The composition of probiotic supplements and the disease condition varied between the studies, complicating the comparison of the results. In addition, as most of the studies used combinations of bacteria, drawing conclusion from a single probiotic, e.g. the effect or the mechanism, is not possible.

Lactobacillus rhamnosus GG (LGG) in children with cryptosporidial diarrhea has been shown to improve intestinal permeability (Sindhu et al. 2014). In another study in children with moderate and severe atopic dermatitis, decrease in intestinal permeability was observed after administration of *L. rhamnosus* 19070-2 and *Lactobacillus reuteri* DSM 12246 (Rosenfeldt et al. 2004). Decreased intestinal

permeability has been detected in a study with preterm infants, supplemented with preterm formula containing *Bifidobacter lactis* (Stratiki et al. 2007). In children with short bowel syndrome, *L. rhamnosus* had no impact on intestinal permeability (Sentongo et al. 2008). The impact of probiotics on intestinal permeability are widely studied *in vitro*. The effect detected depends on whether living or dead probiotic cells or cell free medium, ie. metabolites produced by probiotics are used (Klingberg et al. 2005, Bischoff et al. 2014, Ulluwichewa et al. 2011).

The mechanisms by which probiotics enhance intestinal epithelium integrity may be related to antimicrobial metabolites produced by the probiotics, which inhibit the disruptive impact of pathogens on intestinal epithelium. Probiotics may also protect the epithelium from pathogens by colonizing the mucosa or by affecting the toxins produced by pathogens (Bron et al. 2017). In human studies, the TJ proteins, zonula occludens (ZO)-1 and occludin has been shown to increase in the vicinity of the tight-junction (TJ) structures after *Lactobacillus plantarum* administration in the duodenum for 6 h by means of a feeding catheter in healthy subjects (Karczewski et al 2010). This indicates that *L.plantarum* enhances intestinal epithelium by regulating TJs, possibly through activating TLR2 signaling (Karczewski et al 2010).

Both probiotics and fiber are suggested to induce beneficial effects on intestinal permeability, however only few studies have investigated the symbiotic effects on intestinal permeability. A preparation consisting of four probiotics and oligofructose had no impact on intestinal permeability (L/M-ratio) in critically ill patients (Jain et al. 2004). Administration of a symbiotic with 8 probiotic strains and fructo-oligosaccharides did not affect intestinal permeability in healthy humans, measured using the L/M-ratio and plasma zonulin concentration (Wilms et al. 2016).

Table 1. Outcomes from all found randomized clinical trials in adults investigating the effect of probiotics on L/M-ratio, serum zonulin concentration or recovery of enterally administered polyethylene glycols.

Intestinal permeability measurement method	Subjects/type of the study	Probiotics	Effect of probiotics compared to control	Reference
L/M-ratio, sucralose excretion	Diarrhea-predominant irritable bowel syndrome patients, n=30 (probiotics=14, placebo=14), RCT, single-blind	Probiotic fermented milk; *Streptococcus thermophilus, Lactobacillus bulgaricus, L. acidophilus* and *Bifidobacterium Longum* or milk without probiotics, 4 weeks	L/M-ratio ↓, no effect on sucralose excretion	Zeng et al. 2008
L/M-ratio	Patients with colorectal cancer, n=100 (probiotic=50, control=50), RCT, double-blind	*Lactobacillus plantarum (CGMCC No. 1258), Lactobacillus acidophilus (LA-11)* and *Bifido-bacterium longum,* 6 days preoperatively and 10 days post-operatively	L/M-ratio ↓	Liu et al. 2011
L/M-ratio	Patients with obstructive jaundice undergoing biliary drainage, n=17 (probiotic=5, inactivated probiotic=5, water=7), RCT, bouble-blind	*Lactobacillus plantarum 299v* or inactivated *LP299v* (placebo) and group with water, 7 days after biliary drainage.	L/M-ratio ↓	Jones et al. 2013
L/M-ratio	Critically ill patients, n=103 (probiotics=52, no probiotics=51), RCT	*Lactobacillus plantarum* v299 and conventional therapy or conventional therapy alone, 9 (IQR 3-17) days	no effect	McNaugth et al. 2005
L/M-ratio	Patients with multiple organ dysfunction syndrome, n=28 (probiotics=10, probiotic sonicates=9, placebo=9), RCT, double-blind	*Lactobacillus (L. casei, L. plantarum, L. acidophilus,* and *L. delbrueckii subsp. Bulgaricus),* 3 strains of *Bifidobacterium (B. longum, B. breve, and B. infantis)* and *Streptococcus salivarius subsp. Thermophilus,* 7 days	no effect	Albersa et al. 2007

Intestinal permeability measurement method	Subjects/type of the study	Probiotics	Effect of probiotics compared to control	Reference
L/M-ratio, saccharose excretion	Metabolic syndrome-patients, n= 28 patients (probiotics =13, standard therapy =15), 10 healthy, RCT, open label	*Lactobacillus casei Shirota*, 4 months	no effect	Leber et al. 2012
L/M-ratio	Patients with untreated celiac disease, n=22 (probiotics=12, placebo=10), RCT, double-blind	*Bifidobacterium infantis* natren life start strain super strain (Lifestart 2), 3 weeks	no effect	Smecuol et al. 2013
L/M-ratio	Chronic liver disease-patients, n=53 (probiotics=25, control=25), RCT, double-blind	*B. bifidum, B. lactis, B. longum, L. acidophilus, L. rhamnosus,* and *Streptococcus thermophiles*, 4 weeks	no effect	Kwak et al. 2014
L/M-ratio	Chirrosis-patients, n=80 (probiotics=44, control=36), RCT, double-blind	*Bifidobacterium bifidum W23, Bifidobacterium lactis W52, Lactobacillus acidophilus W37, Lactobacillus brevis W63, Lactobacillus casei W56, Lactobacillus salivarius W24, Lactococcus lactis W19* and *Lactococcus lactis W58,* 6 months	no effect	Horvath et al. 2016
Recovery of enterally administered polyethylene glycols	Severe acute pancreatitis, n= 141 (probiotics=69, placebo=72), RCT, double-blind	Ecologic 641 (6 *lactobacillus, lactococcus*, or *bifidobacteriae species*), 7 days	no effect	Besselink et al. 2009

Intestinal permeability measurement method	**Subjects/type of the study**	**Probiotics**	**Effect of probiotics compared to control**	**Reference**
Serum zonulin	Colorectal carcinoma – patients, n=150 (probiotics=75, control=75), RCT, double-blind	*L. plantarum, L. acidophilus*-11, and *B. longum*-88, 6 d preoperatively and 10 d postoperatively of colectomy for colorectal cancer.	serum zonulin ↓	Liu et al. 2013
Serum zonulin	Colorectal liver metastases - patients, n= 150 (probiotics=66, control =68), RCT, double-blind	Perioperative treatment (6 days preoperatively and 10 days postoperatively) after colectomy for colorectal cancer *L. plantarum, L. acidophilus-11* and *B. longum-88*	serum zonulin ↓	Liu et al. 2015
Serum zonulin	Metabolic syndrome-patients, n=35 patients, 16 healthy, probiotics=13, control=15, RCT	*L. casei Shirota,* 12 weeks	no effect	Stadbauer et al. 2015
Serum zonulin	Overweight and obese subjects, n=225, probiotics=48, placebo=56, RCT, double-blind	*Bifidobacterium animalis,* ssp. *lactis* 420, 6 months	no effect	Stenman et al. 2016

The trials are listed according to the method used. RCT= randomized clinical trial.

2.1.5 Associations between increased intestinal permeability and health

Alterations in intestinal permeability and the consequences for the host's health have been widely studied in animal models. In humans, the focus has been on investigating the role of intestinal epithelium on local (intestinal) chronic inflammatory diseases. Indeed, alterations in intestinal epithelium have been detected in intestinal inflammatory diseases, such as inflammatory bowel diseases ulcerative colitis and Crohn's disease (Bischoff et al. 2014, König et al. 2016).

Increased intestinal permeability, measured both using L/M-ratio and serum zonulin concentration has been associated with various metabolic disorders. Increased L/M-ratio has been detected in patients with metabolic syndrome (Leber et al. 2012) and in obese women, in which the L/M-ratio was further associated with increased blood insulin and the insulin resistance, measured by Homeostatic model assessment, HOMA, index (Teixeira et al. 2012). Recent study has shown higher L/M- ratio in obese patients with moderate or severe steatosis compared to those without any steatosis (Damms-Machado et al. 2017). After weight loss, the gut permeability decreased, suggesting intestinal permeability is associated with obesity or diet. In patients with a non-alcoholic fatty liver disease, NAFLD, increased intestinal permeability, measured by urinary excretion of ^{51}Cr -EDTA, has been detected compared to healthy controls (Miele et al. 2009). Another study revealed higher intestinal permeability, measured by an L/M-test, in 35% of patients with liver cirrhosis (Benjamin et al. 2013).

Increased serum zonulin concentrations have been observed in obesity, in various metabolic disorders (Table 2) and in association with several autoimmune diseases, such as celiac disease and type 1 diabetes (Fasano 2012, Fasano 2011). In a recent study with healthy subjects, higher zonulin levels were associated with higher waist circumference, diastolic blood pressure, and glucose levels. Higher zonulin levels were also associated with increased risk of overweight, obesity and hyperlipidemia, but not with gastrointestinal symptoms or gastrointestinal diseases (Ohlsson et al. 2017a).

2.1.6 Intestinal permeability in pregnancy

Initial evidence exists that intestinal permeability is altered during pregnancy. When analyzed by L/M- test at gestational week 25, higher intestinal permeability was found in pregnant women compared to non-pregnant women (Kerr et al. 2015). Another study has shown higher excretion of lactulose in pregnant women in the third trimester compared to non-pregnant women (Reyes et al. 2006).

Table 2. Metabolic diseases associated with serum zonulin in adults

Disease	Study subjects	Disease association	Reference
Obesity	123 Caucasian men, BMI>25 Kg/m2	Serum zonulin ↑ • correlated with higher BMI, waist to hip ratio, fasting insulin, fasting triglycerides, uric acid and IL-6 • inverse correlation with HDL-cholesterol and insulin sensitivity	Moreno-Navarrete et al. 2012
Obesity	50 obese and 30 normal weight subjects without concomitant diseases.	Serum zonulin ↑ • in obese compared to normal weight subjects • correlated positively with age, body mass, BMI, fat mass and fat percentage	Zak-Golab et al. 2013
Patients with impaired fasting glucose	35 patients with impaired glucose tolerance and 35 healthy matched subjects	Serum zonulin ↑ • in patients with impaired glucose tolerance • correlated positively with LPS	Carnevale et al. 2017
Type 2 diabetes	143 newly diagnosed type 2 diabetes patients, 124 with impaired glucose tolerance, 121 subjects with normal glucose tolerance	Serum zonulin ↑ • in Type 2 diabetes compared to impaired or normal glucose tolerant subjects. • correlated positively with BMI, waist-to-hip ratio, triglyceride, total cholesterol, fasting plasma glucose, 2h plasma glucose, HbA1c, tumor necrosis factor α, interleukin 6, HOMA-IR • inverse correlation with HDL-C and insulin sensitivity	Zhang et al. 2014
Type 2 diabetes	45 subjects with normal glucose tolerance and 45 with type 2 diabetes.	Serum zonulin ↑ • in Type 2 diabetes compared to control subjects	Jayashree et al. 2014

Disease	Study subjects	Disease association	Reference
Type 1 diabetes	339 type 1 diabetic patients	Serum zonulin ↑ • in 141 (42%) subjects compared to control subjects • in 70% of subjects in pre–type 1 diabetes phase	Sapone et al. 2006
NAFLD	40 obese children with non-alcoholic fatty liver disease (NAFLD) and 40 obese children without NAFLD	Serum zonulin ↑ • in obese subjects with NAFLD compared to those without NAFLD • correlated positively with steatosis	Pacifico et al. 2014
NAFLD	56 adults with NAFLD and 20 healthy controls	Serum zonulin ↑ • in NAFLD group compared to control • correlated positively with BMI, aspartate transaminase , triglycerides, fasting insulin, HOMA-IR, liver histopathology, and serum IL-6, inversely with HDL-C	Hendy et al. 2017
Metabolic syndrome	35 patients, 16 healthy	No difference in serum zonulin between healthy subjects and patients	Stadbaeur et al. 2015
PCOS	8 women with Polycystic Ovary Syndrome (POCS) and 63 age-matched healthy controls	Serum zonulin ↑ • in PCOS women compared to healthy controls • correlated positively with HOMA-IR and insulin sensitivity after adjustment for age and BMI	Zhang et al. 2015

The studies are listed according to the metabolic disease.

Increase in intestinal permeability in the non-pregnant population is suggested to be involved in metabolic complications. It is currently not known whether intestinal permeability changes during pregnancy and if the alterations are normal, pregnancy associated physiological changes. Similarly, the factors affecting intestinal permeability during pregnancy and the significance of the intestinal permeability change for maternal health, e.g. metabolic risk factors including low grade inflammation, are poorly known.

2.2 Low grade inflammation and pregnancy

2.2.1 Definition of low grade inflammation

Recently, maternal low grade inflammation has been suggested to contribute to pregnancy related metabolic complications (Pantham et al. 2015). Low grade inflammation, a condition characterized by increased concentrations of inflammatory markers including C-reactive protein (CRP), tumor necrosis factor-α (TNFα), and interleukin -6 (IL-6), is typically detected in overweight and obese non-pregnant individuals (Calder et al. 2011). Previous studies suggest low grade inflammation as an underlying mechanism for a range of metabolic disturbances such as metabolic syndrome, type 2 diabetes, dyslipidemia, and cardiovascular diseases (Santos et al. 2005, Wang et al. 2013, Pfutzner et al. 2006).

2.2.2 Adipose tissue origin low grade inflammation

The organs involved in low grade inflammation include the pancreas, adipose tissue, muscles and liver. Adipose tissue in lean individuals is a strictly regulated anti-inflammatory milieu (Han and Levings 2013). The mechanism of adipose tissue inflammation is not fully understood, but the expansion of adipose tissue, changes in immune regulation and macrophage content is involved. Multiple inflammatory mediators are produced by adipose tissue and the inflammatory cells, the most abundant being the macrophages, accumulated in adipose tissue. TLR4 is one of the pattern recognition receptors (PRR) that recognizes pathogens. Activation of TLR4, by gram-negative bacterial endotoxin, lipopolysaccharide (LPS), and fatty acids released from adipose tissue may also be important (Han and Levins 2013). The mediators produced include hormones, such as pro-inflammatory leptin and anti-inflammatory adiponectin, acute-phase proteins including haptoglobin and alfa-I glycoprotein, cytokines, such as IL-6 and TNFα produced by pro-inflammatory type macrophage M1, various chemokines, transforming growth factor- beta and components of the complement system (Calder et al. 2011, Esser et al. 2014).

2.2.3 Placenta origin low grade inflammation

The placenta is an active inflammatory organ and secretes various inflammatory mediators critical for the implantation and the maintenance of early pregnancy (Pantham et al. 2015, Lekva et al. 2016). Higher expression of the inflammatory cytokines IL-1, TNFα and IL-6 have been detected in the placenta of obese compared to lean women (Challier et al. 2008). Maternal inflammatory lesions has also been observed at higher rate in the placenta of obese than normal-weight women (Bar et al. 2012). It is not clear whether the inflammatory mediators are transported to the fetus or if the inflammation impacts the placental function, such as nutrition transport to the fetus (Ingvorsen et al. 2015). Similarly, the possible contribution of maternal inflammatory mediators on fetal e.g. metabolic programming is unclear. Increased levels of CRP have been detected in non-obese twelve years old children born to obese mother (Leibowitz et al. 2005) and in offspring (56-57 years old) with both parents being obese (Lieb et al. 2009) compared to those born to lean parents, suggesting a transfer of maternal inflammatory status to offspring.

2.2.4 The gut origin low grade inflammation

The gut has a vital role both in the development and maintenance of immune tolerance and immune defense. In addition, the gut has been proposed to act as a source of low grade inflammation (Boroni Moreira and De Cassia Goncalves 2012, Cani et al. 2008, 2012). Increased intestinal permeability appears to be a key factor contributing to the formation of low grade inflammation by allowing the passage of gut components, such as bacterial LPS, into circulation. The subsequent metabolic endotoxemia, i.e. a 2-3- times increase in the levels of LPS in circulation compared to normal (Cani et al. 2008), may induce inflammatory responses (Moreira et al. 2012, Cani et al. 2008). TLR4 is an important mediator of LPS induced inflammation. LPS binding protein (LBP) and CD14 are proteins that coordinate the delivery of LPS to the adipose tissue. In adipose tissue, LPS is presented to the TLR4-MD-2 complex, which in turn activates multiple inflammatory pathways, including nuclear factor kappa-light –chain –enhancer of activated B-cells (NF-kB), interferon regulatory factors (IRFs) and MAPK, resulting into production of pro-inflammatory factors (Neves et al. 2013, Park and Parl 2013, Tand and Kagan 2014) (Figure 3). The inflammatory factors produced may further impair glucose metabolism by different ways: they may induce cellular stress in the pancreas resulting in islet dysfunction and failure (O'Neill et al. 2013) or inhibiting the insulin signaling through altered phosphorylation of the insulin receptors (Robbins et al. 2014).

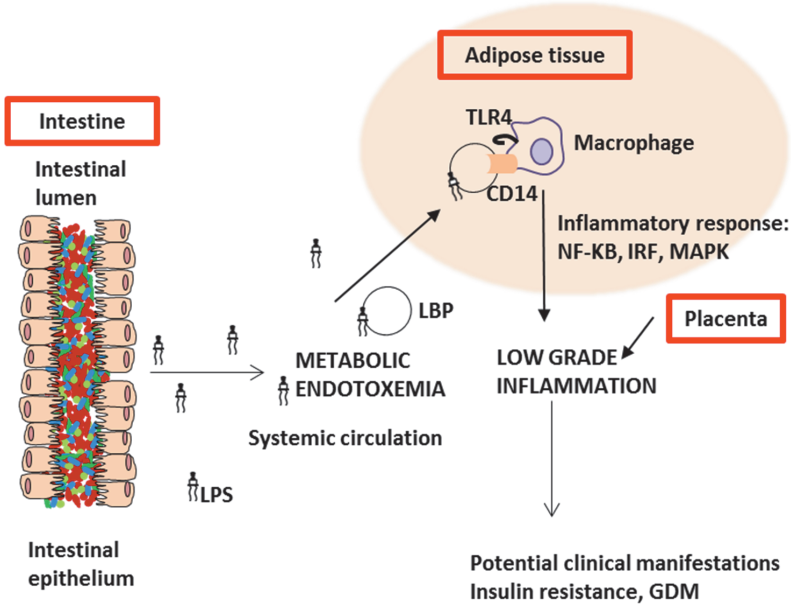

Figure 3. The hypothesis of how the intestine may act as a possible source of low grade inflammation (modifed from Mokkala et al. 2016). Increased intestinal permeability enhances passage of LPS into blood circulation resulting in metabolic endotoxemia. LPS binding proteins (LBP) bind to LPS and delivers the LPS into the adipose tissue where it binds to CD14 on the surface of the macrophage. This then initiates a cascade, resulting in a TLR4 dependent inflammatory response with potential clinical manifestations. Other sources for low grade inflammation include adipose tissue and the placenta. GDM; gestational diabetes.

2.2.5 Low grade inflammation in pregnancy

Highly regulated changes take place in maternal inflammatory status during pregnancy. In the early phase of pregnancy, during implantation, a pro-inflammatory state is required to secure the repair of cells and tissues involved in implantation of the blastocyst (Mor and Cardenas 2010). Later, the innate immune system is stimulated while the adaptive immune system is suppressed to prevent the rejection of the developing fetus. During pregnancy, the balance of T helper (Th) cells is shifted from the pro-inflammatory Th1 responses towards less pro-inflammatory Th2 responses in order to allow fetal growth and development (Mor and Cardenas 2010, Rizzo and Sen 2014, Pantham et al. 2015, Lekva et al. 2016). Before birth, there is an increase in inflammation; this pro-inflammatory milieu promotes the birth of the fetus and the rejection of the placenta (Mor and Cardenas 2010).

Studies investigating the profile of inflammatory mediators during pregnancy have yielded unclear and controversial results. In obese women, the CRP values may already be elevated before pregnancy, which may influence the findings. Increase

in CRP during pregnancy has been detected in women of normal weight (Larsson et al. 2008), while other studies have shown higher CRP levels in obese compared to lean pregnant women, values decreasing from the first to the third trimester in both categories (Stewart et al. 2007, Christian and Porter 2014). Further studies have demonstrated higher CRP values in obese women compared to women of normal weight at various stages of pregnancy (Basu et al. 2010, Sen et al. 2013, Catalano et al. 2009, Kac et al. 2011, Challier et al. 2008). Association between CRP and maternal adiposity has been detected in early pregnancy, but towards the end of pregnancy CRP did not differ between BMI categories (Friis et al. 2013). In normal weight and obese women, the concentrations of IL-6 increase during pregnancy (Pantham et al. 2015, Pendeloski et al. 2017, Stewart et al. 2007 and Christian and Porter 2014). IL-6 has been shown to correlate with maternal adiposity in many studies (Christian and Porter 2014, Ramsay et al. 2002, Stewart et al. 2007, Basu et al. 2010, Catalano et al. 2009, Challier et al. 2008) but not in all (Kac et al. 2011) or not at the end of the pregnancy (Friis et al. 2013). TNFα, which is lower in pregnant compared to non-pregnant women, has been shown to elevate from early to late pregnancy both in normal weight and obese women (Christian and Porter 2014, Melczer et al. 2002). Inconsistent findings are associated with the correlation between TNFα and maternal BMI (Pantham et al. 2015, Basu et al. 2010, Challier et al. 2008, Christian and Porter 2014, Farah et al. 2012, Steward et al. 2007, Vega-Sanchez et al. 2010).

Increased CRP values have been associated with maternal complications, such as gestational diabetes (GDM) in some (Ozgu-Erdic et al. 2015, Maged et al. 2014 and Salmi et al. 2012) but not in all studies (Pöyhönen-Alho et al. 2011, Kim et al. 2014, Syngelaki et al. 2016 and Retnakaran et al. 2003). Findings on the correlation between TNFα and development of insulin resistance are also inconclusive. Some studies do however suggest that circulating TNFα is related to the development of GDM, independently of BMI (Kirwan et al. 2002, Ategbo et al. 2006, Xu et al. 2014). Elevated levels of IL-6 has been linked to GDM both in normal weight and obese women, but the findings are divergent and differ depending on the stages of pregnancy (Pantham et al. 2015, Pendeloski et al. 2017).

2.3 Summary and hypothesis

Pregnancy is an important period for the health of the mother and child. Obesity and overweight are well characterized contributors of maternal and child's health complications. Emerging data indicates that increase in intestinal permeability may be a factor related to metabolic complications in a non-pregnant population. Intestinal epithelium integrity is tightly regulated and gut microbiota and dietary factors have shown both beneficial and adverse effects on the barrier integrity. The possible connection between increase in intestinal permeability and metabolic

disorders is low grade inflammation, induced by circulation entering gut components, such as LPS. Whether pregnancy is accompanied with alterations in intestinal permeability is poorly known. It is also unclear whether maternal gut microbiota and dietary factors impact intestinal permeability, as well as the association between intestinal permeability and maternal metabolic risk factors.

The hypothesis of this thesis is that in overweight and obese pregnant women, gut microbiota and diet have impact on intestinal permeability, seen as change in serum zonulin concentration. Further, serum zonulin concentration increases along with pregnancy and correlates with metabolic risk markers during early pregnancy.

3 AIMS OF THE STUDY

The overall aim of this thesis was to investigate whether maternal gut microbiota and dietary factors contribute to intestinal permeability, measured by the serum zonulin concentration and to further study the association between serum zonulin concentration and maternal metabolic risks factors in early pregnancy.

The specific aims were to study in overweight and obese women:

I the impact of gut microbiota and diet on serum zonulin concentration in early pregnancy (Study I)

II the association between serum zonulin concentration and metabolic risk markers in early pregnancy (Study II)

III changes in serum zonulin concentrations during pregnancy (Study III)

IV the impact of supplementation with probiotics and/or n-3 LC-PUFA on serum zonulin concentration during pregnancy (Study III) and on intestinal permeability *in vitro* (Study IV)

4 MATERIALS AND METHODS

4.1 Clinical studies I-III

4.1.1 Study subjects and design

The study population in the studies I and II comprised of 100 overweight pregnant women, and in the study III (Figure 4) of 200 overweight pregnant women; all of whom were participating in a larger on-going mother-infant dietary intervention trial (ClinicalTrials.gov, NCT01922791) conducted in Southwest Finland. The inclusion criteria for the intervention trial were: prepregnancy BMI≥25, age >18 years, and early pregnancy (<17 weeks of gestation). The exclusion criteria were: gestational diabetes diagnosed in the current pregnancy, multifetal pregnancy, and the presence of metabolic or inflammatory disease including type 1 and type 2 diabetes, celiac disease and inflammatory bowel disease. The study measurements and samples in the studies I and II were obtained in early pregnancy (the first study visit, <17 weeks of gestation), which was the baseline of the intervention trial. In the study III, the measurements and samples obtained from both the early and the late (the second study visit, at 34-36 weeks of gestation) pregnancy visits were used. The women in the studies I and II were the first 100 who provided blood and fecal samples at the first study visit, and in the study III, the first 200 women, who provided blood samples both in the early and late stages of pregnancy.

At the first visit of the intervention trial, the women were randomized into four intervention groups: the first group received probiotics and a placebo for n-3 LC-PUFA, the second group n-3 LC-PUFA and a placebo for probiotics, the third group both probiotics and n-3 LC-PUFA and the fourth group a placebo for both of the intervention products (the control group, Figure 4). The intervention products are described in section 4.1.9. The randomization was conducted by a statistician leaving the research personnel blinded. The mothers were guided not to consume any probiotics or n-3 LC-PUFA containing products during the intervention. Women were to consume, according to the study protocol, the capsules from the first study visit until the baby was 6 months of age. The mother-infant dietary intervention trial is conducted according to the guidelines in the Declaration of Helsinki, and all the procedures involving human subjects were approved by the Ethics Committee of the Hospital District of Southwest Finland (permission number 115/180/2012). Written informed consent was obtained from all of the women. The overview of the clinical studies I-III is presented in the Figure 4.

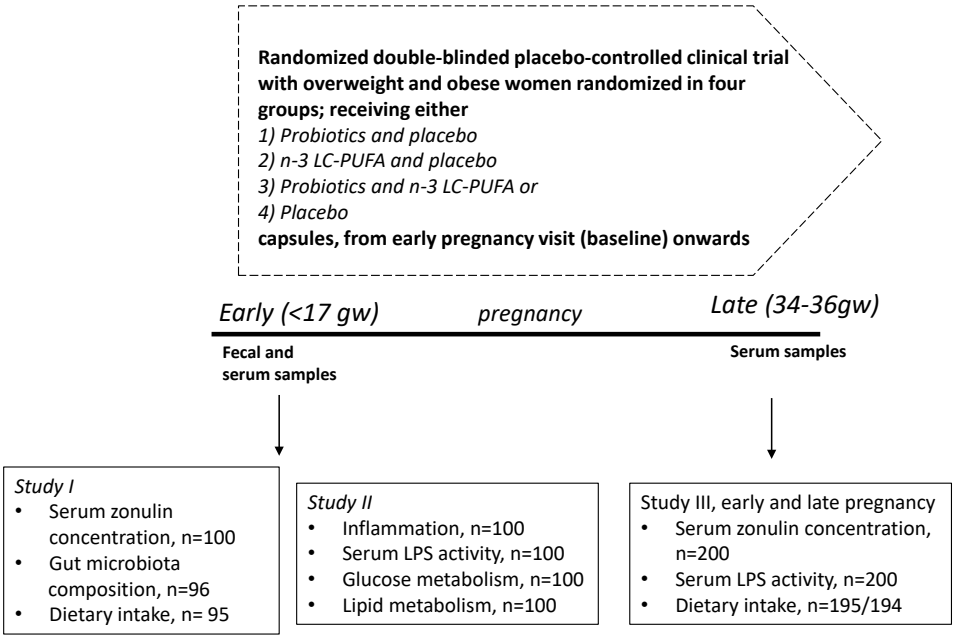

Figure 4. Overview of the clinical studies I-III. GW; gestational weeks.

4.1.2 Serum and fecal samples

On the morning of the study visit, a fasting blood sample was drawn from the antecubital vein of mothers, the serum was separated and analyzed for insulin, glucose, and hs-CRP, and the rest of the samples were frozen in aliquots at -80°C until analyzed for zonulin, LPS, metabolomics and markers for lipid metabolism. Fecal samples were collected from mothers in sterile plastic pots. Samples were collected either on the morning of the study visit or the previous evening, delivered to the study unit and kept at +4 °C until DNA extraction. The extracted DNA samples were stored at -80°C until analyzed for gut microbiota composition.

4.1.3 Serum zonulin and LPS measurement

Intestinal permeability was evaluated using serum zonulin concentration. Serum zonulin concentration was measured using the Zonulin ELISA kit (Immundiagnostik AG, Bernsheim, Germany). The interassay variation was 10.6% in the studies I and II and <8% in the study III. Since no reference values exist for serum zonulin concentration, zonulin median (<46.4 ng/ml) was used for classifying the study subjects in the study I into two groups (the low and the high zonulin groups), the higher group reflecting a higher intestinal permeability. In the study II, zonulin quartiles were used for classification of the study subjects into

four study groups (Q1-Q4), with the highest quartile reflecting the highest intestinal permeability.

LPS activity, a marker of metabolic endotoxemia, was analyzed (University of Helsinki, Finland) using a Limulus amebocyte lysate assay coupled with a chromogenic substrate (HyCult Biochemistry B.V., Uden, the Netherlands). The interassay coefficient of variation was 5.9% in the study II and 5.1% in the study III.

4.1.4 Inflammatory markers

CRP and glycoprotein acetylation (GlycA) were used as serum markers of inflammatory status. Hs- CRP levels were determined using an automated colorimetric immunoassay on the Dade Behring Dimension RXL autoanalyzer (Siemens Healthcare, Camberly, Surrey, UK) to in an accredited Turku University Hospital Laboratory according to the quality control system. The lower limit of detection was 0.1 mg/L. GlycA is a composite nuclear magnetic resonance (NMR) biomarker of systemic inflammation. GlycA contains N-acetyl sugar groups originating from multiple acute phase circulating glycoproteins: α1- acid glycoprotein, haptoglobin, α1- antitrypsin, α1-antichymotrypsin and transferrin (Otvos et al. 2015). GlycA was quantified from serum samples using a commercial high-throughput proton NMR, metabolomics platform (Brainshake Ltd, Helsinki, Finland) as previously described (Soininen et al. 2015).

4.1.5 Fasting glucose and insulin and calculation of insulin resistance and sensitivity index

Serum glucose and insulin concentrations, a homeostatic model assessment-method (HOMA-2IR) and the Qualitative Insulin Sensitivity Check Index (QUICKI) were used to evaluate glucose metabolism. Glucose and insulin concentrations were measured in an accredited Turku University Hospital Laboratory according to the quality control system. Glucose was determined using an enzymatic method utilizing hexokinase (Cobas 8000 automatic c702-analyzer, Roche Diagnostics GmbH, Mannheim, Germany), and insulin was determined with an immunoelectrochemiluminometric assay (a modular E170 automatic analyzer, Roche Diagnostics GMbH, Mannheim, Germany). HOMA2-IR was calculated from fasting plasma glucose and fasting insulin using HOMA calculator (http://www.dtu.ox.ac.uk/) (The Oxford Centre for Diabetes). QUICKI was calculated as = 1 / (log(Fasting Insulin) + log(Fasting Glucose)) (Katz et al. 2000). Serum triglycerides and total low density lipoprotein (LDL)-, and high density

lipoprotein (HDL)-cholesterol for lipid metabolism was analyzed using NMR metabolomics as described above.

4.1.6 Body weight and BMI

Pre-pregnancy weight was self-reported and was obtained from the maternal welfare clinic records. The weight was measured using an electronic scale (the Bod Pod system, COSMED, Inc., Concord, CA, USA). The weight gain was calculated as the difference between the weight measured at the late and early pregnancy visits. Prepregnancy BMI (kg/m^2) was calculated by dividing self-reported weight in kilograms, obtained from welfare women clinic records, by height measured with a wall stadiometer to the nearest 0.1 cm during early pregnancy.

4.1.7 Gut microbiota composition

DNA was extracted from 50 mg of homogenized feces using GTX stool extraction kit and fully automated GenoXTract machine (Hain Lifescience, Nehren, Germany) as previously described (Toivonen et al. 2014). Prior to extraction, mechanical lysis was performed by bead-beating the samples in ceramic bead tubes with MOBIO PowerLyzerTM 24 Bench Top Bead-Based Homogenizer. The DNA samples were sequenced in Sequencing and Bioinformatics Service at Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana (FISABIO) (Valencia, Spain). The 16S ribosomal amplicons were amplified following the 16S Metagenomic Sequencing Library Preparation Illumina protocol (Part # 15044223 Rev. A). The gene-specific sequences used in this protocol targeted the 16S V3 and V4 region. Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences. The primers were selected from Klindworth et al. (Klindworth et al. 2012). The full length primer sequences, using standard IUPAC nucleotide nomenclature to follow the protocol targeting this region were: 16S Amplicon polymerase chain reaction (PCR) Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

16S Amplicon PCR Reverse Primer = 5'

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTA TCTAATCC

Microbial Genomic DNA (5 ng/µl in 10 mM Tris pH 8.5) was used to initiate the protocol. After 16S amplification, the multiplexing step was performed using a Nextera XT Index Kit (FC-131-1096). 1 µl of the PCR product were run on a Bioanalyzer DNA 1000 chip to verify the size. The expected size on a Bioanalyzer

trace was ~550 base pairs (bp). After size verification, the libraries were sequenced using a 2x300pb paired-end run (MiSeq Reagent kit v3 (MS-102-3001) on a MiSeq Sequencer according to the manufacturer's instructions (Illumina). Raw sequences were processed using the QIIME software package (Kuczynski et al. 2011). Operational Taxonomic units (OTUs) were chosen at 97% similarity against the Greengenes database and matched with known bacterial genomes to identify members of the fecal community. Microbiota composition was determined using OTUs. To compare the gut microbiota diversity and richness, we chose to use four measures of diversity: Chao1 (species based index), observed OTUs, phylogenetic diversity (PD, phylogenetic differences among species), and Shannon index (diversity index) (Lozupone et al 2008.). The diversity and richness estimators were calculated at alpha rarefaction sequence depth 36382.0

4.1.8 Dietary intake

Three-day-food diaries including the intake of food supplements were recorded by the women in the week prior to the study visit. The subjects were given instructions on how to record their food intake and during the study visit diaries were checked by trained project coordinator or researcher for completeness and accuracy with the help of a portion picture booklet. Mean daily intake of energy and nutrients (excluding food supplements) were calculated using computerized software, Aivo diet 2.0.2.3 (Aivo, Turku, Finland), which uses the Food and Nutrient Database of the National Institute for Health and Welfare (Fineli 2016).

4.1.9 Intervention supplements

The intervention supplements and their doses were selected based on current scientific knowledge, to be optimal for the purpose of the larger on-going trial (ClinicalTrials.gov, NCT01922791), in which the outcome variables include glucose metabolism and child's allergy. The probiotics used were a combination of two bacteria, *Bifidobacterium animalis* ssp. *lactis* 420 (DSM 22089; Dupont, Niebuell, Germany) and *Lactobacillus rhamnosus* HN001 (ATCC SD5675; Dupont, Niebuell, Germany). Capsules contained 10^10 cfu of each bacteria and 1 capsule was consumed/day. The n-3 LC-PUFA capsule (Croda Europe Ltd, Leek, England) consisted of 1.2 g of n-3 LC-PUFA (79.6% DHA and 9.7% EPA). Two capsules were consumed per day to give a total daily dose of 2.4 g. The placebo for the probiotics consisted of microcrystalline cellulose and for the n-3 LC-PUFA, medium chain fatty acids (capric acid C8 54.6% and caprylic acid C10 40.3%). The probiotic capsules were stored at -20 °C until given to the subjects, who were instructed to store the capsules in a refrigerator. The viability of the probiotic capsules was confirmed by regular analysis by the manufacturer. The n-

3 LC-PUFA capsules were stored at room temperature and the quality was controlled by regular analysis by the manufacturer. The capsule containers were numbered according to the randomization list by a person not involved in the study. Compliance with the consumption of study capsules was assessed by interview. Of the total of the participating women, 97.5% (194 out of 200) reported that they consumed the capsules regularly daily. The women considered as not consuming the capsules, either consumed less than 5 capsules a week (one women), discontinued the consumption (three women) or had a 12- day break before the late pregnancy visit.

4.2 *In vitro* study (study IV)

4.2.1 Epithelial cell culture

Human intestinal Caco-2 cells (ATCC HTB-39, LGC Standards, Manassas, USA) were cultured according to ATCC instructions and Natoli et al. (2012). Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Paisley, United Kingdom #21969-035) supplemented with 4 mM L-glutamine (Gibco, # 25030), penicillin 100 U/l and streptomycin 100 µg/l (Gibco, #15140-122), 1% non-essential amino acids (Gibco, # 11140-050) and 20% heat-inactivated fetal bovine serum (Gibco#10270-106) was used as a cell growth medium (CGM). Passage numbers 25 and 28 were used for differentiating the cells for experiments.

4.2.2 Preparation of bacterial culture, cell free medium and fish oil capsules.

Both bacteria, *Bifidobacterium animalis* ssp. *lactis* 420 and *Lactobacillus rhamnosus* HN001were grown in MRS broth (Oxoid, Hampshire, United Kingdom, # CM0359B) until the colony forming units (CFU) reached ~10^8 /ml, which corresponded to an OD^{600} of 0.86 for *L. rhamnosus* HN001 and 0.75 for *B. lactis* 420. The bacterial supernatant was used in experiments to examine the potential effect of compounds excreted by bacteria on the Caco-2 cells. As in a previous study (Putaala et al. 2008), the bacterial growth medium was centrifuged to collect cell-free supernatant (CFS). CFS was filtered through a 0.2 µm sterile filter and used in cell culture experiments in either as 10% (*B.lactis* and *L. rhamonusus*) or 20% (*B.lactis* 420) concentration. In this study, the n-3 LC-PUFAs used were fatty acid ethyl esters, the form which have also been used in previous study (Elamin et al. 2013). The capsules consisted of 9.4% EPA and 79.1% DHA as ethyl esters, with a total n-3 LC-PUFA content of 88.5%. The content of the capsule was suspended in 1 ml of 99.5% ethanol and diluted to CGM to correspond to a 1 mM DHA/0.12 mM EPA concentration, which was then added to the cells (ethanol concentration in final dilution < 0.05%). As a control for the effects of

the MRS broth, 10% MRS broth in CGM was used. To be able to compare the n-3 LC-PUFA treated cells to those treated with CFS, 10% MRS broth was added to the final n-3 LC-PUFA dilution.

4.2.3 Transepithelial electrical resistance (TEER) measurements to study intestinal epithelial integrity

For differentiation of the cells for the TEER measurement, a previously described short cell growth protocol (Putaala et al. 2008, Andersson et al. 2010) with slight modifications was used. Briefly, 6.7 x 10^5 CaCo-2 cells/cm^2 were seeded on collagen (Rat tail collagen, Gibco, #A1048301) coated Transwell ThinCert™, 12W Multiwell Plate Inserts (Greiner Bio-One, Frickenhausen, Germany # 703-665640) with translucent membrane filters (growth area: 113.1 mm^2). The growth medium was changed every second day. After 6 days, cells were confluent as observed by light microscopy. In confluent Caco-2 cells TJs are already formed (Andersson et al. 2010), which is indicated as a TEER value exceeding 250 Ω (Cai et al. 2014). The experiments were performed 6 days after differentiation of the cells on cell culture inserts. TEER was measured with Millipore Millicell-ERS before (time 0 h) and after (time 24 h) adding 1 ml of 10% CFS or n-3 LC-PUFA (1 mM DHA, 0.12 mM EPA) in CGM to the apical side of the cells. Bacterial CFS and n-3 LC-PUFAs were tested separately. In addition, bacterial CFSs were tested in combination with n-3 LC-PUFA. The results from TEER measurements were obtained by subtracting the TEER (Ω/cm^2) from the experiment with the TEER value of the empty insert. The data were expressed as the change in TEER 24 h post cell culture treatment.

4.2.4 Quantification of the expression of tight junction and MLCK mRNA

Total RNA were extracted from the cultured cells using the RNeasy Mini-kit (Qiagen, Hilden, Germany, # 74101). To study the possible contribution of tight junction mRNA expression to the increased integrity of the intestinal barrier, only cells that showed changes in TEER (cells treated with *B. lactis* 420 and n-3 LC-PUFA) were chosen for RNA extraction and and real time quantitative, RT qPCR. RNA was reverse-transcribed using SuperScript III (Invitrogen, Carlsbad, USA, # 18080-400) with random hexamer primers. PCR amplification was performed using a SYBR select master mix (Applied Biosystems, Warrington, United Kingdom, # 4472903) with primers (Life Technologies, Gent, Belgium) for occludin, claudin-1, JAM-1, zonulin-1, and myosin light chain kinase (MLCK) as previously published (Zhou et al. 2010, Liu et al. 2012). Glyceraldehyde 3-phosphate dehydrogenase, GAPDH, was used as an endogenous PCR control.

ΔΔCq values were normalized to non-treated cells and relative mRNA expression calculated as in Haimes and Kelley (Haimes and Kelley).

4.3 Statistics

The power calculations for the studies I-III were as follows: as no a priori data were available on serum zonulin concentrations (primary outcome in study I) in pregnant women, the sample size estimation in the study I was based on data from two studies in which the difference in zonulin concentration was measured between obese and normal weight subjects (21% difference) (Zak-Golab et al. 2013) and healthy young subjects before and after consuming inulin-enriched pasta (32% difference) (Russo et al. 2012). When calculating with 90% power and at a 5% significance level, the sample size needed to observe 30% difference was 35 and 22, respectively. On the basis on these calculations, we estimated a sample size of 100 would be adequate to observe sufficient variation (30%) between the study subjects, thus allowing division of the data between high and low zonulin groups (n=50 in each group) based on the median zonulin value.

The sample size for the study II was calculated on the expected difference (40%) in LPS values between the highest and lowest serum zonulin quartiles, based on data from previous studies (Basu et al. 2010) that demonstrated a 50% difference in serum LPS between normal weight and obese women. The sample size was calculated with 90% power and at a 5% significance level. The required sample size was 19 subjects/group, but we chose to include 25 subjects in each group.

For the study III, the primary outcomes were the change in serum zonulin concentration (intestinal permeability) from early to late pregnancy and the impact of consuming dietary probiotics and/or n-3 LC-PUFA supplements on such change. The secondary outcomes were the change in serum LPS activity from early to late pregnancy and the impact of consuming dietary probiotics and/or n-3 LC-PUFA supplements on such change. No a priori data exists on how serum zonulin concentrations are altered, nor for the effects of probiotics or n-3 LC-PUFA on serum zonulin concentration during pregnancy. The statistical power of the study was calculated using data from a previous study (Liu et al. 2015) in which probiotics compared to placebo resulted in ~70% decrease in serum zonulin in patients undergoing colorectal liver metastases surgery, and that of the study II in this thesis. To detect a 15% difference in serum zonulin concentration from early to late pregnancy or between the control and intervention groups in late pregnancy, the required sample size was 39/group (80% power, α-level of 0.05). For the power calculations regarding the secondary outcome we used data from a study comparing lean and obese pregnant women (Basu et al. 2011) where a 50% difference in LPS between the groups was detected, and also data from study II in

this thesis. To detect a 15% difference in serum LPS from early to late pregnancy or between control and intervention groups in late pregnancy, the required sample size was calculated to be 25/group. As this is the first study in this population with the depicted dietary intervention, we decided to analyze samples from 50 subjects per each group.

For the *in vitro* study, the sample size was chosen based on previous similar studies (Anderson et al. 2010, Miyauchi et al. 2012, Donato et al. 2010).

All the data were evaluated for normality by using the Kolmogorov-Smirnov-test and/or by visual inspection of the histograms. The statistical analyses were as follows: in the study I, as not all of the data were normally distributed, the Mann-Whitney U-test was used to compare the differences in the gut microbiota composition, the richness, and dietary intake between the high and the low zonulin groups. In the study I, the variables are presented as medians (interquartile range, IQR). In Table 4 and Supplemental Table 1 (in the original publication I), in addition to the medians (IQR), the means (standard deviation, SD) are presented in order to evaluate the relative abundance of bacteria. Univariable linear regression was conducted to study the relationship between serum zonulin (outcome) concentration and nutrient intake (continuous predictors). To further evaluate which of the nutrients best predicted serum zonulin concentration, multiple linear regression analyses were performed. A Spearman's nonparametric correlation was conducted to study the relationship between serum zonulin and gut microbiota relative abundance.

In the study II, to compare the outcome variables according to the serum zonulin quartiles, a Kruskal-Wallis test with Bonferroni corrections was used to analyze the variables that were not normally distributed, and one-way ANOVA with Tukey's corrections for normally distributed variables. To particularly investigate the subjects with low grade inflammation having an increased risk for metabolic disturbances, a group of women with a cut-off for hs-CRP>3mg/L (Pearson et al. 2003, Ridker et al. 2003) was formed. For this investigation, subjects with hs-CRP<3mg/L were excluded (n=25), resulting in a group of 75 subjects. Subsequently, an adjusted linear regression analysis was performed for the evaluation of the association between zonulin and serum markers for inflammation, glucose, and lipid metabolism, with possible confounding factors being considered. For this analysis, maternal prepregnancy BMI and gestational weeks at sampling were used as covariates. Variables that were not normally distributed were natural log-transformed. Spearman's correlation analysis was used to evaluate the relationships between LPS activity and inflammation (GlycA) with hs-CRP, and markers of glucose and lipid metabolism.

In the study III, the difference in the baseline characteristics and dietary intake between the study groups were evaluated using One-Way ANOVA for the parametric and a Kruskal-Wallis Test for the nonparametric variables and Pearson Chi-Square for categorized variables. The differences in dietary intake between the study visits were evaluated using paired sample t-test. When evaluating the change in serum zonulin concentration and LPS activity from early to late pregnancy study visit, a one-way ANOVA was conducted in order to compare both the difference among the study groups and within the study groups. Similarly, when comparing the serum zonulin concentration and LPS activity among the study groups in late pregnancy, a one-way ANOVA was performed. Adjustments for any baseline characteristics (Table 11) were not done, since no differences were detected between the study groups in those variables evaluated as possible confounding factors for serum zonulin concentration and LPS activity. To further study the possible factors affecting the serum zonulin concentration and LPS activity, as well as the changes from early to late pregnancy, a Pearson correlation was conducted. Since intervention had no impact on serum zonulin and LPS levels, correlation was performed without adjustments in the intervention groups.

Pearson's correlation was used to analyze the association between serum zonulin and pregrenancy BMI (studies I-III).

In the *in vitro* study, as the data from TEER measurements was not normally distributed, the significant differences in TEER among the groups were analyzed using the Kruskal-Walls nonparametric test with Bonferroni corrections. The nonparametric Mann-Whitney U-test was used to analyze the change in TEER between baseline and 24 hours after incubation. The results from the TEER experiments are shown as median (interquartile ranges (IQR)). Statistics for qPCR were calculated using the Mann-Whitney U-test from $2^{-\Delta Cq}$ values and values shown as the mean ± SD.

In all the studies, P-values of less than 0.05 were considered as statistically significant. Statistical analyses were performed with SPSS for Windows, version 21.0- 23.0 (IBM Corp., Armonk, NY).

5 RESULTS

5.1 Clinical characteristics of the study subjects

The clinical characteristics of the women are presented in Table 3. All the women were Caucasian and 50% of the women in the studies I and II and 63% in the study III were highly educated with a college or university degree. Most of the women were non-smokers, 97% in the studies I and II and 96% in the study III. Of the women, 44% were primipara in the studies I and II and 43% in the study III.

Table 3. Clinical characteristics of the study subjects.

	Study I and II	**Study III**
Number of subjects	100	200
Prepregnancy BMI, mean (SD)	30.3 (4.4)	30 (5)
Obese subjects (prepregnancy BMI>30) (% of all)	48 (48%)	86 (44%)
Weeks of gestation at early pregnancy, mean (SD)	13 (3)	14 (2)
Weeks of gestation at late pregnancy, mean (SD)		35 (1)
Mean age at early pregnancy, mean (SD)	29 (5)	30 (5)

5.2 Serum zonulin concentration

The mean (SD) serum zonulin concentration in the studies I and II was 46.6 (11.1) ng/ml and 62.7 (12.9) ng/ml in early and 68.1 (14.6) ng/ml in late pregnancy in the study III. The increase detected in the values (between the studies) is possibly due to the serum zonulin kit properties. When comparing studies (Zak-Golab et al. 2013, Moreno-Navarrete et al. 2012) using the same kit, a considerable variation in serum zonulin concentration is detected between the studies. The manufacturer recommends that each laboratory should establish its own reference values. In this thesis, to avoid the interassay variation, the serum zonulin concentration used in the studies I and II were analyzed in the same batch, using the same kit lot. Similarly, the serum zonulin concentrations used in the study III were analyzed in the same batch, using the same lot of the kit.

5.3 The relationship between gut microbiota and serum zonulin concentration in early pregnancy (Study I)

The total number of subjects whose fecal sample was analyzed for microbiota composition was 96 of which 4 samples were discarded due to low yield of sequences, leaving 92 samples (with at 41 000 to 118 000 sequences /sample) for statistical analysis. On the basis of the sequences, a total of 731 OTUs were detected.

When comparing the gut microbiota estimators, Chao1, observed species, PD and Shannon, between the high and the low zonulin groups, statistically significant differences were detected with all the richness estimators, but not with the Shannon index (diversity index) (median (IQR): 5.5 (5.3-5.9) in the low and 5.4 (5.0-5.8) in the high zonulin group, P=0.3). Compared to the high zonulin group, higher indices for Chao1 (409 (363-438) vs. 376 (338-415), P= 0.01), observed species (361 (309-382) vs. 331 (294 -363), P= 0.01) and PD (38.8 (33.6-42.1) vs. 34.9 (30.0-39.6), P = 0.01) were found in the low zonulin group (Figure 5).

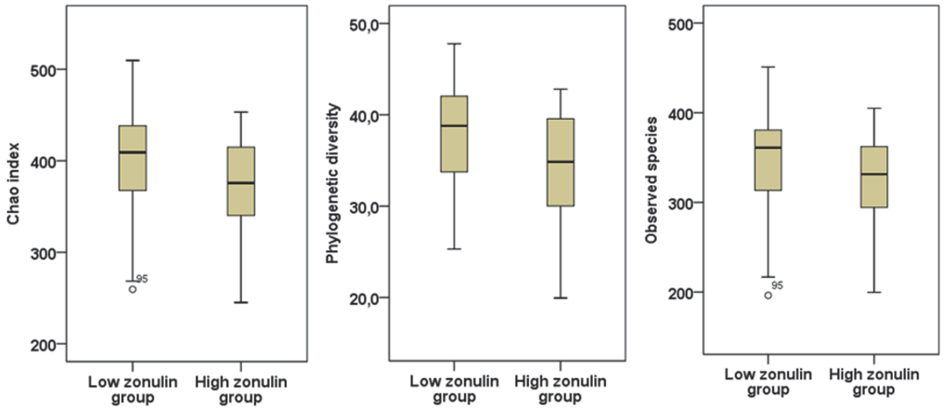

Figure 5. Gut microbiota richness in the high and the low zonulin groups. The plots represent the indexes for gut microbiota richness. Line: median, whiskers: 1.5x IQR. N=46 in both groups.

Bacteroidetes and Firmicutes accounted for most of the total sequences, (median) 94% of the low zonulin group and 95% of the high zonulin group. When comparing the relative abundance of bacteria in different taxonomic levels between the high and the low zonulin groups, statistically significant differences were detected in the relative abundance of 42 taxonomic groups. No statistically significant difference was found (P=0.2) in the ratio of Bacteroides to Firmicutes between the two zonulin groups.

Of the 42 taxonomic groups, 35 had relative abundance <1% and 7 had relative abundance >1%, which were taken into further analysis. The *Faecalibacterium* genus and *Faecelibacterium prausnitzii* species were significantly higher and the *Bacteroidaceae* and *Veinnonellaceae* families, *Bacteroides* and *Blautia* genera and unidentified species of *Blautia* genus were lower in the low zonulin group compared to the high zonulin group (Table 4).

Table 4. Relative abundance (%) in the low and the high zonulin group. (Original publication I).

Relative abundance %	Low zonulin group		High zonulin group		P-value[1]
	Median (IQR)	Mean (SD)	Median (IQR)	Mean (SD)	
Family level					
Bacteroidaceae	29 (21-39)	30±11	35 (25 -46)	36±13	0.02
Veillonellaceae	0.95 (0.52-1.6)	1.2±0.91	1.5 (0.74-2.9)	1.9±1.6	0.03
Genus level					
Bacteroides	29 (21-39)	30±11	35 (25-46)	36±13	0.02
Blautia	1.3 (0.87-2.0)	1.9±2.0	2.0 (1.4-2.6)	2.4±1.6	0.001
Faecalibacterium	5.5 (3.8-7.5)	6.0±3.1	4.5 (2.6-6.6)	4.9±3.0	0.04
Species level					
Blautia	1.3 (0.87-2.0)	1.8±1.9	2.0 (1.4-2.6)	2.4±1.6	0.001
F. prausnitzii	5.5 (3.8-7.5)	6.0±3.1	4.5 (2.6-6.6)	4.9±3.0	0.04

Values are medians (IQR) and means (SD). *n*= 46 in both groups (96 samples were analyzed, four samples were discarded due to the low yield of sequences, leaving 92 samples for statistical analysis). [1]Comparison for median (Mann-Whitney U test).

The relationship between serum zonulin and those bacteria that were significantly different between the high and the low zonulin group, were further analyzed by Spearman's correlation analysis, showing positive correlation between serum zonulin concentration and relative abundance of *Blautia* (ρ =0.29, P=0.005) and inverse correlation with relative abundance of *F.prausnitzii* (ρ =-0.25, P=0.018) (Figure 6).

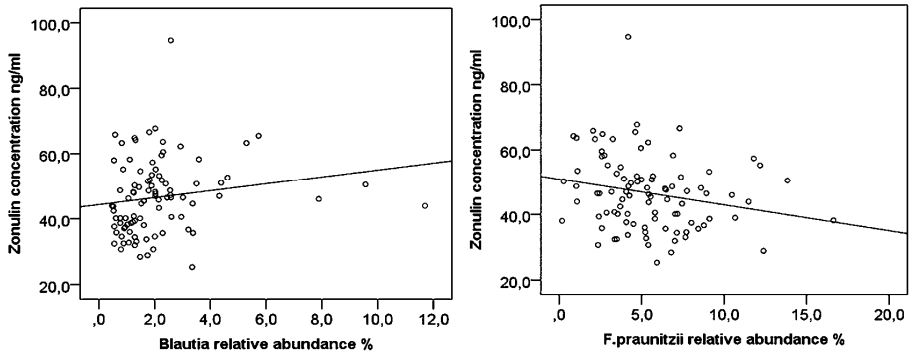

Figure 6. Correlation between serum zonulin concentration and *Blautia* and *Faecalibacterium prausnitzii*.

5.4 The association between maternal diet and serum zonulin concentration in early pregnancy (Study I)

The dietary intake between the high and the low zonulin group was significantly different in absolute intake (in grams) of energy yielding nutrients and several vitamins and minerals (Table 5 and 6). No significant differences were detected in energy yielding nutrients as a percentage of energy intake (% of energy). No difference was detected between the two groups in the adherence to the dietary reference values (Table 5 and 6).

The daily absolute intake of fiber (g) and n-3 LC-PUFA was higher in the low zonulin group compared to the high zonulin group. Those nutrients having a trend (P<0.06) in the difference between the groups were further analyzed using linear regression; significant inverse regression was detected between serum zonulin and intake of protein, LC-PUFA and n-6 LC-PUFA, with protein being the significant factor in predicting serum zonulin in multiple linear regression model (Model 1) with energy yielding nutrient protein and LC-PUFA (Table 7).

Table 5. Daily intakes (calculated from 3-day food diaries) of energy and energy yielding nutrients in the low and the high zonulin groups. (Original publication I).

Nutrient	Low zonulin group	High zonulin group	*P*-value[1]	Dietary reference values
Energy MJ	8.3 (7.0-9.5)	8.0 (6.4-9.0)	0.14	
Protein				10-20 E%
g	86.2 (75.3-99.0)	80.0 (57.6-92.2)	0.06	
% of energy	17.5 (15.7-19.0)	16.9 (14.5-20.2)	0.47	
Carbohydrates				45-60 E%
g	215 (183-274)	204 (164-254)	0.20	
% of energy	46.2 (41.9-50.2)	46.0 (41.4-51.5)	0.89	
Saccharose				<10 E%
g	44.8 (35.9-60.6)	42.8 (31.1-65.5)	0.98	
% of energy	9.70 (7.85-11.8)	9.90 (8.05-13.4)	0.93	
Fat				25-40 E%
g	79.1 (63.2-92.2)	74.9 (59.0-87.5)	0.50	
% of energy	33.5 (29.5-39.3)	35.7 (30.4-38.7)	0.51	
Saturated fatty acids				<10 E%
g	28.3 (20.8-32.0)	25.7 (20.4-32.5)	0.88	
% of energy	11.5 (9.9-13.7)	12.7 (10.7-14.3)	0.21	
Polyunsaturated fatty acids				5-10 E%
g	12.0 (10.0-16.2)	10.5 (8.6-13.7)	0.06	
% of energy	5.6 (4.6-6.7)	5.1 (4.4-5.7)	0.12	
Monounsaturated fatty acids				10-20 E%
g	26.0 (21.3-33.2)	24.1 (19.8-31.1)	0.28	
% of energy	11.2 (10.1-14.0)	11.1 (9.6-13.6)	0.96	
n-3 polyunsaturated fatty acids				1 E%
g	3.6 (2.7-4.5)	3.01 (2.4-3.9)	0.03	
% of energy	1.50 (1.3-1.9)	1.5 (1.2-1.7)	0.14	
n-6 polyunsaturated fatty acids				
g	9.6 (7.0-12.6)	8.0 (6.4-10.7)	0.06	
% of energy	4.0 (3.4-5.4)	4.1 (3.2-4.4)	0.17	
Fiber				25-35g
g	22.2 (16.7-26.1)	18.2 (13.6-21.6)	0.001	
% of energy	2.0 (1.7-2.5)	1.9 (1.5-2.2)	0.12	

Values are medians (IQR), n= 45 in the low and 50 in the high zonulin group (five out of 100 subjects were excluded due to missing food diaries). [1]Comparison for median (Mann-Whitney U test). Nutrition reference values according to Nordic recommendations (Nordic recommendations 2012).

The intake of several vitamins and minerals were significantly higher in the low zonulin group compared to the high zonulin group (Table 6). In the linear regression model, including those vitamins and minerals with significant differences between the groups, it was found that vitamin E, magnesium, niacin, iron and potassium, had a significant inverse regression with serum zonulin. None of the single micronutrients had a significant association with serum zonulin in the multiple linear regression model (Model 2) (Table 7).

Table 6. Daily intakes (calculated from 3-day food diaries) of vitamins and minerals (excluding dietary supplements) in the low and the high zonulin groups[1]. (Original publication I).

Nutrient	Low zonulin group	High zonulin group	*P*-value	Dietary reference values
Vitamins				
Thiamine, µg	1.3 (1.1–1.5)	1.1 (0.9-1.4)	0.01	1.5
Niacin, mg	33.7 (27.0-37.6)	28.1 (21.3-35.3)	0.03	17
Pyridoxine , mg	2.1 (1.5-2.4)	1.6 (1.3-2.2)	0.01	1.6
Riboflavin, mg	1.8 (1.6–2.3)	1.7 (1.3–2.1)	0.009	1.6
Vitamin B12, µg	4.8 (3.8–5.9)	4.3 (2.9–5.6)	0.20	2.0
Vitamin C, mg	126 (75.0–191)	103 (70.0–159)	0.18	85
Folate, µg	240 (195–309)	220 (182–259)	0.08	500
Vitamin A[1], µg	632 (515–829)	544 (405–764)	0.05	800
Vitamin D, µg	6.9 (5.3–10.7)	6.2 (3.5–9.3)	0.05	10
Vitamin E, mg	11.1 (8.3–12.7)	8.8 (6.9–10.9)	0.01	10
Vitamin K, µg	109 (83.6–134)	89.2 (58.7–124)	0.03	
Minerals				
Magnesium, mg	336 (953–1.4)	294 (242–335)	0.008	280
Potassium, g	3.7 (3.2–4.2)	3.1 (2.6–3.7)	0.001	3.1
Calcium, g	1.1 (1.0-1.4)	0.9 (0.7–1.3)	0.01	0.9
Iodine, µg	208 (165–250)	182 (141–218)	0.02	175
Phosphorus, g	1.5 (1.3–1.7)	1.3 (1.1–1.5)	0.009	700
Iron, mg	11.0 (9.2–13.2)	10.2 (8.2–11.8)	0.05	-[2]
Copper, mg	1.2 (1.04–1.4)	1.1 (0.9–1.4)	0.10	1
Selenium, µg	64.2 (56.1–77.2)	62.3 (48.8–72.5)	0.26	60
Zinc, mg	11.2 (9.9-12.9)	10.8 (8.8-12.9)	0.28	9

Values are medians (IQR), *n*= 45 in the low and 50 in the high zonulin group (five out of 100 subjects were excluded due to missing food diaries).[1]Vitamin A: Retinol equivalent comprising of retinols and carotenoids. [2]Iron: iron intake according to individual need. Nutrition reference values according to Nordic recommendations (Nordic recommendations 2012).

Table 7. Association between daily intake of nutrients and serum zonulin in a linear regression and multiple linear regression[1]. (Original publication I).

Nutrient	Linear regression			Multiple linear regression Model 1[2]		Multiple linear regression Model 2[3]	
	β (95%CI)	P	R^2	β (95%CI)	P	β (95%CI)	P
Energy yielding nutrients							
Energy, MJ	-0.001 (-0.002, 0.000)	0.07	0.033				
Protein, g	-0.158 (-0.265, -0.051)	0.004	0.085	-0.139 (-0.247, 0.031)	0.01		
PUFA, g	-0.554 (-1.053, -0.055)	0.03	0.050	-0.419 (-0.915, 0.078)	0.09		
n-6 PUFAs, g	-0.738 (-0.141, -0.141)	0.01	0.061				
Vitamins and minerals, mg							
Vitamin E	-0.801 (-1.507, -0.095)	0.02	0.052			-0.253 (-1.090, 0.583)	0.54
Magnesium	-0.044 (-0.073, -0.014)	0.004	0.085			0.010 (-0.061, 0.080)	0.78
Niacin	-0.381 (-0.641, -0.122)	0.004	0.084			-0.180 (-0.523, 0.164)	0.30
Iron	-0.952 (-1.794, -0.111)	0.02	0.052			-0.050 (-1.390, 1.290)	0.94
Potassium	-0.005 (-0.008, -0.002)	0.001	0.118			-0.004 (-0.010, 0.002)	0.18

[1] The regression coefficient (β) represents the change in serum zonulin concentration associated with the increase in unit of each nutrients. n=95. [2] P=0.004, adjusted R^2 =0.093. [3] P= 0.024, adjusted R^2 = 0.085.

5.5 The association between serum zonulin concentration and metabolic risk markers in early pregnancy (Study II)

Serum zonulin concentration was associated with multiple metabolic risk markers (Table 8). When comparing the serum zonulin quartiles, all measured metabolic markers, except serum glucose, total, LDL- and HDL- cholesterol differed according to zonulin quartiles (Table 8). Further, positive relationship was observed between zonulin and LPS activity, hs-CRP, GlycA, insulin, triglycerides, total and LDL-cholesterol concentrations and HOMA2-IR, and negative between zonulin and QUICKI in linear regression model including subjects with low grade inflammation (hsCRP>3mg/L) (Table 9). These associations remained significant after adjusting with prepregnancy BMI and number of gestational weeks (Table 10). In addition, no associations were detected between serum zonulin and maternal prepregnancy BMI (Pearson's correlation r =0.016, P= 0.891).

To study the possible role of serum LPS activity in increased levels of markers of inflammation, a correlation analysis was conducted between serum LPS activity and hs-CRP and GlycA concentrations. LPS correlated positively with GlycA, but not with hs-CRP concentration (Spearman's correlation coefficients, ρ=0.40, P<0.001 and ρ=0.023, P=0.842, respectively). In addition, LPS activity correlated with serum triglycerides, LDL-, HDL-, and total cholesterol concentrations (ρ =0.38, P=0.001; ρ=0.31, P=0.006; ρ=0.29, P=0.011; and ρ=0.40, P<0.001, respectively), the findings also confirmed in linear regression model (Table 9). In this model, LPS activity was found to associate positively with serum insulin concentration and HOMA2-IR and negatively with QUICKI (Table 9).

To evaluate the possible contribution of low grade inflammation in alterations in glucose and lipid metabolism, the association of GlycA with these markers were analyzed. GlycA correlated positively with insulin (ρ=0.157, P<0.001), HOMA2-IR (ρ=0.50, P<0.001) and triglycerides (ρ=0.79, P<0.001), LDL- (ρ=0.35, P=0.002), and total cholesterol (ρ=0.34, P=0.003) and inversely with QUICKI (ρ =-0.50, P<0.001), further analysis in linear regression confirming the results (Table 9).

Table 8. Median [IQR] and mean values (SD) for serum metabolic markers according to zonulin quartiles. n=100. (Original publication II).

Serum variables	Q1	Q2	Q3	Q4	P-value[1]
Zonulin ng/ml (mean (range))	33.9 (25.3-38.2)	42.3 (38.7-46.3)	49.0 (46.5-52.6)	61.2 (53.2-94.7)	
Inflammatory markers					
hsCRP mg/l[1]	4.0 [2.0-6.3][Q4]	3.6 [2.5-6.6][Q4]	4.7 [2.7-6.6][Q4]	8.7 [6.0-13.7]	<0.001
GlycA mmol/l[1]	1.4 [1.3-1.5][Q3, Q4]	1.4 [1.3-1.5][Q3, Q4]	1.6 [1.5-1.6]	1.6 [1.5-1.7]	<0.001
Metabolic endotoxemia					
LPS EU/ml[2]	0.36 (0.06)	0.35 (0.06) [Q4]	0.37 (0.007)	0.41 (0.074)	0.023
Glucose metabolism					
Insulin mU/l[1]	7.0 [5.5-11.5][Q3, Q4]	9.0 [7.0-11.0][Q4]	11.0 [9-13.5]	14.0 [55.2-64.5]	<0.001
Glucose mmol/l[2]	4.74 (0.35)	4.74 (0.27)	4.80 (0.03)	4.83 (0.71)	0.706
HOMA2- IR[1]	0.91 [0.70-1.48][Q3,Q4]	1.15 [0.91-1.42][Q3, Q4]	1.43 [1.18-1.74]	1.8 [1.2-2.3]	<0.001
QUICKI[1]	0.36 [0.33-0.38][Q3,Q4]	0.35 [0.34-0.36][Q3, Q4]	0.33 [0.32-0.34]	0.32 [0.31-0.35]	0.001
Lipid metabolism					
TG mmol/l[1]	0.96 [0.88-1.09][Q3,Q4]	1.00 [0.76-1.44]	1.29 [1.17-1.45]	1.32 [0.95-1.82]	0.001
Total-C mmol/l[1]	4.5 [4.0-4.8]	4.5 [4.1-5.7]	4.9 [4.1-5.7]	4.9 [4.3-5.8]	0.087
LDL-C mmol/l[2]	1.46 (0.38)	1.63 (0.73)	1.74 (0.48)	1.73 (0.55)	0.235
HDL-C mmol/l[2]	1.81 (0.29)	1.77 (0.30)	1.79 (0.33)	1.99 (0.37)	0.062

n=100. Statistically significant differences (P<0.005) between the quartiles are shown as superscript between the quartiles. [1] Kruskal-Wallis nonparametric test with Bonferroni corrections. [2]One-way ANOVA with Tukey's corrections.

Table 9. Association of zonulin, LPS and GlycA with markers of metabolic endotoxemia, inflammation, glucose and lipid metabolism in subjects with hs-CRP>3mg/L (n=75) in a linear regression model. (Original publication II).

	β (95% CI)	R^2	P-value	β (95% CI)	R^2	P-value	β (95% CI)	R^2	P-value
	LPS EU/ml			**hsCRP mg/l[1]**			**GlycA mmol/l[1]**		
zonulin	0.002 (0.001, 0.004)	0.14	0.001	0.006 (0.002, 0.011)	0.11	0.004	0.005 (0.003, 0.006)	0.25	<0.001
LPS				0.018 (-1.734, 1.771)	0.000	0.983	0.590 (0.260, 0.920)	0.15	0.001
GlycA[1]	0.269 (0.153, 0.386)	0.18	>0.001	2.150 (1.122, 3.178)	0.19	<0.001			
	Insulin mU/l[1]			**HOMA2-IR1**			**QUICKI[1]**		
zonulin	0.015 (0.007, 0.023)	0.15	0.001	0.015 (0.007, 0.023)	0.14	0.001	-0.002 (-0.004, 0.001)	0.13	0.001
LPS	1.740 (0.274, 3.206)	0.07	0.02	1.655 (0.188, 3.121)	0.07	0.02	-0.249 (-0.485, -0.014)	0.06	0.03
GlycA[1]	2.221 (1.374, 3. 67)	0.27	<0.001	2.180 (1.332, 3.029)	0.26	<0.001	-0.339 (-0.477, -0.202)	0.25	<0.001
	Triglycerides mmol/l[1]			**total cholesterol mmol/l[1]**			**LDL-cholesterol mmol/l**		
zonulin	0.011 (0.006, 0.017)	0.18	<0.001	0.006 (0.002, 0.010)	0.11	0.004	0.013 (0.002, 0.024)	0.07	0.021
LPS	1.9901 (0.923, 2.879)	0.06	<0.001	1.105 (0.467, 1.742)	0.14	0.001	2.255 (0.398, 4.123)	0.07	0.01
GlycA[1]	2.338 (1.898, 2.778)	0.61	<0.001	0.626 (0.201, 1.050)	0.11	0.004	1.453 (0.223, 2.673)	0.07	0.02
	HDL-cholesterol mmol/l								
zonulin	0.006 (-0.001, 0.012)	0.03	0.111						
LPS	1.221 (0.077, 2.365)	0.058	0.03						
GlycA[1]	0.024 (-0.745, 0.793)	0.000	0.951						

The regression coefficient (β) represents the one-unit change in zonulin (ng/ml), LPS (EU/ml) or a log-transformed serum GlycA (mmol/l) associated with the change of serum marker. R^2 = R-square, 95% CI: 95% confidence interval for β. [1] Values are natural log-transformed for linear regression analysis i.e. the regression coefficient (β) represents the one-unit increase in serum marker in the natural log-scale associated with the change of zonulin, LPS or GlycA in natural log-scale.

Table 10. Association among zonulin and markers of inflammation, metabolic endotoxemia, and glucose and lipid metabolism within subjects with hs-CRP>3mg/L (n=75) in a multiple linear regression model adjusted for log-transformed BMI and gestational weeks. (Original publication II).

	β (95% CI)	R^2/P[1]-value	P-value	β (95% CI)	R^2/P[1]-value	P-value	β (95% CI)	R^2/P[1]-value	P-value
	LPS EU/ml			**hs-CRP mg/l[1]**			**GlycA mmol/l[1]**		
zonulin	0.002 (0.001, 0.003)	0.21/0.001	0.002	0.013 (0.003, 0.023)	0.17/0.004	0.015	0.004 (0.002, 0.006)	0.38/<0.001	<0.001
BMI	-0.088 (-0.213, -0.037)		0.164	1.087 (0.122, 2.052)		0.028	0.322 (0.152, 0.492)		<0.001
weeks	0.061 (-0.010, 0.132)		0.090	0.144 (-0.403, 0.691)		0.601	0.073 (-0.023, 0.169)		0.135
	Insulin mU/l[1]			**HOMA2-IR[1]**			**QUICKI[1]**		
zonulin	0.015 (0.007, 0.022)	0.38/<0.001	<0.001	0.015 (0.007, 0.022)	0.38/<0.001	<0.001	-0.002 (-0.003,- 0.001)	0.36/<0.001	<0.001
BMI	1.407 (0.684,2.129)		<0.001	1.394 (0.674, 2.114)		<0.001	-0.211 (-0.329, -0.094)		0.001
weeks	-0.492 (-0.901, -0.082)		0.019	-0.511 (-0.919, -0.103)		0.015	0.087 (0.021, 0.154)		0.011
	Triglycerides mmol/l[1]			**Total cholesterol mmol/l[1]**			**LDL- cholesterol mmol/l**		
zonulin	0.009 (0.003, 0.015)	0.28/<0.001	0.003	0.004 (0.000, 0.007)	0.32/<0.001	0.032	0.008 (-0.003, 0.019)	0.21/0.001	0.135
BMI	0.572 (0.022, 1.122)		0.022	0.076 (-0.268, 0.419)		0.662	0.528 (-0.513, 1.569)		0.315
weeks	0.450 (0.139, 0.762)		0.139	0.447 (0.253, 0.642)		<0.001	1.044 (0.454, 1.634)		0.001

The regression coefficient (β) represents a one-unit (ng/ml) increase in zonulin, a one-unit (kg/m^2) increase in BMI, or a one week increase in gestational weeks associated with the change of serum marker. R^2 = R-square, 95% CI: 95% confidence interval for β.P [1] – value=P-value for adjusted linear regression. [1] Values are natural log-transformed for linear regression analysis, i.e., the regression coefficient (β) represents the one-unit (ng/ml) increase in zonulin, one-unit (kg/m2) increase in BMI, or one week increase in gestational weeks associated with the change in serum marker in the natural log-scale.

5.6 Variation in serum zonulin concentration and LPS activity during pregnancy (study III)

5.6.1 Baseline characteristics and dietary intake among the intervention groups

Of all the women, 44% were obese, and 63% were highly educated with a college or university degree (Table 11). No statistically significant differences were detected in clinical characteristics among the four intervention groups of the intervention trial (Table 11). Similarly, no differences were found among the groups in the dietary intake of energy, energy yielding nutrients and fiber at either study visit (Original publication III, S1). When evaluating the dietary intake between the study visits, the intake of carbohydrates as a proportion of energy intake (E%) (mean (SD) difference -1.5 (8.2) E% (95%CI: -2.6 to -0.3), P=0.015) decreased, while intake of fat (1.5 (7.6) E% (95%CI: 0.5-2.6), P=0.006), SFA (0.6 (3.4) E% (95%CI: 0.07-1.0), P=0.025) and monounsaturated fatty acids, MUFAs, as absolute (1.7 (11.1) g (95%CI: 0.1-3.3), P=0.036) and as proportion of energy intake (E%) (0.7 (3.5) E% (95%CI: 0.18-1.20), P=0.008) increased from early to late pregnancy. All measured nutrients, except the intake of fiber (early pregnancy: 20.5 (6.9) g, late pregnancy: 20.0 (7.2) g), which was lower, and SFAs (early pregnancy: 12.5 (3.9) E%, late pregnancy 13.1 (3.2) E%), which was higher, were within the dietary reference values (Nordic recommendations for fiber: 25-35g/daily, <10 E% SFAs/daily) at both study visits.

5.6.2 Changes in serum zonulin concentration and LPS activity from early to late pregnancy

During the follow-up period, serum zonulin concentration increased by (mean (SD)) 5.3 (11.6) ng/ml from early pregnancy (Table 12). The serum zonulin concentration increased in 67.8% (135/199) of the women. Similarly, a LPS activity increased (0.04 (0.07) EU/ml) significantly (Table 12). The LPS activity increased in 83.5% (167/200) of the women.

Table 11. Baseline characteristics in the study groups. (Modified from the original publication III).

	probiotics + placebo	LC-PUFA+ placebo	probiotics+ LC-PUFA	control group	all	P-value
Characteristics						
Subjects/group	51	49	49	51	200	
Age (years)	30.5 (4.9)	30.7 (5.5)	30.1 (5.3)	30.2 (3.9)	30.4 (4.9)	0.9
Prepregnancy BMI (kg/m^2)	30.3 (5.1)	30.3 (4.4)	30.0 (4.1)	29.8 (4.5)[2]	30.1 (4.5)	0.9
Highly educated	31/47 (66%)	34/48 (71%)	27/48 (56%)	31/51 (61%)	123/194 (63%)	0.4[1]
Primipara	43%	45%	41%	43%	43%	0.9[1]
Non-smoking/early pregnancy	44/46 (96%)	47/48 (98%)	46/48 (96%)	48/51 (94%)	185/193 (96%)	0.9[1]
Non-smoking/late pregnancy	47/51 (92%)	48/49 (98%)	47/49 (96%)	50/51 (98%)	192/200 (96%)	0.4[2]
Weight gain (kg)	8.6 (3.8)	8.9 (4.0)	9.0 (4.2)	8.8 (4.4)	8.8 (4.0)[1]	0.9
Weeks/early pregnancy	16.5 (2.2)	13.7 (2.4)	13.96 (2.1)	13.9 (2.1)	13.8 (2.2)	0.8
Weeks/late pregnancy	35.2 (1)	35.2 (1)	35.2 (1)	35.2 (1.2)	35.2 (1)	0.9
Weeks of supplementation	21.8 (2.6)	21.5 (2.5)	21.3 (2.5)	21.3 (2.3)	21.4 (2.5)	0.7

[1]Pearson Chi-Square. Others: One-way Anova between groups, values are mean (SD) [2]One measure in the study III is missing at the second visit due to hospitalization.

Table 12. Early and late pregnancy serum zonulin and LPS concentrations (mean (SD)) and 95% Confidence intervals for mean (95% CI). (Original publication III).

	probiotics + placebo	LC-PUFA+ placebo	probiotics+ LC-PUFA	control group	All	P-value[1]
Serum zonulin ng/ml						
Early pregnancy	61.9 (11.7)	64.7 (16.4)	61.7 (11.3)	62.6 (11.8)	62.7 (12.9)	
Late pregnancy	68.4 (12.4)	70.0 (18.5)	67.2 (13.6)	66.8 (13.5)	68.1 (14.6)	0.7
Mean change (SD)	6.5 (12.3)	5.2 (11.2)	5.5 (11.9)	4.0 (11.2)	5.3 (11.6)	0.8
95%CI	3.0-10.0	2.0-8.5	2.1-9.0	0.8-7.2	3.7-6.9	
Serum LPS EU/ml						
Early pregnancy	0.15 (0.04)	0.15 (0.04)	0.16 (0.05)	0.16 (0.04)	0.16 (0.04)	
Late pregnancy	0.18 (0.04)	0.21 (0.11)	0.19 (0.05)	0.20 (0.05)	0.19 (0.07)	0.2
Mean change (SD)	0.03 (0.05)	0.06 (0.11)	0.03 (0.05)	0.04 (0.05)	0.04 (0.07)	0.2
95%CI	0.018-0.041	0.023-0.088	0.012-0.042	0.027-0.054	0.028-0.048	

[1] One-way ANOVA; tests between the intervention groups

5.6.3 Correlations between serum zonulin concentration, LPS activity and maternal characteristics

As both serum zonulin concentration and LPS activity increased during pregnancy, we investigated the interrelations between these factors. A significant positive correlation was observed between serum zonulin concentration and LPS activity in early pregnancy (Pearson's correlation coefficient r=0.216, P=0.002) but not in late pregnancy (r=0.013; P=0.8). In addition, the change in serum zonulin concentration and LPS activity from early to later pregnancy did not correlate (r=0.02, P=0.7)

Furthermore, the association of serum zonulin concentration and LPS activity with maternal prepregnancy BMI, weight gain from early and to late pregnancy, weeks of supplementation and gestational weeks at the early pregnancy visit were investigated (Table 13).

Table 13. Correlations between serum zonulin concentration and LPS activity with maternal prepregnancy BMI, weight gain from early and to late pregnancy, weeks of supplementation and gestational weeks at the early pregnancy visit.

	Prepregancy BMI	**Weight gain (kg) from early to late pregnancy**	**Weeks of supplementation**	**Gestational weeks at early pregnancy visit**
Serum zonulin concentration ng/ml				
Early pregnancy	r=-0.06, P=0.4			r=0.12, P=0.10
Late pregnancy	r=0.04, P=0.5	r=-0.17, P=0.01	r=0.114, P=0.1	r=0.093, P=0.2
Change from early to late pregnancy	r=0.11, P=0.1	r=-0.09, P=0.2	r=0.25, P<0.001	
LPS activity EU/ml				
Early pregnancy	r=0.043, P=0.5			r=0.21, P=0.003
Late pregnancy	r=-0.079, P=0.2	r=0.12, P=0.09	r=-0.058, P=0.4	r=-0.045, P=0.5
Change from early to late pregnancy	r=-0.103, P=0.1	r=0.19, P=0.008	r=0.12, P=0.09	

r= Pearson's correlation coefficient

Several correlations were detected (Table 13). Maternal weight gain was negatively associated with with serum zonulin concentration in late pregnancy and positively with the change in serum LPS activity. Weeks of supplementation correlated with the change in serum zonulin concentration. Number of gestational weeks at early pregnancy associated positively with early pregnancy LPS activity. Maternal prepegnancy BMI did not associate with serum zonulin concentration or LPS activity either in early or late pregnancy or with changes in these levels between the study visits.

Intake of fat as a proportion of energy intake did not correlate with serum zonulin concentration or LPS activity in early pregnancy (r=0.04, P= 0.6; r=0.03, P=0.6). A trend in correlation between the intake of fat as a proportion of energy intake and serum LPS activity was detected in late pregnancy (r=0.14, P=0.053), but not with serum zonulin concentration (r=-0.02, P= 0.77).

5.7 The impact of supplementation with probiotics and n-3 LC-PUFA on serum zonulin concentration and LPS activity during pregnancy (Study III) and on intestinal permeability *in vitro* (Study IV)

Consuming probiotics and/or n-3 LC-PUFA supplements had no impact on either zonulin concentration or serum LPS activity, as no statistically significant differences were detected among the intervention groups from early to late pregnancy (P=0.8 and P=0.2, respectively) or in late pregnancy (P=0.7 and p=0.2, respectively) (Table 12).

In the *in vitro* study (study IV), intestinal barrier integrity, measured as TEER, increased significantly (P<0.05) from the baseline to 24 hours with both *B.lactis* CFS concentrations (10% CFS: median (IQR) +8.7 (7.1, 13.0) %, P<0.001, 20% CFS: +16.7 (14.4, 24.3) %, P=0.019) and n-3 LC-PUFA (+8.2 (2.5, 14.8) %, P=0.008). A statistically significant decrease from baseline to 24 hours in TEER was detected with the control (-1.9 (-10.3, 0.1) %, P=0.023). No significant change in TEER were found with *L.rhamnosus* (+4.6 (-4.0, 7.6) %, P=0.41). When comparing the change in TEER between the control and test compounds; statistically significant differences were detected with both of the CFS concentrations of *B.lactis* (10%: 15.7%, P<0.001, 20%: +24.8, P=0.002) and n-3 LC-PUFA (+14.0, P=0.02), with no difference between *L.rhamnosus* and the control (+6.8, P=0.07) (Figure 7). No statistically significant changes after a 24-hour incubation or differences in changes, compared to the control, were detected, with the combinations of the test compounds.

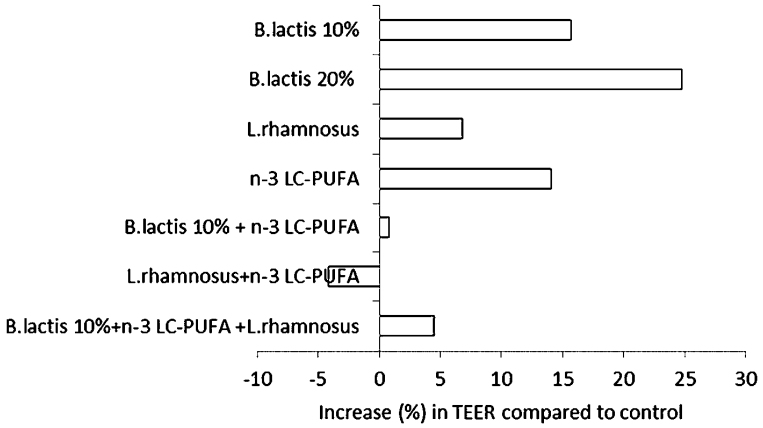

Figure 7. Change in TEER between control and test compounds; *Bifidobacterium lactis* 420 10% CFS (n=10, two separate experiments, n=5+5 in each); *B. lactis* 420 20% CFS (n=5); *Lactobacillus rhamnosus* HN001 10% (n=10, two separate experiments, n=5+5 in each); n-3 LC-PUFA (n=5); *B. lactis* 420 10% CFS+n-3 LC-PUFA (n=5); *L. rhamnosus* HN001 10% CFS+n-3 LC-PUFA (n=5); *B. lactis* 420 10% CFS + *L. rhamnosus* HN001 10% CFS+n-3 LC-PUFA (n=5); control CGM+10% MRS (n=10, two separate experiments, n=5+5 in each) in Caco-2 cells. (Modified from original publication IV).

When the *B.lactis* CFS concentration was increased from 10% to 20%, TEER increased by +7.9% (P=0.019), suggesting a dose-response effect for *B.lactis* CFS (R^2=0.71, P<0.001) (Figure 8).

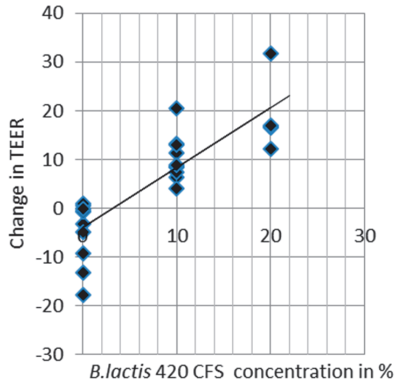

Figure 8. Dose-response effect with *B.lactis* 420 CFS. (Original publication IV).

N-3 LC-PUFA induced statistically significant increase in MLCK mRNA expression, with ΔΔCq being 1.53±0.4 (P=0.032, calculated as $2^{-\Delta Cq}$ differences between n-3 LC-PUFA and untreated cells). In the *B. lactis* 420 and n-3 LC-PUFA treated group, no statistically significant changes were detected in the mRNA expression of the following TJ proteins studied: occludin, claudin-1, JAM-1 and zonulin-1.

6 DISCUSSION

These studies show a novel relationship between maternal gut microbiota and diet with serum zonulin, a marker for intestinal permeability, which further correlated with maternal metabolic risk factors in overweight and obese women in early pregnancy. Importantly, the detected increase in serum zonulin concentration during pregnancy suggests an increase in intestinal permeability over the course of pregnancy in overweight and obese women. Supplementation with probiotics and n-3 LC-PUFA had no impact on the change in serum zonulin concentration and LPS activity in the clinical study, although *B. lactis* 420 and n-3 LC-PUFA enhanced intestinal epithelium *in vitro*.

6.1 Association between maternal gut microbiota and diet with serum zonulin

A higher gut microbiota richness was detected in subjects with lower serum zonulin levels suggesting an association between richer gut microbiota and lower intestinal permeability. A higher gut microbiota richness has recently been shown to correlate with a healthier metabolic phenotype (LeChatelier et al. 2013, Cotillard et al. 2013). The mechanism may originate from the enhancing effects of the epithelial integrity of bacterial metabolites, such as SCFAs, produced by more variable gut microbiota. Additional mechanism may arise from the production of anti-inflammatory components and colonization of anti-inflammatory bacteria in the epithelium, as higher proportions of anti-inflammatory bacteria are detected in richer gut microbiota compared to less-rich microbiota (LeChatelier et al. 2013).

The subjects with higher and lower serum zonulin differed according to the relative abundance of their gut microbiota; higher abundance of *F.prausnitzii* and lower abundance of *Blautia* was detected in the low serum zonulin group. In previous studies, *F. prausnitzii* has been associated with healthy metabolic outcomes (LeChatelier et al. 2013, Barlow et al. 2015), however, in contrast, a higher relative abundance of *Blautia* has been linked with glucose intolerance (Egshatyan et al. 2016). Both bacteria have also been related to alterations in intestinal permeability; in alcohol-dependent subjects, a lower abundance of *F.prausnitzii* and a higher abundance of *Blautia* was related to high intestinal permeability, measured by ^{51}Cr-EDTA in urine (Leclercq et al. 2014). The possible intestinal epithelium enhancing mechanism of *F.prausnitzii* may be related to increased production of butyrate, an important energy source for intestinal epithelial cells. *F.prausnitzii* is suggested to be one of the most abundant species in the gut and as a fiber fermenting bacteria, thus the major supplier of butyrate (Duncan et al. 2002).

The dietary intake of nutrients had a significant impact on serum zonulin concentrations. When comparing the intake of energy yielding nutrients and fibre with serum zonulin concentration, the most striking difference was detected with the intake of fiber, which was higher in subjects with lower serum zonulin levels. A similar finding has been observed in a study with normal healthy young subjects, in which inulin enriched pasta decreased serum zonulin (Russo et al. 2012). The possible mechanism accounting for this is presumably related to the indirect effect of fiber, i.e. an increased production of SCFAs due to bacterial fermentation. The association between the intake of fiber and intestinal permeability is of importance, since in our study, the intake of fiber was below the recommended levels (20-25g/day) in both the low and high serum zonulin group. We did not detect differences in total fat intake between the zonulin groups. The intake of fat in our study subjects was at the level typically observed in clinical trials with Finnish women and lower when compared to animal experimental diets; e.g. in mice, a diet containing about 20 times the typical fat intake was shown to increase intestinal permeability (Cani et al. 2008). Intake of PUFA, n-3 and n-6 LC-PUFAs correlated inversely with serum zonulin, highlighting the importance of the quality of the fat to its impact on serum zonulin. n-3 LC-PUFA may influence the intestinal epithelium by serving as a precursor to anti-inflammatory eicosanoid synthesis and by regulating the TJs, enhancing the intestinal epithelial integrity, as shown in *in vitro* studies (Xiao et al. 2013, Li et al. 2008). Further correlations with serum zonulin were detected with the intake of protein and several vitamins and minerals. The mechanism by which protein induces a beneficial effect may be related to single amino acids, such as glutamine, or other products of breakdown or fermentation (Fan et al. 2015) with nourishing effects. The mechanism by which vitamins and minerals induce their effects may be many; these include their direct impact on TJs and also the possible anti-inflammatory effects on intestinal epithelium. As the intake of dietary nutrients previously linked with heathy outcomes was positively associated with serum zonulin, the intake of an overall healthy diet seems important. This is supported by the finding of higher intake of nutrient rich fibre in the low zonulin group, the intake which may also explain the higher intakes of vitamins and minerals detected in this group. The findings here include the dietary intake of vitamins and minerals, while the impact of food supplements and total intake from both diet and food supplements remains to be determined.

Zonulin is released from the intestinal epithelium by triggers, such as gliadin and pathogens (Fasano 2000). Loss of barrier integrity due to zonulin induced disassembly of TJs, leads to an increase in intestinal permeability. The overall mechanism by which dietary nutrients and gut microbiota impact the release of serum zonulin may be related to the protection of the intestinal epithelium. Dietary nutrients may enhance the epithelial integrity or modulate gut microbiota to

contain more anti-inflammatory bacteria with beneficial impact on barrier integrity. PUFA, as an example, has been shown to induce a beneficial effect on intestinal epithelium integrity (Li et al. 2008, Xiao et al. 2013) and to modify gut microbiota composition into a more anti-inflammatory type in mice (Kaliannan et al. 2015) and thus by reinforcing the barrier integrity, may prevent the release of zonulin.

6.2 Association of serum zonulin concentration with metabolic risk markers

Serum zonulin concentration was associated with LPS activity, markers of inflammation, and glucose and lipid metabolism in early pregnancy, independently of prepregnancy BMI, suggesting that intestinal permeability may be of importance in regulating maternal metabolic health.

Serum zonulin concentration associated with serum LPS activity and the levels of inflammatory markers hs-CRP and GlycA. Previous studies have shown similar associations between serum zonulin and inflammatory markers, such as IL-6, in a non-pregnant population (Moreno-Navarrete et al. 2012, Zhang et al. 2014). In this study, serum zonulin was also directly linked to markers of glucose and lipid metabolism, a finding which is also in line with previous studies in a non-pregnant population (Moreno-Navarrete et al. 2012, Zhang et al. 2014, Zhang et al. 2015, Ohlsson et al. 2017b). The mechanism linking serum zonulin to increased inflammation and alterations in glucose metabolism may involve the interplay between LPS, low grade inflammation, and glucose metabolism. Increased circulating LPS levels, due to increased intestinal permeability, may stimulate TLR4, with the subsequent induction of an inflammatory response (Neves et a. 2013, Park and Parl 2013, Tan and Kagan 2014)). The cytokines produced, such as IL-1β, may impair insulin function by inhibiting the action of insulin receptors, resulting in interference in insulin recognition (Robbins et al. 2014). The association of serum LPS with metabolic disorders have been shown in previous studies: higher LPS activities, insulin resistance and inflammatory markers CRP and IL-6, but not TNFα, were detected in obese compared to lean pregnant women (Basu et al. 2011). The association of higher LPS activity with obesity suggest that increase in LPS activity is related to obesity, rather than pregnancy itself (Basu et el. 2010). In a non-pregnant population, increased LPS activities and levels have been detected in type 2 diabetes patients (Creely et al. 2007, Pussinen et al. 2011, Jayashree et al. 2014) as well as in cardiovascular diseases (Kallio et al. 2015). The clinical implications of increased LPS activities during pregnancy remains to be studied.

Serum zonulin also correlated with serum triglycerides, total cholesterol, and LDL-cholesterol. The mechanism behind the correlation is unclear, but possibly includes crosstalk between LPS and the endocannabinoid system, a mechanism which is suggested to contribute to adipogenesis in obesity (Muccioli et al. 2010). Nevertheless, as GlycA was also associated with lipid metabolism, interrelation between all the metabolic risk markers is possible.

The novel finding of association of GlycA complex with metabolic risk markers suggests that elevated levels may predict the risk for metabolic disorders, such as insulin resistance in pregnancy. GlycA is a recently described inflammatory marker and detected in association with several metabolic disorders in non-pregnant populations (Ritchie et al. 2015, Akinkuole et al. 2013, 2014, Connelley et al. 2016). A recent study of pregnant women detected an increase in GlycA over the duration of pregnancy when compared to non-pregnant women (Wang et al. 2016), suggesting that GlycA may reflect the pregnancy associated changes in maternal inflammatory status. Other previous studies have only investigated the levels of individual glycoproteins in women with a normal pregnancy and with gestational diabetes with variable outcomes (e.g. Pöyhönen-Alho et al. 2011, Larsson et al. 2008, Ruhaak et al. 2014, Chu et al. 1981, Honda et al. 1990, Yaghmaei et al. 2009). The relevance of this new marker as regards its clinical implications, such as a potential use as a predictive marker for metabolic diseases, needs further studies.

The concentrations of the risk markers detected in this study were within normal reference values. Nonetheless, the correlation between serum zonulin and metabolic risk factors indicate that an increase in intestinal permeability may precede adverse metabolic conditions detected during pregnancy.

6.3 Pregnancy accompanied with an increase in serum zonulin concentration and LPS activity

Serum zonulin concentration increased from early to late pregnancy, suggesting that intestinal permeability increases over pregnancy, which is a novel finding. This increase was independent of maternal prepregnancy BMI and weight gain between the study visits. Increased intestinal permeability, compared to non-pregnant women was observed in a previous study using L/M-test (Kerr et al. 2015) and thus together with our findings suggest that the elevated permeability may be a normal physiological process during pregnancy. Similar to other organs, the intestine adapts to pregnancy by enhancing the absorption of nutrients to support the fetal development (Astbury et al. 2015). In addition, the passage of maternal gut components could allow the education of the fetal immune system towards immune tolerance, reducing the burden of inflammatory-related diseases,

(Khan et al. 2015) or by shaping the immune system, thus benefitting the offspring when encountering pathogens (Agüero et al. 2016). The underlying mechanism and trigger for zonulin release during pregnancy needs further studies; this may involve the contribution of maternal hormones and maternal gut microbiota composition, which have been suggested to alter over pregnancy (Koren et al. 2012).

The increase in the intestinal permeability, particularly if occurring in excess, may challenge the mother to elevated passage of gut components, such as LPS, into circulation. In study III, LPS activity increased from early to late pregnancy, but as no correlation between serum zonulin and LPS in late pregnancy was detected, other mechanism than paracellular transport may have been involved. LPS is also transported in chylomicrons (Hersoug et al. 2016) and hence the intake of fat may influence the results. However, this may not be the case as only weak correlation between the intake of fat and serum LPS activity was detected. LPS is cleared in the liver by hepatocytes or Kupffer cells (Hersough et al. 2016) and another possible mechanism explaining the increase LPS concentration may relate to pregnancy or maternal weight gain induced alterations in hepatic LPS clearance.

6.4 Impact of probiotics and fish oil *in vivo* and *in vitro*

6.4.1 Impact of probiotics and/or n-3 LC-PUFA on serum zonulin concentration and LPS activity

In the clinical study (III) no impact of probiotics and/or n-3 LC-PUFA on serum zonulin concentration or LPS activity was detected among the study groups either when comparing the change from early to late pregnancy or in late pregnancy. This is surprising as we hypothesized, based on previous, although not unanimous experimental and human studies, that supplementation with probiotics and/or n-3 LC-PUFA, might enhance the integrity of intestinal epithelium.

There are several possible explanations why the dietary intervention induced no effects on intestinal permeability in this study. The women in this study were well-nourished, healthy women, suggesting that the function and integrity of their intestinal epithelium was normal and thus the impact of the intervention may be less than that in compromised cases. It is also clear that the consumed probiotic strains and LC-PUFA composition and concentration, as well as the duration of the intervention, may be influencing factors. Further, the detected changes in serum zonulin concentrations and LPS activities at the group level were small, and in this sense, it may be that the number of subjects in the study was not sufficient to detect differences across the intervention groups. Based on the findings here,

probiotics and n-3 LC-PUFA had no impact on serum zonulin concentration. Yet, there are other markes for intestinal permeability, such as L/M-ratio, which were not investigated in this study.

In non-pregnant populations, the studies investigating the effect of probiotics on intestinal permeability have revealed contradictory results on L/M-ratio (Horvath et al. 2016, Kwak et al. 2014, Sharma et al. 2011, Zeng et al. 2008, McNaught et al. 2005) and on serum zonulin concentrations (Liu et al. 2013, Liu et al. 2015, Stadbauer et al. 2015). In a recent study with *Bifidobacterium animalis* ssp. *lactis* 420, the same probiotic as in our study, serum zonulin concentration tended to be lower in the *B.lactis* 420 than in the placebo treated subjects after six months of supplementation in overweight and obese non-pregnant adult males and females (Stenman et al. 2016). However, no other studies were found investigating the effect of *B.lactis* 420 or *L.rhamnosus*, the probiotics used in our study, on intestinal permeability in humans. No previous clinical studies investigating the effects of n-3 LC-PUFA on intestinal permeability or serum zonulin were found. To the best of our knowledge, this is the first study to evaluate this relationship in humans. The impact of probiotics and LC-PUFA on LPS activity have been studied in humans only after an induced LPS inflammation or in disease conditions such as alcoholic hepatic patients, where a 7-day supplementation with *Bacillus subtilis/Enterococcus faecium* (Han et al. 2015) decreased the serum LPS.

6.4.2 Impact of probiotics and/or n-3 LC-PUFA on intestinal permeability in vitro

In the *in vitro* study, increase in intestinal epithelial integrity was detected when the Caco-2 cells were supplemented with *B. lactis* 420 growth medium. This finding suggests that during bacterial growth *in vitro*, *B.lactis* 420 produces active metabolites with epithelium improving properties. This finding is in line with previous study with the same probiotic (Putaala et al. 2008), however, the mechanism remains to be further studied since no alterations in TJ and MLCK protein expression were detected. Another possible mechanisms may include the impact of these metabolites on localization of TJs or expression of other proteins related to regulation of adjacent epithelial cells. In addition, n-3 LC-PUFA, EPA and DHA as fatty acid ethyl ester forms, enhanced intestinal epithelial integrity *in vitro* along with the increase in MLCK protein expression. Increased MLCK expression has in previous studies (Hecht et al. 1996, Shen et al. 2006) been linked to increased intestinal permeability. The opposite finding in our study may be related to the study model we used; the Caco-2 cells in our short 6-day protocol may still be differentiating and thus the role of MLCK may differ between the fully differentiated cells and those still in a differentiating phase. The concentration we used *in vitro*, was 1 mmol/L DHA and 0.12 mmol/L EPA, which we consider as

being in the physiological range. However, the proportion of free fatty acids *in vitro* is not known, which may limit the translation of these results *in vivo*, where fatty acids are free, as a consequence of hydrolysis by lipase.

Interestingly, no added benefit were detected when B.*lactis* and n-3 LC-PUFA were supplemented as combination. Instead, the combination appeared to negate the individual beneficial impact of B.*lactis* and n-3 LC-PUFA. This was unexpected since based both on the previous studies and our findings, supplementation with probiotics and/or n-3 LC-PUFA, might enhance intestinal epithelium. In the clinical study, the synergistic effect was neither seen.

In the clinical study, the dietary intervention induced no effects on intestinal permeability, although the effect was seen *in vitro*. This may explained by the difference between *in vivo* and *in vitro* approaches. Contrary to cell models, human intestine contains other bacteria and metabolites which may restrict the production of metabolites of the selected probiotic. In addition, although Caco-2 cells are widely used as a model for the intestinal epithelial barrier due to formation of TJs in cell culture, they lack the important mucus layer, which mediates the relationship between the host and the microbes.

6.5 Strengths and limitations of the study

The well characterized study population, i.e. pregnant women with good clinical data, adds significantly to this study. The number of subjects needed was estimated by power calculations and we considered that the duration of the intervention period was sufficient to observe the possible influence of supplementation.

In this study, three-day-food diaries were used as a dietary assessment method. This method requires motivated study subjects and detailed description of the daily food intake. To achieve the accurate food intake, the subjects were instructed on how to record the food diary and at the study visit, the diaries were carefully checked with the help of a portion size booklet. However, the limitations, such as underreporting and within-person variation e.g. in day-to-day and seasonal intake, may weaken the validity of this method in terms of reflecting the usual dietary intake of the study subjects. Further, these limitations may weaken the possibility for detecting the associations between dietary intake and investigated variables. The other limitation in this study is related to the use of serum zonulin. It has recently been used as marker for intestinal permeability in several studies and in our study, due to study population, the zonulin assay was a reasonable choice to use as a marker for intestinal permeability; L/M-ratio test is challenging, especially when considering that the women in our study were pregnant. In type 1 diabetic patients, a direct correlation ($R = 0.36$, $P = 0.0004$) between serum zonulin and

L/M-ratio has been detected (Sapone et al. 2006). Decreased concentration of serum zonulin has been shown to associate with decreased L/M-ratio (Liu et al. 2013, Russo et al. 2012), yet the correlation between serum zonulin and L/M-test during pregnancy needs to be elucidated.

Zonulin is a pre-haptoglobulin 2, which, based on the allele distribution, is not expressed in all subjects (Figure 1) (Tripathti et al. 2009). In our analyses serum zonulin was detected in all study subjects, suggesting that the assay was not specific for zonulin, but may also recognize other unspecific forms of prehaptoglobin. Further, in addition to enterocytes, other tissues, such as lung, liver, adipose tissue, and heart have been shown to secrete zonulin (Wang et al. 2000, Vanuytsel et al. 2013). Since the promoter of zonulin gene, haptoglobin 2, is under IL-6 control (Oliviero and Cortese 1989), inflammatory status may influence serum zonulin concentrations and thus hamper its' use as a marker of intestinal permeability. Indeed, further studies are needed to evaluate the sources of zonulin in circulation and its role as a marker of intestinal permeability during pregnancy. We did not measure IL-6, which would have been of interest since it has an association with zonulin and also has a role as an inducer of CRP (Pendeloski et al. 2017).

7 SUMMARY AND CONCLUSIONS

The main findings of this study are:

I Maternal gut microbiota composition and dietary intake was related to serum zonulin concentration in overweight and obese pregnant women in early pregnancy: richer gut microbiota, a higher abundance of *Faecalibacterium prausnitzii*, a higher dietary intake of fiber, as well as PUFA and multiple minerals and vitamins, were associated with lower serum zonulin levels.

II Serum zonulin concentration was related to metabolic risk markers in early pregnancy: serum zonulin concentration correlated directly with serum LPS activity, hs-CRP, GlycA, triglycerides, serum insulin concentrations and insulin resistance and inversely with insulin sensitivity.

III Serum zonulin concentration increased over the period of the pregnancy.

IV Probiotics and n-3 LC-PUFA, administered separately or combined, had no impact on the change detected in serum zonulin concentration. *In vitro, B.lactis* 420 and n-3 LC-PUFA enhanced epithelial integrity.

Based on these findings, intestinal permeability, measured as serum zonulin concentration, may be a possible factor contributing to maternal health. The finding of association between gut microbiota and overall healthier dietary intake with serum zonulin concentration is novel and important, as it implies that intestinal permeability could behave as a target for modification to improve maternal health. However, the supplementation with probiotics and n-3 LC-PUFA in this study had no impact on serum zonulin levels. As the change in serum zonulin concentration was small and thus possibly indicates a physiological change, it might be that the impact of the intervention may have been difficult to observe. Further studies are needed to evaluate the impact of probiotics and n-3 LC-PUFA on other markers, such as the L/M-ratio of intestinal permeability, low grade inflammation, and on other maternal risk factors.

The conclusions drawn in this thesis are based on the associations detected between diet, gut microbiota, serum zonulin and metabolic risk markers. The hypothesis is that increased serum zonulin, due to dietary factors and gut microbiota, and thus increase in intestinal permeability contributes to metabolic risk factors. However, since causality was not studied, it cannot be ruled out that the effect reflects the opposite, e.g. increase in inflammation drives the alteration in the intestinal epithelium and the subsequent release of zonulin. Zonulin is also

synthesized in liver and other tissues (Wang et al. 2016) and may thus have tissue specific functions. Threfore role of serum zonulin as a marker of intestinal permeability during pregnancy needs further studies.

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