

Gut Microbiota Richness and Composition and Dietary Intake of Overweight Pregnant Women Are Related to Serum Zonulin Concentration, a Marker for Intestinal Permeability^{1–3}

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Abstract

Background: Increased intestinal permeability may precede adverse metabolic conditions. The extent to which the composition of the gut microbiota and diet contribute to intestinal permeability during pregnancy is unknown.

Objective: The aim was to investigate whether the gut microbiota and diet differ according to serum zonulin concentration, a marker of intestinal permeability, in overweight pregnant women.

Methods: This cross-sectional study included 100 overweight women [mean age: 29 y; median body mass index (in kg/m²): 30] in early pregnancy (<17 wk of gestation; median: 13 wk). Serum zonulin (primary outcome) was determined by using ELISA, gut microbiota by 16S ribosomal RNA sequencing, and dietary intake of macro- and micronutrients from 3-d food diaries. The Mann-Whitney *U* test was used for pairwise comparisons and linear regression and Spearman's nonparametric correlations for relations between serum zonulin and other outcome variables.

Results: Women were divided into "low" (<46.4 ng/mL) and "high" (≥46.4 ng/mL) serum zonulin groups on the basis of the median concentration of zonulin (46.4 ng/mL). The richness of the gut microbiota (Chao 1, observed species and phylogenetic diversity) was higher in the low zonulin group than in the high zonulin group (*P* = 0.01). The abundances of *Bacteroidaceae* and *Veillonellaceae*, *Bacteroides* and *Blautia*, and *Blautia* sp. were lower and of *Faecalibacterium* and *Faecalibacterium prausnitzii* higher (*P* < 0.05) in the low zonulin group than in the high zonulin group. Dietary quantitative intakes of n-3 (ω-3) polyunsaturated fatty acids (PUFAs), fiber, and a range of vitamins and minerals were higher (*P* < 0.05) in women in the low zonulin group than those in the high zonulin group.

Conclusions: The richness and composition of the gut microbiota and the intake of n-3 PUFAs, fiber, and a range of vitamins and minerals in overweight pregnant women are associated with serum zonulin concentration. Modification of the gut microbiota and diet may beneficially affect intestinal permeability, leading to improved metabolic health of both the mother and fetus. This trial was registered at clinicaltrials.gov as NCT01922791. *J Nutr* 2016;146:1694–700.

Keywords: dietary intake, gut microbiota composition, intestinal permeability, pregnancy, zonulin

Introduction

Emerging data indicate that increased intestinal permeability is involved in several disorders associated with low-grade inflam-

mation, including obesity, obesity-associated insulin resistance, and type 2 diabetes (1–3). The proposed mechanism in low-grade inflammation-associated disorders is presumed to involve heightened passage of inflammation-initiating factors, such as bacterial LPS, through the intestinal barrier into the circulation. Subsequently, increased circulating concentrations of bacterial LPS can lead to metabolic endotoxemia, a potential mediator of low-grade inflammation and subsequent metabolic disorders (4–6).

The intestinal epithelium forms a critical barrier against invading pathogens and other harmful entities, and consists of adjacent epithelial cells joined together by junctional complexes. Tight junctions are the most apical complexes and are responsible for regulating paracellular transport of ions, solutes, and

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³ Supplemental Table 1 and Supplemental Figure 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

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water. Tight junctions are composed of multiple proteins, including cytosolic zonula occludin (7). Zonulin, a protein detectable in human serum (8), has been shown to reflect intestinal permeability (9–12). Serum zonulin has been used as a serum marker for intestinal permeability in several studies (1–4); and further increased serum concentrations have been detected in a range of metabolic conditions, including obesity (1), obesity-associated insulin resistance (2), and type 1 (9) and type 2 (3) diabetes.

Environmental components, such as diet, may have an important role in regulating intestinal epithelium and gut health. Nutritional deficiencies, such as a deficiency in glutamine, the key energy source for gut epithelial cells, and zinc, appear to affect intestinal permeability (13, 14). The effect of dietary nutrients on intestinal permeability in conditions other than malnutrition is less well known. The dietary FAs EPA and DHA in *in vitro* studies (15), vitamin D (16), and other components, such as probiotics in humans (17), have been linked to intestinal permeability with potential health effects. In addition, the gut microbiota has been shown to influence intestinal permeability, mainly in animal studies (6, 8, 18, 19).

Pregnancy is accompanied by numerous metabolic alterations that support fetal growth and development. Initial results based on the lactulose/mannitol test suggested that healthy pregnant women exhibited an increase in intestinal permeability compared with nonpregnant women (20). However, little is known about the effects of pregnancy on intestinal permeability and whether this could lead to subsequent health consequences. In the nonpregnant population, being overweight has been associated with both increased intestinal permeability and metabolic disorders, such as increased inflammatory markers and decreased insulin sensitivity (1, 2). We postulate that being overweight or obese during pregnancy may present additional challenges that lead to increased intestinal permeability, because, in nonpregnant population, overweight was associated with both increased intestinal permeability and subsequent metabolic disorders (1, 2).

The aim of the study was to investigate the extent to which the gut microbiota, which is altered during pregnancy (21), and diet differ in pregnant overweight women according to serum zonulin concentration, a marker of intestinal permeability. We hypothesized that a higher abundance of certain beneficial gut bacteria along with higher intakes of nutrients with known benefits for gut health are related to intestinal permeability, as measured by concentrations of serum zonulin.

Methods

Study participants. This cross-sectional study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of the Hospital District of Southwest Finland (permission 115/180/2012). Written informed consent was obtained from all participants. This study included 100 overweight women in early pregnancy (<17 wk of gestation; median: 13 wk; IQR: 11–15 wk of gestation) living in southwest Finland. The data were collected from healthy overweight women participating in a mother-infant dietary intervention trial (clinicaltrials.gov, NCT01922791) and who provided fecal and serum samples. Women's mean age was 29 ± 5 y, and 50% (47 of 95) were highly educated with college or university degrees. The median prepregnancy BMI (in kg/m^2) was 30 (IQR: 27–33). Fifty-two percent of the participants were overweight (BMI: 25–30) and 48% were obese (BMI >30). The study measurements and samples were obtained at the first study visit, which was considered the baseline of the intervention trial.

Serum zonulin assay. On the morning of the study visit, after at least a 9-h fast, blood samples were drawn from the antecubital vein and the

sera were frozen at -80°C until analysis. Serum zonulin was measured by using a zonulin ELISA kit (Immundiagnostik AG). The interassay variation in zonulin assay was 10.6%, which is below the interassay values reported in the manufacturer's manual and in line with previous studies that used the same kit (1, 2). Because no reference values for zonulin exists, we used the median of serum zonulin (46.4 ng/mL) as a cutoff for classifying the study participants into 2 groups: "low" (<46.4 ng/mL) and "high" (≥ 46.4 ng/mL) serum zonulin groups.

Gut microbiota assessment. Fecal samples from mothers were collected in sterile plastic pots. Samples were collected the morning of the study visit or the previous evening, delivered to the study unit, and kept at $+4^\circ\text{C}$ until DNA extraction. Four of 100 fecal samples were excluded from analysis due to the analytical method used. DNA was extracted from 50 mg homogenized feces by using a GTX stool extraction kit and a fully automated GenoXtract machine (Hain Lifescience) as previously described (22). Before extraction, mechanical lysis was performed by bead-beating the samples in ceramic bead tubes with a MOBIO PowerLyzer 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, Inc., Carlsbad, CA). The DNA samples were sequenced with the use of the Sequencing and Bioinformatics Service at Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana (FISABIO). 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 regions. Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences. The primers were selected from Klindworth et al. (23). The full-length primer sequences, with the use of standard International Union of Pure and Applied Chemistry (IUPAC) nucleotide nomenclature to follow the protocol targeting this region, were as follows: 16S amplicon PCR forward primer = $5'$ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 16S amplicon PCR reverse primer = $5'$ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

Microbial genomic DNA (5 $\text{ng}/\mu\text{L}$ in 10 mM Tris, pH 8.5) was used to initiate the protocol. After 16S amplification, the multiplexing step was performed by using the Nextera XT Index Kit (FC-131-1096). One microliter of the PCR product was analyzed on a Bioanalyzer DNA 1000 chip to verify the size. The expected size on a Bioanalyzer trace was ~ 550 bp. After size verification, the libraries were sequenced by using a 2×300 -bp 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 by using the QIIME software package. 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. Relative abundance was determined by using OTUs. Rarefaction curves were generated for Chao1, observed species, phylogenetic diversity (PD), and Shannon index; α diversity was calculated at an α rarefaction sequence depth of 36382.0.

Dietary intake. Three-day food diaries were recorded by the women within 1 wk before the study visit. Five of 100 participants were excluded due to missing food diaries. The participants were given instructions on how to record their food intake and during the study visit diaries were checked for completeness and accuracy with the help of an illustrated portion booklet. Mean daily intakes of energy and nutrients were calculated by using computerized software (Aivo diet 2.0.2.3; Aivo). To evaluate adherence to the recommended dietary intake, the intake of different nutrients ($n = 27$) was categorized as either intake within or outside of the Nordic nutrition recommendations (24). The proportion of different nutrients consumed as recommended was calculated for each study participant.

Statistical analysis. Because no *a priori* data were available on serum zonulin concentrations (primary outcome) in pregnant women, the sample size estimation in this study was based on data from 2 studies in which the difference in zonulin concentration was measured between obese and normal-weight participants (1) or in healthy young individuals before and after consuming inulin-enriched pasta (25). On the basis of

the differences in serum zonulin concentrations between the study groups in these studies, we estimated that a sample size of 100 would be adequate to observe sufficient variation between the study participants, thus allowing division of the data between high and low serum zonulin groups based on the median zonulin value. All of the statistical analyses were performed by using SPSS version 21.0 (IBM Inc.), with $P < 0.05$ considered significant. The normality of the data was tested by using the Kolmogorov-Smirnov test and by visual inspection of histograms. Because not all of the data were normally distributed, the Mann-Whitney U test was used to compare the differences in dietary intakes of nutrients, the percentage of nutrients consumed within the recommendations, and gut microbiota diversity and abundance between the high and the low zonulin groups. Variables are presented as medians (IQRs), with the exception of Table 1 and Supplemental Table 1 in which both medians (IQRs) and means (SDs) are presented for relative abundances of bacteria. Univariable linear regression was conducted to study the relation between serum zonulin (outcome) concentration and nutrient intake (continuous predictors). The results were reported as the change in serum zonulin associated with an increase per unit of each predictor. To further evaluate which of the nutrients best predicted the serum zonulin concentration, adjusted multiple linear regression analyses were performed. Spearman's nonparametric correlation was conducted to study the relation between serum zonulin and relative abundance of the gut microbiota.

Results

Serum zonulin concentrations. Serum zonulin concentrations ranged from 25 to 95 ng/mL ($n = 100$). Median (IQR) zonulin concentrations were 38 (35–43) ng/mL and 53 (49–60) ng/mL in the low (<46.4 ng/mL) and high (≥ 46.4 ng/mL) zonulin groups, respectively.

Association of gut microbiota with serum zonulin. Of the fecal DNA samples analyzed, 4 samples (out of 96) were discarded due to the 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. At the phylum level, *Bacteroidetes* and *Firmicutes* accounted for 94% of total sequences in the low zonulin group and 95% in the high zonulin group. There were significant differences in the relative abundance of 42 taxonomic groups between the low and the high zonulin groups (Supplemental Table 1). The abundance of bacteria >1% of total microbiota

was considered to be reliable. Of the 42 taxonomic groups, 35 had a relative abundance <1% and 7 had a relative abundance >1%, which were taken for further evaluation. At the family level, the abundance of *Bacteroidaceae* and *Veillonellaceae* was lower in the low zonulin group than in the high zonulin group (Table 1). At the genus level, *Bacteroides* and *Blautia* had lower and *Faecalibacterium* higher abundances in the low zonulin group than in the high zonulin group. At the species level, the abundance of *Blautia* was lower and *Faecalibacterium prausnitzii* higher in the low zonulin group than in the high zonulin group. No significant difference was found ($P = 0.2$) in the ratio of *Bacteroides* to *Firmicutes* between the low (1.0; 0.7–1.4) and the high (1.1; 0.8–1.6) zonulin groups.

To further study the relation between serum zonulin and gut microbiota composition, Spearman's correlation was conducted for those bacteria that were significantly different ($P < 0.05$) in abundance between the high and the low zonulin groups. At the genus level, *Faecalibacterium* was negatively and *Blautia* positively associated with serum zonulin [$\rho = 0.29$ ($P = 0.004$) and $\rho = -0.25$ ($P = 0.018$), respectively]. At the species level, *F. prausnitzii* was negatively and *Blautia* positively associated with serum zonulin [$\rho = 0.29$ ($P = 0.005$) and $\rho = -0.25$ ($P = 0.018$), respectively]. No association was found between any other bacteria and serum zonulin.

To compare the overall gut microbiota composition by diversity and richness, we examined the following estimators: Chao1, observed species, PD, and Shannon index (26). Compared with the high zonulin group, higher indexes for Chao1 [409 (363–438) compared with 376 (338–415); $P = 0.01$], observed species [361 (309–382) compared with 331 (294–363); $P = 0.01$], and PD [38.8 (33.6–42.1) compared with 34.9 (30.0–39.6); $P = 0.01$] were found in the low zonulin group, suggesting higher gut microbiota richness in the low zonulin group. Higher α rarefaction curves further supported higher richness in the low zonulin group than in the high zonulin group (Supplemental Figure 1).

Association of diet with serum zonulin. Differences in dietary intakes of various nutrients were detected between the high and the low zonulin groups (Table 2). These were attributable to significantly higher absolute intakes (g) of n-3 PUFAs and dietary fiber and a trend for a higher intake of n-6

TABLE 1 Relative abundance of gut microbiota in overweight pregnant women by serum zonulin concentration¹

	Low zonulin (<46.4 ng/mL), % of total bacteria		High zonulin (≥ 46.4 ng/mL), % of total bacteria		P^2
	Median (IQR)	Mean \pm SD	Median (IQR)	Mean \pm SD	
Family level					
<i>Bacteroidaceae</i>	29 (21–39)	30 \pm 11	35 (25–46)	36 \pm 13	0.02
<i>Veillonellaceae</i>	0.95 (0.52–1.6)	1.2 \pm 0.91	1.5 (0.74–2.9)	1.9 \pm 1.6	0.03
Genus level					
<i>Bacteroides</i>	29 (21–39)	30 \pm 11	35 (25–46)	36 \pm 13	0.02
<i>Blautia</i>	1.3 (0.87–2.0)	1.9 \pm 2.0	2.0 (1.4–2.6)	2.4 \pm 1.6	0.001
<i>Faecalibacterium</i>	5.5 (3.8–7.5)	6.0 \pm 3.1	4.5 (2.6–6.6)	4.9 \pm 3.0	0.04
Species level					
<i>Blautia</i>	1.3 (0.87–2.0)	1.8 \pm 1.9	2.0 (1.4–2.6)	2.4 \pm 1.6	0.001
<i>Faecalibacterium prausnitzii</i>	5.5 (3.8–7.5)	6.0 \pm 3.1	4.5 (2.6–6.6)	4.9 \pm 3.0	0.04

¹ $n = 46$ in both groups (96 samples were analyzed and 4 samples were discarded due to the low yield of sequences, leaving 92 samples for statistical analysis).

² Comparison for median (Mann-Whitney U test).

PUFAs, total PUFAs, and protein in the low zonulin group compared with the high zonulin group. Although the intake of n-3 PUFAs was different ($P = 0.03$) between the zonulin groups, no differences ($P = 0.8$) were found in the intakes of the individual n-3 FAs DHA or EPA between the groups. No significant difference was found in the intake of energy-yielding nutrients as a percentage of energy intake (% of energy) or energy intake between the low and the high zonulin groups. The intakes of thiamin, niacin, pyridoxine, carotenoids, riboflavin, vitamins E and K, magnesium, potassium, calcium, iodine, and phosphorous were higher in the low zonulin group than in the high zonulin group (Table 3). The percentage of nutrients consumed within the recommendations (24) was higher in the low zonulin group than in the high zonulin group [67% (56–76%) in the low zonulin group compared with 57% (40–65%) in the high zonulin group; $P = 0.004$].

To further study the relation between serum zonulin and dietary intakes of nutrients, linear regression was performed for nutrients with a P value <0.06 , which reflected the difference between the intake of nutrients in the high and the low zonulin groups. $P < 0.06$ was chosen to include also nutrients with a trend in difference between the groups. For all linear regression

TABLE 2 Daily intakes of energy and energy-yielding nutrients in overweight pregnant women by serum zonulin concentration¹

Nutrient	Low zonulin (<46.4 ng/mL)	High zonulin (≥ 46.4 ng/mL)	P
Energy, MJ	8.33 (7.03–9.54)	8.0 (6.37–9.0)	0.14
Protein			
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			
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			
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			
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
SFAs			
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
PUFAs			
g	12.0 (10.0–16.2)	10.5 (8.59–13.7)	0.06
% of energy	5.60 (4.55–6.70)	5.10 (4.35–5.70)	0.12
MUFAs			
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 PUFAs			
g	3.63 (2.74–4.47)	3.01 (2.44–3.93)	0.03
% of energy	1.50 (1.30–1.90)	1.45 (1.20–1.70)	0.14
n-6 PUFAs			
g	9.59 (6.95–12.6)	8.02 (6.44–10.7)	0.06
% of energy	4.0 (3.40–5.35)	4.05 (3.18–4.40)	0.17
Fiber			
g	22.2 (16.7–26.1)	18.2 (13.6–21.6)	0.001
% of energy	2.0 (1.65–2.50)	1.90 (1.5–2.23)	0.12

¹ Values are medians (IQRs) calculated from 3-d food diaries; $n = 45$ in the low zonulin group, and $n = 50$ in the high zonulin group (5 of 100 participants were excluded due to missing food diaries).

TABLE 3 Daily intakes of vitamins and minerals in overweight pregnant women by serum zonulin concentration¹

Nutrient	Low zonulin (<46.4 ng/mL)	High zonulin (≥ 46.4 ng/mL)	P
Vitamins			
Thiamin, μ g	1.27 (1.09–1.54)	1.08 (0.92–1.41)	0.01
Niacin, mg	33.7 (27.0–37.6)	28.1 (21.3–35.3)	0.03
Pyridoxine, mg	2.06 (1.53–2.35)	1.62 (1.29–2.15)	0.01
Riboflavin, mg	1.82 (1.60–2.31)	1.65 (1.30–2.10)	0.009
Vitamin B-12, μ g	4.76 (3.76–5.89)	4.27 (2.86–5.59)	0.20
Vitamin C, mg	126 (75.0–191)	103 (70.0–159)	0.18
Folate, μ g	240 (195–309)	220 (182–259)	0.08
Vitamin A, ² μ g	632 (515–829)	544 (405–764)	0.05
Vitamin D, μ g	6.94 (5.32–10.7)	6.20 (3.47–9.31)	0.05
Vitamin E, mg	11.1 (8.26–12.7)	8.78 (6.87–10.9)	0.01
Vitamin K, μ g	109 (83.6–134)	89.2 (58.7–124)	0.03
Minerals			
Magnesium, mg	336 (953–1.4)	294 (242–335)	0.008
Potassium, g	3.68 (3.18–4.21)	3.10 (2.62–3.71)	0.001
Calcium, g	1.09 (0.95–1.38)	0.92 (0.74–1.32)	0.01
Iodine, μ g	208 (165–250)	182 (141–218)	0.02
Phosphorus, g	1.47 (1.34–1.68)	1.28 (1.11–1.54)	0.009
Sodium, g	2.61 (2.25–2.96)	2.38 (2.01–2.81)	0.09
Iron, mg	11.0 (9.21–13.2)	10.2 (8.22–11.8)	0.05
Copper, mg	1.24 (1.04–1.44)	1.08 (0.9–1.39)	0.10
Selenium, μ g	64.2 (56.1–77.2)	62.3 (48.8–72.5)	0.26
Zinc, mg	11.2 (9.9–12.9)	10.8 (8.82–12.9)	0.28

¹ Values are medians (IQRs) calculated from 3-d food diaries; $n = 45$ in the low zonulin group, and $n = 50$ in the high zonulin group (5 of 100 participants were excluded due to missing food diaries).

² Retinol equivalents comprising retinols and carotenoids.

models, the normal distribution of standardized residuals was confirmed. A significant inverse relation was identified between serum zonulin concentration and absolute intakes of protein, total PUFAs, n-6 PUFAs, vitamin E, niacin, magnesium, iron, and potassium (Table 4) in univariable linear regression. When energy-yielding nutrients (protein and total PUFAs) were included in adjusted multiple linear regression analysis, protein was found to be a significant factor (β : -0.14 ; 95% CI: -0.25 , -0.03 ; $P = 0.01$) for predicting serum zonulin, with the whole model explaining 9.3% of the variability in serum zonulin ($P = 0.004$; adjusted multiple linear regression model 1, Table 4). The adjusted multiple linear model for vitamin E, niacin, magnesium, iron, and potassium explained 8.5% of the variability in serum zonulin ($P = 0.02$), although no significant associations with serum zonulin were found for any single micronutrient (adjusted multiple linear regression model 2, Table 4).

Discussion

We showed that gut microbiota composition, including both microbiota richness and the abundance of specific microbiota, and dietary intakes of n-3 PUFAs, fiber, and certain vitamins and minerals are linked to concentrations of serum zonulin. We suggest that modifying the composition of the gut microbiota and diet could beneficially influence intestinal permeability and thus may affect maternal and child health.

We found that gut microbiota richness differed between the high and the low zonulin groups, as exhibited by higher microbiota richness in the low zonulin group. To our knowledge, this is the first

TABLE 4 Associations between daily intakes of nutrients and serum zonulin concentrations in overweight pregnant women in linear regression and multiple linear regression analyses¹

Nutrient	Linear regression			Multiple linear regression			
	β (95%CI)	<i>P</i>	<i>R</i> ²	Model 1 ²		Model 2 ³	
				β (95%CI)	<i>P</i>	β (95%CI)	<i>P</i>
Energy, MJ	−0.001 (−0.002, 0.000)	0.07	0.033				
Energy-yielding nutrients							
Protein, g	−0.158 (−0.265, −0.051)	0.004	0.085	−0.139 (−0.247, 0.031)	0.01		
PUFAs, 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 nutrient. *n* = 95 (5 of 100 participants were excluded due to missing food diaries).

² *P* = 0.004, adjusted *R*² = 0.093.

³ *P* = 0.024, adjusted *R*² = 0.085.

study to suggest that gut microbiota richness is associated with intestinal permeability in humans *in vivo*. In 1 previous study, bacterial diversity was associated with intestinal barrier function in patients with ulcerative colitis and chronic pouchitis. In this study, a correlation was found between a higher passage of *Escherichia coli* K12 and higher bacterial diversity (Shannon index) *ex vivo* in the Ussing chambers (27). Recent studies have shown a correlation between low gut microbiota richness and adverse metabolic conditions, including adiposity, insulin resistance, and a more pronounced inflammatory phenotype (28, 29), although intestinal permeability was not taken into account. We hypothesize that the interplay between the gut microbiota and intestinal permeability is a potential mechanism linking the gut microbiota to the inflammation associated with the metabolic disorders identified in these studies. Abundant and volatile anti-inflammatory metabolites, such as SCFAs generated by variable bacteria, are essential for the maintenance of intestinal epithelium integrity (30). Low microbiota richness with less variable metabolites or the presence of certain bacteria may result in increased intestinal permeability and subsequent leakage of LPS or other harmful components into the circulation, resulting in inflammatory responses and low-grade inflammation.

In addition to the difference in gut microbiota richness, microbiota abundance differed between the high and the low zonulin groups. Specifically, a higher abundance of *F. prausnitzii* together with a lower abundance of *Bacteroides* in the low zonulin group indicate that these bacteria may play a role in intestinal epithelial integrity. This observation may be of importance with regard to human health, because a high presence of pro-inflammatory species, such as *Bacteroides*, in relation to potentially anti-inflammatory species, such as *F. prausnitzii*, has been associated with adverse metabolic outcomes, such as insulin resistance (28). A higher abundance of the genus *Blautia* has also been associated with glucose intolerance (31). In alcohol-dependent participants, a lower abundance of *Faecalibacterium*, particularly *F. prausnitzii*, and a higher abundance of the genus *Blautia* have been linked to high intestinal permeability, as assessed by the radioactive probe ⁵¹Cr-EDTA measurement in urine (32). In our study, we found a similar relation between serum zonulin and *F. prausnitzii* and *Blautia* in pregnant overweight women.

In addition to gut microbiota composition, we found that the intakes of certain dietary components and several nutrients were different between the low and the high zonulin groups. Because there was no difference in total energy intake between the low and the high zonulin groups, the overall magnitude of nutrient intake appears to be important. This is further supported by the finding that women in the low zonulin group were more likely to consume the recommended intake of nutrients than were women in the high zonulin group, highlighting the importance of diet quality for gut health. Few clinical studies, to our knowledge, have investigated the impact of diet on intestinal permeability in well-nourished conditions. Similar to our finding, an inverse correlation between daily dietary protein intake as a proportion of energy intake and serum zonulin was detected in a study in normal-weight and overweight participants (1). How proteins confer their effect is not fully understood; however, several amino acids, such as glutamine, are known to provide nourishment to gut epithelia. In addition, protein fermentation products and metabolites originating from protein breakdown by microbiota may also be involved in the regulation of intestinal epithelial growth (33), thus influencing intestinal permeability.

In our study, we did not observe differences in total fat intake between the zonulin groups. In the study by Zak-Gotåb et al. (1), a positive correlation between serum zonulin and energy intake, which was associated with higher fat intake, was found (1). Our finding may be explained by the fact that the intake of fat for all of the women was at an amount typically observed in Finnish women and was low compared with animal experimental diets. Indeed, in animals, a high-fat diet containing about 20 times the typical fat intake than the usual feed pellet diet was shown to increase intestinal permeability (6). Instead of the amount of total fat, our study highlights the importance of the quality of dietary fat for intestinal permeability. We found a higher intake of total PUFAs and n-3 PUFAs to be associated with lower serum zonulin concentrations. In maintaining intestinal epithelial integrity, PUFAs influence the inflammatory status of the gut by serving as precursors to anti-inflammatory eicosanoid synthesis, or they may enhance intestinal integrity through the regulation of tight junction functions (15, 34–36). Previous *in vitro* studies with EPA and DHA (15) and animal studies with

n-3 PUFAs (34–37) suggested a beneficial effect of these FAs on intestinal permeability. We found no association between intakes of the individual n-3 FAs DHA or EPA and serum zonulin concentration. This may be due to the high variation in and relative low intake of DHA and EPA in the women who did not receive dietary supplements at the study baseline.

Consistent with our findings, dietary fiber in the form of inulin-enriched pasta has been shown to lower serum zonulin concentrations in healthy young participants (25). The fermentation products of fiber, such as butyrate, an energy source for epithelial cells, may be the mechanism by which fiber exerts its beneficial impact on intestinal epithelium. In addition to dietary macronutrients, micronutrients may also affect intestinal permeability. In our study, we found that the intake of several vitamins and minerals benefited intestinal permeability, further supporting the beneficial role of a healthier diet. In certain disease conditions, intakes of zinc and vitamin D have been shown to beneficially affect intestinal epithelial integrity (14, 16). In our study, there was no association between serum zonulin concentration and zinc intake in well-nourished women, but a trend was detected for vitamin D. How micronutrients confer a beneficial impact on intestinal permeability may be related to immunomodulatory or nutritional effects and remains to be studied.

The limitations of our study relate to the choice of study population, overweight women in early pregnancy, because it is possible that intestinal permeability is further altered as pregnancy proceeds toward delivery. At the same time, this is a strength of our study because all of the women were studied at the same stage of pregnancy. It remains for future studies to show the extent to which intestinal permeability is altered over the whole course of pregnancy, preferably as follow-up from early to late pregnancy, and whether the influencing factors are the same as in early pregnancy. Similarly, because we studied only overweight pregnant women, it is uncertain whether the findings can be translated to normal-weight pregnant women. The limitations of the study also relate to the methods used. Established scientific procedures were used while performing the study, which included the instructions and personal guidance for sampling and for recording dietary intakes. Nevertheless, it is possible that typical errors in measuring dietary intake, such as underreporting or inaccuracy due to self-reporting, occurred.

How specific dietary components confer beneficial effects on intestinal permeability is not completely known. The rapid turnover of intestinal epithelial cells and the preservation of intestinal integrity necessitate a constant supply of nutrients, and thus a high intake of nutrients in general may support this renewal process. Importantly, we showed that meeting the recommended intake of nutrients was related to serum zonulin and appears to be essential for intestinal integrity. There is emerging evidence that diet plays an essential role in determining gut microbiota composition and metabolic activity (29, 38). Thus, in addition to having a direct effect on intestinal epithelial integrity, dietary components may affect the intestinal barrier by modulating the gut microbiota. Further studies are needed to elucidate the details of these interactions and the mechanisms underlying the interplay between the gut microbiota, diet, and intestinal permeability.

In conclusion, we showed that there is a relation in humans between gut microbiota richness and the abundance of specific species and dietary intakes of fiber, n-3 PUFAs, certain vitamins and minerals, along with the overall recommended diet, and serum zonulin, a marker of intestinal permeability. Our findings provide an important basis for future well-executed intervention trials to further investigate how diet and the gut microbiota should be modified to beneficially influence intestinal permeability and

subsequently affect the metabolic health of obese pregnant women and their offspring.

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